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CRANFIELD UNIVERSITY

Louise Claire Youngs

EVALUATION OF *IN SILICO* AND *IN VITRO* SCREENING METHODS FOR CHARACTERISING ENDOCRINE DISRUPTING CHEMICAL HAZARDS

SCHOOL OF ENERGY, ENVIRONMENT AND AGRIFOOD
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Doctor of Philosophy
Academic Year: 2014

Supervisor: Dr Ruth Bevan
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Institute for Environment, Health, Risk and Futures

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ABSTRACT

Anthropogenic activities have drastically altered chemical exposure, with traces of synthetic chemicals detected ubiquitously in the environment. Many of these chemicals are thought to perturb endocrine function, leading to declines in reproductive health and fertility, and increases in the incidence of cancer, metabolic disorders and diabetes.

There are over 90 million unique chemicals registered under the Chemical Abstracts Service (CAS), of which only 308,000 were subject to inventory and/or regulation, in September 2013. However, as a specific aim of the EU REACH regulations, the UK is obliged to reduce the chemical safety initiatives reliance on *in vivo* apical endpoints, promoting the development and validation of alternative mechanistic methods. The human health cost of endocrine disrupting chemical (EDC) exposure in the EU, has been estimated at €31 billion per annum. In light of the EU incentives, this study aims to evaluate current *in silico* and *in vitro* tools for EDC screening and hazard characterisation; testing the hypothesis that *in silico* virtual screening accurately predicts *in vitro* mechanistic assays. Nuclear receptor binding interactions are the current focus of *in silico* and *in vitro* tools to predict EDC mechanisms. To the author's knowledge, no single study has quantitatively assessed the relationship between *in silico* nuclear receptor binding and *in vitro* mechanistic assays, in a comprehensive manner.

Tripos ® SYBYL software was used to develop 3D-molecular models of nuclear receptor binding domains. The ligand binding pockets of estrogen (ERα and ERβ), androgen (AR), progesterone (PR) and peroxisome proliferator activated (PPARγ) receptors were successfully modelled from X-ray crystal structures. A database of putative-EDC ligands (n= 378), were computationally 'docked' to the pseudo-molecular targets, as a virtual screen for nuclear receptor activity. Relative to *in vitro* assays, the *in silico* screen demonstrated a sensitivity of 94.5%. The SYBYL Surflex-Dock method surpassed the OECD Toolbox ER-Profiler, DfW and binary classification models, in correctly identifying endocrine active substances (EAS). Aiming to evaluate the current *in vitro* tools for endocrine MoA, standardised ERα transactivation (HeLa9903), stably transfected AR transactivation (HeLa4-11) assays in addition to novel transiently transfected reporter gene assays, predicted the mechanism and potency of test substances prioritised from the *in silico* results (n = 10 potential-EDCs and 10 hormone controls). In conclusion, *in silico* SYBYL molecular modelling and Surflex-Dock virtual screening sensitively predicted the binding of ERα/β, AR, PR and PPARγ potential EDCs, and was identified as a potentially useful regulatory tool, to support EAS hazard identification.

Keywords: *Endocrine Disrupt*, Test Methods, Prioritisation, Regulation, SYBYL Surflex-Dock*

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"*From the world we actually live in, the world that is given by our senses, our intuitions of beauty and goodness, our emotions and impulses, our moods and sentiments, the man of science abstracts a simplified and private universe of things possessing only those qualities which used to be called "primary". Arbitrarily, because it happens to be convenient, because his methods do not allow him to deal with the immense complexity of reality, he selects from the whole of experience only those elements which can be weighed, measured, numbered, or which lend themselves in any other way to mathematical treatment. By using this technique of simplification and abstraction, the scientist has succeeded to an astounding degree in understanding and dominating the physical environment. The success was intoxicating and, with an illogicality which, in the circumstances, was doubtless pardonable, many scientists and philosophers came to imagine that this useful abstraction from reality was reality itself*" Aldous Huxley.

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TABLE OF CONTENTS

ABSTRACT i
ACKNOWLEDGEMENTS iii
LIST OF FIGURES ix
LIST OF TABLES xi
LIST OF ABBREVIATIONS xiii
1 INTRODUCTION 1
1.1 The Scientific Context 1
1.1.1 A Human Health Concern? 2
1.1.2 Mechanism of Action 3
1.2 A Regulatory Conundrum 6
1.2.1 Chemical Safety Testing Strategies 6
1.3 The EDC Problem 8
1.4 Research Aims and Objectives 9
2 BACKGROUND INFORMATION 13
2.1 The '*Start*' of the Science 13
2.1.1 Endocrinology 13
2.1.2 The Rise of Environmentalism 15
2.1.3 Wildlife Effects 16
2.1.4 Summary 20
2.2 Evidence for Endocrine Disruption in Humans: A Human Health Concern? 21
2.2.1 Reproductive Health 21
2.2.2 Hormonal Cancers 25
2.2.3 Metabolism and Development 26
2.2.4 Summary 27
2.3 Chemical Regulation and Testing 28
2.3.1 Legislation & Regulation 28
2.3.2 A Defined Mode-of-Action? 29
2.3.3 Chemical Safety Test Guidelines 30
2.3.4 Alternative (Non-Animal) Methods 33
2.3.5 Exposure 35
2.3.6 Summary 36
2.4 Chemical Prioritisation & Screening 36
2.4.1 Prioritisation 36
2.4.2 EDC Screening 37
2.4.3 The OECD Toolbox ER Profiler 38
2.5 Summary & Discussion 40
3 BIOLOGICAL MECHANISMS 43
3.1 Endocrine Receptors and the Classical Genomic Pathway 43
3.1.1 The Nuclear Receptor Superfamily 43

3.1.2 Estrogen Receptor 47
3.1.3 Androgen Receptor 49
3.1.4 Progesterone Receptor 50
3.1.5 Peroxisome Proliferator Activated Receptor-gamma 51
3.2 Nuclear Receptor Regulation 51
3.2.1 Heat Shock Proteins (Hsp) 51
3.2.2 Co-Regulation 52
3.2.3 Post-Translational Modification 54
3.2.4 Inter- & Intra- Receptor Cross-Talk 54
3.2.5 Agonism vs. Antagonism 55
3.3 Summary & Discussion 56
4 ENDOCRINE ACTIVITY *IN SILICO* 59
4.1 The theory of *in silico* Nuclear Receptor Binding 59
4.2 *In silico* Methodology 61
4.3 *In silico* Materials & Methods 62
4.3.1 *In silico* Software and Programs 62
4.3.2 Nuclear Receptor Phylogeny Modelling 62
4.3.3 Chemical Prioritisation for *in silico* Virtual Screening 66
4.3.4 SYBYL Molecular Modelling 67
4.3.5 SYBYL Surflex-Dock Virtual Screening 70
4.3.6 Statistical Methods 74
4.3.7 Summary of *in silico* Methods 75
4.4 *In silico* Results & Discussion 75
4.4.1 Nuclear Receptor Phylogeny 75
4.4.2 SYBYL Site ID Nuclear Receptor Molecular Modelling 79
4.4.3 SYBYL X-Surflex-Dock *in silico* Virtual Screening 83
4.4.4 Regression Analysis of Virtual Screening Results 96
4.4.5 SYBYL Surflex-Dock Virtual Screening – A 'Good' *in silico* Tool? 99
4.5 *In silico* Summary and Conclusions 101
4.5.1 *In silico* Approaches to EAS: Study Milestones 102
4.5.2 Future Work for Development of *in silico* EDC Methods 103
5 ENDOCRINE ACTIVITY *IN VITRO* 107
5.1 Introduction to *in vitro* Toxicology 107
5.1.1 Testing for Estrogenicity 109
5.1.2 Testing for Androgenicity 110
5.1.3 Testing for PR and PPARγ Endocrine Modes of Action 111
5.1.4 The Caveats of *in vitro* Investigation 112
5.1.5 Summary 113
5.2 *In vitro* Materials and Methods 113
5.2.1 *In vitro* Test Compounds 114
5.2.2 Plasticware and Laboratory Equipment 119
5.2.3 Cell Culture Materials 120

5.2.4 *In vitro* Cell Lines and Maintenance 122
5.2.5 Cell Viability and Cytoxicity Methods 124
5.2.6 Stably-Transfected Transactivation Assay (STTA) Method 125
5.2.7 Transient-Transfection Transactivation Assay (TTTA) Method 128
5.2.8 Luciferase Assay Method 130
5.2.9 Dual Luciferase Assay Method 130
5.2.10 Statistical Analysis 131
5.3 *In vitro* Results and Discussion 132
5.3.1 Cell Viability and Cytotoxicity 132
5.3.2 HeLa9903 ERα Agonism STTA 136
5.3.3 BG1Luc4E2 ERα/β Agonism STTA 144
5.3.4 HeLa4-11AR Agonism STTA 145
5.3.5 HeLa4-11 AR Antagonism STTA 154
5.3.6 Transient-Transfection Transactivation Assays (TTTA) 163
5.4 *In vitro* Summary and Conclusions 165
5.4.1 *In vitro* Summary and Milestones 172
5.4.2 Further Work to Aid Development of *in vitro* Methods for EDC Hazard Characterisation 173
6 GENERAL DISCUSSION AND CONCLUSIONS 175
6.1 Future Research and Requirements 179
REFERENCES 183
APPENDICES 217
Appendix A List of Prioritised Endocrine Disrupting Chemicals 217
Appendix B Supplementary Phylogeny Results 251
Appendix C SYBYL *in silico* Molecular Modelling of NR 253
Appendix D Amino Acid Properties 259
Appendix E SYBYL Surflex Dock Potential EDC Receptor Binding Score Data 261
Appendix F *In vitro* Testing Plate Layout 275
Appendix G Alternative *in vitro* Methods 277
Appendix H Supplementary *in vitro* STTA Results 279

LIST OF FIGURES

Figure 1.1 Ligand-Dependent Transactivation of Nuclear Receptors 4
Figure 2.1 Organs and Glands Associated with the Human Endocrine System..... 14
Figure 2.2 Integration of AOP Paradigm and OECD Conceptual Framework with Suggested Endocrine Relevant Modifications 31
Figure 2.3 Animals used in UK Scientific Procedures in 2010 and 2011 32
Figure 3.1 Androgen Receptor Signalling Pathways 44
Figure 3.2 Chemical Structure of Estrogen Receptor Binders (E2, DES and RAL)..... 49
Figure 4.1 SYBYL NR Protein Preparation 69
Figure 4.2 SYBYL Surflex-Dock Protomol Generation for Virtual Screening 72
Figure 4.3 SYBYL Surflex-Dock Virtual Screening Parameters..... 73
Figure 4.4 SYBYL Surflex-Dock Results Browser..... 74
Figure 4.5 Phylogeny of the Estrogen Receptor (ERα/β)..... 76
Figure 4.6 Phylogeny of the Androgen Receptor (AR)..... 77
Figure 4.7 Tripos™ SYBYL 3D-Molecular Modelling of Estrogen Receptor-α (ERα 3DT3) Monomer and Surflex-Dock Protomol..... 80
Figure 4.8 SYBYL SiteID Human Estrogen Receptor (3DT3) Ligand Binding Pocket 81
Figure 4.9 SYBYL SiteID Human Androgen Receptor (3V49) Ligand Binding Pocket 83
Figure 4.10 Regression Analysis of Physicochemical Properties of Chemical Database 97
Figure 4.11 Correlation Between *in silico* Nuclear Receptor (ERα, ERβ, AR, PR and PPARγ) Binding Score ($-\log K_d$) and Ligand Molecular Volume..... 98
Figure 4.12 Chemical Structure of Phthalates and Polychlorinated biphenyls (PCBs) 100
Figure 5.1 Assessment of Test Substance Cytotoxicity in HeLa4-11 and HeLa9903 Cells with PrestoBlue® Cell Viability Assay 133
Figure 5.2 Cytotoxicity of Coexposure to Test Substance and 1fM Testosterone in HeLa4-11 Cells Measured with PrestoBlue® Cell Viability Assay 134
Figure 5.3 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to 17β-Estradiol (E2)..... 137
Figure 5.4 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to Synthetic Estrogens (EE2 and DES)..... 139
Figure 5.5 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to Bisphenol-A or its' Metabolite 4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBPA)..... 140
Figure 5.6 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to Phthalates (DBP, DEHP and BBP)..... 141
Figure 5.7 Inadequate Response of BG1Luc4E2 Estrogen Receptor (α/β) Transactivation Assays in 24-Well and 96-Well Formats 145

Figure 5.8 Androgen Receptor Transactivation in Response to 24h Testosterone Exposure in HeLa4-11 Cells in 24- and 96- Microtitre Plates 146
Figure 5.9 Androgen Receptor Transactivation in HeLa4-11 Cells in Response to 24h Estrogen Exposure (E2 and DES) 148
Figure 5.10 Androgen Receptor Transactivation in HeLa4-11 Cells in Response to 24h Progestagin Exposure (PRG and 19-ND) 149
Figure 5.11 Androgen Receptor Transactivation in HeLa4-11 Cells in Response to MBPA and DDE Exposure 151
Figure 5.12 Androgen Receptor Transactivation in HeLa4-11 Cells in Response to 24h Consumer Chemical Exposure (MeP, TCN and OMC) 152
Figure 5.13 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Co-Exposed to Flutamide and 1fM Testosterone for 24 Hours 155
Figure 5.14 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Estrogens (E2, EE2 and DES) for 24 Hours 156
Figure 5.15 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Progestagins for 24 Hours 157
Figure 5.16 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Bisphenols (BPA and M-BPA) for 24 Hours 159
Figure 5.17 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Phthalates (DBP, DEHP and BBP) for 24 Hours 160
Figure 5.18 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Phenolic Chemicals (4-BP and 4-NP) for 24 Hours 161
Figure 5.19 Roach Estrogen Receptor alpha (ERα) Transactivation Assay 163
Figure 5.20 Roach Androgen Receptor Beta (ARβ) Transactivation Assay 164
Figure 5.21 Agonistic ER Transactivtion Concentration-Response in the HeLa9903 Stably Transfected Cell Line and Hek293 Cells Transiently Transfected with *Rutilus rutilus* (common roach) ERα 166
Figure 5.22 Agonistic AR Transactivation Concentration-Response in HeLa4-11 Cells Consequent to 24 Hour Chemical Exposure 168
Figure 5.23 Antagonistic AR Transactivation Concentration Responses in HeLa4-11 Cells Consequent to 24 Hour Chemical Exposure 168

LIST OF TABLES

Table 1.1 Summary of Thesis Aims and Objectives 10
Table 2.1 Classes of Hormone..... 13
Table 2.2 Scientific Definitions of EDCs Adopted by Regulatory and Advisory Jurisdictions 29
Table 2.3 OECD ER Binding QSAR Constraints 39
Table 3.1 Nuclear Receptor Domains..... 46
Table 4.1 Software and Programs for *in silico* Approaches 63
Table 4.2 ER Sequence Accession Numbers, Latin Taxonomy and Common Name ... 64
Table 4.3 AR Sequence Accession Numbers, Latin Taxonomy and Common Name ... 65
Table 4.4 Protein Database NR X-ray Crystallography Accession Information..... 68
Table 4.5 Surflex-Dock Score Variation using Default Minimisation for Ligand Database 71
Table 4.6 *In silico* Nuclear Receptor Natural and Synthetic Hormone Binding Results 86
Table 4.7 *In silico* Nuclear Receptor Fungicide Binding Screening Results 88
Table 4.8 *In silico* Nuclear Receptor Herbicide Binding Results..... 89
Table 4.9 *In silico* Nuclear Receptor Organophosphate Insecicide Binding Results..... 91
Table 4.10 *In silico* Nuclear Receptor Organochloride Insecticide Binding Results..... 92
Table 4.11 *In silico* Nuclear Receptor Pyrethrin & Pyrethroid Insecticide Binding Results..... 92
Table 4.12 *In silico* Nuclear Receptor Phenolic Industrial Chemical Binding Results.. 93
Table 4.13 *In silico* Nuclear Receptor HPV Chemical Binding Results..... 94
Table 4.14 *In silico* Nuclear Receptor Consumer Product Binding Results 96
Table 4.15 SYBYL Surflex-Dock Virtual Screening Sensitivity and Summary Data. 102
Table 5.1 Natural Endogenous Hormones for *in vitro* Assessment 115
Table 5.2 Synthetic Xenobiotic Hormones for *in vitro* Assessment 116
Table 5.3 Environmental Contaminants identified for *in vitro* Assessment..... 117
Table 5.4 HPV Industrial Chemicals for *in vitro* Assessment..... 118
Table 5.5 Test Substance Identifiers and Stock Solution Molarity 119
Table 5.6 Technical Apparatus and Plasticware Suppliers..... 120
Table 5.7 Laboratory Equipment Required for *in vitro* Methods..... 121
Table 5.8 *In vitro* Cell Culture and Assay Material Suppliers 121
Table 5.9 HeLa9903 and HeLa4-11 Cell Line Maximum Non-cytotoxic Test Substance Concentrations Identified in PrestoBlue® Cell Viability Assays..... 135
Table 5.10 Results of HeLa9903 Stably Transfected ER Transactivation Assay to Detect Agonism of Estrogenic Signalling 143
Table 5.11 Results of HeLa4-11 Stably Transfected AR Transactivation Assay to Detect Agonism of Androgenic Signalling 153
Table 5.12 Results of HeLa4-11 Stably Transfected AR Transactivation Assay to Detect Antagonism of Androgenic Signalling 162

Table 5.13 Summary of Stably Transfected Transactivation Assay Results for ER Agonism (HeLa9903) and AR Agonism and AR Antagonism (HeLa4-11)..... 170
Table 6.1 Concordance of *in silico* human ERα and AR binding scores (SYBYL®) and *in vitro* transactivation assays 178

LIST OF ABBREVIATIONS

α-H	α-Helices
Å	Angstrom
AD	Activation Domain
ADME	Adsorption, Distribution, Metabolism & Excretion
AF	Activation Function
AhR	Aryl Hydrocarbon Receptor
Ala	Alanine (A)
AOP	Adverse Outcome Pathway
AR	Androgen Receptor
Arg	Arginine (R)
Asp	Aspartic acid (D)
BLAST	Basic Local Alignment Search Tool
BLOSUM	BLOcks of amino acid SUbstitution Matrix
BPA	Bisphenol A
CAS	Chemical Abstracts Service
CERI	Chemicals Evaluation and Research Institute (Japan)
CI	Confidence Interval
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DfW	Derek for Windows
DNA	Deoxyribonucleic acid
EAS	Endocrine Active Substance
EC	European Commission
ECVAM	European Centre for Validation of Alternative Methods
ED	Endocrine Disruptor
EDC	Endocrine Disrupting Chemical
EDSTAC	Endocrine Disruptor Screening & Testing Advisory Committee
EDTA	Endocrine Disrupters Testing and Assessment
EFSA	European Food Safety Authority
ER	Estrogen Receptor
EU	European Union
FUB	False Unfavourable Binder
GC/MS	Gas Chromatography Mass Spectometry
GD	Guidance Document
GFP	Green Fluorescent Protein
Glu	Glutamine (E)
Gly	Glycine (G)
GRIP1	Glucocorticoid receptor-interacting protein 1
His	Histidine (H)
HPG	Hypothalamic-Pituitary-Gonadal (HPG axis)
HPV	High Production Volume

HRE	Hormone Response Element
Hsp	Heat-shock protein
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
Ile	Isoleucine (I)
LBD	Ligand Binding Domain
LBP	Ligand Binding Pocket
Leu	Leucine (L)
MEGA	Molecular Evolutionary Genetic Analysis
MoA	Mechanism of Action
MoS	Margin of Safety
mRNA	Messenger Ribonucleic acid
MUSCLE	Multiple Sequence Comparison by Log Expectation
MW	Molecular Weight
NCoA-2	Nuclear Receptor Coactivator 2
NCoR	Nuclear Corepressor
NIBB	National Institute of Basic Biology
NINS	National Institute of Natural Sciences
N_{is}	Negative *in silico*
NOAEL	No Observed Adverse Effect Level
NR	Nuclear Receptor
NTD	N-Terminal Domain
NTP	National Toxicology Programme
OECD	Organisation for Economic Cooperation and Development
PBTG	Performance Based Test Guideline
PC	Positive Control
PCP	Personal Care Product
PDB	Protein Database
Phe	Phenylalanine (F)
P_{is}	Positive *in silico*
PPAR	Peroxisome Proliferator Activated Receptor
PR	Progesterone Receptor
PXR	Pregnane-X-Receptor
qHTS	Quantitative High-Throughput Screening
QSAR	Quantitative Structure Activity Relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic acid
SAICM	Strategic Approach to International Chemicals Management
SD	Standard Deviation
SEM	Standard Error of the Mean
SRC	Steroid Receptor Coactivator
STTA	Stably Transfected Transactivation Assay
TA	Transactivation Assay
TDI	Tolerable Daily Intake
TG	Test Guideline
TGCC	Testicular Germ Cell Cancer
TIF2	Transcritptional Intermediary Factor 2

TNR	True Negative Rate
TP	True Positive
TPR	True Positive Rate
TR	Thyroid Receptor
TS	Test Substance
TSD	Temperature-dependant Sex Determination
TTTA	Transient Transfected Transactivation Assay
UN	United Nations
UPGMA	Unweighted Pair Group Method with Arithmetic
VC	Vehicle Control
WHO	World Health Organisation
WoE	Weight of Evidence
XDS	Xenobiotic Detection Systems

1 INTRODUCTION

1.1 The Scientific Context

The use and manufacture of chemicals is fundamental to the western mode of living and economy, subsequently synthetic chemicals are ubiquitous to the modern environment (OECD Observer, 2010a). Annually, the chemical industry turnover is €1.8 trillion; accounting for 7% of global trade (OECD Observer, 2010b). In October 2014, over 90 million unique organic and inorganic chemicals were CAS registered, of which only 311,000 were subject to inventory or regulation (CHEMLIST®)[1]. However, chemicals are not restricted by national borders and the reporting of bioaccumulation of lipophilic synthetic chemicals in the polar regions, due to aerial and aquatic migratory movements, emphasises the potential global environmental threat of the chemical industry (Czub *et al.* 2008).

Traces of synthetic chemicals are found ubiquitously in the environment. An estimated 50,000 chemicals are thought to prevail in UK surface waters (Matthiessen and Johnson, 2007), contaminating wildlife habitats and potable water sources. The ubiquity of human exposure to chemicals, via food, water, consumer products and household agents, has led to growing concern regarding the potential health threats. Biomonitoring studies have added to concerns by demonstrating the absorbance and accumulation of anthropogenic chemicals in human tissues. The High Production Volume (HPV) plasticiser Bisphenol A (BPA), has been detected in human autopsy adipose (3.78 ng/g), liver (1.48 ng/g) and brain (0.91 ng/g) tissues by gas chromatography mass spectrometry (GC/MS) (Geens *et al*., 2012). In addition, polybrominated biphenyl ether (PBDE) flame retardants have been detected in the hair of newborns (0.048-1.01 pg/mg) and children (0.208-2.695 ng/mg) by GC/MS (Aleska *et al*., 2013).

Worldwide production of the organochlorine pesticide, Dichlorodiphenyltrichloroethane (DDT), exceeded 40,000 tonnes per annum for thirty years (1950-1980) (Geisz *et al*., 2008). Despite the worldwide ban of DDT for agricultural purposes (1972-2004[2]), it's metabolite, Dichlorodiphenyldichloroethylene (4,4'-DDE), can still be detected in human serum and adipose tissues (Ozen *et al.*, 2012), highlighting the long-term consequences of lacklustre chemical regulation. Furthermore, significant differences in the concentration of environmental pollutants (Cd, Ni, PCB-153 and 4,4'-DDE) detected in blood suggest regional differences in exposure and pollutant body burden

[1] http://www.cas.org/content/regulated-chemicals
[2] Restricted worldwide under the Stockholm Convention

(Foster *et al.*, 2012). These regional differences have been supported by Braun *et al.'s* (2012) study, which demonstrated regional differences in urinary BPA concentration, with urban and rural Egyptian locations having levels of 1.0 and 0.6 ng/ml, respectively. Age matched American girls participating in the NHANES (US National Health and nutrition Examination Survey) typically presented higher BPA concentrations 2.60ng/ml.

The sewage sludge contaminants Nonylphenol (50-1070 μg NP kg^{-1}), Di-(2-ethylhexyl) phthalate (30-4920 μg DEHP kg^{-1}), Polychlorinated biphenyl (0.007–89.19 μg ΣPCB kg^{-1}), PBDE (0.07-24.91 μg ΣPBDE kg^{-1}) and Benzo(a)anthracene (<1.0-235 μg BaP kg^{-1}) have been detected in livestock tissue (Rhind *et al*., 2011). Bellingham *et al*. (2012) reported a significant ($p<0.001$) association between spermatogenic abnormalities and pastoral sewage sludge exposure[3] in sheep; *in utero* and postnatal exposure reduced germ cell numbers per testis, impairing sperm production. Subsequently, the wider consequences of exposure to anthropogenic chemicals for the environment have become of paramount concern, and adverse effects observed in exposed wildlife have added weight to human health concerns.

1.1.1 A Human Health Concern?

In 1992, Carlsen *et al*. suggested a global 0.8% annual decline in sperm count (1938-1990), reporting a reduction from 113 x 10^6/ml to 66 x 10^6/ml over 50 years. Sperm concentrations below 48 x 10^6/ml may hinder the potential fertility of males (Guzick *et al*., 2001), although lower thresholds have also been proposed (40 x 10^6/ml by Bonde *et al*., 1998; 20 x 10^6/ml by Paasch *et al*., 2008). Founding biological plausibility in the testicular dysgenesis syndrome (Skakkebaek, 2001), a number of epidemiology studies have drawn associations between declines in male reproductive health and exposure to anthropogenic chemicals (see Bergman *et al*., 2012).

Andersen *et al*., (2012) demonstrated a link between maternal occupational pesticide exposure and impaired reproductive function; a significant association between *in utero* pesticide exposure and male offspring reproductive tract abnormalities (cryptorchidism and hypospadias) ($p=0.047$). Abnormalities observed in the male offspring cohort persisted into adolescence (6-11 years of age), and re-examination by Wohlfarh-Veje *et al.* (2012a) showed significantly reduced testicular volume ($p=0.05$), 24% smaller testes and 9.4% shorter penile length. Furthermore, a relationship between cryptorchidism incidence and PDBE exposure has been reported (Krysiak-Baltyn *et al*., 2012) and increased DEHP concentrations have been associated with male infertility and altered sex steroid hormone circulation (Mendiola *et al*., 2012).

[3] Application of 2.25 tonnes dry matter per hectar twice per year to pastoral land

In 2010, 48.5 million couples sought *in vitro* fertilisation (IVF) treatment (ART, 2011) and 2% of UK births were due to IVF (HFEA, 2011). Ehrlich *et al.* (2012) identified a statistically significant positive dose-response relationship between urinary BPA concentration and IVF implantation failure (p=0.06). Epidemiology and toxicology studies have identified a plethora of chemicals that may interfere with reproductive health. 4,4'-DDE has been associated with increased incidence of spontaneous abortion, foetal loss and preterm birth (Longnecker *et al*., 2005; Weselak *et al*., 2008). Furthermore, perturbation of the sex ratio was observed in Seveso (Italy), subsequent to the accidental release of dioxins in 1976. A significant skew in the sex ratio (48 females to 26 males) was reported, which Mocarelli *et al*. (1996) attributed to maternal and paternal dioxin exposure. Thus, chemical exposure may have long-term effects on reproductive health and population fecundity.

Testicular Germ Cell Tumour (TGCT) incidence is increasing in Caucasian populations, rendering it the most commonly diagnosed malignant neoplasm in men aged 15-35 years of age (Chia *et al*., 2010). Multifactorial in aetiology, the development of TGCT is linked to insufficient androgen action *in utero* (Rajpert-de Meyts, 2006). A number of epidemiological studies have reported positive associations between anthropogenic chemicals and TGCT (Hardell *et al*., 2003; Hardell *et al*., 2004). Chlordanes, 4,4'-DDE and some PCB congeners are believed to antagonise the androgen receptor, dampening androgen responses (Kojima *et al*., 2004; Vinggaard *et al*., 2008).

Exogenous chemicals capable of perturbing endogenous endocrine function have been termed endocrine disrupting chemicals (EDCs). The endocrine system encompasses a number of pathways and chemical cues (hormones) that control developmental events such as proliferation, growth, differentiation (histogenesis and organogenesis) and regulatory processes including metabolism, homeostasis, respiration, excretion, movement, reproduction and sensory perception. Subsequently, any substance capable of interfering with the synthesis, secretion, transport, binding, action or elimination of endogenous hormones, may be an endocrine disruptor. Human health end-points associated with endocrine disruption include, reproductive and developmental toxicity; teratogenicity; carcinogenicity; neurotoxicity; and, metabolic syndrome (Holt and Hanley, 2006).

1.1.2 Mechanism of Action

The mechanistic pathways underlying EDC activities encompass a plethora of biological receptors, such as: (1) nuclear hormone receptors; (2) non-nuclear steroid hormone receptors; (3) non-steroid receptors; and, (4) orphan receptors, such as the Aryl Hydrocarbon Receptor (AhR) (Filby *et al*., 2006). Hormones and their respective agonists (chemicals that mimic the action of endogenous hormones causing a cellular response) and antagonists (chemicals that can bind, block or dampen agonist mediated responses) interact with these receptors to initialise (or block) signal transduction, gene

transcription and mRNA translation, which ultimately leads to a cellular response. It is the ratio and interplay of all of these pathways that leads to a *'normal'* homeostatic balance (Diamanti-Kandarakis *et al*., 2009).

Binding interactions of EDCs can be important in elucidating the biological mechanisms by which toxicological endpoints are elicited. For example, an estrogenic phenotype may be the result of agonistic activity at the estrogen receptor (ER) or by competitively binding to the androgen receptor (AR), thereby blocking the action of endogenous androgens, creating an estrogenic environment (Sohoni and Sumpter, 1998). Furthermore, a substance may mediate phenotypic effects by multiple pathways; Prochloraz and Linuron lead to an antiandrogenic phenotype, by both antagonising the androgen receptor and by inhibiting the biosynthesis of testosterone (Hotchkiss *et al*., 2008).

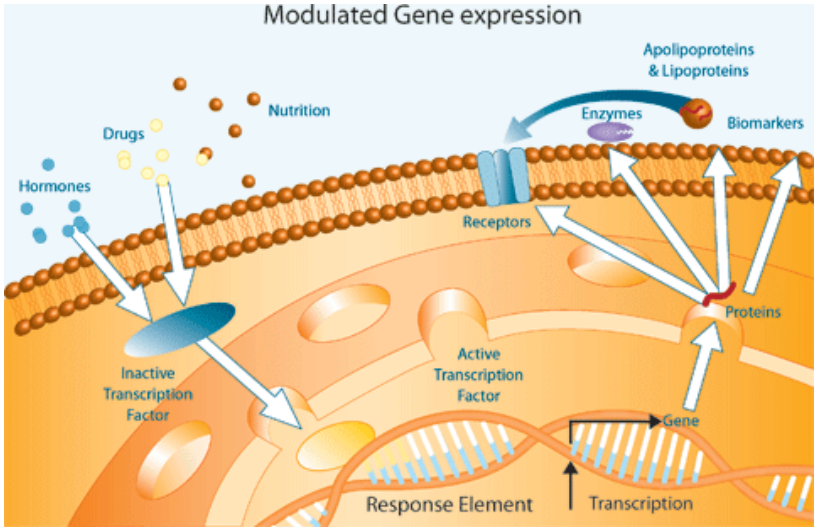


Figure 1.1 Ligand-Dependent Transactivation of Nuclear Receptors

Hormones and their respective agonists (chemicals that mimic the action of endogenous hormones causing a cellular response) and antagonists (chemicals that can bind, block or dampen agonist mediated responses) interact with nuclear receptors (ligand-dependent transcription factors) to initialise (or block) signal transduction, gene transcription and mRNA translation, which lead to cellular response

The endocrine system is highly conserved throughout vertebrates, which mirrors and justifies the inter-species extrapolations fundamental to conventional toxicology. However, extrapolating *in vivo* endpoints in one species to another is not without its caveats. Differences in the ontogeny of reproductive functions and structures; in the metabolism of xenobiotics and endogenous sex steroids; and, in variable body burdens, can lead to significant interspecies differences. Assessment of the efficacy of the OECD two-year carcinogenicity test guideline, suggested less than 70% concordance between rats and mice (Omenn, 2001). Considering phylogenetic distances, human to rodent tumour induction concordance may be even lower. Consequently, lifetime rodent bioassays, commonly utilised in chronic toxicity testing, may not always correctly predict the risk to humans and other species (Selkirk *et al*., 2005).

Potential inter- and intra-species variance in biological machinery complicates regulatory risk assessment (Hartig *et al*., 2007; 2008). Species differences in nuclear receptor structure, functionality and xenobiotic binding affinity have been reported. Variations in toxic effect as a consequence of genetic polymorphisms and endocrine receptor isoforms have also been observed. Västermark *et al*. (2011) identified an association between polymorphic variation in the human androgen receptor gene and Testicular Germ Cell Cancer (TGCC) (Odds Ratio 2.07; CI 95% 1.03 – 4.15). The authors suggest that minor differences in ligand binding domain may confer elevated susceptibility to the effect of endocrine disruptors, leading to androgen insensitivity. Comparative analysis of the agonistic activity of pesticides in human and mouse pregnane X receptors (PXR)[4] *in silico*, demonstrated that a wide range of pesticides possess PXR mediated transcriptional activity in both humans and mice. Furthermore, pesticide binding to the PXR receptor was shown to be species dependent (Kojima *et al*., 2011).

Ishiniwa *et al*. (2010) sequenced the homology of the AhR gene in Japanese field mice (*Apodemus speciosus*) identifying 49 functional alleles expressing phenotypic variance. The authors suggest that the AhR polymorphisms detected may alter an individual's susceptibility to disruption by agonists and antagonists of the AhR, such as the common chemical by-product, dioxin. Furthermore, functional variation in mouse and human AhR unliganded cytoplasm-nucleus shuttling and chaperone proteins, may alter ligand binding potential; C57BL/6 mice transfected with human AhR showed lower induction of cytochromes 1A1 and 1B1, than wildtype models (Bergman *et al*., 2011), highlighting the uncertainty in extrapolating toxicological findings within species to another. This provides further evidence to suggest that rodent species, such as rats and mice, may not always be good predictive models of toxic effects in humans, due to significant variations in the receptor binding that mediates cellular response, and subsequent toxicity (Kojima *et al*., 2011).

Endocrine receptor polymorphisms have also been identified in ecological and wildlife models; Wells and Van Der Kraak (2000) demonstrated varying binding affinities of several EDCs to rainbow trout and goldfish androgen receptors (ARs), emphasising the need for multiple models in chemical risk assessment, while Wilson *et al*. (2007) identified similar species variance in the fathead minnow, rainbow trout and human ARs. Eco-epidemiological and toxicological studies in birds have found similar variances in the development of toxic endpoints within the genus and throughout the Aves (birds) class. Dietary exposure to DDE resulted in eggshell thinning in some avian species, including the peregrine falcon (*Falco peregrines*), sparrow hawk (*Accipiter nisus*) and golden eagle (*Aguila chrysaetos*). However, laboratory studies demonstrated that susceptibility to 2,4'-DDE toxicity, and the manifestation of endocrine endpoints, was dependent on the avian species considered (Vos *et al*., 2000).

[4] Nuclear receptor involved in the metabolism of xenobiotics.

It is thus clear, that as a relatively new mode of toxicity, the basic biology and mechanisms of endocrine disruption are still to be elucidated. Furthermore, if toxicity in one species is not necessarily predictive of toxicity in another, the efficacy of extrapolations integral to toxicological study and regulatory risk assessment may be compromised.

1.2 A Regulatory Conundrum

The uncertainty in endocrine disruptor science has been exploited by the media to generate headlines such as "Poison" (Girling, 2004), "Ban gender bender used in baby bottles" (Derbyshire, 2010a) and "Babies in womb exposed to 'Gender-bending' chemicals" (Cook, 2006). Public perception of risk is influenced by the media and scientific uncertainty (Chryssochoidis *et al.*, 2009). This uncertainty is mirrored and enhanced by the varied regulatory stances of European Union (EU) jurisdictions; BPA is banned in some US states and Denmark, but remains legal in other EU member states, such as the UK. Consequently, endocrine disruption has become a field of hot public, regulatory and scientific debate.

Between 1997 and 2007 it was estimated that global spending on endocrine research exceeded $100 million. A figure that has undoubtedly increased in light of the European Commission's (EC) strategy under the Fifth Framework Programme, funding over €60 million on 23 projects under the auspices of CREDO (Cluster of Research into Endocrine Disruption in Europe) and €50 million under the Sixth Research Framework Programme. Thus, endocrine disruption presents a cost burden at both national and international levels.

The EC has defined EDCs as "*exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub) populations*" (EC, 1998; ECETOC, 2009). However, there is currently no globally accepted regulatory definition of an EDC between jurisdictions, consequent to epistemological problems, not only in the science, but regarding the regulatory and economic implications of a stringent definition. Furthermore, a lack of coherence in acceptance criteria and terminology has curtailed the distinction between an endocrine active substance (EAS) and an endocrine disrupter (ED). Nuclear receptor binding or '*endocrine activity'*, is not necessarily indicative of endocrine disruption, presenting no hazard in itself, but rather the mechanism to a hazard.

1.2.1 Chemical Safety Testing Strategies

The focus on apical endpoints utilised by *in vivo* studies limits their ability to elucidate specific biological mechanisms. Subsequently, there is debate regarding the efficacy of animal models in accurately predicting possible human health and ecological impacts. However, irrespective of the legitimacy of extrapolating animal data to predict interspecies health risks, these studies remain the focus of regulatory safety assessment.

Understanding the uncertainty and applicability of testing methods is essential in effectively characterising and communicating risks.

Contrary to a conventional toxicological study, which generally extrapolates high dose toxicity to low dose exposure, it is generally accepted that endocrine disruption is a mechanism that may result in a hazard, rather than being a hazard itself, and subsequently may not conform to archetype dose-response relationships; demonstrating low-dose toxicity or hormesis (EC, 1999). Non-monotonic relationships have been observed for a number of EDCs; induction of metabolising enzymes or conjugation substances may result in U-shaped dose responses. Effects at low and at high levels of exposure, and diminished or non-existent toxicity at intermediate exposure levels due to increased metabolic breakdown or elimination, further complicates testing strategies and regulatory risk assessment, which balances chemical safety with economic feasibility.

Furthermore, due to cost and time restraints, only a limited number of chemicals are investigated in chronic two-year toxicity studies, such as those utilised for the assessment of carcinogenicity or reproductive and developmental toxicity (e.g. only if production exceeds 1000t/year) (Borlak, 2006). Consequently, there is uncertainty in the risks of chronic exposure to most chemicals.

Low tier testing is deliberately over-responsive, detecting chemicals with the capacity to interact with biological receptors without necessarily inducing adverse endpoints, in order to minimise the risk that EDC's will go undetected; i.e. presenting a low false negative and high false positive rate. It is unlikely that *in vitro* and *in vivo* assays will supersede *in vivo* tests in a weight-of-evidence (WoE) assessment, due to limited pharmacokinetic and pharmacodynamics parameters (e.g ADME). However, a two-year carcinogenicity study uses approximately 800 animals per species (N3CRs, 2014; Long, 2007) and costs in excess of $1 million (~£601,214) (Schmidt, 2006). Thus, utilising endocrine-relevant *in vivo* test guidelines for the screening and prioritisation of chemicals on the market (>12,399 unique registered substances[5] in excess of 1 tonne/year), is not feasible. Furthermore, a positive bioassay result is not necessarily indicative of a positive result in an intact organism, and the European Food Safety Authority (EFSA) stress that all evidence should be assessed in a WoE approach (EFSA, 2010).

Aiming to develop alternative non-animal approaches to toxicological study, the 2010 European Partnership for Alternative Approaches to Animal Testing (EPAA) '*Harnessing the Chemistry of Life: Revolutionising Toxicology*' workshop, gathered international experts in chemistry, systems biology and toxicology to discuss the challenges and potential solutions these sciences may offer. Assurance of *in silico* and

[5] As of the 19th March 2014, the European Chemical Agency (ECHA) REACH database contained 12,399 unique substances, from 47,731 dossiers (http://echa.europa.eu/information-on-chemicals/registered-substances [Accessed 31/03/2014 12.10am]).

in vitro predictions, in lieu of chronic animal studies, was highlighted as a significant challenge to future work (Kimber *et al*., 2011). Furthermore, the necessity of incorporating metabolism, toxicodynamics and toxicokinetics into non-animal models was stressed.

An ideal screening tool should be rapid, cost effective and reflective of multiple mechanisms of concern (Ankley *et al*., 1998). Many *in vitro* screening programs have been developed to identify potential EDCs and assess both ecological and human health risk, by assessing competitive binding of xenobiotics to receptors (Van Der Kraak, 2000). More recently, alternative *in silico* bioinformatics approaches have been suggested to predict mechanisms and prioritise chemicals for *in vitro* and *in vivo* screening (Bohl *et al.*, 2007; Schilter *et al.*, 2014).

1.3 The EDC Problem

Chemicals are detected ubiquitously in the environment, wildlife and humans. Chemical exposure, at environmentally relevant concentrations, has been linked to declines in wildlife ecology. Furthermore, the breadth of research has started to bridge the gap between effects observed in wildlife and human epidemiological speculation (Bergman *et al.*, 2012).

In the EU, the human health costs of EDC exposure has been estimated at €31 billion per annum (HEAL, 2014). In crude calculations by economists, Hunt and Ferguson, evaluated the monetary cost of: reproductive disorders; fertility problems; cryptorchidism; hypospadias; cancer of the breast; prostate and testes; behavioural disorders, such as autism and attention deficit hyperactivity disorder (ADHD); and, metabolic disorders such as obesity and diabetes (HEAL, 2014). Of the €636-637 bn/y cost identified, 2-5% was attributed to EDC exposure. The estimation is biased and does not account for potential health costs incurred by banning chemicals; via reduction in cleanliness, pest control and economic factors, which can also negatively impact health quality. Nevertheless, the calculation emphasises the potential health cost of inadequate chemical regulation.

EDC's are restricted by EU law, but without specific scientific criteria to enable adequate regulation, abidance to the legislation is questionable. The EC missed the 2013 deadline for defining an EDC for regulatory purposes, undoubtedly due to the implications of a stringent definition. For example, the EC definition necessitates "*adverse health effects in an intact organism…*" Adopting this definition would mean that lower tier testing, including some *in vivo* bioassays, *in vitro* assays and *in silico* and QSAR predictions, would not be sufficient to regulate chemicals, requiring a tier 5 *in vivo* test (~2 years). This reliance on *in vivo* toxicology studies conflicts with national (UK/EU) and international (OECD) obligations to reduce scientific procedures on animals. The economic, ethical and time infeasibility of testing 311,000 chemicals *in vivo*, demands the development of alternative methods (*in silico* and *in vitro*) to

elucidate endocrine mechanisms of action. Data gaps currently add significant uncertainty to the applicability of mechanistic studies in regulatory risk assessment.

1.4 Research Aims and Objectives

To the author's knowledge, no single study has quantitatively assessed the relationship between *in silico* nuclear receptor binding and *in vitro* mechanistic assays, in a comprehensive manner. This study aims to evaluate current *in silico* and *in vitro* tools for EDC screening and hazard characterisation; testing the hypothesis that *in silico* virtual screening accurately predicts *in vitro* mechanistic assays, which are more adept to elucidate endocrine mechanism of action (MoA) than traditional *in vivo* toxicity tests. Detailed review of EDC literature aims to identify caveats and considerations, knowledge of which may reduce the uncertainty of *in silico* and *in vitro* analyses. The historical context and molecular mechanisms underpinning endocrine disruptor science are elucidated, assessing their relevance to chemical screening and regulation.

Nuclear receptor (NR) agonism and antagonism is a biological mechanism by which adverse endocrine endpoints may be incurred. Binding interactions of NRs are currently the focus of *in vitro* and *in silico* tools used to predict EDC mechanisms. As previously alluded to, it is not feasible to assess '*all*' potential effects putatively ascribed to endocrine active substances. Consequently, *in silico* and *in vitro* approaches were prioritised based on their regulatory acceptance, biological plausibility and availability.

The purpose of this thesis is not to provide a detailed review of all MoA and test guidelines associated with EDCs, but to evaluate the current state of the science regarding adoption of *in silico* and *in vitro* tools, to characterise EDC hazards for regulatory purposes. Consequently, it is anticipated that the methods adopted herein will reflect the current assumptions regarding EDC mechanisms. The aims and objectives of this study are summarised in Table 1.

Computational chemistry can characterise the dynamics, energetics and structure of ligand-receptor interactions (Taft *et al*., 2007). Thus, computational chemistry presents a solid foundation for *in silico* toxicological study, particularly with regard to receptor-mediated activity. It is anticipated that *in silico* molecular modelling of nuclear receptors will generate computational 3D-representations of nuclear receptor binding domains. Aiming to evaluate the possibility of adopting *in silico* molecular modelling to EDC hazard characterisation, modelled nuclear receptors were virtually screened against a chemical database of potential EDC's. Curated on the basis of regulatory concern, exposure, structural variability and assumed functionality, the chemical database aims to include a diverse array of potential-EDCs, for virtual screening. The sensitivity of *in silico* molecular modelling for EDC hazard characterisation will be assessed relative to published *in vitro* bioassay results.

A number of *in vitro* studies have shown that EDC binding affinity is species specific (Lange *et al.*, 2012; Wilson *et al.*, 2007). Interspecies variation in NR homology and binding affinity has also been reported *in silico* (Wu *et al.*, 2010; Kojima *et al.*, 2011). Phylogenetic analysis of nuclear receptor sequence homology aims to identify species differences at the most basic biological level. The harvesting of nuclear receptor (ER, AR, etc.) sequences published on databanks[6], using DNA base-pair homology search terms (BLAST), will be used to phylogenetically map evolutionary changes in receptor sequence, which may impede comparisons between species. However, receptor sequence variance is not necessarily representative of receptor function variance; many amino acid substitutions, deletions or insertions may not affect the binding interactions. Thus, the aforementioned objective of phylogenetic analysis is inadequate to determine functional differences in receptor binding.

Table 1.1 Summary of Thesis Aims and Objectives

As a whole, this study aims to evaluate the '*state-of-EDC-science'* and currently available *in silico* and *in vitro* tools for EDC screening and hazard characteriation. Contributory aims are identified on the left side in bold, while objectives are stated on the right.

Aim	Objectives
Evaluate the literature pertaining to the historical context, biological mechanisms and regulatory confounders of endocrine disrupter science.	- Review the basic biology of endocrine disruption - Review the epidemiological and toxicological evidence - Review the regulatory drivers of EDC initiatives - Detail nuclear receptor interactions and functioning - Identify caveats of alternative toxicological approaches
Evaluate *in silico* tools for EDC hazard characterisation	- Literature review of *in silico* theory - Review available methods - Create chemical database of potential EDCs - Assess sequence phylogeny of nuclear receptors - Model nuclear receptors using *in silico* software - Screen chemical database against *in silico* receptors - Assess sensitivity of molecular methods
Evaluate *in vitro* tools for EDC hazard characterisation	- Literature review of *in vitro* theory - Review available methods - Prioritise test substances for *in vitro* assessment - Test compounds using identified *in vitro* tools
Assess applicability of *in silico* and *in vitro* tools for EDC hazard characterisation	- Assess the contribution of *in silico* and *in vitro* data in hazard profiles (case-studies) - Test the null hypothesis that *in silico* tools accurately predict *in vitro* mechanistic assays, to elucidate endocrine MoA.
Identify areas for further research	- Identify data gaps in EDC science and testing strategies

NR structures from different species shall be virtually screened against the compound database, potentially enabling *in silico* predictions of interspecies differences in EDC binding. However, it should be noted that, the objectives, of phylogenetic modelling and molecular modelling, are inherently caveated by the availability of NR sequence and structure information, potentially limiting the evaluation.

[6] UniProt (http://www.uniprot.org/uniprot/)

Aiming to evaluate the current *in vitro* tools for endocrine modes-of-action, it is anticipated that potential-EDCs, prioritised on the basis of *in silico* screening results and *in vitro* assay requirements, will be tested in standardised *in vitro* mechanistic assays. *In vitro* transactivation assays are significantly cheaper (~£1,500–£2,000 per chemical) than *in vivo* exploration (OECD, 2012). However, the cost renders testing '*all'* chemicals *in vitro* infeasible – cheaper, high-throughput virtual screening, as suggested herein, is thus required. Virtual screening has been suggested as a tier 1 regulatory test (OECD conceptual framework level 1), driving the development of molecular methods (Worth *et al*., 2014; Kojima *et al.* 2011; Cronin & Worth, 2008).

The *in vitro* methods aim to provide mechanistic and potency information for EDC hazard characterisation. Recently adopted by the OECD, *in vitro* estrogen receptor agonism transactivation assays, in addition to more novel *in vitro* methods, will be used to assess the MoA of potential EDCs. The *in silico* and *in vitro* results will be compared to the literature and *in vivo* evidence, to characterise the hazard of potential EDCs, and assess more formal adoption of *in silico* tools.

In summary, merging the interfaces of bioinformatics, computational chemistry, endocrinology, *in vitro* cell culture and toxicology, this study aims test the null hypothesis that *in silico* and *in vitro* mechanistic tools, are more adept to elucidate endocrine MoA, than traditional *in vivo* toxicity tests. The sensitivity of adopted methods, relative to published bioassay results and OECD validated *in vitro* assays, will determine the feasibility of incorporating the novel *in silico* and *in vitro* tools into regulatory screening and prioritisation. It is not expected that the methods detailed herein, will conclusively predict endocrine disruption, but it is anticipated that the *in silico* and *in vitro* screens may predict endocrine activity, to prioritise chemicals for more conclusive higher tier testing.

2 BACKGROUND INFORMATION

2.1 The '*Start*' of the Science

2.1.1 Endocrinology

'*Hormone*' is derived from the Greek word '*hormaein*' meaning to '*arouse*' or '*excite*', and was first coined by Sir Ernest Starling in 1905. Endocrinology is the study of the endocrine system, its diseases, and the biosynthesis and secretion of hormones that modulate development (histogenesis and organogenesis), metabolism, respiration, excretion, movement, reproduction and sensory perception. Co-ordinating internal physiology, the endocrine system regulates development and homeostasis, enabling adaptation to a milieu of nutritional and environmental changes (Figure 2.1). The hormones and receptors that compile the endocrine system are ubiquitous to all vertebrates and some invertebrates (WHO, 2002). Hormones bind to specific receptors, either on the surface or within the target cells, to initiate a cascade of intracellular reactions, which amplifies the original stimulus and generates a cellular response (Brook and Marshall, 2005).

Table 2.1 Classes of Hormone
Based on their chemical composition, Griffin and Ojeda (1996) identified three classes of hormone: amines, peptides and steroid hormones.

Amine	Amines are derived from single amino acids, such as tyrosine, which founds the formation of norepinephrine, epiphrine and dopamine. However, thyroid hormones such as 3,5,3'-triiodothyronine (T3) and 3,5,3',5'-tetraiodothyronine (thyroxine T4) make up a subset of this class because they derive from the combination of two iodinated tyrosine amino acid residues.
Peptide	Peptide hormones (or protein hormones), are endogenously manufactured chemicals consisting of three (in the case of thyrotropin-releasing hormone) to more than 200 (in the case of follicle-stimulating hormone) amino acid residues, and can subsequently have molecular weights as large as 30,000 kDa. All hormones secreted by the pituitary gland are peptide hormones, as are leptins from adipocytes, ghrelin from the stomach and insulin from the pancreas.
Steroid	Steroid hormones are derived from cholesterol and in mammalian systems can be classified into five groups by the receptor to which they bind (glucocorticoids, mineralocorticoids, androgens, oestrogens and progestagens). The steroid hormones are produced by steroidogenic enzymes in the adrenal gland, testis and ovary (Brook and Marshall, 2005).

There are two superfamilies of receptors: receptors for water soluble hormones such as insulin, which can only enter the cell via active transport; and, nuclear intracellular receptors that interact with the lipophilic steroid and thyroid hormones, which enter the cell passively. Located in either the cytosol or the nucleus of the target cell, the receptors mediate responses to three classes of hormone (Table 2.1). Hormones may be enzymically modified by their target cells (e.g. the deiodination of T4 to T3 by 5'-deiodinase) in order to bind and stimulate nuclear receptors. Cellular modification provides an important mechanism for the local regulation of hormone action at the target tissue. Defects in the components of these pathways, receptors or intracellular mediators can lead to an array of endocrinopathies (Brook and Marshall, 2005).

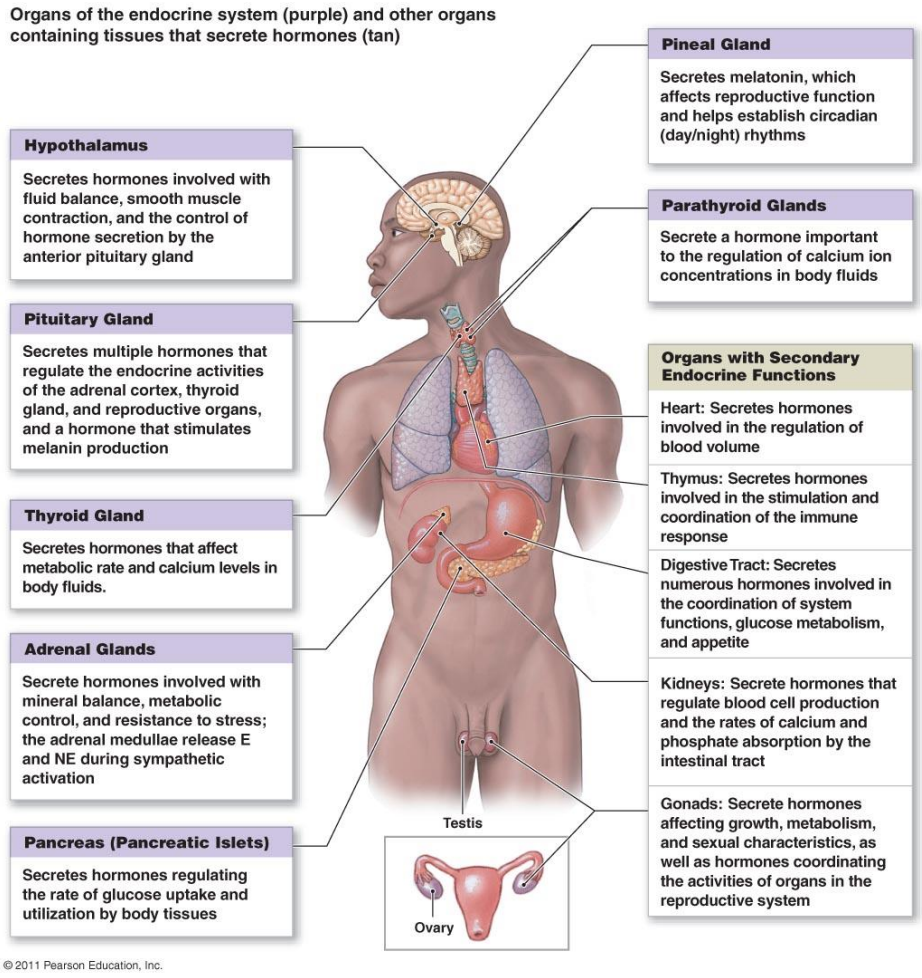


Figure 2.1 Organs and Glands Associated with the Human Endocrine System
Co-ordinating internal physiology, the endocrine system regulates development and homeostasis via a multitude of organs and glands, to enable adaptation to a milieu of nutritional and environmental changes. Diagram details the role of: hypothalamus; pituitary; thyroid; adrenal; pancreas; pineal; and parathyroid glands.

The endocrine system is generally regulated by negative feedback; the hormone stimulates a pathway and inhibits production of the initiating hormone. However, under more unusual circumstances hormone feedback can enhance, rather than inhibit, secretion of the initiating hormone - termed positive feedback. For example, estrogen induced ovulatory surges of luteinising hormone and follicle stimulating hormone during childbirth (Holt and Hanley, 2006).

Logistically, the structure of hormone-receptor complexes highlights the biological plausibility of endocrine perturbation; larger structurally complex hormones, such as insulin, may be harder for xenobiotics to mimic. Furthermore, water soluble xenobiotics may not be actively transported across the cell membrane, unless mistaken by channel ion proteins. Endocinology validates the primary focus of endocrine disruption on the steroid hormones (estrogens and androgens) and amines (thyroid hormones). However, endocrine disruptor science did not begin under the auspices and biological plausibility

of endocrinology, but rather through adverse effects observed in wildlife populations. Subsequently, the science has developed under the bias of observation, in which mechanisms were assumed by reported apical endpoints, in a top-down manner.

2.1.2 The Rise of Environmentalism

As detailed in the introduction (Section 1), anthropogenic activities have drastically altered the pattern of chemical exposure. Concerns over the repercussions of synthetic chemicals on wildlife were initially highlighted in 1962, by the publication of Rachel Carson's '*Silent Spring'*. The book, depicting a barren world resultant of the intensive use of organochlorine pesticides, prompted the then President of the USA J. F. Kennedy to order an examination into the misuse of pesticides (Lear, 1998). Adding evidence to Carson's concerns, in 1967 Ratcliffe reported that eggshell thickness in bird species indigenous to Great Britain, such as the peregrine falcon (*Falco peregrines*), sparrow hawk (*Accipiter nisus*) and golden eagle (*Aguila Chrysaetos*), had declined since 1946 - coinciding with the introduction of the pesticide DDT (Vos *et al*., 2000). Exposure to DDE, the degradation product of DDT, has since been demonstrated to also reduce eggshell thickness (Struger *et al*., 1985) and reduce fecundity, which nearly resulted in the extinction of several avian species in North America. DDT was banned in Western Europe and North America in the 1970s (Kime, 1998). Consequent to the ban of organochlorine pesticides and polychlorinated biphenyls (PCBs), notable increases in population number and eggshell thickness have been reported.

Exposure to Tributyltin (TBT), the active ingredient of antifouling paints used on ship hulls, has been shown to cause imposex in 150 species of marine snail (Matthiessen *et al*., 1995). High prevalence of intersex, a condition in which the sexual phenotype of molluscs is disturbed, was also reported in the German Wadden Sea periwinkle (*Littorina littorea*) following exposure to environmentally relevant concentrations of TBT (10 – 15 ng L^{-1}) (Bauer *et al.,* 1995; Bauer *et al*., 1997). The effects of TBT and Triphenyltin (TPT), on mollusc sexual development and mollusc populations, are considered one of the best documented incidences of ecologically relevant endocrine disruption to date. Matthiessen *et al*. (1995) demonstrated significant recovery in imposex-affected populations as a result of the restriction of TBT in 1987 and subsequent ban in 2000, highlighting the positive impact of good environmental regulation on biodiversity.

The adverse effects observed in birds and molluscs, as a result of exposure to DDT and TBT, respectively, have now been attributed to perturbation of the endocrine system. The subsequent section (2.1.3 Wildlife Effects) aims to highlight the ecological impacts of inadequate chemical governance; summarising the epidemiological and laboratory evidence for endocrine disruption in wildlife (by phylogenetic Class).

2.1.3 Wildlife Effects

2.1.3.1 Invertebrates

Comprising 95% of faunal species, with 30 phyla, invertebrates represent the most mechanistically diverse endocrine system (WHO, 2002; Ketata *et al*., 2008). However, vertebrate neurotransmitters, neurohormones and steroid hormones have also been detected in invertebrates (Lafont & Mathieu, 2007). In 1996 Bettin *et al.* reported that masculinisation of female gastropods was due to elevated Testosterone, consequent to inhibition of an aromatase enzyme that metabolises T to 17β-estradiol (E2). More recently, it has been shown that the accumulation of TBT in nerves and ganglia is associated with neurotoxicity which stimulates the production of Penis Morphogenic Factor (PMF), inducing the development of male secondary sex characteristics (Oberdörster and McClellan-Green, 2002). Testicular ooctyes and intersex have been reported in populations of lobsters (*Homarus americanus*) living near sewage outfalls (Sangalang & Jones, 1997). Sewage and industrial effluent discharged into rivers, a source of EDCs, has been demonstrated to affect numerous invertebrate species (Oetken *et al*., 2003; Tillmann *et al.,* 2001).

Terpenoid and ecdysone hormones play a vital role in the physiology, morphology and behaviour of invertebrates, generating a wide range of phenotypic variation. Oda *et al*. (2011) investigated antipredatory responses in cladiceran, *Daphnia galeata*, in response to Methyl fenosoatote (MF) (1.9 to 30 µg/L) and the juvenile hormone-mimicking pesticide, fenoxycarb (12 to 200 ng L^{-1}). Animals developed a longer helmet at doses of 1.9 µg L^{-1} and 25 ng L^{-1} fenoxycarb, in a dose dependent fashion, suggesting that MF affects allometrical growth, altering development of *Daphnia* defensive morphology. As helmet size and phenotypic plasticity is believed to be beneficial to organisms, conferring adaptation, the authors suggest shifts in the biological interaction between predator and prey, consequent to terpenoid hormone exposure. Perfluoroctane sulfonic acid exposure (2184±365 ng PFOS/g body weight) in the bumblebee, *Bombus terrestris*, reduced survival and reproductive capacity (Mommaerts *et al*., 2011). The authors report antagonism of the EcR-b.act.luc reporter construct, suggesting an endocrine related mechanism of action.

2.1.3.2 Fish

Water is a key pathway for contaminant exposure and fish play an important role in assessing the ecological consequences of pollution. There are approximately 28,000 species of fish, comprising teleosts, chondrichyhyes and lampreys, which may be either gonochoristic or hermaphroditic (Scholz and Kluver, 2009). Sumpter (2005) showed that environmental estrogens exposure increased vitellogenin concentration and decreased reproductive potential. Increased plasma levels of the egg yolk precursor vitellogenin can be used as a specific biomarker of estrogen exposure in fish. Elevations

in vitellogenin have been observed in the wild roach (*Rutilus rutilus*), fathead minnow (*Pimephales promelas*), carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) among other fish species (Bergman *et al*., 2012).

Effluents from municipal waste treatment plants frequently contain high concentrations of estrogenic, pharmaceutical and anti-androgenic compounds (Purdom *et al*., 1994). The concentrations of 17β-Estradiol (E2) in Sewage Treatment Work (STW) effluents ranges from 3.7 to 80 ng L^{-1}. Induction of vitellogenin and intersex in response to EDC's has been reported in a number of locations worldwide (Desforges *et al*., 2010). Lange *et al.* (2011) exposed *R. rutilus* to either 50% or 100% STW effluent from 35 days post hatch, for up to 3.5 years post exposure. The study demonstrated a predominance of the female phenotype and, subsequently, declines in spawning. Cotton and Wedekind (2009) suggest that feminisation could potentially result in moderate population increase, as females are a limiting factor in breeding success, however, little is known about the reproductive capability of sex reversed male offspring. Alterations in testicular histopathology, including alterations in spermatozoa parameters, have been observed in stickleback (*Gasterosteidae*) exposed to STW effluent (Bjorkblom *et al*., 2009). Furthermore, the potential loss of genetic variability in affected populations may have serious repercussions on biodiversity (Jobling *et al*., 2006).

Johnstone *et al*. (1978) demonstrated dietary administration of 17β-Estradiol (E2) suppressed both weight and length of rainbow trout (*Oncorhynchus mykiss*). Population survival is intrinsically linked to growth, due to larger fish being more successful at competing for resources. Thus, exposure to chemicals that perturb individual growth may affect population success (Ashfield *et al*., 1998; Elliott, 1990). Lavelle and Sorensen (2011) assessed the effects of EDC exposure on *P. promelas* breeding, using competitive spawning experiments. Males exposed to E2 (44 ng L^{-1}) for three weeks failed to compete with unexposed fish, while male fathead minnows exposed to the lowest dose (4 ng L^{-1}) outcompeted and sired more young than unexposed individuals ($p<0.05$), indicative of hormesis[7]. The authors conclude that estrogenic effluents may determine the reproductive success of male fishes. Supporting this, 5 ng EE2 L^{-1} exposure in *D. rerio* led to complete sex reversal and reproductive failure, however, no statistically significant effects were observed at 0.5 ng EE2 L^{-1} or 5 mg EE2 L^{-1} (Gutjahr-Gobell *et al*., 2006). Presenting an inverted 'U-shaped' dose response curve, Gutjahr-Gobell *et al*.'s study adds further weight to EDC hormesis hypotheses.

2.1.3.3 Amphibians and Reptiles

Tetrapod in descent, amphibians and reptiles present diverse mechanisms of metamorphosis and reproduction. An estimated 32% of amphibian species are

[7] Hormesis refers to a biphasic dose response, characterised by low dose stimulation, and high dose inhibition

threatened with extinction (GAA, 2004). Xenobiotic chemicals have been shown to alter amphibian vitellogenin expression, metamorphosis, osmoregulation and migrational behaviour (WHO, 2002; Palmer & Palmer's, 1995). Bishop *et al*. (1991) identified a significant correlation between PCB exposure in snapping turtles (*Chelydra spentinia sepentinia*) and hatchling success and developmental abnormalities.

Devoid of distinct sex chromosomes, some amphibians and reptiles determine gender by incubation temperature during organogenesis (temperature-dependant sex determination (TSD)) (Crain & Guillette, 1998). TSD is mediated by the up-regulation of SOX9 at male-producing temperatures and aromatase up-regulation at female producing temperatures, resulting in elevated 17β-Estradiol (E2) levels *in ovo* (Pieau & Dorizzi, 2004). Exogenous hormones have been shown to alter TSD (Crain *et al.*, 1997).

As a Class of approximately 8225 species, Reptilia is undoubtedly the most under-represented ectotherm in EDC research. However, accidental contamination of Lake Apopoka, Florida (1980), exposed alligators to high pesticide concentrations (Crain *et al*., 1998) which stimulated research. Elevated levels of dicofol, DDT and their metabolites were found in alligator eggs and surviving juveniles – in which there was a 90% decline (Heinz *et al.*, 1991; Guillette *et al*., 1996; Guillette & Gunderson, 2001). Increased incidence of developmental abnormalities were observed in male juvenile alligators; these included abnormal gonads, altered hormone concentrations and an average 24% reduction in penis size (Guillette *et al*., 1996). Male testosterone levels were depressed to levels comparable to females from a reference site, while females from the contaminated site showed nearly twice the E2 plasma concentrations considered as normal. Unresponsive gonadal steroidogenesis, in exposed males treated with exogenous luteininsing hormone, indicated permanent *in ovo* changes in the gonads. However, due to the ecoepidemiological nature of the study, causality is difficult to define (WHO, 2002). Similar observations in alligator gonadal morphology have been observed in other contaminated wetlands (Hamlin *et al*., 2010). However, contradicting previous conceptions, the potential role of nitrates was also identified (Edwards and Guillette, 2007). Cadenas *et al*. (2000) demonstrated disruption of key steroidogenic enzymes and P450 enzymes involved in steroidogenesis and liver clearance, following exposure to nitrate, which is converted to nitric oxide by vertebrate mitochondria in mosquitofish. This impairment in liver function has been associated with an increase in circulating steroid hormones (Hamlin *et al.*, 2008).

2.1.3.4 Birds

The complex social behaviours, neurodevelopment and reproductive success of birds, is intrinsically linked to hormone levels, rendering the species susceptible to endocrine disruption. Contrary to mammal sexual development, the male phenotype is dominant (the default), while the female phenotype of birds relies on the synthesis of estrogen by

the ovaries during embryogenesis (Brunström *et al*., 2003). Testosterone exposure has been linked to a male-biased skew of the sex ratio in spotless starlings, *Sturnus unicolor* (Veiga *et al*., 2004) and homing pigeons, *Columba liva* (Goerlich *et al*., 2009). Furthermore, Erikstad *et al*. (2009) identified a female-biased skew of the sex ratio of lesser black-backed gull (*Larus Fuscus*) populations with high organochlorine burdens.

Other adverse reproductive effects observed in birds include eggshell thinning, embryonic foot, bill and spine deformities, chick death and retarded growth (Bowerman *et al*., 2000; Best *et al*., 2010). Asymmetrical primary feathers reported in great tits (*Parus major*) exposed to metal smelter fumes, indicative of elevated stress, suggest that the adrenocorticoid axis may also be susceptible to endocrine disruption in birds. Regarding social behaviour, laboratory studies have shown *in ovo* DDT, Diethylstilbesterol (DES) and EE2 exposure leads to alterations in adult male Japanese quail (*Coturnix japonica*) sexual behaviour (Bryan *et al*., 1989; Van den Berg *et al.*, 1998). Similarly, studies on Ring Doves (*Streptopelia risoria*) found that mixtures of DDE, PCBs, Mirex and Photomirex, led to altered sex hormone production, resulting in females failing to respond to male courtship behaviours (McArthur *et al*., 1983). Prior to this, changes in wildlife behavioural patterns were noted in gulls native to Southern California, in which female-female pairing was observed (Hunt and Hunt, 1977). The colonies under observation were in areas contaminated with high levels of organochlorines, leading to the suggestion that this behaviour was attributable to endocrine disruption (Fry and Toone, 1981). Similarly, in North American gull populations skewed sex ratios and female-female pairing have been observed in regions contaminated with DDT (Fox, 1992).

2.1.3.5 Mammals

Bioaccumulation of PCBs, DDT and their persistent methylsulfone metabolites has been reported in Baltic Grey Seals (*Halichoerus grypus*) and Ringed seals (*Pusa hispida*) native to the Baltic Sea. One-fortieth of the volume of The Baltic Sea is attributable to run-off from adjacent countries, leaving it particularly susceptible to xenobiotic pollution (Thulin and Andrushaitis, 2003). This bioaccumulation has been associated with significant declines in seal populations, uterine stenosis[8] and occlusions in 30% of adult grey seals and 70% of ringed seals that were autopsied (Bergman *et al.*, 1994). The Helsinki Commission (HELCOM) 1988 Ministerial Declaration reduced the burden of chemicals emitted into the Baltic Sea by 20 – 50%. Baltic Seal fecundity has subsequently improved and population numbers increased (Bergman, 1999). The Semifield Reproduction laboratory studies later confirmed these ecoepidemiological findings – demonstrating that both reproduction and immune systems were impaired by PCBs in common seals (Reijinders, 1986). This immune dysfunction was associated

[8] which is an abnormal narrowing of a tubular organ or structure, also referred to as a stricture or coarctation

with mass mortalities as a result of morbillivirus infections (Vos *et al.* 2000). The reported association between immune dysfunction and EDCs has been strengthened by an epizootic[9] in striped dolphins (*Stenella coeruleoalba*) consequent to xenobiotic exposure; Aguilar and Borrell (1994) suggested that mobilised concentrations of PCBs led to an increase in susceptibility to the Mediterranean morbillivirus in 1990.

Alterations in reproductive function consequent to PCB exposure, has also been reported in mustelids, including the European otter (*Lutra lutra*) and the Mink (*Mustela vision*) (Kihlström *et al.*, 1992; Leonards, 1997; Roos *et al*., 2001). Laboratory studies in mink demonstrated a dose-response relationship between fecundity and concentration of PCB consumed per day (Brunstrom *et al*., 2001). Exposure to EDCs has also been tenuously associated to high levels of cryptorchidism in the male Florida panther (*Felis concolor coryi*). Sperm abnormalities, thyroid dysfunction and sterility have also been reported (Facemire *et al*., 1995). More recently, as a model of high trophic level carnivore organohalogen susceptibility, Kirkegaard *et al*. (2011) exposed post-weanling female Greenland sledge dogs to 128 µg PCB/day. Relative to sister controls, lower thyroid hormone (T3 and T4) levels were observed in bitches at 10 months of age. Authors reported a significant negative correlation between thyroid gland weight and ∑DDT and a positive association between total T3 and dieldrin - supporting the hypothesis that organohalochlorines (OHCs) may adversely affect thyroid function. Alterations in thyroid function may have significant behavioural, neurological, neuropsychological and thermoregulational consequences throughout all periods of development.

Hejmej *et al.* (2011) assessed the *in vivo* effects of exposure to 4-*t*-Octylphenol (OP) (200 mg kg^{-1} bw) on male testes and seminal vesicle development in bank vole (*Clethyrionomys glareolus*). Histological examination identified elevated expression of 3β-hydroxysteroid dehydrogenase and androgen receptor, in addition to increased testosterone levels. Interestingly, the observed endpoints were more evident in voles kept under long photoperiods, suggesting that the susceptibility to OP toxicity varied with external zietgebers such as light, highlighting hormonal interplay with circadian rhythms.

2.1.4 Summary

A plethora of developmental, reproductive and behavioural perturbations have been reported in wildlife exposed to anthropogenic chemical contaminants. From the birds to the bees, endocrine disruption appears to be omnipresent throughout phylogenic classes. The similarity of the endocrine system and its components throughout phyla, in combination with the aforementioned disruption in wildlife, has led to mounting concern regarding the consequences of EDC exposure on human health (Bergman *et al.*,

[9] the unprecedented increase of a disease in an animal population, equivalent to an epidemic in humans

2012; Colborn and Clement, 1992). The following section 'Evidence for Endocrine Disruption in Humans: A Human Health Concern?' summarises the literature pertaining to human endocrinology, biological plausibility, epidemiology and endocrine disruption in humans.

2.2 Evidence for Endocrine Disruption in Humans: A Human Health Concern?

The average human is comprised of over 10^{14} cells of more than 200 different cell types, which require an effective communication system to concord function (Holt and Hanley, 2006). As in any complex regulatory system, functional perturbation of the endocrine system has consequences; for example, lack of growth hormone (GH) in children causes dwarfism, while excess GH hormone leads to gigantism (Brook and Marshall, 2005). In this section, the biological plausibility and evidence pertaining to human endocrine disruption is captured under three headings: reproductive health; hormonal cancers; and, metabolism and developmental health.

2.2.1 Reproductive Health

Genetic sex is determined by the paternal X or Y chromosome during fertilisation. However, the mechanism translating a zygotes genetic sex, into the sexually dimorphic male and female phenotypes (gender), is dependent on a plethora of genetic, hormonal, psychological and social factors. The regression of the Müllerian duct and virilisation[10] of the Wolfian duct are vital to male development; fetal testis secrete anti-Müllerian hormone (AMH), a glycoprotein synthesised by the Sertoli cells during foetal life. The action of AMH is ipsilateral[11] and the Müllerian duct is only sensitive to AMH between the 7th and 8th weeks of intrauterine life. In the absence of these testicular secretions, the female phenotype persists into postnatal life.

Testosterone is responsible for virilisation of the Wolffian duct. Conversion of Testosterone to 5α-Dihydrotestosterone necessitates virilisation of the foetal external genitalia and the development of the prostate, which is dependent upon the 5α-reductase enzyme in those foetal derivatives of the urogenital sinus. Failure of one of these components, such as a deficiency of 5α-reductase, may lead to abnormal sexual differentiation (Brook and Marshall, 2005). Hermaphriditism, the presence of both functional testicular and ovarian tissue, and pseudohermaphroditism, which details either abnormal male or female sexual development, are usually indicative of early *in utero* disruption.

[10] Virilistion is the development of male secondary sex characteristics
[11] Located or affecting on the same side of the body

The maintenance of reproductive capability is reliant on the secretion of a milieu of hormones. In males, the control of gametogenesis, or spermatogenesis, is dependent on Luteinising Hormone (LH) and Follicle-Stimulating Hormone (FSH) secretion, which is inhibited by the negative feedback of Testosterone and Inhibin. FSH and LH also mediate the production of female ova, which is regulated by negative feedback of the ovarian hormones (estrogen, progesterone and inhibin). Ovulation is initiated by a surge of luteinising hormone mid-cycle, temporarily switching to positive feedback by estrogenic action on the pituitary, causing follicular development, luteolysis and menstruation. Estrogens also maintain secondary sexual characteristics (Holt and Hanley, 2006). Excess gonadotrophins usually reflect a loss of negative feedback from the testis or ovary; for example during the female menopause, depleted ova ends cyclical ovarian hormone production. Inappropriately timed GnRH secretion can cause central precocious puberty. Cyclical gonadotrophin secretion in women is exceptionally vulnerable to perturbation - exercise, excessive dieting and stress, can all be sufficient to temporarily silence the reproductive axis (Holt and Hanley, 2006). The hormonal regulation and sensitivity of the reproductive axis, scientifically justifies for a role of EDC's in the aetiology of reproductive disorders.

2.2.1.1 Male Reproductive Health

Male infertility can be consequent to defective spermatogenesis and reduced sperm concentrations are believed to be the cause of infertility in 20% of males (Yong *et al.* 1998). Spermatogenesis may fail due to reductions in LH and FSH secretion and high levels of prolactin can cause testicular involution and impotence, due to its role in gonadotropin release. However, male infertility is typically attributed to a primary fail of the testis, resultant of cryptorchidism[12] or testicular damage. Incidence rates of cryptorchidism, hypospadias and reductions of sperm parameters, generally coincide, leading to speculation of a common aetiology. The testicular dysgenesis syndrome (TDS) hypothesis suggests that perturbation of androgen levels during foetal development of Sertoli cells (the cells supporting germ cells) and Leydig cells (the site of androgen biosynthesis), negatively impacts the functioning and development of male reproductive endpoints (Skakkebaek *et al.*, 2001).

Spermatozoa production is under hormonal control, potentially rendering it susceptible to endocrine disruption (WHO, 2002). In 1992, Carlsen *et al*. suggested global 0.8% annual declines in sperm count since 1938; reporting a reduction from 113 x 10^6/ml to 66 x 10^6/ml over 50 years. Sperm concentrations below 48 x 10^6/ml are generally considered to hinder the potential fertility of males (Guzick *et al.,* 2001), although, lower thresholds, of 40 x 10^6/ml (Bonde *et al.,* 1998) and 20 x 10^6/ml (Paasch *et al.*, 2008) have also been proposed. The applicability of sperm counts as a biomarker of

[12] the failure of either one or both testicles to descend, representative of the incomplete movement of testis from an abdominal position to the *ipsilateral scrotum*

male reproductive health is however questionable, due to natural variation and fluctuation in counts as a result of abstinence, ethnicity, infectious disease, season, clothing and drug abuse (Carlsen *et al.,* 2005). Nonetheless sperm count can be used as an indicator of spermatogenesis and Sertoli cell number, size and activity.

Cryptorchidism, affects 2-4% of boys, making it the most common congenital birth abnormality (Bergman *et al*., 2011). Kristensen *et al*. (2010) conducted a prospective birth cohort study on *in utero* analgesic exposure, in which 2570 male newborns were assessed for variances in anogenital distance and testicular position. Analgesic use was found to be associated with congenital cryptorchidism incidence (Odds Ratio (OR) =1.43; 0.73, 2.79) in a dose-dependent manner. To verify associations found in the epidemiological study, Kristensen *et al*. exposed pregnant Wistar rats to subtoxic doses of Paracetemol (150, 250 and 350 mg kg^{-1}/day) and Acetylsalicylic acid (150, 200 and 250 mg kg^{-1}/day). Intrauterine exposure to the analgesics led to a decrease in anogenital distance in male offspring, leading the authors to suggest that intrauterine exposure to mild analgesics is a risk factor for the development of male reproductive disorders.

In concordance with Kristensen *et al.*'s (2010) study, occupational epidemiology has identified higher frequencies of orchidopexy (the surgical treatment of cryptorchidism) in boys from regions of intensive farming and pesticide use (Garry *et al*., 1996). Montes *et al*. (2010) found that mothers who bore sons with cryptorchidism generally had higher 4,4'-DDT and β-Hexachlorocyclohexane (β-HCH) levels (0.464 vs. 0.269 mg kg^{-1} and 0.263 vs 0.192 mg kg^{-1}, respectively). *In utero* exposure to anti-androgenic EDCs may contribute to the aetiology of cryptorchidism, corroborating the conclusions of Anderson *et al*. (2008), who noted elevated cryptorchidism, decreased penis length, lower testis volume and lowered serum testosterone in levels in sons born to Danish greenhouse workers exposed to pesticides.

In addition to cryptorchidism, reductions in foetal androgen action have been linked to hypospadias, a condition in which the urethra opens on the underside of the gland penis. Affecting an estimated 0.2 – 4% of boys at birth, the incidence of hypospadias is believed to be increasing (Nassar *et al*., 2007), raising speculation regarding the role of EDC's in disease aetiology. *In utero* DES/progestin exposure has been associated with hypospadias (Klip *et al*., 2002). Giordano *et al.* (2010) reported an association between elevated maternal serum Hexachlorobenzene (HCB) levels and the likelihood of bearing a son with hypospadias. However, a number of other risk factors for cryptorchidism and hypospadias have been identified; including low birth weight, premature birth, gestational diet and alcohol consumption (Akre *et al.*, 2008; Berkowitz and Lapinski, 1996; Damgaard *et al.*, 2008; Pierik *et al.*, 2004).

In light of the evidence, it is not infeasible to suggest that other hormonally regulated male reproductive parameters may be susceptible to endocrine disruption. For example, it has been estimated that approximately 5 – 20% of men (2.3 million) suffer from moderate-to-severe erectile dysfunction (Kubin *et al.*, 2003). Adult male exposure to

estrogenic compounds is believed to promote gynecomastia and interfere with the hypothalamus-hypophyseal-gonadal axis, resulting in a loss of libido, impotence and low sperm counts, in men occupationally exposed to 4,4'-DDT. This association has also been demonstrated in the male Wistar rat; Brien *et al.* (2000) reported significant perturbation of erectile functioning for 2 weeks following a single sub-cutaneous dose of 500 mg 4,4'-DDE kg^{-1}. Fruthermore, acute exposure to the antiandrogen Flutamide (50 mg kg^{-1}) significantly decreased apomorphine-induced erections (>50%) for 12 to 48 hours, leading authors to suggest that exogenous hormones may play a role in erectile dysfunction.

2.2.1.2 Female Reproductive Health

Female fecundity is determined by hormonal profile, menstruation, early pregnancy loss, ovarian reserve and failure, and reproductive senescence or menopause. The female reproductive system is vulnerable to a plethora of environmental stressors (including smoking, alcohol and caffeine consumption), psychological stressors and physiological factors such as age and weight. In addition, perturbations of kisspeptins (KiSS), pivotal hypothalamic signals for the preovulatory surge of gonadotropins required for cyclicity and ovulation, have been identified as possible targets of endocrine toxicity (Roa *et al*., 2008; Castellano *et al.*, 2006; Crain *et al*., 2008). Disruption of AhR signalling cascades, that modulate follicular steroidogenesis, have also been implicated in reduced fecundity (Hernandez-Ochoae *et al*., 2009).

Chemicals that prevail in the environment have been implicated in adverse fertility and pregnancy outcomes; 4,4'-DDT has been associated with spontaneous abortion (Venners *et al*., 2005); and, 4,4'-DDE has been associated with spontaneous abortion, foetal loss (Longnecker *et al*., 2005) and preterm birth. In an assessment of colostrum[13] organochlorine pesticide concentrations (n=63), Cioroiu *et al.* (2010) identified higher mean 4,4'-DDE (470 ng g^{-1}) and γ-HCH (99 ng g^{-1}) levels in women with preterm, relative to full term, labour (268 ng g^{-1} and 96 ng g^{-1}, respectively). Furthermore, household pesticide usage has been associated with a six-fold increase in spontaneous abortion risk (Weselak *et al.*, 2008). Further complicating the mechanisms, Kishi *et al*. (2008) demonstrated genetic susceptibility and racial differences in fertility endpoints in response to polyaromatic hydrocarbon exposure, possibly as a result of endocrine of xenobiotic metabolism receptor polymorphisms. In addition, Wohlfahrt-Veje *et al* (2012b) identified early breast development in girls prenatally exposed to non-persistent pesticides.

The biological plausibility for the role of EDCs in driving precocious puberty has been enhanced by identification of kisspeptin and G-protein coupled receptor GPR54 regulation of reproduction (Navarro *et al*., 2004). The elucidation of peripheral

[13] The first postnatal mammary gland secretion.

regulators of KiSS-1 expression, such as the adipose hormone leptin (Roa *et al*., 2008), has uncovered the pathway for metabolic control of puberty onset and GnRH secretion, and a number of studies discuss the role of oxytocin neurones and prostaglandin E2 production in puberty progression (Parent *et al*., 2008; Ojeda *et al.*, 2003). Adewale *et al.* (2009) reported *in utero* BPA exposure (50µg kg^{-1} or 50 mg kg^{-1}) accelerated pubertal timing and disrupted ovarian development at low and environmentally relevant doses, without affecting the ability of GnRH neurones to respond to steroid-positive feedback. An *in vivo* rat study reported by Navarro *et al.* (2009) concluded that the hypothalamic KiSS-1 system was altered by BPA exposure (100 or 500 µg rat^{-1}), disrupting gonadotrophin secretion and potentially affecting onset later in life. Furthermore, Sprague Dawley rats exposed to BPA (500 µg/50 µL or 50 µg/50 µL) demonstrated dose-dependent acceleration of the onset of puberty and altered oestrous cyclicity, which was corroborated by *in vitro* analysis of dosed animal pituitary cells, which showed impaired GnRH-induced LH secretion (Fernàndez *et al*., 2009). Supporting the mechanistic studies, a positive correlation ($p<0.001$) between increased plasma levels of phthalates and patients with pubertal gynecomastia (DEHP: 4.66 ± 1.58 µg/ml; MEHP: 3.19 ± 1.41 µg/ml), relative to age matched controls (DEHP: 3.09 ± 0.90 µg/ml; MEHP 1.37 ± 0.36 µg/ml) has been reported (Durmaz *et al.*, 2010).

2.2.2 Hormonal Cancers

Consequent to increased understanding of the role of steroidal hormones in disease progression, human exposure to environmental EDC's has been implicated in the aetiology of cancer (Davis *et al*., 1993). Epidemiological studies have inconsistently reported a 3-4 fold increase in testicular germ cell cancer (TGCC) incidence in Caucasians over the past 30-40 years. TGCC development has been linked to inhibition of androgen receptor and/or biosynthesis, and reduced testosterone production in foetal tissues. Subsequently, it is suggested that *in utero* exposure to xenobiotics and polymorphisms in the androgen receptor gene, may play a role in the aetiology of TGCC (Bergman *et al.*, 2011). In an attempt to identify genetic polymorphisms in the AR that might confer elevated risk of TGCC development, Västermark *et al.* (2011) genotyped 11 halotype-tagging single nucleotide polymorphsisms (SNPs) of CAG and GGN repeats in AR's. For the non-coding G variant tag SNP, rs12014709 in the androgen receptor, the minor genotype was found in 10% of cases and in 5.1% of controls, suggesting the polymorphism may confer elevated TGCC risk (odds ratio (OR) = 2.7; 95% CI; 1.03 – 4.15). Furthermore, short GGN (<23) was associated with an increased risk of metastatic disease (OR: 2.15; 95% CI: 1.04 – 4.45). In support of the hypothesis that AR function is linked to the aetiology of TGCC, the authors concluded that the AR polymorphisms identified in their study may be involved in gene-environment interactions, increasing the susceptibility of some individuals to the effects of EDCs.

The potential role of EDCs in breast tumour proliferation has been demonstrated in a number of studies. Bidgoli *et al.* (2010) evaluated the interaction of genes associated with the development of breast cancer (p53, K-Ras, ER and PR), with the AhR, which mediates the effects of many environmental EDCs, contributing to losses in normal ovarian function. Data reported increases in epithelial cell expression of AhR, resulting in elevated susceptibility to environmentally induced tumours. The early onset of breast malignancy in Iranian women has been attributed to interactions between hormonal and environmental factors. These findings agreed with those from an *in vitro* examination of total estrogenicity of adipose tissue extract, separated on polarity, which suggested that breast cancer was more frequent among women with higher levels of estrogenicity, measured in terms of estrogen equivalents (Fernandez *et al.*, 2007; Ibarluzea *et al.*, 2004).

In vivo toxicology models have corroborated human epidemiological evidence. Zearalenone (ZEA) is an estrogenic secondary metabolite produced by some *Giberella* species, and a regulated contaminant in 32% of 5018 mixed cereal samples, with suspected carcinogenicity (Metzler *et al.*, 2010). Phenotypic alterations in Wistar rat mammary tissues following exposure to 0.2 µg kg^{-1} ZEA suggest that exposure could contribute to the induction of breast disorders (Belli *et al.*, 2010). Doherty *et al.* (2010) identified a 2-3 fold increase in expression of Enhancer of Zeste Homolog 2 (EZH2), in the mammary gland of mice, following *in utero* exposure to DES or BPA at levels approximating human exposure. The authors suggest developmental programming of EZH2 as a novel epigenetic mechanism regulating the mammary gland. In Sprague Dawley CD rats, exposure to butyl benzyl phthalate (BBP; 120 or 500 mg kg^{-1}/day) modified the architecture and proliferative index of the mammary gland in a dose dependent fashion (Moral *et al.*, 2011). BBP exposure modified genes related to immune function, cell signalling, proliferation, differentiation and metabolism, suggesting that *in utero* exposure to BBP may result in delayed pubertal onset and an increased susceptibility to mammary carcinogenesis.

2.2.3 Metabolism and Development

Perturbation of neurodevelopmental processes can impact sensory, motor and cognitive functions, and neurobehaviour, potentially leading to mental retardation, cerebral palsy, psychoses, epilepsy, altered maturational milestones, cognitive defects, sensory dysfunction and perturbed sexual dimorphism (Tilson, 1998). Choi *et al.* (2004) suggested that 50% of EDC's may have neurotoxic potential. Some authors have suggested that this toxic potential may play a role in the aetiology and prevalence of psychiatric illnesses such as bipolar disease, depression, personality and obsessive-compulsive disorders and psychoses (Genius, 2008). Estimates in the US suggest learning difficulties may affect 10% of school children, of which 17% may be affected by conditions such as deafness, blindness, epilepsy, speech deficits and emotional and behavioural problems (Schettler, 2001). In addition, the incidence of attention deficit

disorder (ADD) and autism spectrum disorders (ASD), have increased in Western child populations (Gore and Patisaul, 2010).

It is hypothesised that there are two specific endocrine mechanisms of developmental neurotoxicity; perturbation of the hypothalamus-pituitary axis, which is key to reproductive and sexually dimorphic behaviour, and interference with circulating hormones (estrogen, androgens and thyroid hormones), which modulate neurodevelopment (Ahmed *et al*., 2008). Further implicating the endocrine system in the aetiology of neurodevelopment, hypothyroidism – a congenital or acquired deficiency of thyroid hormone – has been linked with clinical and subclinical neuronal defects. Untreated, hypothyroidism may result in cretinism[14], mental retardation, deafness, short stature and facial deformities (Roberts and Ladenson, 2004). The incidence of congenital hypothyroidism is believed to be increasing; in the US, a 73% increase in incidence was reported between 1987 and 2002. However, 40% of this is believed to be attributable to demographic factors, including ethnicity, sex, birth plurality, birth weight and maternal age (Hinton *et al*., 2010).

Low molecular weight phthalate (LMWP) metabolites and BPA were detected in >90% urine samples of third-trimester women (n=404) in American 1998 and 2002 cohorts, presenting mean concentrations of 419 µg L^{-1} and 1.2 µg $L^{-1,}$ respectively (Miodovnik *et al.*, 2011). Follow-up assessment of offspring at age 7 and 9 years (n=137) using the Social Responsiveness Scale (SRS), identified an association between increasing log-transformed LMWP metabolite concentration and larger social deficits, including poorer social cognition, social communication and social awareness.

The metabolic toxicity of EDC's has also been suggested to occur through the profiling of estrogenic interference, metabolism-related effects and stress responses using integrated transcriptomic and proteomic characterisation techniques (De Wit *et al.*, 2010). Furthermore, Lee *et al.* (2010) examined whether *in vivo* levels of several POPs could prospectively predict occurrence of type-2 diabetes, using a nested case-control study within the U.S. Coronary Artery Risk Development in Young Adults (CARDIA) cohort. Results suggested that environmental POP exposure may increase the risk of developing type-2 diabetes in a non-linear fashion, particularly in obese persons.

2.2.4 Summary

Endocrine disruption has been observed in wildlife populations and demonstrated in laboratory studies. Yet, while epidemiology studies have identified associations in human populations, the epistemological limitations of the studies hinder defining causality. Furthermore, the endocrine system presents mechanisms of potential low dose toxicity (hormesis), sexual dimorphism, transgenerational effects and variable

[14] Cretinism terms physical and mental stunting

endpoints, depending on the timing and duration of exposure and complicating health impact assessments. Subsequently, assessing the impact of EDC exposure on human health presents significant challenges.

Research has started to bridge the gap between wildlife observations and human epidemiological speculation. A decade following the reproductive abnormalities observed in Lake Apopka alligators, research into the effects of occupational pesticide exposure in pregnant green-house workers identified similar human endpoints. Anderson *et al*. (2008) reported decreases in serum testosterone and testicular volume, with notable reductions in penile length, in sons born to mothers exposed to pesticides. Similar to the alligators, a reduction in inhibin B expression from the Sertoli cells elevated the levels of female sex hormones. Thus, steroidogenesis is expected to be permanently altered by pesticide-induced effects on gonadal cells during embryonic development (review by Hamlin *et al*., 2010). Similarly, sons born to mothers with elevated phthalate concentrations showed reduced anogenital distances and smaller penis volumes (Swan *et al*., 2005; Marsee *et al*, 2006). Seifert-Klauss *et al*. (2006) postulated that these results highlight the molecular and cellular conservation in endocrine function throughout the vertebrates, increasing the concern, and necessity for further assessment of the role environmental contaminants play in congenital disorders (Hamlin *et al*., 2010).

However, the approach to characterising regulatory risk in ecotoxicity differs to that of human toxicology. Ecological risk is assessed at population level, rather than the individual; adverse affects observed in individual organisms are not considered, unless the population is significantly affected. In human assessments, any elevation in risk to a toxicological endpoint is considered a concern. Subsequently, the regulatory burden of human EDC exposure must be greater. The chemical regulation, testing and prioritisation strategies, in addition to the policy drivers of EDC research are detailed in the subsequent section.

2.3 Chemical Regulation and Testing

The ubiquity of anthropogenic chemicals in the environment, from food and water to consumer products and household agents, in addition to the potential health impacts, has increased public concern and led to premature legislative actions by regulatory jurisdictions.

2.3.1 Legislation & Regulation

Despite lacking regulatory criterion, endocrine disruptors are prohibited by EU law. In lieu of the Commission's measure, regarding specific scientific criteria in the determination and classification of endocrine disruption, substances registered under the Plant Protection Products (PPP) legislation can only be approved provided there is evidence that the substance, safener or synergist does not have "*an inherent capacity to*

cause endocrine disrupting, neurotoxic or immunotoxic effects" in non-target organisms (Regulation (EC) No 1107/2009). The PPP legislation regulates the agrochemical industry, which is mainly data rich. However, consumer products are generally regulated under the REACH (Registration, Evaluation and Authorisation of Chemicals) Regulations ((EC) No 1907/2006) or Cometics Directive (Regulation (EC) No 1223/2009). Under REACH there are currently no testing strategies or guidance on how to identify EDCs. However, EDCs can be authorised for use under the provisions stated in REACH Article 57, pertaining the authorisation of CMRs and PBTs ('substances possessing carcinogenic, mutagenic or reproductive toxicity' and 'Persistent, bioaccumulative and toxic substances' respectively). CMRs and PBTs constitute chemicals that are of high concern, and subsequently may be listed in Annex XIV of REACH, preventing their release onto the market. These endpoints of toxicity may be indicative of an endocrine mode of action, but at the current time there is no formal requirement to screen for endocrine disruption under REACH. It is therefore possible, that some EDCs may already be in wide use throughout society - unless compounds are potent carcinogens, mutagens, and/or reproductive toxins or persist and bioaccumulate in the environment, they will not be captured by the data requirements of REACH.

2.3.2 A Defined Mode-of-Action?

As previously alluded to, there are a number of epistemological problems in the definition of an EDC. As a recently identified mechanism of toxicity, it is generally recognised that endocrine disruption is a mechanism that may result in a hazard, rather than being a hazard in itself; US EPA "*…does not consider endocrine disruption to be an adverse effect per se, but rather to be a mode or mechanism of action potentially leading to other outcomes, for example, carcinogenic, reproductive, or developmental effects, routinely considered in reaching regulatory decisions*". Thus, there is difficulty in defining endocrine specific endpoints, which has lead to multiple scientific definitions (Table 2.2).

Table 2.2 Scientific Definitions of EDCs Adopted by Regulatory and Advisory Jurisdictions

Weybridge (1996)
"*An endocrine disrupter is an exogenous substance that causes adverse health effects in intact organisms, or its progeny, secondary (consequent) to changes in endocrine function. A potential endocrine disruptor is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism*" (ECETOC, 2009)
US Environmental Protection Agency (1996)
"*An exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development and/or behaviour*" (Kavlock *et al.*, 1996)
European Commission (1998)
"*Endocrine disruptors are exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effect in an intact organism, or its progeny, or (sub)populations*" (ECETOC, 2009)
WHO/IPCS (2002)
"*An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations*"

The phraseology and differences between definitions highlights the discordance between regulatory jurisdictions, academia and industry regarding EDC criteria, in addition to the economic implications of a stringent definition. The inclusion of the word '*adverse'* requires the observed effect to elicit changes that fall outside the normal range of physiological variation within a population, and was included in order to distinguish between endocrine active and endocrine disruptive substances. However, the inclusion of '*adverse*' may simply shift the assumptions, presumptions and epistemological problems onto the word, retaining possible subjectivity in the relevance of observed endpoints. Furthermore, with regards to a regulatory definition, incorporating '*intact organism'* contradicts the use of castrated animals and increasing desire to use *ex vivo* and *in vitro* assessments, which irrespective of intactness, may provide useful information pertaining to risk assessment and regulation (Bergman *et al*., 2011). Nevertheless, there is an urgent need for a regulatory definition of an EDC, particularly in light of amendments to EU legislation; Plant Protection Products, Biocides and REACH Regulations (Marx-Stoelting *et al*., 2011).

2.3.3 Chemical Safety Test Guidelines

Over 100,000 chemicals are listed on the European Inventory of Existing Chemicals Substances (EC, 1996). International harmonisation of endocrine activity testing strategies is vital for hazard identification and risk assessment (EFSA, 2010; 2006). In an attempt to harmonise member state methodologies and safeguard the environment, over 150 Guidelines for the Testing of Chemicals have been developed by the OECD since their formation in 1981 (OECD, 2013). The OECD Test Guideline Programme and Council Decision on the Mutual Acceptance of Data (MAD) has saved governments and industry an estimated €153 million (OECD, 2010a); this has been achieved by avoiding the duplication of testing, reducing the number of laboratory animals and avoiding non-tariff trade barriers. Furthermore, due to the UN's Strategic Approach to International Chemicals Management (SAICM), all instruments necessary for the management of chemicals are universally available at no cost to the user, allowing conformation and regulatory acceptance of data by non-members (OECD, 2010b).

As a result of the national and international chemical safety initiative a number of *in vivo* and *in vitro* assays have been developed (OECD, 1998; EDSTAC, 1998). The OECD adopted the first branch of chemical test guidelines for the elucidation of endocrine-mediated toxicity, relevant to both human health and biotic systems (wildlife), in 2009. Figure 2.2 compares hierarchies for the OECD Conceptual Framework, Adverse Outcome Pathways (AOP) and Test Guideline Programme initiatives, for chemicals interacting with the hypothalamus-pituitary-gonad (HPG) axis (OECD, 2012). As previously alluded to, in sections 2.1 and 2.2, observations in wildlife populations skewed EDC research focus on estrogenicity and androgenicity (HPG axis); subsequently, testing initiatives have focused on these MoAs. In

concordance with the study of carcinogenicity and reproductive and developmental toxicity, core EDC strategy entails two fundamental stages of mechanistic *in vitro* and *in vivo* assays (EDSTAC tier 1; OECD CF tier 1-3), and *in vivo* tests to establish dose-response data (EDSTAC tier 2; OECD CF tier 4 & 5) (Lee *et al*., 2012).

Adverse Outcome Pathway	OECD Conceptual Framework	New Assays/ Modified OECD Test Guidelines
	Level 1 Existing Data and Non-Test Information	
Initiating event: ER-mediated signaling	**Level 2** *In vitro* assays providing data about selected endocrine mechanism(s) / pathways(s) (Mammalian and non mammalian methods)	ER transactivation assay (TG 455); embrane binding assay
Tissue-level responses Gene pathway responses in defined tissues or cultured cells	**Level 3** *In vivo* assays providing data about selected endocrine mechanism(s) / pathway(s)[1] (Relevant cell-based assays were included in this level)	Microarray analysis using estrogen-responsive tissues derived from *in vivo* exposures (could be applied to any *in vivo* exposure assays); microarray analysis using estrogen-responsive cultured cells
Organ-level responses Disruption of brain or gonad development	**Level 4** *In vivo* assays providing data on adverse effects on endocrine relevant endpoints[2]	GnRH neuron development in brain of chronically exposed fish (fish life cycle toxicity test)
Whole organism responses Disruption of brain or gonad development	**Level 5** *In vivo* assays providing more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism [2]	Gonad histopathology in chronically exposed amphibians (TG 231) GnRH neuron development in brain of chronically exposed fish (fish life cycle toxicity test)

* See section 1.2

[1] Some assays may also provide some evidence of adverse effects.

[2] Effects can be sensitive to more than one mechanism and may be due to non-endocrine mechanisms.

Figure 2.2 Integration of AOP Paradigm and OECD Conceptual Framework with Suggested Endocrine Relevant Modifications

Presenting proposed Adverse Outcome Pathway (January, 2012) maps with the OECD Conceptual Framework and promising assays to detect and characterise chemical effects on novel estrogen signalling pathways, the figure shows the progress and direction of testing for chemicals that may perturb the hypothalamus-pituitary-gonad (HPG) axis.

However, the development and adoption of OECD Test Guidelines is a costly and time consuming process, particularly for complex modes of toxicity such as endocrine disruption, as validation stages may take several years. Furthermore, a widely accepted consideration is that "*ecological importance of an effect may differ widely between species*". Thus, it is stipulated that conducting tests in several species, of different taxa,

is required to gain some indication of natural variability (OECD, 2006). *In vivo* assessment is typically regarded as the gold standard of toxicological investigation (see Figure 2.2). Nevertheless, there is subjectivity in what constitutes a significant effect (OECD, 2006), a factor further complicated by the debate regarding endocrine active versus endocrine disruptive classifications. Thus, the number and scope of the current OECD Test Guidelines may not be sufficient to elucidate the plethora of endocrine endpoints, nor economically and ethically feasible to utilise in large scale chemical screening assessments.

The species used in chemical safety assessments are typically based on logistics such as, ease of breeding; purchasing; animal husbandry; duration of life cycle; growth/development; and, the availability of historical toxicity data, which enables and reduces uncertainty in extrapolation. When determining mammalian toxicity, rodent models are typically utilised, at least for non-pharmaceutical products (see Figure 2.3). In ecological risk assessment, fish (*Danio rerio*, *Pimephales promelas* or *Oncorhynchus mykiss*), bird (*Coturnix japonica*) or collembolan (*Folsomia fimetaria*) models are recommended.

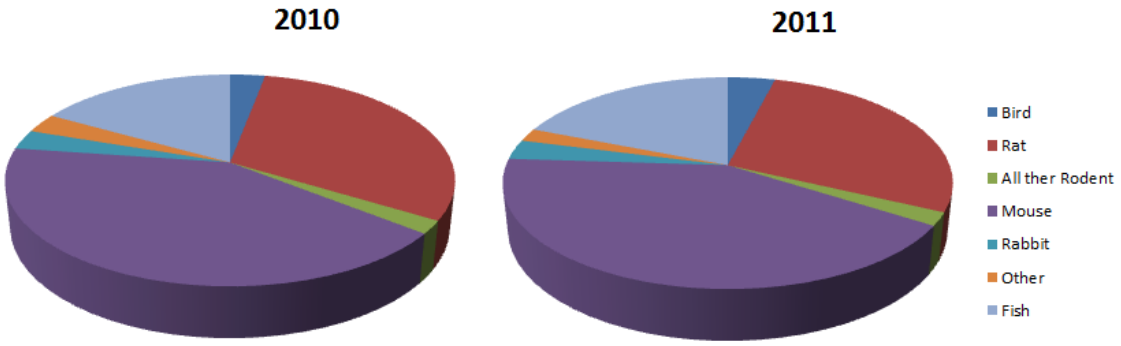


Figure 2.3 Animals used in UK Scientific Procedures in 2010 and 2011
In 2011, of the 3.79 million procedures conducted in animals, 399,000 were for toxicological/safety evaluation purposes, increasing 2% from the 2010 baseline (+7,932). The proportion of animal species investigated were: Bird 3%; Rat 31%; All other Rodent 2%; Mouse 42%; Rabbit 3%; Other 3% and Fish 17% in 2010. In 2011, Bird 4%; Rat 28%; All other Rodent 2%; Mouse 42%; Rabbit 3%; Other 2% and Fish 19% (Home Office Statistics, 2010; 2011).

However, the reliability of extrapolating toxicity observed in one species to another is limited by differences in pharmacokinetics (ADME[15]) and pharmacodynamics (e.g. binding affinities at receptor). The species bias of regulatory toxicology is highlighted by the UK Home Office statistics on animal usage (Figure 2.3). In 2010, 75% of *in vivo* toxicological procedures were conducted in rodent models; representing a very limited assessment of the potential inter- and intra-species variation, and subsequently a large amount of uncertainty.

[15] **A**borption, **D**istribution, **M**etabolism and **E**xcretion

Regulatory bodies currently mitigate for this uncertainty in two ways. Firstly, where toxicological assessment of a chemical is deemed necessary (i.e. for a high production volume chemical under REACH), a minimum of two species are required. Secondly, assessment factors (also called uncertainty or safety factors) are applied during extrapolation of toxicological findings to predict and ensure safe levels of exposure. However, the efficacy and relevance of two species in determining toxicity and the adequacy of safety factors, in safeguarding individual humans and multiple wildlife species is questionable. A greater understanding of the inter- and intra-species variation in endocrine function is undoubtedly vital to understanding the applicability of extrapolating toxicological findings from one species to another. Thus, it may be concluded, under the present state of knowledge, that conventional *in vivo* approaches to elucidating toxicity may not be appropriate for inferring endocrine mediated mechanisms.

2.3.4 Alternative (Non-Animal) Methods

In addition to any concerns regarding the applicability of *in vivo* animal studies, consequent to animal welfare issues, initially highlighted by Russel and Burch's (1959) paper detailing the 3R's concept (Replacement, Reduction and Refinement of animals), the UK is obliged by EU and OECD incentives, to reduce the number of animals used in chemical testing strategies. The predominance of apical endpoints, in addition to amended chemical safety requirements (REACH), demands the development and validation of alternative methods, which incorporate mechanisms of toxicity. A battery of toxicity tests (*in vitro* and *in vivo*) has been demonstrated to provide "*scientific rigor and flexibility*" when identifying chemical toxicity; focussing efforts to major chemicals of concern (ECETOC, 2009). Furthermore, the AOP framework, which aims to map molecular initiating events (MIE) through to adverse outcomes (AO), by elucidating the mechanism and mode of action (Tørsløv *et al*., 2011), requires mechanistic *in vitro* studies to define MIE. .

The EC ReProTect was a project which aims to develop alternative *in vitro* methods to elucidate reproductive toxicity. In a critical assessment of the efficacy of the ReProTect Feasibility study, Schenk *et al*. (2010) comparatively analysed *in vitro* testing results of 10 blinded toxicologically well-defined chemicals. The vast majority of the predictions made based on *in vitro* testing correlated with *in vivo* finds. Thus, combining nearest neighbour statistical analysis with WoE approaches may effectively guide toxicity testing. Systems biology is increasing the availability and applicability of *in silico* tools. For example, launched in 2011, ChemProt[16] compiled multiple chemical-protein annotation resources and disease-associated protein-protein interactions (PPIs). The database assembled over 2-million interactions for over 700,000 distinct chemicals with 30,578 proteins, assisting *in silico* assessments of environmental chemicals, natural

[16] http://www.cbs.dtu.dk/services/ChemProt/

products and approved drugs (Taboureau *et al*., 2011). However, regardless of the development and scientific validation of *in silico* and *in vitro* tools, their adoption into regulatory toxicology remains controversial.

2.3.4.1 Regulatory Acceptance of Alternative Methods

In a workshop held in Kansas City, Missouri (1997), thirty international scientists discussed the methods for detecting estrogenic/androgenic effects in the context of reproductive and developmental toxicity. Delegates concluded that (Quantitative) Structure Activity Relationships ((Q)SAR) and *in vitro* test systems, although useful in a weight-of-evidence approach could not, at the time, replace *in vivo* tests as the sole basis for screening (Ankley *et al.*,1998).

Later in 2003, an evaluation of the *in vitro* test methods for detecting potential endocrine disruptors was conducted by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)[17]. Collating historical data from the NTP Interagency Centre for Evaluation of Alternative Toxicological Methods (NICEATM), ER and AR binding and transcriptional activation *in vitro* assays were appraised based on their incorporation to EDSP Tier 1 screening. ICCVAM concluded that there was little consistency amongst *in vitro* protocols, with no adequately detailed or standardised test method protocols. Recombinant rat or human nuclear receptors were superior to crude cytosolic preparations in binding assays, due to standardisation and significantly reduced contamination risk, enabling greater reproducibility and inter/intra-laboratory comparison (ICCVAM, 2003). Transiently transfected transactivation systems were demonstrated to have a higher level of responsiveness. However, stably transfected cell lines were more amenable to high-throughput screening, highlighting the need for a comparative study to determine whether transiently or stably transfected cell lines are more appropriate for EDC screening.

Progression in the reliability and relevance of some *in vitro* tools has led to validation and regulatory acceptance. Developed by the Chemicals Evaluation and Research Institute (CERI, Japan), the HeLa9903 Stably Transfected ERα Transcriptional Activation (STTA) assay was adopted by the OECD as a level 2 *in vitro* screen for ERα agonistic activity in 2009 (OECD, 2003, 2009). In addition, developed under the auspices of the US EPA EDSP, the BG1Luc (ERα/β) STTA for ER agonists and antagonists, was also included in the OECD Test Guidelines Programme as a tier 2 mechanistic *in vitro* assay in 2012 (OECD, 2012).

[17] ICCVAM contributes to the US EPAs legal requirement to safeguard public health in the Food Quality Protection Act (FQPA) of 1996 (Public Law [P.L.] 104-170) and the 1996 amendments to the Safe Drinking Water Act (SDWA) (P.L. 104-182)

In 2012 the OECD published a Guidance Document[18] (GD) to aid EDC testing strategy, subsequent to generating results in assays adopted by the OECD Test Guidelines Programme (OECD, 2012). Reflective of the state of the science, the GD included estrogenic, androgenic, thyroid hormone and steroidogenic endocrine modalities. However, less characterised modes of toxicity, such as corticosteroid disruption, were not included and may not be detected by current ED assays (Trenzado *et al.*, 2003). The interpretation of *in vitro* and/or *in vivo* endocrine-relevant endpoints in a WoE approach, requires an evaluation into the nature, quantity and quality of existing data and data requirements (Borgert *et al.*, 2011). Nevertheless, it is accepted that there is generally "*no single 'right' approach"* to the interpretation and progression from conflicting ED data (OECD, 2012). The complexity of endocrine physiology and variations in endocrine-mediated pathogenesis requires expertise in both endocrinology and toxicology to effectively assess risk.

2.3.5 Exposure

One further aspect that should be noted is that of mixture toxicity. Contrary to standard toxicological assessments, which evaluate chemical toxicity in isolation, environmental exposure is most likely to occur as a complex chemical mixture. Chemicals with similar modes of action, present in a mixture at concentrations independently below No-Observed-Adverse-Effect-Level (NOAEL) thresholds, may collectively cause adverse effects, complicating regulatory efforts. Silva *et al*. (2002) demonstrated responses of up to 40% maximal estrogenic effect by combining eight xenestrogens at levels equivalent to 50% of their individual no-observed-effect-concentrations (NOECs) in the yeast estrogen screen. Moreover, Rajapakse *et al.* (2002) identified a dose addition relationship following the combined exposure of 11 estrogens. Additionally, *in vivo* studies in an extended rat developmental toxicity model demonstrated three similarly acting androgen receptor antagonsists (at levels below their NOEC) to cause significant signs of feminisation (reduced anogenital index, retained nipples) (Hass *et al*., 2007).

A review by Kortenkamp (2007) concluded that dose addition is generally a predictive tool for assessing the combined effects of EDCs acting via the same biological mechanism (e.g. estrogenic, anti-androgenic, androgenic or thyroid disrupting agents). However, this may underestimate observed effects, as independent action may not always be representative of mixture effects (synergism or potentiation). In order for mixture toxicity to be assessed, detailed mechanistic studies of the mode of action of

[18] Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption: Throughout OECD documentation a 'test method' is defined as "*an experimental system that can be used to obtain a range of information from chemical properties through the adverse effects of a substance*". Thus, the term 'test method' and 'assay' are synonyms within the context of ecotoxicity and human health studies. A 'screen' defines an *in vitro* or *in vivo* assay which provides mechanistic information, but not general information on apical endpoints.

individual components is required, further highlighting the inadequacy of current *in vivo* regulatory tools.

2.3.6 Summary

The premature ban of EDCs by EU legislation has complicated the implementation of regulatory criteria. Testing *'all'* chemicals for endocrine MoA in animals is both ethically and monetarily infeasible, necessitating the development of *in silico* and *in vitro* screening and prioritisation methods. Furthermore, these mechanistic studies found concentration-addition calculations aiming to assess complex environmental exposure. The ubiquitous exposure and limitations of regulatory chemical safety testing highlight risks to both the environment and human health. Subsequently, despite scientific concerns for over 20 years, EDCs remain a policy conundrum.

2.4 Chemical Prioritisation & Screening

2.4.1 Prioritisation

With the plethora of legislation, schemes and definitions, but lack of universally accepted criterion, there is uncertainty in terming a substance as an EDC for regulatory purposes. However, in 2000, the European Commission (EC) published an initial list of 564 chemical substances with known or potential ED properties; 146 substances were identified as either persistent or of high production volume, of which 66 had been demonstrated to disrupt the endocrine system of animals *in vivo* and 52 showed potential ED properties (BKH, 2000). The Environment Agency consolidated a list of 966 chemicals with "*some degree of ED activity*", of which 539 were of anthropogenic origin, 225 biocides, 62 naturally occurring substances, 58 pharmaceuticals, 54 metallo-complex substances and 28 consumer products. Nonylphenol (NP), BPA, TBT, E2 and EE2 were the most frequently used substances in EDC studies.

Internationally, a prioritised list of 65 chemicals was published by the Ministry of Environment (MoE, Japan) for full risk assessment, including: TBT; triphenyl tin (TPT); 4-octylphenol (OP); NP; DBP; octachlorostyrene; benzophenone; di-cyclohexyl phthalate; DEHP; BBP, diethylphthalate (DEP); di-(2-ethylhexyl)adipate; BPA; 2,4-dichlorophenol; 4-nitrotoluene; diphentyl phthalate; di-hexylphthalate; dipropyl phthalate; HCB; HCH; chlordane; oxychlordane; trans-nonachlor; DDT; DDE; and, DDD (SPEED'98; ExTEND 2005).

However, international priority substances are not necessarily indicative of, or relevant to, UK exposure. In 2010, the UK Drinking Water Inspectorate (DWI) commissioned an analysis of peer reviewed EDC literature; 509 journal articles were reviewed, highlighting 325 '*potential*' EDC's that had been detected or modelled in groundwaters, surface waters, freshwaters, drinking/potable waters or sewage treatment effluent (IEH, 2012). The authors assessed the identified chemicals based on IUPAC, EU or EUROPA

listing; UK pesticide registration; conjugated hormone or phytestrogen; assessment by clinical endocrinologist; and, OECD QSAR Toolbox ER-binder or protein binder classification. Environmental fate, behaviour, and water treatment modelling was conducted for 207 compounds. Modelled average daily intakes of chemicals were compared to Tolerable Daily Intakes (TDI), in order to approximate a Margin of Safety (MoS) for drinking water. The IEH (2012) study balanced exposure with potential ED potency, logically prioritising chemicals for further UK investigation and EDC screening. However, the prioritisation method was not high-throughput and success was reliant on potentially biased and retrospective published literature. Thus, the method would not be appropriate for prospective chemical safety assessments.

2.4.2 EDC Screening

As a primary goal of the US EPA ToxCast™ Program, 309 environmental chemicals were asessed in high-throughput (HT) screening assays and categoried in a weight-of-evidence Toxicological Priority Index (ToxPi) score (Reif *et al*., 2010). Relative contributions of *in vitro* assays, chemical descriptors and biological pathways were visualised, with potential to incorporate exposure constraints (Gangwal *et al*., 2012). However, even at <1% of the cost of full-scale animal testing (Dix *et al*., 2007), the ToxCast™ program is far from categorising the >300,000 chemicals on the market, and subsequently requires the, inherently caveated, prioritisation strategies detailed previously (Section 2.4.1).

Virtual *in silico* screening of large chemical libraries has played a significant role in pharmaceutical lead identification and optimisation, adopting both ligand based (e.g. 3D QSAR) and structure based (e.g. automated docking) screening methods (Balaji *et al.*, 2013). Verdani *et al*. (2012) detailed an *in silico* technology for estimating toxic potential (linear function from 0.0 (none) to 1.0 (extreme)) by quantifying the thermodynamic binding of small molecules towards a battery of proteins (10 nuclear receptors, 4 cytochrome P450's, the AhR and potassium ion channel (hERG)). The authors suggest that the '*VirtualToxLab*' could be used to generate toxicity alerts, but that compounds with high toxic potential should be further investigated for the kinetic stability of protein-ligand interactions using molecular dynamics (Verdani *et al*., 2012). However, the utilisation of molecular dynamic virtual screening methods for toxicological prioritisation is still in its infancy.

Conversely, the adoption of the European REACH regulations has required the implementation of a framework for read-across and data-gap filling, and prompted the development of Quantitative Structure Activity Relationship (QSAR) models (EC, 2006), such as the OECD (Q)SAR Application Toolbox (Devillers *et al*., 2006; Jensen *et al*., 2008; Mombelli, 2012).

2.4.3 The OECD Toolbox ER Profiler

The wealth of information pertaining to functional interactions of the estrogen receptor (see Chapter 1) have highlighted several structural alerts for ER binding (Tong *et al*., 2004; Bignon *et al.*, 1989), leading to the successful development of the OECD Toolbox ER-profiler. Ligand triphenylethylene and dielthylstilbestrol backbone (Tong *et al*., 2004; Bignon *et al*., 1989) and number of hydroxyl groups, have been identified of particular significance in predicting binding affinities, as they are indicative of the number of intramolecular bonds that can be formed with the ER binding pocket (Li & Gramatica, 2010).

Positive associations between molecular complexity and planarity with increasing binding potency have also been reported; phenolic compounds presenting low affinity, while steroids demonstrate high affinity (Liu *et al.*, 2006). Substitutions of aromatic hydroxyl groups and electronegative heteroatoms at the ortho position have been noted to decrease binding affinity (Bradbury *et al*., 2000; Mekenyan *et al.*, 2000; Liu *et al*., 2006). The OECD (Q)SAR Application Toolbox ER-profiler utilises the aforementioned relationships, and those stipulated in Table 2.3, to quickly categorise chemicals on their propensity to bind to ER (Cronin & Worth, 2008; Tong *et al*., 2004; Schmieder *et al*., 2003; Schultz *et al*., 2002). Two-dimensional structures are classified as binders according to cyclicity and a molecular weight of <500 Da, with either hydroxyls or NH_2 groups. Non-binders are chemicals that do not satisfy this criteria, or if the OH/NH_2 groups are impaired by ortho-di-substitutions (Mombelli, 2012).

In a QSAR context, sensitivity pertains to the proportion of true positives correctly identified, specificity details the number of non-binders correctly identified by the tool, and concordance quantifies the proportion of chemicals correctly identified. The predictive performance of the OECD (Q)SAR Toolbox ER profiler has been evaluated by comparing predictions with human and rat experimental binding affinities (Mombelli, 2012). Regardless of strength, chemicals identified as having possible interactions in the QSAR Toolbox, were considered positive, while non-binders were negative. Mombelli (2012) reported OECD Toolbox ER-profiler sensitivity of 84.1% (116 true positives, 22 false negatives), 68.3% specificity (71 true negatives, 33 false positives) and concordance of 77.3% for human datasets. Combining the rat and human datasets (Mekenyan *et al*., 2002), on the basis of significant homology and similar binding behaviour (Koike *et al.*, 1987), reduced sensitivity to 70.6%, specificity to 73.1% and concordance to 72.1% (Mombelli, 2012).

The predictive performance of the Stably Transfected Transcriptional Activation Assay (STTA test) in detecting estrogenicity, relative to the *in vivo* Immature Rat Uterotrophic Assay as a gold standard, presented sensitivity, specificity and concordance of 91%, 88% and 90%, respectively (Takeyoshi *et al*., 2002).

Table 2.3 OECD ER Binding QSAR Constraints

Table shows the criteria utilised by the OECD Toolbox ER-Profiler in determining a chemicals propensity to bind with the ER.

QSAR Prediction	Criteria
Moderate, binder NH_2 group	Chemicals with a single five- or six-member carbon ring structure with an unhindered amino group (-NH_2) (one in the para- or meta- position on the ring) are ER binders. Binding potency is related to the size and shape of non-amino-substituted-ring aspect of the molecule, which can be grossly measured by molecular weight.
Moderate, binder OH group	Chemicals with a five- or six- member carbon ring structure with an unhindered hydroxyl-group (-OH) (on in the para- or meta- position on the ring) are ER binders. Binding potency is related to the size and shape of non-hydroxylated ring aspect of the molecule, which can be grossly measured by molecular weight.
Non-binder, impaired OH or NH_2 group	'With impaired OH or NH_2 group' chemicals with cycles and MW ≤ 500 Da and all their OH and NH_2 groups attached to five or six carbon-atom ring are impaired.
Non binder, MW > 500 Daltons	Chemicals that are too large (MW > 500) cannot bind to the receptor regardless of structure or shape.
Non binder non-cyclic structure	Chemicals that have a molecular weight of less than 500, but do not possess a cyclic structure are reported to be non-binders to the receptor.
Non binder, without OH or NH_2 group	Chemicals that have a molecular weight less than 500, and possess a cyclic structure but one without a hydroxyl or amino group are reported to be non-binders to the receptor.
Strong binder, NH_2 group	MW > 200 Daltons and MW ≤ 500 Daltons and with a non-impaired NH_2 groups attached to five or six carbon-atom ring.
Strong binder, OH group	MW > 200 Daltons and MW ≤ 500 Daltons and with a non-impaired OH group attached to five or six-carbon atom ring.
Very strong binder, OH group	MW > 200 Daltons and MW ≤ 500 Daltons and with two non-impaired OH groups attached to two different five or six carbon-atom rings. Binding potency is strong because of the interactions at both the A and B sites of the receptor.
Weak binder, NH_2 group	MW < 170 Daltons and with a non-impaired NH_2 group attached to a five or six carbon-atom ring.
Weak binder, OH group	MW < 170 Daltons and with a non-impaired OH group attached to a five or six carbon-atom ring.

Thus, the predictive performance of the ER-profiler is lower than that of *in vitro* and *in vivo* testing. Mombelli (2012) compared predictivity of the OECD ER-profiler to the of the Murine Local Lymph Node Assay (LLNA) and Episkin® protocols, which are validated skin irritation models (ECVAM, 2010; Portes *et al*., 2002), highlighting the potential use of the OECD Toolbox ER-profiler as a screening tool. However, the inefficacy of the ER-profiler in detecting moderate binders, including the environmentally relevant phthalates, PCBs and tin-containing compounds, hinders stringent use as a screening tool.

2.5 Summary & Discussion

From the birds to the bees, a plethora of developmental, reproductive and behavioural perturbations have been reported in wildlife exposed to anthropogenic chemicals. The omnipresence of endocrine disruption throughout phylogenetic classes, in addition to the homology of biological machinery, has mounted concern regarding the role of EDC exposure in the aetiology of human disease. In the EU, health costs associated with EDC exposures have been estimated at €31 billion per annum (HEAL, 2014).

The literature review aimed to provide the foundation of EDC knowledge, assessing the historical context, caveats, epidemiological and toxicological concerns, and current knowledge which contribute to the understanding of *in silico* and *in vitro* approaches, and the context which has driven, and will continue to drive, EDC science.

The published EDC research has focused primarily on estrogenicity and androgenicity of the HPG axis, which is in part justified by the endocrinology. However, endocrine disruptor science did not develop under the auspices of endocrinology, but through wildlife observations. As a result, historically the science presented bias, by assuming mechanisms from apical endpoints in a top-down manner. For example, intersex in invertebrates was attributed to estrogenicity and/or anti-androgenicity, however later mechanistic studies suggested significant differences in invertebrate endocrinology, potentially rendering observed effects incomparable. While research has started to bridge the gap between wildlife observations and human epidemiology, it has also highlighted the complexity of EDC MoA(s). A complexity stressed further by the potential intraspecies variation of EDC susceptibility, due to polymorphisms in biological receptors mediating responses.

The aforementioned publication bias undoubtedly constrains the development of *in silico* and *in vitro* methods to estrogenic and androgenic MoAs. This may be a significant short-falling of EDC screening, which currently neglects effects on the amine and peptide hormones, in addition to glucocorticoid, mineralocorticoid and progestagen steroid hormones. Understanding endocrine mechanisms, and their potential disruption by exogenous chemicals, is vital to assess the impacts on environment and health, due to the significant costs of inadequate chemical regulation, both in terms of monetary cost and, mortality and morbidity of wildlife and human populations. Development, adoption and validation of *in silico* and *in vitro* tools are necessary for effective regulation.

The ban of EDCs by some EU legislation has complicated the implementation of regulatory criteria. In addition, the adoption of the REACH regulations has demanded the implementation of frameworks for read-across and data-gap filling, and prompted the development of QSAR models (EC, 2006); such as the OECD QSAR Application Toolbox (Devillers *et al*., 2006), which includes a crude estrogen receptor profiler, and may be used in chemical screening. However, the inability of the OECD ER profiler to

detect moderate binders, in addition to its limited scope, suggests virtual screening tool developments are required. The caveats of the current prioritisation and screening methods, in addition to the lack of regulatory criteria, also hinder the evaluation of *in silico* and *in vitro* tools for EDC hazard characterisation, which will inevitably present the same bias.

Having achieved the aim of elucidating the historical context and drivers of endocrine disruptor science, the following section summarises the molecular mechanisms underpinning the endocrine system. Detailing the nuclear receptor classic genomic pathway and contributory mechanisms, the biological mechanisms founding the biological plausibility of *in silico* molecular docking, are elucidated. Focusing on the data-rich ER and AR, in addition to the progesterone receptor (PR) and Peroxisome Proliferator Activated Receptor-γ (PPARγ), which were prioritised based on literature review findings (sections 2.1.1, 2.1.3 and 2.2), this studies' objective of *in silico* and *in vitro* comparison with published literature, has been refined.

3 BIOLOGICAL MECHANISMS

3.1 Endocrine Receptors and the Classical Genomic Pathway

The Nuclear Receptors regulate the transcription of target genes, controlling a gamut of physiological processes, ranging from metabolism to neuronal development and sexual differentiation (Fang *et al*., 2008; Fang *et al*., 2001). As a result, the nuclear receptors are among the most successful molecular targets in drug discovery history - binding a plethora of pharmacophores (Li *et al*., 2003). In parallel, the NRs were identified as molecular targets for endocrine disruption (Bergman *et al*., 2012). The following section details the mechanisms of nuclear receptor function, identifying pathways susceptible to disruption by xenobiotics and exogenous hormones.

3.1.1 The Nuclear Receptor Superfamily

The nuclear receptors are structurally related ligand-dependent transcription factors, ubiquitous to vertebrates and invertebrates, which play a vital role in endocrine signalling (Baker, 2004). Forty-eight nuclear receptors, including classic receptors and orphan receptors, have been reported in the human genome (Jin & Li, 2010; McEwan, 2009).

Phylogenetic analysis of the steroid receptors suggests that the receptors arose by a series of gene duplications from an ancestral nuclear receptor in a primitive vertebrate, at least 540 million years ago, and in concordance with the steroidogenic and steroid-inactivating enzymes, such as cytochrome P450s and hydroxysteroid dehydrogenases (HSDs) (Baker, 2004). Thornton (2001) suggested that a low specificity estrogen receptor (ER) was the ancestral steroid receptor, a duplication of which led to the 3-ketosteroid receptor family (Eick & Thornton, 2010). The evolution of PR, TR, glucocorticoid and mineralocorticoid receptors is thought to have evolved via a process of '*ligand exploitation'*, whereby gene duplication enabled evolution of new receptors that were '*liganded*' by intermediates of the steroidogenic pathway (Thornton, 2001).

Nuclear receptors (NR) influence the interaction of transcriptional machinery with target genes - conformational changes, usually stimulated by hormone ligands, enable recruitment of coregulatory molecules and the cell's chromatic modifying machinery. Uncoordinated nuclear receptors form '*inactive*' complexes with heat shock proteins (HSPs) in the cytoplasm or nucleus. Consequent to ligand binding, the alpha-helices of the NRs present either an agonist or antagonist conformation, depending on the ligand. In positively regulated genes, ligand binding causes the dissociation of HSPs and corepressors, hetero- or homo-dimerisation, activation, recruitment of coactivators and translocation to nucleus (Brzozowski *et al*., 1997; Shiau *et al*., 1998).

The NRs interact with DNA by binding to hormone response elements (HREs) in the promoter sequence of target genes, or by binding to other transcription factors associated with target genes. Conformational changes consequent to steroid binding and dimerisation, reveal two polypeptide loops stabilised by zinc ions (termed zinc fingers), which bind to target DNA at a specific HRE. HREs are typically repeated hexanucleotides, separated by a spacer of a variable number of nucleotides, arranged as a direct repeat; for example, the AR, PR, glucocorticoids and mineralocorticoids receptors tend to act via response elements comprising an inverted repeat of AGAACA, whist estrogen receptors tend to act on elements with an inverted repeat of AGGTCA (Eick & Thornton, 2010).

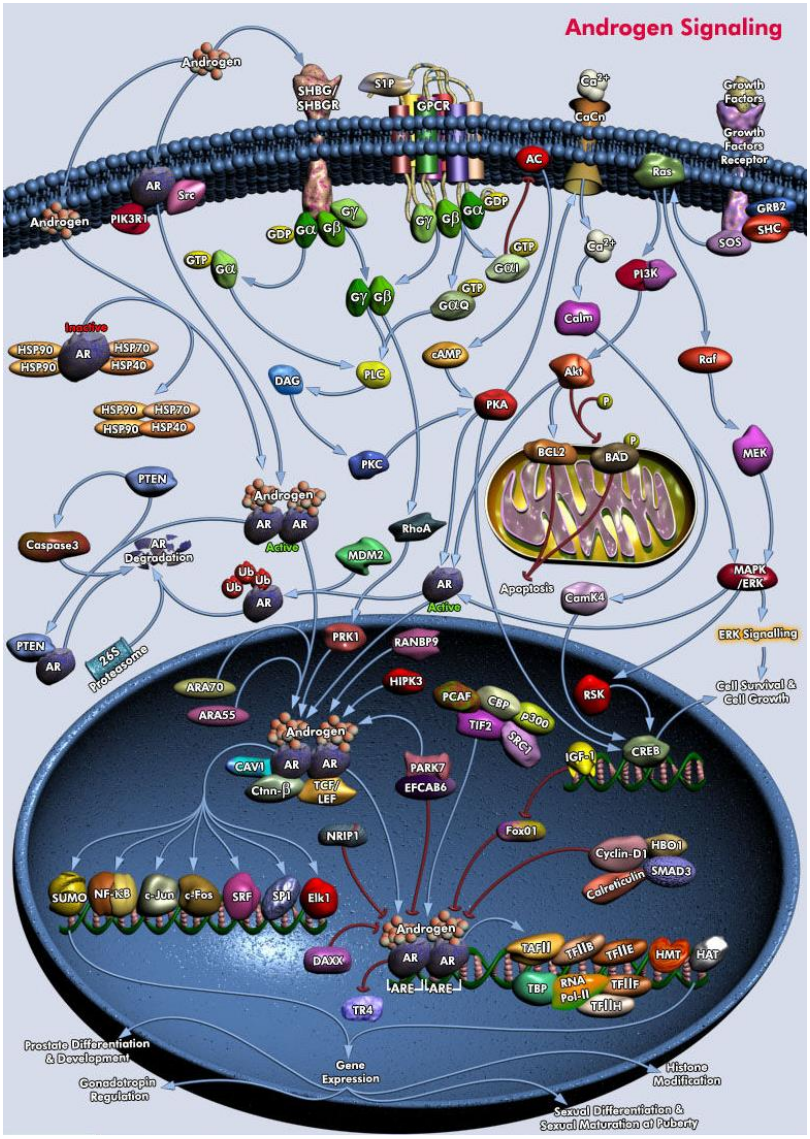


Figure 3.1 Androgen Receptor Signalling Pathways

The nuclear receptors utilise a complex array of feedback loops, coregulators and inter-receptor cross-talk; the diagram presented here shows the the AR signalling pathway, identifying a number of pathways, that may be disrupted by exogeneous chemicals (Qiagen Androgen Signalling Pathway Navigator, http://www.qiagen.com).

Upon binding to the DNA promoter or enhancer, NR AF-1 and/or AF-2 recruit (i) proteins (or protein complexes) with chromatin remodelling enzymatic activity; and, (ii) components of the general transcriptional machinery, forming the pre-initiation complex (Acevedo *et al*., 2004; Rosenfield *et al*., 2006), thereby activating the transcription of target genes. NR coactivators with enzymatic activity include CREB-binding protein (CBP), p300/CBP associated factor (P/CAF) and the histone acetyltransferase Tip60, the kinase CDK7 and ubiquitin/SUMO-1 conjugating enzyme (McEwan, 2009). However, in addition to these a number of NR have been demonstrated to directly bind general transcription factors (TATA-binding proteins, TFIIB, TFIIF and RNA Polymerase 2) (Lavery *et al*., 2005). A visual representation of the androgen receptor signalling pathway is shown in Figure 3.1. Additional NR signalling complexity resides in the dual-activity of agonists, which can bind to receptors to produce a full agonistic response, or partial depending on the relative binding affinity. Partial agonists may also act as antagonists in the presence of a stronger agonist, by competitively binding to the LBD of nuclear receptors (Hotchkiss *et al.,* 2008).

The requirement of transcription and translation to elicit an effect means that endocrine biological responses are generally slow, relative to cell surface receptor signalling (Holt and Hanley, 2006), with a characteristic lag period between the time of exposure and the onset of *in vivo* biological response (Brook and Marshall, 2005). It has been demonstrated that endogenous nuclear receptor function can be perturbed by exogenous environmental pollutants, by interaction with the ligand binding domain. Chemicals act as NR ligands because their stereochemistry allows them to fit, by chance, into the NR ligand binding pocket. McLachlan (2001) suggested that many plant and industrial chemicals, including pesticides, plastic components and xenobiotic drugs may interact with LBDs and thereby mimic, block or otherwise disrupt the natural activity of nuclear receptors. Subsequently, understanding the basic biology of NR is vital to understanding the biological plausibility of receptor mediated endocrine toxicity.

3.1.1.1 Nuclear Receptor Structure

All members of the steroid hormone class of the nuclear receptor family have a similar modular structure comprising of 5 homologous domains, which are lettered from A to E, from the N terminal to the C terminal (Table 3.1). X-Ray Crystallography[19] studies have identified significant structural homology between nuclear receptors, suggesting similar functionality of domains. Contrary to this, the A/B domain (AF-1) varies, demonstrating distinct structural features with flexibility that renders structural determination difficult. Subsequently, the molecular basis of AF-1 function is uncertain, although a role in ligand-independent transcription has been reported (Jin & Li, 2010).

[19] X-ray Crystallography can be used to determine the atomic and molecular structure of proteins by measuring the angles and intensities of diffracted incident X-rays. The density of electrons can then be used to determine the mean positions of atoms, enabling the generation of the 3D structure.

Table 3.1 Nuclear Receptor Domains

The nuclear receptor family present homologous modular domains, lettered from A to E, which contain the activation funcitons (1 and 2), DNA binding domain, and coactivator binding sites. The role of each domain is detailed in the table.

A/B Domains
Containing the transcription factor, AF1, the A/B domains can act autonomously from ligand binding to influence transcription. The A/B domains also interact with coactivators and other transcription factors (Gronemeyer *et al.*, 2004).
C Domain
The DNA binding domain (DBD) is highly conserved, containing a core of 66 residues that interacts with specific DNA response elements and characterised by two 'zinc fingers', which are two polypeptide loops of approximately 10-20 amino acids long. The zinc fingers are essential for interlocking the receptor with the HRE. Changes in a single residue can convert the receptor from recognising one response element to another. The DBD is also the target for post-translational modifications, and plays a role in nuclear localisation and interactions with transcription factors and coactivators (Brook and Marshall, 2005).
D Domain
Is a poorly conserved hinge region, enabling the DBD to rotate relative to the LBD, which may allow receptor conformations that would otherwise suffer steric hindrance.
E Domain
The ligand binding domain (LBD) is involved in transcriptional activation (Beato *et al.*, 1995), and a highly conserved domain, containing four surfaces: i) a variable ligand binding pocked (LBP); ii) a dimerisation surface through which interaction with partner LBDs occurs; iii) a co-regulatory binding surface; and, iv) the ligand dependent transcriptional activation function, AF-2. The AF-2 corresponds to helix 12 (H12 of the LBD), the position of which is flexible and dependent on ligand binding; the position of H12 influence the population of co-regulators that bind, driving either agonist or antagonist action (Heldring *et al.*, 2007).

The NR E-domain (LBD) is typically composed of 11-13 α-helices (H) arranged into a three-layer antiparallel ridge; helices 3, 7 and 10 forming the external surface, while the structure of α-helices 4, 5, 8 and 9 create a hydrophobic cavity, termed the ligand binding pocket (LBP). Bound ligands energetically stabilise the protein conformation via contacts with H3, H5, H6, H7 and H10 (Jin & Li, 2010). The hydrophobicity of the LBP allows NR to interact with lipid soluble ligands, leading to promiscuity of NR and contributing to the biological plausibility of NR mediated endocrine disruption. However, there is discrepancy in the response generated in different cells consequent to a ligand binding, restricting a cohesive model of NR action (McDonnell *et al.*, 2002).

There is limited structural homology in NR LBP, the cavity ranging from 100 $Å^3$ (ERα) to 1400 $Å^3$ (PPARs) (Jin & Li, 2010). The size of the LBP is indicative of the specificity; for example, the large binding pocket of PXR (1200 $Å^3$), is able to bind both the antibiotic Rifampicin (MW 822.94) (Chrencik *et al.*, 2005) and the cholesterol lowering drug SR12813 (MW 504.53) (Watkins *et al.*, 2001). Studies on the PPARs have also identified the importance of LBP shape in NR specificity (Xu *et al.*, 1999). Conformational changes in the LBP, consequent to ERα agonism, have been demonstrated to expose the AF-2 (Tora *et al.*, 1989), permitting interaction with coactivators (Shiau *et al.*, 1998). Conversely, in antagonism, the AF-2 is translocated into a different position, enabling the recruitment of corepressors, rather than coactivators (Shiau *et al.*, 1998; Smith *et al.*, 1997; Barkhem *et al.*, 2002).

Subsequently, the most pronounced feature of NR LBD is their plasticity; accommodating a number of specific ligands in a variety of conformations (Jin & Li, 2010).

A hydrophobic surface enclosed by α-helices 3, 4 and 12, consequent to ligand binding, provide a site for coregulator binding (Jin & Li, 2010; Hur *et al*., 2004). X-ray structures of NR complexes suggest a conserved mechanism of coregulator binding; the position of α-helices 12 critically defining coregulator binding selectivity (Xu *et al*., 2002). Coactivators typically contain multiple LXXLL[20] motifs, which adopt a two-turn alpha-helix conformation, with the three leucine side chains fitting into the hydrophobic LBD. The coactivator-NR complex is further stabilised by '*clamping*' to a conserved H12 glutamate residue and H3 lysine residue. Corepressors bind to the LBD via the conserved LXXXIXXXL/I motif which, unlike coactivators, adopts a three-turn alpha-helix conformation, forcing conformational change of the AF-2 and blocking LXXLL activation (Jin & Li, 2010).

Interestingly, the mechanism for hormone-dependent Androgen Receptor (AR) activation (AF-2) varies from other NR, as AR does not interact with LXXLL motifs, or the associated family of coactivators (SRC-1/p160 family) (He *et al*., 2002; Hsu *et al*., 2003). Coactivators of the AR typically contain FXXLF motifs, which alters the conformation of AR side chains through an induced-fit mechanism (He *et al*., 2004; Lee *et al*., 2001). Understanding the variances in structure, function and coregulation of the NR's under investigation (ERα, ERβ, AR, PR and PPARγ) is vital for the *in silico* analysis. The following sections summarise the literature pertaining to specific structural and functional features of each receptor to be investigated in this study.

3.1.2 Estrogen Receptor

Estrogens, such as the endogenous 17β-estradiol (E2), interact with the estrogen receptor (ER) to elicit the transcription of associated genes regulating human physiology, including development, reproduction, metabolism and homeostasis (Ascenzi *et al*., 2006). Thus, perturbation of '*normal*' estrogenic signalling may trigger adverse health effects. ER interacts with a number of cell check stop points and cellular regulators, such as; FOXC2, MAP1, SLC30A9, UBE1C and NCOA3[21]. Mammals express two ER subtypes, ERα and ERβ (Ramsey *et al*., 2004); however, a further subtype (ERγ) has been detected in fish (Hawkins *et al*., 2000; Ma *et al*., 2000; Menuet *et al*., 2002; Shi *et al*., 1997). Located on distinct chromosomes, ERα and ERβ are the products of separate genes (ESR1 and ESR2, respectively), which maintain 8 exons separated by 7 introns (Green *et al*., 1986). ERα is larger than ERβ (Kumar *et al*., 2004) and Pike *et al*. (1999, 2000) suggested that ERα and ERβ bind E2 differently, with the

[20] Where L = Leucine, I = Isoleucine and X = any amino acid.
[21] http://www.uniprot.org/uniprot/P03372

ligand '*upside down'* in the latter. Interestingly, ERβ can act as a dominant inhibitor of ERα transcriptional activity when co-expressed (Metivier *et al*., 2003). Mutations of ER have been identified in a number of diseases, while polymorphisms in ERα have been associated with bone mineral density (BMD); commonly detected in post-menopausal women, low BMD is associated with increased osteoporosis risk (Ascenzi *et al*., 2006).

ERs bind a plethora of structurally diverse chemicals (Ascenzi *et al*., 2006), possibly as a consequence of the large discrepancy in volume between the ER binding cavity (450Å) and E2 (245Å). Consistent with the other NRs, the LBD is folded into a three-layered antiparallel α-helical sandwich. E2 binds diagonally across the hydrophobic core of the LBD, formed by αH3 (Met342 to Leu354), αH6 (Trp383 to Arg394), the loop and αH8 (Val418 to Leu428), αH11 (Met517 to Met528), αH12 (Leu539 to His547) and with S1/S2 hairpin (Leu402 to Leu410), adopting a low-energy conformation (Brzozowski *et al*., 1997). The phenolic hydroxyl of the E2 A-ring (Figure 3.2) forms hydrogen bonds to the ER Glu353 carboxylate and guanidinium group of Arg394, and a water molecule. The A-ring itself poses between the side chains of Ala350, Leu387 and Phe404. Located at the other end of the LBP, the E2 D-ring forms non-polar contacts with Ile424, Gly521 and Leu525, while the 17β-hydroxyl (O17) group forms a hydrogen bond with His524 (Brzozowski *et al.*, 1997). In E2 binding, αH12 clamps over the LBP, projecting its inner hydrophobic surface (Asp538, Asp545) towards E2, and projecting Glu542 away (Brzozowski *et al.*, 1997).

DES bound ER, closely resembles the conformation of the E2-ER complex defined by Brzozowski *et al*. (1997), Tanenbaum *et al*. (1998) and Shiau *et al*. (1998). However, the DES (structure C in Figure 3.2) ethyl groups projecting from the phenolic rings, fit into unoccupied cavities of the ER, forming non-polar contacts with Ala350, Leu384, Phe404 and Leu428, possibly accounting for higher affinity (Kuiper *et al*., 1997; Shiau *et al.*, 1998). Furthermore, the A^1 ring of DES forms van der Waals contacts with Met343, Leu346 and Met421, in addition to Gly521 and Leu525, further stabilising the complex (Shiau *et al.*, 1998). Stabilisation is important for dimerisation and cofactor recruitment (Chapter 4.1).

The ER antagonist, Raloxifene (RAL), utilises the same hydrophobic pocket of the LBD, mimicking the interaction of the A-ring hydroxyl, however, bonding between αH8 and αH11 is significantly different, with a 5.1Å displacement of the His524 hydrogen bond to RAL's phenolic hydroxyl (O11). The RAL side chain forms hydrophobic contacts with αH3, αH5/H6, αH11 and the connecting loop, which is stabilised by a hydrogen bond between Asp351 and the piperazine ring (N26 in Figure 3.2). However, 11Å in length, the RAL side chain is not contained within the LBP, protruding from the pocket and displacing αH12 (Brzozowski *et al*., 1997), and subsequently coactivator binding.

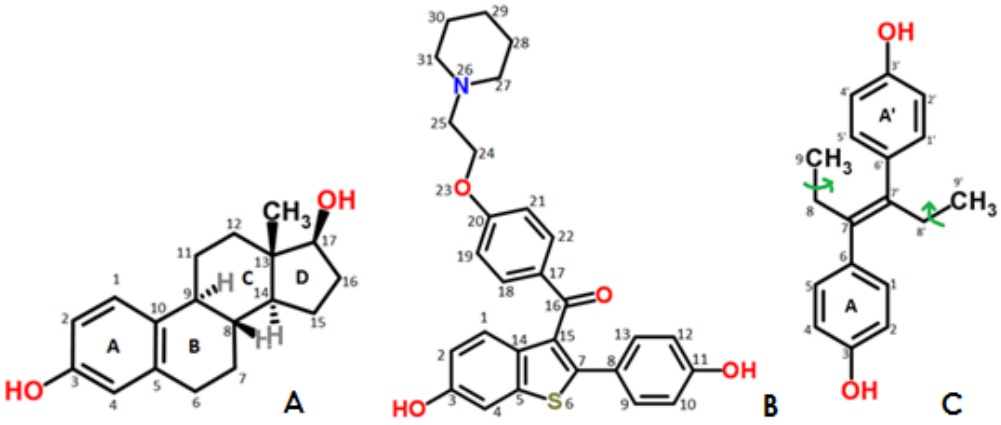


Figure 3.2 Chemical Structure of Estrogen Receptor Binders (E2, DES and RAL)
The chemical structures of the estrogen receptor agonists 17β-estradiol (A) and Diethylstilbestrol (C) and antagonist raloxifene (B), demonstrate the plethora of structures that interact with ER. The ethyl groups involved in DES non-ionic bonding are labelled in green.

Ligand binding drives the recruitment of coregulatory molecules via the NR Box II peptide-LBD interface (Phillips *et al*., 2011); buried 1000Å into the ERα LBD and comprised of residues Leu345, Val355, Ile358, Ala361 and Lys362 (αH3), Phe367, Val368 (αH4), Leu372, Gln375, Val376, Leu379, Glu380 (αH5) and Asp538, Leu539, Glu542 and Met545 (αH12) (Shiau *et al*., 1998). However, the LBD interacts primarily with Ile689, and the LXXLL motif leucines (Leu690, Leu693 and Leu694); the side chain of Leu690 forms van der Waals contacts with the side chains of Ile358, Val376, Leu379, Glu380 and Met543 (Shiau *et al.*, 1998). Subsequently, the AF-2 surface is believed to be formed from grove of αH3, αH4, αH5 and αH12 (Brzozowski *et al*., 1997; Shiau *et al*., 1998). The blocking of AF-2 function (by antagonists, such as RAL, Figure 3.2) is a consequence of αH12 interfering with the static region of the coactivator recognition groove (Shiau *et al.*, 1998).

3.1.3 Androgen Receptor

The androgen receptor (AR) is a ligand activated transcription factor that triggers the expression of genes involved in the development of the male phenotype and male secondary sex characteristics. Insensitivity to the action of androgens, consequent to the failure of androgenic compounds eliciting the AR genomic pathway, is a common cause of under-masculinisation in 46 XY individuals (Werner *et al*., 2010). Furthermore, as a result of location on the X chromosome (locus q11-12 in humans), males are hemizygous, thus mutations in the gene directly affect male sexual development. Carrying two copies of the X chromosome, heterozygous women are usually unaffected (Spencer *et al*., 1991). However, the formation of Barr bodies[22], via a process of

[22] Named after their discoverer, Murray Barr, a Barr body is an inactive compressed X chromosome in female somatic cells.

lyonisation (X-inactivation), means that women may express two AR, complicating extrapolations from genotype to phenotype.

The N-Terminal Domain (NTD) of AR contains glutamine (Glu/Q) and glycine (Gly/G) polymorphic repeats, termed polyQ and polyG repeats, respectively. If the polygutamate repeat exceeds 40 residues, the late onset neurodegenerative disorder, spinal and bulbar muscular atrophy (SBMA or Kennedy disease) develops (LaSpada *et al*., 1991). The length of the polyQ and polyG repeats in healthy men usually ranges from 9-36 and 10-27 residues, respectively. The polyQ repeat induces AR transactivity (Mhatre *et al*., 1993) and the polyG increases AR activity. Thus, the number of these repeats may predispose an individual to virilisation disorders and infertility, depending on baseline testosterone levels. Over 600 mutations of the AR have been identified (Gottlieb *et al*., 2004); furthermore, activating mutations that lead to LBD promiscuity have been reported in prostate cancer tissues (Werner *et al*., 2010).

The AR ligand binding domain is constructed of a hydrophobic region of 12 α-helices and four β-strands, which seal the binding pocket consequent to ligand activation (He *et al.*, 2004), and enables AR response elements and coactivators to be recruited. However, adding uncertainty to the extrapolation of receptor binding studies, almost 200 protein coregulators of the AR have been described (Heemers & Tindall, 2007).

3.1.4 Progesterone Receptor

Progesterone, the endogenous agonist of the progesterone receptor, is a female reproductive hormone, renowned for its role in uterine receptivity, implantation and pregnancy (Schumacher *et al.*, 2012). However, more recently the neuroprotective properties of progestagens have been demonstrated in experimental models of nervous system injury, such as middle cerebral artery occlusion, excitotoxic neuron death and traumatic brain injury (Garcia-Segura *et al*., 2001; Stein, 2001; Wise, 2002; De Nicola *et al*., 2009), suggesting multiple functions beyond reproduction.

The progesterone receptor (PR) encoded by the PGR gene located on chromosome 11q22 has two main forms, A and B, the latter of which includes the transcription activation function TAF-3 (>165 a.a.) in a B-upstream segment (BUS) of the N-terminal (Kastner *et al*., 1990). Though the remainder of the protein sequence is similar, the two PR are functionally distinct, mediating different response elements and physiological effects. In the human PR, a number of variable sites have been identified, including four polymorphisms and five common haplotypes. For example, +331G/A polymorphism in the promotor region alters the PR transcription site, which led to increases in PR gene transcription in Ishikawa endometrial cancer cells *in vitro* (Terry *et al*., 2005).

3.1.5 Peroxisome Proliferator Activated Receptor-gamma

The Peroxisome Proliferator Activated Receptors (PPARs) are a class of nuclear receptor transcription factors (Michalik *et al*., 2006) of genes involved in cellular differentiation, development, metabolism and tumourigenesis (Belfiore *et al.*, 2009) of higher organisms (Berger & Moller, 2002; Feige *et al*., 2006). PPARs heterodimerise with the retinoid X receptor (RXR) and bind to specific DNA promoters, termed peroxisome proliferator hormone response elements (PPREs). Consistent with other NR, the function of PPARs is mediated by conformational changes consequent to ligand binding and a number of coactivator and corepressor proteins, which can activate or inhibit PPAR function, respectively (Yu & Reddy, 2007).

Three subtypes of peroxisome proliferator activated receptor have been identified: PPARα, PPARδ and PPARγ (Berger & Moller, 2002). PPARα is expressed predominantly in the liver, kidney, heart, muscle and adipose tissue, while PPARδ is expressed in all tissues, but markedely in the lipid rich tissues of the brain, adipose tissues and skin. There are three alternative splice variants of the PPARγ, splice variant γ is expressed in all tissues, γ2 is mainly expressed in adipose tissues, while γ3 is expressed in macrophages, the large intestine and white adipose tissue. Each PPAR subtype has distinct cellular functions. Due to its agonism with TBT and TPT, leading to imposex in marine molluscs and adipogenesis in mammals, PPARγ will be the focus of this investigation.

A number of hereditary disorders in PPARs have been identified, generally resulting in PPAR loss of function, which has been associated with concomitant lipodystrophy[23], insulin resistance and acanthosis nigricans[24] (Meirhaeghe & Amouyel, 2004). In PPARγ, a Pro12Ala gain of function mutation has been extensively studied and associated with decreased risk of insulin resistance. However, Pro115Gln has been associated with obesity; increased prevalence of PPARγ polymorphisms have been identified in a number of obese populations (Buzzetti *et al*., 2004).

3.2 Nuclear Receptor Regulation

3.2.1 Heat Shock Proteins (Hsp)

Prior to ligand binding, nuclear receptors are non-covalently associated with a number of chaperone[25] molecules, including heat shock proteins (Hsp), which are requisite for effective ligand binding (Pratt *et al*., 2004; Zoubeidi *et al*., 1007). Ligand binding

[23] Medical condition characterised by abnormal or degenerative adipose tissue.
[24] Brown-black hyperpigmentation of the skin, associated with obesity or endocrinopathies such as hyperthyroidism, hyperthyroidism, acromegaly, Polycystic Ovaran Syndrome (PCOS) and type II diabetes. Pigmentation is usually found in the lateral folds of the neck, axilla, groin, forehead etc.
[25] Chaperone proteins aid in the non-covalent folding (and unfolding) of macromolecules, preventing newly synthesised and assembled proteins from aggregating into non-functional structures.

causes a conformational shift (activation) in the NR, leading to dissociation of Hsp and dimerisation (Tetel *et al.*, 2009). Heat shock proteins are up-regulated in response to physiological stress (heat, toxins and UV) and are vital for protein folding; enhancing the ability of cells to cope with denatured proteins (Jäättelä, 1999). Named according to their weight (i.e. Hsp60 is 60 kilodaltons in size), Hsps have been implicated in an array of cellular processes (Smith *et al.*, 1997). The NR typically associate with two Hsp90 (Chambraud *et al*., 1990), low levels of which have been associated with dysfunctional nuclear receptor function in yeast mutants (Picard *et al*., 1990), one molecule of Hsp70 and one molecule of either Hsp56, Hsp26 or Hsp40, to retain a non-DNA binding state (Morimoto *et al*., 1998).

The complete binding site of Hsp to NR remains uncertain, however, Pratt *et al.* (1988) suggested that Hsp90 binds to the ligand binding domain (LBD), consequenet to the isolation of Hsp90-GR LBD complexes in mutant cDNA transfection studies in Cos7 cells (Denis *et al*., 1988). Additionally, Chambraud *et al*. (1990) demonstrated an association between the C-terminal extremity of the DBD (residues 251-271), suggesting that multiple regions are involved in the formation of Hsp90 complexes. Typically positively charged, residues 251-271 are conserved in the NR of various species, adding to the plausibility of its interaction with the negatively charged '*A-region'* of Hsp90 (Chambraud *et al.*, 1990).

3.2.2 Co-Regulation

Nuclear Receptor (NR) activity is mediated by approximately 300 NR coregulators (Lonard *et al*., 2007), including p160 factor and steroid-receptor-coactivator (SRC) family activators, and SMART (silencing mediator for retinoid and thyroid hormone receptors) and N-CoR (nuclear corepressors) repressors (Glass *et al*., 2000; Nettles *et al*., 2005). The functionality of nuclear receptors is largely determined by coregulators, which are themselves recruited on the basis of specific conformational changes in the LBD consequent to ligand binding (Jin & Li, 2010). Interestingly, *'stapled'* synthetic peptides modulating NR behaviour, via coregulatory mechanisms have become an area of drug-design research (Phillips *et al*., 2011). The activity of NRs and their auxiliary coregulators is further controlled by kinases, ligases and covalent modifications (Goodson *et al*., 2009). *In vitro* studies suggest that coactivator recruitment is a rate limiting step in NR-mediated gene transcription (Rosenfield *et al*., 2006; Torchia *et al*., 1997). Additionally, *in vitro* repression of NR transcriptional activity through cross-talk with other NR, or '*squelching*', is reversed by the addition of coactivators (Oñate *et al*., 1995). The NR auxiliary coregulators can influence target gene transcription through acetylation, methylation, phosphorylation, chromatin remodelling and mRNA splicing (Rosenfield *et al*., 2006; Lonard *et al*., 2005).

The P160 family includes steroid receptor coactivator-1 (SRC-1/NCoA-1), which was one of the first ligand-dependent transcription factor coactivators reported (Oñate *et al*.,

1995), SRC-2 (also referred to as GRIP1, TIF2 and NCoA-2), and SRC-3 (also termed AIB1, TRAM-1, p/CIP, ACTR and RAC3) (Suen *et al*., 1998). The SRC coactivators physically associate with the LXXLL motifs (NR-box) of nuclear receptors (ER, PR and GR), in a ligand dependent fashion (Rosenfield *et al*., 2006; Oñate *et al*., 1995). The P160 family of coactivators contain two activation domains, AD1 and AD2, which mediate CBP association (Chen *et al*., 1997) and coactivator-protein interactions, for example with arginine methyltransferase CARM1 (Chen *et al* ,1999), respectively. Consequent to binding, the SRC coactivators recruit other coactivators, such as CBP and p300/CBP-associated factors, which can remodel the chromatin via histone acetyltransferase activity (McKenna *et al*., 1998). However, estrogen receptor-associated protein-140 (ERAP140) enhances the transcriptional activity of ERα, ERβ, TR, PPARγ and RARα in the absence of LXXLL motifs (Tetel *et al*., 2009), highlighting incongruity in coactivator mechanism.

Depletion of SRC-1 *in vitro* has been demonstrated to hinder nuclear receptor-dependent transcription (Torchia *et al*., 1997). Furthermore, decreased responsiveness to progesterone target tissues, partial thyroid resistance and delayed cerebellar Purkinje cell development, have been demonstrated in SRC-1 knockout mice *in vivo* (Tetel *et al*., 2009). However, interestingly, SRC-2 was up-regulated in steroid-sensitive tissues, indicative of compensation for the loss of SRC-1 (Xu *et al*., 1998). SRC-2 ablation, knockout and microarray studies have highlighted roles in fertility, mammary gland development (Fernandez-Valdivia *et al*., 2007), cell cycle and immunity (Jeong *et al*., 2007).

Gene silencing is resultant of DNA methyltransferase (DNMT) methylation of DNA CpF sites (Reik *et al*., 2001; Klose *et al*., 2006), which subsequently recruit corepressors and histone deacetylase (HDAC) repressor complexes; e.g. Sin3, NuRD, CoREST and NCoR/SMRT (Tetel *et al*., 2009). Corepressors aid gene silencing by removing acetyl groups on histones, restoring a positive charge on the histone tails enabling tighter binding with DNA and DNA compression (Cunliffe, 2008). However, the mechanism of corepression in its entirety is still under elucidation. Interestingly, global deletion of nuclear receptor corepressors (NCoR) is embryonic lethal (Jepsen *et al*., 2000), suggesting that loss of NCoR function cannot be compensated for by another coregulator (Tetel *et al*., 2009).

Coactivator and corepressor messenger RNA (mRNA) is ubiquitously expressed (McDonnell *et al*., 2002). However, Misiti *et al*., (1998) reported significant variation in tissue-specific expression patterns of SRC-1, p300, SMRT and NCoR, dependent on hormonal regulation. For example, SRC-1 levels in the anterior pituitary (AP) were regulated by triiodothyronine (T3) and E2. Furthermore, coregulator expression patterns present sexual dimorphism; female AP samples contained an average of 40% less SRC-1 mRNA, than male rats (Misiti *et a*l., 1998). Variations in the relative expression of

coactivator and corepressors genes may add heterogeneity to hormonal responses (Misiti *et al*., 1998), and subsequently to the plethora of toxic responses.

3.2.3 Post-Translational Modification

Post-translational SUMOylation[26] of the NR has been identified as a modulator of hormone response, in a gene and receptor specific manner (McEwan, 2009). Post-translational SUMOylation of the AR on lysines 386 and 520 by Ubc9 and E3 ligases (PIASI and PIASAxa) represses AR-dependent transactivation at specific promoters. Conversely, SUMOylation of ERα at the hinge region lysines (K266 and K268), which are also subject to acetylation, enhances receptor activity (Faus *et al*., 2006; Popov *et al*., 2007). Subsequently, post-translational modifications provide a '*fine-tuning*' mechanism of NR transactivation (McEwan, 2009).

In addition to the NR proteins themselves, coactivators can be phosphorylated, methylated, ubiquitinated, SUMOylated and acetylated, altering the functionality of the transcriptional complex (Tetel *et al*., 2009). For example, ERα is phosphorylated on Serine-118 in response to epidermal growth factor, enhancing coactivation (McEwan, 2009).

3.2.4 Inter- & Intra- Receptor Cross-Talk

There is functional cross-talk between NR (inter-receptor); for example, EDCs can impact ER signalling indirectly by binding to the AhR, which is activated by a wide variety of hydrocarbons. Cross-talk between the steroid receptors and constitutive androstane receptors (CAR), pregnane X receptors (PXR), peroxisome proliferator-activated receptors (PPAR's) and retinoid X receptors (RXR's) may alleviate or aggravate responses *in vivo* (EFSA, 2010). The transactivation of PR is reduced by coexpression of ERα, possibly as a result of '*squelching*' or shared coactivators (Oñate *et al*., 1995; Tetel *et al*, 2009). Inter-domain communication poses a further conundrum when elucidating NR modes of action (McDonnell *et al*., 2002). The AF-1 domain is required for the partial agonistic activity of tamoxifen at the LBP (AF-2), and can be positively affected by MAPK-directed phosphorylation (Kato *et al.*, 1995), suggesting that inter-domain communications play a role in the NR response phenotype.

Dimerisation (homo- or hetero-) of monomeric NR units is essential for transcription. The principle dimerisation surface of NR consists of a large contact area (1703 $Å^2$ in ERα) on the surface of the LBD (Brzozowski *et al.*, 1997). The dimerisation stability is regulated by ligand binding; agonists, antagonists and mixtures having characteristic effects on the rate of monomer dissociation. Dimer stability is implicated in the efficacy of the transcriptional complex to associate downstream factors, and thus may be

[26] SUMOylation is a directed enzymatic cascade involving **S**mall **U**biquitin-like **Mo**difier (SUMO) proteins

important in defining the *in vivo* potency of NR responses (Tamrazi *et al*., 2002); this demonstrates that intra-receptor communications (conformational responses) play a role in inter-receptor communications.

As discussed in Section 3.2.2, understanding '*allostery*'[27] is vital to understanding molecular signalling in cellular physiology. Bidirectional allostery has been observed between the DBD and LBD NR domains, termed '*interdomain allostery'* or intra-receptor cross-talk (Bapat *et al*., 2003; Kong *et al*., 2005). Melamed *et al*. (1996) reported variances in ER-ERE interaction consequent to LBD truncation; LBD truncated ER still formed dimer complexes and attached to DNA response elements, but less tightly. Subsequently, the conformational alterations induced by ligand binding are not only important for the interaction with cofactors, but also the dimerisation and DNA binding.

Furthermore, in addition to receptor cross-talk, functional mutations and ligand dynamics, communications between coregulatory complexes highlight the complexity and intricacy of the ligand-dependent NR transcription (McKenna *et al*., 2002) that is pivotal to the *in silico* and *in vitro* analysis undertaken in this study.

3.2.5 Agonism vs. Antagonism

A number of studies have reported dual agonism/antagonism of endogenous hormones and xenobiotics (Jackson *et al*., 1997; Melamed *et al*., 1997; Srinivasan *et al*., 2013). For example, the endogenous hormone, estriol (E3), acts as a weak estrogen in ovariectomised laboratory animals at a single dose, and produces full estrogenic response upon chronic exposure. However, E3 has also been shown to antagonise the ER when co-administered with E2 (Melamed *et al*., 1997). Cell-free transcription assays highlighted the dose-dependency of antagonism, as a result of competitive binding kinetics (Melamed *et al*., 1997). The weak estrogenicity of E3 has been attributed to impaired association between hER and ERE, as the E3 16αhydroxyl group interferes with the 17α-hydrogen bond to Glutamate-419 of ERα, engendering altered LBD conformation (Lewis *et al*., 1995). Interestingly, Quartz-Crystal Microbalance Dissipation (QCM-D) and Surface-Plasmon Resonance (SPR) spectroscopy of ERα have demonstrated significant differences in viscoelastic[28] behaviour subsequent to 4-hydroxytamoxifen (4HT) or E2, LBD binding (Peh *et al*., 2007). Both ligands altered the biolayer water content, however, relative to E2, 4HT bound ER-ERE complexes were dispersed and less dense (Peh *et al*., 2007), consistent with the previous findings of

[27] Derived from allosteric, '*allostery*' defines the alterations in enzyme shape and activity consequent to molecular binding of a coregulator or regulatory substance, at a site other than the LBD.

[28] Viscoelasticity is the property used to define the interface between viscosity and elasticity; folded proteins, present both amorphous solid and crystal-like properties, thus viscoelasticity provides an Angstrom-resolution picture of protein properties (Wang & Zocchi, 2011).

impaired hER-ERE interaction of partial agonist complexes (Lewis *et al*., 1995; Melamed *et al*., 1997).

The anti-inflammatory ERα agonist, WAY-166916, has been demonstrated to present dynamic binding (multiple conformations in the active site.); the bulky side group orientating towards H12, disrupting the activation function (AF-2), or, towards the hydrophobic side pocket and H8, supporting an active conformer, depending on orientation (Srinivasan *et al*., 2013). Subsequently, the same ligand can cause either agonistic or antagonistic responses. Adding to the complexity of this process, Brunning *et al*. (2010) also reported '*gain of allostery'* mutations that mimic ligand-dependent responses with WAY-169916, with both the canonical active and inactive conformations of the ERα – binding the ligand in different orientations. In other words, nuclear receptor surface mutations can lead to stabilisation of specific protein conformations, which might otherwise be energetically unfavourable. Srinivasan *et al*. (2013) concluded that such ligand dynamics explain the varied pharmaceutical phenotypes observed *in vivo*, subsequent to simple-binding and *in vitro* reporter gene assay observations, further stipulating that such dynamics may be exploited to modulate signalling specificity. However, with regard to endocrine toxicity, if ligand dynamics define specific signalling and subsequently ERα phenotypes, the plethora of health effects associated with NR binding may be more varied than currently anticipated.

Interestingly, the agonistic or antagonistic characteristics of a ligand are also defined by the concentration of coregulatory molecules; Smith *et al* (1997) reported activation and inhibition of ER consequent to 4-hydroxytamoxifen exposure, depending on coactivator or corepressor concentration, respectively. The bifunctionality of dynamic ligands, such as WAY-169916 (Brunning *et al*., 2010; Srinivasan *et al*., 2013), increases the complexity of ligand-dependent NR transactivation, suggesting a more complex Michaelis-Menten kinetics[29], susceptible to stereoisomerism[30] and conformation of both the receptor and ligand.

3.3 Summary & Discussion

Nuclear Receptors regulate the transcription of target genes, controlling a gamut of physiological processes, ranging from metabolism to neuronal development and sexual differentiation (Fang *et al*., 2008), and have subsequently been identified as molecular targets for endocrine disruption (Bergman *et al*., 2012). This chapter aimed to elucidate the biological mechanisms of nuclear receptor ligand-dependent transcription factors, and in particular the classical genomic pathway, which play vital roles in vertebrate

[29] In biochemistry, Michaelis-Menten Kinetics provides a descriptor of enzyme activity, relating the rate to substrate concentration.

[30] Stereoisomerism reflects chemicals of the same molecular formula and functional groups, differing only in their three-dimensional shape, including enantiomers (varying only by reflection) and diastereomers (non-optically active enantiomer).

endocrine signalling (Baker, 2004). Concomitantly, a number of considerations for *in silico* and *in vitro* analyses are detailed.

Uncoordinated (no ligand) nuclear receptors form 'inactive' complexes, non-covalently associating to chaperone molecules, such as HSP (Pratt *et al*., 2004). A plethora of structurally diverse chemicals interact with the LBD (E-domain), which stimulate conformational changes, enabling dissociation from HSP. The LBD consists of four surfaces: 1) a variable ligand binding pocket (LBP); 2) a dimerisation surface through which interaction with partner LBD occurs; 3) a co-regulatory binding surface; and, 4) the ligand dependent transcriptional activation function, AF-2. The AF-2 corresponds to helix 12, the position of which is dependent on ligand binding, and influences the recruitment of coregulators, driving either agonism or antagonism (Heldring *et al*., 2007). Nuclear receptor dimer complexes bind to hormone response elements, which subsequently bind to the DNA promoter or enhancer, while the AF recruit chromatin remodelling enzymes and transcriptional machinery, forming the pre-initiation complex and thereby activating the transcription of target genes.

The nuclear receptor structure and binding information (section 3.1.1), and in particular the detailed interactions of ER (section 3.1.2), aid the assessment of *in silico* NR molecular modelling. Less information is published regarding specific ligand interactions of AR, PR and PPARγ, which may hinder the assessments of their *in silico* counterparts at a molecular level. However, the polymorphisms and isoforms (detailed in sections 2.2 and 3.1), suggest that inter- and intra-species differences in LBD may play a role in EDC susceptibility.

Additional NR signalling complexity resides in the fact that agonists may bind to receptors to produce a full agonistic response, or partial, depending on the relative binding affinity. Partial agonists may also act as antagonists in the presence of a stronger agonist, by competitively binding to the LBD of nuclear receptors (Hotchkiss *et al*., 2008). Functional cross-talk between NR further complicates the EDC biological mechanism. For example, transactivation of PR is reduced by coexpression of ERα, which may be due to 'squelching' or shared coactivators (Tetel *et al*., 2009). EDC toxicology and epidemiology studies (see section 2.1 and 2.2) have focused primarily on single MoAs (e.g. estrogenicity *or* androgenicity), thereby ignoring potential ligand promiscuity, or transactivation variation due to alterations in the concentration of co-regulatory molecules. There are approximately 300 nuclear receptor coregulators (Lonard *et al*., 2007), concentrations of which vary between cells, tissues, organs and individuals. *In vitro* studies suggest that coactivator recruitment is a rate-limiting step in NR-mediated gene transcription, thus, the susceptibility to EDC mediated toxicity, consequent to molecular initiation by nuclear receptor binding, is likely to be highly variable.

While toxicity studies mitigate for natural variation by using selected mutant strains, with fixed diet and age, extrapolating observations to other taxonomic classes may

present significant challenges, due to variations in supporting biological machinery. Furthermore, the importance of chaperones, co-regulatory molecules, transcriptional machinery and phosphorylation state detailed in the literature, condones the inconsistency of epidemiological tools, which attempt to elucidate relationships in highly variable cohorts (i.e. human and wildlife populations). The molecular 'fingerprint' subsequent to toxicant exposure, revealed in comparative genomic analysis and toxicogenomics, may provide detailed MoA information and elucidate DNA sequences and polymorphisms which confer elevated risk; highlighting susceptible individuals and demonstrating the efficacy of toxicological models (Borlak, 2006). Though out of the scope of this study, adoption and validation of toxicogenomic approaches, which combine gene expression, proteomic and metabonomic profiling with bioinformatics and toxicology (Borlak, 2006; Tennant, 2002), may improve the predictive accuracy of human risk assessment by elucidating genomic responses to environmental toxicants.

Having elucidated the foundations of endocrine disruption, from the historical context to basic biological mechanisms, the founding theory of *in silico* and *in vitro* approaches has been introduced. The subsequent chapters aim to evaluate the available *in silico* (Section 4) and *in vitro* (Section 5) tools for EDC hazard characterisation.

4 ENDOCRINE ACTIVITY *IN SILICO*

A plethora of virtual databases (Endocrine Disruptor Knowledge Base), QSAR (T.E.S.T.: The Toxicity Estimation Software Tool; OECD QSAR Toolbox), molecular modelling (VirtualToxNet) and decision tree (binary classification models) approaches, have been adopted, to computationally predict toxicity. The term '*in silico*' refers to performance via computer or computer simulation; thus, endocrine disruption *in silico* refers to the prediction of endocrine disruption via computational methods. Endocrine disruption is not a toxic hazard *per se*, but a mechanism by which toxic hazards may be incurred (Section 1.1 and 2.1). The Nuclear Receptors (ER, AR, PR and PPAR) have been identified as important molecular targets for endocrine disruption (Section 3.1). However it is important to note that, QSAR, molecular modelling and decision-trees of NR binding, do not provide sufficient information on *in vivo* cellular implications, neccessary to determine endocrine disruption, or toxic phenotypes (Piparo & Worth, 2010).

The purpose of this study is not to provide a detailed review of the *in silico* tools available, but rather to explore the theory and application of molecular modelling and dynamics, to EDC hazard characterisation. The *in silico* methods developed herein may provide useful mechanistic information, for EDC hazard characterisation, but are not able to determine the toxic potencies required for risk assessment.

4.1 The theory of *in silico* Nuclear Receptor Binding

The toxicological and/or pharmacological activity of a compound is dependent on the spatial arrangement and electronegativity[31] of atoms, and their subsequent interaction with endogenous biological systems. The interaction between a hormone and a receptor located in or on the target cell, forms the first step in classical genomic endocrine responses (see section 3.1). Subsequently, characterisation of these molecular initiation events has formed an important area for understanding the mechanisms that govern the responses of target cells to hormones (Walters *et al*., 1998).

Computational chemistry can characterise the dynamics, energetics and structure of these ligand-receptor interactions (Taft *et al*., 2007). Advances in computer-aided drug design (CADD) technologies, provide a solid foundation for *in silico* toxicological study. This is particularly true with regards to receptor-mediated toxicity, in which systems biology has been used to identify ligand binding domains (LBDs) and pharmacophores (the group of atoms in a xenobiotic substance responsible for biological action). LBD's are encoded by DNA, thus based on the assumption that proteins related in sequence share similar structure (Dolan *et al.*, 2008), the biological function and '*global*' similarities of proteins can be

[31] Electronegativity is the tendency of an atom to attract bonding pairs of electrons, group 17 elements or halogens (fluorine, chlorine and bromine) are typically very electronegative, as are oxygen and nitrogen. Electronegativity is also indicative of polarity, due to the unbalanced distribution of electrons between components of a bond.

estimated by sequence and structural homology (Taft *et al.*, 2007) – thereby justifying taxonomic ranking and phylogenetic approaches.

Computational ligand-receptor interactions can be estimated by molecular mechanisms which provide static structures, or by molecular dynamics which incorporate an approximation of bond rotation and degrees of freedom (i.e. induced fit). There are a variety of molecular dynamics-based docking programs available to predict ligand-receptor interactions and docking. For example, QXP (quick explore) uses a superposition force field to automatically assign short-range attractive forces to similar atoms in different molecules in combination with Monte Carlo flexible docking estimations (Kövesdi *et al*., 1999; McMartin and Bohacek, 1997), while ICM (Internal Coordinate Molecular) dynamics models conformations based on empirical force fields with fixed degrees of freedom (Mazur *et al*., 2009). However, such timely physicochemical estimates of bond energy and orbital theory may be considered out of the scope of this study. Evolutionary methods for docking which incorporate genetic algorithms are utilised by software such as: PRO-LEADS (Murray *et al*., 1999); GOLD (Jones *et al*., 1997); and, AutoDock (Morris *et al.*, 1998), which use Gaussian functions to fit and score the ligand into the LBD. Scoring usually represents an approximation of the binding free energy of the ligand-LBD complex by a sum of electrostatic and van der Waals interactions (Taft *et al*., 2007; Paulsen & Anderson, 2009). Genetic algorithm docking models provide a quick and easy tool to screen for ligand-receptor interactions, and may be more efficient in the investigation of endocrine nuclear receptor binding (Wu *et al*., 2010).

A number of recent studies have utilised these computational techniques to predict endocrine receptor binding. In 2010, Wu *et al*. assessed the interspecies variation of androgen receptor binding *in silico*; the MODELLER 9V7 program was used to construct homology models of LBD's collected from the Swiss-Prot Database[32], and the Tripos™ SYBYL Surflex-Dock program (see Section 4.3.5) scored binding affinities of six model EDCs (Nonylphenol; Butyl benzylphthalate, BPA, 4,4'-Dichlorodiphenyldichloroethylene; 2,2'4,4'5,5'-Hexabromodiphenyl ether, Linuron) and Testosterone. Cluster analysis demonstrated that the predicted binding affinities were species specific, consequent to variations in LBD hydrogen bonding, corroborating a number of *in vivo* and *in vitro* ecotoxicology studies, which compared the binding of EDCs to ARs (Wells and Van Der Kraak, 2000; Wilson *et al*., 2007). In a more recent study, Kojima *et al*. (2011) characterised the human pregnane X receptor (hPXR) and mouse PXR (mPXR) agonistic activity of 200 pesticides *in silico*, demonstrating significant interspecies variation, thereby increasing the uncertainty of the mouse model when deciphering human risk.

However, in order for a predictive *in silico* model to be a useful alternative method, extrapolating the results to a defined endpoint must be possible (OECD, 2009). Currently the reliability[33] and relevance[34] of *in silico* tools is uncertain (section 2.3.4.1), hindering validation attempts and regulatory acceptance (section 2.3). The OECD published validation

[32] Available at: http://www.uniprot.org/
[33] The intra- and inter- laboratory reproducibility of results
[34] Provides meaningful and useful information for a particular purpose

criteria for QSAR development in Guidance Document No. 69. There are a number of ER QSAR tools which estimate receptor binding based on the steric hindrance and presence of phenolic hydroxyl groups (section 2.4.3; OECD, 2012). However, QSAR studies for other nuclear receptors (AR, TR, PR etc.) are limited, and the OECD toolbox is not capable of identifying all potential EDCs, adding importance to the research conducted herein. Regarding the OECD principles of alternative methods, *in silico* prediction of nuclear receptors-ligand binding dynamics is concordant with the expected chemistry *in vivo*, i.e. the physical interaction of the ligand and the receptor. Consequently *in silico* binding has the potential to provide a useful alternative to *in vitro* binding studies, as a representation of the molecular initiating event *in vivo*.

4.2 *In silico* Methodology

The *in silico* research aims to elucidate the interaction between potential EDCs and nuclear receptors, while evaluating the potential variability of NR. Homology modelling works under the premise that protein sequence is indicative of protein function, thus, differences in sequence homology may indicate differences in protein function. Phylogeny explores the evolution of genetically related organisms, assuming that similar sequences derive from a common ancestor. Assessment of protein homology and phylogeny, therefore, provides a tool to visualise the fundamental inter- and intra-species differences in molecular machinery (i.e. NR).

Basic Local Alignment Search Tool (BLAST) can be used to infer the functional and evolutionary relationship of proteins, in addition to detecting orphan receptors and gene families. Originally devised by Altschul *et al.* (1990), BLAST identifies regions of local similarity between nucleotide or protein sequences and calculates the statistical significance of matches. BLOSUM 62 (Blocks of Amino Acid Substitution Matrix) is a scoring method devised by Henikoff and Henikoff utilised by BLAST, which effectively aligns evolutionary distinct gapped sequences (Durbin *et al*., 1998). Results obtained using the NCBI BLAST tool, can subsequently be analysed in Molecular Evolutionary Genetic Analysis (MEGA); an integrated tool for conducting sequence alignment, estimating rates of molecular evolution, inferring ancestral sequences and phylogenetic trees and testing evolutionary hypotheses. Agglomerative/hierarchical clustering can be conducted using Unweighted Pair Group Method with Arithmetic mean (UPGMA) or Neighbour-joining, the latter of which assumes a constant rate of evolution (unsuitable for the current study). Created by Naiton & Nei, Neighbour-joining clusters protein sequence data; starting with an unresolved tree, with topology corresponding to a start network, formula iterates repeatedly over the data until all of the branch lengths are known.

With regard to virtual screening, Tripos ® SYBYL software for '*molecular modelling from sequence through lead optimisation*' is a commercially available bioinformatics tool for small molecule modelling and simulation, macromolecular modelling and simulation, lead identification and molecular design. SYBYL 7.3 provides "*unique, competitive advantages"* in 3D Quantitative Structure Activity Relationships (3D QSAR), cheminformatics, ligand-based virtual screening and docking. *'Surflex-Dock couples a unique scoring function with a*

patented state-of-the-art search engine. The combination has been shown to yield excellent results in terms of docking accuracy and distinctly superior results in terms of screening enrichment'[35].

Surflex-Dock incorporates an empirical scoring function based on the Hammerhead docking system, with negative training data and a surface-based molecular similarity search engine, to accurately score ligand-receptor interactions. Scoring functions are derived from QSAR binding affinity data and negative training data to increase credibility and reduce false positives. Surflex-Dock can quickly score a library of ligands against a protein structure, predicted either in SYBYL or with experimentally defined X-ray crystal structures. Independent validation studies have identified Surflex, GLIDE and GOLD docking techniques as the most successful tools, in accurately ranking known inhibitors, in virtual screening experiments (Verdonk *et al*., 2003; Kellenberger *et al*., 2004; Warren *et al*., 2006). However, small variations in protein proton positions lead to large variations in SYBYL performance, highlighting the importance of protein preparation and local optimisation (Jain, 2007).

4.3 *In silico* Materials & Methods

This section details the materials and methods utilised for the evaluation of *in silico* methods. The assessment of NR mechanisms in this study, were split into two core stages: (1) analysis of relevant NR sequence phylogeny; and (2) ligand-docking and virtual screening. Adopted methods for NR phylogeny modelling; chemical prioritisation for virtual screening; NR molecular modelling; molecular docking (virtual screening); and, methods of statistical analysis, are detailed in the following sub-sections.

4.3.1 *In silico* Software and Programs

The software, programs and databases used in the *in silico* method are listed in Table 4.1. As detailed in sections 4.1 and 4.2, a number of bioinformatics tools have been developed in response to high-throughput screening requirements. However, the use, reliability and scope of *in silico* methods remain uncertain. Funded (US and EU) public biotechnology information hubs (UniProt, ZINC, ChemSpider, PubChem and PDB) were used in combination with commercial Tripos™ SYBYL molecular modelling software, to evaluate endocrine activity in a cost-effective manner.

4.3.2 Nuclear Receptor Phylogeny Modelling

Reviewed human estrogen (ER: P103372), androgen (AR: P10275), progesterone (PR: P06401) and Peroxisome Proliferator Activated Receptor-γ (PPARγ: P37231) protein sequences, downloaded from UniProt (Table 4.1), were used to initiate BLAST homology searches. The BLAST search parameters were set at: BLOSUM62 Matrix; No filtering; and, Gapped with a 250 hit cap (Wu *et al.*, 2010). To mitigate for potential human bias, NR

[35] SYBYL Brochure

sequences from other taxonomic Classes, identified in the first search, were also used to initiate BLAST. Retrieved nuclear receptor sequences (Table 2 and Table 3) were renamed to reflect common species names and exported into active MEGA5 data files. The nuclear receptors retrieved for the estrogen receptor and androgen receptor are detailed (accession number, latin and common name) in Table 4.2 and Table 4.3 respectively. Accession numbers with an asterisk (*) denote sequences of functional receptor mutations linked to endocrine disorders. Due to limited verified inter-species sequence publications, PR and PPARγ were excluded from the phylogenetic analysis.

Table 4.1 Software and Programs for *in silico* Approaches

Table shows the information hubs, databases and software used in the method adopted for *in silico* evaluation of nuclear receptor homology/phylogeny, molecular modelling and virtual screening. Excluding the commercial Tripos™ SYBYL® software, which requires a license, all tools are free. [All accessed 11.11.14]

Software Tool	Functionality	Available from
UniProt Knowledge Base	UniProt, a collaboration between the European Bioinformatics Institute (EMBL-EBI), Swiss Institute of Bioinformatics (SIB) and Protein Information Resource (PIR), provides a hub of annotated functional protein information, capturing core data on amino acid sequence, protein name, taxonomy and citations.	http://www.uniprot.org/blast/
BLAST (Altschul *et al*., 1990)	Basic Local Alignment Search Tool (BLAST) identifies regions of similarity between biological sequences. Assessing the statistical significance of nucleotide or protein sequence homology, BLAST, can be used to infer functional and evolutionary relationships.	http://blast.ncbi.nlm.nih.gov/Blast.cgi
MEGA (Tamura *et al*., 2011)	Molecular Evolutionary Genetics Analysis (MEGA v.5) integrates tools for sequence alignment, phylogenetic analysis, data mining, ancestry inference and estimating divergence.	http://www.megasoftware.net/
MUSCLE (Edgar, 2004)	The Multiple Sequence Comparison by Log Expectation (MUSCLE) program aligns multiple sequences with accuracy and speed, exceeding CLUSTALW.	http://www.drive5.com/muscle/
ZINC[12] (Irwin *et al*., 2005)	Created by the Shoichet Laboratory at the University of California, ZINC is a free-database of over 35 million purchasable compounds in 'ready-to-dock' 3D formats.	http://zinc.docking.org/
ChemSpider RSC	Royal Society of Chemistry (RSC) ChemSpider is a free chemical structure database with annotations on over 32 million structures.	http://www.chemspider.com/
PubChem US NCBI	National Center for Biotechnology Information (NCBI) PubChem provides information on the biological activities of small molecules within the Entrez information retrieval system.	https://pubchem.ncbi.nlm.nih.gov/
PDB RCSB	Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) provides an information portal to biological macromolecular structures, containing over 104371 structures.	http://www.rcsb.org/pdb/home/home.do
Tripos ™ SYBYL® 7.3 / X CERTARA	SYBYL is a molecular modelling tool for high-throughput screening and optimisation, aiding structure and ligand based design, cheminoformatics and protein modelling.	http://www.certara.com/products/molmod/sybyl-x

Table 4.2 ER Sequence Accession Numbers, Latin Taxonomy and Common Name

Table shows the retrieval information for estrogen receptor sequences, identified by BLAST homology searches, assessed for phylogenetic relationships (section 4.4.1).

ER Protein Accession	Latin Name	Common Name
P03372, Q92731,	*Homo sapiens*	Human
XP002817538,	*Pongo abelii*	Sumatran orangutan
XP003311596,	*Pan troglodytes*	Chimpanzee
XP003811543,	*Pan Paniscus*	Pygmy chimpanzee
XP001097228,	*Macaca mulatta*	Rhesus monkey
ABY64728,	*Gorilla gorilla*	Western gorilla
Q97171	*Callithrix jacchus*	White-tufted-ear marmoset
ABY64719, ABY64730	*Hylobates lar*	Common gibbon
ABY64722, ABY64731	*Colobus guereza*	Mantled Guereza
AEY83591	*Rhinocerous unicornis*	Greater Indian Rhinoceros
Q9TV98	*Equus ferus caballus*	Horse
P49884, Q9XSB5	*Bos taurus*	Bovine
Q29040, Q9XSW2	*Sus scrofa*	Wild boar
Q9TU15	*Ovis aries*	Sheep
Q9QZJ5	*Mesocricetus autatus*	Golden hamster
AAY53653, ADD91583	*Microtus ochrogaster)*	Prairie vole
P06211, Q62986	*Rattus norvegicus*	Brown rat
P19785, Q08537	*Mus musculus*	House mouse
BAD08348, BAJ15429	*Alligator mississippiensis*	American alligator
BAE45626	*Crocodylusniloticus*	Nile crocodile
ACF28457	*Lepidochelys olivacea*	Pacific ridley
BAJ15428	*Elaphe quadrivirgata*	Japanese striped snake
BAE79505	*Eublepharis macularius*	Leopard gecko
BAH01724	*Torgos tracheliotos*	Lappet-faced vulture
P06212, Q9PTU5	*Gallus vulgaris*	Reg junglefowl
ADK26789	*Zonotrichia albicollis*	White-throated sparrow
Q91250	*Taeniopygia guttata*	Zebra finch
Q9PVE2	*Sturnus vulgaris*	Common starling
O93511	*Zonotrichia albicollis*	Japanese quail
BAJ05025	*Cynops pyrrhogaster*	Japanese firebelly newt
P81559, BAG31997	*Xenopus laevis*	African clawed frog
AAJ71194	*Xenopus tropicalis*	Western clawed frog
AAL82743	*Acanthopagrus schegeliii*	Japanese black porgy
O42132	*Pagrus major*	Red seabream
P50241, NP001098172	*Oryzias latipes*	Japanese medaka
P16058, P57782	*Oncorhyunchus mykiss*	Rainbow trout
Q9YHZ7, Q9IAK1	*Ictalurus punctatus*	Channel catfish
BAF99812	*Cyprinus carpio*	Common carp
P57717, AAK16742	*Danio rerio*	Zebra fish
P57781	*Micropoogonias undulates*	Atlantic croaker
O13012	*Anguilla japonica*	Japanese eel
Q9W6M2	*Sparus aurata*	Gilthead seabream
Q9W669	*Carassius auratus auratus*	Goldfish

Table 4.3 AR Sequence Accession Numbers, Latin Taxonomy and Common Name

Table shows the retrieval information for androgen receptor sequences, identified by BLAST homology searches, assessed for phylogenetic relationships (section 4.4.1). * indicates mutated androgen receptor sequences.

AR Protein Accession	Latin Name	Common Name
Q25AW7	*Brachytarsomys albicauda*	White-tailed antsangy
Q25AW8	*Macrotarsomys ingens*	Greater big-footed mouse
Q25AW3	*Cricetomys gambianus*	Gambian pouched rat
Q25AW1	*Otomys angoniensis*	Angoni vlei rat
Q25AW6	*Myodes glareolus*	Bank vole
Q25AW5	*Calomyscus mystax*	Great balkham hamster
PA5207	*Rattus norvegicus*	Brown rat
P19091	*Mus musculus*	House mouse
Q25AX1	*Spalax ehrenbergi*	Middle east blind mole rat
B0BF02	*Sciurus vulgaris*	Red squirrel
Q25AV9	*Cavia porcellus*	Guinea pig
Q25AV2	*Hapalemur simus*	Great bamboo lemur
Q25AV4	*Lemur catta*	Ring-tailed lemur
Q25AU8	*P. deckenii coronatus*	Crowned sifake
O97776	*Eulemur fulvus collaris*	Collared brown lemus
Q25AU6	*Tarsius bancanus*	Horsefield's tarsier
Q2MDI3	*Micropotamogale lamottei*	Nimba otter shrew
Q2MDH5	*Limnogale mergulus*	Web-footed tenrec
Q2MDH3	*Amblysomus hotentotus*	Hottentot golden mouse
Q2MDI5	*Elephas maximus*	Asian elephant
G3TXN4	*Loxodonta Africana*	African bush elephant
D211X7	*Ailuropoda melanoleuca*	Giant panda
Q9TT90	*Canis familiaris*	Dog
Q8MIK0	*Crocuta crocuta*	Spotted hyena
Q2MDK0	*Felis catus*	Cat
Q2MDJ7	*Suricata suricata*	Meerkat
Q9GKL7	*Sus scrofa*	Wild boar
F1N2B6	*Bos Taurus*	Bovine
A4LAN9	*Saimiri boliviensis*	Black-capped squirrel monkey
E3SWD5	*Callithrix jacchus*	Common marmoset
Q6QT55	*Macaca mulatta*	Rhesus macaque
O97775	*Pan troglodytes*	Chimpanzee
P10275, D5M8Q2*	*Homo sapiens*	Human
P49699	*Oryctolagus cuniculus*	European rabbit
E0D582	*Elaphe quadrivirgata*	Japanese striped snake
E0D581	*Protobothrops flavoridis*	Habu snake
Q2VP86	*Taeniopygia guttata*	Zebra finch
Q2ACE0	*Gallus gallus*	Red jungleowl
P70048	*Xenopus laevis*	African clawed frog
F6W9U4*	*Xenopus tropicalis*	Western clawed frog
D4AFY1	*Glandirana rugosa*	Japanese wrinkled frog
Q7T1K4	*Rana catesbeiana*	Bullfrog
E1U2A4	*Raja erinacea*	Little skate
E1XUC9	*Anguilla anguilla*	European eel
B9P3Q7, F1R0F2, A4GVF3	*Danio rerio*	Zebrafish
Q8QFV2	*Carassius auratus*	Goldfish
Q918F5	*Pimephales promelas*	Fathead minnow
O93245, O93244	*Oncorhynchus mykiss*	Rainbow trout
O93497	*Pagrus major*	Red seabream
Q66VR6	*Micropogonias undulates*	Atlantic croaker
D1MV73, Q76LM5	*Oryzias latipes*	Japanese medaka
Q06I32, Q5NU07	*Gambusia affinis*	Mosquitofish

Phylogenetic analyses were conducted using distance based phenetic[36] methods, in which phenograms (dendograms) were calculated from sequence similarity. Sequences were aligned using MUSCLE software (Table 4.1) to produce a distance matrix, clustering branches by successively linking the taxa with minimal distances. Bootstrapped (500 replicates) phenograms of alignments were constructed using the Neighbour-Joining algorithm. The phenogram presents genetic change as horizontal lines, which represent evolutionary lineages and the branch lengths are proportional to amino acid substitutions per site (0.05 scale represents 5% difference between species). Phenogram sum branch lengths, defined as the phylogenetic diversity (PD) of the assembled sequence data (Faith, 1992), were stated as a measure of evolutionary divergence since the most recent common ancestor (Chao *et al*., 2010). Phenograms were labelled and colour coded according to taxonomic clusters identified according to UniProt KB annotations. Species taxonomy was compared with phenogram topology to assess whether evolutionary distinct species present more sequence divergence, and potentially functional differences.

4.3.3 Chemical Prioritisation for *in silico* Virtual Screening

A database of potential EDC's for virtual screening was assembled by the following methods. The EDC definition adopted for EDC classification is stated in section 4.3.3.1, while prioritisation methods, for construction of a chemical database, are detailed in section 4.3.3.2.

4.3.3.1 EDC Definition

A lack of scientific concordance regarding the significance of apical endpoints, has hindered the term *'model EDC'*, complicating prioritisation methods. For the purpose of this study, the WHO/IPCS EDC definition has been adopted (see section 2.3.2). Classified as a working definition by the Community Strategy for Endocrine Disruptors and acknowledged by OECD member states as a top-level definition, the scientific criteria are applicable to both human and ecological hazard and risk assessment. For chemicals with inconclusive *in vivo* data, but mechanistic *in silico* or *in vitro* evidence, the term 'potential endocrine disruptor' has been used.

4.3.3.2 Prioritisation Method for *in silico* Assessment

A basic prioritisation process was utilised to identify chemicals for *in silico* screening and subsequent *in vitro* assessment. The aim of prioritisation was to identify chemicals economically and scientifically relevant to EDC screening, while ensuring the inclusion of data rich chemicals with relevance to UK regulation. A chemical database (n=378) was curated from the prioritisation lists reported in section 2.4.1; 166 chemicals from the EC candidate list and 241 from the DWI report, of which 36 were EC duplicates, in addition to 9 hormones.

[36] Phenetics (taximetrics) is the classification of organism based on overall similarity.

To enable a crude assessment of sensitivity, published bioassay data for each of the 378 chemicals, were retrieved from PubChem (Table 4.1). Full details of the chemical database, summarising the published high-throughput agonism and antagonism *in vitro* testing results, are presented in 6.1Appendix A; Table_A 1. Collating information from ChemSpider, PubChem and ZINC[12], Table_A 1 also summarises the classification, usage and assumed primary endocrine mode-of-action. For the purposes of analysis, agonists and antagonists were assumed positive, while compounds without positive *in vitro* data were treated as unknowns. Thus, limited in data source, due to the vast number of chemicals investigated, the reliability of the *in silico* sensitivity (%), approximated in section 4.4.3, is intrinsically linked to the reliability of PubChem *in vitro* bioassay publications. Due to the epistemological problems of identifying 'negative' activity, specificity was deemed outside the scope of this study.

Three-dimensional structures of prioritised chemicals were downloaded from ZINC[12], the freely available database of >35 million commercially available 3D chemical structures, in Mol2 format (Irwin *et al.*, 2005).

4.3.4 SYBYL Molecular Modelling

In silico molecular modelling aims to generate 3D computational representations of receptor binding domains, which can be used to characterise the dynamics, energetics and structure of potential EDC ligand-receptor interactions (see sections 3.1 and 4.1).

4.3.4.1 Nuclear Receptor Crystal Structure Selection

It is possible to predict tertiary protein structure from sequence information. However, methods are caveated and the accuracy of molecular modelling is greatly enhanced by using X-ray and NMR protein crystallography information.

The reliability of the *in silico* screen is linked to the reliability of NR modelling, thus, for the purpose of this study, only proteins with known crystal structures were included, as detailed in Table 4.4. The protein structures for NRs previously identified of interest (ER, AR, PR, and PPARγ) were downloaded into SYBYL 7.3 in .pdb format, and saved as *mol2* files.

4.3.4.2 Protein Preparation

X-ray crystallography selectively favours the protein conformations most likely to crystalise (Srinivasan *et al.*, 2013). Subsequently, most structural X-ray crystallography studies of NR are dimers with bound ligands. However, ligand binding occurs in NR LBD monomers, stabilised by HSP, dimerisation occurring consequently (sections 3.1 and 3.2). Subsequently, the cofactors and ligands bound to NR protein structures identified in Table 4.4 were removed to represent the biological scenario.

Table 4.4 Protein Database NR X-ray Crystallography Accession Information

The receptor type, species, PDB accession number, ligand and any bound coregulators in addition to the publication, of all the X-ray crystal structures used in this *in silico* analysis are detailed.

Receptor	Species	Identifier	Reference
Oestrogen Receptor alpha (ERα)	Human ERα LBD	3DT3 Ligand: GW368	Fang *et al.* (2008) Synthesis of 3-alkyl naphthalenes as novel estrogen receptor ligands. *Bioorg. Med. Chem. Lett.* 18: 5075-5077.
	Human ERα LBD	2QZO Ligand: WAY1669916 Coregulator: NCO2	Brunning *et al.* (2010) Coupling of receptor conformation and ligand orientation determine graded activity. *Nat. Chem. Biol.* 6 (11): 837-843.
	Human ERα LBD	2IOK Ligand: Antagonist*	Dykstra *et al.* (2007) Estrogen receptor ligands. Part 16: 2-Aryl indoles as highly subtype selective ligands for ERalpha. *Bioorganic and Medicinal Chemistry Letters* 17: 2322-2328.
	Human ERα LBD	IX7R Ligand: Genistein Coregulator: SRC-1	Manas *et al.* (2004) Understanding the selectivity of genistein for human estrogen receptor-beta using X-ray crystallography and computational methods. *Structure* 12: 2197-2207.
Oestrogen Receptor beta (ERβ)	Human ERβ LBD	1X7J Ligand: Genistein Coregulator: SRC-1	Manas *et al.* (2004) Understanding the selectivity of genistein for human estrogen receptor-beta using X-ray crystallography and computational methods. *Structure* 12: 2197-2207.
	Rat ERβ LBD	1HJ1 Ligand: ICII64,384	Pike *et al.* (2001) Structural insights into the mode of action of a pure antiestrogen *Structure* 9: 145.
Androgen Receptor (AR)	Human AR LBD	3V49 Ligand: SARM I1	Nique *et al.* (2012) Discovery of diarylhydantoins as new selective androgen receptor modulators. *J. Med. Chem* 55: 8225-8235.
	Chimp AR LBD	1T7T Ligand: DHT Coregulator: FxxFF motif	Hur *et al.* (2004) Recognition and accommodation of the androgen receptor coactivator binding interface. *Plos Biol.* 2: E274-E274.
	Rat AR LBD	2IHQ Ligand: N-AHBH**	Sun *et al.* (2006) Discovery of potency, orally-active, and muscle-selective androgen receptor modulators based on an N-aryl-hydroxybicyclohydantoin scaffold. *J. Med. Chem.* 49: 7596-7599
	Mouse AR LBD	2QPY Ligand: 4HY/DHT	Estébanez-Perpiña *et al.* (2007) A surface on the androgen receptor that allosterically regulates coactivator binding. *Proc. Natl. Acad. Sci. USA* 104: 16074-16079.
Progesterone Receptor (PR)	Human PR LBD	1SQN Ligand: Norethindrone	Madauss *et al.* (2004) Progesterone receptor ligand binding pocket flexibility: crystal structures of the norethindrone and mometasone furoate complexes. *J. Med. Chem.* 47: 3381-3387.
PPARγ	Human PPARγ LBD	1PRG	Nolte *et al.* (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* 395: 137-143.
* Ligand: N[(1R)-3-(4-hydroxyphenyl)-1-methylpropyl]-2(2-phenyl-1H-indo-3-yl)acetamide ** Ligand: N-aryl-hydroxybicyclohydantoin			

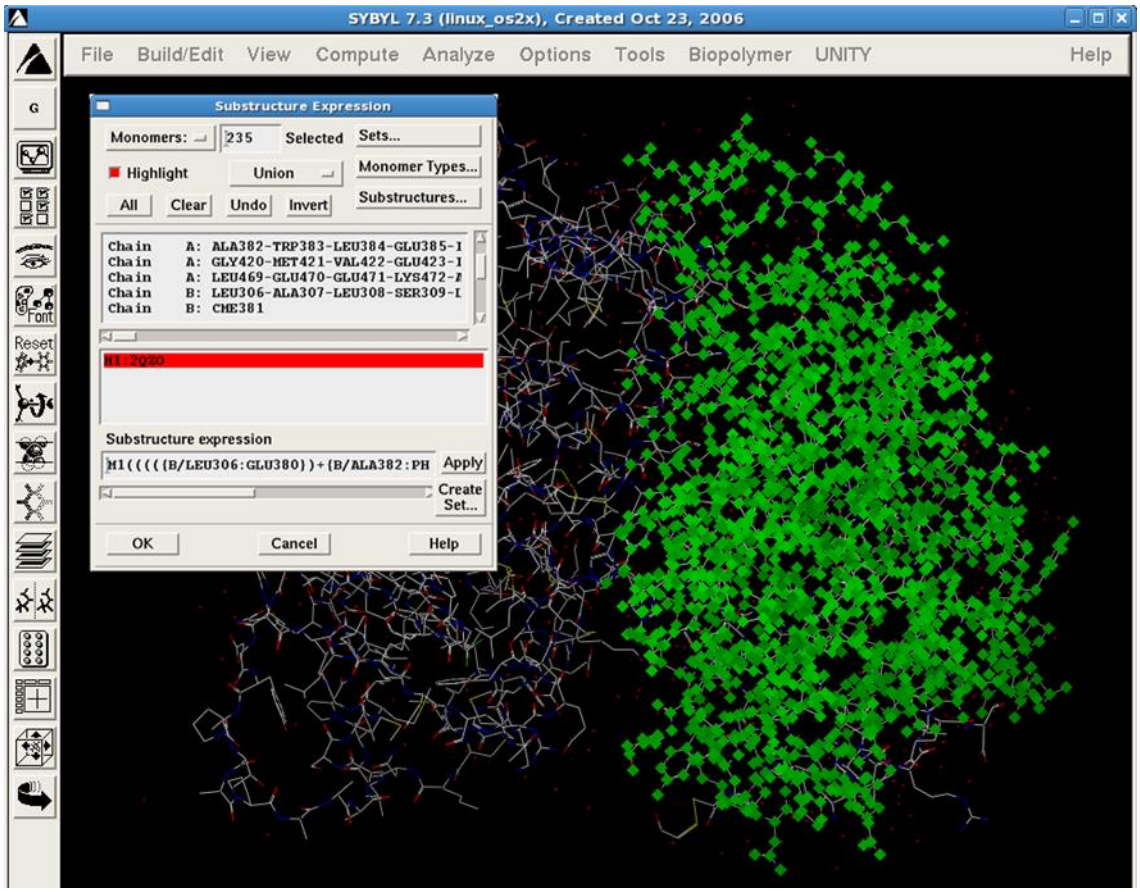


Figure 4.1 SYBYL NR Protein Preparation

This screen-shot shows the SYBYL interface and display screen. Subsequent to ligand binding NR's dimerise. However, ligand binding occurs in monomers, consequently one of the monomers should be removed for docking studies. Removal can be achieved under the Build/Edit>>Delete>>Substructure Expression function. The monomer unit highlighted in green was deleted.

Removal of NR monomers, for accurate SYBYL Surflex-Dock simulations, was achieved using the Build/Edit>>Delete>>Substructure Expression command (Jain, 2007). Figure 4.1 shows the SYBYL interface, with one of NR monomers highlighted in green. NR LBD monomers were prepared via the interface command: Biopolymer>>Prepare structure>>Structure Preparation Tool, at which point unrecognised atoms were renamed[37], hydrogens were added and the energy constraints of sidechain, backbone and termini positions were checked (highlighting energetically unfavourable regions).

4.3.4.3 SYBYL SiteID LBP Molecular Model

The monomeric structures of the LBDs, predicted as per 4.3.4.2, represent a number of sub-domains (see section 3.1.1.1 and Table 3.1), many of which do not play a direct role in ligand binding. The PDB publishes ligand binding pocket (LBP) annotations for a number of the NR-superfamily; however, the detail is variable and often predicted on *in silico* homology modelling. Thus, to enable evaluation of less characterised NR, the SYBYL 7.3 (Table 4.1) SiteID application was used to infer the LBP

[37] Based on structural constraints, i.e. 4 bonds = carbon atom.

SiteID adopts flood-fill solvation techniques to locate potential hydrogen bonds, calculate solvent accessible surface area and determine hydrophobic pocket volume and depth; the protein is solvated with a single layer of water, thereby identifying pockets by counting the number of non-hydrogen atoms (75 spheres), within a specified radius (default 8Å) (Ho *et al*., 1990). As standard, the ligand binding pocket identified is filled with solvent spheres, surrounding residues are coloured yellow and the exposed atoms are coloured red. SYBYL 7.3 interface commands were: Biopolymer>Analyse protein>SiteID Find Pockets. The accuracy of the SiteID predictions were assessed relative to PDB annotations and the published literature summarised in section 3.1.

4.3.5 SYBYL Surflex-Dock Virtual Screening

Surflex-Dock enables flexible molecular docking by the incorporation of small-molecule force fields, which evaluate Cartesian coordinates[38] constrained by ligand energetics (Jain, 2007, 2003, 2000), supporting dynamic ring flexibility and optimisation of docked ligand poses. Surflex-Dock was used to create a novel virtual NR-binding screen, using the proteins detailed in section 4.3.4, to identify endocrine active substances for more conclusive *in vitro* testing, and further investigation. The Surflex-Dock virtual screening method was split into three main categories: database preparation; Surflex docking; and, post-processing.

4.3.5.1 Database Preparation & Minimisation

Energy is a function of atomic coordinates. Minimisation generates coordinates which correspond to a minimum energy; abiding to the chemical bonding theory that stipulates net inter-atomic forces should near zero (Peng *et al*., 1996). Jain (2007) stated that "*Completely ignoring the strain issues of input ligands results in docking performance worse than random*". Thus, the minimisation of ligand databases is essential for Surflex-Dock performance. Table 4.5 shows the preliminary binding scores of NR receptors with randomly selected chemicals from prioritised database, minimised using default SYBYL Powell and Simplex methods, with a termination gradient of 0.05 kcal/mol and maximum iterations of 100 (default settings).

Repeating the simulation (R2), the standard deviations of scores generated under the same Surflex-Dock parameters ranged from 0.01-1.66 ($-\log(K_d)$). Such large variance between binding scores leads to uncertainty and overlap of the *in silico* NR binding scores; thereby reducing significance.

[38] Cartesian coordinates detail the point at which perpendicular lines, measured in the same unit, meet (i.e. ordered pair, [0.0]).

Table 4.5 Surflex-Dock Score Variation using Default Minimisation for Ligand Database

The Standard Deviation (SD) of NR-ligand scores for randomly selected chemicals in the Database, generated in runs of SYBYL Surflex-Dock under the same parameters, showed variation ranging from 0.01–1.66 -log(K_d), questioning the applicability of the default minimisation settings. R1=Replicate 1, R2=Replicate 2

Chemical Name		ERa 3DT3	AR 3V49	AR 1T7T	AR 2QPY	PR 1SQN	PPARγ 1PRG
		Score	Score	Score	Score	Score	Score
4-t-Butylphenol	R1	3.13	6.04	3.61	3.66	4.16	3.69
	R2	3.60	3.69	3.37	3.69	3.73	3.57
	SD	0.33	1.66	0.17	0.02	0.30	0.08
						Average SD	0.429
Bis(4-Hydroxy phenyl) methane	R1	3.72	4.67	5.17	4.29	4.88	4.47
	R2	3.85	4.42	4.99	3.80	4.65	4.59
	SD	0.09	0.18	0.13	0.35	0.16	0.08
						Average SD	0.16
DDT	R1	2.36	1.16	-0.88	0.38	2.49	2.98
	R2	1.76	1.17	-1.28	0.86	2.98	1.97
	SD	0.42	0.01	0.28	0.34	0.35	0.71
						Average SD	0.3524

To reduce the output variability of results generated herein, the chemical database (n=378) was minimised to 10,000 iterations, 0.001 kcal/mol termination gradient and T. Giegmesiter Huckle Energy, using a novel minimisation script (289.new1.spl)[39]. Using this script, chemicals in the database were minimised closer to zero and replications of the simulation showed no variance in binding score (SD 0.00 -log(K_d)) (Table_A 1). All Surflex-Dock screens were run on three occasions (day repeats). Chemical properties, including molecular_weight (Da), CLogP (logarithm of the $P_{octanol/water}$ partition coefficient) total_area ($Å^2$) and molecular_volume ($Å^3$), assimilated in SYBYL Molecular Spreadsheets, were exported.

4.3.5.2 SYBYL Surflex-Dock Ligand-Receptor Docking (Sybyl 7.3/X)

Surflex-Dock inputs must be protonated as expected at physiological pH, including non-polar hydrogens (protonation state strongly affects docking). Proteins and ligands were prepared as per sections 4.3.4.2 and 4.3.5.1., additional protein checks were conducted in the Surflex application: Surflex-Dock>Protein structure>Mol2 File>prepare.

Surflex-Docking requires a ligand, protomol and protein. A protomol (pseudo-molecule or docking target) consists of molecular probes (CH_4, C=O and N-H) which provide an object-orientated framework for prototyping novel algorithms for molecular dynamics (Matthey *et al.*, 2004; Balaji *et al.*, 2013), or more simply a representation of the binding cavity to which putative ligands are aligned. The protomol intends to mimic the ideal interactions of the '*perfect ligand*' to the protein active site (Jain, 2007).

[39] Script created by Tripos® SYBYL for Kal Karim (kk256@le.ac.uk) Leicester University Department of Chemistry, College of Science and Engineering.

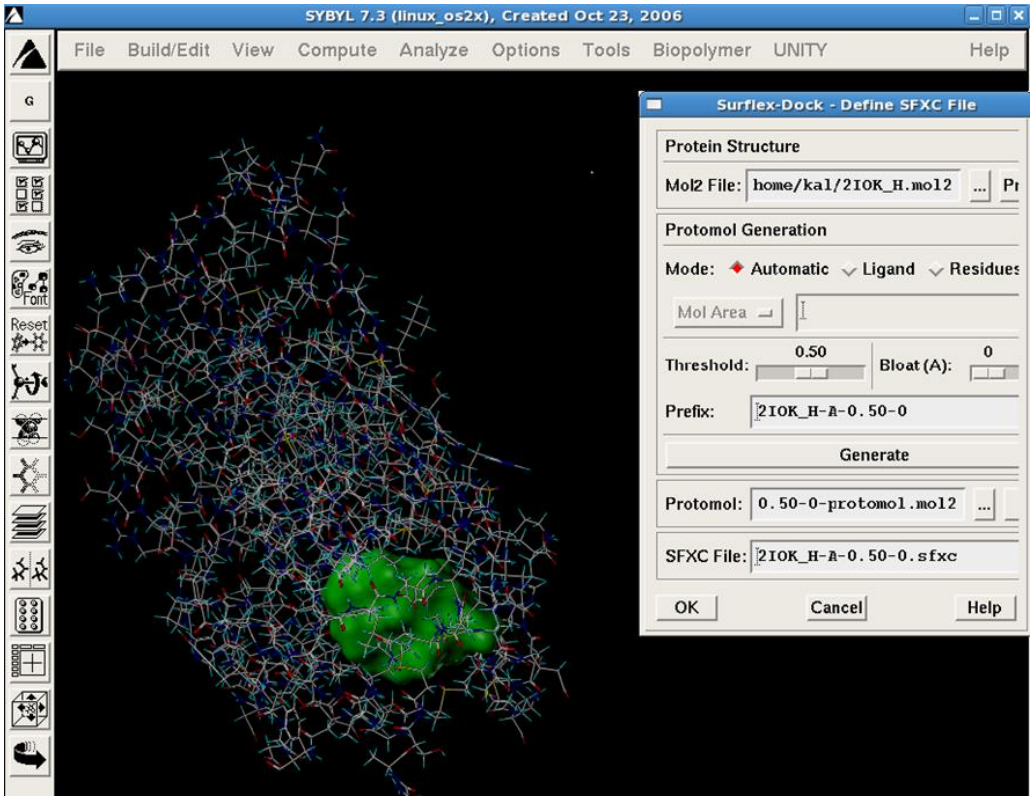


Figure 4.2 SYBYL Surflex-Dock Protomol Generation for Virtual Screening
The NR LDB monomer is visualised in stick & line format (white = carbon; navy = nitrogen; cyan = hydrogen; and, red = oxygen), while the protomol generated in Surflex-dock is represented as a glomerular green mass.

There are three methods available for protomol generation: automatic, ligand-based and residue based. When generating a protomol using a ligand to specify the active site, the voxels[40] occupied by the ligand are explored by the protomol by default, even if they are not highly buried**.** However, as detailed in sections 3.1 and 4.3.4.1, the LBP of NR are notoriously large and promiscuous, consequently generating the protomol using endogenous hormones (e.g. E2) could bias docking scores towards ligand similarity, rather than propensity to bind with the receptor. Generating the protomol from LBP residues, identified in PDB sequence annotations, would limit the virtual screening to data-rich NR.

NR LBD protomols were generated using Surflex's automatic function, in which the largest hydrophobic pocket of the receptor is identified using the solvation method detailed previously (section 4.3.4.3). Default values of 0.5 for Threshold[41] and 0 for bloat[42] were used. The impact of bloat on protomol generation is shown in Figure_Apx 2. Figure 4.7 shows the SYBYL Surflex interface for protomol generation, highlighting the protomol in green and

[40] A voxel (volume pixel) is the 3D counterpart of a 2D pixel. In computer-based modelling a voxel is an array of elements of volume that constitute a notional three-dimensional space (i.e. representing a value on a regular grid in 3D space).
[41] Threshold is a factor ranging from 0-1 determining the penetration the protomol into the protein – increasing the threshold, decreases the volume of the protomol. Low thresholds greatly increase the computational demands of protomol generation.
[42] The bloat inflates the protomol to include nearby crevices; see Figure_Apx 2.

showing the spatial location of the hydrophobic pocket within the ligand binding domain. Generated protomols were saved as SFXC files.

Default docking parameters were adopted for virtual screening; 0 additional starting conformations, 6Å expanded search, 20 conformations per fragment, up to 100 rotatable bonds and 10 poses per ligand. Enhancements in docking accuracy have been reported when incorporating ring confirmation flexibility and pre- and post- minimisation (Jain, 2007). Therefore, additional flags, including ring flexibility and pre- and post- dock minimisation were added. The Surflex-Dock details interface options are shown in Figure 4.3; the constraints highlighted were used to dock the NR protomols (section 4.3.5.1), for each protein, against the minimised ligand database (section 4.3.3 and 4.3.5.1).

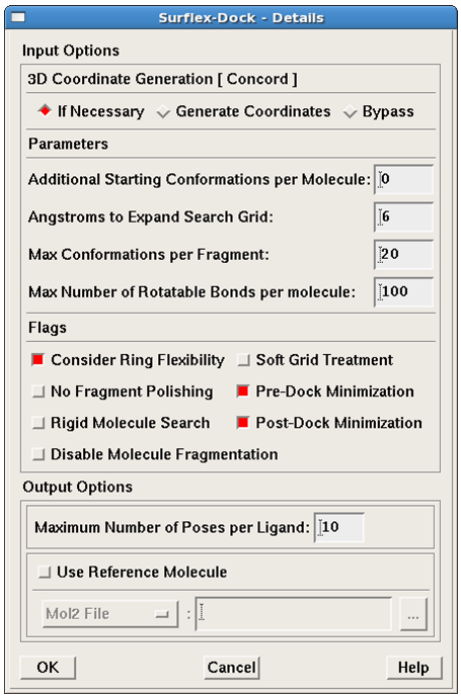


Figure 4.3 SYBYL Surflex-Dock Virtual Screening Parameters
SYBYL Surflex interface shows parameters undertaken in docking studies, default parameters; 0 additional starting conformation, 6Å expanded search, 20 conformations per fragment, up to 100 rotatable bonds and 10 poses per ligand. However, in addition to this flags, such as ring flexibility and pre- and post- dock minimisation were included.

4.3.5.2.1 Surflex-Dock Results Processing

Surflex-Dock employs an empirical scoring function, combining Hammerhead and Böhm approaches, which consider hydrophobic contact, polar interactions and entropic fixation costs for loss of torsional, translational and rotational degrees of freedom (Böhm, 1994; Eldridge *et al*., 1997). Contrary to other scoring functions, Surflex-Dock finds the nearest local optimum, rather than restricting calculations to a precise pose (Jain, 2007, 1996).

Two scores were provided for each docked conformation: and affinity ($-\log(K_d)$) and a crash score (pK_d units). The crash score represents the degree of inappropriate penetration into the protein by the ligand, in addition to the internal steric hindrance of the ligand – in other words unfavourable energy states. Crash scores that are nearing zero are favourable. The Surflex-

Dock Results Browser, Figure 4.4, shows the superimposition of the potential-EDC database (n=378) aligned against the ER protomol. The score presented for each ligand, in the browser interface, represents the highest calculated -log(K_d) value achieved from 10 poses of each ligand, irrespective of crash score. Scores were highlighted, saved and exported in all formats (mol2, SDF, HTML, MSS).

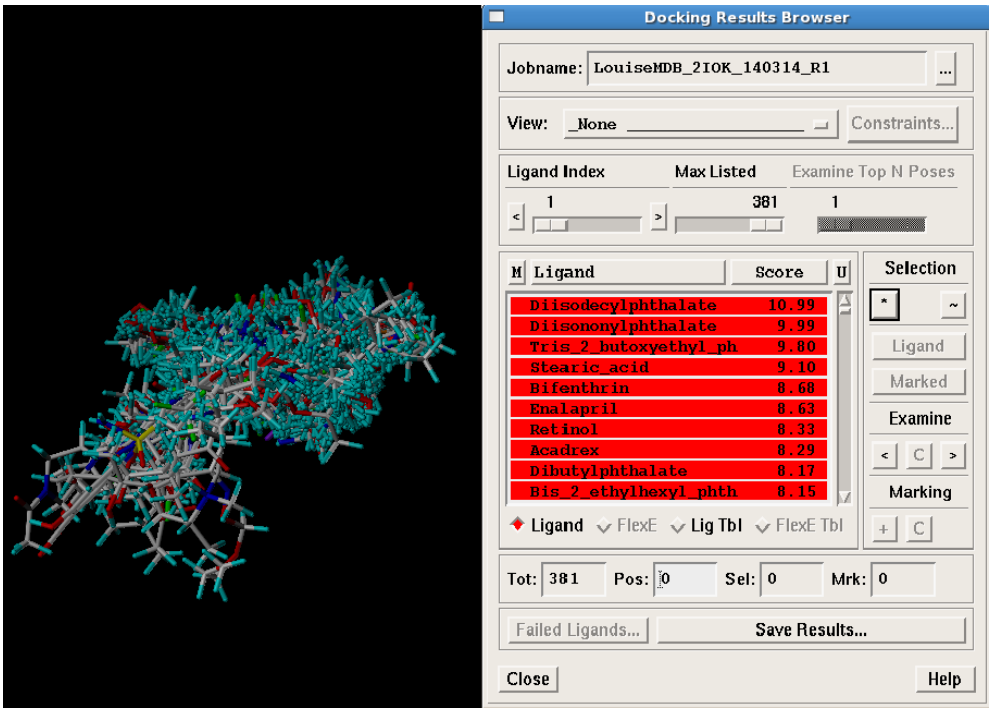


Figure 4.4 SYBYL Surflex-Dock Results Browser
Image shows the database of potential EDC ligands aligned (3 duplicates, n=378), as per the constraints determined by the protomol. The Surflex-Docking browser is on the left, all results were highlighted, saved and exported in mol2, SDF, HTML and MSS formats.

4.3.6 Statistical Methods

Phylogeny was identified using consensus bootstrapped Neighbour-Joining analysis. Bootstrapping enabled estimation of sampling distribution (Ader *et al*., 2008), by creating new alignments to replace the original dataset, thereby identifying the most probable topology. The number of possible topologies is proportionate to the orthologs under investigation; i.e. the more branches, the more scope for error. To mitigate for the large number of protein sequences (Table 4.2 and Table 4.3), results were bootstrapped to 500 replicates to create a consensus phylogram, presenting the most probable topology; the more times a particular branch point occurred, the more valid the branching point.

A number of epistemological problems hinder the statistical evaluation of virtual screening. Virtual screening is reliant on the ranking of molecular docking and scoring, however, validation and statistical evaluation of *in silico* methods are lacking (Zhao *et al*., 2005). The sensitivity, or efficacy, of identifying NR binders was evaluated by comparing the number of predicted positives, with true positives *in vitro*. However, as a MoA rather than a hazard in itself, endocrine activity may not be identified by *in vivo* animal tests, traditionally

considered a gold-standard. The virtual screen is not predicting endocrine toxicity, but interaction with nuclear receptors that form part of the biological response. Thus, *in silico* true positives must interact with NR, but may not be endocrine disruptive. Consequently, observed *in vivo* endocrine endpoints (toxicity) were not considered in the *in silico* sensitivity analyses, which were evaluated relative to published *in vitro* high-throughput (HTP) bioassay data (Table_A 1). Agonism and/or antagonism of ER(α/β), AR, PR or PPARγ pathways *in vitro*, were considered positive binders. Structurally diverse, the chemical database (n=378) included 118 ER, 111 AR, 6 PR and 74 PPARγ true positives *in vitro* (Table_A 1). Limited true-negative data prevented evaluation of *in silico* specificity.

Regression analysis, a statistical method of inferring relationships among variables, was used to assess the importance of chemical MW, ClogP, total area and molecular volume, on NR binding. IBM SPSS and R statistics software were used to elucidate coefficients and plot vaues, respectively.

4.3.7 Summary of *in silico* Methods

Phenograms of ER and AR sequences (Table 4.2 and Table 4.3) identified using UniProt BLASTp homology searches, were conducted using the MEGA software, to present a crude visualisation of genotypic species variation. X-ray crystallography structures of human ERα (3DT3; 2Q2O; 2IOK; and, IX7R), human ERβ (IX7J), rat ERβ (IHJI), human AR (3V49), chimpanzee AR (IT7T), rat AR (2IHQ), mouse AR (2QPY) and human progesterone (1SQN) and PPAR (1PRG), were modelled *in silico* (Table 4.4). A prioritised chemical database of potential-EDC's (n=378 section 4.3.3) were virtually screened against the *in silico* nuclear receptor LBP models, in Tripos™ SYBYL Surlfex-Dock software. Sensitivity of the *in silico* model was assessed relative to published bioassay results (6.1Appendix A). Regression analysis evaluated associations between *in silico* binding scores and chemical structural features (section 4.3.5.1).

4.4 *In silico* Results & Discussion

Reflective of the materials and methods (section 4.3), the *in silico* results arising from this study are detailed in order of NR phylogeny (4.4.1), molecular modelling (4.4.2) and virtual screening (4.4.3).

4.4.1 Nuclear Receptor Phylogeny

The boostrapped consensus phenogram, Figure 4.5, constructed using Neighbor-joining p-distance amino acid substitution statistical methods, shows the evolutionary divergence of ERα and ERβ sequences, throughout taxonomic classes. The phenogram corroborates the literature on ERα and ERβ (Thornton, 2001) by suggesting gene duplication of a common ancestral estrogen receptor, prior to the divergence to all Chordate. The scale of 0.05 represents 5% difference between two species; calculated as amino acid differences per site. The optimal tree presented (Figure 4.5) has a Σbranch length of 0.4565, indicative of the phylogenetic diversity of the receptor (Chao *et al*., 2010; Faith, 1992).

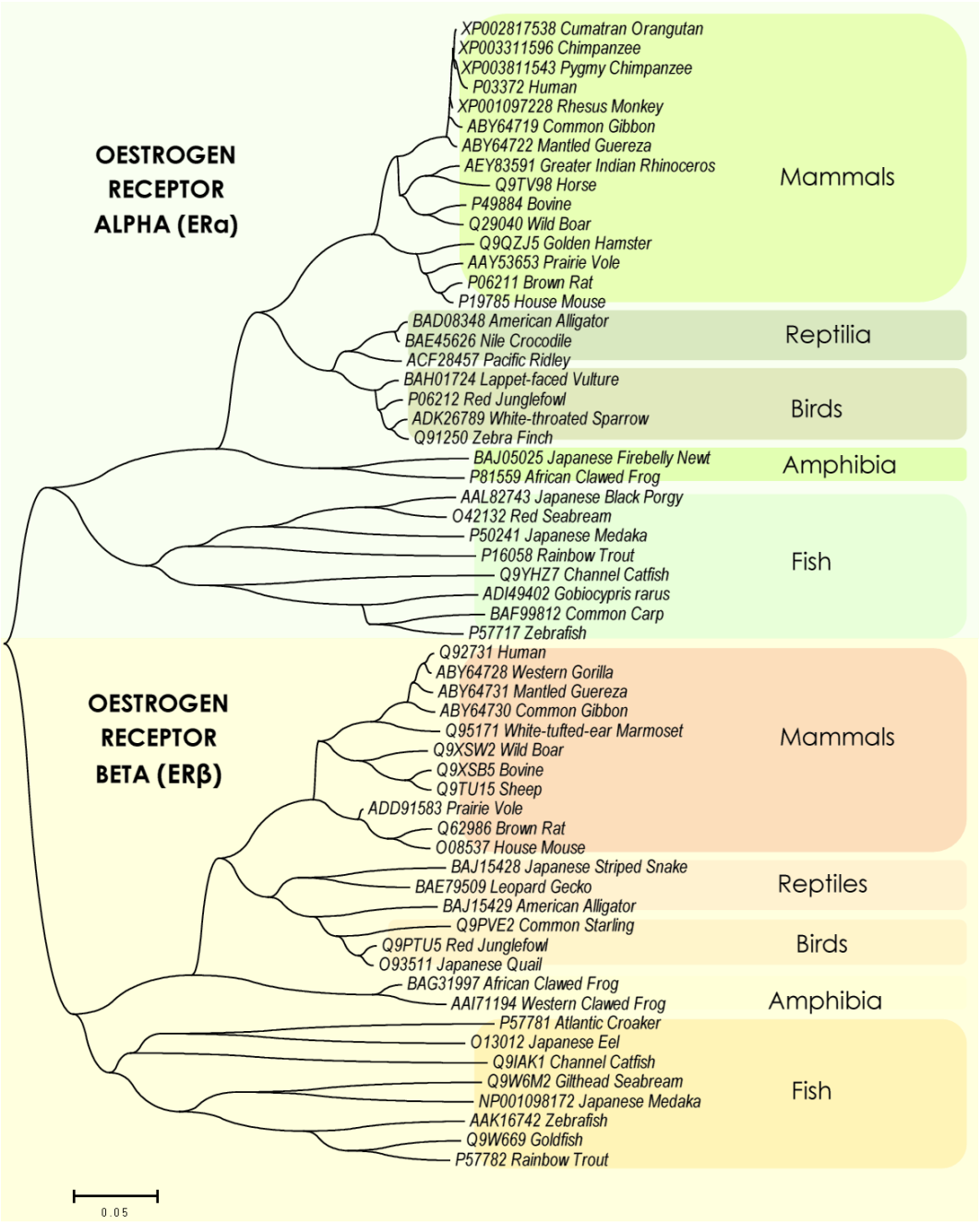


Figure 4.5 Phylogeny of the Estrogen Receptor (ERα/β)
The bootstrap consensus (n=500) unrooted phenogram, constructed in MEGA5, using Neighbor-joining amino acid substitution models (p-distance), homogeneous lineage and pairwise deletion, shows the evolutionary relationship of estrogen receptor sequences. The phenogram identifies the gene duplication of a common ancestral estrogen receptor, prior to the divergence to all Chordate, corroborating the literature on ERα and ERβ (Thornton, 2001; Eick and Thornton, 2010). Assuming protein sequence homology is indicative of function, branch lengths and topology, highlight potential inter- and intra-species differences (labelled as protein accession# and common name). The branch length 0.05 scale represents 5% difference in amino acid sequence between species. Σbranch length = 4.565.

Nuclear receptor phylogeny, presented as the topology, branch length and root, suggests that the variance observed in protein sequence is consistent with taxonomic rank i.e. evolutionary distinct species present more sequence divergence. Thus, assuming protein sequence is indicative of function, uncertainty in extrapolating endocrine toxicological mechanisms may increase with evolutionary distance.

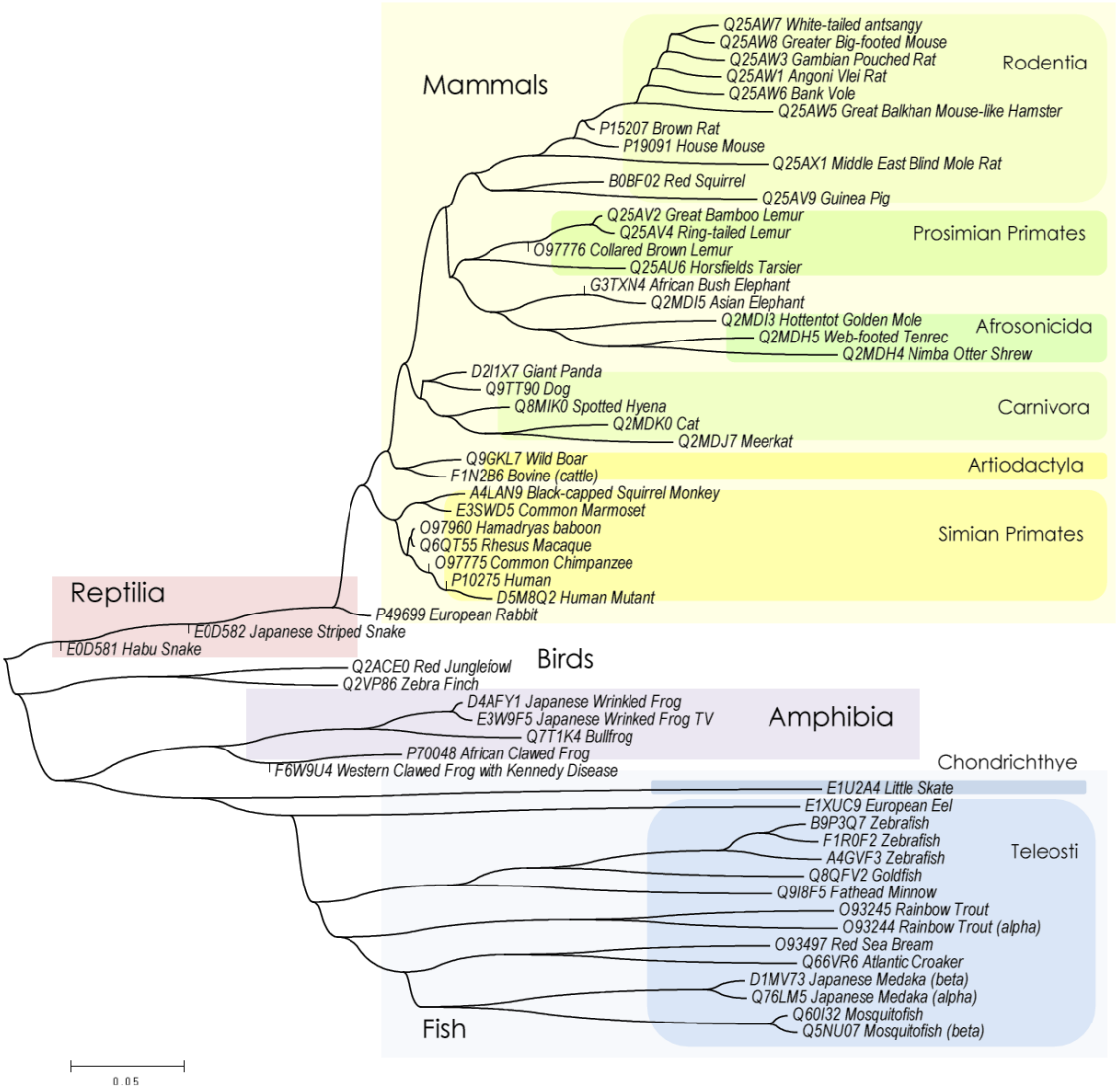


Figure 4.6 Phylogeny of the Androgen Receptor (AR)

The bootstrap consensus (n=500) unrooted phenogram, constructed in MEGA5, using Neighbor-joining amino acid substitution models (p-distance), homogeneous lineage and pairwise deletion, shows the evolutionary relationship of androgen receptor sequences. The topology of the AR sequence phylogeny is consistent with taxonomic ranks, and divergence is clustered into clades as small as the artiodactyla order. Assuming protein sequence homology is indicative of function, branch lengths and topology, highlight potential inter- and intra-species differences (labelled as protein accession# and common name). The branch length 0.05 scale represents 5% difference in amino acid sequence between species. Σbranch length = 4.468.

Conversely, the clustering of ER sequence by class increases the certainty of extrapolating nuclear receptor binding in one mammal to another, as sequences present significant homology (Figure 4.5). The topology presented in Figure 4.6 is consistent with taxonomic ranks; the phylogenetic divergence of AR sequence clustered into clades as small as the artiodactyla order. Thus, the phylogram demonstrates sequence divergence within mammals, which may be indicative of divergence in protein function. The AR phenogram Σbranch length was calculated to be 4.468 (Figure 4.6), which is slightly smaller than that established for the estrogen receptors (Figure 4.5). Interestingly, birds and reptiles, demonstrate relatively short branch lengths, suggesting that their AR are more homologous to the ancestral gene (i.e.

the root). The importance of taxonomic Class, in inferring sequence homology, was further corroborated by a bootstrapped consensus sub-tree of primate AR sequences (Figure_Apx 1). The phenogram Σbranch length = 0.439, suggesting conservation between primate androgen receptors. However, consistent with other NR topologies, sequence clustering predicted infraorder, such as human, simian or prosimian (Figure_Apx 1). The inclusion of mutant hAR sequences, splice variants and polymorphisms in Figure_Apx 1, highlight the potentially significant effect of small changes in sequence, on EDC susceptibility. In agreement with Västermark *et al.*'s (2011) study on genetic predictors of TGCT risk, the limited sequence divergence between AR phenotypes highlights the potential caveats of extrapolating mechanistic observations in one species, to another.

The phenograms (Figure 4.5 and Figure 4.6) corroborate reported interspecies variation in nuclear receptor binding affinity. In particular, the long branch lengths connecting fish species highlight androgen receptor sequence divergence (Figure 4.6), reflected in trout, goldfish and minnow AR transactivation and binding *in vitro* (Lange *et al.*, 2012; Wells & Van Der Kraak, 2000). The potentially significant receptor divergence between fish models presented, compromises the assumptions of regulatory ecotoxicology testing, which predominantly extrapolate findings in *D. rerio*, *P. promelas* or *O. mykiss* to assess environmental risk (Section 2.1.3 and 2.3).

Contrary to the divergence identified, assessment of LBP cDNA sequence homology has identified significant homology between different metazoans (Krust *et al*., 1986; Koike *et al*., 1987; White *et al.*, 1987). The LBP of two metazoan ER subtypes (ERα and ERβ) are near identical; Leu384/Met421 in ERα corresponds to Met336/Ile373 in ERβ (Ascenzi *et al.*, 2006). Figure 4.5 identifies ERα and ERβ ligand binding domain sequence divergence, conflicting with the aforementioned literature. However, as alluded to in Section 1.4, phylogenetic analysis of ligand binding domain variability, may not reflect the ligand binding pocket; conservation of functional regions.

The results obtained in the study reported here suggest that the uncertainty of extrapolation increases with species divergence. Furthermore, the phenograms produced highlight the potential folly of species bias in regulatory toxicology; 75% of *in vivo* toxicological procedures for human health are conducted in rats, while ecotoxicology limits itself to three fish (above), bird (*C. japonica*) and/or collemnolan (*F. fimetaria*) models, representing a very limited assessment of potential inter- and intra- species variation. Reptilia, one of the most under-represented ectotherms in EDC research, presents the most sequence conservation of the ancestral AR (Figure 4.6).

Endocrine disruption appears omnipresent throughout phylogenetic classes, which is reflected by the homology of endocrine machinery. However, no invertebrate sequences were identified in BLAST searches (5 searches, 250 hits; section 4.3.2). The homology of the vertebrate nuclear receptor-superfamily, which presents >60% sequence conservation, led to retrieval of other members prior to retrieval of invertebrate NR-orthologs. The effets of TBT, on mollusc sexual development and population, are considered one of the best documented incidences of ecologically relevant endocrine disruption (Matthiessen *et al*., 1995). The

molluscan intersex was initially attributed to TBT estrogenicity, which spurred concerns regarding vertebrate exposure. However, mechanistic studies later identified significant differences in invertebrate endocrinology, rendering the observed effects incomparable (Scott, 2013). The lack of invertebrate sequence retrieval, suggests large NR sequence divergence, supporting reports that invertebrate and vertebrate endocrine mechanisms are structurally and functionally distinct. The role of terpenoid hormones in insect physiology, morphology and behaviour (Section 2.1.3.1) further stresses the mechanistic diversity of invertebrate endocrinology (WHO, 2002; Ketata *et al*., 2008). Thus, the relevance of the 21 NR genes identified in *Drosophilia melanogaster* flies (Adams *et al*., 2000) and 270 in *Caenorhabditis elegans* nematode worms (Sluder *et al*., 1999), to the 48 NR genes identified in humans (Robinson-Rechavi *et al.*, 2001), remains uncertain.

Throughout taxonomic classes, NR present divergence in the amino acid sequences defining functionality (Figure 4.5, Figure 4.6 and Figure_Apx 1). However, the phylogeny does not consider functional conservation, highlighting the need for molecular modelling and virtual screening to assess structural features.

4.4.2 SYBYL Site ID Nuclear Receptor Molecular Modelling

The interaction between hormones and their target receptors forms the first step in classical genomic endocrine responses (Section 1), which found the understanding of mechanisms that govern cellular responses to hormones. Computational chemistry can characterise the dynamics, energetics and structure of these ligand-receptor interactions (Taft *et al*., 2007). Ligand binding pockets, identified and modelled in SYBYL SiteID, from X-ray crystallography structures, were used to generate hypothetical pseudo-docking targets (protomol). The protomol is fundamental to virtual screening in Surflex-Dock. To identify NR ligand binding pockets for protomol generation, dimers, cofactors and ligands were removed, and the resulting monomer was evaluated via solvation method (See 4.3.4 and 4.3.5). In this section, the efficacy of SYBYL-SiteID solvation method (Section 4.3.4.3), is evaluated relative to the literature (See Section 3.1). Concordance of the *in silico* LBP's to published structural information, supports the use of the computational molecular models to infer binding regions and ligand-binding relationships.

As detailed in Sections 2 and 3, EDC research has predominantly focused on the ER and AR, leading to a wealth of structural information for comparison with *in silico* models (Sections 3.1.2 and 3.1.3). Conversely, the neglect of other NRs, such as PR and PPARγ, hinders direct validation of SYBYL SiteID molecular models. Figure 4.7 shows the 3DT3 human estrogen receptor-α (ERα), rendered in SYBYL according to secondary structure (α-helices and β-sheets), with the Surflex-Dock protomol superimposed. The α-helices form the three-layer antiparallel ridge previously detailed (Jin & Li, 2010; Brzozowski *et al*., 1997). The protomol generated for hERα (Figure 4.7:B) presents many of the structural features quantified in the OECD Toolbox ER-profiler (Section 2.4; Table 2.3). The 'red' oxygens of the protomol reflect the strong and moderate hydroxyl groups required for ER-binding, 'white' carbon regions form the triphenylethylene backbone, while 'navy' atoms identify the NH groups necessary for strong binding (Tong *et al*., 2004; Li & Gramatica, 2010). Thus, the SYBYL

protomol created appears to provide a 3D-molecular model of the 2D-parameters stipulated by the OECD Toolbox ER-profiler.

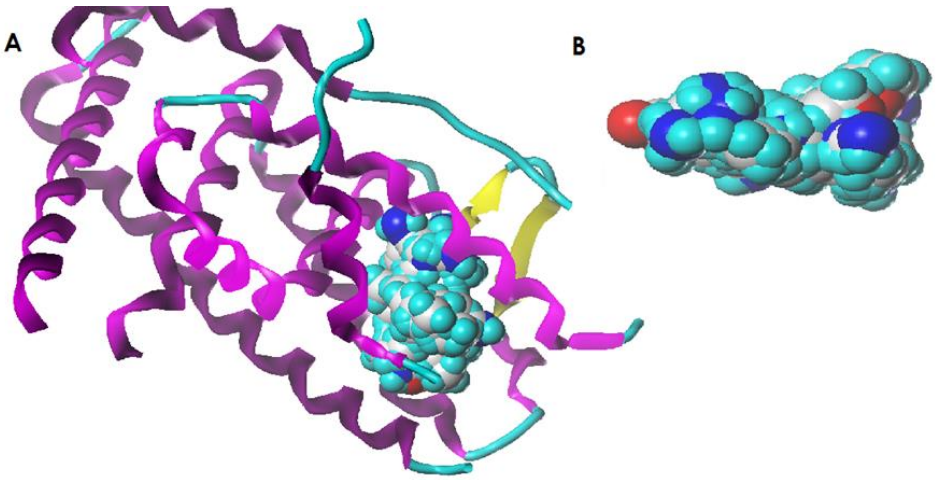


Figure 4.7 Tripos™ SYBYL 3D-Molecular Modelling of Estrogen Receptor-α (ERα 3DT3) Monomer and Surflex-Dock Protomol

A shows the rendered secondary protein structure of 3DT3 in SYBYL; α-Helices are shown as magenta coils, while β-sheets are as yellow arrows. The binding region (superimposed protomol) is represented as a spacefil model. Generated using Surflex-Dock default settings – 0.5 Threshold and 0 Bloat – the protomol is colour coded by atom: white = carbon; navy = nitrogen; cyan = hydrogen; and, red = oxygen. B' shows the protomol separated from the protein.

To enable closer inspection of SYBYL solvation method efficacy in predicting docking regions (see Sections 4.3.4.3 and 4.3.5.2), residues within 8Å of the 3DT3 ERα solvent spheres are highlighted in Figure 4.8, for comparison with residues identified in Section 3.1.2. Functional groups and classification of amino acid residues are presented in 6.1Appendix D: Figure_Apx 10. The hydrophobic core of the agonist ERα LBD conformation is formed by α-helices 3 (αH3: Met342-Leu354), αH6 (Trp383-Arg394), αH8 (Val418-Leu428), αH11 (Met517-Met528), αH12 (Leu539-His547) and S1/S2 hairpin (Leu402-Leu410) (Brzozowski *et al*., 1997). Of the 24 residues highlighted in Figure 4.8, twelve correspond to structural features reported in the literature (Section 1): Leu349 and Ala350 residues of αH3; Leu384, Ille386, Leu387, Met388, Ile389, Gly390, Leu391 and Trp393 of αH6; and, Leu403 and Phe404 of the S1/S2 hairpin. The SYBYL molecular modelling identified Glu353 and Arg394 residues (Figure 4.8) which form hydrogen bonds to phenolic hydroxyl agonists, with carboxylate and guanidinium groups, respectively. The A-ring of E2 has been demonstrated to pose between the Ala350, Leu387 and Phe404 residues, also highlighted (Brzozowski *et al*., 1997).

Furthermore, in DES studies, higher affinity has been accredited to non-polar contact of phenolic ethyl groups with Ala350, Leu384, Phe404 and Leu428 (Shiau *et al*., 1998; Kuiper *et al*., 1997), all of which are highlighted in Figure 4.8. In light of the concordance, it can be concluded that SYBYL software accurately identified the ligand binding pocket of ERα, using the solvation method.

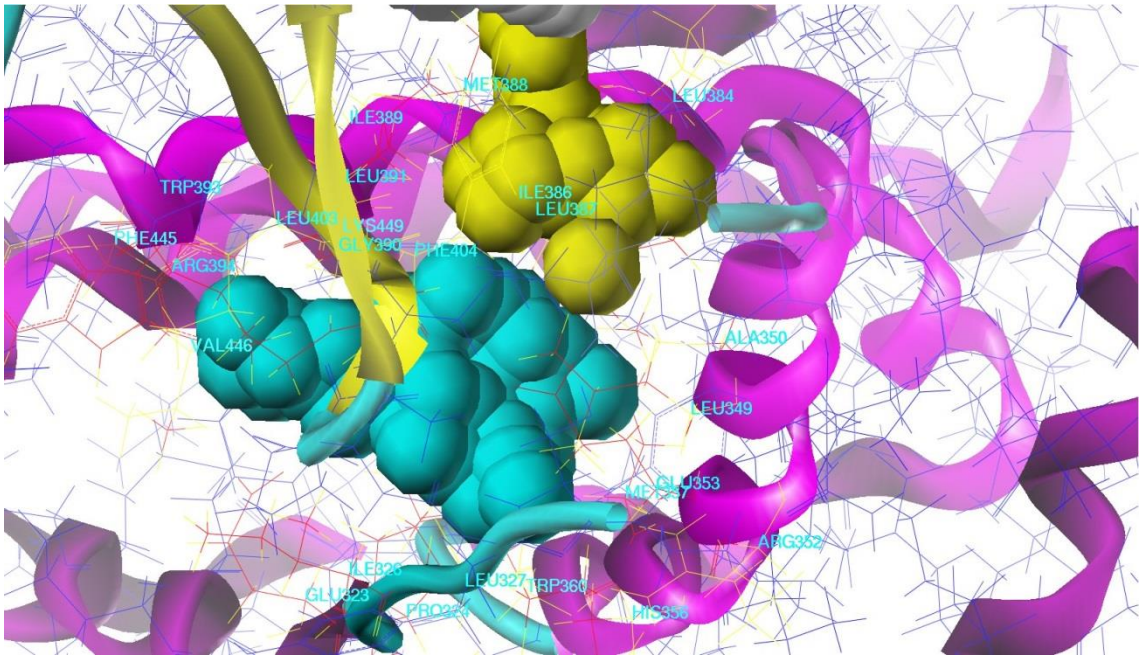


Figure 4.8 SYBYL SiteID Human Estrogen Receptor (3DT3) Ligand Binding Pocket

The Human ERα (3DT3) monomer SYBYL ribbon representation, identifying α-helices and β-sheet secondary structure, was originally complexed to the GW368 agonist (5-(4-hydroxyphenoxy)-6-(3-hydroxyphenyl)-7-methylnaphthalen-2-ol) for X-ray crystallography. Stick-representation of the 3DT3 molecular backbone (blue) highlights residues surrounding the solvent spheres yellow and exposed atoms red. Residues identified within 8Å of the SiteID solvents spheres are labelled aqua; including: Pro224, Leu327, Arg352, Glu353, His356, Met337, Leu349, Ala350, Trp360, Leu384, Ile386, Leu387, Met388, Ile389, Gly390, Leu391, Trp393, Arg394, Leu403, Phe404, Phe445, Val446, Lys449 and Glu623.

The SYBYL SiteID model for $_{human}$ERβ and $_{rat}$ERβ are shown in Figure_Apx 5. Residues identified in the $_{human}$ERβ (1L2J) ligand binding pocket: Lys401, Leu339, Glu305, Thr299, Leu298, Glu276 and Met295. Solvent spheres in the $_{rat}$ERβ LBP identified residues Ile331, Tyr352, Phe311, Arg301, Gly297, Met295, Val293, Met291, Leu254 and Leu253. The hERα ligand binding site contains 4 residues with hydrophobic side chains (Ala350, Leu384, Phe404 and Leu428), an arginine with a positively charged NH_2 side group (Arg394) and a negatively charged glutamic acid residue (Glu353) (Shiau *et al*., 1998; Brzozowski *et al*., 1997). As shown in Figure 4.5, the ERα and ERβ paralogs arose subsequent to gene duplication, in the chordate common ancestor. However, the sequences have diverged, complicating direct comparison. Nevertheless, conservation of the ligand binding pocket may be expected as both receptors bind the endogenous hormone 17β-estradiol (E2). Interestingly, although the human and rat ERβ vary from human ERα, both of the identified LBPs (Figure_Apx 5) contain 2 leucine residues, a positively charged arginine or lysine and residues with hydrophobic side chains (Met295 in $_{human}$ERβ and Ile331, Phe311, Met295, Val293 and Met291 in $_{rat}$ERβ); highlighting requirements for conservation of function.

Figure 4.9 shows the hydrophobic ligand binding pocket of the androgen receptor E-domain identified. Consistent with ERα, the antiparallel docking site consists of αH8, αH9, αH11 and αH12 (Sack *et al*., 2001; Brzozowski *et al*., 1997). Of the 21 residues highlighted in Figure

4.9, Gln711, Met745 and Arg752 have been shown to interact with the DHT A-ring, while Leu704 interacts with the DHT C-ring (Sack *et al*., 2001). The replacement of Thr877 with alanine (mutation T877A) is the most frequent mutation observed in prostate cancer patients *in vivo* and in LNCaP cells *in vitro* (Taplin *et al.*, 1995; Stevens *et al*., 1996). Identified using the *in silico* molecular model (Figure 4.9), Thr877 plays a regulatory role in the LBP; substitution with alanine enables the LBP to accommodate other ligands such as progestins, estrogens and cortisol (Sack *et al.*, 2001). Chimpanzee (*P. troglodytes*), rat (*R. norvegicus*) and mouse (*M. musculus*) AR LBP's were also evaluated using SYBYL SiteID Solvation method (Figure_Apx 6 and Figure_Apx 7). However, as previously alluded to, validation of the molecular models is hindered by uncertainty of animal NR LBP interactions.

The SYBYL SiteID model for $_{chimp}$AR, $_{rat}$AR and $_{mouse}$AR are shown in Figure_Apx 5 and Figure_Apx 7. Residues identified in the $_{chimp}$AR ligand binding pocket: Pro682, Gln711, Val715, Leu744 and Met745. Solvent spheres in the $_{rat}$AR LBPs identified residues Gln711, Val715, Arg752, Ala748 and Met745. Residues Leu701, Leu704, Asn705, Gly708, Val715, Met742, Met745, Val746, Ala748, Arg752, Phe764 and Leu873 were identified in the $_{mouse}$AR. All AR LBP contained a residue with an uncharged polar side chain (Gln/Asn) and at least two residues containing a hydrophobic side chain (one residue of methionine or alanine and a leucine), consistent with the literature (Sack *et al.*, 2001).

In addition, a valine residue at 715 was conserved between all investigated AR, suggesting that the hydrophobic-side chain amino acid is important to function. The Thr877 residue which determines ligand promiscuity in human AR (Sack *et al*., 2001; Taplin *et al*., 1995) was not detected in the chimp, rat or mouse AR LBP, which supports the intra- and iner-species differences detailed in Sections 0 and 1 at a molecular level. Whether the slight differences in LBP, translate into differences in ligand binding, is evaluated in Section 4.4.3.

The accuracy of the SYBYL solvation method in determining the ER and AR LBP validates the method (Section 4.3.4) and adds weight to the residues identified for protomol generation in PR and PPARγ LBP molecular modelling; detailed in 6.1Appendix C: Figure_Apx 8 and Figure_Apx 9. Furthermore, the accuracy of the SYBYL solvation method demonstrated herein supports the Surflex-Dock virtual screening, which is reliant on chemical database minimisation and *in silico* modelling of the protomol and protein.

The results demonstrate that Tripos™ SYBYL 7.3 software can 'blindly' predict the LBP of nuclear receptors, using the solvation method to identify hydrophobic cavities and interacting residues, in lieu of structural information and annotation, which is requisite for traditional QSAR approaches. Section 4.4.3 SYBYL X-Surflex-Dock *in silico* Virtual Screening, utilises the aforementioned *in silico* LBP's to screen the potential-EDC chemical database (n=378).

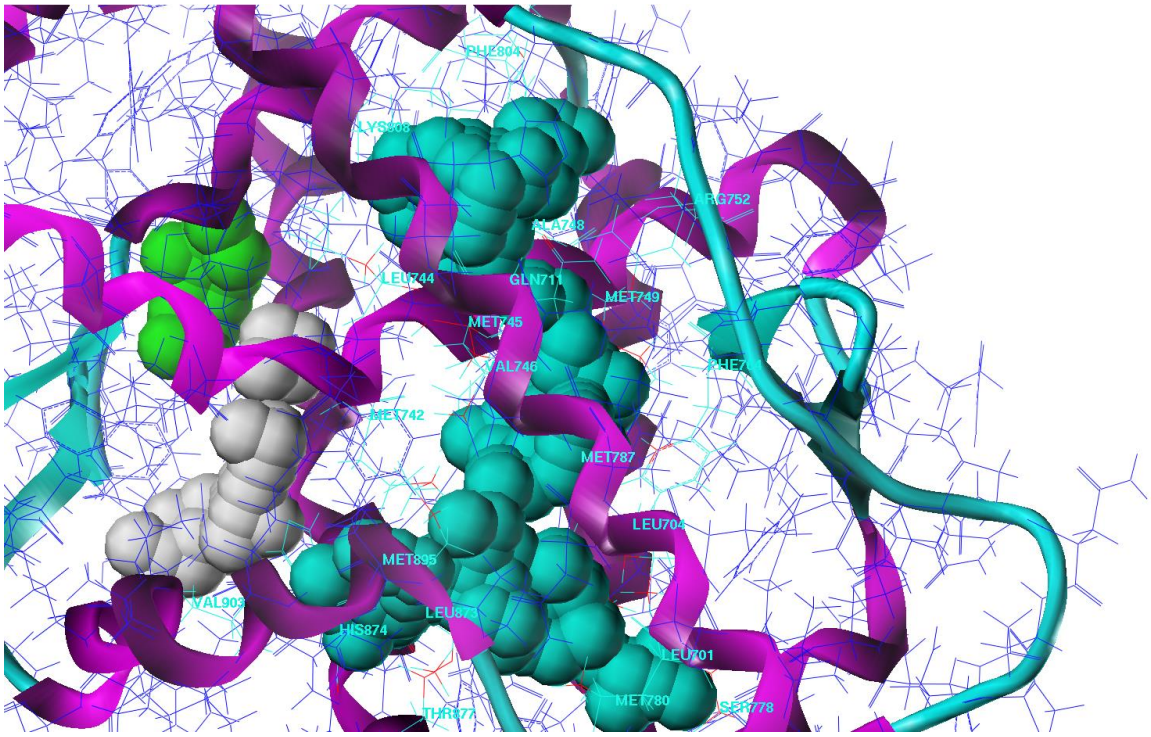


Figure 4.9 SYBYL SiteID Human Androgen Receptor (3V49) Ligand Binding Pocket

The Human AR (3V49) monomer ribbon representation in SYBYL, identifying α-helices and β-sheet secondary structure, was originally complexed to the PK0 selective AR modulator 4-[(4R)-4-(4-hydroxyphenyl)-3,4-dimethyl-2,5-dioxoimidazoidin-1-yl]-2-(trifluoromethyl) benzonitrile for X-ray crystallography. Stick-representation of the 3V49 molecular backbone (blue) highlights residues surrounding the solvent spheres yellow and exposed atoms red. Residues identified within 8Å of the SiteID solvents spheres are labelled aqua; including: Leu701, Leu704, Gln711, Met742, Leu744, Met745, Val746, Ala748, Met749, Arg752, Phe764, Ser778, Met780, Met787, Phe804, Lys808, Leu873, His874, Thr877, Met895 and Val903.

4.4.3 SYBYL X-Surflex-Dock *in silico* Virtual Screening

Evaluating the Cartesian coordinates constrained by ligand energetics, Tripos™ SYBYL software enables flexible and dynamic molecular modelling and screening *in silico* (Jain, 2007). This section details the results of screening NR ligand binding pockets modelled in SYBYL (Section 4.3.4 and 4.4.2), against a prioritised list of potential EDC's (n=378, Section 4.3.3) in Surflex-Dock. The complete *in silico* binding data, for $_{h}$ER (3DT3), $_{h}$ER (1X7J), $_{rat}$ER (1HJ1), $_{h}$AR (3V49), $_{chimp}$AR (1T7T), $_{rat}$AR (21HQ), $_{mouse}$AR (2QPY), $_{h}$PR (1SQN) and $_{h}$PPARγ (1PRG), is presented in 6.1Appendix E: Table_Apx 2. The scores represent the highest calculated -log(Kd) value achieved from 10 poses of each ligand, irrespective of the crash score, which denotes unfavourable penetration of the ligand.

As detailed in the method (Section 4.3), a number of epistemological problems hinder the statistical evaluation of virtual screening (Zhao *et al*., 2005). The sensitivity of SYBYL Surflex-Dock in correctly identifying NR binders was evaluated by comparing the number of *in silico* predicted positives with true positives. The *in silico* analyses were evaluated relative to published *in vitro* high-throughput (HTP) bioassay data (Table_A 1); agonism and/or antagonism of ER(α/β), AR, PR or PPARγ pathways *in vitro*, were considered positive binders. Structurally diverse, the chemical database (n=378) included 118 ER, 111 AR, 6 PR

and 74 PPARγ true positives *in vitro* (309 TP total: Table_A 1); however, epistemological limitation in categorising a "true-negative" hindered evaluation of *in silico* specificity. PubChem Bioassay identifiers (AID#) which can be used to retrieve results are detailed in 'List of Prioritised Endocrine Disrupting Chemicals' Table_A 1.

The results were categorised as follows: true positive (TP); positive *in silico* (P_{is}); false negative (FN); and, negative *in silico* (N_{is}). Additionally, a category for predicted low-affinity binders was included to prioritise positive results: unfavourable binding *in silico* (FUB_{is}); and false unfavourable binding (FUB).

4.4.3.1 SYBYL Surflex-Dock Sensitivity and Specificity

Sensitivity[43] pertains to the proportion of correctly identified true positives, while specificity[46] details the number of non-binders correctly identified by the tool. Assessing human predictivity relative to *in vitro* binding and transactivation bioassays, SYBYL Surflex-Dock Nuclear Receptor (ER, AR, PR & PPARγ) virtual screening demonstrated a sensitivity of 94.5% ([309 TP/ (309 TP + 18 FN)] x100 = %). Of the 309 true positives, 291 chemicals were correctly identified, 18 were incorrectly scored as non-binders, and 21 were identified as low-affinity (6.8% of TP scored 0>2 –log(Kd)) (Table_Apx 2).

The difficulty of 'proving' inactivity was highlighted during the evaluation of *in vitro* HTP bioassay data (Table_A 1); assessments were limited to active data, thereby ignoring the proportion of inactive and inconclusive results. For example, Endosulfan has been detected as positive in 3 of 16 AR *in vitro* bioassays (19%), consequently, Endosulfan was considered positive for the purpose of this study. However, the uncertainty in the published *in vitro* data increases the uncertainty of the sensitivity analysis conducted herein. Classifying chemicals as true negatives on comparable numbers of inactive results may be erroneous. Subsequently, the strength of *in vitro* MoA data is uncertain and prevents categorisation of chemicals as non-binders, thereby limiting specificity[44] analyses. The lack of true negatives' prevents the estimation of false positives, which can also be used to calculate precision[45] and specificity[46]. Nevertheless, a crude maximum specificity of 80.7% was calculated, by assuming the absence of reported activity as inactivity ([75 TN/ (75 TN + 18 FN)] x100 = %).

Evaluating SYBYL Surflex-Dock predictability, by receptor class, suggested $_{h}$ERα virtual screening sensitivity of 97.5% ([118 TP/ (118 TP + 3 FN)] x100 = %); correctly identifying 115 true positives and incorrectly scoring 3 chemicals as non-binders. Ninety-four percent of the correctly identified TP had *in silico* binding scores >2 –log(Kd); 7 true positives were identified as low affinity *in silico* (unfavourable binders 0<2 –log(Kd)). Of the 378 chemicals screened, 360 were shown to interact with the hERα LBP *in silico* (TP, P_{is}, UFB, UFB_{is}), while 18 presented repellent energy states (N_{is} non-binders) (Table_A 1Table_Apx 2). The human androgen receptor ($_{h}$AR) presented sensitivity of 88.1% ([111 TP/ (111 TP + 15 FN)]

[43] Sensitivity (true positive rate (TPR)) = true positives / (true positives + false negatives)
[44] Specificity (true negative rate (TNR)) = true negatives / (true negatives + false negatives)
[45] Precision (positive predictive value (PPV)) = true positives / (true positives + false positives)
[46] TNR = true negatives / (false positives + true negatives)

x100 = %); 96 of the 111 true positives were correctly identified, while 15 were incorrectly identified as negatives. Eighty-eight percent of the potential-EDC database was shown to interact with $_{h}$AR *in silico*; of the 378 chemicals investigated only 45 demonstrated repellent $_{h}$AR binding energetics. All $_{h}$PR and $_{h}$PPARγ true positives were correctly identified (6/6 and 74/74, respectively), the $_{h}$PR screen identified 351 (27 N_{is}) *in silico* binders, while the $_{h}$PPARγ interacted with 376 (2 N_{is}).

The prioritisation for database inclusion (Section 2.4 and 4.3.3) was biased towards high-exposure biologically-active potential-EDCs, and thus not a representative sample of all registered chemicals. Consequently, it is not justifiable to conclude that similar proportions of other chemical databases would present the same skew towards binding. Evaluating the results via chemical class, to identify any areas of weak *in silico* predictivity, the following sections categorise *in silico* ligand-receptor interactions on the basis of usage – natural hormones, pesticides, consumer products exetera.

4.4.3.1.1 Natural and Synthetic Hormone *in silico* Docking

As the endogenous hormones by which the nuclear receptor superfamily evolved (Thornton, 2001), high sensitivity and specificity of *in silico* ligand-receptor interactions would be expected for the natural and synthetic hormones. The SYBYL Surflex-Dock screening, showed a sensitivity of 94.8% ([55 TP/ (55 TP + 3 FN)] x100). However, excluding low affinity binders (FUB) from the positives dataset (classifying the ambiguity as a negative) reduced sensitivity to 90.2% ([55 TP/ (55 TP + 3 FN + 3FUB)] x100). Including presumed positive binders ($_{\Sigma}$PP), on the basis of interspecies extrapolation, did not significantly affect sensitivity ([97 $_{\Sigma}$PP/ (97 $_{\Sigma}$PP + 6 $_{\Sigma}$PN)] x100 = 94.2% sensitivity).

Interestingly, five of the six false negative binders (FN and $_{\Sigma}$PN) originated from the rat androgen receptor (21HQ) screening, highlighting either significant inter-/intra- species variance in rat AR, or in the reported sequence and computational modelling. The colour coding highlights the ligand promiscuity of endogenous hormones, corroborating the literature detailed in Section 1.

Table 4.6 *In silico* Nuclear Receptor Natural and Synthetic Hormone Binding Results

Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Natural & Synthetic Hormone Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
Estrone (E1)	6.2 (-0.75) TP	5.55 (-1.86) TP	8.13 (-0.85) TP	4.69 (-2.9) TP	4.87 (-1.5) P_{is}	3.78 (-4.41) P_{is}	5.84 (-1.7) P_{is}	6.21 (-0.95) TP	5.22 (-1.16) P_{is}
17β-Estradiol (E2)	5.09 (-1.21) TP	5.47 (-3.85) TP	5.19 (-1.82) TP	4.42 (-3.39) TP	4.49 (-2.84) P_{is}	-0.13 (-7.94) N_{is}	4.32 (-2.73) P_{is}	5.21 (-0.66) P_{is}	4.28 (-1.27) TP
Estriol (E3)	6.47 (-0.99) TP	5.78 (-2.15) TP	7.91 (-1.18) TP	5.37 (-3.01) TP	7.49 (-1.08) P_{is}	4.34 (-5.28) P_{is}	6.53 (-1.78) P_{is}	6.18 (-1.35) P_{is}	2.81 (-2.11) P_{is}
Progesterone (PRG)	4.1 (-3.55) TP	3.59 (-6.91) P_{is}	3.55 (-4.91) P_{is}	2.05 (-6.41) TP	5.99 (-3.54) P_{is}	-2.13 (-12.3) N_{is}	6.56 (-2.96) P_{is}	8.35 (-2.37) TP	3.86 (-0.85) TP
Stanolone/ Dihydrotestosterone (DHT)	4.08 (-2.67) TP	3.5 (-5.51) P_{is}	4.44 (-3.91) P_{is}	3.58 (-5.94) TP	9.38 (-0.57) TP	-0.1 (-9.16) FN	8.18 (-0.97) TP	7.15 (-2.22) P_{is}	4.52 (-1.46) P_{is}
Androstanedione (ANSD)	3.47 (-2.63) P_{is}	3.49 (-4.6) P_{is}	3.45 (-4.01) P_{is}	2.76 (-4.61) TP	7.74 (-0.95) TP	0.48 (-8.71) FUB	6.99 (-1.11) TP	5.31 (-2.22) P_{is}	3.77 (-5.81) P_{is}
Androsterone (ANS)	4.1 (-4.36) P_{is}	1.9 (-6.1) UB_{is}	2.45 (-6.16) P_{is}	2.86 (-4.89) TP	7.44 (-1.64) TP	1.58 (-8.71) FUB	3.12 (-3.46) TP	5.51 (-4.39) P_{is}	3.3 (-1.18) P_{is}
Epiandrosterone (EANS)	3.52 (-4.68) P_{is}	2.44 (-6.0) P_{is}	4.13 (-5.36) P_{is}	4.04 (-4.46) TP	7.94 (-1.46) TP	2.83 (-7.85) TP	6.76 (-1.18) TP	4.22 (-2.72) P_{is}	4.39 (-0.87) P_{is}
Cortisone (CRT)	4.58 (-3.91) P_{is}	0.92 (-8.72) UB_{is}	3.95 (-4.9) P_{is}	-0.7 (-10.6) FN	5.27 (-4.44) TP	-3.38 (-14.4) FN	5.54 (-4.68) TP	7.89 (-3.33) P_{is}	4.51 (-0.84) P_{is}
Liothyronine/ Triiodothyronine (T3)	1.17 (-6.93) FUB	-0.01 (-6.75) N_{is}	-2.55 (-9.07) N_{is}	-0.95 (-7.1) N_{is}	-4.81 (-11.3) N_{is}	-0.88 (-9.61) N_{is}	0.96 (-7.04) UB_{is}	0.42 (-6.08) UB_{is}	5.15 (-2.38) P_{is}
Levothyroxine/ L-Thyroxine (T4)	-0.62 (-7.93) N_{is}	-6.6 (-13.6) N_{is}	0.94 (-6.39) UB_{is}	-3.02 (-10.3) N_{is}	-2.53 (-8.67) N_{is}	-8.27 (14.8) N_{is}	-4.1 (-10.9) N_{is}	-0.48 (-9.44) N_{is}	3.59 (-3.57) TP
Diethylstilbestrol (DES)	5.71 (-3.87) TP	6.08 (-2.13) P_{is}	6.35 (-2.46) P_{is}	3.76 (-2.73) TP	5.85 (-1.56) P_{is}	4.32 (-5.54) P_{is}	6.26 (-1.14) P_{is}	5.19 (-2.7) TP	5.56 (-2.43) P_{is}
Ethinylestradiol (EE2)	6.04 (-1.34) TP	2.81 (-5.17) P_{is}	6.35 (-2.99) P_{is}	4.64 (-3.98) TP	5.51 (-3.65) P_{is}	2.7 (-7.32) P_{is}	6.3 (-2.78) P_{is}	7.14 (-1.98) TP	2.8 (-1.27) TP
Levonorgesterel (LVG)	4.71 (-3.91) TP	1.06 (-8.04) UB_{is}	5.4 (-5.11) P_{is}	5.55 (-4.34) TP	5.01 (-4.54) P_{is}	2.49 (-9.42) P_{is}	4.44 (-4.11) P_{is}	8.27 (-1.85) P_{is}	3.88 (-4.92) P_{is}
Norethindrone (19-ND)	3.88 (-2.94) TP	1.04 (-6.53) UB_{is}	3.98 (-4.44) P_{is}	5.12 (-3.55) TP	6.44 (-3.43) P_{is}	2.16 (-8.25) P_{is}	4.83 (-2.82) P_{is}	6.61 (-1.38) TP	2.78 (-2.04) P_{is}
Methyltrienolone (R1881)	4.72 (-1.83) P_{is}	2.73 (-5.79) P_{is}	6.66 (-1.96) P_{is}	5.28 (-4.38) TP	6.63 (-1.35) P_{is}	6.12 (-4.4) P_{is}	6.79 (-1.17) P_{is}	6.9 (-1.01) P_{is}	3.99 (-0.99) P_{is}
Boldenone/ Dehydrotestosterone (DHT)	5.06 (-0.91) P_{is}	1.26 (-5.62) UB_{is}	5.05 (-1.97) P_{is}	3.84 (-4.81) TP	7.14 (-0.86) P_{is}	-3.2 (-11.9) N_{is}	6.14 (-1.43) P_{is}	5.31 (-2.22) P_{is}	3.77 (-5.81) P_{is}

4.4.3.1.2 Plant Protection Product & Biocide *in silico* Docking

The EU Plant Protection Products (PPP) and Biocide legislation regulates pesticide use and the agrochemical industry, and demand that a substance, safener or synergist, does not cause endocrine disrupting, neurotoxic or immune effects in non-target organisms. As for high production volume (HPV) chemicals there is a wealth of information pertaining to pesticide

bioactivity. As a broad chemical category including 112 unique compounds, PPP and Biocides *in silico* screening results are presented below under the umbrellas of fungicides (thiazole, benzimidazole, imidazole, oxazole and thiocarbamate), herbicides (chloroacetanillide, azole, triazine, aniline and thiazide) and insecticides (organophosphate, organochloride, pyrethrin and pyrethroid); however, the full dataset is available in Table_Apx 2.

As a whole (n=112), pesticide screening sensitivity was 96.4% ([106 TP/ (106 TP + 4 FN)] x100); the sensitivities of $_{h}$ER, $_{h}$AR and $_{h}$PPAR were 97.4%, 93.5% and 100%, respectively (Table_Apx 2). No progesterone receptor (PR) interactions had been reported[47] for the pesticides investigated, preventing an estimation of sensitivity. Lacking EDC regulatory criteria complicates adherance to EU law (Section 2.3). Agrochemical companies typically only investigate endocrine mechanisms on the basis of adverse endpoints observed *in vivo* (i.e. top-down approach), consequently, many marketed chemicals may present endocrine activity. The SYBYL Surflex-Dock virtual screening presented here suggests many of the uncharacterised pesticides present the 3D-spatial arrangements of functional groups, required to bind at the ligand-binding pockets of $_{h}$ER (n=66), $_{h}$AR (n=56), $_{h}$PR (n=97) and $_{h}$PPARγ (n=84). Similar to the endogenous hormones, many of the pesticides are promiscuous, interacting with multiple receptors of the nuclear-receptor superfamily, both *in vitro* and *in silico* (Table_Apx 2).

Sub-categorising the pesticides, Table 4.7 presents the *in silico* SYBYL Surflex-Dock fungicide screening results, which accurately predicted all azole (triazole, imidazole and oxazole), benzimidazole and thiocarbamate fungicide '*true*' positives; sensitivity of 100% ([21 TP / (21 TP + 0 FN)] x100). However, the thiocarbamate fungicide, Thiram, was detected as a weak AR binder (FUB 1.5 –log(Kd)), reclassifying the ambiguous result as a false negative reduced sensitivity to 95.5% ([21 TP/ (21 TP + 1 FUB)] x100). The predicted binding affinities vary with species, which concords with the slight differences in protomol detailed in Sections 4.3.4.3 and 4.3.5.2. Prochloraz has been demonstrated to interact with $_{h}$AR (3V49) both *in silico* and *in vitro* (Table_A 1 and Table_Apx 2). However, interactions with $_{chimp}$AR (1T7T) and $_{rat}$AR (21HQ) *in silico* were less favourable; 1.84 and 0.39 –log(Kd), respectively. The crash scores, for the $_{chimp}$AR (-4.2) and $_{rat}$AR (-6.5) receptor-ligand interactions reported are more negative, suggesting inappropriate penetration into the protein (energetically unfavourable). Species differences in predicted ligand-NR binding were also identified for difenoconazole with $_{h}$ERβ and $_{rat}$ERβ; -0.49 and 6.19 –log(Kd), respectively. In addition, the *in silico* results suggest that Maneb and Carbendazim may be endocrine active, consistent with other thiocarbamate and benzimidazole fungicides. Though evolutionary distinct paralogs, ERα and ERβ LBP's are believed to be conserved due to ligand specificity (see Section 3.1.1 and 3.1.2). However, marked differences in $_{h}$ERα, $_{h}$ERβ and $_{rat}$ERβ binding affinities are shown in Table 4.7 and Table 4.8. Virtual screening correctly identified all herbicide binders (Table 4.8); 100% sensitivity ([15 TP/ (15 TP + 0 FN)] x100).

[47] Only published HTP bioassay data reported on PubChem were evaluated; no academic papers were included as per the contraints detailed in Section 4.3.3.

Table 4.7 *In silico* Nuclear Receptor Fungicide Binding Screening Results

Results of the *in silico* screening of commonly used fungicides, split into the chemical classes: triazoles; benzimidazoles; imidazole & oxazole; and thiocarbamates. Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Fungicide Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
Triazole Fungicide									
Difenoconazole	5.65 (-1.64) TP	-0.49 (-9.43) N_{is}	6.19 (-1.55) P_{is}	4.86 (-4.23) P_{is}	-1.05 (-9.34) N_{is}	0.31 (-10.5) UB_{is}	3.65 (-5.23) P_{is}	3.12 (-4.75) P_{is}	5.96 (-1.93) TP
Epoxiconazole	5.29 (-1.24) TP	2.93 (-3.94) P_{is}	3.76 (-2.91) P_{is}	3.95 (-3.97) TP	3.71 (-2.57) P_{is}	1.45 (-6.26) P_{is}	4.09 (-1.03) P_{is}	4.91 (-1.44) P_{is}	5.09 (-0.82) P_{is}
Penconazole	4.96 (-1.61) TP	4.33 (-1.65) P_{is}	5.23 (-1.98) P_{is}	4.93 (-1.28) TP	4.24 (-1.39) P_{is}	2.91 (-2.62) P_{is}	4.88 (-2.06) P_{is}	5.63 (-1.87) P_{is}	4.86 (-2.51) P_{is}
Propiconazole	5.93 (-2.31) TP	3.63 (-4.02) P_{is}	5.22 (-3.07) P_{is}	5.35 (-2.31) TP	4.49 (-2.32) P_{is}	3.36 (-4.48) P_{is}	3.78 (-2.48) P_{is}	4.47 (-2.74) P_{is}	4.34 (-3.55) P_{is}
Tebuconazole	4.73 (-1.42) TP	5.22 (-2.81) P_{is}	4.36 (-2.91) P_{is}	3.63 (-4.3) TP	4.85 (-1.68) P_{is}	3.25 (-4.34) P_{is}	4.6 (-2.42) P_{is}	4.78 (-2.4) P_{is}	6.23 (-2.2) P_{is}
Mycobutanil	6.1 (-0.96) TP	4.35 (-1.72) P_{is}	5.26 (-2.01) P_{is}	5.35 (-2.15) P_{is}	4.97 (-1.61) P_{is}	5.85 (-2.31) P_{is}	4.46 (-1.74) P_{is}	4.19 (-2.9) P_{is}	5.2 (-1.21) P_{is}
Benzimidazole Fungicide									
Benomyl	3.2 (-1.73) TP	3.76 (-2.14) P_{is}	4.54 (-1.91) P_{is}	2.87 (-2.66) TP	4.59 (-2.6) P_{is}	3.41 (-4.43) P_{is}	3.91 (-1.35) P_{is}	2.38 (-3.14) P_{is}	3.41 (-2.17) P_{is}
Carbendazim	3.0 (-0.42) P_{is}	1.87 (-0.12) UB_{is}	2.97 (-1.13) P_{is}	3.16 (-0.87) P_{is}	2.19 (-0.43) P_{is}	3.23 (-0.47) P_{is}	3.64 (-1.95) P_{is}	2.29 (-1.13) P_{is}	3.0 (-0.67) P_{is}
Imidazole & Oxazole Fungicide									
Enilconazole	5.98 (-1.62) TP	6.08 (-1.25) P_{is}	5.59 (-1.46) P_{is}	5.35 (-1.63) TP	5.11 (-2.03) P_{is}	5.46 (-1.33) P_{is}	4.14 (-1.7) P_{is}	5.06 (-1.59) P_{is}	5.63 (-1.79) TP
Prochloraz	4.69 (-1.91) TP	2.89 (-5.02) P_{is}	3.86 (-2.7) P_{is}	3.9 (-2.96) TP	1.84 (-4.2) UB_{is}	0.39 (-6.15) UB_{is}	4.14 (-2.78) P_{is}	3.72 (-3.5) P_{is}	6.15 (-1.18) P_{is}
Vinclozolin	2.66 (-1.76) P_{is}	1.8 (-2.35) UB_{is}	2.66 (-1.69) P_{is}	4.13 (-0.7) TP	2.58 (-1.21) P_{is}	4.18 (-1.74) P_{is}	2.49 (-1.37) P_{is}	2.35 (-0.96) P_{is}	5.13 (-0.64) TP
Thiocarbamate Fungicide									
Maneb	2.6 (-0.77) P_{is}	2.26 (-1.36) P_{is}	2.87 (-1.24) P_{is}	2.47 (-1.07) P_{is}	2.02 (-0.37) P_{is}	2.46 (-1.04) P_{is}	2.55 (-0.25) P_{is}	2.6 (-1.41) P_{is}	2.45 (-0.51) P_{is}
Thiram	2.96 (-1.17) TP	3.32 (-1.61) P_{is}	3.78 (-1.31) P_{is}	1.5 (-4.21) FUB	2.94 (-1.03) P_{is}	3.53 (-1.95) P_{is}	2.96 (-0.5) P_{is}	3.72 (-1.89) P_{is}	2.9 (-1.8) P_{is}

However, Bromoxynil and Alachlor, were detected as low affinity binders (0<2 –log(Kd)) for $_{h}$ERα and $_{h}$AR, respectively. Bromoxynil is a nitrile herbicide which controls weeds by inhibiting photosynthesis, while Alachlor is a chloroacetanilide herbicide which inhibits geranylgeranyl pyrophosphate (GGPP) cyclisation enzymes. Reclassifying the ambiguity of true positive low affinity *in silico* binding as false negatives, reduced sensitivity to 88.2% ([15 TP/ (15 TP + 2 FN)] x100).

Table 4.8 *In silico* Nuclear Receptor Herbicide Binding Results

Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Herbicide Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
3,5-Dibromo-4-hydroxybenzonitrile/ Bromoxynil	1.95 (-0.32) FUB	1.21 (-0.58) UB_{is}	1.46 (-0.11) UB_{is}	3.29 (-0.49) TP	1.43 (-0.39) UB_{is}	1.77 (-1.03) UB_{is}	1.3 (-0.42) UB_{is}	0.84 (-1.01) UB_{is}	3.05 (-0.74) TP
N-(3,4-dichlorophenyl) Propanamide	3.81 (-1.06) P_{is}	1.9 (-0.33) UB_{is}	3.21 (-0.93) P_{is}	3.13 (-0.6) TP	2.32 (-0.45) P_{is}	3.47 (-0.46) P_{is}	2.09 (-0.58) P_{is}	3.04 (-1.55) P_{is}	3.15 (-0.83) P_{is}
Pronamide	2.04 (-1.69) P_{is}	1.86 (-2.62) UB_{is}	1.89 (-2.36) UB_{is}	2.97 (-0.54) TP	3.44 (-0.79) P_{is}	2.81 (-1.7) P_{is}	3.24 (-0.66) P_{is}	1.96 (-1.05) UB_{is}	2.66 (-0.52) P_{is}
4-(2,4-dichlorophenoxy) Butanoic acid	3.74 (-0.43) P_{is}	3.87 (-0.9) P_{is}	3.81 (-0.47) P_{is}	4.68 (-2.23) TP	4.72 (-0.96) P_{is}	4.47 (-0.46) P_{is}	5.11 (-0.99) P_{is}	4.42 (-0.81) P_{is}	5.21 (-0.71) P_{is}
Chloroacetanilide Herbicide									
Alachlor	4.5 (-1.02) TP	3.33 (-3.9) P_{is}	4.02 (-1.76) P_{is}	0.7 (-4.58) FUB	2.96 (-3.86) P_{is}	1.92 (-5.28) UB_{is}	3.51 (-1.05) P_{is}	4.06 (-1.92) P_{is}	3.86 (-3.41) P_{is}
Acetochlor	5.4 (-1.03) TP	3.45 (-3.36) P_{is}	4.27 (-2.54) P_{is}	3.22 (-3.5) TP	2.66 (-3.3) P_{is}	3.02 (-5.03) P_{is}	3.51 (-2.66) P_{is}	5.09 (-1.4) P_{is}	4.65 (-2.24) P_{is}
Azole Herbicides									
3-amino-1,2,4-triazole/ Amitrole	3.49 (-0.25) TP	2.98 (-0.2) P_{is}	3.09 (-0.31) P_{is}	2.83 (-0.14) P_{is}	2.04 (-0.3) P_{is}	2.25 (-0.17) P_{is}	2.35 (-0.19) P_{is}	2.69 (-0.83) P_{is}	2.72 (-0.06) P_{is}
Triazine Herbicide									
Atrazine	3.76 (-0.83) P_{is}	3.57 (-1.04) P_{is}	3.84 (-0.66) P_{is}	4.26 (-1.18) P_{is}	3.51 (-1.09) P_{is}	3.53 (-2.25) P_{is}	3.69 (-1.3) P_{is}	4.69 (-0.77) P_{is}	3.91 (-1.14) P_{is}
Propazine	2.82 (-1.22) P_{is}	3.73 (-1.28) P_{is}	4.98 (-0.69) P_{is}	4.6 (-1.86) P_{is}	3.33 (-1.58) P_{is}	3.5 (-3.12) P_{is}	3.69 (-1.65) P_{is}	4.62 (-1.01) P_{is}	3.27 (-2.28) P_{is}
Prometryn	3.94 (-1.94) P_{is}	4.64 (-1.7) P_{is}	3.91 (-0.46) P_{is}	3.19 (-4.05) P_{is}	3.99 (-1.79) P_{is}	2.13 (-5.63) P_{is}	3.56 (-2.39) P_{is}	4.12 (-1.99) P_{is}	4.26 (-1.4) P_{is}
Simazine	2.92 (-1.17) P_{is}	2.79 (-0.96) P_{is}	3.35 (-2.01) P_{is}	4.3 (-1.0) P_{is}	2.52 (-1.01) P_{is}	4.46 (-1.09) P_{is}	2.99 (-0.63) P_{is}	3.22 (-0.63) P_{is}	3.26 (-0.57) P_{is}
Terbutryne	3.63 (-2.0) P_{is}	4.75 (-1.73) P_{is}	4.96 (-0.76) P_{is}	4.26 (-1.49) TP	4.97 (-1.70) P_{is}	3.98 (-3.28) P_{is}	4.05 (-2.37) P_{is}	4.83 (-0.68) P_{is}	3.44 (-1.05) TP
Terbutylazine	6.66 (-2.89) TP	7.02 (-2.51) P_{is}	5.76 (-2.25) P_{is}	6.49 (-2.76) P_{is}	6.7 (-1.85) P_{is}	5.47 (-4.94) P_{is}	6.53 (-1.94) P_{is}	6.5 (-1.58) P_{is}	7.52 (-1.79) P_{is}
Aniline Herbicides									
Pendimethalin	6.07 (-1.65) TP	3.22 (-4.41) P_{is}	5.46 (-2.01) P_{is}	3.8 (-2.31) P_{is}	1.44 (-3.34) UB_{is}	3.86 (-3.69) P_{is}	4.99 (-1.75) P_{is}	3.65 (-1.95) P_{is}	6.2 (-3.5) P_{is}
Trifluralin	6.34 (-1.39) P_{is}	-0.58 (-7.82) N_{is}	5.43 (-1.57) P_{is}	0.73 (-5.7) UB_{is}	3.02 (-3.47) P_{is}	1.64 (-4.84) UB_{is}	3.37 (-2.63) P_{is}	4.71 (-1.63) P_{is}	7.06 (-3.29) P_{is}
Thiazide Herbicides									
Bentazone	3.38 (-0.55) P_{is}	2.98 (-2.11) P_{is}	3.88 (-0.83) P_{is}	2.6 (-2.62) P_{is}	3.91 (-0.89) P_{is}	3.24 (-1.07) P_{is}	4.04 (-0.51) P_{is}	2.05 (-3.38) P_{is}	3.49 (-1.02) P_{is}

To varying degrees all investigated herbicides presented the spatial arrangement of functional groups required to interact with the LBP of nuclear receptors (Table 4.8). It should be noted that the binding score is not necessarily predictive of *in vitro* agonism or antagonism, as

SYBYL scoring evaluates the chemistry of functional groups, not protein functionality. However, academic publications corroborate the endocrine activity of triazine herbicides detected *in silico*. Atrazine has been identified as an EDC *in vitro* (Basini *et al*., 2012; Kucka *et al.*, 2012) and *in vivo* (De La Casa-Resino *et al*., 2012; Shelley *et al.,* 2012), while simazine has been identified as an ER antagonist in Sprague-Dawley rats (Tennant *et al.*, 1994).

Surflex-Dock correctly identified organophosphate insecticides with known endocrine activity (Table 4.9); sensitivity = 100%. However, only weak chemical interactions were identified for Dicofol, which structurally related to DDT, has been shown to antagonise the AR signalling pathway *in vitro* at 48.97 μM (Table_A 1). The absence of colour coding highlights the lack of *in vitro* endocrine MoA (ER, AR, PR & PPARγ) studies for organophosphates, nevertheless, the *in silico* model identified potential ED MoA for a number of insecticides, such as Chlorfenvinos, Fenitrothion and Parathion (Table 4.9). This ambiguity of Dicofol scoring was mirrored in the organochlorides which presented the lowest sensitivity of 72.7% (Table 4.10).

In particular, Toxaphene scoring was ubiquitously unfavourable (-7.53 to 0.79 –log(Kd)), which conflicts with reports of *in vitro* ER signalling agonism in BG1 cells and androgen receptor antagonism (Table_A 1). However, as a mixture of at least 177 C10 polychloroderivates, toxaphene highlights a limitation of the virtual screening, which utilised one conformation of one component the mixture, rather than deconstructing the mixture for individual assessment. Relative to the organophosphate insecticides, herbicides and fungicides, the organochlorides, notorious for their persistence and toxicity, presented very low affinity for the ligand binding pockets of nuclear receptors.

Derived from *Chrysanthemum cinerariifolium*, pyrethrins are natural insectides, while pyrethroids are synthetic analogues. As reactive compounds which degrade in sunlight, the pyrethrins and pyrethroids do not persist in the environment and are widely used despite their neurotoxicity. SYBYL Surflex-Dock screening of pyrethrins and pyrethroids correctly identified *in vitro* $_{h}$ER, $_{h}$AR and $_{h}$PPARγ binders (Table 4.11).

Interestingly, large differences in the *in silico* binding affinity of different species are reported; AR binding of fenvalerate varies from -17.0 to 3.78 –log(Kd). Interspecies variance in virtual screening scores suggests that molecular differences in LBP structure (Section 1 and 4.4.2) affected protomol generation and *in silico* binding, increasing the uncertainty of extrapolating mechanistic information from one species to another. The *in silico* results (Table 4.11), suggest that human receptors may be more susceptible to perturbation of the classic genomic signalling pathway by pyrethrins and pyrethroids, than the chimpanzee, rat and mice models, typically used to infer adverse effects on human health.

Table 4.9 *In silico* Nuclear Receptor Organophosphate Insecicide Binding Results

Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Organophosphate Insecticide Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
Acephate	4.33 (-0.6) P_{is}	1.97 (-0.67) UB_{is}	3.24 (-0.53) P_{is}	3.24 (-0.56) P_{is}	2.11 (-1.0) P_{is}	3.53 (-1.31) P_{is}	2.57 (-1.24) P_{is}	3.56 (-0.55) P_{is}	3.42 (-1.66) P_{is}
Chlorfenvinfos	6.11 (-1.28) P_{is}	3.89 (-2.56) P_{is}	5.54 (-1.16) P_{is}	4.57 (-2.01) P_{is}	3.36 (-2.99) P_{is}	1.55 (-5.36) UB_{is}	3.21 (-2.16) P_{is}	4.43 (-1.43) P_{is}	3.97 (-1.02) P_{is}
Demeton-S-methyl	4.46 (-1.05) P_{is}	4.2 (-1.35) P_{is}	5.06 (-0.86) P_{is}	4.01 (-1.28) P_{is}	4.09 (-0.61) P_{is}	4.7 (-1.79) P_{is}	3.68 (-2.0) P_{is}	4.75 (-1.66) P_{is}	4.67 (-1.35) P_{is}
Diazinon	6.12 (-1.6) P_{is}	5.17 (-3.72) P_{is}	6.54 (-1.94) P_{is}	6.24 (-1.81) P_{is}	4.16 (-3.28) P_{is}	4.46 (-4.41) P_{is}	6.14 (-1.28) P_{is}	6.56 (-2.1) P_{is}	5.54 (-3.72) TP
Dichlorvos	2.65 (-0.57) TP	2.06 (-0.65) P_{is}	3.0 (-0.64) P_{is}	2.78 (-1.49) TP	1.99 (-0.8) UB_{is}	2.86 (-0.76) P_{is}	2.29 (-1.1) P_{is}	4.07 (-0.71) P_{is}	3.85 (-1.13) TP
Dicofol	1.25 (-4.17) UB_{is}	0.51 (-3.68) UB_{is}	2.23 (-2.74) P_{is}	0.28 (-3.58) FUB	-0.58 (-4.63) N_{is}	-1.48 (-5.86) N_{is}	-0.31 (-2.89) N_{is}	2.05 (-1.76) P_{is}	2.25 (-1.32) P_{is}
Dimethoate	4.68 (-0.67) P_{is}	3.06 (-0.79) P_{is}	5.13 (-1.08) P_{is}	3.47 (-1.91) P_{is}	3.11 (-0.79) P_{is}	2.34 (-2.34) P_{is}	2.95 (-1.34) P_{is}	3.32 (-2.35) P_{is}	4.74 (-0.87) P_{is}
Fenitrothion	4.62 (-1.38) P_{is}	5.04 (-1.36) P_{is}	5.67 (-0.78) P_{is}	4.37 (-1.42) TP	3.86 (-1.25) P_{is}	5.51 (-1.40) P_{is}	4.36 (-0.88) P_{is}	3.54 (-1.74) P_{is}	4.47 (-1.71) P_{is}
Malathion	5.48 (-1.48) P_{is}	4.73 (-2.46) P_{is}	4.81 (-4.24) P_{is}	5.89 (-2.17) P_{is}	5.31 (-2.86) P_{is}	3.89 (-4.57) P_{is}	3.81 (-2.05) P_{is}	5.59 (-1.80) P_{is}	5.28 (-2.98) P_{is}
Mevinphos	4.44 (-0.73) P_{is}	4.14 (-0.96) P_{is}	4.63 (-0.88) P_{is}	5.13 (-1.05) P_{is}	4.3 (-0.82) P_{is}	4.52 (-1.53) P_{is}	4.55 (-0.98) P_{is}	5.21 (-0.95) P_{is}	6.69 (-1.15) P_{is}
Omethoate	4.01 (-0.77) P_{is}	4.56 (-0.76) P_{is}	4.14 (-0.67) P_{is}	4.44 (-1.0) P_{is}	3.25 (-1.02) P_{is}	3.87 (-0.86) P_{is}	3.92 (-1.67) P_{is}	4.92 (-1.27) P_{is}	4.03 (-0.7) P_{is}
Oxydemeton-methyl	4.79 (-0.98) P_{is}	4.35 (-1.18) P_{is}	4.88 (-0.72) P_{is}	4.49 (-1.31) P_{is}	3.82 (-1.70) P_{is}	4.84 (-1.28) P_{is}	3.79 (-1.63) P_{is}	3.91 (-0.99) P_{is}	5.56 (-1.32) P_{is}
Parathion	4.17 (-0.59) P_{is}	5.76 (-2.17) P_{is}	4.62 (-1.61) P_{is}	4.36 (-1.30) P_{is}	3.68 (-1.82) P_{is}	4.60 (-2.37) P_{is}	4.49 (-1.64) P_{is}	3.71 (-1.26) P_{is}	5.36 (-1.19) P_{is}
Tetrachlorvinphos	4.05 (-1.31) TP	3.17 (-2.35) P_{is}	3.81 (-1.91) P_{is}	3.19 (-2.31) TP	3.08 (-1.81) P_{is}	2.29 (-3.46) P_{is}	2.46 (-1.62) P_{is}	3.72 (-1.89) P_{is}	3.42 (-1.45) TP
Trichlorfon	2.74 (-1.40) P_{is}	2.67 (-0.80) P_{is}	2.62 (-0.77) P_{is}	2.27 (-1.38) P_{is}	1.57 (-1.58) UB_{is}	0.85 (-2.0) UB_{is}	1.95 (-1.19) UB_{is}	3.23 (-0.41) P_{is}	2.72 (-0.75) P_{is}

The ubiquitous governance of pesticides has led to a wealth of *in vivo*, *in vitro* and *in silico* data, which enabled evaluation of the novel *in silico* screen. However, the majority of chemicals are not required to provide such extensive assurances of safety. The following sub-sections, present the virtual screening results of industrial chemicals (REACH regulated), pharmaceuticals and personal-care-products.

Table 4.10 *In silico* Nuclear Receptor Organochloride Insecticide Binding Results

Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Organochloride Insecticide Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
DDE	2.4 (-2.12) TP	1.31 (-2.88) UB_{is}	2.57 (-0.5) P_{is}	2.0 (-2.09) TP	-1.36 (-4.34) N_{is}	1.2 (-2.19) UB_{is}	0.81 (-2.08) UB_{is}	2.61 (-0.88) P_{is}	3.96 (-0.9) P_{is}
DDD	3.7 (-0.89) TP	2.41 (-2.15) P_{is}	2.69 (-1.39) P_{is}	3.19 (-1.38) TP	0.1 (-3.32) UB_{is}	0.98 (-2.81) UB_{is}	1.75 (-1.70) UB_{is}	3.02 (-0.94) P_{is}	3.76 (-1.35) P_{is}
DDT	2.78 (-0.87) TP	1.55 (-2.72) UB_{is}	1.66 (-2.27) UB_{is}	1.27 (-2.95) UB_{is}	-0.5 (-3.64) N_{is}	-2.3 (-6.76) N_{is}	-0.21 (-3.41) N_{is}	2.46 (-1.4) P_{is}	3.41 (-1.29) P_{is}
Dieldrin	0.57 (-3.63) UB_{is}	0.11 (-3.69) UB_{is}	-2.02 (-5.10) N_{is}	-7.51 (-10.6) FN	-1.95 (-4.74) N_{is}	-8.43 (-13.4) N_{is}	-1.19 (-4.88) N_{is}	0.68 (-2.64) UB_{is}	0.3 (-4.62) UB_{is}
Lindane	1.79 (-0.66) UB_{is}	2.02 (-0.7) P_{is}	1.42 (-0.83) UB_{is}	1.19 (-2.14) UB_{is}	1.03 (-2.15) UB_{is}	0.95 (-2.44) UB_{is}	1.03 (-0.74) UB_{is}	1.35 (-0.49) UB_{is}	1.99 (-0.4) FUB
Mirex	-.6.25 (-8.58) N_{is}	-7.70 (-9.73) N_{is}	-4.53 (-7.45) N_{is}	-13.3 (-17.8) N_{is}	-9.33 (-12.1) N_{is}	-18.3 (-20.9) N_{is}	-7.83 (-10.6) N_{is}	-4.27 (-6.35) N_{is}	-1.18 (-0.45) N_{is}
Toxaphene	0.68 (-3.37) FUB	-2.58 (-6.13) N_{is}	-1.75 (-5.37) N_{is}	-1.98 (-8.26) FN	-1.0 (-5.42) N_{is}	-7.53 (-11.9) N_{is}	-2.47 (-6.07) N_{is}	0.79 (-1.98) UB_{is}	0.53 (-3.16) FUB

Table 4.11 *In silico* Nuclear Receptor Pyrethrin & Pyrethroid Insecticide Binding Results

Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Pyrethrin & Pyrethroid Insecticide Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
Bifenthrin	4.48 (-5.19) TP	0.02 (-10.3) UB_{is}	1.91 (-2.86) UB_{is}	-4.37 (-15.3) N_{is}	-1.57 (-12.0) N_{is}	-11.6 (-20.9) N_{is}	1.07 (-8.16) UB_{is}	5.49 (-4.99) P_{is}	6.33 (-2.34) P_{is}
Deltamethrin	5.74 (-1.61) P_{is}	-4.21 (-12.8) N_{is}	3.32 (-4.96) P_{is}	0.33 (-9.22) UB_{is}	-7.43 (-15.2) N_{is}	-5.33 (-15.6) N_{is}	-4.39 (-12.6) N_{is}	0.18 (-8.31) UB_{is}	5.53 (-1.92) P_{is}
Etofenprox	6.94 (-1.61) P_{is}	2.06 (-8.11) P_{is}	5.34 (-1.65) P_{is}	0.75 (-8.75) UB_{is}	1.69 (-9.30) UB_{is}	-10.4 (-21.4) N_{is}	0.85 (-8.14) UB_{is}	3.93 (-6.90) P_{is}	8.23 (-1.53) P_{is}
Fenvalerate	4.83 (-3.48) TP	-4.09 (-15.9) N_{is}	5.17 (-3.58) P_{is}	3.78 (-7.39) TP	-6.75 (-17.0) N_{is}	-17.0 (-25.6) N_{is}	-0.83 (-10.2) N_{is}	-2.61 (-13.7) N_{is}	6.79 (-2.59) N_{is}
Fluvalinate	5.25 (-3.45) P_{is}	-3.52 (-16.7) N_{is}	7.92 (-4.35) P_{is}	-7.92 (-4.35) N_{is}	-3.59 (-15.9) N_{is}	-18.6 (-30.4) N_{is}	-4.11 (-15.2) N_{is}	0.44 (-11.8) UB_{is}	6.51 (-2.25) P_{is}
Permethrin	3.11 (-7.21) P_{is}	-0.54 (-9.62) N_{is}	4.71 (-3.72) P_{is}	-0.32 (-10.3) N_{is}	-6.09 (-12.6) N_{is}	-4.38 (-14.1) N_{is}	0.54 (-7.49) UB_{is}	4.30 (-6.04) P_{is}	5.93 (-1.29) P_{is}
Resmethrin	6.5 (-4.86) P_{is}	1.42 (-10.1) UB_{is}	5.97 (-3.69) P_{is}	6.01 (-5.35) TP	-3.04 (-13.8) N_{is}	-3.87 (-13.8) N_{is}	1.32 (-8.08) UB_{is}	3.64 (-6.89) P_{is}	7.18 (-1.82) TP

The large degree of inappropriate protein penetration (crash score (pKd)), of pyrethrin and pyrethroid insecticides, is reflective of their long functionalised backbone, which support a reactive cycloalkane and phenoxy groups and electronegative halogens (Cl or Br).

4.4.3.1.3 Industrial Chemical *in silico* Docking

Produced from a plethora of raw materials, 'industrial chemicals' or 'commodity chemicals' term the polymers, peterochemicals, inorganics and fertilisers that form the building blocks of manufacturing, construction, consumer goods and services. Sensitivity of SYBYL Surflex-Dock in correctly identifying industrial chemicals known to interact with ERα, AR, PR or PPARγ (n=52), was 98.1% ([52 TP/ (52 TP + 1 FN)] x100) (see Table_Apx 2). A number of additional industrial chemicals interacted with the *in silico* protomols. Modelled on nuclear receptor LBP (45 ERα; 52 AR; 69 PR and 62 PPARγ), binding of industrial chemicals to the protomol pseudo-molecular target, suggests that exposure may perturb classic-genomic signalling (endocrine mechanisms). Table 4.12 and Table 4.13 show the virtual screening results for phenolic and phthalate industrial chemicals, respectively.

Table 4.12 *In silico* Nuclear Receptor Phenolic Industrial Chemical Binding Results

Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Phenolic Industrial Chemical Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
Phenols									
1-Naphthol	3.88 (-0.2) P_{is}	3.62 (-0.8) P_{is}	3.84 (-0.49) P_{is}	3.81 (-0.14) P_{is}	3.63 (-0.99) P_{is}	4.38 (-0.67) P_{is}	3.15 (-0.83) P_{is}	3.61 (-0.64) P_{is}	3.9 (-0.49) P_{is}
2-Naphthol (Naphthalen-2-ol)	3.75 (-1.11) TP	3.7 (-0.76) P_{is}	3.75 (-0.87) P_{is}	4.21 (-0.42) P_{is}	3.2 (-0.59) P_{is}	2.9 (-0.86) P_{is}	3.39 (-0.45) P_{is}	3.59 (-0.74) P_{is}	4.17 (-1.29) P_{is}
2-Benzylphenol	3.46 (-0.38) P_{is}	4.61 (-2.19) P_{is}	4.71 (-1.57) P_{is}	3.44 (-0.35) P_{is}	4.56 (-0.33) P_{is}	4.83 (-0.87) P_{is}	3.78 (-0.52) P_{is}	4.5 (-0.75) P_{is}	4.27 (-0.77) P_{is}
2,4-Dinitrophenol	3.52 (-0.42) P_{is}	2.24 (-0.57) P_{is}	4.09 (-0.41) P_{is}	2.67 (-0.39) TP	1.45 (-0.6) UB_{is}	3.34 (-0.62) P_{is}	2.67 (-0.35) P_{is}	1.99 (-0.4) UB_{is}	4.53 (-0.09) P_{is}
4-Isopentylphenol	4.14 (-0.63) P_{is}	4.57 (-1.12) P_{is}	4.77 (-0.57) P_{is}	4.51 (-1.12) P_{is}	4.47 (-0.23) P_{is}	5.18 (-1.38) P_{is}	4.71 (-0.51) P_{is}	4.14 (-0.83) P_{is}	5.31 (-0.47) P_{is}
4-Nitrophenol	3.44 (-0.31) P_{is}	3.07 (-0.17) P_{is}	2.68 (-0.21) P_{is}	2.29 (-0.33) TP	2.89 (-1.12) P_{is}	2.14 (-0.53) P_{is}	2.63 (-1.24) P_{is}	3.72 (-0.46) P_{is}	4.6 (-0.16) P_{is}
4-Phenylphenol	4.03 (-0.76) TP	3.5 (-0.79) P_{is}	3.47 (-0.59) P_{is}	2.92 (-0.49) TP	4.2 (-0.39) P_{is}	4.49 (-1.26) P_{is}	3.19 (-0.29) P_{is}	3.17 (-0.48) P_{is}	3.22 (-0.29) P_{is}
Alkylphenols									
4-t-Butylphenol	3.54 (-0.53) TP	3.92 (-0.68) P_{is}	3.75 (-0.58) P_{is}	5.86 (-2.17) P_{is}	4.03 (-0.83) P_{is}	3.37 (-0.8) P_{is}	3.89 (-0.55) P_{is}	4.1 (-0.48) P_{is}	3.64 (-0.58) P_{is}
4-t-Octylphenol	6.14 (-0.64) TP	5.89 (-0.86) P_{is}	5.84 (-1.29) P_{is}	5.54 (-2.21) TP	5.72 (-2.01) P_{is}	5.64 (-2.2) P_{is}	5.13 (-0.63) P_{is}	5.57 (-0.58) P_{is}	5.07 (-0.86) P_{is}
4-t-Nonylphenol	6.36 (-0.81) TP	6.55 (0.67) P_{is}	6.79 (-0.97) P_{is}	7.45 (-1.18) TP	6.46 (-0.81) P_{is}	6.41 (-2.84) P_{is}	7.13 (-0.78) P_{is}	7.33 (-0.79) P_{is}	6.04 (-2.98) P_{is}

Table 4.13 *In silico* Nuclear Receptor HPV Chemical Binding Results

Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Large Production Volume Bisphenol & Phthalate Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
Bisphenols									
Bisphenol A	4.88 (-0.62) TP	5.76 (-0.63) P_{is}	5.21 (-0.82) P_{is}	4.97 (-0.87) TP	3.11 (-1.66) P_{is}	3.65 (-3.17) P_{is}	3.46 (-1.66) P_{is}	4.39 (-0.98) P_{is}	4.8 (-0.91) TP
Bisphenol B	5.17 (-1.26) TP	4.06 (-2.33) P_{is}	4.36 (-2.58) P_{is}	4.05 (-1.76) TP	3.04 (-2.35) P_{is}	2.89 (-5.82) P_{is}	4.54 (-2.07) P_{is}	6.16 (-0.98) P_{is}	5.47 (-1.24) TP
Bisphenol E	3.93 (-1.32) P_{is}	4.48 (-0.53) P_{is}	5.3 (-0.66) P_{is}	4.41 (-1.48) P_{is}	3.19 (-2.53) P_{is}	4.08 (-2.96) P_{is}	3.01 (-1.61) P_{is}	4.76 (-2.09) P_{is}	4.87 (-1.03) P_{is}
Bisphenol F	4.28 (-1.01) TP	4.08 (-1.46) P_{is}	4.87 (-0.65) P_{is}	3.63 (-1.14) P_{is}	4.04 (-0.81) P_{is}	4.92 (-1.42) P_{is}	4.07 (-0.73) P_{is}	4.91 (-0.4) P_{is}	4.32 (-0.83) P_{is}
Phthalates									
Benzylbutyl phthalate	7.00 (-0.7) TP	6.84 (-2.18) P_{is}	5.56 (-1.45) P_{is}	3.6 (-3.92) TP	6.96 (-1.84) P_{is}	3.64 (-6.04) P_{is}	5.25 (-1.54) P_{is}	5.03 (-1.38) P_{is}	7.11 (-2.27) TP
Bis(2-ethylhexyl) phthalate	8.76 (-2.99) P_{is}	0.02 (-13.4) UB_{is}	6.35 (-4.99) P_{is}	0.34 (-11.7) UB_{is}	-1.5 (-14.8) N_{is}	-10.6 (-23.6) N_{is}	-2.84 (-12.7) N_{is}	4.47 (-8.01) P_{is}	8.29 (-0.76) P_{is}
Dicyclohexyl phthalate	6.61 (-2.03) P_{is}	4.12 (-6.2) P_{is}	5.93 (-1.78) P_{is}	-0.75 (-9.04) N_{is}	-0.21 (-9.56) N_{is}	-6.68 (-16.8) N_{is}	0.6 (-6.88) UB_{is}	2.13 (-6.87) P_{is}	6.82 (-1.46) TP
Dibutyl phthalate	7.15 (-2.02) TP	4.35 (-4.21) P_{is}	6.55 (-1.74) P_{is}	5.49 (-3.72) P_{is}	5.9 (-1.74) P_{is}	5.96 (-3.9) P_{is}	5.68 (-1.58) P_{is}	7.3 (-1.55) P_{is}	6.22 (-0.9) P_{is}
Diethyl phthalate	4.13 (-1.98) P_{is}	4.59 (-0.69) P_{is}	5.24 (-0.98) P_{is}	3.95 (-1.37) P_{is}	3.7 (-0.85) P_{is}	3.98 (-1.99) P_{is}	4.0 (-0.81) P_{is}	3.99 (-1.27) P_{is}	4.41 (-0.66) P_{is}
Diisobutyl phthalate	5.81 (-1.42) TP	5.02 (-2.86) P_{is}	6.62 (-1.32) P_{is}	3.95 (-5.64) P_{is}	4.36 (-2.72) P_{is}	3.99 (-5.1) P_{is}	4.76 (-2.06) P_{is}	6.01 (-2.67) P_{is}	6.43 (-1.11) P_{is}
Diisodecyl phthalate	8.13 (-3.4) P_{is}	-0.59 (-15.2) N_{is}	5.84 (-5.97) P_{is}	-6.38 (-21.4) FN	-18.1 (-31.6) N_{is}	-19.1 (-35.2) N_{is}	-1.49 (-13.8) N_{is}	-0.74 (-16.0) N_{is}	8.42 (-4.93) P_{is}
Diisononyl phthalate	11.0 (-3.48) P_{is}	-5.6 (-19.8) N_{is}	7.17 (-4.08) P_{is}	5.47 (-8.98) P_{is}	-6.84 (-17.9) N_{is}	-16.0 (-29.3) N_{is}	0.29 (-13.7) UB_{is}	2.52 (-11.4) P_{is}	9.62 (-1.09) P_{is}
Monobutyl phthalate	6.24 (-0.73) P_{is}	5.6 (-1.25) P_{is}	5.19 (-1.55) P_{is}	4.96 (-0.93) P_{is}	6.19 (-0.77) P_{is}	5.55 (-0.66) P_{is}	4.15 (-0.59) P_{is}	6.72 (-1.17) P_{is}	5.77 (-0.38) P_{is}
Structurally Diverse Industrial Chemicals									
Benzophenone	3.75 (-0.53) TP	4.0 (-1.0) P_{is}	3.3 (-1.4) P_{is}	3.33 (-0.95) TP	3.47 (-0.34) P_{is}	4.05 (-0.44) P_{is}	3.13 (-0.34) P_{is}	3.7 (-0.48) P_{is}	3.78 (-0.87) P_{is}
Biphenyl	3.71 (-0.33) P_{is}	3.89 (-0.86) P_{is}	3.11 (-0.41) P_{is}	4.56 (-0.36) P_{is}	2.91 (-0.31) P_{is}	3.7 (-1.13) P_{is}	2.55 (-0.26) P_{is}	2.99 (-0.35) P_{is}	2.5 (-0.53) P_{is}
Perfluorodecanoic acid	4.17 (-1.27) TP	3.9 (-1.75) P_{is}	5.34 (-1.17) P_{is}	3.89 (-1.75) P_{is}	5.5 (-1.83) P_{is}	4.34 (-3.41) P_{is}	5.58 (-1.34) P_{is}	5.11 (-1.19) P_{is}	6.05 (-1.95) P_{is}

Chemicals with an unhindered hydroxyl group (-OH), in the meta- or para- position of a phenyl ($-C_6H_5$) or cyclopentene ($-C_5H_4$) ring, are considered ER-binders by the OECD QSAR Toolbox ER-profiler (Section 2.4; Table 2.3). Resonance in the aromatic ring (delocalisation of pi electrons) stabilises the acidity of the hydroxyl group, distinguishing activity from alcohols (IUPAC, 2006), and defining binding potency (Li and Gramatica, 2010). The SYBYL *in silico* screening results of phenolic industrial chemicals are presented

in Table 4.13. As expected, all compounds were scored as potential binders; the model correctly identifying 10 true positives.

The esters of phthalic acid, Phthalates, are used in the manufacturing of plastics. Bisphenols and phthalates are not archetypical NR-ligands, and inappropriate penetration of side-chains into NR proteins is reflected in the large crash scores (e.g. Diisodecylphthalate and DEHP). Nevertheless, the SYBYL *in silico* molecular model correctly identified all 14 bisphenol and phthalate NR binders (Table 4.13), superceeding the OECD QSAR Toolbox ER-profiler (Section 2.4.3).

4.4.3.1.4 Pharmaceutical *in silico* Docking

Pharmaceuticals, define pharmacologically active substances used in disease diagnosis, treatment and prevention. Regulated by the European Medicines Agency (EMA), Directives 2001/83/EC and Regulation (EC) No.726/2004 stipulate the quality, manufacturing, pharmacovigilance and safety thresholds for pharmaceuticals, generating a plethora of mechanistic studies. However, these studies assess pharmacological dose-ranges, which may not reflect indirect environmental exposure, leading to mounting concern regarding the health implications of pharmaceuticals detected in potable water (IEH, 2012). The *in silico* molecular model correctly identified 56 of the 68 true positives; sensitivity = 85.0% ([68 TP/ (68 TP + 12 FN)] x100) (Table_Apx 2). Eleven of the twelve false negatives were incorrect classification of $_{h}$AR (3V49) binders *in silico*; sensitivity of $_{h}$AR model = 68.6% ([24 TP/ (24 TP + 11 FN)] x100). This inefficiency in detecting hAR binders may reflect problems with the *in silico* molecular modelling and screening, but could also reflect the presence of metabolisation and/or deconjugation of pharmaceuticals *in vitro* and/or *in vivo*. Tamoxifen citrate and atorvastatin calcium were $_{h}$AR non-binders *in silico*, which contradicted the *in vitro* bioassays. Assessing the information logically, the scoring error may reside in virtually screening the pharmaceuticals as their salts, which as mild chelating agents, prevent coagulation of pharmaceuticals, but may also prevent accurate binding assessments; thereby highlighting the limitation of evaluating chemicals in the absence of a metabolic system. Of the 114 pharmaceuticals investigated, 45 $_{h}$ERα, 52 $_{h}$AR, 69 $_{h}$PR and 62 $_{h}$PPARγ new potential nuclear receptor interactions were identified *in silico*.

4.4.3.1.5 Natural Compounds and Consumer Products *in silico* Docking

SYBYL virtual screening correctly identified all investigated cosmetics (n=12), food additives (n=2) and natural compounds (n=19) that bind with nuclear receptors *in vitro* (Table_Apx 2); sensitivity = 100% ([33 TP/ 33TP] x100). Presenting a subset of the data , Table 4.14 shows the *in silico* scoring of cosmetics and personal care products. The *in silico* model correctly identified the $_{h}$ERα binding of parabens, but also suggested interaction with hAR. Used in a plethora of cosmetics and personal hygiene products, Cashmeran (6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone) and 4,4-Dihydroxy-benzaphenone presented unhindered (low inappropriate protein penetration) *in silico* nuclear receptor activity. Natural extracts and consumer products present interesting regulatory considerations, as exposure will vary greatly on lifestyle choices (i.e. diet, bathing routine and product use). However, the

in silico activity suggests many natural extracts and PCP components present endocrine activity, which may be currently overlooked in EDC exposure and risk assessments.

Table 4.14 *In silico* Nuclear Receptor Consumer Product Binding Results

Top values reflect binding score (-log(kD)) and the degree of inappropriate protein penetration (crash score) is shown in parentheses TP=True Positive; P_{is}= Positive *in silico*; FUB=False Unfavourable Binding; UB_{is}=Unfavourable Binding *in silico*; N_{is}=Negative *in silico;* FN=False Negative. See section 4.3.6. and Appendix A for information on true positive criteria. Colour coding: TP's are highlighted green; "assumed" TP's on the basis of interspecies extrapolation are light green; False Negatives are red; *in silico* negatives "assumed" positive are in pink. Unfavourable binders are in orange.

Cosmetics and Personal Care Product Nuclear Receptor Binding (–log(kD))									
Chemical Name	hERα	hERβ	rERβ	hAR	chAR	rAR	mAR	hPR	PPARγ
Cosmetic									
Cashmeran	4.2 (-0.58) P_{is}	4.35 (-1.54) P_{is}	4.34 (-0.96) P_{is}	3.19 (-3.11) P_{is}	3.37 (-1.33) P_{is}	3.02 (-5.0) P_{is}	3.96 (-1.36) P_{is}	3.70 (-1.64) P_{is}	4.51 (-0.99) P_{is}
Celestolide	4.95 (-2.62) P_{is}	2.22 (-4.13) P_{is}	3.61 (-2.86) P_{is}	0.36 (-6.74) UB_{is}	-1.94 (-8.31) N_{is}	-0.15 (-8.02) N_{is}	2.32 (-4.0) P_{is}	1.43 (-5.39) UB_{is}	4.16 (-1.58) TP
Galaxolide	3.36 (-3.31) TP	4.47 (-3.28) P_{is}	4.76 (-2.34) P_{is}	2.05 (-5.0) P_{is}	4.59 (-2.62) P_{is}	-0.73 (-8.96) N_{is}	5.27 (-1.75) P_{is}	4.13 (-2.66) P_{is}	4.01 (-1.51) P_{is}
Propyl-4-hydroxy benzoate	4.24 (-1.50) TP	5.02 (-0.41) P_{is}	4.65 (-0.28) P_{is}	3.67 (-0.49) TP	3.70 (-0.64) P_{is}	4.22 (-0.98) P_{is}	3.56 (-0.38) P_{is}	4.12 (-0.36) P_{is}	3.35 (-0.86) P_{is}
2,4-Dihydroxy benzophenone	4.34 (-0.48) TP	4.02 (-1.68) P_{is}	4.71 (-0.64) P_{is}	4.37 (-0.79) TP	4.69 (-0.48) P_{is}	4.03 (-0.47) P_{is}	3.95 (-0.36) P_{is}	4.18 (-0.38) P_{is}	4.26 (-1.53) TP
Musk Ketone	4.35 (-2.92) TP	3.89 (-3.33) P_{is}	3.94 (-2.01) P_{is}	-0.83 (-6.49) N_{is}	3.12 (-2.03) P_{is}	1.04 (-6.31) UB_{is}	0.64 (-3.83) UB_{is}	3.42 (-2.24) P_{is}	3.0 (-2.09) P_{is}
4-Methoxycinnamic Acid	6.09 (-1.44) TP	4.26 (-4.74) P_{is}	5.51 (-3.12) P_{is}	6.55 (-3.94) P_{is}	2.04 (-6.87) P_{is}	5.05 (-6.66) P_{is}	6.87 (-2.45) P_{is}	6.64 (-2.81) P_{is}	6.3 (-1.68) P_{is}
4,4'-Dihydroxy benzophenone	3.97 (-0.72) P_{is}	4.44 (-1.08) P_{is}	3.84 (-0.83) P_{is}	4.08 (-1.2) P_{is}	5.54 (-0.60) P_{is}	4.2 (-1.73) P_{is}	4.01 (-0.53) P_{is}	4.5 (-0.86) P_{is}	4.33 (-0.86) P_{is}
Parabens									
Butyl-4-hydroxy benzoate	4.56 (-1.13) TP	4.93 (-0.56) P_{is}	4.69 (-0.88) P_{is}	3.88 (-0.63) TP	5.17 (-0.60) P_{is}	4.33 (-0.73) P_{is}	5.1 (-0.47) P_{is}	4.07 (-0.13) P_{is}	4.75 (-0.91) P_{is}
Ethyl-4-hydroxy benzoate	3.95 (-0.73) TP	3.89 (-0.27) P_{is}	3.76 (-0.90) P_{is}	2.9 (-0.84) P_{is}	3.17 (-0.92) P_{is}	3.17 (-0.82) P_{is}	3.2 (-0.19) P_{is}	4.36 (-0.58) P_{is}	4.59 (-0.19) P_{is}
Methyl-4-hydroxy benzoate	3.57 (-1.42) TP	3.8 (-0.72) P_{is}	3.34 (-0.44) P_{is}	3.38 (-0.55) P_{is}	3.05 (-0.31) P_{is}	3.41 (-0.48) P_{is}	3.18 (-0.3) P_{is}	3.32 (-0.62) P_{is}	3.48 (-0.85) P_{is}
Benzyl-4-hydroxy benzoate	4.76 (-0.87) TP	4.17 (-1.04) P_{is}	4.2 (-0.53) P_{is}	4.28 (-0.43) P_{is}	4.64 (-0.34) P_{is}	5.3 (-0.66) P_{is}	4.31 (-0.53) P_{is}	4.47 (-0.61) P_{is}	2.8 (-0.43) TP

4.4.4 Regression Analysis of Virtual Screening Results

A number of structural features have been demonstrated to affect ligand binding. For example, the MW and partition coeffication (LogP) have been utilised as QSAR binary classification predictors, for ER-binding (Netzeva *et al.*, 2006; Piparo & Worth, 2010). In accordance with these hypotheses, the SYBYL Surflex-Dock virtual screening results were analysed relative to their physicochemical properties. The physicochemical properties of the potential-EDC database (n=378) were calculated in SYBYL Molecular Spreadsheets, and exported into excel for regression analysis with IMB SPSS and R statistics software.

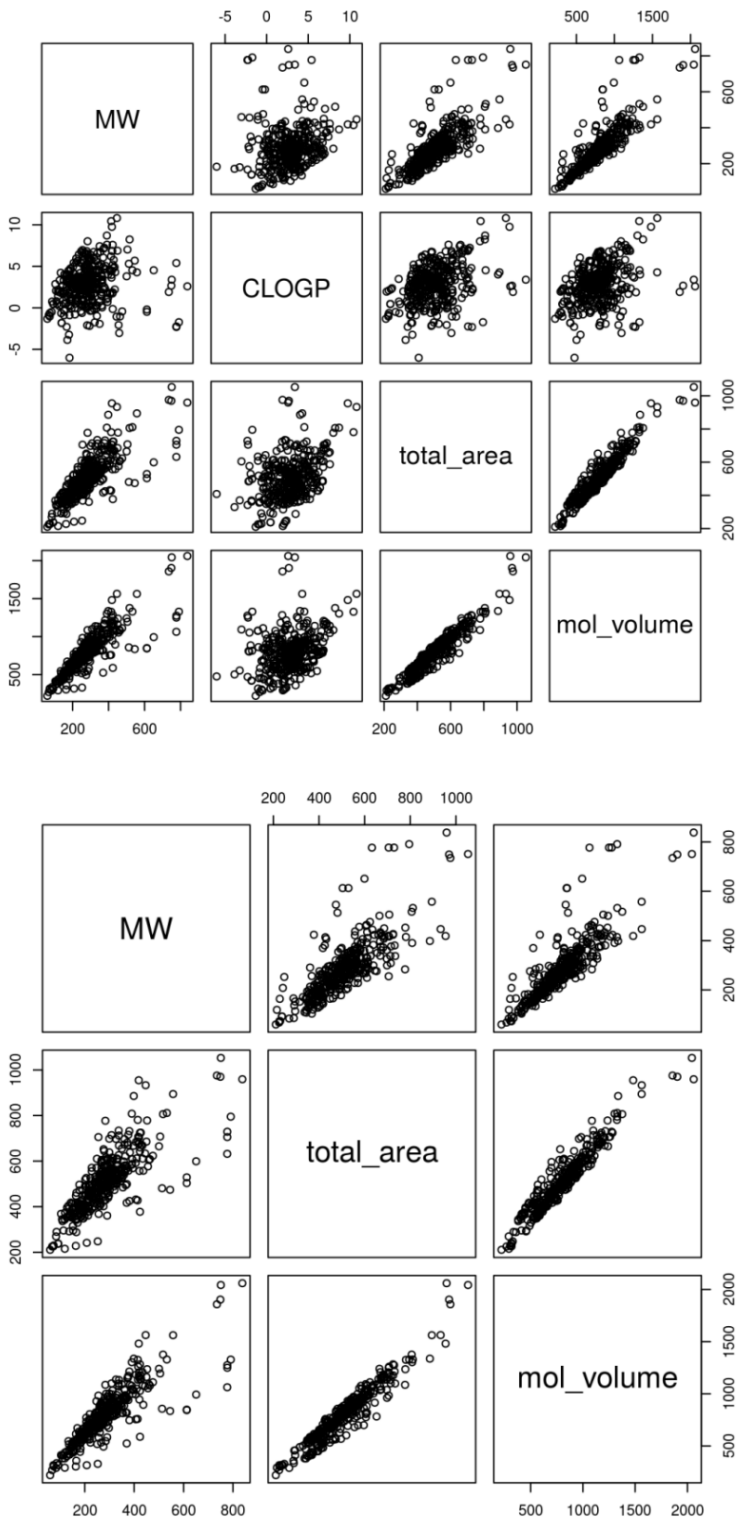


Figure 4.10 Regression Analysis of Physicochemical Properties of Chemical Database

Plots show the regression analysis of the physicochemical properties of the prioritised chemical database (n=378). Indices of Molecular Weight (Da); ClogP (partition coefficient); total area ($Å^2$); and, molecular volume ($Å^3$) were included in coefficient analysis and plotting in R-statistics software. From the left-hand plot it is clear to see that there are positive correlations between all variables, however, the data was relatively dispersed for ClogP. The right hand plot shows the strong relationship between MW, total area and molecular volume, as expected, these variables are dependent.

Figure 4.10 shows the relationship between the physicochemical properties of the prioritised chemical database (n=378). Regression of MW (Da), ClogP (calculated partition coefficient), total area ($Å^2$), and, molecular volume ($Å^3$) in R statistics software, identified positive correlations between all variables, suggesting non-independence and limiting the use of multiple regression. The ClogP coordinate data was dispersed; however, strong relationships between MW, total area and molecular volume were identified.

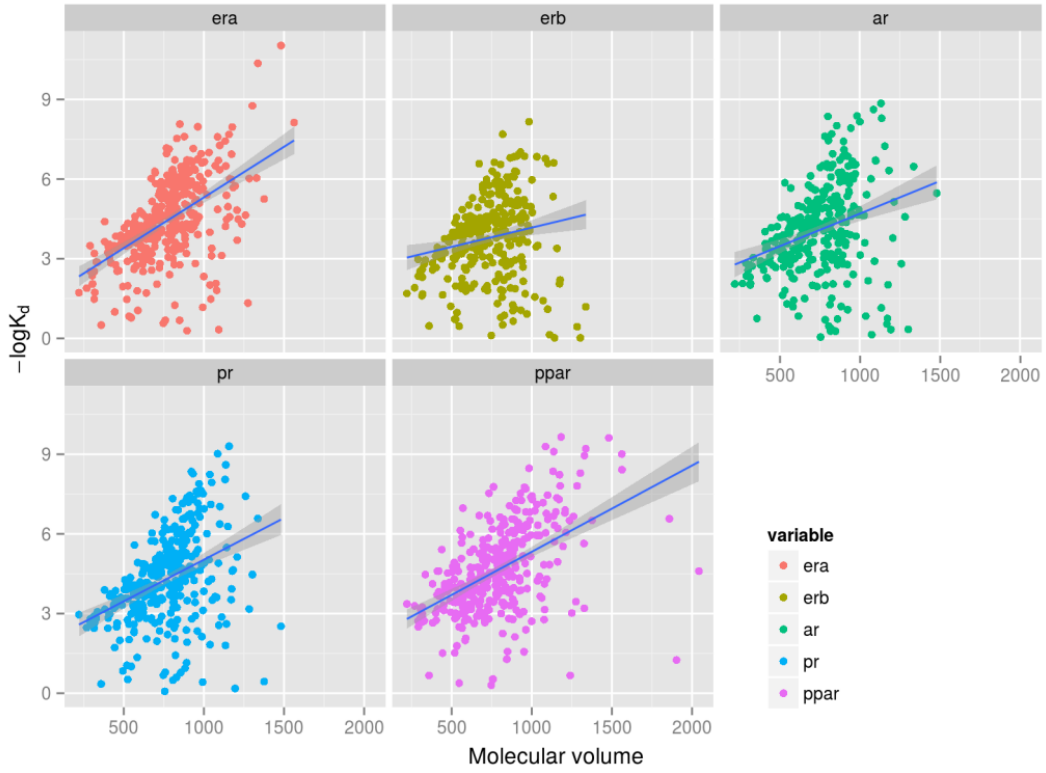


Figure 4.11 Correlation Between *in silico* Nuclear Receptor (ERα, ERβ, AR, PR and PPARγ) Binding Score (-logK_d) and Ligand Molecular Volume

Plot and regression analysis in R demonstrated positive correlections between molecular volume and *in silico* SYBYL NR binding: estrogen receptor-α (era): $y = 1.412 + 0.00382x$ [R^2 = 0.284, p-value <2e-16]; estrogen receptor-β (erb): $y = 2.72 + 0.00145x$ [R^2 = 0.0312, p-value 0.000994]; androgen receptor (ar): $y = 2.23 + 0.00246x$ [R^2 = 0.0907, p-value 1.98e-08]; progesterone receptor (pr): $y = 1.892 + 0.00314x$ [R^2 = 0.155, p-value 2.15e-14]; and, peroxisome proliferator activator receptor (ppar): $y = 2.078 + 0.00325x$ [R^2 = 0.248, p-value <2e-16]. Energetically unfavourable binding scores (<0–logKd) were exluded from the analyses. The dispersion of molecular volume coordinates, highlights the variability of the chemical database and putative ligands.

The variables downloaded (MW, CLogP, area and volume) were interdependent, limiting the suitability of multivariate analysis (Figure 4.10). Excluding energetically unfavourable interactions (<0–logKd), Figure 4.11 illustrates the positive correlections between molecular volume and *in silico* SYBYL NR binding: estrogen receptor-α (era): $y = 1.412 + 0.00382x$ [R^2 = 0.284, p-value <2e-16]; estrogen receptor-β (erb): $y = 2.72 + 0.00145x$ [R^2 = 0.0312, p-value 0.000994]; androgen receptor (ar): $y = 2.23 + 0.00246x$ [R^2 = 0.0907, p-value 1.98e-08]; progesterone receptor (pr): $y = 1.892 + 0.00314x$ [R^2 = 0.155, p-value 2.15e-14]; and, peroxisome proliferator activator receptor (ppar): $y = 2.078 + 0.00325x$ [R^2 = 0.248, p-value <2e-16]. Thus, regression analysis suggests that the SYBYL Surflex-Dock NR virtual screening agreed with MW and LogP binding predictors.

4.4.5 SYBYL Surflex-Dock Virtual Screening – A 'Good' *in silico* Tool?

The development of alternative (non-animal) methods for EDC hazard characterisation has become a high political priority, leading to a plethora of computational databases, QSAR, molecular modelling and decision tree approaches, to identify endocrine active substances (Piparo & Worth, 2010). Derek for Windows (DfW) detects structural alerts for developmental toxicity (n=3), teratogenicity (n=5), testicular toxicity (n=1) and estrogenicity (n=4), which indicated 72% sensitivity and 100% specificity, in a pilot validation study of 34 chemicals (Pearl *et al.*, 2001). In a similar vein, Toxmatch clusters chemicals on the assumption that structurally similar chemicals act by similar mechanisms (Enoch *et al.*, 2009). Quantifying interactions with *in silico* macromolecular targets, VirtualToxLab combines multidimensional QSAR with flexible docking (Verdani *et al*., 2012). While, a binary classification model of ER-binding, which utilised logP and hydrogen bond donor descriptors, was developed by Netzeva *et al*. (2006). Evaluation of the Netzeva *et al*. (2006) decision tree indicated sensitivity of 84% and specificity of 69%, relative to *in vitro* reporter gene assays (Gallegos-Saliner *et al*., 2006), which supplemented the development of the OECD QSAR Management group (OECD, 2009).

The OECD QSAR Toolbox ER-profiler categorises two dimensional chemical structures on the basis of cyclicity, molecular weight (<500 Da) and hydroxyl and/or NH_2 groups (Liu *et al*., 2006; Mombelli, 2012). Non-binders are chemicals which do not satisfy these criteria, or present steric hindrance of OH/NH_2 groups by ortho-disubstitutions (see Section 2.4.3). The development of the ER-profiler required a wealth of structural and functional information (Tong *et al*., 2004; Bignon *et al*., 1989; Bradbury *et al.*, 2000; Cronin & Worth, 2008; Schmieder *et al*., 2003). On the contrary, in agreement with VirtualToxLab flexible docking technologies (Verdani *et al*., 2012), this study generated novel three dimensional pseudo-molecular docking targets from molecular probes (CH_4, C=O and N-H), which were identified by rolling solvent spheres (Section 4.3.4.3) over the surface of published X-ray crystallography protein structures ($_{h}$ERα, $_{h}$ERβ, $_{rat}$ERβ, $_{h}$AR, $_{chimp}$AR, $_{rat}$AR, $_{mouse}$AR, $_{h}$PR and $_{h}$PPARγ) in Tripos™ SYBYL 7.3 software. An energetically minimised potential-EDC 3D database was screened against generated protomols in Surflex-Dock, which scored the *in silico* interactions according to Hammerhead and Bohn functions; accounting for hydrophobic contact, polar interactions and entropic fixation costs for loss of torsional and rotationa degrees of freedom (Bohn, 1996; Eldrige *et al*., 1997). Conceptually, 3D molecular modelling and dynamics, provide an object orientated framework for ligand-binding (Matthey *et al*., 2004; Balaji *et al*., 2013) which are advantageous over 2D QSAR statistical analyses.

- ✓ **The SYBYL Surflex-Dock virtual screening conducted in this study demonstrated mean sensitivity of 94.5% (291/309); representative of $_{h}$ERα = 97.5% (115/118), $_{h}$AR = 88.1% (96/111), $_{h}$PR = 100% (6/6) and $_{h}$PPARγ = 100% (74/74).**

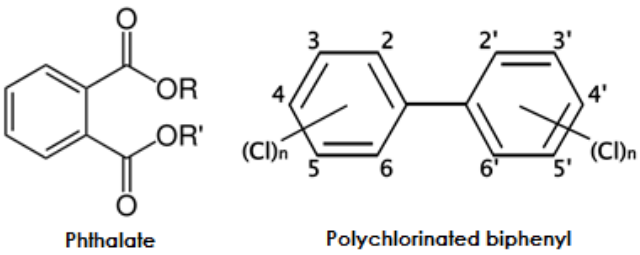


Figure 4.12 Chemical Structure of Phthalates and Polychlorinated biphenyls (PCBs)
Esters of Phthalic acid, Phthaltes are produced by the reaction of phthalic anhydride with alcohols. The alcohol carbon chain length, ranging from methanol (C1) to tridecyl alcohol (C13), defines the R variable alkyl chain, which is used as a crude predictor of hazard; low Molecular Weight (LMW) and high MW phthalates, range from 194.18Da (Dimethyl phthalate) to 530.82Da (Disotridecyl phthalate). Polychlorinated biphenyls are synthetic compounds manufactured by reaction of biphenyl with 1 to 10 chlorine atoms. There are 209 PCB configurations.

Specificity of 80.7% was calculated, however, epistemological limitations in the science and adopted method, hindered the definition of true negatives, which hinder the calculation (Section 4.4.3.1). The OECD Toolbox ER-profiler sensitivity has been reported at 84.1% (116/138) and 68.3% specificity (71/104) for human datasets (Mombelli, 2012). However, the aforementioned criterion has led to inefficiency in detecting moderate binders, such as phthalates and PCBs. This inefficiency can be explained by the 2D-structure of phthalates and PCBs (Figure 4.12). Phthalates and PCB's do not have the NH_2 or OH groups used to predict moderate and strong ER binding (Table 2.3), furthermore many exceed 500Da, leading to immediate classification as non-binders by the OECD Toolbox.

Scoring interactions on the basis of three-dimensional hydrophobic contact, polar interaction, and degrees of freedom, the SYBYL Surflex-Dock method adopted in this study provides 3D-docking flexibility, which correctly identificatied all investigated phthalate binders (Table 4.13).

- ✓ **The SYBYL Surflex-Dock method, demonstrated higher sensitivity and specificity in correctly identifying endocrine active substances (EAS), than the OECD Toolbox ER-profiler (Mombeli, 2012), DfW (Pearl *et al*., 2001) and binary classification model (Netzeva *et al*., 2006).**

Superceeding currently available tools, Tripos™ SYBYL macromolecular modelling of NR and Surflex-Dock screening of putative ligands', has been identified as a potentially useful regulatory tool to support EAS hazard identification. However, the sensitivity observed is at a cost of specificity. SYBYL Surflex-Dock scoring does not assess the functional importance of molecular probes and thus neglects the conformational changes required to define agonism or antagonism (Section 3.1.1.1 and 3.2.2). Predictivity was calculated relative to cell proliferation in dependent cell lines, reporter gene transactivation, binding assays and dissociation constants *in vitro.* Thus, sensitivity was calculated relative to endocrine activity (*in vitro*), rather than endocrine disruption (*in vivo*). The predictive performance of *in vitro* STTA, relative to the *in vivo* Immature Rat Uterotrophic Assay, presented sensitivity, specificity and concordance of 91%, 88% and 90%, respectively (Takeyoshi, 2006). The Uterotrophic Assay is OECD Conceptual Framework Level 3, i.e *in vivo* assay evaluating one

MoA (Figure 2.2), and therefore does not conclusively test for endocrine disruption. Highlighting the gap between *in silico*, *in vitro* and *in vivo* mechanistic studies, with conclusive *in vivo* chronic toxicity tests (Level 4 and 5). Nevertheless, the predictivity of the SYBYL Surflex-Dock Virtual Screening surpassed that of the OECD ER-profiler, which was comparable in performance to validated skin irriation models (Mombeli, 2012); Murine Local Lymph Node Assay (LLNA); and, Episkin® protocols (ECVAM, 2010; Portes *et al*., 2002). SYBYL Surflex-Dock virtual screening has been demonstrated as a potentially useful and superior *in silico* screening tool for EDC hazard characterisation.

4.5 *In silico* Summary and Conclusions

This chapter aimed to explore the theory and application of *in silico* molecular modelling and dynamics to EDC hazard characterisation. Identified in Section 3.1, nuclear receptor molecular targets (ER, AR, PR and PPAR) were prioritised for investigation.

Bootstrap-consensus phenograms, founded on Neighbour-Joining p-distance amino acid substitution statistical methods, successfully presented the evolutionary divergence of ERα (n=32) and ERβ (n=27) paralog and AR (n=58) orthologs (Thornton, 2001). Nuclear receptor phylogeny, presented as the topology, branch length and root, suggested sequence variance was consistent with taxonomic rank i.e. evolutionary distinct species present more sequence divergence. Thus, assuming protein sequence is indicative of function, uncertainty in extrapolating endocrine toxicological mechanisms may increase with evolutionary distance. The phenograms highlight the potential folly of species bias in regulatory toxicology; 75% of *in vivo* toxicological procedures for human health are conducted in rats (*R.norvegicus*), while ecotoxicology limits itself to three fish (*D. rerio*, *P. promelas* or *O. mykiss*), bird (*C. japonica*) and/or collembolan (*F. fimetaria*) models, representing a very limited assessment of potential inter- and intra- species variation. No invertebrate NR sequences were identified in BLAST searches, which suggests vertebrate paralogs (>60% sequence conservation) are more homologous, than invertebrate NR "orthologs", corroborating suggestions that invertebrate endocrinology is incomparable to that of vertebrates (Scott, 2013).

The Tripos™ SYBYL solvation method accurately predicted the LBP of hERα and hAR.; identifying the amino acid residues and molecular probes demonstrated to define binding (Brzozowski *et al*., 1997; Shiau *et al*., 1998; Sack *et al*., 2001). X-ray crystallography structures for hER (3DT3), hER (1X7J), ratER (1HJ1), hAR (3V49), chimpAR (1T7T), ratAR (21HQ), mouseAR (2QPY), hPR (1SQN) and hPPARγ (1PRG), were modelled. The SYBYL molecular modelling of chimpanzee, rat and mouse ER and AR identified conservation of hydrophobic (Ala, Leu and Phe) and uncharged polar (Gln/Asn) residues. In addition, a valine residue at 715 was conserved between all investigated AR, suggesting that the hydrophobic-side chain amino acid is important to function. However, the Thr877 residue detected in human AR, which determines ligand promiscuity (Sack *et al*., 2001; Taplin *et al*., 1995), was not detected in the chimp, rat or mouse AR LBP, supporting phylogeny results and the intra- and inter-species differences detailed in Sections 2 and 3; however, demonstration of LBP differences *in vitro* and *in vivo* are required to clarify concerns. The accuracy of the solvation method in determining ER and AR LBP validates the method

(Section 4.3.4) for *in silico* predictions of less characterised NR LBP, such as PR and PPARγ; detailed in Appendix C: Figure_Apx 7 and Figure_Apx 8. The results suggest that Tripos™ SYBYL 7.3 software can 'blindly' predict the LBP of NR for protomol generation and virtual screening, in lieu of structural information and annotation, which is requisite for traditional QSAR approaches.

Table 4.15 SYBYL Surflex-Dock Virtual Screening Sensitivity and Summary Data

Table **4.15** provides a summary of the number of new potential ERα, AR PR and PPARγ binders identified in the SYBYL Surflex-Dock virtual screening. True positive *in vitro* EAS were excluded from the tally, however, the sensitivity in correctly identifying true positives is shown in the second column.

Category	Sensitivity	*in silico* 0<2 –log(Kd)				*in silico* >2 –log(Kd)			
		ER	AR	PR	PPAR	ER	AR	PR	PPAR
Hormones	**94.8%**	0	0	1	0	6	0	9	13
Pesticides	**96.4%**	6	6	13	2	66	56	97	84
Industrial	**98.1%**	6	4	4	3	45	52	69	62
Pharmaceuticals	**85.0%**	2	4	5	3	85	74	94	88
Consumer goods	**100%**	0	1	1	0	9	9	14	11
Phytochemicals	**100%**	0	0	0	0	5	10	16	13

Conceptually, 3D molecular modelling and dynamics, provide an object orientated framework for ligand-binding (Matthey *et al.*, 2004; Balaji *et al.*, 2013) which are advantageous over 2D QSAR statistical analyses. SYBYL Surflex-Dock virtual screening demonstrated mean sensitivity of 94.5%; representative of $_{h}$ERα = 97.5%, $_{h}$AR = 88.1%, $_{h}$PR = 100% and $_{h}$PPARγ = 100%. Specificity of 80.7% was calculated, however, epistemological limitations in the science and adopted method, hindered the definition of true negatives, which then hinders the calculation (Section 4.4.3.1). Scoring interactions on the basis of three-dimensional hydrophobic contact, polar interaction, and degrees of freedom, the SYBYL Surflex-Dock method demonstrated higher sensitivity and specificity in correctly identifying EAS, than the OECD Toolbox ER-profiler (Mombeli, 2012), DfW (Pearl *et al.*, 2001) and binary classification model (Netzeva *et al.*, 2006).

The study reported here demonstrates important *in silico* developments since the initial use of SYBYL macromolecular modelling by Wu *et al.* (2010) in which interspecies variation of nonylphenol, BBP, BPA, 4'4-DDE, hexabromodiphenyl ether, Linuron and testosterone AR binding *in silico* were investigated. However, further investigation of interspecies ligand binding *in vitro* (Lange *et al.*, 2012; Wilson *et al.*, 2007; Wells & Van Der Kraak, 2000), is required to validate the sensitivity of molecular modelling in predicting interspecies variation. In conclusion, SYBYL molecular modelling and Surflex-Dock virtual screening have been identified as a potentially useful regulatory tool to support EAS hazard identification. Furthermore, a number of chemicals were shown to present endocrine activity *in silico*, justifying further investigation *in vitro*.

4.5.1 *In silico* Approaches to EAS: Study Milestones

- ✓ Phenograms identified evolutionary divergence of ER and AR amino acid sequences;

- ✓ A structurally diverse chemical database of 378 potential-EDCs was curated, including 118 ERα, 111 AR, 6 PR and 74 PPARγ EAS *in vitro* (n=309);
- ✓ The ligand binding pockets of $_{h}$ER (3DT3), $_{h}$ER (1X7J), $_{rat}$ER (1HJ1), $_{h}$AR (3V49), $_{chimp}$AR (1T7T), $_{rat}$AR (21HQ), $_{mouse}$AR (2QPY), $_{h}$PR (1SQN) and $_{h}$PPARγ (1PRG), were successfully modelled in SYBYL SiteID using the solvation method;
- ✓ Interspecies variation in virtual ER and AR ligand binding pockets were demonstrated;
- ✓ *In silico* pseudo-molecular NR LBP targets (ERα, AR, PR and PPARγ protomols) were virtually screened against a compound library of 378 potential-EDCs;
- ✓ SYBYL Surflex-Dock Nuclear Receptor (ER, AR, PR & PPARγ) virtual screening demonstrated a sensitivity of 94.5%; of 309 true positives, 291 chemicals were correctly identified, 18 were incorrectly scored as non-binders;
- ✓ The SYBYL Surflex-Dock method superseded the OECD Toolbox ER-Profiler, DfW and binary classification models, in correctly identifying EAS;
- ✓ A number of chemicals were shown to present endocrine activity *in silico*, highlighting the need for further *in vitro* assays and conclusive testing;
- ✓ Regression analysis identified significant correlations between binding score, molecular volume, MW, molecular area and ClogP; and
- ✓ Tripos™ SYBYL molecular modelling and Surflex-Dock virtual screening has been identified as a potentially useful automated tool for EDC hazard characterisation.

4.5.2 Future Work for Development of *in silico* EDC Methods

Whilst exploring the theory and application of *in silico* molecular modelling and dynamics to EDC hazard characterisation, a number of difficulties, caveats and potential developments of *in silico* methods were identified.

The curation of a potential-EDC database from publically available information hubs highlighted an array of biotechnology tools. However, software and bioinformatics tools varied with jurisdiction, increasing disparity and disconcordance of *in silico* approaches. A harmonised data hub, pooling UK (EndiChem; RSC ChemSpider), EU (PubChem; RCSB PDB; VirtualToxLab; ChemProt) and US (EDKB; ZINC) sources would aid the accessibility and consistency of *in silico* approaches; under the current paradigm, the sourcing and significance of online data is uncertain.

The strategy for positive NR-binding classification, for inclusion in this study, required a demonstration of *in vitro* NR activity; this comprised cell proliferation of hormone-dependent cell lines, reporter gene studies and radiolabelled-binding/dissociation assays (PubChem Bioassay databank). However, this method presented a number of caveats, including the potential classification of false positive binders (*in vitro*), on the basis of ligand-independent increases in transactivation or stimulation of cellular proliferation via alternative mechanisms. Furthermore, the significance of observations *in vitro* remained uncertain; for example, Endosulfan was classified as a positive binder of ER and AR for *in silico* sensitivity analysis on the basis of 8/16 and 3/16 positive *in vitro* results, respectively. This highlights the question, what burden of inactive *in vitro* data justifies classification as a non-binder? The

OECD stipulates that statistically significant responses in the *in vitro* ER STTA assay, repeated on 3 separate days, provide conclusive evidence that a chemical influences ER signalling pathways. Two day repeats provides strong evidence, while 1 day repeat suggests ER interaction. However, most academic research is not conducted to OECD standard, limiting the application of these constraints. Development of decision criteria to categorise academic *in vitro* bioassay data, would promote consistency among research groups, which may be evaluating *in silico* predictivity with inconsistent constraints. Equally, excluding the Uterotrophic Assay, observations of endocrine disruption *in vivo* are not directly comparable to *in vitro* and *in silico* binding predictions. Harmonised translucent bioinformatic mapping of *in silico*, *in vitro* and *in vivo* evidence is vital to understand adverse outcome pathways and the relevance of alternative (non-animal) lower tier testing.

A significant limitation of *in silico* molecular modelling is the inability to predict cellular metabolism. Incorporation of metabolic simulator, such as T.I.M.E.S Tissue Metabolism Simulator (Mekenyan *et al*., 2003; Schmieder *et al*., 2003), may increase the sensitivity of nuclear receptor virtual screening

The reliability of the *in silico* SYBYL screening is intrinsically linked to the reliability of NR modelling. For the purpose of this study, only proteins with known crystal structures were included, which limited the number of NR's investigated. X-ray crystallography selectively favours the nuclear receptor conformations most likely to crystalise (Srinivasan *et al*., 2013); i.e. dimers with bound ligands and coregulators. X-ray crystallography of sentinel species NR would enable virtual screening of a larger array of species. For example, as an ecologically important species, a wealth of mechanistic and ecotoxicology studies have investigated EDC's in fish. Interspecies variation has been reported in teleost fish ligand-dependent ER, AR and PXR transactivation *in vitro* (Lange *et al*., 2012; Kojima *et al*., 2011; Wilson *et al*., 2007). X-ray crystallography of the aforementioned fish NR LBP would enable virtual screening, with an evaluation of sensitivity relative to *in vitro* reporter gene assays.

Nuclear receptor LBP structure can be predicted from amino acid sequence *in silico*, in the absence of X-ray crystallography via structural homology modelling of protein databanks. However, this functionality was beyond the Tripos™ SYBYL licence available for this study. Accurate prediction of NR LBP, without specific X-ray crystallography studies, would enable 3D *in silico* nuclear receptor screening of any NR sequence data; there are over 628 ER and 394 AR reviewed sequences available on UniProt, including a plethora of taxonomic ranks. Validation of 'blind' molecular modelling, in addition to the 'blind' molecular screening demonstrated in this study, would enable the virtual screening of diverse nuclear receptor libraries with large chemical databases; automatically screening inter- and intra-species NR susceptibility to putative ligands.

However, a number of advancements would need to be made, prior to achieving this, including the confirmation of *in silico* interspecies variation *in vitro*. Further investigation of interspecies ligand binding *in vitro*, is required to validate the sensitivity of molecular modelling in predicting species variation.

Sructural interactions of NR, identified in Section 1, highlight potential *in silico* developments. SYBYL Surflex-Dock creates a pseudo-molecular target of the molecular probes identified in the ligand binding pocket, thereby evaluating binding independent of the protein as a whole. Although ligand binding occurs in NR LBD monomers, justifying this method, the structural implications of monomer stabilisation by HSP association are uncertain. Furthermore, the conformational repercussions of predicted ligand-binding on the dimerisation site, DNA-binding site and cofactor regulatory site are ignored and thereby prevent prediction of agonism or antagonism. SYBYL Surflex-Dock pilot studies of active NR ligand binding domains for interaction with known cofactors conducted herein (Figure_Apx 3 and Figure_Apx 4), suggest that the *in silico* model is not appropriate to predict surface affinity. Development of a multi-stage NR binding model, which enabled prediction of the conformational repercussions of ligand-binding and cofactor binding, would enable predictions on agonism/antagonism. This would not be possible without collaboration with Tripos™ SYBYL software developers or creation of a supplementary tool.

4.5.2.1 Summary of Future Work

- ✓ Development of a harmonised biotechnology software hub with evaluated, translucent *in silico*, *in vitro* and *in vivo* evidence;
- ✓ Criterion for positive/negative binding classification from pooled *in vitro* data;
- ✓ Inclusion of *in silico* metabolic prediction software
- ✓ Elucidation of diverse sentinel species NR X-ray crystallography structures;
- ✓ Validation of LBP molecular modelling for inter-/intra- species variation;
- ✓ SYBYL Surflex-Dock screening of a larger mechanistically diverse database; and,
- ✓ Development of Surflex-Dock to incorporate multi-stage assessment.

5 ENDOCRINE ACTIVITY *IN VITRO*

In its entirety, this study aims to evaluate the current *in silico* and *in vitro* tools for EDC screening and hazard characterisation. The application of *in silico* molecular modelling and virtual screening to EDC hazard characterisation was detailed in Section 1. This chapter aims to evaluate the currently available *in vitro* tools for endocrine modes-of-action, via literature review and the testing of potential-EDCs in standardised *in vitro* mechanistic assays. The *in vitro* methods adopted herein provide mechanistic and potency information for EDC hazard characterisation.

5.1 Introduction to *in vitro* Toxicology

Cell culture, the *in vitro* growth of cells derived from multi-cellular organisms, enables investigation into cell physiology and function in a defined environment, and has been utilised in the study of: disease processes; receptor-ligand interactions; drug binding assays; mutagenicity; the production of recombinant proteins; biology; and toxicology.

The classical genomic pathway is initiated by endogenous hormones activating the LBD of nuclear receptors, which causes dissociation of heat shock proteins and dimerization, leading to transcriptional activation and ultimately cellular response (Section 3.1). The steroid hormones and NRs regulate transcription of specific genes, which regulate cell proliferation, foetal development and reproductive function. EDC research has focused primarily on estrogenicity and androgenicity of the HPG axis, consequent to observations in exposed wildlife. A number of *in vitro* tests have been developed to identify ER and AR agonists and antagonists, including: receptor binding; cell proliferation; gene expression; and inhibition/stimulation of hormone synthesis (Gray *et al.*, 1998).

Assays evaluating the direct interaction of potential-EDCs with nuclear receptors (binding affinity) successfully characterised ERα and ERβ using radiolabelled competitive binding assays (Kuiper *et al.*, 1998). However, similar to the *in silico* assessments (Section 1), receptor binding assays do not distinguish between agonistic and antagonistic binding but may minimise false negatives (Vonier *et al.*, 1996). High throughput techniques utilising nonradioactive fluorescence have been developed, in which changes in polarisation of fluorescently tagged E2 can be assessed in response to increasing concentrations of competitor compounds (Bolger *et al.*, 1998). Alternatively, Fluorescence or Bioluminescence Resonance Energy Transfer (FRET or BRET, respectively) assays can measure the dimerization of nuclear receptors (Tamrazi *et al.*, 2002), or receptor-coregulator interactions (Liu *et al.*, 2003), subsequent to ligand binding. BRET assays are characterised by an enzyme catalysed bioluminescent donor such as luciferase, which emits a photon of light in the presence of substrate and can be fused to the nuclear receptor of interest (Xu *et al.*, 2007).

Cellular proliferation assays measure *in vitro* proliferation of hormonally dependent cell lines, on the premise that agonistic xenobiotics will cause cells to proliferate, while antagonists will inhibit the proliferation in response to endogenous hormones. The E-screen, for example, utilises the estrogen-dependent human breast cancer cell line, MCF-7, to detect xenestrogens (Ankley *et al*., 1998). However, guidance documents published by the OECD (2012a) and ICCVAM (2003) did not recommend proliferation assays, due to the potential proliferation through cellular pathways, other than those involving the transcriptional activation of hormone responsive genes. Nevertheless, the US EDTA is leading development of a standardised test guideline for the '*MCF-7 Cell Proliferation Assay for the Detection of Estrogen Receptor Agonist and Antagonist*' (OECD, 2013), which would be used in a battery of tier 1 tests (Figure 2.2).

Ultimately cellular response is the result of gene transcription. Gene expression assays measure gene expression induced by hormone receptor activation (Zacharewski, 1997), via the measurement of endogenous gene products (mRNA), or the induction of response elements, stably or transiently, transfected with a reporter enzyme (e.g. luciferase or β-galactosidase). Reporter gene assays utilise *in vitro* eukaryotic cell lines which catalyse chemillumiscent, radioactive or colorimetric enzymatic reactions consequent to response element induction, which quantitatively represents gene expression. A derivative of the MCF-7 cell line, MCF-7 MVLN, has been used to detect estrogenic activity of single chemicals and complex mixtures in a reporter gene assay (Routledge & Sumpter, 1996; Zacharewski *et al.*, 1995). Gene expression assays can distinguish between agonists and antagonists, as a consequence of the coregulatory mechanisms detailed in Section 3.2 (i.e. downsteam signalling). Nuclear receptor transactivation is considered one of the key mechanisms of endocrine disruption (ED). However, the transient transfection of plasmids is integral to novel reporter gene assays, which is labour intensive and introduces inter-assay variation (ICCVAM, 2003).

However, ligand-dependent transactivation is not the only mechanism of endocrine disruption, which may also occur through: inter-receptor interations; hormone synthesis; metabolic activation and/or inactivation of hormones; distribution of hormones to target tissues; and, clearance of hormones from the body. The OECD H295R Steroidogenesis Assay utilises NCI-H295R cells, which resemble undifferentiated human foetal adrenal cells and express steroidogenic enzymes, to detect substances that affect the production of E2 and testosterone. The assay incorporates biosynthetic reactions that are stage specific, thus no *in vivo* tissue would express all the enzymes at once. Albeit not predictive of specific *in vivo* responses, validation studies demonstrated that chemicals were accurately flagged as reproductive toxicants or disruptive of steroidogenesis, (OECD, 2012a).

5.1.1 Testing for Estrogenicity

Perturbation of estrogen homeostasis may trigger adverse effects on normal development (ontogenesis), reproductive health and the integrity of the reproductive system. The OECD TG455 Stably Transfected human estrogen receptor alpha (ERα) Transcriptional Activation Assay (ER STTA) detects chemicals that activate ERα; the receptor-ligand complex binds to specific DNA response elements and transactivates a reporter gene, such as luciferase.

A number of studies have compared the efficacy of test methods to detect estrogenic chemicals. Lee *et al.* (2012) compared the estrogenic activity of DEHP, DBP, BBP, Diethyl phthalate (DEP), Tetrabromobisphenol A (TBBPA), Bisphenol A (BPA) and Nonylphenol (NP) in ERα STTA, ER binding (Akahori *et al*., 2008), E-Screen (Soto *et al*., 1995) and Yeast screening (Coldham *et al.*, 1997) assays. In the HeLa9903 STTA, the estrogenic activities of BBP (PC_{50} 4.32 $x10^{-6}$ M), BPA (PC_{50} 1.26 $x10^{-7}$ M) and NP (PC_{50} 2.92 $x10^{-6}$ M), were significantly lower than 17β-Estradiol (E2 PC_{50} 2.43 $x10^{-11}$M). DEHP, DBP and DEP did not show any estrogenic activity. The ERα binding affinities of BBP, BPA and NP were approximately 200000-fold (IC_{50} 4.91 x 10^{-4} M), 8000-fold (IC_{50} 1.92 $x10^{-5}$M) and 1400-fold (IC_{50} 3.34 x 10^{-6}M) less than E2 (IC_{50} 2.45 $x10^{-9}$M), in competitive human ER binding assays. Thus, the relative potencies of STTA assay were comparable to ER binding, E-SCREEN, and Yeast screening assays (Lee *et al*., 2012).

The relative potencies of potential-EDCs have been compared in luciferase reporter gene assays (MVLN and HGELN cell lines), competitive binding assay (hERα and hERβ) and proliferation of MCF-7 cells (E-Screen). The authors reported assay sensitivity decreased in the order of: MVLN cells ~ E-Screen> HGELN cells> binding ERα> binding to ERβ. Potencies obtained in cell free binding assays were 1-2 orders of magnitude higher than those obtained in cell culture assays. However, plotting the relative potencies obtained in the luciferase reporter gene assays demonstrated a slope of 0.957 (R=0.999). Results obtained in the E-Screen and MVLN cell line were significantly correlated (1.049, R=0.9891). Regardless of sensitivity, all assays ranked the chemical potencies in the same order (Witters *et al*., 2010; Gutendorf & Westendorf, 2001).

Numerous stably transfected human estrogen receptor alpha (ERα) transcriptional activation assays have been reported (Anderson *et al*., 2002; Escande *et al*., 2006; Takeyoshi *et al*., 2002; Du *et al*., 2010). Recently adopted by the OECD, Test Guideline 455 identifies chemicals that induce downstream transactivation of ERα and ERα/β, in HeLa9903 and BG1Luc cells, respectively. Vertebrates predominantly express ERα, which mediates the classic estrogenic response (Anderson *et al.*, 1972) and ERβ is coexpressed to a lesser extent. The BG1Luc STTA incorporates stably transfected ER in the human ovarian adenocarcinoma cell line (BG-1), to provide concentration-response data for *in vitro* ER agonism and/or antagonism (Rogers & Dennison, 2000; Cavailles,

2002). Expressing both ERα and β, the BG1Luc ER STTA surpasses the HeLa9903 ER STTA, which only measures interaction with ERα. Validation studies have shown the TG455 ER STTA to be sensitive and specific, relative to the *in vivo* Uterotrophic Assay; predictive performance of the HeLa9903 STTA in detecting estrogenicity, relative to the *in vivo* Immature Rat Uterotrophic Assay presented sensitivity, specificity and concordance of 91%, 88% and 90%, respectively (Takeyoshi, 2006).

Both the ER STTA and BG1Luc ER TA quantify transactivation of responsive genes by measuring chemiluminescence. Luciferase activity can be quickly evaluated using a microplate luminometer, generating the RLU (relative luciferase units), required to establish a positive or negative result. However, it is important to note that some chemicals inhibit luciferase enzymes by protein stabilisation, which confounds chemiluminescence measurements (Thorne *et al.,* 2010). Both OECD STTA assays are hindered by the potential for false positives, via chemicals that increase chemiluminescence without interacting with ERα; such as Genistein and Daidzein (Kuiper *et al*., 1998; Escande *et al*., 2006).

5.1.2 Testing for Androgenicity

There are currently no OECD Test Guidelines to assess mechanisms of androgenicity or AR binding. The US EPA EDSP has validated and adopted a rat AR binding assay (US EPA.OPPTS 890.1150) as a Tier 1 test (US EPA, 2009). Rat ventral prostate tissue homogenate is cultured *in vitro*, to assess the ability of chemicals to displace radiolabelled Metribolone (R1881), which is a synthetic agonist, from the androgen receptor. Consistent with other binding assays, the assay cannot distinguish agonists and antagonists. Conservation of the AR ligand binding domain reported in the literature (Section 3.1), justifies the extrapolation of positive results. However, the assay requires castration of male rats and may be considered outdated, in light of developments in stably transfected cell lines.

More recently, Chatterjee *et al*. (2007) constructed a yeast-based beta-galactosidase reporter assay, by transfecting *S. cerevisiae* with human androgen receptor (hAR) and androgen-responsive elements (ARE), to assess the androgenicity of environmental pulp and paper mill effluents. The assay reported testosterone (EC_{50} 16nM) and Dihydrotestosterone (EC_{50} 4nM) sensitivity comparable to previously conducted *in vitro* assays (Leskinen *et al*., 2005; Michelini *et al*., 2001). Bovee *et al.* (2008) developed an androgen bioassay with a yeast-enhanced green fluorescent protein (yEGFP) reporter. The assay was sensitive (EC_{50} 50nM), reliable and reproducible. Furthermore, the author noted that the relative androgenic potencies were in agreement with optimised QSAR calculations; suggesting a lack of metabolism and crosstalk in the yeast model and the applicability of the QSAR model (Bovee *et al.,* 2008).

However, mammalian cell line-based luciferase reporter assays have been demonstrated to show higher sensitivity (Svovodova & Cajthaml, 2010). Kim *et al*. (2006) identified

22Rv1 prostate cells as an appropriate model for hAR-mediated reporter gene assays (Sun *et al*., 2007). Whereas Owens *et al*. (2006) detected the androgenic activity of diesel fuel and biomass combustion emissions, using MDA-kb2 cells transfected with androgen-responsive promoter-luciferase gene constructs.

Consequent to the development of the MCF-7 proliferation assay (E-Screen), Sonnenschein and Soto (1998) developed the androgen proliferative screen assay (A-Screen). Human prostate adenocarcinoma LNCaP-FGC cells were demonstrated to exhibit biphasic proliferation in response to androgens; at low androgen doses, cells increase their proliferation rate, while at high doses, androgens inhibited proliferation. The LNCaP-FGC cell proliferation assay verified DDE AR antagonism in *in vivo* rodent studies (Gray *et al*., 1995). However, a point mutation in the ligand binding domain of the LNCaP-FGC androgen receptor, associated with increased ligand promiscuity, has spurred the development of a stable androgen receptor transfectant of MCF-7 cells (MCF7-ARI), which responds to androgens by decreasing its proliferation rate (Szelei *et al.*, 1997).

In 2002, Andersen *et al*. used Chinese hamster ovary cells (CHO K1) to assess androgenicity and antiandrogenicity of 24 pesticides[48]. None of the pesticides were AR agonists; however, co-exposure with R1881 identified anti-androgenicity of several pesticides, including: Dieldrin; Endosulfran; Methiocarb; and, Fenarimol. Many of which have been demonstrated to agonise the estrogen receptor, highlighting ligand promiscuity and variable MoA, which may elicit variable *in vivo* endpoints (Anderson *et al*., 2002). A cellular conformation-based screen for AR inhibitors, which exploits fluorescence resonance energy transfer (FRET) consequent to conformational changes in the androgen receptor on ligand binding, has been developed by Jones *et al.* (2008). The study demonstrated comparably sensitive to transcription-based reporter assays.

Albeit not validated for use by regulatory authorities, a number of stably transfected AR transactivation assays have been developed for pharmacophore investigation i.e. drug identification and lead optimisation. For example, Cignal manufactures an Androgen[49] Receptor Reporter (luc) kit (CCS-1019L), which can be transfected into LNCaP cells to monitor increases and/or decreases in androgen dependent transcriptional activation.

5.1.3 Testing for PR and PPARγ Endocrine Modes of Action

In collaboration with estrogens, progestagens regulate proliferation and differentiation of reproductive tissues. However, only a limited number of studies have developed methods for screening agonism or antagonism of progesterone signalling, such as:

[48] Endosulfan, Methiocarb, Methomyl, Pirimicarb, Propamocarb, Deltamethrin, Fenpropathrin, Dimethoate, Chlorpyriphos, Dichlorvos, Tolchlofosmethyl, Vinclozolin, Iprodion, Fenarimol, Prochloraz, Fosetyl-aluminium, Chlorothalonil, Daminozid, Paclobutrazol, Chlormequatchlorid and Ethephon

[49] http://www.sabiosciences.com/reporter_assay_product/HTML/CCS-1019L.html

bioassays utilising stably transfected yeasts (Li *et al*,. 2008; Chatterjee *et al*., 2008) and mammalian cell lines (Molina-Molina *et al*., 2006). *S. cerevisiae* yeast transfected with human PR, a prolactin promoter and two copies of the progesterone response element (PRE), to drive GFP expression in the presence of PR agonists has been developed by Chatterjee *et al.* (2008). The authors demonstrated a significant elevation in fluorescence in response to 0.1 nM progesterone (EC_{50} 1nM). Human embryonic kidney (HEK 293) cells cotransfected with human PR and a luciferase reporter gene regulated by PRE, have been utilised in the development of a stably transfected hPR transactivation assay (Viswanath *et al*., 2008). A dual-luciferase reporter assay was utilised to monitor the PR dependent concentration of luciferase and Renilla luciferase control, demonstrating a comparable sensitivity to the aforementioned yeast screen (Svobodova & Cajthaml, 2010).

Disruption of peroxisome proliferator activated receptors (PPARs) has largely been ignored by endocrine disruption testing. However, consequent to the unprecedented increase in obesity worldwide and the elucidation of PPAR transcriptional regulation of gene networks controlling intracellular lipid flux, adipocyte proliferation and differentiation, the potential for disruption by xenobiotics has become a high research priority (Grün & Blumberg, 2009). PPARγ agonists have been utilised to improve insulin sensitivity and glycaemic control in diabetics (Golberg, 2007). Conversely, persistent weight gain was observed with prolonged use (Larsen *et al*., 2003). PPARγ antagonists (SR-202, GW9662 and JTP-426267) have been demonstrated to prevent high-fat diet induced weight gain in rodents (Rieusset *et al*., 2002; Nakano *et al*., 2006). Regarding environmental and regulatory relevance, tributyl and triphenyl tin (TBT, TPT, respectively) have been demonstrated to agonise the PPARγ.

5.1.4 The Caveats of *in vitro* Investigation

A major limitation of *in vitro* test systems is the inability to replicate metabolic processes, integral to toxicity *in vivo*. However, inclusion of metabolic activation systems to ER and AR binding TA assays is not without caveat, and uncertainty regarding the differences in xenobiotic metabolism, bioavailability and toxicokinetics in *in vivo*, versus *in vitro* tests systems (Ankley *et al*., 1998), prevents incorporation into standardised protocols. In the 2012 guidance document, the OECD stresses the varying relevance and significance of *in vitro* metabolising systems, from one species to another, suggesting that the addition of a metabolising system should only be considered when *in vivo* metabolism is certain. A number of systems, such as '*Metapath*' are being developed, as part of the OECD Working Group on Pesticides, to predict xenobiotic metabolism *in silico*. Metapath[50] also provides the foundation for the development of metabolic simulators. Conversely, *in vitro* tests entail the advantage of

[50] http://yosemite.epa.gov/sab/sabproduct.nsf/373C1DB0E0591296852579F2005BECB3/$File/OPP+SAP+document-May2011.pdf

investigating interactions of interest independent of the complexity inherent to an entire organism (e.g. ADME), which may confound the ability to detect mechanisms of action *in vivo*. However, it is important to note that chemicals which are bio-transformed to endocrine active metabolites may not be detected by *in vitro* assays, if phase I and II metabolism is not considered. Thus, *in vitro* results should not be directly extrapolated to the complex signalling and regulation of the intact endocrine system *in vivo*.

Futher complicating *in vitro* investigation, sensitivity has been demonstrated to vary with host cell line. There is evidence to suggest STTA results vary significantly in yeast and mammalian cells, as a result of differences in pharmacokinetics and pharmacodynamics (Zacharewski, 1997).

5.1.5 Summary

In vitro EDC research has prioritised estrogenic and androgenic mechanisms of action, following apical endpoints observed in exposed wildlife. A number of *in vitro* tests have been developed to identify ER and AR agonists and antagonists, including receptor binding, cell proliferation, gene expression and inhibition/stimulation of hormone synthesis (Gray *et al*., 1998). The OECD Stably Transfected human ERα Transcriptional Activation Assay detects chemicals that activate ERα/β. Agonist bound receptor conformations bind to specific DNA response elements and transactivate a luciferase reporter gene. Validated for detecting estrogenicity, transactivation of stably transfected cell lines, may be considered the best current available *in vitro* tool to detect nuclear receptor interaction.

The scope, validation and variety of *in vitro* assays to elucidate endocrine mechanisms, has reflected the state of the science. Hence, tools to decipher less conventional MoA are limited, while a plethora of assays evaluate ER agonism/antagonism. Time and monetary constraints prevent evaluation of all the aforementioned techniques in this study, which will focus on ER and AR STTA, for regulatory relevance and comparison with *in silico* results (Section 1).

The remainder of this section details the *in vitro* evaluation of potential-EDCs, prioritised via *in silico* screening, in Stably Transfected Transcriptional Activation Assays (ER and AR), in addition to experimentation with transient transfection of NR, for novel reporter gene assays.

5.2 *In vitro* Materials and Methods

Adopted by the OECD, Test Guideline 455 defines STTA experimental method, to identify chemicals that induce *in vitro* transactivation of ERα and ERα/β elements, in HeLa9903 and BG1Luc cells, respectively. As a validated regulatory tool, the concepts and approaches detailed in TG455 were adhered to when possible. All experiments were conducted with aseptic technique and appropriate personal protective equipment (PPE;

EN374-3 standard gloves, Howie labcoat and eye protection). Contamination was controlled via experimentation in laminar air-flow cabinets sterilised with 70% Isopropanol and fortnightly laboratory cleaning of local areas with Virkon (2%) and Isopropanol (70%). The prioritisation and sourcing of test compounds, *in vitro* materials, including plasticware, laboratory equipment, cell culture reagents, cell lines and assay kits, are detailed prior to descriptions of experimental methods.

5.2.1 *In vitro* Test Compounds

5.2.1.1 Prioritisation Method for *in vitro* and Case Study Inclusion

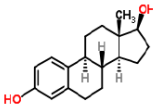
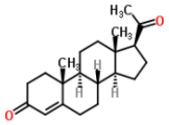
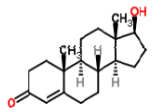
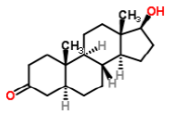
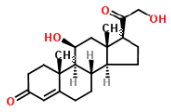
In silico screening identified a number of potential endocrine active substances (Section 4.4.3). However, testing all potential-EDCs *in vitro* was outside the scope of this study, hence, compounds representative of chemical exposure scenarios, were prioritised for *in vitro* assessment (n=20). Assay controls, environmental contaminants and HPV industrial chemicals of varying regulatory data requirements (i.e. data-rich vs. data-poor) were included: 5 endogenous hormones; 4 synthetic hormones; 5 consumer chemicals (preservatives, pesticides and antibiotics); 5 industrial chemicals; and, 1 UV-filter (PCP ingredient). Concurrent assay controls serve as an indication that assays are operative and verify sensitivity. OECD TG455 requires demonstration of laboratory standards, via the testing of 14 proficiency chemicals[51]. However, the purpose of the study is not to inform the regulation of specific chemicals, but rather to assess the application of *in silico* and *in vitro* tools. Thus, strict adherance to the OECD standard was deemed unnecessary; 3 proficiency and 2 positive controls (PC) from TG455 were included. The endogenous and synthetic hormone controls tested *in vitro* were: 17β-Estradiol (E2), Progesterone (PRG), Testosterone (T), Dihydrotestosterone (DHT), Corticosterone (C), Metribolone (R1881), Diethylstilbestrol (DES) and 17α-Ethinylestradiol (EE2). The structure, use and affiliative nuclear receptor mechanisms, of the natural and synthetic hormones, are detailed in Table 5.1 and 5.2 respectively.

In silico results (Section 4.4) highlighted the ligand promiscuity of endogenous hormones detailed in the literature (Section 3.2). Progesterone and the synthetic analogue 19-Norethindrone were included to assess promiscuity of ER/AR-regulated transactivation *in vitro*, in response to 'non-target' hormones (Table 5.1 & 5.2). High-priority chemicals identified by OECD member states (Section 2.4) were prioritised on the basis of their environmental relevance, human exposure, structural diversity and breadth of toxicological data. Reflective of the regulatory scope of EDCs, preservatives, antibiotics, pesticides, PCP components, plasticisers and industrial chemicals were included.

[51] OECD Proficiency Chemicals: Diethylstilbestrol (DES); 17α-Estradiol; meso-Hexestol; 4-tert-Octylphenol (4-OP); Genistein; Bisphenol A (BPA); Kaempferol; Butylbenzylphthalate (BBP); p,p'-Methoxychlor; Ethylparaben; Atrazine (ATZ); Spironolactone; Ketoconazole; and, Reserpine.

Table 5.1 Natural Endogenous Hormones for *in vitro* Assessment

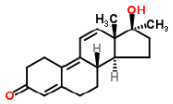
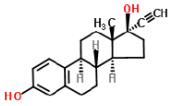
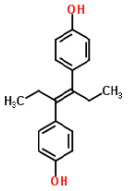
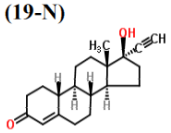
Positive controls and endogenous hormones were identified from the literature and the OECD Test Guideline TG455 proficiency chemical and control lists. PubChemBioassay (Section 4.3.1; Table 4.1) was used as a hub for mechanistic data; AID identifiers provide a reference to the bioassay publication. Reported nuclear receptor interactions are summarised, highlighting potential ligand promiscuity.

Natural Endogenous Hormones			
17β-Estradiol (E2) 	An aromatised C18 steroid with a hydroxyl group at the 3-β and 17-β position. As the most potent endogenous mammalian estrogenic steroid, E2 is the most predominant circulating hormone during reproductive years.	Agonist of human ERα signalling pathway, 0.00052 µM (AID:743077). Antagonist of the Androgen receptor (AR), 0.06 µM (AID:743063) and agonist at 2.23 µM (AID:743036). Antagonist of Glucocorticoid Receptor signalling, 7.795 µM (AID:588533). Thyroid receptor signalling pathway antagonist, 23.71 µM (AID:743065). Antagonist of PPARγ signalling pathway, 39.8 µM (AID:588537).	ER+ PPAR+ AR+ TR+ GR+
Progesterone (PRG) 	Progestational steroid secreted primarily by the corpus luteum and placenta, progesterone is required for implantation, pregnancy maintenance and the development of mammary tissue. Converted from pregnenolone, PRG is an intermediate in steroid hormone and corticosteroid biosynthesis.	Activation of progesterone receptor in human T47D cells by PRE-tagged luciferase, 0.001 µM (AID:469431). Agonistic activity of PR, 0.0005 µM (AID:339656). qHTS AR agonist signalling, 0.0251 µM (AID:588515). Displacement of dexamethasone from GR, Ki 0.0305 µM (AID:74227). Antagonist ER activity in CV-1 cells, IC_{50} 10 µM (AID:162110). Antagonist of the TR, 16.15 µM (AID:743067). PPARγ signalling antagonist, 43.396 µM (AID:743194) and PPARδ, 48.69 µM (AID:743211).	PR+ AR+ ER+ AR+ GR+ TR+ PPARγ+ PPARδ+
Testosterone (T) 	Potent androgen secreted by the testis Leydig cells, which are stimulated by luteinising hormone produced in the pituitary gland. Testosterone can be further converted to dihydrotestosterone or estradiol.	qHTS AR signalling agonist, 0.0014 µM (AID:743053). Displacement of [3H]5α-dihydrotestosterone from human sex hormone binding globulin, Kd 0.00063 µM (AID:318680). ERα signalling pathway agonist in the BG1 cell line, 0.0215 µM (AID:743079).Antagonist of GR signalling, 7.0795 µM (AID:588533). Thyroid Receptor (TR) signalling pathway, 30.42 µM (AID:743065).	AR+ ER+ GR+ TR+
Dihydro-testosterone (DHT) 	Also known as Androstanolone and Stanolone, dihydrotestosterone is a potent androgenic metabolite of testosterone produced by the action of the enzyme 3-Oxo-5α-steroid 4-deyhydrogenase.	AR agonist in mouse C2C12 cells, EC_{50} 5e-05 µM (AID:569780), activity in human Saos2 cells, IC_{50} 5e-05 (AID:319592). Displacement from human sex hormone binding globulin, Kd 0.000182 µM (AID:318680) and binding to human AR in CV1 cells, Ki 0.0002 µM (AID:290231). qHTS TR signalling pathway antagonist, 0.0014 µM (AID:743065). Agonist of ERα signalling pathway in BG1 cell line, 0.42 µM (AID:743079). Displacement of progesterone from rabbit PR, IC_{50} 0.44 µM (AID:578353). Progesterone radio-ligand binding, IC_{50} 1.25 µM (AID:625172). Antagonist of the GR signalling pathway, 1.396 µM (AID:743077). Agonist of Retinoid X Receptor alpha signalling, 7.94 µM (AID:588544). Antagonist of the thyroid receptor (TR) signalling pathway, 26.6 µM (AID:743067).	AR+ ER+ GR+ PR+ RXR+ TR+
Corticosterone (C) 	Adrenocortical 21-carbon steroid produced in the adrenal glands with mineralocorticoid and glucocorticoid activities – involved in immune, metabolic and stress responses.	Agonist of the glucocorticoid receptor (GR) signalling pathway, 0.0693 µM (AID:720691). Agonist of AR signalling in the MDA cell line, 0.1087 µM (AID:743040). Thyroid Receptor signalling, 19.33 µM (AID:743067).	GR+ AR+ TR+

Anthropogenic chemicals for *in vitro* assessment included: 4-Benzylphenol (4-BP); 4-Nitrophenol (4-NP); Triclosan (TCN); Methylparaben (MP); *o,p*-Dichlorophenyldichloroethane (*o,p*-DDE); Bisphenol A (BPA); Dibutylphthalate (DBP); Di-(2-ethylhexyl)phthalate (DEHP); Butylbenzylphthalate (BBP); and, Octyl-4-Methoxycinnamate (OMC).

Table 5.2 Synthetic Xenobiotic Hormones for *in vitro* Assessment

Synthetic hormones and hormone analogues utilised in pharmacology identified in the literature review and OECD Test Guidelines TG455 proficiency chemical list. PubChemBioassay (Section 4.3.1; Table 4.1) was used as a hub for mechanistic data; AID identifiers provide a reference to the bioassay publication. Reported nuclear receptor interactions are summarised, highlighting potential ligand promiscuity.

Synthetic Hormones & Pharmaceuticals			
Metribolone (R1881) 	Also known as R1881 and methyltrienolone, metribolone is a potent non-aromatisable androgen (anabolic steroid).	Dissociation of [3H]R1881 to human Androgen Receptor, Kd 0.00052 µM (AID:238057). Inhibition of rat AR-mediated reporter gene expression in COS7 cells, IC_{50} 0.0015 µM (AID:265578).	AR+
17α-Ethinylestradiol (EE2) 	Semi-synthetic alkylated estradiol (E2) with high oral bioavailability, used in contraceptive pills.	qHTS ERα signalling pathway agonist, 0.00077 µM (AID:743077), binding affinity to ERα IC_{50} 0.008 µM (AID:478658) and ERβ IC_{50} 0.0081 µM (AID:265000). Displacement of 5α-dihydrotestosterone from human sex hormone binding globulin, 0.155 µM Kd (AID318680). AR signalling pathway, 0.687 µM (AID:743035). Progesterone radio-ligand binding, 1.067 µM (AID:625172). Glucocorticoid radio-ligand binding, 1.71 µM (AID:625228). Thyroid receptor signalling pathway, 9.689 µM (AID:743065). PXR signalling pathway, 12.59 µM (AID:720659). PPARδ antagonist (AID:743226).	ER+ AR+ PR+ GR+ TR+ PXR+ PPARδ+
Diethylstilbestrol (DES) 	A synthetic nonsteroidal estrogen used in the treatment of menopausal and postmenopausal disorders. DES was given to cattle as a growth supplement and misguidingly given to pregnant women to reduce the risk of birth complications.	*In vitro* agonist of ERα transcriptional activation in MCF-7 cells at 10 pM (EC_{50} 7e-0.6) (AID:102438). Activation of Estrogen Response element in HeLa cells stably transfected with human ERα, EC_{50} 2e-0.5 µM (AID:70505). Displacement of 0.5 nM E2 from human ER , Ki 0.00049 µM (AID:70002). DRUGMATRIX Progesterone radio-ligand binding, IC_{50} 4.68 µM (AID:625172) and Glucocorticoid Radio-ligand binding, IC_{50} 10.6 µM (AID:625263). qHTS TR signalling pathway antagonist, 13.69 µM (AID:743065). Inhibitor of recombinant rat AR in *E. coli* using R1881, IC_{50} 14.125 µM (AID:255211), human AR antagonism confirmed in qHTS assay in MDA cell line, 27.72 µM (AID:743054).	ER+ PR+ TR+ AR+
Norethindrone (19-N) 	A synthetic progestational hormone with action similar to progesterone, functioning as an inhibitor of ovulation in the contraceptive pill. Norethindrone has also been used to treat amenorrhea, functional uterine bleeding and endometriosis.	Dissociation constant for progesterone receptor, Kd 0.0004 µM (AID:162459) and for the rat uterine estrogen receptor alpha, Kd 0.00063 µM (AID:69387). Modulation of hPR-B in co-transfected CV-1 cells, EC_{50} 0.0022 µM (AID:161792). Agonist of the Androgen receptor signalling pathway, 0.0022 µM (AID:743053). IC_{50} against recombinant rat AR in *E. coli* using R1881, 0.12 µM (AID:255211). DRUGMATRIX: Glucocorticoid radio-ligand binding, 0.397 µM (AID:625263). Thyroid Receptor signalling pathway antagonist, 6.86 µM (AID:743065).	PR+ ER+ AR+ GR+ TR+

As detailed previously (Section 5.1.4), the absence of metabolic systems hinders *in vitro* investigation. Yoshihara *et al*. (2001) demonstrated increased estrogenicity of phthalates treated with liver S9 fragments (human, monkey, rat and mouse), suggesting metabolites potentially play a significant role in the potency of chemicals. Subsequently, 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBPA), the predominant metabolite of BPA (Yoshihara *et al.*, 2004), was also included *in vitro*. The structure, use and published in vitro bioassay data for environmental contaminants and HPV industrial chemcials, are shown in Table 5.3 and Table 5.4, respectively.

Table 5.3 Environmental Contaminants identified for *in vitro* Assessment

Table shows the chemicals that were prioritised for *in vitro* mechanistic toxicity testing, including information on use and current assumed endocrine mechanisms. PubChemBioassay (see Section 4.3.3 & Table_A 1) was used as a hub for mechanistic data; AID identifiers provide a reference to the bioassay publication.

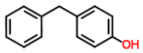
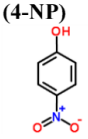
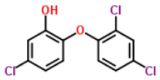
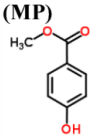
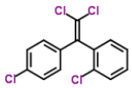
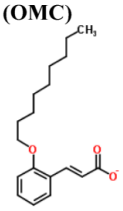
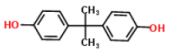
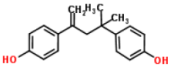
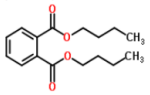
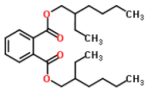
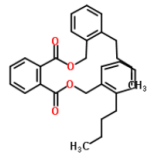
Consumer Products, Pesticides & Antibacterial Agents			
4-Benzylphenol (4-BP) 	Used as a germicide, antiseptic and preservative.	Active in qHTS ERα signalling pathway agonist assay, 50.88 µM (AID:743077) and 57.06 µM (AID:743075).	ER+
4-Nitrophenol (4-NP) 	4-NP is used in the manufacture of methyl and ethyl parathion, acetominophen, dyestuffs and leather treatment agents. 4-NP is the degradation product and impurity of parathion, the insecticide.	Active in qHTS assay to identify AR signalling pathway agonists, potency 0.001µM (AID:743053).	AR+
Triclosan (TCN) 	Diphenyl ether derivative used in cosmetics and toilet soaps as an antiseptic – bacteriostatic and fungistatic action.	PPARδ signalling pathway antagonist, 4.47 µM (AID:743213) and PPARγ antagonist, 6.30 µM (AID:743194). Thyroid Receptor signalling antagonist, 8.83 µM (AID:743065). ERα signalling pathway antagonist, 11.22 µM (AID:743074). Antagonist of the AR signalling pathway, 12.59 µM (AID:743033). Activator of the AhR signalling pathway, 19.78 µM (AID:743086). Glucocorticoid Receptor signalling antagonist, 22.39 µM (AID:720693).	PPARγ+ PPARδ+ TR+ ER+ AR+ AhR+ GR+
Methylparaben (MP) 	Methylparaben, or Methyl 4-hydroxybenzoate, is the methyl ester of p-hydroxybenzoic acid, naturally found in fruits, which is commonly artificially added to cosmetics, personal care products and food items (E number E218).	qHTS activator of the Aryl Hydrocarbon Receptor signalling pathway, 69.29 µM (AID:743085). Listed in the DSSTox (NCTRER) National Center for Toxicological Research Estrogen Receptor Binding Database (AID:1204).	AhR+ ER+
***o,p*-DDE (2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene)** 	2,2-(2-chlorophenyl-4'-chlorophenyl)-1,1-dichloroethene.	Inhibitory concentration against recombinant AR expressed in *E. coli* against R1881, IC_{50} 20.42 µM (IAD:255211). DSSTox (NCTRER) Estrogen Receptor Binding Database (AID:1204).	AR+ ER+
Octyl-4-Methoxycinnamate (OMC) 	Octylmethoxycinnamate (OMC)/ Ethylhexyl methoxycinnamate is a UV-B filter used in sunscreens and lip balms.	Schlumpf *et al.* (2001) detected *in vitro* estrogenicity.	ER+

Table 5.4 HPV Industrial Chemicals for *in vitro* Assessment

Table shows the High Production Volume (HPV) industrial chemicals prioritised for *in vitro* mechanistic toxicity testing. PubChemBioassay (Section 4.3.1; Table 4.1) was used as a hub for mechanistic data; AID identifiers provide a reference to the bioassay publication. Reported nuclear receptor interactions are summarised, highlighting potential ligand promiscuity.

Industrial Chemicals			
Bisphenol A (BPA) 	As a biphenol, BPA has two hydroxyphenyl functionalities. BPA is used as a plasticiser and in epoxy resins, commonly found in consumer goods. World production capacity of BPA was 1 million tons in the 80's and more than 2.2 million tons in 2009 (Fiege *et al.*, 2000).	qHTS assay in the BG1 cell line for ER signalling agonists, 0.22 µM (AID:743079). Lipoxygenase 15-LO enzyme inhibition, IC_{50} 1.30 µM (AID:625146). Serotonin (5-hydroxytryptamine) 5-HT6 radio-ligand binding, IC_{50} 5.42 µM (AID:625221). Antagonist of human PPARγ signalling pathway (AID:743199). Antagonist of ER signalling pathway, 8.71 µM (AID:743069). Activator of human PXR signalling pathway, 19.95 µM (AID:720659). Agonist of PPARδ signalling pathway, 22.27 µM (AID:743211). Antagonist in qHTS stufy of glucocorticoid receptor signalling pathway, 24.26 µM (AID:720692). Activator of the Aryl Hydrocarbon Receptor (AhR), 30.64 µM (AID:743219). qHTS AR antagonist, 39.96 µM (AID:743063). Antagonist of the TR signalling pathway, 61.13 µM (AID:743065).	ER+ PPARγ+ AR+ AhR+ PPARδ+ PXR+
4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBPA) 	Metabolite of bisphenol A.	Generated by rat liver S9 fractions demonstrated to be more potent than BPA (Yoshihara *et al.*, 2004).	ER+
Di-n-butylphthalate (DBP) 	A plasticiser used in most plastics, which is also used in adhesives and printing inks.	qHTS ERα signalling pathway agonist in the BG1 cell line, 30.64 µM (AID:743079).	ER+
Di-(2-ethylhexyl)phthalate (DEHP) 	Also known as Bis(2-ethylhexyl) phthalate, an ester of phthalic acid, DEHP is an odourless liquid plasticiser for resins and elastomers. DEHP is the most common class of phthalate plasticisers, accounting for 54% of the market share (Ceresana, 2013).	Activator of the rat pregnane X receptor signalling pathway, 11.22 µM (AID:651751). Human Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 24.34 µM (AID:743085).	AhR+ PXR+
Butylbenzyl phthalate (BBP) 	Benzylbutyl phthalate, BBP is an ester of phthalic acid, benzyl alcohol and n-butanol, commonly used as a plasticiser for PVC and cellulose resins.	Agonists of ER signalling, 6.31 µM (AID:588514) and 10.87 µM in the BG1 luc cell line (AID:743079). Aryl Hydrocarbon Receptor (AhR) signalling pathway, 7.70 µM (AID:743085). Agonist of the PPARδ signalling pathway, 19.49 µM (AID:743211). qHTS antagonist of the human AR signalling pathway, 21.70 µM (AID:743035).	ER+ AhR+ PPARδ+ AR+

5.2.1.2 Test Compound Solutions

Chemicals were purchased from Sigma-Aldrich Company Ltd. (Dorset) and dissolved in Dimethyl sulfoxide (DMSO), to make molar (0.1–1 mol/dm^3 = M = mol/m^3) stock solutions. Molarity was calculated using molecular weight (MW); 1 mole = MW (g) in 1 L (dm^3/m^3). Chemical batch lot#, purity (%) and molar stock solution calculations are shown in Table 5.5. Presenting cytotoxicity at high doses, DMSO concentrations were

constrained to <0.1% (v/v), which does not interfere with assay performance (OECD, 2012). Serial 1:10 100% DMSO dilutions of stock solutions (1mM – 0.1pM) were produced in 1ml aliquots (100µL TS in 900µL DMSO) and stored at -20C.

Table 5.5 Test Substance Identifiers and Stock Solution Molarity

All *in vitro* test substances were purchased from Sigma-Aldrich Company Ltd., The Old Brickyard, New Road Gillingham, Dorset, SP8 4XT (UK). Sigma catalogue, lot and CAS numbers are shown. Purity for all test substances was ≥97%. Test substances were solvated in ≥99.9% dimethyl sulfoxide (DMSO) to make molar (0.1–1 mol/dm^3 = M = mol/m^3) stock solutions. Molarity was calculated using molecular weight (MW); 1 mole = MW (g) in 1 L (dm^3/m^3).

Compound	Sigma Identifiers	Stock Solution
17β-Estradiol **E2 ≥98%** CAS: 50-28-2	E2758-1G Lot#: SLBB9733V	MW: 272.38 0.27g in 10mL DMSO ~ 0.1 Molar
17α-Ethinylestradiol **EE2 ≥98%** CAS: 57-63-6	E4876-1G Lot#: 071M1492V	MW: 296.40 0.29g in 10mL DMSO ~ 0.1 Molar
Diethylstilbestrol **DES ≥ 99%** CAS: 56-53-1	D4628-1G Lot#: BCBH3774V	MW: 268.35 0.27g in 10mL DMSO ~ 0.1 Molar*
Progesterone **PRG ≥ 99%** CAS: 57-83-0	P8783-1G Lot#: SLBD9445V	MW: 314.46 0.315g in 10mL DMSO ~ 0.1 Molar
19-Norethindrone **19-ND ≥ 98%** CAS: 68-22-4	N4128-250MG Lot#: 061M1488V	MW: 298.42 0.03g in 10mL DMSO ~ 0.01 Molar
4,4'-DDE (Pestanal®) **DDE ≥99.9%** CAS: 72-55-9	35487-100MG Lot#: SZB9209XV	MW: 318.03 0.10g in 3144 µL DMSO ~ 0.1 Molar
4-Benzylphenol **4-BP ≥ 99%** CAS: 101-53-1	H25808-25G Lot#: MKBH7945V	MW: 184.23 1.84g in 10mL DMSO ~ 1 Molar
4-Nitrophenol **4-NP ≥ 99%** CAS: 100-02-7	241326-50G Lot#: MKBF5564V	MW: 139.11 1.39g in 10mL DMSO ~ 1 Molar
Bisphenol A **BPA ≥ 99%** CAS: 80-05-7	239658-50G Lot#: MKBF3852V	MW: 228.29 2.29g in 10mL DMSO ~ 1 Molar
2,4-bis(4-hydroxyphenyl)-4-methyl-1-pentene (M-BPA) CAS: 13464-24-9	S706213-1G Lot#: N/A Aldrich[CPR]	MW: 268.359 0.27g in 10mL DMSO ~ 0.1 Molar
Dibutylphthalate **DBP ≥ 99%** CAS: 84-74-2	524980-500mL Lot#: MKBL8501V	MW: 278.34 Density: 1.043g/mL 2.7mL in 7.3mL DMSO ~ 1 Molar
Dioctylphthalate **DEHP ≥ 99.5%** CAS: 117-81-7	D201154-100mL Lot#: S42805V	MW: 390.56 Density: 0.985g/mL 4mL in 6mL DMSO ~ 1 Molar
Butylbenzylphthalate **BBP ≥ 98%** CAS: 85-68-7	308501-250mL Lot#: MKBH8959V	MW: 312.36 Density: 1.1g/mL 2.9mL in 7.1mL DMSO ~ 1 Molar
Methyl paraben **MeP ≥ 99.7%** CAS: 99-76-3	PHR1012-1G Lot#: P500012	MW: 152.15 0.15g in 10mL DMSO ~ 0.1 Molar
Octylmethoxycinnamate **OMC 98%** CAS: 5466-77-3	78848-50G Lot#: BCBF2152V	MW: 290.40 Density: 1.01g/mL 2.87mL in 7.13mL DMSO ~ 1 Molar
Triclosan (Irgasan®) **TCN ≥ 97%** CAS: 3380-34-5	72779-5G-F Lot#: BCBG0479V	MW: 289.54 2.89g in 10mL DMSO ~ 1 Molar
5α-Androstan-17β-ol-3-one **DHT ≥ 99%** CAS: 521-18-6	10300-1G-F Lot#: BCBK6042V	MW: 290.44 0.29g in 10mL DMSO ~ 0.1 Molar
Metribolone **R1881 ≥ 98%** CAS: 965-93-5	R0908-10MG Lot#: 112M4617V	MW: 284.39 10mg in 351.63µL DMSO ~ 0.1 Molar
Testosterone **T ≥ 98%** CAS: 58-22-0	T1500-5G Lot#: 070M1626V	MW: 288.42 2.88g in 10mL DMSO ~ 1 Molar*
Corticosterone **C ≥ 98.5%** CAS: 50-22-6	27840-500MG Lot#: BCBK7907V	MW: 346.46 0.35g in 10mL DMSO ~ 0.1 Molar
Flutamide **FLU ≥ 99%** CAS: 13311-84-7	F9397-1G Lot#: SLBC6159V	MW: 276.21 0.276g in 10mL DMSO ~ 0.1 Molar
** Limited solubility - saturated at high molarity as indicated by precipitate formation*		

5.2.2 Plasticware and Laboratory Equipment

The sourcing of plasticware is of particular importance for EDC studies, as many plastics contain biphenol and phthalate plasticisers that can leech into cell culture

medium. All cell culture vessels (96, 24 and 12 microtitre plates), falcon tubes and stripipettes were purchased as sterile single use plasticware; to ensure sterility and quality assurance. Additionally, sterilised plasticware offers a cost effective alternative to recycling glassware, which would demand validation of cleaning and sterilisation procedures. Tissue culture flasks and microtitre plates were TC-treated to provide a hydrophilic surface to facilitate attachment of anchorage dependent cells. Plasticware consumables and suppliers are detailed in Table 5.6, while the laboratory equipment utilised for *in vitro* experimentation, is detailed in Table 5.7.

Table 5.6 Technical Apparatus and Plasticware Suppliers

Table details the suppliers of technical apparatus and plasticware; catalogue numbers are in square brackets. All assays were consistently conducted in the same brands, for consistency or error in the event of plasticware contamination.

Supplier	Item
Fisher Scientific UK ***Bishop Meadow Road*** ***Loughborough*** ***Leicestershire,*** ***LE11 5RG***	Corning Incorporated Costar® 3606 sterile black clear bottom polystyrene TC-treated 96 well plates **[1312-8713]** X32 TC NUNC Flask straight neck radiation sterilised 500cm^2 case **[1075-7811]** Costar® 3595 sterile clear flat bottom with low evaporation lid polystyrene TC-treated 96 well plates **[07-200-103]** Corning costar® 3524 sterile clear flat bottom TC-treated 24-well plate **[07-200-84]** Corning costar® 3512 sterile clear flat bottom TC-treated 12-well plate **[07-200-81]** Thermo Scientific™ Finnpipette GLP Pipette Kit (4x F1 pipettes: 100-1000µL; 20-200µL; 2-20µL; and, 0.2-2µL) **[4700860]** Thermo Scientific™ Matrix™ Multichannel (12 channel model) Electronic 2-125µL pipettor **[14-387-117]** Thermo Scientific™ Matrix™ racked sterile 125µL tips **[14-387-198]** Corning 4487 costar® sterile stripette 5mL **[12333715]** Corning 4488 costar® sterile stripette 10mL **[10159192]** Corning 4489 costar® sterile stripette 25mL **[10445243]** Corning 4490 costar® sterile stripette 50mL **[10611604]** Sterilised cryogenic vial cryo tube 1.8mL **[10674511]** Sterile polypropylene 15mL centrifuge tube **[11849650]** Sterile polypropylene 50mL centrifuge tube **[11849650]** Microcentrifuge Eppendorf tube 1.5mL snap top **[11532293]** Sterile polypropylene pipettor tip 1-200µL **[10124314]** Sterile polypropylene pipettor tip 200-1000µL **[10787524]** T75 Nunclon sterile culture flask 75cm^2 **[15227915]**
Anachem Ltd. **Laporte Way** **Luton** **LU4 8EF**	Sterile racked tips 0.1 – 10 µL **[RT-10S]** Sterile racked Tips 200 – 300 µL **[RT-L300F]**
Microlab Technologies Ltd. ***Endway*** ***SS7 2AN***	25ml Microlab sterile reagent reservoirs, pyrogen-free, RNaes/DNaes certified **[S3054-1004]**
Invitrogen ***5791 Van Allen way*** ***PO Box 6482,*** ***Carlsbad, CA 92008***	Countess® cell counting slides **[C10228]**

5.2.3 Cell Culture Materials

The suppliers of the *in vitro* cell culture material are detailed in Table 5.8. Particular care was taken in the selection of fetal bovin serum (FBS), which is a complex mixture of albumins, growth factors and growth inhibitors. Susceptible to variation and contamination, the EU ECACC guidance on sourcing Zone 1 origin (BSE-free), sterile filtered and cell culture-tested FBS, was adhered to. Sigma-Aldrich charcoal-stripped

sterile-filtered FBS and Gibco® FBS were used for *in vitro* testing and cell maintenance, respectively. Charcoal-stripped dextran-treated FBS (DCC-FBS) is treated to reduce serum hormone concentratons, which may interfere with *in vitro* assay components (i.e. potentiate or synergise observed effects). Regulatory standard DCC-FBS was used in the STTA reporter gene assays (Table 5.8).

Table 5.7 Laboratory Equipment Required for *in vitro* Methods

Table details the standard laboratory equipment adopted for use during cell maintenance, Presto® Blue cell viability assays, transient- and stably- transfected transactivation assays, luciferase assays and MCF-7 proliferation assays (Appendices: 6.1G.1 MCF-7 GFP Proliferation Assay).

Laboratory Equipment
• **Invitrogen Life Technologies™ Countess® Automated Cell Counter**
• **LSM 510 ZEISS Laser Scanning Confocal Microscope**
• **Leica Microsystems Microscope DFC-295**
• **Sanyo CO_2 Incubator (37 ℃, 5% CO_2, 20% O_2)**
• **LEC Laboratory Fridge (4 ℃)**
• **Bosch Economic Freezer (-20 ℃)**
• **Sanyo VIP Series Freezer (-80 ℃)**
• **Sanyo UltraLow VIP Series Freezer (-150 ℃)**
• **Fisherbrand Whilimixer**
• **Stuart Scientific 3D rocking platform STR9**
• **Thermo Scientific ™ Fisher Motorised Pipetting Aid**
• **Thermo Scientific ™ MSC Advantage Laminar flow hood**
• **Thermo Scientific ™ Varioskan® Flash machine**
• **Thermo Scientific ™ Heraeus Megafuge 16R Centrifuge**
• **Grant Scientific JB Aqua Plus Waterbath**

Table 5.8 *In vitro* Cell Culture and Assay Material Suppliers

Table details the sourcing and catalogue number of cell culture reagents and assay kits used in the *in vitro* cell culture, maintenance and testing.

Supplier	Item
Fisher Scientific UK ***Bishop Meadow Road*** ***Loughborough*** ***LE11 5RG***	Invitrogen PrestoBlue® Cell viability reagent **[VXA13261]**
Sigma Aldrich Company Ltd. ***The Old Brickyard*** ***Gillingham*** ***Dorset, SP8 4XT***	Penicillin Streptomycin **[P0781-100mL]** Dimethyl Sulfoxide **99.9% A.C.S. [472301-500ML]** Fetal Bovine Serum charcoal stripped sterile-filtered **[F6765]**
Invitrogen Life Technologies ***5791 Van Allen way*** ***PO Box 6482*** ***Carlsbad*** ***CA 92008***	DMEM F12, Glutamax **[10565-016]** Gibco® MEM no glutamine, no phenol red **[51200-087]** Gibco® MEMα nucleosides, no glutamine, no phenol red **[41061-029]** L-Glutamine 200mM **[25030024]** Gibco® Kanamycin sulphate **[15160-047]** Gibco® RPMI 1640 Medium **[11835-030]** Gibco® Fetal Bovine Serum **[10106-169]** Gibco® Blasticidin S HCl **[R210-01]** Gibco® Geneticin (G418 sulfate) selective antibiotic **[10131-035]** Gibco® 0.5% Trypsin-EDTA (10x) **[15400-054]** D-PBS **[14190-136]**
Promega UK Limited ***Southampton Science Park*** ***Southampton*** ***SO16 7NS***	Dual-Luciferase® Reporter Assay System **[E1910]** Luciferase Assay System **[E1501]** Reporter Lysis 5x Buffer **[E3971]** FuGene® HD Transfection Reagent **[E2311]**

5.2.4 *In vitro* Cell Lines and Maintenance

Aiming to evaluate the currently available *in vitro* tools for endocrine MoA, an objective of testing potential-EDCs (n=20) in HeLa9903 and BG1Luc4E2 cells, validated by the OECD for dectection of estrogenicity (TG455), was defined. Developed by the Japanese Chemical Evaluation and Research Institute (CERI), the hERα-HeLa-9903 cell line is an immortalised cervical cancer cell line, stably transfected with hERα and a luciferase construct, bearing 5 tandem repeats of an estrogen response element (ERE) driven by a mouse metallothionein (MT) promoter. The BG-1Luc4E2 cell line, developed by Michael Denison in collaboration with the US NTP ICCVAM and NICEATM, is derived from human adenocarcinoma cells stably transfected with plasmid pGudLuc7.ERE, which endogenously express ERα and ERβ. Plasmid dGudLuc7.ERE contains a synthetic oligonucleotide (4x) ERE upstream of a mouse mammary tumor viral (MMTV) promoter and firefly luciferase gene. Both cell lines are appropriate for use in ER transactivation test methods, to identiy ER agonists.

Excluding the US EPA EDSP rat AR binding assay, which is reliant on rat ventral prostate tissue, there are currently no regulatory Test Guidelines to assess androgenicity. However, mammalian cell based luciferase reporter assays have been demonstrated to show sensitivity (Svovodova & Cajthaml, 2010). Testing AR transactivation *in vitro* was an objective of the current study, thus, an STTA of comparable standard to TG455 was sought. Deposited by the same research group as the HeLa9903 cell line, HeLa4-11 cells are stably transfected with a hAR expression construct and a firefly luciferase reporter gene under the control of the MMTV promoter. The HeLa4-11 cell line was developed to detect androgenic activity and adopted to assess the androgenicity of potential-EDCs (n=20).

In agreement with the literature (Lange *et al*., 2012; Kojima *et al*., 2011), HEK293 cells housed the evaluation of novel transiently-transfected nuclear receptor reporter gene assays. Human embryonic Kidney-293 (HEK293) cells are susceptible to liposomal-transfection and an appropriate cellular host for *in vitro* NR reporter gene assays.

Authenticated cryopreserved HeLa9903 [#11033105] and HeLa4-11 [#11033103] cell lines were purchased from Public Health England and the European Collection of Cell Cultures (ECACC). ECACC authenticated cryopreserved HEK293 cells [#85120602], were purchased from Sigma-Aldrich, the Brick Yard, Dorset (UK). Received as a live culture, BG1Luc4E2 cells were obtained from Michael Denison at the University of California[52].

To maintain the integrity of assay responses, TG55 advises culture in conditioned media for at least one passage from frozen stock in cell lines under 40 passages. Experimental

[52] Michael S. Denison, Ph.D. Professor, Dept. of Environmental Toxicology, 4241 Meyer Hall, One Shelds Ave, University of California, CA 95616, E: msdenison@ucdavis.edu (530) 754-8649.

assays were conducted in cells cultured for at least 3 passages from frozen stock (~1 week). To avoid cross-contamination of cultures, cell lines were handled separately with allocated cell-line specific reagents, airflow cabinets were sterilised with 70% Isopropanol and 30-minutes ultraviolet (UV) radiation and gloves were changed, before handling a new cell line. Live cell culture maintenance, cryopreservation and resuscitation methods, are detailed in Section 5.2.4.1, Section 5.2.4.2 and Section 5.2.4.3, respectively.

5.2.4.1 Cell Culture Maintenance Method

HeLa4-11, HeLa9903, BG1Luc4E2 and HEK293 cells were maintained in Eagle's Minimum Essential Medium (EMEM) without phenol red (due to oestrogenicity), supplemented with 60 mg/L of kanamycine antibiotic and 10% Gibco® DCC-FBS (Table 5.8) in T25 or T75 culture flasks (Table 5.6) in a 5% CO_2 incubator at 37±1ºC (Table 5.7). As adherent cell lines, to maintain logarithmic growth, cells were subcultured upon reaching 75-90% confluence (splitting 1:4, 1:5 or 1:6). Media was discarded into Virkon (2%) and cell monolayers were washed with Gibco® D-PBS (without Ca^{2+}/Mg^{2+}), using volumes equivalent to half the volume of culture medium (i.e. ~7.5–10mls). Adherent cell monolayers were detached using the protease enzyme trypsin; 1ml trypsin 0.5% EDTA per 25cm^2 surface area, incubated for 2-10 minutes. Cells were examined under the microscope to check for detachment and floating; in suspension the morphology of cells change, become spherical masses. Serum-containing medium was added to inactivate the trypsin and cell suspensions were centrifuged at 100xg for 5 minutes to separate and discard trypsin-contaminated media. Cells were resuspended in pre-warmed supplemented EMEM and seeded at 2 x 10^4 cells/cm^2 density (cell counting methods are detailed in Section 5.2.5.1).

5.2.4.2 Cryopreservation Method

Cryopreservation provides long-term storage of cells, thereby reducing the risk of microbial contamination, cross-contamination, genetric drift and morphological changes, reducing costs and enabling experimentation at consistent passage numbers. The basic principle of successful cryopreservation and resuscitation is slow freeze and quick thaw; cooled at a rate of -1ºC to -3ºC per minute and thawed quickly by incubation in a 37ºC water bath for 3-5 minutes. HeLa4-11, HeLa9903, BG1Luc4E2 and HEK293 log phase cell cultures of >90% viability (2 x 10^6 – 4 x 10^6 cells/ml) were suspended in 90% FBS and 10% DMSO cryoprotectant, to protects cells from ice-crystal rupture. 1ml aliquots were pipetted into cryoprotective ampoules (cryovials) and slowly frozen (i.e. -20ºC to -80ºC to -150ºC freezers over 3 days).

5.2.4.3 Method of Cell Line Resuscitation from Frozen Stock

Frozen ampoules were swabbed with 70% isopropanol, defrosted in a 37ºC water bath and wiped again with 70% isopropanol prior to opening. Ampoule contents were

pipetted into sterile falcon tubes (15 or 50 ml) and suspended in media. Suspensions were centrifuged at 100xg for 5 minutes to separate and discard DMSO-contaminated media. Cells were resuspended in pre-warmed supplemented EMEM and seeded at 2 x 10^4 cells/cm^2 density.

5.2.5 Cell Viability and Cytoxicity Methods

The presence of increasing levels of cytotoxicity can significantly alter or eliminate NR agonism sigmoidal concentration-response curves. Cells should present 80% viability; test substance concentrations that reduce cell number by >20%, should be regarded as cytotoxic and excluded from *in vitro* assessment. Confluency can be used as a general measure of cell growth, while a Hemocytometer can be used to count cells; however, both present subjectivity and lack precision. In this study, cell numbers were calculated using Invitrogen's Countess® Cell Counter (Section 5.2.5.1) and 80% cell viability was assessed in Invitrogen's PrestoBlue® assays (Section 5.2.5.2).

5.2.5.1 Invitrogen Countess® Cell Counter

Cells were counted using the Invitrogen Countess Slide system (Table 5.7 & Table 5.6); 10µl of suspended cells were added to 10µl of Trypan blue in a 1.5ml sterile ampoule. 10µl of the stained cell mixture was pipetted into a glass channel and measured using the Countess® Counter machine, which calculates cell number and viability on the basis of trypan-blue staining. Seeding density varies with cell line, however, throughout this study 1.5 x 10^6 cells per T75 flask (20mls of 7.5 x 10^4 cells/ml) was kept as standard for cell maintenance. Cell concentrations were calculated using the following equation:

$$\frac{\text{Cell Concentration (cells/ml)} \times \text{Volume (mls)}}{\text{Desired Concentration (cells/ml)}} - \text{current volume} = \text{Volume of media to add.}$$

5.2.5.2 Invitrogen PrestoBlue® Cell Viability Assay

PrestoBlue™ Cell Viability Reagent is a ready-to-use reagent for rapidly evaluating the viability and proliferation of a wide range of cell types. PrestoBlue™ reagent is quickly reduced by metabolically active cells, providing a quantitative measure of viability and cytotoxicity. When cells are viable, they maintain a reducing environment within their cytosol. PrestoBlue® reagent is a resazurin-based solution that functions as an indicator of cell viability, by measuring resazurin reduction, as a quantitative measure of cell proliferation. The cell-permeant non-fluorescent PrestoBlue® reagent is converted to resorufin, by the reducing environment of the viable cells. Turning red and highly fluorescent, the reduction of resazurin to resorufin can be detected by measuring fluorescence. Conversion is proportional to the number of metabolically active cells and thus can be measured quantitatively. PrestoBlue® cell viability reagent has been demonstrated to significantly outperform other resazurin-based assays, including MTT and CellTiter-Glo® assays (Gloeckner *et al.*, 2001; Squatrito *et al*., 1995)

Cytotoxicity can significantly alter or eliminate NR ligand-dependent transactivation, thus assurances of 80% cell viability were run in conjunction with STTA assays. Cells were suspended in 10% DCC-FBS-EMEM at a concentration of 1 x 10^5 cell/ml, dispensing 1 x 10^4 cells/100µL volume per well, with a multichannel pipette into Costar™ Clear 96-well microtitre plates (Table 5.6 & Table 5.7). Testing culture medium was not supplemented with antibiotics (Kanamycin sulphate, G418 and/or Blasticidin-HCl), due to assay interference. Cells were allowed to attach, in a 5% CO2 incubator at 37±1ºC, for 3 or 12 hours prior to chemical exposure, for HeLa4-11 and HeLa9903 cells, respectively. On adherence, 100 µL of 37±1ºC 10% DCC-FBS-EMEM testing medium was added to each well (Σvolume = 200µL), in addition to 0.2µL (200nL) of serially-diluted molar solutions of test substances in 100% DMSO (Table 5.5). Avoiding the need for diluents, final concentrations were achieved via dilution; for example, 0.2µL of 1x10^{-2} molar (10mM) stock solution was added to 200µL of culture medium, to produce a final concentration of 1x10^{-5} molar (10µM). The plate layouts of *in vitro* PrestoBlue® cell viability assays are detailed in 6.1Appendix G, which included no-cell and cell-only assay controls. Subsequent to 24-hour exposure 22µL PrestoBlue® reagent was added to each well and incubated at 37±1ºC for an additional 45 minutes (~1hour). Reduction of resazurin was measured by top-read 200ms fluorescence at 535nm excitation and 615nm emission (12nm bandwidth), in the Varioskan plate-reader automatic dynamic range setting (Table 5.7).

Results were normalised by subtracting the fluorescence of a no-cell MEM control and adjusted by dividing by the fluorescence of cells exposed to the DMSO vehicle control; to generate percentage cell viability relative to unexposed cell controls, results were multiplied by 100. Results were analysed using GraphPad Prism® Log(inhibition) vs. Response (three parameters) function, graphs show the % cell viability, measured by the reduction of the PrestoBlue™ resazurin-based solution by metabolically active cells, producing a quantifiable fluorescence.

5.2.6 Stably-Transfected Transactivation Assay (STTA) Method

As a performance-based test guideline (PBTG), TG455 details the methodology of *in vitro* transactivation assays and in particular, to detect ERα (HeLa9903) or ERα/β (BG1Luc4E2) agonists. Validation studies of STTA and BG1Luc TA demonstrated relevance and reliability in detecting estrogenic responses (ICCVAM, 2011; Rogers & Denison, 2000); ER transactivation results of 34 chemicals, in HeLa9903 and BG1Luc4E2 cell line methods, were in 100% agreement. Comprising several mechanistically and functionally similar test methods, the PBTG facilitates the development of new test methods in accordance with the validated principles. Thus, all *in vitro* TA assays were conducted in concordance with the principles defined in TG455; however, all method variations are detailed herein.

In agreement with TG455, all cells for *in vitro* experimentation were obtained from 1-week old cultures (Section5.2.4.1), conditioned in hormone-stripped media (Sigma DCC-FBS) without phenol-red for 1-2 passages, which has been shown to increase the sensitivity of NR reporter gene assays (ICCVAM, 2011). Cells were suspended in 10% DCC-FBS-EMEM at a concentration of 1 x 10^5 cell/ml, dispensing 1 x 10^4 cells/100µL volume per 96-well of Costar™ Black Clear-bottom microtitre plates (Table 5.6 & Table 5.7). Testing culture medium was not supplemented with antibiotics (Kanamycin sulphate, G418 and/or Blasticidin-HCl), due to potential assay interference. Cells were allowed to attach in a 5% CO2 incubator at 37±1ºC, however, evaluation under light-microscope showed HeLa9903 and BG1Luc4E2 cells did not attach within 3 hours. Therefore, deviating from TG455, HeLa9903 and BG1Luc4E2 cell lines were allowed to attach for 12 hours prior to chemical exposure. Cell concentration and plating were kept constant, however the method of chemical exposure varied with ER agonism (Section 5.2.6.1), AR agonism (Section 5.2.6.2) or AR antagonism (Section 5.2.6.3) mechanisms.

5.2.6.1 STTA Method for ER Agonism

TG455 suggests dilution of 1.5µL of test chemical in the 100% DMSO solvent (Table 5.5) with 500µL of DCC-FBS-EMEM media to create a diluent, 50 µL of which is added to each assay well (n=3) to create a final volume of 150µL per well. The volume cap is required for Promega Steady-Glo® Luciferase Assays, which measure the conversion of luciferin to oxyluciferin in the presence of media components (150µL media to 150µL luciferase reagent ∴ total volume 300µL). However, opting for standard luciferase assays (Promega E1500, Table 5.8), the cell medium is removed prior to cell lysis and luciferase measurement (Section 5.2.8); thus, a larger final volume of 200µL was adopted herein. On cell adherence, 100 µL of 10% DCC-FBS-EMEM testing medium was added to each of the plated 96-wells. Within the standard error of the F1 0.2-2µL Finnpipette (Table 5.6), 0.2µL of serially-diluted test substances (Table 5.5) were added to each well; achieving a final concentration 1000^{th} of the starting stock.

In the presence of intraplate variation, the method was upscaled, to minimise error. In which case, HeLa9903 cells from a 1-week old culture suspended in 10% DCC-FBS-EMEM at a concentration of 1x10^5 cell/ml and plated in 1000µL (1ml) volumes per 24-microtitre plate well (Table 5.6). Cells were allowed to attach for 12 hours prior to chemical exposure. Test substances were pipetted into each well in 1µL volumes; e.g. 1µL of 1x10^{-3} M 100% DMSO stock solution in 1mL cell media produces a final concentration of 1x10^{-6} M at 0.1% DMSO.

The plate layouts of *in vitro* ER Agonism transactivation assays (HeLa9903 and BG1Luc4E2) are detailed in Appendix H. Evapouration of outer 96-wells was observed, thus all assays were conducted in wells B-G, 2-11. Each 96-plate included 1nM E2

positive controls (n=12) and DMSO vehicle controls (n=6), which were plated strategically to monitor plate reader-effects. Three replicates of 7 test substance concentrations (range: 1mM, 100µM, 10µM, 1µM, 100nM, 10nM, 1nM, 100pM and 10pM (10^{-3}–10^{-11} molar)) enabled evaluation of 2 test substances per plate. All 96-wells presented final DMSO concentrations of 0.1%. The plate layout varied from TG455, which evaluated 3 test substances per plate, using outside wells and with fewer positive controls.

Cells were exposed to test substances for 40 hours, rather than the 20-24 hours recommended by the OECD, to increase assay sensitivity; 1nM E2 failed to consistently drive luciferase induction after 20-24 hours, possibly as a result of slight variations in media components. Subsequent to 40hr exposure test media was removed from each microtitre plate, via pipetting and inversion. The monolayers of exposed cells were washed with D-PBS (~100µL/well) and lysed with 20µL/well Promega 1x Reporter Lysis Buffer (Table 5.8). In accordance with RLB manufacturers' instructions a freeze-thaw cycle (-40°C for 24 hours) was adopted to ensure cell lysis. Defrosted microtitre plates were calibrated to room temperature on a 3D-rocking platform (Table 5.7), and measured in a luciferase assay (Section 5.2.8).

5.2.6.2 STTA Method for AR Agonism

The HeLa4-11 stably-transfected AR transactivation assays were conducted as for HeLa9903 and BG1Luc4E2 (Section 5.2.6.1), with slight methodological variations to account for differences in cell growth and mechanism. Cells were suspended in 10% DCC-FBS-EMEM and plated at 1 x 10^4 cells/100µL per 96-well, of Costar™ Black Clear-bottom microtitre plates (Table 5.6 & Table 5.7). Cells were alowed to attach for 3 hours prior to the addition of 100 µL of 10% DCC-FBS-EMEM and 0.2µL of test substances in 100% DMSO (Table 5.5) to each well and incubated at 5% CO2 at 37±1ºC. The plate layouts of *in vitro* AR Agonism transactivation assays (HeLa4-11) are detailed in Appendix H; all assays were conducted in wells B-G numbers 2-11. Each 96-plate included 1nM Testosterone positive controls (n=12), DMSO vehicle controls (n=6) and 3 replicates of 7 concentrations. Full responses were observed following 20-24 hour chemical exposure. Media was removed post-exposure and monolayers of exposed cells were washed with D-PBS (~100µL/well) and lysed with 20µL/well Promega 1x Reporter Lysis Buffer (Table 5.8). In accordance with RLB manufacturers' instructions a freeze-thaw cycle (-40°C for 24 hours) was adopted to ensure cell lysis. Defrosted microtitre plates were calibrated to room temperature on a 3D-rocking platform (Table 5.7) and measured in a luciferase assay (Section 5.2.8).

5.2.6.3 STTA Method for AR Antagonism

To modify the STTA method to detect antagonism rather than agonism, the method detailed in 5.2.6.2 was modified. Cells were suspended in 10% DCC-FBS-EMEM and plated at 1 x 10^4 cells/100µL per 96-well, in Costar™ Black Clear-bottom microtitre

plates (Table 5.6 & Table 5.7). Cells were alowed to attach for 3 hours prior to the addition of 100 µL of 10% DCC-FBS-EMEM supplemented with 1nM testosterone and 0.2µL of test substance in 100% DMSO (Table 5.5) to each well. The plate layouts of *in vitro* AR Antagonism transactivation assays are detailed in Appendix H. Each 96-plate included 1nM Testosterone positive controls (n=6), 10µM Flutamide antagonist controls (n=6), DMSO vehicle controls (n=6) and 3 replicates of 7 concentrations. Assay plates were incubated at 5% CO2 at 37±1ºC. Full responses were observed after 20-24 hour chemical exposure. Media was removed, monolayers of exposed cells were washed with D-PBS (~100µL/well) and lysed with 20µL/well Promega 1xRLB (Table 5.8). In accordance with Promega's RLB instructions, a freeze-thaw cycle (-40℃ for 24 hours) was adopted to ensure efficient cell lysis. Defrosted microtitre plates were calibrated to room temperature on a 3D-rocking platform (Table 5.7) and measured in a luciferase assay (Section 5.2.8).

5.2.7 Transient-Transfection Transactivation Assay (TTTA) Method

Aiming to explore the current *in vitro* tools for endocrine MoA, the evaluation of transient transfection technologies for novel reporter gene assays was an objective. Transfection is the transfer of nucleic acids to cells via artificial, non-viral methods. Including the transfer of plasmid DNA, oligonucleotides, yeast artificial chromosomes and RNA, transfection technologies are routinely used in the study of cellular metabolism, transcriptional control and protein function. Transient transfection refers to the temporality of nucleic acid expression; constructs have not been incorporated into host machinery and will not be expressed in subsequent generations. Reporter gene assays are typically conducted in transiently transfected cell lines, due to limited scope and sensitivity of stably-transfected cell lines (Lange *et al*. 2012; Kojima *et al*., 2011; Katsu *et al.*, 2010, 2007). However, the validation of transient transfection methods has been hindered by vast variability, leading to a lack of standardisation.

Prior to the development of polyplex transfection reagents and synthetic cationic liposomes, such as 1,2- dioleoyloxypropyl-3-trimethyl ammonium bromide (DOTMA) (Felgner *et al*., 1987), diethylaminoethyldextra (DEAE-dextran), calcium phosphate precipitation and RNA/DNA virus vectors were the only methods of mammalian cell nucleic acids transfer (Schenborn, 2000; Graham *et al*., 1973; Vaheri & Pagano, 1965). Demonstrated to be an effective transfection reagent (Lange *et al*., 2012), FuGENE® HD is a novel, nonliposomal formulation, designed to transfect DNA into a wide variety of cell lines, with high efficiency and low toxicity. FuGENE®HD does not require washing or culture medium changing, after introduction of the reagent/DNA complex. For adherent cell lines Promega FuGENE®HD instructions recommend testing 100ng of DNA per well in a 96-well plate format. Increasing the amount of DNA does not necessarily result in higher transfection efficiencies, and a FuGENE® HD Transfection Reagent: DNA ratio of 3:1 or 2.5:1, are recommended.

In concordance with research conducted at the Japanese NINS National Institute of Basic Biology (NIBB) (Oka *et al.*, 2012; Katsu *et al.*, 2007), a novel transiently-transfected roach (*Rutilus rutilus*) ERα and ARβ reporter assay was piloted herein, as ecological *in vitro* mechanistic study, relevant to UK sentinel species. Cloned *R. rutilus* ERα and ARβ DNA constructs, in pcDNA3.1 expression vectors (pcDNA3.1-RoachERα and pcDNA3.1-RoachARβ, respectively), were received as a gift from Taisen Iguchi's group at the NIBB Okazaki, Japan. FuGENE® HD transfection methods for $_{roach}$ERα and $_{roach}$ARβ are detailed in Sections 5.2.7.1 and 5.2.7.2, respectively.

5.2.7.1 Roach ERa Transactivation Assay

HEK293 cells from a 1 week old live culture, were seeded into 24-well plates at 5 x 10^4 cells per well in 900µL Dulbeco's Modified Eagles Medium (DMEM) (Sigma D2902) supplemented with 10% DCC-FBS (HyClone SH30068.03). Twenty-four hours later, cells were transfected with the cloned *R. rutilus* ERα DNA construct (pcDNA3.1-RoachERα); FuGENE® HD transfection reagent diluted in DCC-FBS DMEM (1.8µL FuGENE in 95.2 µL DCC-FBS DMEM) and supplemented with 1µL of pcDNA3.1-RoachERα (0.2 µg/µL), 1µL 4xERE-tκLuc (0.4 µg/L) and 1µL pRL-TK (0.1 µg/L), was added to the wells of a 24-microtitre plate and incubated for 5 hours. pRL-TK contains the *Renilla reniformis* luciferase gene, with the herpes simplex virus thymidine kinase promoter, and was used to normalise reporter gene (firefly luciferase) results for transfection efficiency. Subsequent to transfection, cells were exposed to test substances for 48 hours; E2 (0.1pM - 100nM E2), DES (0.1pM – 100nM DES), DBP (10pM – 1µM DBP), DEHP (0.1pM - 1 µM DEHP), BPA (0.1pM - 10 µM BPA) or 4-benzylphenol (0.1pM - 10 µM 4-BP) in a DMSO vehicle. All chemical exposures were tested in triplicate. Fourty-eight hours post exposure, medium was removed from each well and cells were washed with Dulbeco's Phosphate Buffered Saline (DPBS) (Gibco®) and 100µL of reporter lysis buffer (RLB) (1 volume of Promega 5x RLB diluted in 4 volumes distilled water) was added to each well shook for 15 minutes to enable cell lysis, cell lysates were then frozen. Roach ERα transactivation was measured in a dual luciferase assay (Firefly and Renilla).

5.2.7.2 Roach ARβ Transactivation Assay

On separate occasions, Human hepatocellular carcinoma (HepG2) cells (ATCC® HB-8065™) and HEK293 cells from a 1 week old live culture, were seeded into 24-well plates at 5 x 10^4 cells per well in 900µL Dulbeco's Modified Eagles Medium (DMEM) (Sigma D2902) with 10% DCC-FBS (HyClone SH30068.03). Twenty-four hours later, cells were transfected with cloned R. rutilus ARβ DNA construct (pcDNA3.1-RoachARβ). FuGENE® HD transfection reagent diluted in DCC-FBS DMEM (1.8µL FuGENE in 95.2 µL DCC-FBS DMEM) was supplemented with 1µL of pcDNA3.1-RoachARβ (0.2 µg/µL), 1µL 4xARE-tκLuc (0.4 µg/L) and 1µL pRL-TK (0.1 µg/L),

added to each well, swirled and incubated for 5 hours. Subsequent to transfection, cells were exposed to test substances (0.1pM - 1 μM molar) for 48 hours; 11-Ketotestosterone (11-KT), 17α-Methyltestosterone (17-MT), Testosterone (T) and Dihydrotestosterone (DHT) in 100% DMSO vehicle were tested in triplicate. Fourty-eight hours post exposure, media was removed and the cells were washed with D-PBS and lysed with 100μL Promega 1xRLB, and cell lysates were frozen. Roach ARβ transactivation was measured in a dual luciferase (Firefly and Renilla) assay 48 hours later.

5.2.8 Luciferase Assay Method

Firefly luciferase is a widely used reporter due to high sensitivity and low background chemiluminescence (Wood, 1990). Light is produced by the oxidation of luciferin via firefly luciferase catalysis of electron transition, to form oxyluciferin. Promega Luciferase Assay System (E1500) incorporates coenzyme A for improved kinetics and light stability (Wood, 1991). Luciferase assays are used to identify chemicals that activate the ER or AR, which initiates the binding to specific DNA response elements (i.e. ERE) and transactivates the luciferase reporter gene, resulting in increased cellular expression. Luciferase activity was measured by adding 100μL of lyophilised Luciferase Assay Reagent to each 96-well, containing 20μL of defrosted cell lysate equilibrated to room temperature. Plates were read immediately in the Varioskan plate reader with automatic dynamic range and 500ms measurement time (Table 5.7).

5.2.9 Dual Luciferase Assay Method

Conducted at the NIBB (Japan), Promega Dual-Luciferase ® Reporter Assay (E1960) reagents were prepared as per the manufacturers' instructions; firefly luciferase assay reagent (LARII) was made by resuspending lyophilised Luciferase Assay Substrate in Luciferase Assay Buffer II, while the Renilla luciferase Stop & Glo® reagent was resuscitated by adding Stop and Glo® Buffer to Stop and Glo® Substrate. For the assay, 20μL volumes of cell lysate were transferred to 96-well Costar™ Black Clear-bottom microtitre plates. Chemiluminescence was measured in a Promgea GloMax®- Multi Microplate Multimode Reader with dual injectors set to dispense 100μl of LARII and Stop & Glo® Reagent, measurements were read at a 2 second delay and 10 second read time.

Duplicate experiments, conducted in the Cranfield laboratories were measured manually, in the Varioskan machine (without injectors). Firefly luciferase activity was measured on the addition of 100μl of LARII, to each 96-well containing 20μL cell lysate. Stop & Glo® Reagent (100μL) was subsequently added to each well and the relative light units measured, as a control for transfection efficiency.

5.2.10 Statistical Analysis

The OECD suggests that *in vitro* transactivation results should be based 2-3 independent runs (i.e. day repeats) of comparable and therefore reproducible results. Acceptability criteria for ER STTA (HeLa9903 and BG1Luc4E2) suggest mean relative light units (RLU) of 1nM E2 should be at least 4-fold the mean RLU of VC. For a result to be considered positive, the RPC_{max}[53] must exceed 10% of 1nM E2 in at least 2/3 runs, while TG455 stipulates at least 20% of the 1nM E2 maximal response, for positive classification. Data outliers of the E2 reference standard were identified on the basis of RLU ±20% of those in the historical database. All assay plates were quality checked via evaluation of positive and negative control adherence to OECD principles.

As a novel stably transfected tool, without regulatory guidance, additional statistical quality checks were undertaken for HeLa4-11 AR agonism and antagonism transactivation assays. Z'prime, or Z'factor, is a measure of statistical effect size, adopted in high-throughput screening to assess the significance of an assay. The Z'prime is defined by the means (μ) and standard deviations (σ) of positive (p) and negative (n) controls:

$$Z'Prime = 1 - \left[\frac{3(\sigma p + \sigma n)}{\mu p - \mu n}\right]$$

Z'prime scores of 1 are ideal, Z' between 0.5 to 1 can be interpreted as an excellent assay; if σp = σn, a Z' of 0.5 is equivalent to a 12 standard deviation separation between mean positive and negative controls. Z'primes between 0 and 5 can be considered marginal, less than zero signifies significant overlap between positive and negative controls, limiting the use of the assay. The small margin between maximal responses and vehicle controls (4-fold) in ER STTA hinders the applicability of Z'prime statistics, which as a stringent statistical tool requires 99% of values to occur within 3 standard deviations of the mean (i.e. small margin for error). In the event of large error, experimentation was up-scaled to 24-well plates. The data-specifics of statistical analyses for cell viability and transactivation assays are detailed in Section 5.2.10.1 and 5.2.10.2, respectively.

5.2.10.1 Cell Viability:

The % cell viability was calculated by measuring the fluorescence of PrestoBlue™ resazurin-based-solution reduction by metabolically active cells. The mean fluorescent relative light units (RLU) of no-cell MEM control and VC-control were calculated. Results were normalised by subtracting the fluorescence of a no-cell MEM control and adjusted by dividing by the fluorescence of cells exposed to the DMSO vehicle control; to generate percentage cell viability relative to unexposed cell controls, results were

[53] The RPC_{max} is the maximum level of response induced by a test chemical, expressed as a percentage of 1nM E2.

multiplied by 100. Graphs were drawn using GraphPad Prism® Log(inhibition) vs. Response (three parameters) function.

5.2.10.2 Transactivation Assays

The mean relative light units (RLU) of vehicle controls and positive controls were calculated. STTA results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM positive control (E2 or T maximum response). Thus, the final value of each well is the relative transcriptional activity for that well compared to the PC response. Data were presented as mean and standard error of the mean (SEM) of n=3 replicates of day repeat data. Data distribution was presented by the Standard Deviation (SD) or data range (scatter plot). Best fit values were calculated using GraphPad Prism® Log(agonist) vs. response – variable slope (four parameters) and used to calculate the $LogEC_{50}$ and EC_{50} with 95% Confidence Intervals (95% CI). A dotted line at 20% normalised RLU of 1nM PC (y=0.2), highlighted the threshold for categorising positive, negative and/or inadequate data.

5.3 *In vitro* Results and Discussion

This section presents the results of *in vitro* testing of the test substances identified in Section 5.2.1 (Table 5.1; Table 5.2; Table 5.3; and Table 5.4), in standardised mechanistic assays. Stably transfected ER and AR transcriptional activation assays and novel transiently transfected *R. rutilus* (roach) ER and AR reporter gene assay results, are detailed herein. The *in vitro* methods adopted provide mechanistic and potency information for EDC hazard characterisation.

5.3.1 Cell Viability and Cytotoxicity

Cytotoxicity can significantly alter or eliminate NR agonism sigmoidal concentration-response curves. Thus, cells should present at least 80% viability in STTA and TTTA methods. Test substance concentrations that reduced cell viability by >20% were regarded as cytotoxic. To ensure the validity of *in vitro* transactivation assays, PrestoBlue® viability assays were run in parallel. The cell viability detailed herein provides assurance of the STTA concentration ranges.

Figure 5.1 shows the % cell viability of cells exposed to potential-EDC agonists, quantified by fluorescence emitted on reduction of PrestoBlue™ resazurin-based solution by metabolically active cells. Green plots show the cell viability of HeLa4-11 cells, while red plots present HeLa9903 cell viability. The shaded region between 100-80% cell viability shows the threshold for normal variation.

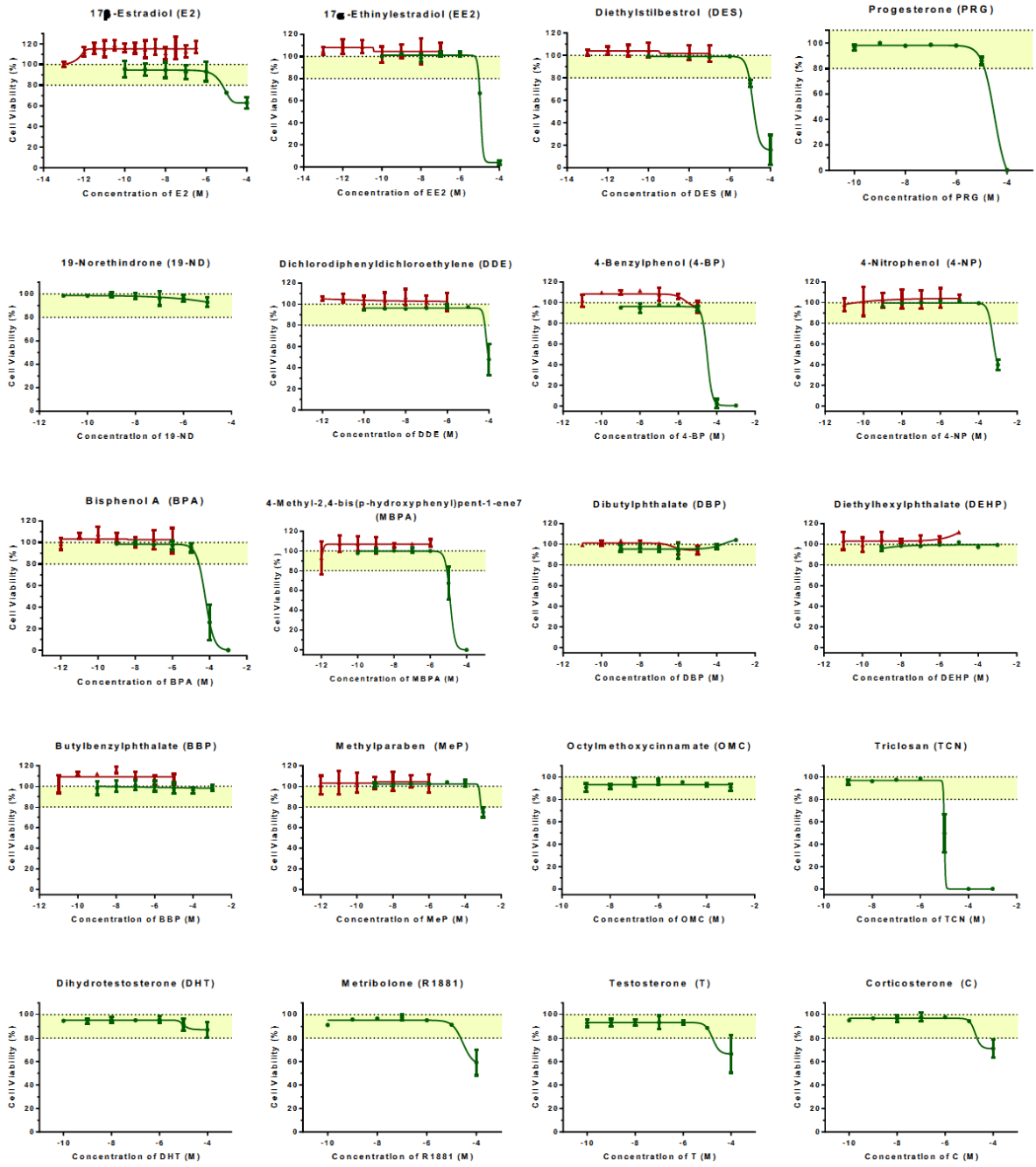


Figure 5.1 Assessment of Test Substance Cytotoxicity in HeLa4-11 and HeLa9903 Cells with PrestoBlue® Cell Viability Assay

Drawn using GraphPad Prism® Log(inhibition) vs. Response (three parameters) line drawing function, graphs show the % cell viability, measured by the reduction of the PrestoBlue™ resazurin-based solution by metabolically active cells, producing a quantifiable fluorescence. Results were normalised by subtracting the fluorescence of a no-cell MEM control and adjusted by dividing by the fluorescence of cells exposed to the vehicle control (DMSO). To generate percentage cell viability, relative to the unexposed cell controls, results were then multiplied by 100. All assays were conducted in 96-well plates at $1x10^4$ cells per well in 200µL supplemented MEM (10% DCC-FBS, 2mM L-glutamine). Test substances were administed in 0.2µL measurements in 100% DMSO ~ all DMSO vehicle concentrations 0.1%. Green plots show the cell viability of HeLa4-11 cells, while red plots present observed HeLa9903 cell viability. The shaded region between 100-80% cell viability shows the threshold for normal variation.

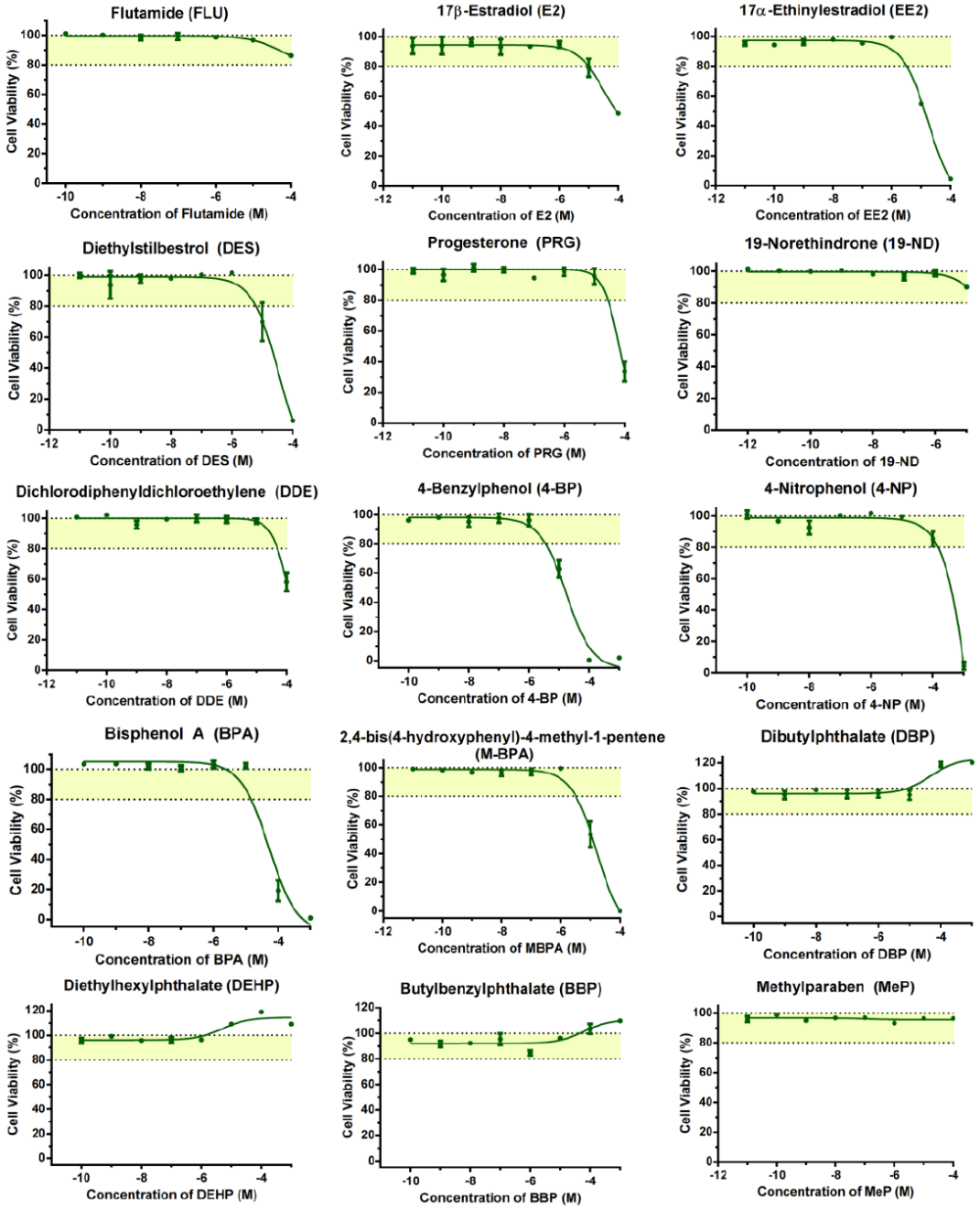


Figure 5.2 Cytotoxicity of Coexposure to Test Substance and 1fM Testosterone in HeLa4-11 Cells Measured with PrestoBlue® Cell Viability Assay

Drawn using GraphPad Prism® Log(inhibition) vs. Response (three parameters) function, graphs show the % cell viability, measured by the reduction of the PrestoBlue™ resazurin-based solution by metabolically active cells, producing a quantifiable fluorescence. Results were normalised by subtracting the fluorescence of a no-cell MEM control and adjusted by dividing by the fluorescence of cells exposed to the vehicle control (DMSO). To generate percentage cell viability, relative to the unexposed cell controls, results were then multiplied by 100. All assays were conducted in 96-well plates at $1x10^4$ cells per well in 100µL supplemented MEM (10% DCC-FBS, 2mM L-glutamine). Testosterone and Test substances were administed 100µL 2fM diluent and 0.2µL measurements in 100% DMSO, respectively. All DMSO concentrations were 0.1%. The shaded region between 100-80% cell viability shows the threshold for normal variation.

Table 5.9 HeLa9903 and HeLa4-11 Cell Line Maximum Non-cytotoxic Test Substance Concentrations Identified in PrestoBlue® Cell Viability Assays

Table shows the test substance (TS) concentrations shown to present at >80% viability in PrestoBlue® Assays. Results for single exposure in HeLa9903 and HeLa4-11 are presented in columns 2 and 3, in addition to coexposure of TS with 1fM of testosterone in column 4 (reflective of antagonistic assays). Excluding 17β-Estradiol (E2 semi-log) all exposures were in 1:10 logarithmic scales. For many TS, cell viability % did not reduce beyond 80% at the concentrations tested: Methylparaben, Butylbenzylphthalate, Diethylhexylphthalate, Dibutylphthalate, 19-Norethrindrone, Flutamide, Dihydrotestosterone and Octylmethoxycinnamate. HeLa9903 cell PrestoBlue® assays were tested at the STTA dose range, none of which presented cytotoxicity. The maximum concentrations tested by ECVAM, XDS and Hyoshi laboratories are presented in column 5.

Test Substance (TS)	HeLa9903 Cell Viability >80%	HeLa4-11 Cell Viability >80%	Antagonism Cell Viability >80%	Max. Testing Concentrations*
17β-Estradiol	≤3.16μM ($10^{-6.5}$M)	≤1μM (10^{-6} M)	<10μM (10^{-5} M)	3.67 x 10^{-3} M
17α-Ethinylestradiol	≤0.1μM (10^{-7} M)	≤1μM (10^{-6} M)	≤1μM (10^{-6} M)	3.37 x 10^{-4} M
Diethylstilbestrol	≤0.1μM (10^{-7} M)	≤1μM (10^{-6} M)	≤1μM (10^{-6} M)	3.73 x 10^{-4} M
Progesterone	-	≤10μM (10^{-5} M)	≤10μM (10^{-5} M)	3.18 x 10^{-4} M
19-Norethindrone	-	≤10μM (10^{-5} M)	≤10μM (10^{-5} M)	-
4'4-DDE	≤1μM (10^{-6} M)	≤10μM (10^{-5} M)	≤10μM (10^{-5} M)	3.14 x 10^{-3} M
4-Benzylphenol	≤10μM (10^{-5} M)	≤10μM (10^{-5} M)	≤1μM (10^{-6} M)	-
4-Nitrophenol	≤10μM (10^{-5} M)	≤100μM (10^{-4} M)	≤100μM (10^{-4} M)	-
Bisphenol A	≤10μM (10^{-5} M)	≤10μM (10^{-5} M)	≤10μM (10^{-5} M)	4.38 x 10^{-3} M
M-BPA	≤1μM (10^{-6} M)	≤1μM (10^{-6} M)	≤1μM (10^{-6} M)	
Dibutylphthalate	≤10μM (10^{-5} M)	≤1mM (10^{-3} M)	≤1mM (10^{-3} M)$^{+}$	3.59 x 10^{-3} M
Diethylhexylphthalate	≤10μM (10^{-5} M)	≤1mM (10^{-3} M)	≤1mM (10^{-3} M)$^{+}$	3.03 x 10^{-3} M
Butylbenzylphthalate	≤10μM (10^{-5} M)	≤1mM (10^{-3} M)	≤1mM (10^{-3} M)$^{+}$	3.20 x 10^{-4} M
Methylparaben	≤1μM (10^{-6} M)	<1mM (10^{-3} M)	≤100μM (10^{-4} M)	-
Octylmethoxycinnamate	-	≤1mM (10^{-3} M)	-	-
Triclosan	-	≤1μM (10^{-6} M)	-	-
Dihydrotestosterone	-	≤100μM (10^{-4} M)	-	3.55 x 10^{-5} M
Metribolone	-	≤10μM (10^{-5} M)	-	-
Testosterone	-	≤10μM (10^{-5} M)	-	3.47 x 10^{-3} M
Corticosterone	-	≤10μM (10^{-5} M)	-	2.89 x 10^{-3} M
Flutamide	-	-	≤100μM (10^{-4} M)	3.62 x 10^{-3} M

* Maximum concentration tested by ECVAM, XDS and/or Hiyoshi published by ICCVAM (2011)
$^{+}$ Cell viability in PrestoBlue® Assay increased at higher doses.

The maximum test substance concentrations that maintained 80% HeLa4-11 and HeLa9903 cell viability are detailed in Table 5.9, with reference to the maximum tested concentrations in STTA conducted by ECVAM, XDS and/or Hiyoshi laboratories (ICCVAM, 2011). Cell viability and cytotoxicity thresholds were used as a reference point for the transactivation assays, detailed in subsequent sections. Assuming concentration addition, elevated cytotoxicity in HeLa4-11 cells exposed to test substances in conjunction with 1fM Testosterone, was unlikely. However, to ensure the cell viability of AR antagonism transactivation assays, Figure 5.2 shows the

PrestoBlue® cell viability assays for coexposure; which are also summarised in Table 5.9. Interestingly, coexposure of phthalates (DBP, DEHP and BBP) and 1fM testosterone does not induce cytotoxicity at high concentrations (1mM to 10μM), but increased cell viability. As detailed previously, PrestoBlue® assays measure fluorescence emitted consequent to the reduction of resazurin-based compounds, indicative of cellular metabolism. Thus, the increase in assumed % cell viability is in fact increased metabolism.

Table 5.9 shows that the maximum concentrations tested by ECVAM, XDS and Hiyoshi laboratories, which reflected test substance solubility, rather than cell viability and exceeded the point of departure (POD) for cytotoxicity identified in this study. Results generated at cytotoxic concentrations should be evaluated with caution; due to possible generation of false negatives and false positives due to altered cellular metabolism. The POD detailed in Table 5.9, reflects the concentration ranges which do not affect cellular metabolism. However, the plots (Figure 5.1 and Figure 5.2) highlight the 'Hill slope' or gradient of the response, which is reflective of the rate of cytotoxicity; i.e. whether cell viability decreased rapidly or gradually with increasing concentration. BPA, 4'4-DDE and 4-Nitrophenol demonstrated POD's for cytotoxicity at $1x10^{-5}$ Molar (Figure 5.2). However, at $1x10^{-4}$ M, the metabolic reduction of Resazurin pigments reflective of cell viability, reduced to 20%, 60% and 80%, respectively. The addition of 1fM testosterone did not appear to affect the cellular metabolism of HeLa4-11 cells (Table 5.9); agonism and antagonism cell viability (%) assays were in agreement. However, coexposure appeared to alleviated the cytotoxicity of E2, Methylparaben and 4-Benzylphenol, which all retained >80% cell viability at higher doses than on single exposure.

5.3.2 HeLa9903 ERα Agonism STTA

Adopted by the OECD, Test Guideline 455 defines STTA experimental method, to identify chemicals that induce *in vitro* transactivation of ERα and ERα/β elements, in HeLa9903 and BG1Luc cells, respectively. With the aim of evaluating currently available *in vitro* tools for endocrine MoA, the objective of testing potential-EDCs (n=20) in HeLa9903 ERα transactivation assays was defined, the results of which are detailed herein.

HeLa9903 cells from a 1-week old culture suspended in 10% DCC-FBS-EMEM at a concentration of $1x10^5$ cell/ml were plated in 100μL or 1000μL volumes, for 96-well and 24-well microtitre plates, respectively. Cells were allowed to attach for 12 hours prior to chemical exposure. Test substances were administered to 96-well assays in 100 μL of 10% DCC-FBS-EMEM with 0.2μL of test substances in 100% DMSO. In 24-well plates, 1μL volumes of test substances (100% DMSO) were added to each well. HeLa9903 cells were exposed to test substances for 40-48 hours, at which point cells were terminated and prepared for luciferase assays (Section 5.2.6.1 and 5.2.8).

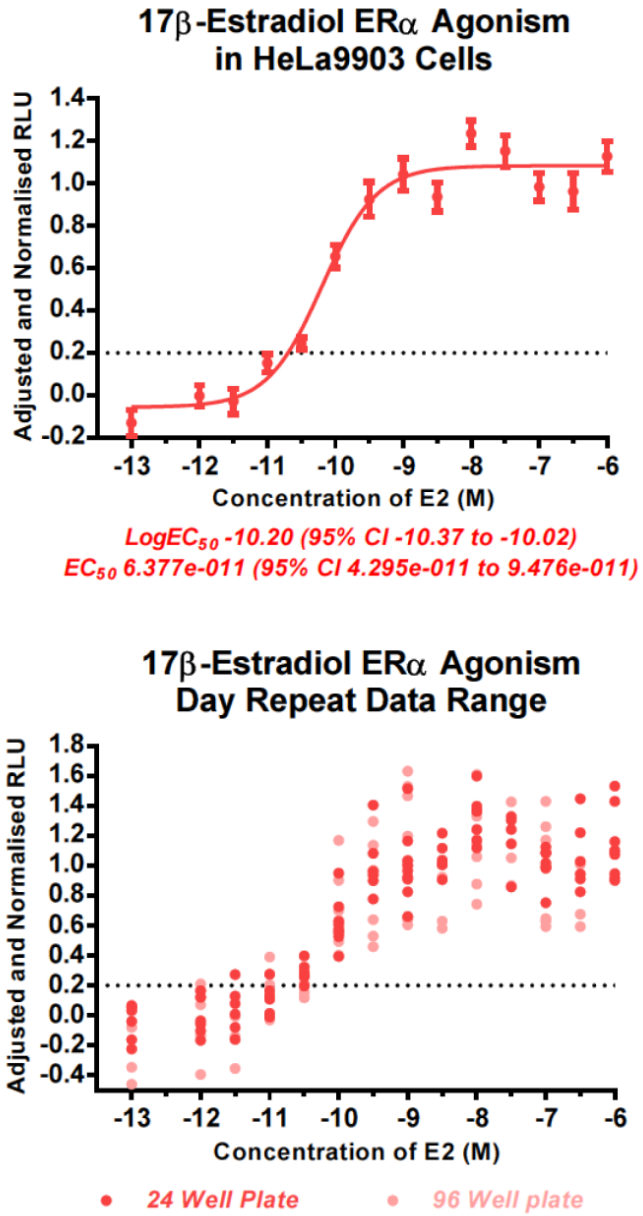


Figure 5.3 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to 17β-Estradiol (E2)

Graphs show the adjusted and normalised relative light units (RLU) emitted in luciferase assays consequent 40-48hr 17β-Estradiol (E2) exposure. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM E2 (maximal agonistic response). Cell concentration was $1x10^4$/well in 96-well (200µL) and $1x10^5$/well in 24-well (1000µL); E2 was solvated in 100% DMSO and administered in 0.2µL and 1µL volumes, respectively. Molar concentrations were made by serial dilutions (log and semi-log). The top graph plots the mean and standard error of the mean (SEM) of the day repeat (n=4) data presented underneath as a scatter plot (showing range). Best fit values were calculated using GraphPad Prism® Log(agonist) vs. response – variable slope (four parameters) and were used to calculate the $LogEC_{50}$ and EC_{50} with 95% Confidence Intervals (95% CI) presented in red font. Mean E2 agonism values: bottom -0.0555 (95% CI -0.153 to 0.0418) and top 1.083 (95% CI 1.025 to 1.141); Hill Slope 1.077 (95% CI 0.674 to 1.481); and R square = 0.80 (n=158). The dotted line (y=0.2) highlights 20% 1nM E2 normalised RLU, as a threshold for categorising positive, negative and/or inadequate data.

Assays were 'upscaled' in the presence of intraplate variation, to minimise error. Throughout Section 5.3.2, data replicates are colour coded according to assay microtitre plate format; highlighting slight differences in method. Chemilluminscence measurements obtained in luciferase assays were normalised by subtracting DMSO vehicle control RLU and adjusted by dividing by the RLU of 1nM E2 (maximal agonistic response).

Figure 5.3 shows the *in vitro* ERα transactivation in HeLa9903 cells exposed to semi-log concentrations of E2 (0.1pM - 1μM). Calculated from the mean and SEM of 4 day repeats (n=158), the E2 $LogEC_{50}$ and EC_{50} (95% Confidence Intervals) were, -10.20 M (95% CI -10.27 to -10.02) and 6.377e-11 M (95% CI 4.295e-11 to 9.476e-11), respectively (R^2=0.80). The Hill slope (gradient) of the plotted data was 1.077 (95% CI 0.7 to 1.5). The Hill slope quantifies the steepness of the curve; a standard slope has a Hill slope of 1.0, a steeper curve is higher (1.5), while a shallow curve has a lower value (0.5). Thus, the E2 dose-response observed followed a standard sigmoidal curve, reflective of the law of mass action (each E2 binds to a single ER).

The US EDSP OPPTS 890.1300 and OECD TG455 define regulatory performance criteria for E2 agonistic responses in HeLa9903 transactivation assays. The E2 responses demonstrated herein are in agreement with EDSP and OECD standards; $LogEC_{50}$ between -11.3 to -10.1 M and a Hill slope value of 0.7-1.5 (ICCVAM, 2011). The applications of HeLa9903 cells, to academic research and publication, have been limited; however, the E2 reference standard results are also in agreement with Coady *et al.* (2014); whom, in collaboration with Dow Chemical Company (US), used the cell line to evaluate the potential endocrine activity of 2,4-Dichlorophenoxyacetic acid. Underneath the mean (±SEM) data (Figure 5.3), the data from intraplate replicates (n=3) and interplate repeats (n=4) are shown (158 data points). Colour coded according to microtitre plate format (i.e. 24 vs 96), the results generated from 96-well plates were more variable.

Figure 5.4 presents the ERα transactivation in HeLa9903 cells consequent to 40 hour exposure to 17α-Ethinylestradiol (EE2) or Diethylstilbestrol (DES). The EE2 $LogEC_{50}$ and EC_{50} were -14.27 (95% CI -14.70 to -13.84) and 5.329e-15 (95% CI 1.776e-15 to 1.421e-14), respectively. The sensitivity of the HeLa9903 cells in detecting EE2 agonism, consequent to 40 hour exposure, surpassed the sensitivity of OECD STTA (PC_{50} <$1.00x10^{-11}$) and BG1Luc ER TA assays (EC_{50} $4.20x10^{-8}$ M), which demand 96-well plates and terminate assays at 24 hours. The right side plot, shows the replicates (i.e. data range) of day repeats (n=6), colour coded according to plate layout. Sigmoidal EE2 dose-response relationships were only detectable via 24-well plate experiments, and the R^2 value of 0.45, highlights the dispersion of data from the mean. The DES $LogEC_{50}$ and EC_{50} were -11.83 M (95% CI -12.07 to -11.60) and 1.464e-12 M (95% CI 8.46e-13 to 2.53e-12), respectively (R^2 = 0.904), which supports values published by the OECD; STTA PC_{50} $2.04x10^{-11}$ M and BG1Luc ER TA EC_{50} $3.34x10^{-11}$ M.

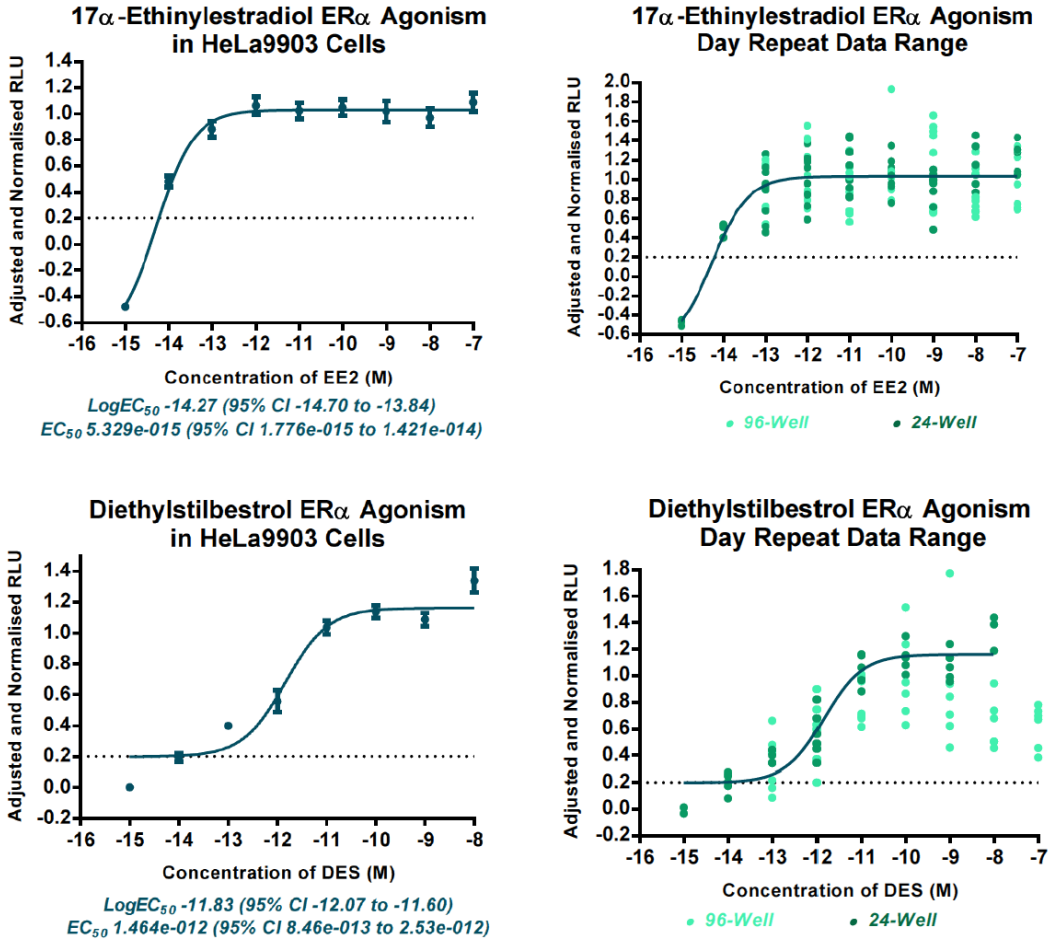


Figure 5.4 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to Synthetic Estrogens (EE2 and DES)

Graphs show the adjusted and normalised relative light units (RLU) emitted by HeLa9903 cells in luciferase assays consequent 40-48hr 17α-Ethinylestradiol (EE2) or Diethylstilbestrol (DES) exposure. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM E2 (maximal agonistic response). Cell concentration was $1x10^4$/well in 96-well (200µL) and $1x10^5$/well in 24-well (1000µL); test chemicals were solvated in 100% DMSO and administered in 0.2µL and 1µL volumes, respectively. Left graphs plots the mean and standard error of the mean (SEM) of repeated data, individual day repeat (n=6) results are shown as a scatter plot to the right (data range). GraphPad Prism® Log(agonist) vs. response (three parameters) software was used to calculate the $LogEC_{50}$ and EC_{50} with 95% Confidence Intervals (95% CI), presented in blue. EE2 bottom -0.7443 (95% CI -1.281 to -0.2077) and top 1.030 (95% CI 0.9784 to 1.081), R square = 0.449 (n=126). DES bottom 0.1982 (95% CI 0.1187 to 0.2778) and top 1.161 (95% CI 1.092 to 1.230), R square = 0.904 (n=42). The dotted line (y=0.2) highlights 20% 1nM E2 normalised RLU, as a threshold for categorising positive, negative and/or inadequate data.

Highlighted by the data replicates in Figure 5.5, the ERα transactivation consequent to 40 hour BPA exposure was highly variable. Subsequently, 96-well plate assays were excluded from the $LogEC_{50}$ and EC_{50} calculations, which were -10.13 (95% CI -10.52 to -9.74) and 7.44e-11 (95% CI 3.037e-11 to 1.824e-10), respectively. Mean values for BPA were calculated from 78 data points, collected over 4 day repeats (R^2=0.589). Forty hour BPA exposure increased the sensitivity of STTA, relative to the STTA PC_{50} $2.94x10^{-7}$ M published by Takeyoshi (2006) and EC_{50} $5.33x10^{-7}$ M reported in the

BG1Luc ER TA assay (ICCVAM, 2011). 4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBPA), is the predominant metabolite of BPA (Yoshihara *et al*., 2004). Yoshihara *et al.* (2001) demonstrated that many phthalate metabolites presented higher estrogenicity than their parent compounds. To explore the potential effects of metabolism on the estrogenicity and androgenicity of BPA, MBPA was evaluated in STTA assays. The $LogEC_{50}$ and EC_{50} of MBPA ER transactivation, relative to 1nM E2, were -10.04 (95% CI -10.64 to -9.44) and 9.068e-11 (95% CI 2.29e-11 to 3.59e-10), respectively (R^2=0.647). MBPA presented comparable agonistic ER transactivation responses to BPA.

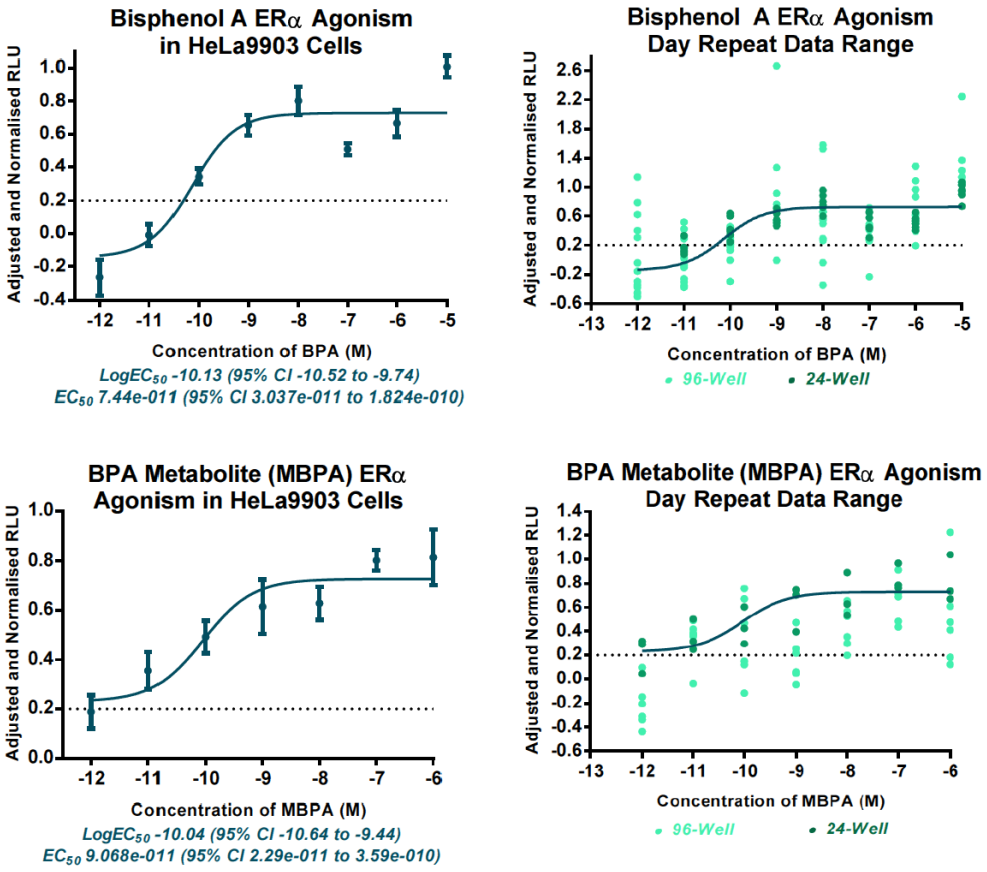


Figure 5.5 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to Bisphenol-A or its' Metabolite 4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBPA)

Graphs show the adjusted and normalised relative light units (RLU) emitted consequent to 40-48hr Bisphenol A (BPA) or 4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene exposure. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM E2 (maximal agonistic response). Cell concentration was $1x10^4$/well in 96-well (200µL) and $1x10^5$/well in 24-well (1000µL); test chemicals were solvated in 100% DMSO and administered in 0.2µL and 1µL volumes, respectively. Left graphs plots the mean and standard error of the mean (SEM) of 24-well microplate data, individual day repeat results, for both 96-well and 24-well experiments, are shown as a scatter plot to the right (data range). GraphPad Prism® Log(agonist) vs. response (three parameters) software was used to calculate the $LogEC_{50}$ and EC_{50} with 95% Confidence Intervals (95% CI). BPA bottom -0.1457 (95% CI -0.3412 to 0.04970) and top 0.7288 (95% CI 0.6563 to 0.8014), R square = 0.589 (n=78). MBPA bottom 0.230 (95% CI 0.0910 to 0.369) and top 0.7275 (95% CI 0.644 to 0.811), R square = 0.647 (n=28). The dotted line (y=0.2) highlights 20% 1nM E2 normalised RLU, as a threshold for categorising positive, negative and/or inadequate data.

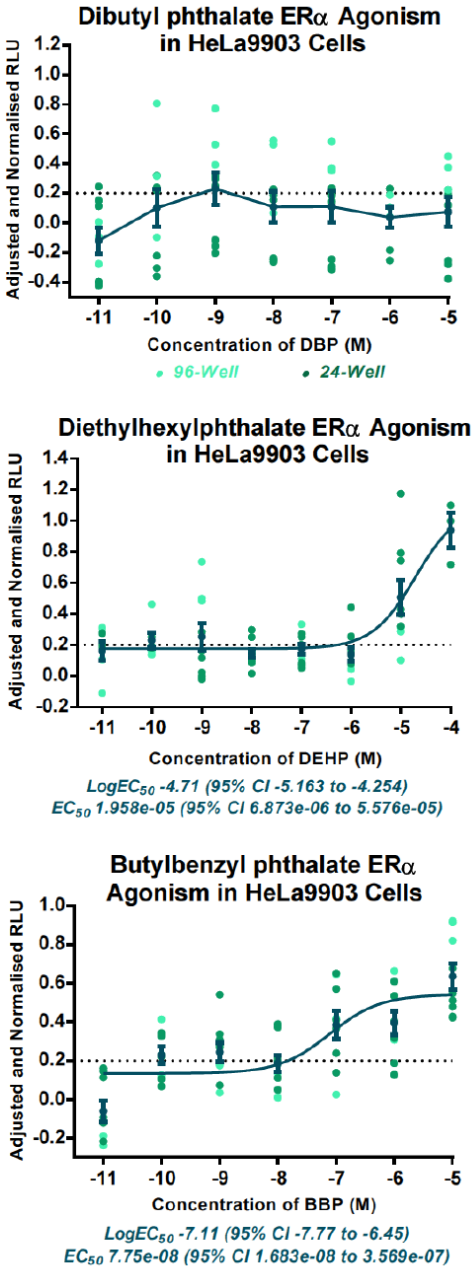


Figure 5.6 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to Phthalates (DBP, DEHP and BBP)

Graphs show the adjusted and normalised relative light units (RLU) emitted consequent to 40-48hr exposre to either Dibutyl phthalate (DBP), Diethylhexyl phthalate (DEHP) or Butylbenzyl phthalate (BBP). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM E2 (maximal agonistic response). Cell concentration was $1x10^4$/well in 96-well (200µL) and $1x10^5$/well in 24-well (1000µL); test chemicals were solvated in 100% DMSO and administered in 0.2µL and 1µL volumes, respectively. Lines of best fit were drawn from the mean and standard error of the mean (SEM). 96-well and 24-well experiment replicates are shown as a scatter plot behind (dark and light teal, respectively). GraphPad Prism® Log(agonist) vs. response (three parameters) was used to calculate the $LogEC_{50}$ and EC_{50} with 95% Confidence Intervals (95% CI). Standard sigmoidal responses are shown for DBP and DEHP. DBP bottom 0.1338 (95% CI 0.05952 to 0.2081) and top 0.5424 (95% CI 0.4347 to 0.650), R square = 0.432 (n=62). DEHP bottom 0.176 (95% CI 0.117 to 0.235) and top 1.097 (95% CI 0.719 to 1.475), R square = 0.4998 (n=60). The dotted line (y=0.2) highlights 20% 1nM E2 normalised RLU, as a threshold for categorising positive, negative and/or inadequate data.

Di-n-butyl phthalate (DBP), while negative in the *in vivo* Uterotrophic assay, has been reported positive in 6 of 11 *in vitro* ER STTA (PC_{10} $4.09x10^{-6}$ M) and BG1Luc ER TA (EC_{50} $4.09x10^{-6}$ M) (ICCVAM, 2011). The data collected for DBP (Figure 5.6) via the method detailed in this study (Section 5.2.6.1), were inadequate. The results of 7 concentrations (100nM to 10µM) with 3 replicates, repeated on 3 days in 96 (n=1) or 24 (n=2) well plates, were inconclusive and no conclusion on either the activity or inactivity could be drawn. Presented in Figure 5.6, the $LogEC_{50}$ and EC_{50} of Diethylhexylphthalate (DEHP) exposure was, -4.71 M (95% CI -5.163 to -4.254) and 1.958e-05 M (95% CI 6.873e-06 to 5.576e-05), respectively (R^2 = 0.50). Sumitomo laboratories (Japan) detected DEHP ER agonism in one of three assays (PC_{10} -5.49 M); however, no ER transactivation was detected in assays conducted at Ceri, Otsuka and Kaneka laboratories (Takeyoshi, 2006). The results generated herein, reflective of 3 day repeats of 7 concenrations with three replicates (100nM to 10µM), support the findings of Sumitomo laboratory, by suggesting that DEHP is an *in vitro* ERα signalling agonist, albeit at a lower potency. Butylbenzylphthalate was the most potent phthalate ER agonist identified in this study (Figure 5.6), the $LogEC_{50}$ and EC_{50} were -7.11 M (95% CI -7.77 to -6.45) and 7.75e-08 M (95% CI 1.683e-06 to 3.569e-07), respectively. These results are comparable to ER STTA (PC_{50} $4.11x10^{-6}$ M) and BG1Luc ER TA (EC_{50} $1.98x10^{-6}$ M) literature values (ICCVAM, 2011). The increase in sensitivity is likely to be a result of increased exposure time.

The mean data for all *in vitro* HeLa9903 stably transfected ER transactivation assays conducted herein, are summarised in Table 5.10. 4'4-DDE agonised ERα transactivation, presenting $LogEC_{50}$ and EC_{50} values of -11.83 M (95% CI -12.38 to -10.35) and 4.35e-12 M (95% CI 4.19e-13 to 4.53e-11), respectively. Transactivation was induced at markedly lower 4'4-DDE concentration ranges, than the EC_{50} $3.00x10^{-6}$ M reported in the literature (ICCVAM, 2011), possibly as a result of longer exposure times (Figure_Apx 15). In addition, 4-Benzylphenol (4-BP) was identified as a potential ER agonist, $LogEC_{50}$ and EC_{50} values were -4.87 M (95% CI -5.284 to -4.460) and 1.343e-05 M (95% CI 5.203-06 to 3.466e-05), respectively; 4 day repeats of experiments at 7 concentrations with 3 in-plate replicates (n=84). Clear negative responses were observed for Triclosan (2 repeats), Methylparaben (2 repeats) and 4-Nitrophenol (3 repeats); all of which were tested at 7 concentrations (10µM - 10pM) with three replicates. The results of ER transactivation, consequent to 40 hour Octylmethoxycinnamate exposure (10µM - 10pM), were inconclusive (Figure_Apx 15).

Supporting the *in silico* predictions of ligand promiscuity and OECD TG455 results, Testosterone (1µM-1pM) was identified as an agonist of ERα transactivation. Surpassing the sensitivity of the 24 hour exposure STTA (PC_{50} $9.78x10^{-6}$ M) and BG1Luc ER TA (EC_{50} $1.75x10^{-5}$), results generated herein showed transactivation with $LogEC_{50}$ -11.16 M (95% CI -11.67 to -10.66) and EC_{50} 6.871e-12 M (95% CI 2.16e-12 to 2.19e-11), consequent to 40 hour testosterone exposure (2 repeats, n=29, R^2=0.72).

Table 5.10 Results of HeLa9903 Stably Transfected ER Transactivation Assay to Detect Agonism of Estrogenic Signalling

Table shows the mean 50% effective concentration (EC50) for test substances in the ER STTA conducted herein. OECD/ICCVAM ER TA values are stated in the third column.

Test Substance (TS)	HeLa9903 ER Agonism (95% Confidence Intervals)	OECD/ICCVAM ER STTA Values*
17β-Estradiol	$LogEC_{50}$ -10.20 M (95% CI -10.37 to -10.02) EC_{50} 6.377e-11 M (95% CI 4.295e-011 to 9.476e-011)	$LogEC_{50}$ -11.3 to -10.1 M
17α-Ethinylestradiol	$LogEC_{50}$ -14.27 M (95% CI -14.70 to -13.84) EC_{50} 5.329e-15 M (95% CI 1.776e-15 to 1.421e-14)	PC_{50} <1.00 x10^{-11} M
Diethylstilbestrol	$LogEC_{50}$ -11.83 M (95% CI -12.07 to -11.60) EC_{50} 1.464e-12 M (95% CI 8.46e-13 to 2.53e-12)	PC_{50} <1.00 x10^{-11} M
4'4-DDE	$LogEC_{50}$ -11.36 M (95% CI -12.38 to -10.35) EC_{50} 4.349e-12 M (95% CI 4.192e-13 to 4.526e-11)	Mean EC_{50} 3.00 x 10^{-6} M
4-Benzylphenol	$LogEC_{50}$ -4.87 M (95% CI -5.284 to -4.460) EC_{50} 1.343e-05 M (95% CI 5.203-06 to 3.466e-05)	-
4-Nitrophenol	Negative [10^{-11} – 10^{-5} M]	-
Bisphenol A	$LogEC_{50}$ -10.13 M (95% CI -10.52 to -9.74) EC_{50} 7.44e-11 M (95% CI 3.037e-11 to 1.824e-10)	PC_{50} 2.02 x 10^{-8} M
M-BPA	$LogEC_{50}$ -10.04 M (95% CI -10.64 to -9.44) EC_{50} 9.068e-11 M (95% CI 2.29e-11 to 3.59e-10)	-
Dibutylphthalate	Inadequate data [10^{-11} – 10^{-5} M]	PC_{50} 4.09 x 10^{-6} M
Diethylhexylphthalate	$LogEC_{50}$ -4.71 M (95% CI -5.163 to -4.254) EC_{50} 1.958e-05 M (95% CI 6.873e-06 to 5.576e-05)	-
Butylbenzylphthalate	$LogEC_{50}$ -7.11 M (95% CI -7.77 to -6.45) EC_{50} 7.75e-08 M (95% CI 1.683e-08 to 3.569e-07)	PC_{50} 1.14 x 10^{-6} M
Methylparaben	Negative [10^{-11} – 10^{-5} M]	-
Octylmethoxycinnamate	Inadequate data [10^{-11} – 10^{-5} M]	-
Triclosan	Negative [10^{-11} – 10^{-5} M]	-
Testosterone	$LogEC_{50}$ -11.16 M (95% CI -11.67 to -10.66) EC_{50} 6.871e-12 M (95% CI 2.16e-12 to 2.19e-11)	PC_{50} 2.82 x 10^{-8} M
Corticosterone	Negative [10^{-11} – 10^{-5} M]	Negative

There are limited data pertaining to the agonistic response of 4-BP, 4-NP, M-BPA, Methylparaben, OMC and Triclosan in the HeLa9903 cell line. However, the dose-repsonse relationships observed for 4-BP (Figure_Apx 15) and MBPA (Figure 5.5), support the ER activity reported in qHTS assays (Table 5.3) and Yoshihara *et al.*'s (2004) study, respectively. Conversely, the inadequate data generated in OMC assays (Figure_Apx 15), do not contribute to the debate regarding the estrogenicity of UV-filters (Schlupf *et al.*, 2001). Triclosan has been shown to antagonise the ER (Table 5.3); however, the results of 40hr exposure in HeLa9903 cells suggest that TCN cannot agonise ER signalling. The chemical structure (see Table 5.3) of TCN may offer an explanation for antagonistic rather than agonistic responses, as 'bulky' chlorine functional groups may prevent the clamping of the ligand binding domain by α-Helices-12, which is required for ERα agonism (See Section 3.1). Methylparaben is listed on the

ER binding database (NCTRER), from the negative results generated herein, binding may result in ER antagonistic MoA, rather than agonistic.

The exposure time was increased, from 20-24 hours to 40 hours, consequent to preliminary testing for laboratory performance and optimisation of 17β-estradiol responses. The rate of HeLa9903 cell growth, appeared to decrease, on 'conditioning' in which the cells were starved of estrogenic hormone components (DCC-FBS, Phenol red-free, antibiotic-free EMEM), the increase in exposure time aimed to mitigate for the delayed growth, without increasing the starting cell concentration. The results generated herein (Table 5.10), suggest that increasing the *in vitro* chemical exposure time, increases the sensitivity of the HeLa9903 stably transfected ER transactivation assay.

5.3.3 BG1Luc4E2 ERα/β Agonism STTA

An objective of the *in vitro* screening, was to test potential-EDCs (n=20) in BG1Luc4E2 cells, recently validated and adopted by OECD TG455. However, preliminary testing demonstrated poor laboratory performance of BG1Luc ER TA; 24 hour E2 or EE2 exposure did not induce ER transactivation. Increasing positive control (E2 or EE2) exposure to 40-48 hours led to an induction of ER transactivation, at 10^{-8} M E2 and 10^{-9} M EE2 (Figure 5.7). Figure 5.7 plots the relative light units (RLU) emitted in the BG1Luc4E2 ER transactivation assays in response to E2 and EE2. The responses to E2 and EE2 reported herein were lower than the TG455 refererance standards; E2 $EC_{50} = 5.63x10^{-12}$ M and EE2 $EC_{50} = 4.20x10^{-8}$ M (ICCVAM, 2011). Validation studies by ECVAM and XDS laboratories identified significant variability in BG1Luc ER TA response; E2 only induced transactivation in 24 of 35 assays. ICCVAM (2011) notes that because DMSO control RLU values are not normalised, they can vary considerably between test plates; ranging from 511 to 9885 RLU (mean 3749). Interestingly, the maximum E2 response reported herein (Figure 5.7), was comparable to that reported in BG1Luc validation studies (10,000 RLU); thus, the ineffectivity of the assay may be as a result of background luminescence.

The BG1Luc ER TA was demonstrated to be an insensitive *in vitro* assay, under the laboratory conditions and equipment specifications (Section 5.2), available for this study. Further testing in the BG1Luc4E2 cell line under these conditions, was not expected to generate statistically significant results. OECD validation has demonstrated concordance between the ERα STTA and the BG1Luc ERα/β TA (OECD, 2012; ICCVAM, 2011). Thus, it is not injudicious to suggest that the BG1Luc ER TA results may have mirrored the HeLa9903 assay results detailed in Section 5.3.2. The difficulty in observing ER responses *in vitro*, in addition to the variability defined in the literature, suggest that utilising the endogenous ERα and ERβ to elicit transactivation of stably transfected response elements in the BG1Luc4E2 cell line, presents more challenges than the STTA, which may be considered a more sensitive assay. Conceptually, the variability of BG1Luc4E2 responses may reside in the feedback mechanisms of ERα

and ERβ responses (i.e. inter-receptor crosstalk). As detailed in Section 3.1, ERβ can act as a dominant inhibitor of ERα transcriptional activity, when co-expressed (Metivier *et al.*, 2003). Thus, the balance of endogenous hormone receptors in BG1Luc4E2 cells may play a significant role in assay sensitivity.

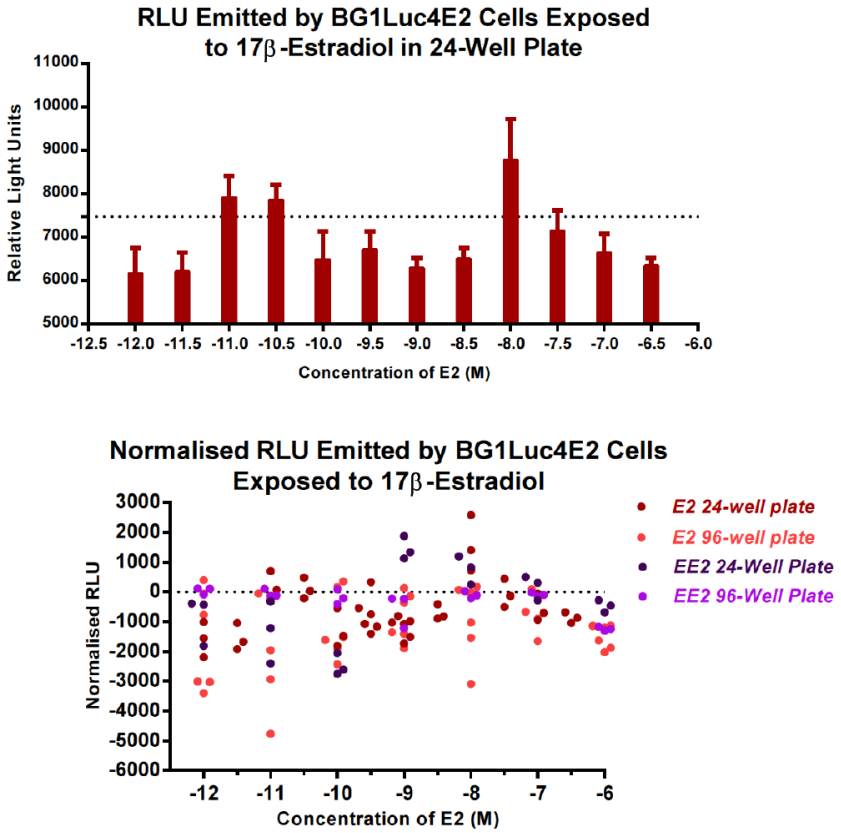


Figure 5.7 Inadequate Response of BG1Luc4E2 Estrogen Receptor (α/β) Transactivation Assays in 24-Well and 96-Well Formats

Graphs show the adjusted and normalised relative light units (RLU) emitted by BG1Luc4E2 Cells consequent to 40-48hr exposre to positive controls (E2 and EE2). Results were normalised by subtracting vehicle control (DMSO) RLU. Cell concentration was $1x10^4$/well in 96-well (200µL) and $1x10^5$/well in 24-well (1000µL); estrogens were solvated in 100% DMSO and administered in 0.2µL and 1µL volumes, respectively. The dotted line (y=7460) on the top graph shows the mean RLU of the DMSO vehicle control, highlighting the limited responses of the BG1Luc4E2 cells observed in this study in response to estrogens.

5.3.4 HeLa4-11AR Agonism STTA

Despite being an objective of ECVAM, there are currently no regulatory test guidelines to assess androgenicity *in vitro*; excluding the rat AR binding study, which is reliant on rat ventral prostate tissue. Reflective of the ER STTA, mammalian cell based luciferase reporter assays have been demonstrated to show sensitivity in detecting androgenicity (Svovodova & Cajthaml, 2010). HeLa4-11 cells are stably transfected with a hAR expression construct and a firefly luciferase reporter gene under the control of the MMTV promoter. The results of stably transfected AR transactivation assays, to detect

AR agonism in HeLa4-11 cells, consequent to 24 hour test substance exposure (n=20), are detailed herein. Figure 5.8 shows the AR transactivation following Testosterone exposure (semi-log dilutions from $1x10^{-6}$ M to $1x10^{-24}$ M Testosterone) in 24 and 96 well microtitre plates. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM testosterone (maximal agonistic response in both plate formats).

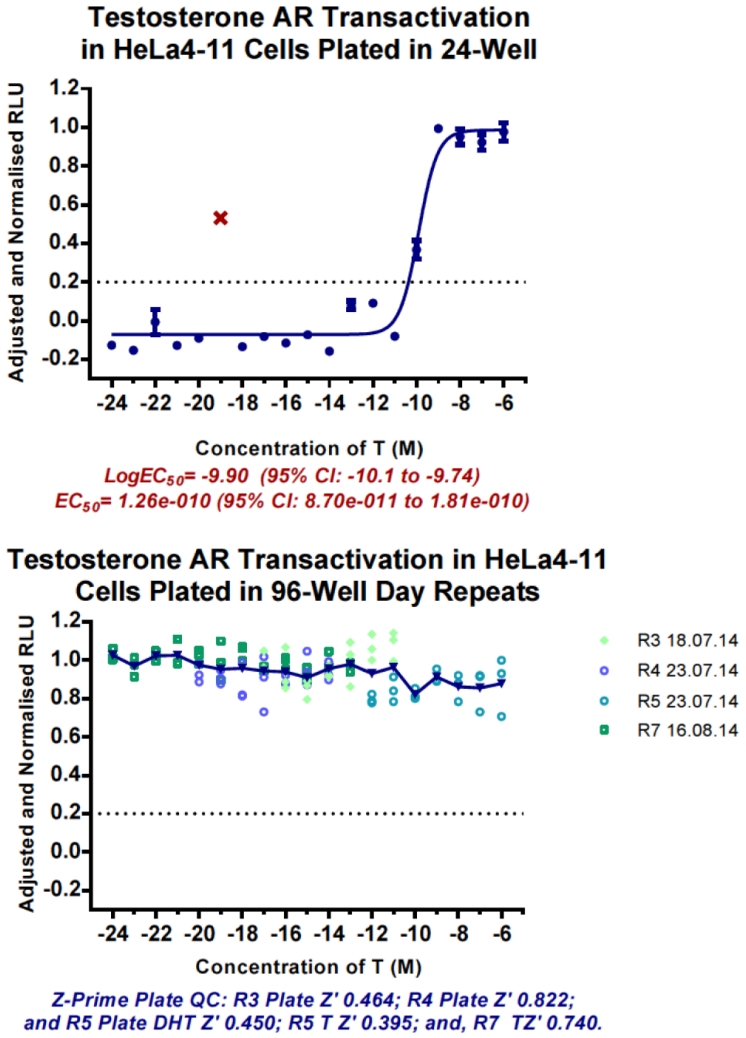


Figure 5.8 Androgen Receptor Transactivation in Response to 24h Testosterone Exposure in HeLa4-11 Cells in 24- and 96- Microtitre Plates

Drawn using GraphPad Prism® Log(agonist) vs. Response (three parameters), plots show the adjusted and normalised relative light units (RLU) emitted in luciferase assays consequent testosterone exposure in either 24- or 96- well plate format. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM testosterone (maximal agonistic response). Cell concentration was $1x10^4$/well in 96-well (200µL) and $1x10^5$/well in 24-well (1000µL); testosterone solvated in 100% DMSO was administered in 0.2µL and 1µL volumes, respectively. 24-well plate data on the left shows the Mean and Standard error of the mean (SEM) of 2 day repeats, with the $LogEC_{50}/EC_{50}$ and 95% Confidence Intervals (95% CI) stated in red. The bottom graph shows the mean and SEM of 96-well day repeat data (n=4), superimposed on the individual data replicates, highlighting the distribution of the data over the different testing days. The dotted line (y=0.2) highlights 20% 1nM testosterone luciferate induction, as a threshold for categorising positive, negative and/or inadequate data. Quality assurance data (Z-prime) for each 96-well plate are stated in blue.

24-well plate Testosterone exposures presented $LogEC_{50}$ and EC_{50} of -9.90 M (95% CI: -10.1 to -9.74) and 1.26e-10 M (95% CI: 8.70e-11 to 1.81e-10), respectively (R^2=0.9599, n=58). The sensitivity of the HeLa4-11 24-well plate assay was comparable to that of the MDA-kb2 cell line, which is stably transfected with an androgen responsive firefly luciferase reporter gene (ARE); 28 hour 0.25 nM DHT exposure induced maximal AR transactivation (Orton *et al*., 2014). The EC_{50} of 0.126nM (1.26e-10 M) identified herein, demonstrated equivalent sensitivity to the *S. cerevisiae* yeast androgen bioassay, developed by Michelini *et al*. (2005); EC_{50} of 10nM for testosterone, with a 0.05nM (50pM) limit of detection.

The lower plot of Figure 5.10 presents the dose-response of Testoserone observed in 96-well plates (i.e. increasing the throughput). All tested Testosterone concentrations ($1x10^{-6}$ M to $1x10^{-24}$ M) induced maximal AR transactivation, consequent to 24 hour exposure (4/4 day repeats); See Table 5.11 for Z'Prime values. Avogadro's constant (N_A) $6.022x10^{23}$ approximates the number of constituent molecules in a mole (Mohr *et al*., 2008), thus, in light of the dilutions, the assay appears to induce maximal AR transactivation in response to trace concentrations of Testosterone. DMSO vehicle controls (n=6 per plate) did not induce AR transactivation, reducing the likelihood of contamination, and comparable AR transactivation sensitivity was observed on 24 hour exposure to R1881 and DHT (Table 5.11). The marked difference observed between plate formats is disconcerting; however, simple explanations may reside in cell concentration and cofactor expression.

The sensitivity of AR STTA in the PALM prostatic cell line, which stably express AR (pSG_5-puro-hAR) and ARE (pMMTV-neo-Luc), was shown to vary with cell concentration (Térouanne *et al*., 2000). PALM cells plated at a density of $8x10^4$, $2x10^4$ and $5x10^3$, led to Testosterone EC50's of 20nM, 3nM and 0.2nM, respectively; thus lower cell concentrations yielded higher Testosterone sensitivity. However, the EC_{50} of the synthetic androgen, Metribolone (R1881) was independent of cell number, suggesting that the effect of cell concentration may not alter sensitivity ubiquitously (Térouanne *et al*., 2000). Cofactors regulate the transactivation of nuclear receptors (Section 3.2.2). ARA70 and ARA55 cofactors have been demonstrated to enhance AR transactivation, in response to residual androgen concentrations (Fujimoto *et al*., 1999; Yeh *et al*., 1999a). Nishimura *et al.* (2003) demonstrated modulation of AR transactivation in response to Gelsolin, the AR-associated protein involved in cytoskeleton reorganisation, cell morphology and motility. Furthermore, Yeh *et al*. (1999b) suggested that cross-talk between AR and HER-2/neu pathway signals, stimulated by growth factors may activate AR.

The HeLa4-11 cell line may express elevated cofactor concentrations, thereby amplifying the response to trace testosterone concentrations. Detailed mechanistic information pertaining to HeLa4-11 cell modifications, were sought via the named distributor, Kazushi Kawaharada (DS Pharma Biomedical Co. Ltd.). However, due to

patenting restrictions, no additional information was provided and the mechanism behind HeLa4-11 AR transactivation sensitivity remains speculative. Consequently, despite the sensitivity of HeLa4-11 STTA in detecting AR agonists *in vitro*, it may be unwise to use the assay to predict potency.

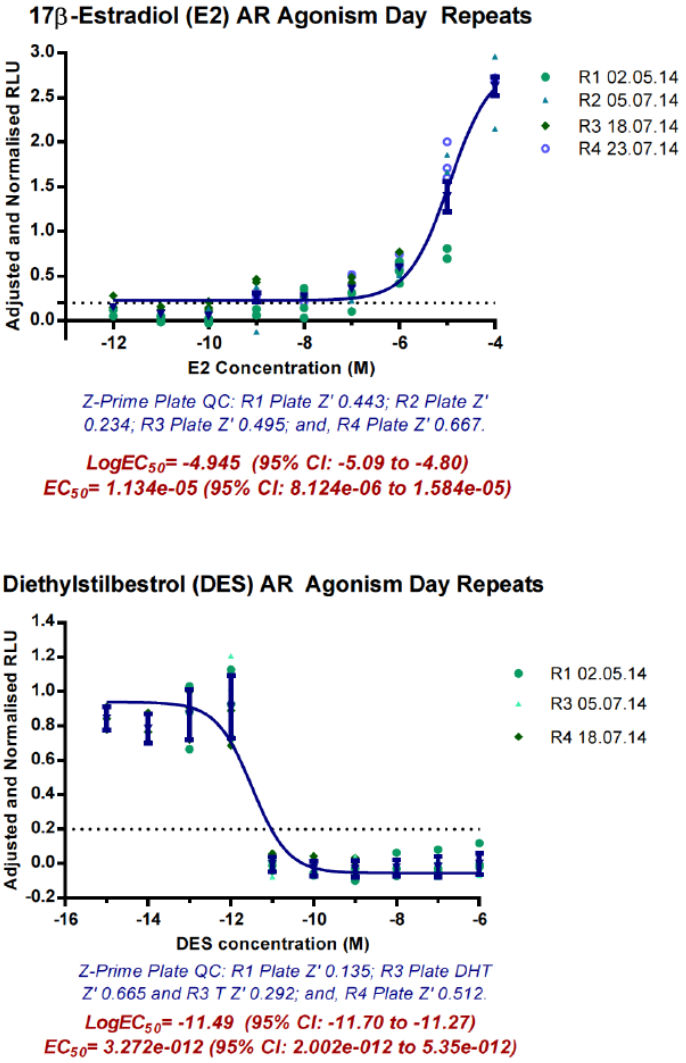


Figure 5.9 Androgen Receptor Transactivation in HeLa4-11 Cells in Response to 24h Estrogen Exposure (E2 and DES)

Drawn using GraphPad Prism® Log(agonist) vs. Response (three parameters) line drawing function. Graphs show the adjusted and normalised relative light units (RLU) emitted during the luciferase assay, consequent to 17β-Estradiol (E2) or Diethylstilbestrol (DES) exposure. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM testosterone (maximal agonistic response). Graphs summarise day repeats replicates and presented the Mean and Standard Error of the mean (SEM) to calculate agonism ($LogEC_{50}$/EC_{50}) with 95% Confidence Intervals (95% CI). Data replicates are presented and colour coded by experiment date (day repeats). The dotted line (y=0.2) highlights 20% 1nM testosterone (maximal response ~ positive control) luciferate induction, as a threshold for categorising positive, negative and/or inadequate data. Quality assurance data (Z-prime) for each 96-well plate are stated in blue.

Figure 5.9 shows the AR transactivation in HeLa4-11 cells consequent to 24 hour E2 or DES exposure. The E2 $LogEC_{50}$ and EC_{50} were -4.945 (95% CI -5.09 to -4.80) and 1.134e-05 (95% CI: 8.124e-06 to 1.584e-05), respectively. Supporting the results detailed herein (15.84µM), agonistic AR transactivation in response to E2, has been reported at 2.23µM (Table 5.1). The comparable sensitivity of HeLa4-11 cells, in detecting estrogenic AR agonists supports Térouanne *et al.*'s (2000) theory, that different agonists present different mechanisms of AR transactivation.

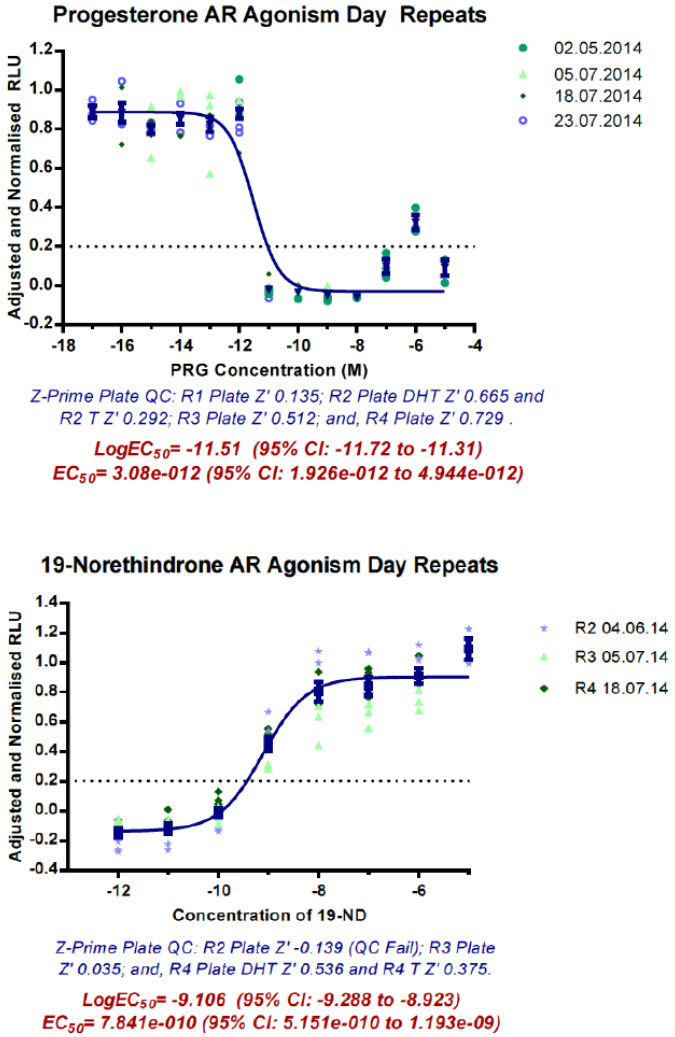


Figure 5.10 Androgen Receptor Transactivation in HeLa4-11 Cells in Response to 24h Progestagin Exposure (PRG and 19-ND)

Drawn using GraphPad Prism® Log(agonist) vs. Response (three parameters) line drawing function. Graphs show the adjusted and normalised relative light units (RLU) emitted during luciferase assays, consequent to progesterone (PRG) or 19-Norethindrone (19-ND) exposure. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM testosterone (maximal agonistic response). The curve plots the Mean and Standard Error of the mean (SEM). Mean data was used to calculate agonism ($LogEC_{50}$/EC_{50}) with 95% Confidence Intervals (95% CI). Data replicates are shown, colour coded by experimental day, to highlight the distribution of data. The dotted line (y=0.2) highlights 20% positive control (1nM testosterone ~ maximal response) luciferate induction, as a threshold for categorising positive, negative and/or inadequate data. Quality assurance data (Z-prime) for each 96-well plate are stated in blue.

The $LogEC_{50}$ and EC_{50} of DES AR transactivation were -11.49 M (95% CI: -11.70 to -11.27) and 3.272e-12 M (95% CI: 2.002e-12 to 5.35e-12), respectively (Figure 5.9). DES has been shown to inhibit recombinant rat AR (IC_{50} 14.13μM) and antagonise transactivation in the MDA cell line (27.72μM); however, the potential agonistic MoA of DES have been neglected (Table 5.2). The low-dose stimulation and high-dose inhibition, of DES induced AR transactivation observed herein, suggests a biphasic dose response (i.e. hormesis), which has not previously been reported.

Figure 5.10 shows the adjusted and normalised relative light units (RLU) emitted during stably transfected AR transactivation assays, consequent to 24 hour progesterone (PRG) or 19-Norethindrone (19-ND) exposure. Similar to DES, Progesterone dose-response appeared to be biphasic. The PRG $LogEC_{50}$ and EC_{50} were -11.51 M (95% CI: -11.72 to -11.31) and 3.08e-12 M (95% CI: 1.926e-12 to 4.944e-12). Interestingly, agonism of AR transactivation has been reported at 0.025 μM (Table 5.1), which bears comparison to a small increase in AR transactivation detected between $1x10^{-8}$ and $1x10^{-5}$ M, herein (Figure 5.10). The $LogEC_{50}$ and EC_{50} for AR transactivation consequent to 19-Norethindrone exposure were -9.12 M (95% CI: -9.29 to -8.92) and 7.841e-10 M (95% CI: 5.15e-10 to 1.19e-09), respectively. The synthetic progestin did not present the biphasic dose-response in AR transactivation observed for Progesterone.

BPA and 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBPA) testing concentrations were 1:10 log dilutions of 1mM to 100pM. BPA did not induce AR transactivation in HeLa4-11 cells; however, MBPA exposure produced a biphastic dose-response (Table 5.11). Figure 5.11 shows the AR transactivation in HeLa4-11 cells consequent to 24 hour MBPA or 4'4-DDE exposure. The MBPA $LogEC_{50}$ and EC_{50} were -11.53 M (95% CI: -11.72 to -11.34) and 2.965e-12 M (95% CI: 1.91e-12 to 4.604-12). To the authors knowledge, the androgenicity of 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene, has not previously been reported.

4,4'-DDE has been demonstrated to inhibit the activity of R1881 in recombinant AR, expressed in *E. coli* ~ IC_{50} 20.42μM (Table 5.3). The antagonistic properties of 4,4-DDE *in utero,* have been associated with cryptorchidism incidence (Montes *et al.,* 2010). Figure 5.11 shows AR transactivation in response to all tested concentrations of 4,4'-DDE ($1x10^{-6}$ M to $1x10^{-18}$ M); inducing maximal agonistic response. Björk *et al.* (2011) identified alterated 4,4'-DDE agonism and antagonism of AR transactivation *in vitro,* relative to androgen receptor CAG length. AR CAG repeats have also been shown to affect AR function *in vivo,* and have been implicated in the aetiology of Testicular Germ Cell Cancer (Västermark *et al.,* 2011; see Section 2.2). The AR transactivation in response to testosterone (Figure 5.8) and 4,4'-DDE exposure reported herein, suggest AR modifications in the HeLa4-11 cell line, which increase sensitivity and transactivational response.

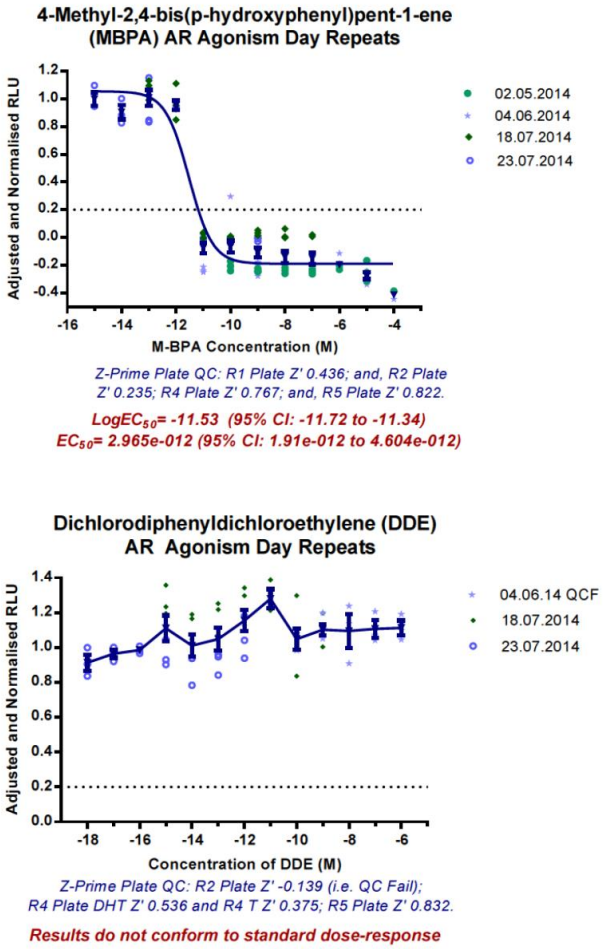


Figure 5.11 Androgen Receptor Transactivation in HeLa4-11 Cells in Response to MBPA and DDE Exposure

Excluding Mean DDE AR Agonism, in which points were connected by lines, all lines of best fit were drawn using GraphPad Prism® Log(agonist) vs. Response (three parameters) function. Graphs show the adjusted and normalised relative light units (RLU) emitted consequent to MBPA or DDE exposure. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM testosterone (maximal agonistic response). Graphs plot the Mean and Standard Error of the mean (SEM). Mean data was used to calculate agonism ($LogEC_{50}/EC_{50}$) with 95% Confidence Intervals (95% CI). Data replicates are colour-coded by experimental date, to show the distribution of data. The dotted line (y=0.2) highlights 20% positive control (1nM testosterone ~ maximal response) luciferate induction, as a threshold for categorising positive, negative and/or inadequate data. Quality assurance data (Z-prime) for each 96-well plate are stated in blue.

The suggestion that AR responses are modified in HeLa4-11 cells is heightened by the *in vitro* AR transactivation observed consequent to Methylparaben (MeP), Octylmethoxycinnamate (OMC) and Triclosan (TCN) exposure (Figure 5.12). Maximal agonistic response was observed for all TCN and MeP concentrations, excluding declines in transactivation observed at cytotoxic concentrations (Figure 5.1). Triclosan had previously been identified as an AR antagonist (Table 5.3). OMC AR transactivation increased at moderately cytotoxic concentrations; exceeding the response of 1nM Testosterone the results hint at ligand-independent AR transactivation.

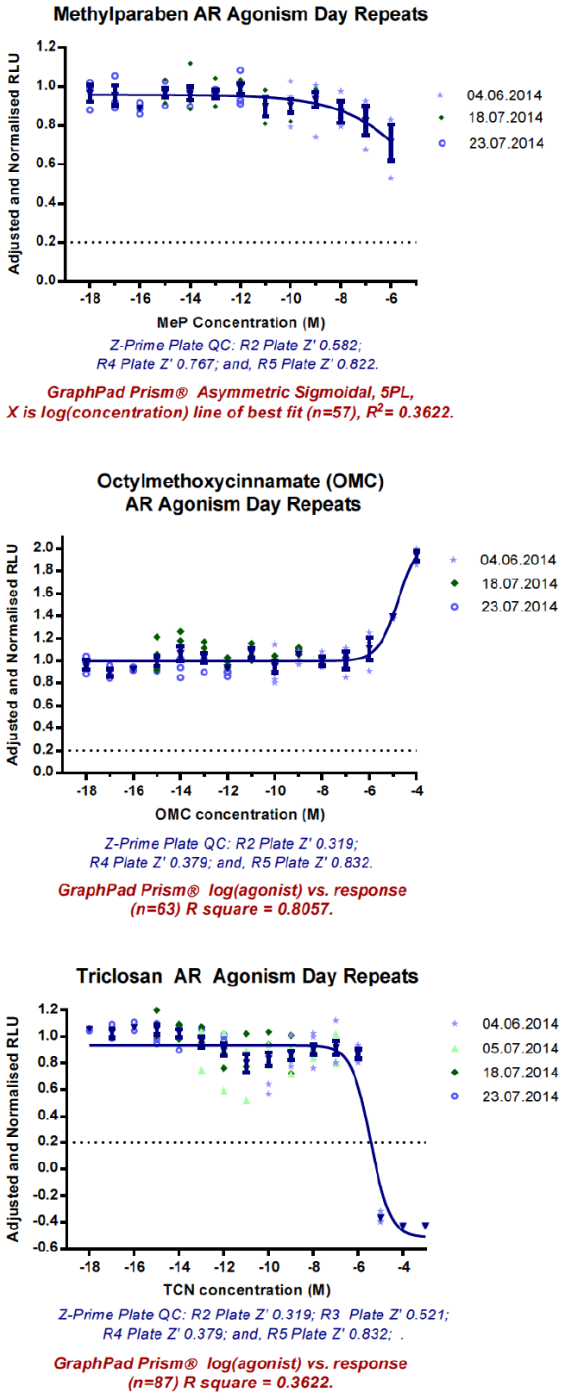


Figure 5.12 Androgen Receptor Transactivation in HeLa4-11 Cells in Response to 24h Consumer Chemical Exposure (MeP, TCN and OMC)

Drawn using GraphPad Prism® Log(agonist) vs. Response (three parameters) function, graphs show the relative light units (RLU) emitted consequent to exposure to either Methylparaben (MeP), Triclosan (TCN) or Octylmethoxycinnamate (OMC). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM testosterone. Graphs show the Mean and Standard Error of the mean (SEM) best fit data used to calculate agonism ($LogEC_{50}/EC_{50}$) with 95% Confidence Intervals (95% CI). Replicates are colour coded according to experiment date.The dotted line (y=0.2) highlights 20% luciferase induction of the positive control (1nM testosterone ~ maximal response), as a threshold for categorising positive, negative and/or inadequate data. Quality assurance data (Z-prime) for each 96-well plate are stated in blue.

Ma *et al*. (2003) did not detect either agonism or antagonism of AR transactivation in the MDA-kb2 cell line, consequent to OMC exposure.

Table 5.11 Results of HeLa4-11 Stably Transfected AR Transactivation Assay to Detect Agonism of Androgenic Signalling

Table shows the mean 50% effective concentration (EC_{50} (95% Confidence Interval)) for test substances in the AR STTA conducted herein. In the absence of regulatory criteria, Z'Prime statistical values for 96-well plates (Mean (range)), the number of data points (N) and day repeats (DR) are detailed in columns 3 and 4. Results highlighted in green presented 'atypical' dose-response relationships, presenting no AR activity at high concentrations, but inducing transactivation at low concentrations. The results for Testosterone are highlighted in red, because a dichotomy in potency was observed, dependent on culture condition (24 vs 96 well plates).

Test Substance (TS)	AR Agonism (95% Confidence Interval)		Z'Prime
17β-Estradiol	$LogEC_{50}$ -4.945 M (95% CI -5.09 to -4.80) EC_{50} 1.134e-05 M (95% CI 8.124e-06 to 1.584e-05)	N=86 DR=4	Mean 0.46 (0.234-0.667)
17α-Ethinylestradiol	Negative 10^{-13} – 10^{-5} M	N=63 DR=3	Mean 0.45 (0.234-0.667)
Diethylstilbestrol	$LogEC_{50}$ -11.49 M (95% CI -11.70 to -11.27) EC_{50} 3.27e-012 M (95% CI 2.00e-12 to 5.35e-12)	N=66 DR=3	Mean 0.44 (0.135-0.665)
Progesterone	$LogEC_{50}$ -11.51 M (95% CI -11.71 to -11.31) EC_{50} 3.08e-12 M (95% CI 1.926e-12 to 4.944e-12)	N=87 DR=4	Mean 0.47 (0.292-0.729)
19-Norethindrone	$LogEC_{50}$ -9.12 M (95% CI -9.288 to -8.923) EC_{50} 7.84e-10 M (95% CI 5.151e-10 to 1.193e-09)	N=64 DR=4	Mean 0.20 (QCF-0.536)
4'4-DDE	96WP Positive 10^{-18}-10^{-6} M All concentrations produced maximum response	N=57 DR=3	Mean 0.40 (QCF-0.832)
4-Benzylphenol	Negative 10^{-10} – 10^{-3} M	N=45 DR=2	Mean 0.24 (0.039-0.420)
4-Nitrophenol	Negative 10^{-10} – 10^{-3} M	N=44 DR=2	Mean 0.24 (0.039-0.420)
Bisphenol A	Negative 10^{-10} – 10^{-3} M	N=48 DR=2	Mean 0.34 (0.235-0.436)
M-BPA	$LogEC_{50}$ -11.53 M (95% CI -11.72 to -11.34) EC_{50} 2.965e-12 M (95% CI 1.91e-12 to 4.604e-12)	N=87 DR=4	Mean 0.57 (0.235-0.822)
Dibutylphthalate	Negative 10^{-10} – 10^{-3} M	N=45 DR=2	Mean 0.33 (0.187-0.476)
Diethylhexylphthalate	Negative 10^{-10} – 10^{-3} M	N=45 DR=2	Mean 0.33 (0.187-0.476)
Butylbenzylphthalate	Negative 10^{-10} – 10^{-3} M	N=45 DR=2	Mean 0.51 (0.438-0.582)
Methylparaben	96WP Positive 10^{-18} – 10^{-6} M NonCT concentrations produced maximum response	N=57 DR=3	Mean 0.72 (0.582-0.822)
Octylmethoxycinnamate	96WP Positive 10^{-18} – 10^{-4} M All concentrations produced maximum response	N=63 DR=3	Mean 0.51 (0.319-0.832)
Triclosan	96WP Positive 10^{-18} – 10^{-6} M NonCT concentrations produced maximum response	N=87 DR=4	Mean 0.51 (0.319-0.822)
Dihydrotestosterone	96WP Positive 10^{-20} - 10^{-7} M All concentrations produced maximum response	N=60 DR=3	Mean 0.774 (0.765-0.786)
Metribolone	96WP Positive 10^{-20} - 10^{-7} M All concentrations produced maximum response	N=63 DR=3	Mean 0.774 (0.765-0.786)
Testosterone	96WP Positive 10^{-23} – 10^{-6} M 24WP $LogEC_{50}$ -9.90 (95% CI -10.1 to -9.74)	N=99 DR=4	Mean 0.57 (0.395-0.822)
Corticosterone	96WP Positive 10^{-20} - 10^{-6} M All concentrations produced maximum response	N=63 DR=3	Mean 0.53 (0.395-0.822)

Table 5.11 summarises the results of HeLa4-11 Stably Transfected AR Transactivation Assays, to detect agonism of androgen receptor signalling. Exposure to 17α-Ethinylestradiol (EE2), Bisphenol A (BPA), 4-Benzylphenol (4-BP), 4-Nitrophenol (4-NP), Dibutylphthalate (DBP), Diethylhexylphthalate (DEHP) or Butylbenzylphthalate (BBP) did not induce agonistic AR responses (Table 5.11; Figure_Apx 16). The data presented herein strongly suggest that BPA, 4-BP, 4-NP, DBP, DEHP and BBP are not androgen receptor agonists in the HeLa4-11 cell line. Slight luciferase induction was observed at 10μM; however, this coincided with cytotoxicity (see Figure 5.1).

Z factors were used to monitor assay sensitivity, by assessing the distance and distribution of 1nM Testosterone AR transactivation positive controls with background DMSO vehicles; inherently stringent, Z' statistics require 99% of values to occur within 3 standard deviations of the mean (Table 5.11; see Section 5.2.10). The mean 96-well plate (n=32) Z'prime value was 0.5, emphasising the statisticaly significant responses generated in the HeLa4-11 AR agonism transactivation assays. However, the sensitivity and disagreement of observed AR transactivation with literature predictions, leads the author to suggest that HeLa4-11 cells have been modified to increase AR transactivation. An array of modifications, including AR CAG repeats, cofactor expression and ARE modulation, may affect AR transactivation responses.

5.3.5 HeLa4-11 AR Antagonism STTA

As an extension to the HeLa4-11 AR transactivation assays, to detect agonists of androgenic signalling, the assay was mofified to detect antagonistic responses. Cells suspended in 10% DCC-FBS-EMEM were plated at 1 x 10^4 cells/100μL per 96-well plate and allowed to attach for 3 hours, prior to the addition of 100 μL of 10% DCC-FBS-EMEM supplemented with 1nM testosterone and 0.2μL of test substance in 100% DMSO. Assay plates were incubated at 5% CO2 at 37±1ºC and terminated after 20-24 hour chemical exposure.

Primarily used in the treatment of prostate cancer, Flutamide antagonises the AR, demonstrating an IC_{50} of $3.62x10^{-6}$ M when coexposed with 0.5nM DHT in MKD-kb2 cells (Ma *et al*., 2003). Figure 5.13 shows the antagonism of AR transactivation in HeLa4-11 cells co-exposed to Flutamide (10fM-100μM) and 1fM Testosterone for 24 hours. The $LogIC_{50}$ and IC_{50} of Flutamide and 1fM Testosterone exposure, identified herein were, -4.315 M (95% CI -4.508 to -4.123) and 4.839e-05 M (95% CI 3.108e-05 to 7.534e-05), respectively (n=99, R^2=0.908). Flutamide was not a strong AR antagonist in the HeLa4-11 cell line, which may reflect the dual activity of antiandrogens detailed by Ma *et al*. (2003). Nevertheless, as a confirmed moderate antagonist, 10μM Flutamide + 1fM Testosterone controls (n=3), were included in each AR antagonism transactivation assay; as a quality control measure all assay plates presented 10-20% reduction in transactivation, consequent to Flutamide exposure.

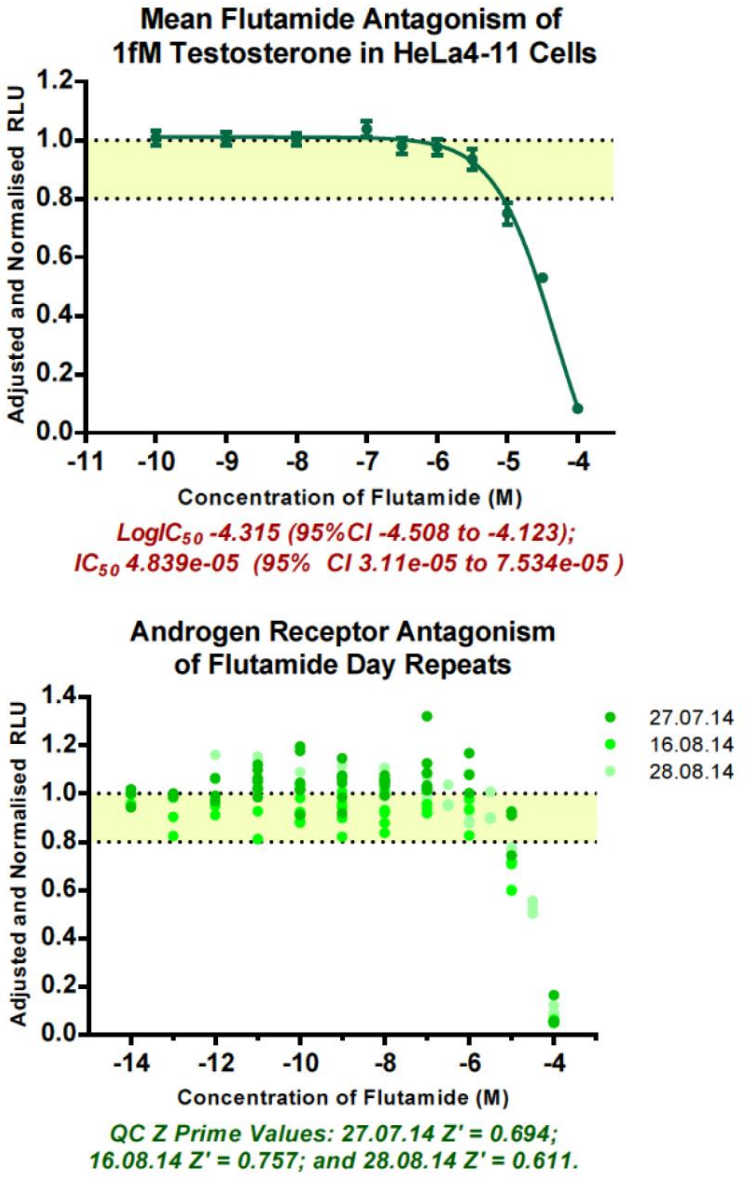


Figure 5.13 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Co-Exposed to Flutamide and 1fM Testosterone for 24 Hours

Drawn using GraphPad Prism® Log(inhibitor) vs. Response (three parameters). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by 1fM testosterone RLU (maximal agonistic response). Top graph summarises day repeat data, presented as the Mean and Standard Error of the mean (SEM). Mean data was used to calculate inhibition ($LogIC_{50}/IC_{50}$) with 95% Confidence Intervals (95% CI) shown in red font. Error bars in the Day Repeat Data represent the standard deviation, highlighting the distribution of the data over the different testing days. Quality assurance data (Z-prime) for each 96-well plate repeat are stated in green. Statistics: bottom -0.3581 (95% CI -0.5982 to -0.1179) and top 1.01 (95% CI 0.9895 to 1.031); $LogIC_{50}$ -4.315 (95% CI -4.508 to -4.123) and IC_{50} 4.839e-05 (95% CI 3.108e-05 to 7.534e-05). R square = 0.908 (n=99).

Interestingly, the endogenous and synthetic hormones inhibited 1fM Testosterone induced AR transactivation, with greater potency than Flutamide (Figure 5.14). The $LogIC_{50}$ and IC_{50} of EE2 were, -8.389 M (95% CI: -8.76 to -8.02) and 4.081e-09 M (95% CI: 1.75e-09 to 9.51e-09), respectively (n=60, R^2=0.745).

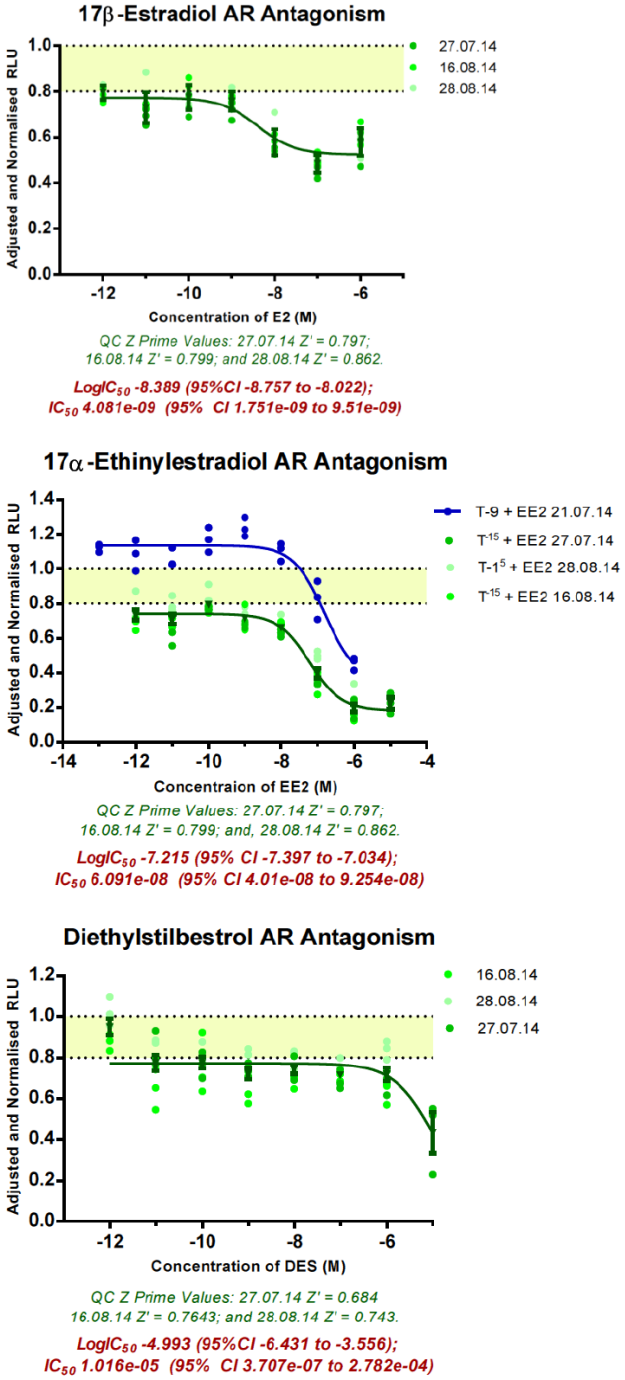


Figure 5.14 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Estrogens (E2, EE2 and DES) for 24 Hours

Drawn using GraphPad Prism® Log(inhibitor) vs. Response (three parameters) line drawing function. Graphs show the adjusted and normalised relative light units (RLU) emitted, consequent to coexposure of testosterone (1fM unless otherwise stated) and estrogens. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by 1fM testosterone RLU. Mean (±SEM) data was used to calculate inhibition ($LogIC_{50}/IC_{50}$) with 95% Confidence Intervals (95% CI). Day repeat data plots raw normalised-data replicates, highlighting the distribution of the data. Z-prime's are stated in green. E2 top 0.7724 (95% CI 0.7465 to 0.7983) and bottom 0.5246 (95% CI 0.4927 to 0.5565). R square = 0.745 (n=60). EE2 top 0.7409 (95% CI 0.7174 to 0.7644) and bottom 0.1814 (95% CI 0.1347 to 0.2281). R square = 0.910 (n=63). DES top 0.7713 (95% CI 0.7397 to 0.803) and bottom 0.09136 (95% CI -1.129 to 1.312). R square = 0.314 (n=63).

Tox21 qHTS assays identified E2 AR antagonism at 0.06µM (Huang *et al*., 2014; see Table 5.1). Thus, the HeLa4-11 AR transactivation assay for antagonistic responses detailed herein, demonstrated higher sensitivity for E2 MoA; IC_{50} = 4.08nM E2 + 1fM Testosterone. Co-exposure of EE2 and 1fM Testosterone presented a $LogIC_{50}$ -7.215 M (95% CI: -7.40 to -7.03) and IC_{50} 6.091e-08 M (95% CI: 4.01e-08 to 9.25e-08); R^2=0.910, n=63 (Figure 5.14).

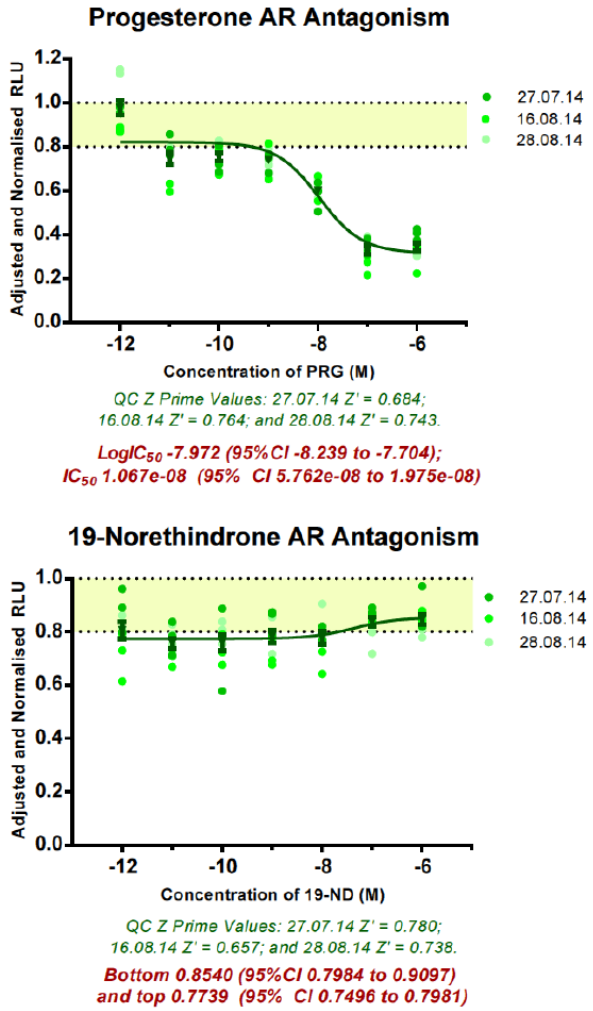


Figure 5.15 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Progestagins for 24 Hours

Drawn using GraphPad Prism® Log(inhibitor) vs. Response (three parameters). Graphs show the adjusted and normalised relative light units (RLU) emitted during the luciferase assay, consequent to coexposure of testosterone (1fM) and either progesterone or 19-Norethindrone. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by 1fM testosterone RLU (maximal agonistic response). Left side graphs summarise day repeat data, presented as the Mean and Standard Error of the mean (SEM), which was used to calculate inhibition ($LogIC_{50}$/IC_{50}) with 95% Confidence Intervals (95% CI). Error shown in the Day Repeat Data (right) is equivalent to the standard deviation, showing the data distribution over the different testing days. Quality assurance data (Z-prime) for each 96-well plate are stated in green. Progesterone (PRG) top 0.8205 (95% CI 0.785 to 0.856) and bottom 0.315 (95% CI 0.2589 to -0.371). R square = 0.8171 (n=63). 19-Norethindrone (19-ND) top 0.7739 (95% CI 0.7496 to 0.7981) and bottom 0.8540 (95% CI 0.7984 to 0.9097). R square = 0.146 (n=63).

The antagonism of AR transactivation in response to 24 hour EE2 and 1nM Testosterone exposure is shown in Figure 5.14; $LogIC_{50}$ and IC_{50} were -6.798 M (95% CI: -7.121 to -6.475) and 1.592e-07 M (95% CI: 7.56e-08 to 3.35e-07), respectively (R^2= 0.903, n=21). The increase in Testosterone concentration decreased the sensitivity of the EE2 AR antagonism transactivation assay. The third graph presented in Figure 5.14, shows the mean (±SEM) of DES + 1fM Testosterone AR transactivation (R^2=0.314, n=63). Demonstrating large confidence intervals, the mean did not accurately reflect data replicates; DES $LogIC_{50}$ -4.993 M (95% CI: -6.43 to -3.56) and IC_{50} 1.016e-05 M (95% CI: 3.71e-07 to 2.78e-04). Nevertheless, the results agreed with the observation of DES AR antagonism at 27.72µM in the MDA cell line (Table 5.2).

Progesterone was identified as a biphasic AR agonist in Section 5.3.4, Figure 5.15 highlights the dual-activity of Progesterone, which demonstrated AR antagonism with a $LogIC_{50}$ of -7.972 M (95% CI: -8.24 to -7.70) and IC_{50} of 1.067e-08 (95% CI 5.762e-08 to 1.98e-08); R^2=0.82, n=63. The synthetic progestin, 19-Norethindrone, did not antagonise AR transactivation in HeLa4-11 cells (Figure 5.15), contradicting the reported antagonism of recombinant rat AR expressed in *E. coli*, at 0.12µM (Table 5.2).

Bisphenol A (IC_{50} 1µM) has been demonstated to antagonise Metribolone (R1881) AR transactivation, in CHO cells transiently transfected with human AR (pSVAR0) (Bonefeld-Jørgensen *et al*., 2007). The BPA $LogIC_{50}$ and IC_{50} antagonism of 1fM Testosterone AR activity, of -5.314 M (95% CI: -5.592 to -5.037) and 4.852e-06 M (95% CI: 2.561-06 to 9.19e-06), respectively, presented comparable potency (Figure 5.16). The predominant metabolite of BPA, 2,4-bis(4-hydroxyphenyl)-4-methyl-1-pentene (M-BPA) antagonised Testosterone AR transactivation at lower concentrations; $LogIC_{50}$ -6.044 M (95% CI: -6.269 to -5.819) and IC_{50} 9.034e-07 (95% CI: 5.38e-07 to 1.517e-06). Yoshihara *et al*. (2004) reported higher estrogenicity in BPA metabolites, such as MBPA, the results generated herein suggest this may also apply to AR antagonism.

The AR transactivation in HeLa4-11 cells consequent to 24 hour co-exposure to 1fM Testosterone with Dibutylphthalate (DBP), Diethylhexylphthalate (DEHP) or Butylbenzylphthalate (BBP), are presented in Figure 5.17. The $LogIC_{50}$ and IC_{50} of DBP exposure was, -5.284 M (95% CI: -6.266 to -5.301) and 5.20e-06 M (95% CI: 5.42e-07 to 4.99e-05), respectively (R^2=0.58, n=42). To the author's knowledge, DBP antagonism of *in vitro* AR transactivation has not been reported previously. DEHP concentrations (100pM-100µM) consistently reduced 1fM Tesosterone AR induction (~20%); however, a dose-response relationship was ambiguous and results were not statistically significant. BBP has been demonstrated to antagonise the androgenic response of 25pM R1881, in the AR-CALUX assay ~ IC_{50} 13µM (Krüger *et al*., 2008). Results generated herein, identified BBP $LogIC_{50}$ and IC_{50}'s of -5.301 M (95% CI -6.20 to -4.40) and 4.997e-06 M (95% CI: 6.32e-07 to 3.95e-05), respectively, thereby supporting Krüger *et al*.'s (2008) report.

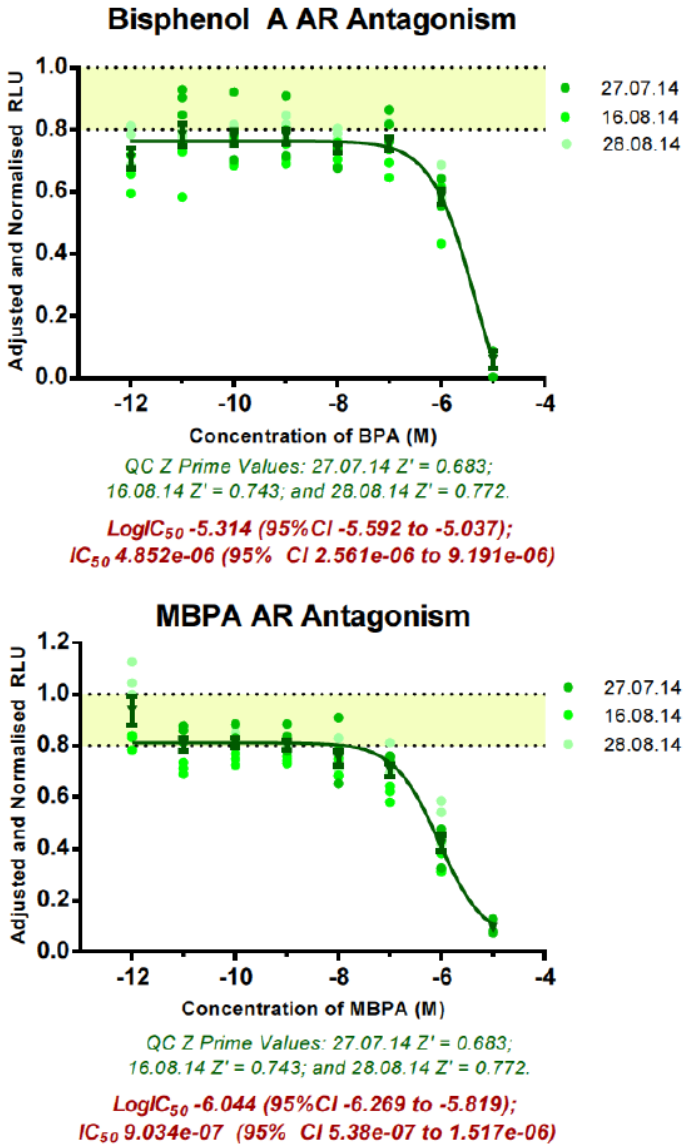


Figure 5.16 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Bisphenols (BPA and M-BPA) for 24 Hours

Graphs, drawn with GraphPad Prism® Log(inhibitor) vs. Response (three parameters), show the adjusted and normalised relative light units (RLU) emitted during the luciferase assay, consequent to coexposure of testosterone (1fM) and either bisphenol A (BPA) or 2,4-bis(4-hydroxyphenyl)-4-methyl-1-pentene (M-BPA). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by 1fM testosterone RLU (maximal agonistic response). Left side graphs summarise day repeat data, presented as the Mean and Standard Error of the mean (SEM), which was used to calculate inhibition ($LogIC_{50}/IC_{50}$) with 95% Confidence Intervals (95% CI). Error shown in the Day Repeat Data (right) is equivalent to the standard deviation, showing the data distribution over the different testing days. Quality assurance data (Z-prime) for each 96-well plate are stated in green. BPA bottom -0.2812 (95% CI -0.5712 to 0.008806) and top 0.7641 (95% CI 0.7425 to 0.7857), R square = 0.8199 (n=63). M-BPA bottom 0.04238 (95% CI -0.0948 to 0.1796) and top 0.8106 (95% CI 0.7835 to 0.8377), R square = 0.8247 (n=63).

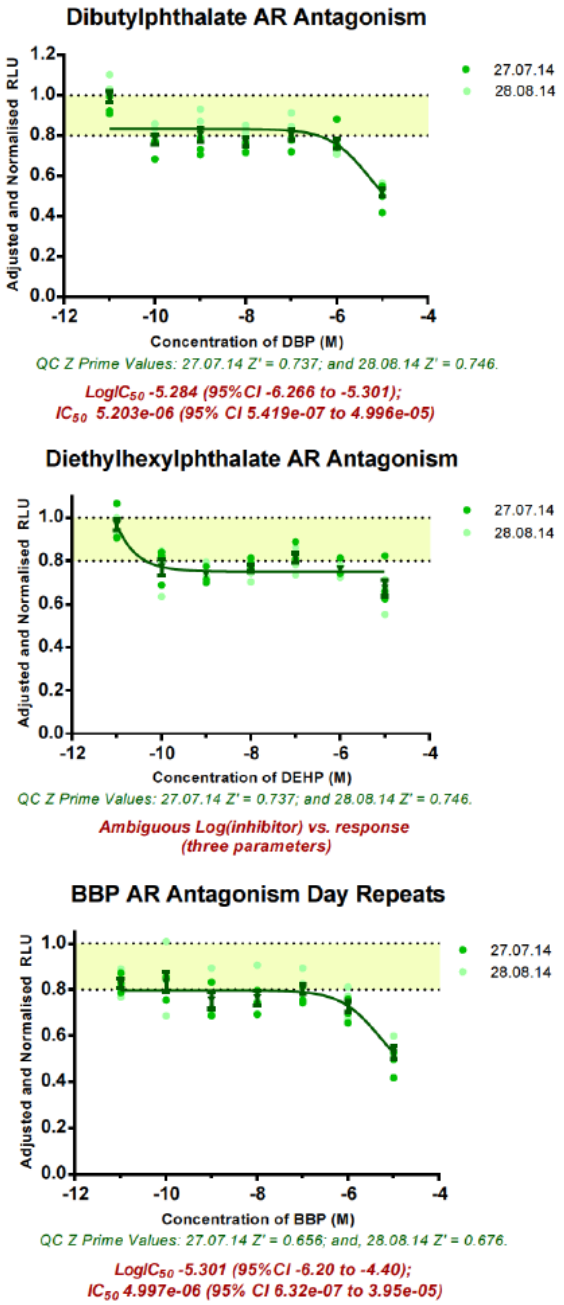


Figure 5.17 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Phthalates (DBP, DEHP and BBP) for 24 Hours

Graphs, drawn with GraphPad Prism® Log(inhibitor) vs. Response (three parameters), show the adjusted and normalised relative light units (RLU) emitted during the luciferase assay, consequent to coexposure of testosterone (1fM) and either Dibutylphthalate (DBP), Diethylhexylphthalate (DEHP) or Butylbenzylphthalate (BBP). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by 1fM testosterone RLU (maximal agonistic response). Left side graphs summarise data, presented as the Mean and Standard Error of the mean (SEM), which was used to calculate inhibition ($LogIC_{50}/IC_{50}$) with 95% Confidence Intervals (95% CI). Error shown in the Day Repeat Data (right) is equivalent to the standard deviation, showing the data distribution over the different testing days. Quality assurance data (Z-prime) for each 96-well plate are stated in green. DBP bottom 0.3554 (95% CI -0.0517 to 0.763) and top 0.8331 (95% CI 0.7968 to 0.870), R square = 0.581 (n=42). DEHP R square = 0.541 (n=42). BBP bottom 0.3924 (95% CI 0.083 to 0.702) and top 0.797 (95% CI 0.768 to 0.826), R square = 0.611.

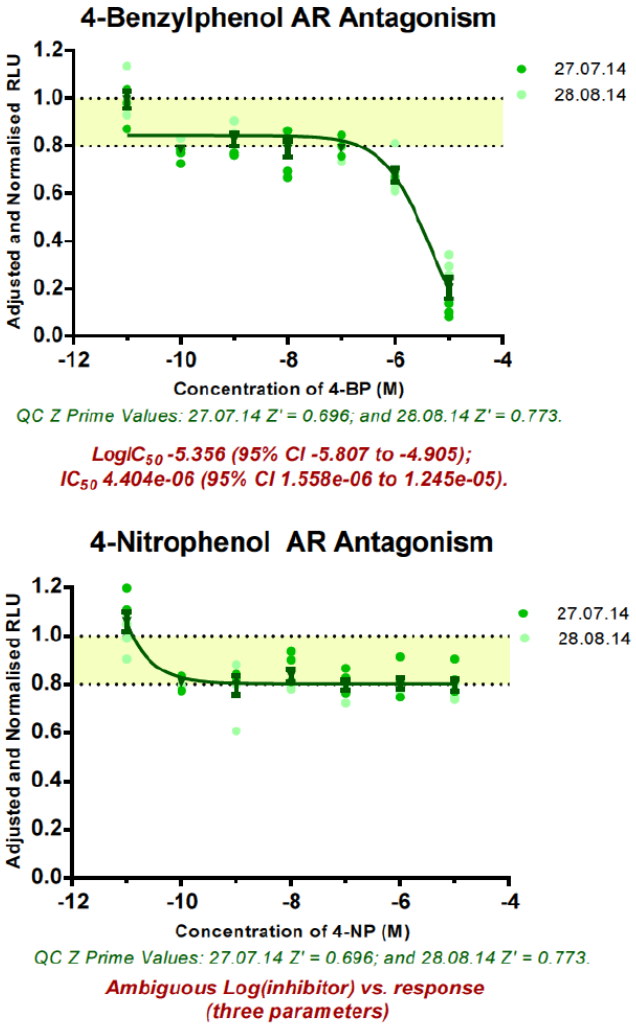


Figure 5.18 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Phenolic Chemicals (4-BP and 4-NP) for 24 Hours

Graphs, drawn with GraphPad Prism® Log(inhibitor) vs. Response (three parameters), show the adjusted and normalised relative light units (RLU) emitted during the luciferase assay, consequent to coexposure of testosterone (1fM) and either 4-Benzylphenol (4-BP) or 4-Nitrophenol (4-NP). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by 1fM testosterone RLU (maximal agonistic response). The Mean and Standard Error of the mean (SEM) was used to calculate inhibition ($LogIC_{50}/IC_{50}$) with 95% Confidence Intervals (95% CI). Error shown in the Day Repeat Data (right) is equivalent to the standard deviation, showing the data distribution over the different testing days. Quality assurance data (Z-prime) for each 96-well plate are stated in green.

Figure 5.18 shows the antagonism of 1fM Testosterone AR transactivation in HeLa4-11 cells exposed to 4-Benzylphenol or 4-Nitrophenol, for 24 hours. The antagonistic response of 4-Nitrophenol was ambiguous, however, 4-Benzylphenol demonstrated a $LogIC_{50}$ and IC_{50} of -5.356 M (95% CI: -5.81 to -4.91) and 4.404e-06 M (95% CI: 1.56e-06 to 1.25e-05), respectively. 4-Nitrophenol has been shown to agonise AR at 0.001µM (see Table 5.3). However, neither agonism nor antagonism of AR transactivation was observed in the HeLa4-11 cell line.

Identified as *in vitro* agonists of AR transactivation in HeLa4-11 cells, in Section 5.3.4, Dichlorodiphenyldichloroethylene (4,4'-DDE), Octylmethoxycinnamate (OMC), Methylparaben (MeP) and Triclosan (TCN) did not antagonise 1fM Testosterone induced AR transactivation (Table 5.12; see Appendix 6.1H.3); all 96-well plates passed quality checks Z' >0.65. Table 5.2 provides a summary of results generated in the HeLa4-11 stably transfected AR transactivation assays, conducted herein. Most results agreed with literature observations, presenting comparable sensitivity to the MDA-k2b cell line. Previously undescribed, Di-n-butylphthalate, 4-Benzylphenol and 2,4-bis(4-hydroxyphenyl)-4-methyl-1-pentene, were identified as androgen receptor antagonists.

Table 5.12 Results of HeLa4-11 Stably Transfected AR Transactivation Assay to Detect Antagonism of Androgenic Signalling

In the absence of standardised *in vitro* assays for AR mechanisms, the table summarises the results of AR transactivation antagonism in the stably transfected HeLa4-11 cell line. N=number of data points, DR=day repeats. >80% refers to the nearest tested concentration exceeding >80% Testosterone induced AR transactivation.

Test Substance (TS)	AR Agonism (95% Confidence Interval)	>80%		Z'Prime
Flutamide	**LogIC$_{50}$ -4.315 M** (95% CI -4.51 to -4.12) **IC$_{50}$ 4.839e-05 M** (95% CI 3.11e-05 to 7.534e-05)	$\leq 10^{-6.5}$ Molar	N=99 DR=3	0.687 (0.61-0.76)
17β-Estradiol	**LogIC$_{50}$ -8.389 M** (95% CI -8.76 to -8.02) **IC$_{50}$ 4.081e-09 M** (95% CI 1.18e-09 to 9.51e-09)	POD 10^{-10} M	N=60 DR=3	0.819 (0.80-0.86)
17α-Ethinylestradiol	**LogIC$_{50}$ -7.215 M** (95% CI -7.40 to -7.03) **IC$_{50}$ 6.091e-08 M** (95% CI 4.01e-08 to 9.25e-08)	POD 10^{-9} M	N=63 DR=3	0.819 (0.80-0.86)
Diethylstilbestrol	**LogIC$_{50}$ -4.993 M** (95% CI -6.43 to -3.56) **IC$_{50}$ 1.016e-08 M** (95% CI 3.71e-07 to 2.78e-04)	POD 10^{-6}M	N=63 DR=3	0.73 (0.68-0.74)
Progesterone	**LogIC$_{50}$ -7.972 M** (95% CI -8.239 to -7.704) **IC$_{50}$ 3.08e-12 M** (95% CI 1.926e-12 to 4.944e-12)	POD 10^{-9} M	N=63 DR=3	0.73 (0.68-0.74)
19-Norethindrone	Negative $10^{-12} - 10^{-6}$ M	Neg	N=63 DR=3	0.725 (0.66-0.78)
4'4-DDE	Negative $10^{-18} - 10^{-7}$ M	Neg	N=63 DR=3	0.731 (0.66-0.78)
4-Benzylphenol	**LogIC$_{50}$ -5.356 M** (95% CI -5.81 to -4.91) **IC$_{50}$ 4.404e-06 M** (95% CI 1.56e-06 to 1.24e-05)	POD 10^{-7} M	N=42 DR=2	0.734 (0.70-0.77)
4-Nitrophenol	Inadequate data $10^{-11} - 10^{-5}$ M	Neg	N=42 DR=2	0.734 (0.70-0.77)
Bisphenol A	**LogIC$_{50}$ -5.314 M** (95% CI -5.59 to -0.504) **IC$_{50}$ 4.852e-06 M** (95% CI 2.56e-06 to 9.19e-06)	POD 10^{-7} M	N=63 DR=3	0.733 (0.68-0.77)
M-BPA	**LogIC$_{50}$ -6.044 M** (95% CI -6.27 to -5.82) **IC$_{50}$ 9.034e-07 M** (95% CI 5.38e-07 to 1.517e-06)	POD 10^{-8} M	N=63 DR=3	0.733 (0.68-0.77)
Dibutylphthalate	**LogIC$_{50}$ -5.284 M** (95% CI -6.27 to -5.30) **IC$_{50}$ 5.203e-06 M** (95% CI 5.42e-07 to 4.99e-05)	POD 10^{-6} M	N=42 DR=2	0.742 (0.74-0.75)
DEHP	Inadequate data $10^{-11} - 10^{-5}$ M	Neg	N=42 DR=2	0.742 (0.74-0.75)
Butylbenzylphthalate	**LogIC$_{50}$ -5.301 M** (95% CI -6.20 to -4.40) **IC$_{50}$ 4.997e-06 M** (95% CI to 6.32e-07 to 3.95e-05)	POD 10^{-7} M	N=42 DR=2	0.666 (0.66-0.68)
Methylparaben	Negative $10^{-13} - 10^{-7}$ M	Neg	N=42 DR=2	0.666 (0.66-0.68)
OMC	Negative $10^{-12} - 10^{-6}$ M	Neg	N=42 DR=2	0.705 (0.68-0.73)
Triclosan	Negative $10^{-12} - 10^{-6}$ M	Neg	N=42 DR=2	0.705 (0.68-0.73)

5.3.6 Transient-Transfection Transactivation Assays (TTTA)

Aiming to explore the current *in vitro* tools for endocrine MoA, the evaluation of transient transfection technologies for novel reporter gene assays was an objective. In line with NIBB (Japan), novel transiently-transfected roach (*Rutilus rutilus*) ERα and ARβ reporter assays were piloted herein, as ecological *in vitro* mechanistic studies relevant to UK sentinel species. The results of $_{roach}$ERα and $_{roach}$ARβ reporter assays are detailed in the following sections.

5.3.6.1 Roach ERa Transactivation Assay

The roach (*R. rutilus*) Estrogen Receptor α (ERα) construct was successfully transfected into the HEK293 cell line, using Fugene HD® transfection reagent (Promega), as demonstrated by the firefly luciferase activity induced by 100 pM of the natural and synthetic hormones, E2 and DES (Figure 5.19). Transfection efficiency was controlled using *Renilla* luciferase activity. Interestingly, E2 appeared slightly more potent than DES, differing from mammalian ER receptors, but in line with the proposed evolution of ERα with the E2 ligand (Thornton, 2001). The anthropogenic chemicals Bisphenol A (>10µM BPA) and 4-Benzylphenol (>10µM 4-BP) induced Roach ERα activity, suggesting a biological mechanism for effects observed in fish *in vivo*. The 50% effective concentration (EC_{50} and $LogEC_{50}$) of the test substances in the Roach ERα Transactivation Assay were: 4.438e-11 E2 (-10.35 log EC50); 5.353e-11 DES (-10.27 log EC50); ~1.329e-6 4-BP (-5.877 $logEC_{50}$); and, 5.797e-7 BPA (-6.237 $LogEC_{50}$).

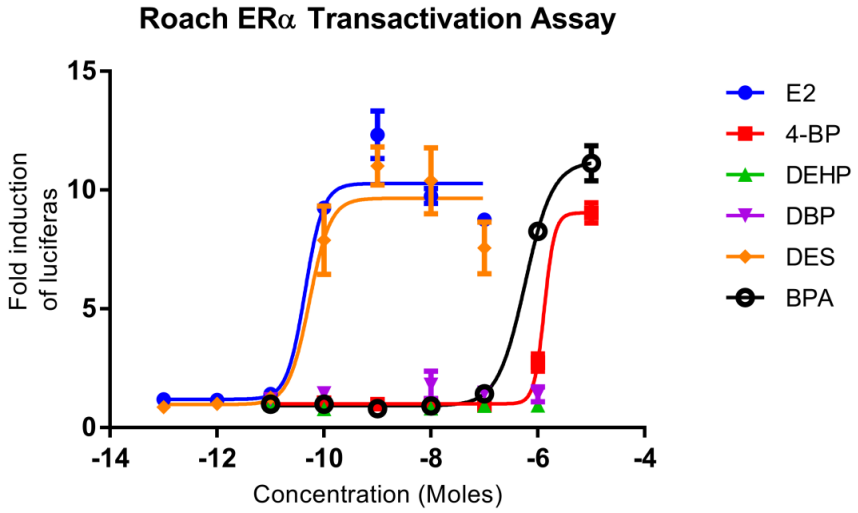


Figure 5.19 Roach Estrogen Receptor alpha (ERα) Transactivation Assay

The Graph shows the fold induction consequent to E2, DES, BPA, DBP, 4-BP and DEHP exposure. Non-linear dose response curves were as variable slope log(agonist) vs. responses (four parameters). Error bars show the Standard Error of the Mean (SEM). As one might expect, the natural and synthetic hormones, E2 and DES, induced activity at the lowest concentrations. Furthermore, <10µM Bisphenol A (BPA) and <10µM 4-Benzylphenol (4-BP) induced Roach ERα activity. Results analysed in GraphPadPrism®.

EC_{50} values were calculated in GraphpadPrism®. It should be noted that Hill slope values ranged between 1.68 and 4.528, indicative of the steep slopes presented in Figure 5.19. The close proximity of the point of departure to maximal response hinders the accuracy of the EC_{50}'s calculated, however, may be considered typical for hormone responses.

5.3.6.2 Roach ARβ Transactivation Assay

The roach (*Rutilus rutilus*) Androgen Receptor β (ARβ) was successfully transfected into the HepG2 cell line, using Fugene HD® transfection reagent (promega), as demonstrated by the firefly luciferase activity induced by 100 pM of the anabolic steroid 17α-Methyltestosterone (17-MT) Figure 5.20. Transfection efficiency was controlled using *Renilla* luciferase activity. None of the other androgenic compounds tested induced activity at the concentrations tested (100nM - 0.1pM). 17-MT has been used in aquaculture to induce sex reversal, skewing the sex ratio towards males, which grow faster and are typically more ornate (Pandian *et al.* 1995). Considered to be more potent than testosterone in fish, the observed activity of 17-MT in this assay is not surprising; 17-MT has been tested *in vivo* in more than 25 species belonging to Salmonidae, Cichlidae, Cyprinidaw, Anbantidae, Poecilidae and Cyprinodontidae (Pandian *et al.*, 1995).

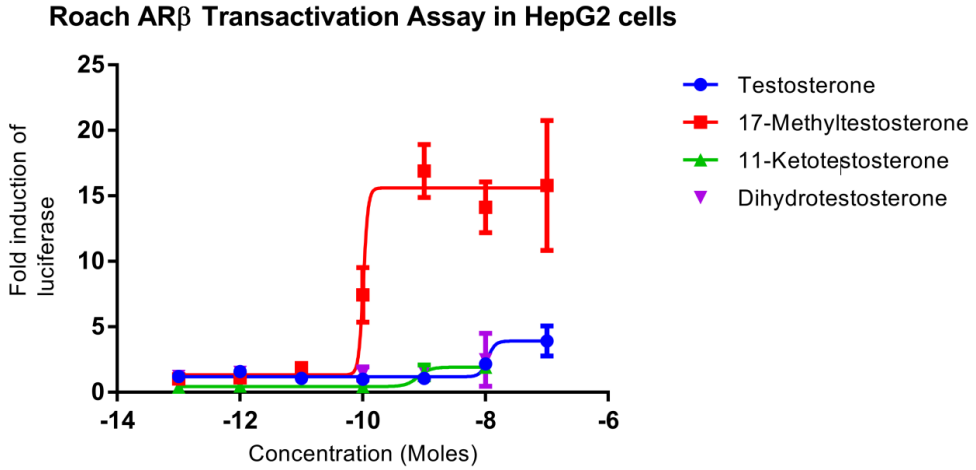


Figure 5.20 Roach Androgen Receptor Beta (ARβ) Transactivation Assay
The non-linear relationship was plotted as a log(agonist) vs. response variable slope (four parameters). Error bars show the Standard Error of the Mean (SEM). The Graph shows the luciferase fold induction consequent to exposure to 100nM – 0.1 pM exposure of testosterone, 17α-Methyltestosterone, 11-ketotestosterone or dihydrotestosterone. Results presented using GraphPadPrism®.

However, it is interesting to note that in human medicine 17-MT is used as a US FDA approved orally active synthetic androgen during menopause, and is considered to have relatively weak affinity to the human androgen receptor (Barrett-Connor *et al.*, 1999). Furthermore, androgen receptor-independent effects in rats have led to confusion regarding the chemicals clinical mode of action (Mor *et al.*, 2001). Collectively, the studies may suggest species variance in the binding affinity between androgenic

compounds and AR. The lack of activity observed for the other compounds, including the endogenous fish androgen, 11-Ketotestosterone (11-KT), may be as a result of inadequate Roach ARβ transfection, or because higher doses (> 100nM) of the compounds are required to induce translational activity in the mammalian assay system.

5.4 *In vitro* Summary and Conclusions

The scope, validation and variety of *in vitro* assays to elucidate endocrine mechanisms, has reflected the state of the science. Consequently, tools to decipher less conventional MoA are limited, while a plethora of assays evaluate ER agonism/antagonism. Validated by the OECD, HeLa9903 and BG1Luc4E2 cell lines were utilised in the ERα and ERα/β transactivation assays conducted herein. The concepts and approaches detailed in OECD TG455 were adhered to where possible. However, the exposure and attachment times were increased, subsequent to preliminary testing for laboratory performance and optimisation of E2 exposure in the HeLa9903 cell line.

- ✓ **The *in vitro* ER transactivation in HeLa9903 cells exposed to semi-log concentrations of E2 (1uM-0.1pM) demonstrated STTA proficiency defined by the US EDSP OPPTS 890 1300 and OECD TG455.**

The response of HeLa9903 ER transactivation in response to semi-log concentrations of E2, presented a $LogEC_{50}$ of -10.2 M with a Hill slope value of 1.07 herein, adhered to the proficiency standards of: $LogEC_{50}$ between -11.3-10.1 M and a Hill slope value of 0.7-1.5 (ICCVAM, 2011). The increase in exposure time led to increased sensitivity in detecting EE2 (EC_{50} $5.33x10^{-15}$ M vs $<1x10^{-11}$ M), DES (EC_{50} $1.46x10^{-12}$ vs $2.4x10^{-11}$ M), BPA (EC_{50} $7.44x10^{-11}$ vs $5.33x10^{-7}$), BBP (EC_{50} $7.75x10^{-8}$ vs $4.11x10^{-6}$), DDE (EC_{50} $4.35x10^{-12}$ vs $3x10^{-6}$ in the BG1Luc ER TA) and Testosterone (EC_{50} $6.87x10^{-12}$ vs $9.78x10^{-6}$). There is limited data pertaining to the agonistic response of 4-BP, 4-NP, M-BPA, Methylparaben, OMC and Triclosan in the HeLa9903 cell line. However, the dose-response relationships observed for 4-BP (Figure_Apx 15) and MBPA (Figure 5.5) support the ER activity reported in the literature (Table 5.3; Yoshihara *et al*., 2004).

- ✓ **The results generated herein, suggest that increasing the *in vitro* chemical exposure time, increases the sensitivity of HeLa9903 stably transfected ER transactivation assay.**

The BG1Luc ER TA was demonstrated to be an insensitive *in vitro* assay, under the laboratory conditions and equipment specifications available herein (Section 5.2); 24 hour E2 or EE2 exposure did not induce any ER transactivation, increasing exposure (48 hours) led to an induction of ER transactivation, at 10^{-8} M E2 and 10^{-9} M EE2 (Figure 5.7). Thus, the transactivation responses reported herein were lower than the TG455 reference standards; E2 EC_{50} $5.63x10^{-12}$ M and EE2 EC_{50} $4.20x10^{-8}$ M (ICCVAM, 2011).

Validation studies by contract laboratories identified significant variability in BG1Luc ER TA response; E2 induced transactivation in 24 of 35 assays. ICCVAM (2011) notes that DMSO vehicle controls vary considerably between test plates; ranging from 511 to 9885 RLU (mean 3749). Interestingly, the maximum E2 response reported herein was comparable to that reported in BG1Luc validation studies (10,000 RLU); thus, the shortfall of the assay may be due to background luminescence.

Received in a mutual transfer agreement, the BG1Luc3E2 cells utilised herein were not authenticated, and estrogen insensitivity may have been a result of morphological changes, inconsistency in media supplementation and/or FBS batch. However, collating the validation studies, literature (Section 3) and *in vitro* assay results, the author suggests that the ubiquitous variability in BG1Luc4E2 ER TA responses may reside in ER feedback mechanisms; Metivier *et al*. (2003) demonstrated dominant inhibition of ERα transactivation by ERβ. Endogenously expressing both ERs, signal transduction in the BG1Luc ERα/β transactivation assay reflects *in vivo* co-expression. However, ERα and ERβ cross-talk may increase the variability of transactivation; highlighting the intricate balance between resembling *in vivo* cellular environments and standardisation of *in vitro* assays.

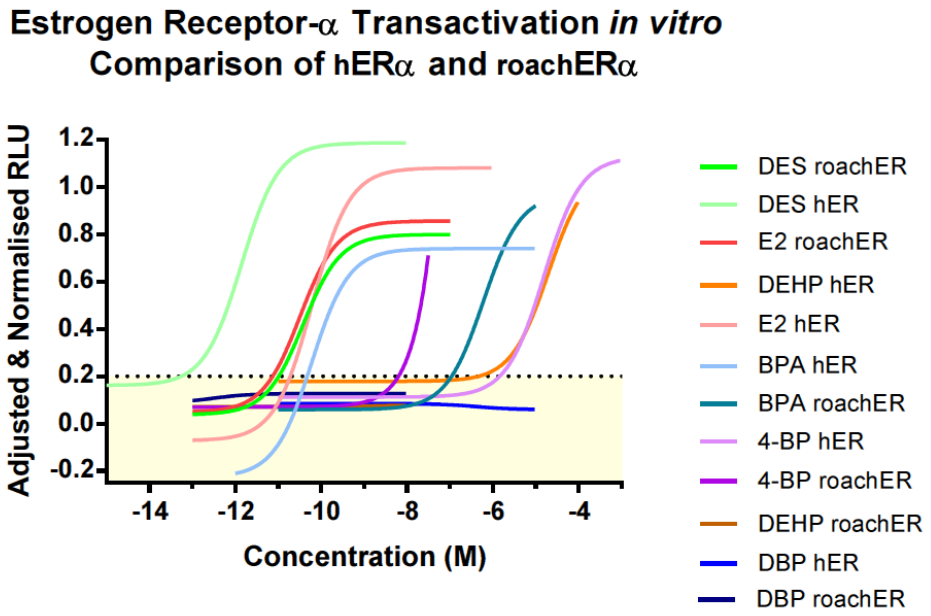


Figure 5.21 Agonistic ER Transactivtion Concentration-Response in the HeLa9903 Stably Transfected Cell Line and Hek293 Cells Transiently Transfected with *Rutilus rutilus* (common roach) ERα

Graphs show the adjusted and normalised relative light units (RLU) emitted by HeLa9903 cells and HEK293 cells in luciferase assays, consequent 40-48hr exposure to DES, E2, DEHP, BPA, 4-BP or DBP. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM E2 (maximal agonistic response). The dotted line (y=0.2) highlights 20% 1nM E2 normalised RLU, as a threshold for categorising positive, negative and/or inadequate data.Data presented is the mean and standard error of the mean (SEM) of data repeats, analysed with GraphPad Prism® Log(agonist) vs. response (three parameters).

A number of *in vitro* studies have shown that EDC binding affinity is species specific (Lang *et al*., 2012; Wilson *et al.*, 2007). Aiming to explore the current *in vitro* tools for interspecies endocrine MoA, transient transfection of novel NR for transactivation assays, was evaluated. Common roach (*Rutilus rutilus*) ERα and ARβ reporter assays were piloted herein, as mechanistic studies relevant to model ecotoxicity species indigenous to the UK.

- ✓ **The roach (*R. rutillus*) Estrogen Receptor α (ERα) construct was successfully transfected and expressed in HEK293 cells.**
- ✓ **Response of transiently transfected HEK293 cells in response to E2, DES, 4-Benzylphenol and BPA, was successfully measured in dual luciferase reporter assays.**

Figure 5.21 presents HeLa9903 $_{h}$ERα STTA assay and $_{roach}$ERα transactivation assay results; highlighting variability in the *in vitro* potency of tested EDC's. In $_{roach}$ERα transactivation assays E2 (EC_{50} 44.4pM) was more potent than DES (EC_{50} 53.5pM), which differed from human ER (E2 EC_{50} 63.7pM vs DES EC_{50} 1.46pM). The anthropogenic chemicals Bisphenol A and 4-Benzylphenol induced Roach ERα transactivation, providing a biological mechanism for *in vivo* apical endpoints observed in fish. The EC_{50} and $LogEC_{50}$ of $_{roach}$ERα transactivation in response to BPA exposure were 0.58μM and -6.24 M, respectively. The $_{h}$ERα in HeLa9903 cell transactivation assays demonstrated higher BPA sensitivity; equivalent exposure presented EC_{50} 74.4pM and $LogEC_{50}$ -10.13 M (Table 5.13) 4-BP exposure in HEK293 cells transiently transfected with $_{roach}$ERα, agonised reporter gene production, presenting EC_{50} and $LogEC_{50}$ values of 1.33μM and -5.88 M, respectively. In HeLa9903 cells, the EC_{50} and $LogEC_{50}$ were 13.43μM and -4.87, respectively; suggesting the common roach is more susceptible to 4-BP agonism of ERα transcativation.

- ✓ **Clear differences in the agonistic responses consequent to 48 hour test substances exposure, in HEK293 $_{roach}$ERα TA and HeLa9903 ST $_{h}$ERα TA, were demontrated**.

The *R. rutillus* Androgen Receptor β (ARβ) was successfully transfected into the HepG2 cell line, as demonstrated by the firefly luciferase activity induced by 100 pM 17α-Methyltestosterone (17-MT) (Figure 5.20). However, none of the other androgens induced AR transactivation at the tested concentrations (100nM-0.1pM), limiting comparisons with HeLa4-11 ST AR TA results. 17-MT has been used in aquaculture to induce sex reversal, skewing the sex ratio towards males (Pandian *et al.* 1995). Demonstrated to be more potent than testosterone in fish, the observed activity of 17-MT in this assay is not surprising (Pandian *et al*., 1995).

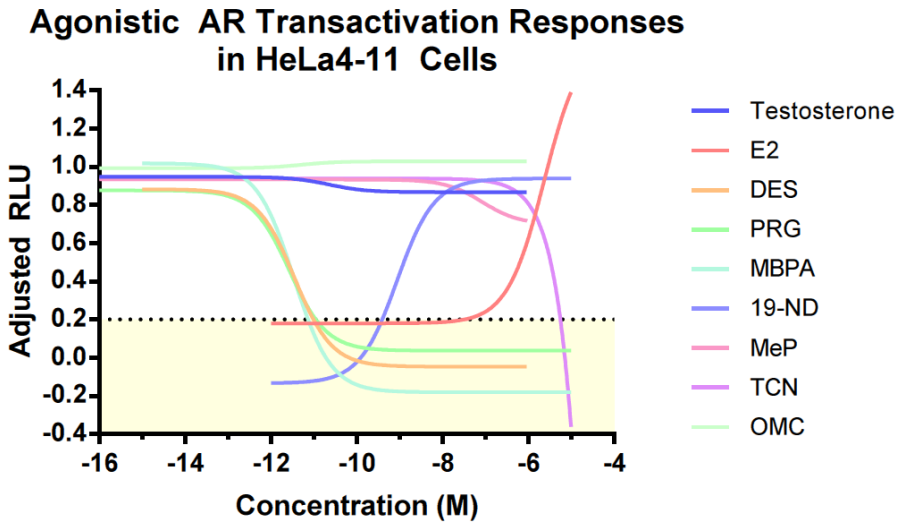


Figure 5.22 Agonistic AR Transactivation Concentration-Response in HeLa4-11 Cells Consequent to 24 Hour Chemical Exposure

Lines of best fit of mean (±SEM) data were drawn using GraphPad Prism® Log(agonist) vs. Response (three parameters). Graphs show the adjusted and normalised relative light units (RLU) emitted consequent to Testosterone, E2, DES, PRG, MBPA, 19-ND, MeP, TCN or OMC exposure. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM testosterone (maximal agonistic response). The dotted line (y=0.2) highlights 20% positive control (1nM testosterone ~ maximal response) luciferate induction, as a threshold for categorising positive, negative and/or inadequate data.

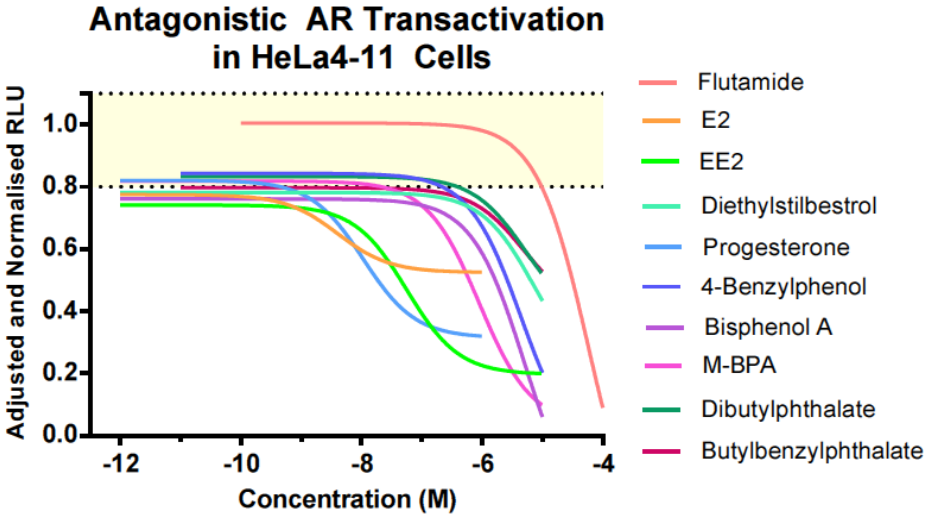


Figure 5.23 Antagonistic AR Transactivation Concentration Responses in HeLa4-11 Cells Consequent to 24 Hour Chemical Exposure

Drawn with GraphPad Prism® Log(inhibitor) vs. Response (three parameters), the graph show the adjusted and normalised relative light units (RLU) emitted by lysed HeLa4-11 cells during luciferase assays, consequent to co-exposure of testosterone (1fM) with 1:10 log dilutions (100µM to 1pM) of either Flutamide, E2, EE2, Diethylstilbestrol, Progesterone, 4-Benzylphenol, Bisphenol A, M-BPA, Dibutylphthalate or Butylbenzylphthalate. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by 1fM testosterone RLU (maximal agonistic response).

Conversely, 17-MT is US FDA approved for use as an orally active weak synthetic androgen during menopause; presenting relatively weak affinity to the human androgen receptor (Barrett-Connor *et al.*, 1999). Furthermore, androgen receptor-independent effects observed in rats, has led to confusion regarding the pharmacological mode of action of 17-MT's (Mor *et al*., 2001).

The lack of AR transactivation, observed for Testosterone, 11-Ketotestosterone (11-KT) and Dihydrotestosterone, may be as a result of inadequate $_{roach}$ARβ transfection, assay insensitivity (higher dose requirements (> 100nM)), or the the mammalian HepG2 cell line may not be a suitable *in vitro* model, to predict *R. rutilus* AR transactivation. Interestingly, pilot reporter gene studies of $_{roach}$ARα transiently transfected into HEK293 cells, identified agonism of AR transactivation, at all tested concentrations of Testosterone (6.1Appendix G).

Developed to detect androgenic activity, HeLa4-11 cells are stably transfected with a hAR expression construct and a firefly luciferase reporter gene under the control of the MMTV promoter.

- ✓ **HeLa4-11 cells stably transfected AR transactivation assays successfully evaluated the *in vitro* androgenicity of potential EDCs.**

In agreement with concepts detailed in OECD TG455, cells were suspended in 10% DCC-FBS-EMEM and plated at 1 x 10^4 cells/100µL per 96-well and allowed to attach for 3 hours, prior to the addition of 100 µL of 10% DCC-FBS-EMEM and 0.2µL of test substances in 100% DMSO. Figure 5.22 summarises the *in vitro* concentration-response results of AR transactivation, in HeLa4-11 cells plated in 96-wells, consequent to 24 hour exposure. Negative responses are detailed in Table 5.13.

However, agonism of AR transactivation in HeLa4-11 cells presented a number of irregularities. In 24-well plates, Testosterone exposures presented LogEC_{50} and EC_{50} of -9.90 M and 0.126nM, respectively (R^2=0.9599, n=58), presenting comparable sensitivity to the MDA-kb2 cell line (Orton *et al*., 2014) and *S. cerevisiae* yeast androgen bioassay (Michelini *et al*., 2005). However, all concentrations of Testosterone ($1x10^{-6}$ M to $1x10^{-24}$ M) induced maximal AR transactivation in 96-well plates (4/4 day repeats). Avogadro's constant (N_A) $6.022x10^{23}$ approximates the number of constituent molecules in a mole (Mohr *et al*., 2008), thus, in light of the dilutions, the assay appears to induce maximal AR transactivation in response to trace concentrations of Testosterone. The mean 96-well plate (n=32) Z'prime value was 0.5, emphasising the statistical significance of responses generated in HeLa4-11 AR transactivation assays. Nevertheless, the marked differences observed between plate formats are disconcerting.

- ✓ **The sensitivity of AR responses reported herein leads the author to suggest modification of AR transcriptional machinery in the HeLa4-11 cell line, via cofactor expression, AR polymorphisms, modifications in the LBD of cross-talk between signalling pathways.**

Table 5.13 Summary of Stably Transfected Transactivation Assay Results for ER Agonism (HeLa9903) and AR Agonism and AR Antagonism (HeLa4-11)

Table shows the results generated herein, in addition to OECD/ICCVAM ER STTA values. The HeLa9903 assay sensitively identified ER agonists; reporting agonism at lower concentrations of BPA, BBP, 4'4-DDE, Testosterone. In the absence of standardised *in vitro* assays for AR mechanisms, AR agonism and antagonism has been demonstrated. Results highlighted green presented atypical dose-response relationships – inactive at high concentrations, but inducing luciferase production at low doses (i.e. reverse curve).

Test Substance (TS)	HeLa9903 ER Agonism	OECD/ICCVAM ER STTA Values*	HeLa4-11 AR Agonism	HeLa4-11 AR Antagonism
17β-Estradiol	$LogEC_{50}$ -10.20 M EC_{50} 6.377e-11 M	PC_{50} <1.00 x10^{-11} M	$LogEC_{50}$ -4.945 M EC_{50} 1.134e-05 M	$LogIC_{50}$ -8.398 M IC_{50} 4.081e-09 M
17α-Ethinylestradiol	$LogEC_{50}$ -14.27 M EC_{50} 5.329e-15 M	PC_{50} <1.00 x10^{-11} M	Negative 10^{-13} – 10^{-5} M	$LogIC_{50}$ -7.215 M IC_{50} 6.091e-08 M
Diethylstilbestrol	$LogEC_{50}$ -11.83 M EC_{50} 1.464e-12 M	PC_{50} <1.00 x10^{-11} M	$LogEC_{50}$ -11.49 M EC_{50} 3.27e-012 M	$LogIC_{50}$ -4.993 M IC_{50} 1.016e-05 M
Progesterone	-	Negative	$LogEC_{50}$ -11.51 M EC_{50} 3.08e-12 M	$LogIC_{50}$ -7.972 M IC_{50} 1.067e-08 M
19-Norethindrone	-	-	$LogEC_{50}$ -9.12 M EC_{50} 7.84e-10 M	Negative 10^{-12} – 10^{-6} M
4'4-DDE	$LogEC_{50}$ -11.36 M EC_{50} 4.349e-12 M	Mean EC_{50} 3.00 x 10^{-6} M	96WP Positive 10^{-18}-10^{-6} M	Negative 10^{-18} – 10^{-7} M
4-Benzylphenol	$LogEC_{50}$ -4.87 M EC_{50} 1.343e-05 M	-	Negative 10^{-10} – 10^{-3} M	$LogIC_{50}$ -5.356 M IC_{50} 4.04e-06 M
4-Nitrophenol	Negative 10^{-11} – 10^{-5} M	-	Negative 10^{-10} – 10^{-3} M	Negative 10^{-11} – 10^{-5} M
Bisphenol A	$LogEC_{50}$ -10.13 M EC_{50} 7.44e-11 M	PC_{50} 2.02 x 10^{-8} M	Negative 10^{-10} – 10^{-3} M	$LogIC_{50}$ -5.314 M IC_{50} 4.852e-06 M
M-BPA	$LogEC_{50}$ -10.04 M EC_{50} 9.068e-11 M	-	$LogEC_{50}$ -11.53 M EC_{50} 2.965e-12 M	$LogIC_{50}$ -6.044 M IC_{50} 9.034e-07 M
Dibutylphthalate	Inadequate data 10^{-11} – 10^{-5} M	PC_{50} 4.09 x 10^{-6} M	Negative 10^{-10} – 10^{-3} M	$LogIC_{50}$ -5.284 M IC_{50} 5.203-06 M
Diethylhexylphthalate	$LogEC_{50}$ -4.71 M EC_{50} 1.958e-05 M	-	Negative 10^{-10} – 10^{-3} M	Inadequate data 10^{-11} – 10^{-5} M
Butylbenzylphthalate	$LogEC_{50}$ -7.11 M EC_{50} 7.75e-08 M	PC_{50} 1.14 x 10^{-6} M	Negative 10^{-10} – 10^{-3} M	$LogIC_{50}$ -5.301 M IC_{50} 4.997e-06 M
Methylparaben	Negative 10^{-11} – 10^{-5} M	-	96WP Positive 10^{-18} – 10^{-6} M	Negative 10^{-13} – 10^{-7} M
OMC	Inadequate data 10^{-11} – 10^{-5} M	-	96WP Positive 10^{-18} – 10^{-4} M	Negative 10^{-12} – 10^{-6} M
Triclosan	Negative 10^{-11} – 10^{-5} M	-	96WP Positive 10^{-18} – 10^{-6} M	Negative 10^{-12} – 10^{-6} M
Dihydrotestosterone	-	Mean EC_{50} 2.50 x 10^{-7} M	96WP Positive 10^{-20} - 10^{-7} M	-
Metribolone	-	-	96WP Positive 10^{-20} - 10^{-7} M	-
Testosterone	$LogEC_{50}$ -11.16 M EC_{50} 6.871e-12 M	PC_{50} 2.82 x 10^{-8} M	96WP Positive 10^{-23} – 10^{-6} M	-
Corticosterone	Negative 10^{-11} – 10^{-5} M	Negative	96WP Positive 10^{-20} - 10^{-6} M	-
Flutamide	-	Negative	-	$LogIC_{50}$ -4.31 M IC_{50} 4.839e-05 M

* Results published in ICCVAM's (2011) ER TA validation study and OECD TG455

The sensitivity of *in vitro* AR transactivation has been shown to vary with cell concentration (Térouanne *et al*., 2000). Cofactors regulate the transactivation of NR, Gelsolin, ARA55 and ARA70 have been demonstrated to enhance AR transactivation, in response to residual androgen concentration (Nishimura *et al*., 2003; Fujimoto *et al*., 1999; Yeh *et al*., 1999a). Furthermore, Yeh *et al*. (1999b) suggested that cross-talk between AR and HER-2/neu pathways stimulated by growth factors, may activate AR.

It is suggested that HeLa4-11 cells express elevated cofactor concentrations and/or elicit inter-cellular signalling, which amplify the response to trace testosterone concentrations, when plated in higher concentrations. However, increased sensitivity may also be consequent to modification in the AR itself. 4,4'-DDE has been demonstrated to inhibit R1881 AR signalling *in vitro*, while the antagonistic properties of 4,4-DDE *in utero*, have been associated with cryptorchidism incidence (Montes *et al.*, 2010). Conversely, 4,4'-DDE ($1x10^{-6}$ M to $1x10^{-18}$ M) induced maximal agonistic response in 96-well HeLa4-11 assays. However, Björk *et al*. (2011) identified alterated 4,4'-DDE AR agonism and antagonism of transactivation *in vitro*, relative to androgen receptor CAG lengths, which have also been implicated in the aetiology of Testicular Germ Cell Cancer (Västermark *et al.*, 2011; see Section 2.2). It may thereore be suggested that HeLa4-11 cells express a more sensitive AR polymorphism.

- ✓ **Biphasic responses, reflecting low-dose stimulation and high-dose inhibition, were identified for DES (EC_{50} 3.27pM), PRG (EC_{50} 3.03pM) and MBPA (EC_{50} 2.97pM).**

Further complicating the *in vitro* HeLa4-11 AR transactivation assay results, biphasic responses were identified. A contentious theory in endocrine disruption science, in light of the atypical sensitivity of the *in vitro* assay, believed to be resultant of modifications in AR transacriptional machinery, the low-dose stimulation observed herein, should be taken with caution. Modulation of *in vitro* AR transactivation may not resemble the scenario *in vivo*, or the response of other *in vitro* cell lines. Nevertheless, the data presented herein strongly suggest that BPA, 4-BP, 4-NP, DBP, DEHP and BBP do not agonise the androgen receptor, in HeLa4-11 cells. Slight luciferase induction was observed at 10μM EE2; however, this coincided with cytotoxicity, and is more likely associated to cellular breakdown (see Figure 5.1).

Results of the HeLa4-11 AR antagonism assays identified in this study, which modified the *in vitro* HeLa4-11 AR transactivation assays, to detect AR signalling antagonists, demonstrated both agonistic and antagonistic modes of action, depending on the concentration and presence of endogenous hormone. The endogenous and synthetic hormones inhibited 1fM Testosterone induced AR transactivation, with greater potency than Flutamide (Figure 5.14); $LogIC_{50}$ and IC_{50} of EE2 were, -8.389 M and 4.08nM, respectively Highlighting, the potential for feedback mechanisms, receptor cross-talk and diversity in cellular response. Tox21 qHTS assays identified E2 AR antagonism at 0.06μM (Huang *et al*., 2014; see Table 5.1). The HeLa4-11 AR transactivation assay for

antagonistic responses detailed herein, demonstrated higher sensitivity for E2 MoA; IC_{50} 4.08nM E2 + 1fM Testosterone.

Table 5.12 provides a summary of STTA results for ER agonism (HeLa9903), AR agonism and AR antagonism (HeLa4-11). Corroborating Yoshihara *et al.*'s (2004) hypothesis, 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBPA) induced transactivation at either equivalent or lower concentrations, relative to the BPA parent compound. In support of the literature, the dual-activity of endogenous hormones, eliciting both agonism and antagonism of NR transactivation, presents potentially significant challenges to EDC hazard characterisation. It may be suggested that while binding and transactivation of NR play vital roles in estrogenicity and androgenicity, the neglect of the supporting regulatory mechanisms has left vast uncertainties regarding the reliability and applicability of *in vitro* results.

The *in vitro* results aimed to provide mechanistic and potency information for EDC hazard characterisation. The stably transfected HeLa9903 and HeLa4-11 cell lines successfully identified estrogenicity and androgenicity, respectively. However, the results simultaneously haze the reliability of *in vitro* responses. In particular, the predicted modulation of HeLa4-11 transactivation highlights an array of biological responses, variability of which which may render predictions of agonism or antagonism futile. Thus, the standardised tools generated standardised responses, but without an understanding of the relevance of these mechanisms to the *in vivo* situation, potency predictions are limited.

5.4.1 *In vitro* Summary and Milestones

- ✓ The HeLa9903 Stably Transfected ER Transactivation assay in this study demonstrated OECD TG455 proficiency in predicting E2 responses;
- ✓ Increasing chemical exposure time to 40 hours significantly increased HeLa9903 cell ERα transactivation sensitivity;
- ✓ HeLa4-11 cells stably transfected AR transactivation assays successfully evaluated the *in vitro* androgenicity of potential EDCs;
- ✓ The sensitivity of AR responses reported herein suggests modification of HeLa4-11 AR transcriptional machinery;
- ✓ Modulated transactivation has been reported in the literature consequent to cofactor expression, AR polymorphisms, modifications in ligand binding domain and cross-talk between signalling pathways;
- ✓ The predominant metabolite of BPA, 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene, demonstrated equivalent or greater potency that the parent compound, in all HeLa ERα and AR transactivation assays;
- ✓ Biphasic responses, reflecting low-dose stimulation and high-dose inhibition, were identified for Diethylstilbestrol, Progesterone and 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBPA); and

- ✓ Chemicals presenting dual agonism/antagonism of NR transactivation present significant challenges to EDC hazard characterisation.

5.4.2 Further Work to Aid Development of *in vitro* Methods for EDC Hazard Characterisation

Genetic polymorphisms, NR cross-talk, co-factors and hormone-response-elements, play a significant role in the classic genomic pathway (Section 3.1). These biological foundations have been demonstrated to vary between individuals, tissues and cells (Västermark *et al*., 2011; Yeh *et al*., 1999a, 1999b; Nishimura *et al*., 2003; Fijimoto *et al*., 1999; Björk *et al*., 2011), and provide a mechanism for variable transactivation, reported in the literature, and observed herein. Further investigation into cellular machinery and signalling pathways, may aid evaluation into the relevance of *in vitro* NR transactivation, from a chemical safety perspective, i.e. evaluate the probability of a possibility.

The *in vitro* HeLa9903, BG1Luc4E2 and HeLa4-11 cells are starved of hormones prior to STTA; to increase the sensitivity of transcriptional response and reduce background estrogenicity/androgenicity. Subsequently, the cells may be considered 'primed' to detect positive responses, which may be improbable *in vivo*. However, amplification and/or inhibition of *in vitro* NR transactivation, in response to mixtures of endogenous hormones remains neglected. The dual-activity of endogenous hormones and xenobiotics, in eliciting agonism and antagonism of NR, presents significant challenges to future work. Evaluating mixtures of the chemicals tested herein could provide invaluable information regarding NR mechanisms and ligand promiscuity. E2, DES, PRG and M-BPA demonstrated ER agonism in HeLa9903 cells, in addition to agonism and antagonism of AR transactivation in HeLa4-11 cells; thus, the mechanistic response appears to vary with concentration, mixture and cell line model.

The increased sensitivity of the HeLa4-11 cell STTA suggests modulated coregulator expression, amplifying the response to trace testosterone concentrations. Alternatively, AR polymorphisms, such as those reported by Björk *et al*. (2011), may increase the sensitivity of AR. Functional loss of the Thr877 residue, identified the hAR *in silico* and associated to prostate cancer aetiology, may also confer increased ligand promiscuity *in vitro* (Sack *et al.*, 2001; Taplin *et al*., 1995). However, the specific modifications of the HeLa4-11 cell remain spectulative, due to patent restrictions (US7537904B1 Ooe & Matsunaga, 2009[54]). A detailed exploration into the mechanism of HeLa4-11 AR transactivation is required, to understand the results generated herein. In particular, further *in vitro* testing of the biphasic responses, observed in HeLa4-11 cells exposed to DES, PRG and M-BPA, at lower concentrations and in different plate formats, may

[54] Cell for measuring the ability to control the activity of a ligand-responsive transcription control factor

provide more conclusive evidence, to either support or deny the low-dose simulation and high-dose inhibition demonstrated herein.

Consequent to the elevated potency of BPA's predominant metabolite, 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene, investigation of metabolites generated in liver S9 fractions for other test substances, may provide useful information regarding the 'true' hazard of a chemical and inform risk assessment. The adoption of gene array technology, to map the fold-induction of gene expression, consequent to chemical exposure, may provide the mechanistic information required to understand the cellular responses observed herein.

6 GENERAL DISCUSSION AND CONCLUSIONS

From the birds to the bees, a plethora of developmental, reproductive and behavioural perturbations have been reported in wildlife exposed to anthropogenic chemicals. The reporting of endocrine disruption throughout phylogenetic classes, in addition to the homology of biological machinery, has mounted concern regarding the role of EDC exposure in the aetiology of human disease. In the EU, health costs associated with EDC exposure has been estimated at €31 billion per annum (HEAL, 2014). However, reflecting a mechanism rather than a hazard in itself, there are complexities in defining endocrine disruption.

Nuclear receptor binding interactions are the current focus of *in vitro* and *in silico* tools used to predict EDC mechanisms. Computational chemistry has been suggested as a surrogate to binding assays, by replicate the dynamics, energetics and structure of ligand-receptor interactions *in silico* (Taft *et al.*, 2007). An established limitation of *in vivo* toxicology is the inability to elucidate MoA from traditional apical endpoints, which may be the result of an array of biological interactions. Thus, there is theoretical plausibility to the hypothesis tested herein, that *in silico* and *in vitro* mechanistic tools are more adept to elucidate endocrine MoA. Aiming to evaluate current *in silico* and *in vitro* tools for EDC screening and hazard characterisation, the literature identified the omnipresence of chemical exposure, in addition to an array of affiliated adverse health outcomes. However, more importantly the literature defined the current understanding of endocrinology and endocrine disruption, which found and support the inconsistencies of in *silico* and *in vitro* results.

Curated on the basis of regulatory concern, exposure, structural variability and assumed functionality, the chemical database (n=378) was successfully docked to nuclear receptor pseudo-molecular targets (protomols). Docking targets were identified using the solvation method. The solvation method accurately predicted the amino acid residues and molecular probes responsible for hERα and hAR binding (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998; Sack *et al.*, 2001). SYBYL Surflex-Dock ER, AR, PR & PPARγ virtual screening demonstrated a sensitivity of 94.5%, relative to *in vitro* bioassay, and surpassing the predictability of the OECD Toolbox ER profiler, DfW and binary classification models.

The study demonstrated important *in silico* developments in the computational prediction of EDC hazards, since the initial application of SYBYL macromolecular modelling to EDCs by Wu *et al.* (2010). However, further investigation of interspecies ligand binding *in vitro* (Lange *et al.*, 2012; Wilson *et al.*, 2007; Wells & Van Der Kraak, 2000), is required to validate the sensitivity of molecular model predictions of

interspecies variation. The novel Common Roach reporter assay, developed to characterise mechanisms of ecological endocrine activity, has been identified as an appropriate method to elucidate species differences in NR. The roach, *Rutilus rutilus* ERα and ARβ constructs were successfully transiently transfected into HEK293 cells and measured, in response to E2, DES, 4-Benzylphenol and BPA, in a dual luciferase transactivation assays. The assay identified interspecies differences in transactivation consequent to ligand binding, in agreement with the differences reported in other sentinel fish species (Lange *et al.*, 2012).

With the aim of evaluating currently available *in vitro* tools for endocrine MoA, the literature was reviewed and potential-EDCs were successfully assessed in stably transfected ERα and AR transactivation assays (HeLa9903 and HeLa4-11 STTA, respectively) *in vitro* assays. The scope, validation and variety of *in vitro* assays reflected the state of the science, which limited the breadth of MoA and species investigated in STTA. However, the aforementioned Common Roach reporter assay successfully explored transient transfection technologies for reporter gene assays, to elucidate novel receptor endocrine activity.

Consequent to increased exposure time, the HeLa9903 ER transactivation assay demonstrated the 17β-Estradiol STTA proficiency defined by the US EDSP OPPTS 890 1300 and OECD TG455; $LogEC_{50}$ -10.2 (Hill slope 1.07). Optimised to the E2 control, the results generated herein, suggest that the 16 hour increase in exposure increased the sensitivity of HeLa9903 cells to the estrogenicity of EE2, DES and BPA. The predominant metabolite of BPA, 4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBPA), demonstrated comparable estrogen transactivation to the parent compound. However, MBPA was shown to agonise androgen receptor transactivation in HeLa4-11 cells, while comparable concentrations BPA did not, supporting the theory that BPA metabolites may have more toxic potential (Yoshihara *et al*., 2004).

Developed to detect androgenic activity, HeLa4-11 cells are stably transfected with a hAR expression construct and a firefly luciferase reporter gene under the control of the MMTV promoter. HeLa4-11 cell transactivation assays successfully evaluated the *in vitro* androgenicity of potential EDCs. However, the sensitivity of the AR responses reported herein suggests modification of AR transcriptional machinery, potentially via cofactor expression, AR polymorphisms, modifications in the LBD or cross-talk between signalling pathways.

Mechanisms of potential low dose toxicity (hormesis), sexual dimorphism, transgenerational effects and variable endpoints, dependent on the time and duration of exposure, have been reported *in vivo*. Non-monotonic or 'biphasic' dose-responses remain a contentious issue in endocrine disruptor science. Biphasic responses, reflecting low-dose stimulation and high-dose inhibition, were identified for Diethylstilbestrol, Progesterone and 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBPA). To the authors' knowledge, these MoA have not been previously reported; however, in light of

the suspected modifications to AR transactivation, these results should be taken cautiously. Collectively, the human epidemiology, *in vivo* toxicological findings (Section 2), biological mechanisms (Section 3) and the *in vitro* transactivation assay results (Section 5), highlight the potential errors in predicting potency from transactivation, which may vary with cellular metabolomics, which vary at cell, tissue, organ and organism levels.

Phylogenetic analysis mapped the differences in the amino acid sequences of ER and AR. These differences were mirrored in the *in silico* binding, which highlighted theoretical functional differences in human, chimpanzee, rat and mouse AR. The *in vitro* transactivational response of hER and roachER, identified in transiently-transfected HEK293 cells, demonstrates functional differences in nuclear receptors from different taxonomic classes. In support of the research conducted at the NIBB (Lange *et al.*, 2012; Katsu *et al*., 2007), the susceptibility to chemical endocrine disruption may be in part defined by functional differences in the receptor, identifying a currently neglected field of interspecies variation.

Understanding endocrine mechanisms, and their potential disruption by exogenous chemicals, is vital to assess the impacts on environment and health. The significant costs of inadequate regulation, both in terms of monetary cost and, mortality and morbidity of wildlife and human populations, highlights the political priority of EDCs. The literature highlights additional NR signalling complexity, while the *in vitro* results demonstrate dual agonism and antagonism of endogenous hormones and MBPA. Thus, the study results agree with Hotchkiss *et al*.'s (2008) research, which demonstrated antagonism of endogenous hormone response by partial agonists; assumed to be consequent to competitive binding. Functional cross-talk between NR further complicates the EDC biological mechanism. There are approximately 300 nuclear receptor coregulators (Lonard *et al.*, 2007), concentrations of which vary between cells, tissues, organs and individuals. Without understanding the variability of supporting systems, understanding the biological consequences in the variability and activation of NR, may not be possible. However, *in vitro* studies suggest that coactivator recruitment is a rate-limiting step in NR-mediated gene transcription, thus, the susceptibility to EDC mediated toxicity, consequent to molecular initiation by nuclear receptor binding, is likely to be highly variable.

Table 6.1 summarises the concordance between *in silico* ER and AR binding with *in vitro* AR transactivation assays. Both the *in silico* and *in vitro* results presented herein, highlight the need for further investigation of cofactor regulation and modulation of nuclear receptor responses. Without this information, predicting the MoA of is severely confounded. In many ways the *in silico* screens and *in vitro* assays surpass traditional apical endpoints, demonstrating excellent sensitivity in detecting either binding or transactivation. However, at the current state in time, the relevance of these observations to the whole organism is uncertain.

Table 6.1 Concordance of *in silico* human ERα and AR binding scores (SYBYL®) and *in vitro* transactivation assays

In silico binding scores are in –log(Kd), while *in vitro* 50% effective concentration (EC50) are presented in moles, concordance between the results for ER and AR are highlighted by a green tick, whilst question marks reflect the need for more data.

Test Substance (TS)	*In silico* hERα binding (-log(Kd))	HeLa9903 ER Agonism (EC_{50})		*In silico* hAR binding (-log(Kd))	HeLa4-11 AR Agonism (EC_{50})	HeLa4-11 AR Antagonism (IC_{50})	
17β-Estradiol	5.09	6.377e-11 M	✓	5.37	1.134e-05 M	4.081e-09 M	✓
17α-Ethinylestradiol	6.04	5.329e-15 M	✓	4.64	Negative	6.091e-08 M	✓
Diethylstilbestrol	5.71	1.464e-12 M	✓	3.76	3.27e-012 M	1.016e-05 M	✓
Progesterone	4.10	-	?	2.05	3.08e-12 M	1.067e-08 M	✓
19-Norethindrone	3.88	-	?	5.12	7.84e-10 M	Negative	✓
4'4-DDE	2.4	4.349e-12 M	✓	2.0	Positive	Negative	✓
4-Benzylphenol	3.46	1.343e-05 M	✓	3.44	Negative	4.04e-06 M	✓
4-Nitrophenol	3.44	Negative	✓	2.29	Negative	Negative	?
Bisphenol A	4.88	7.44e-11 M	✓	4.97	Negative	4.852e-06 M	✓
Dibutylphthalate	7.15	Inadequate data	-	5.49	Negative	5.203-06 M	✓
Diethylhexylphthalate	4.13	1.958e-05 M	✓	3.95	Negative	Inadequate data	?
Butylbenzylphthalate	7.00	7.75e-08 M	✓	3.60	Negative	4.997e-06 M	✓
Methylparaben	3.57	Negative	?	3.38	Positive	Negative	✓
OMC	6.09	Inadequate data	?	6.55	Positive	Negative	✓
Triclosan	3.45	Negative	?	3.60	Positive	Negative	✓
Dihydrotestosterone	4.08	-	?	3.58	Positive	-	✓
Metribolone	4.72	-	?	5.28	Positive	-	✓
Testosterone	3.47	6.871e-12 M	✓	2.76	Positive	-	✓
Corticosterone	-	Negative	?	-	Positive	-	?

Bisphenol A is a political priority as a consequence of an estimated annual production of 4.4 million tonnes in 2012, with approximately 100 tons of BPA being released into the atmosphere (Vandenberg *et al.*, 2010). Multiple sources contribute to human BPA exposure, however dietary exposure is considered the most significant pathway; the US NTP detailed adult oral BPA exposure to range from 0.008 to 1.5 µg kg^{-1} bw/day (Vandenberg *et al.*, 2007). However, ensuring the public of the safety of BPA has generated over 5000 publications, rendering it a good example of scientific and regulatory uncertainty. The *in silico* screening and *in vitro* ER (HeLa9903 and roachER HEK293) and AR (HeLa4-11) assays reported herein, demonstrate the endocrine activity of Bisphenol A, which supports a wealth of *in vivo* toxicity studies. A plethora of studies have investigated the apical endpoints associated with BPA exposure, including increased mammary carcinogenesis, hyperactivity, reduced fertility and alteration of sexually dimorphic juvenile social interactions (Bergman *et al.*, 2012). Many of the observed apical endpoints may be attributed to the potential estrogenicity and androgenicity identified herein. However, there is a vast gap in the knowledge, regarding the pathway from the *in silico*/*in vitro* molecular initiating events to the *in*

vivo adverse outcomes. Elucidating the differences between endocrine activity and endocrine disruption, are vital to our accurately understanding the risk of EDC exposure. Currently it may be suggested, that pathways between the molecular initiating events predicted *in silico* and *in vitro*, and the apical endpoints observed *in vivo*, at the individual and population levels, are clouded by an array of uncertain assumptions.

- ✓ **In conclusion, SYBYL molecular modelling and Surflex-Dock virtual screening sensitively predicted the binding of ERα/β, AR, PR and PPARγ potential EDCs, and could be a useful regulatory tool to support EAS hazard identification.**

- ✓ **The *in vitro* transactivation assays in HeLa9903, HeLa4-11 and transiently-transfected HEK293 cells, sensitively predicted nuclear activity.**

However, in light of the mechanistic complexity of NR signalling identified in the literature, and the transactivation responses of HeLa4-11 cells identified herein, it is suggested that the potency of NR transactivation, is not necessarily predicted by *in vitro* tools, which may present modifications to amplify or dampen expression. This inconsistency appears to be specific to particular ligand-receptor interactions, and as a consequence not predictable.

6.1 Future Research and Requirements

- ✓ Develop a harmonised biotechnology software hub with evaluated, defined *in silico*, *in vitro* and *in vivo* evidence, which elucidates positive/negative binding classification, from pooled *in vitro* data;

- ✓ Elucidation of diverse sentinel species NR X-ray crystallography structures and validation of *in silico* methods to elucidate interspecies variations in ligand binding;

- ✓ Comparison of SYBYL Surflex-Dock *in silico* screening and *in vitro* assays in a larger, more mechanistically diverse chemical databases;

- ✓ Modulated transactivation has been identified as a potentially significant confounder. Elucidating the natural variation of cofactor expression, NR polymorphisms, modifications in ligand binding domain and cross-talk between signalling pathways, may play a significant role in understanding the *in vivo* context of *in silico* ligand binding and *in vitro* transactivation;

- ✓ Evaluate the role of coregulatory molecules to *in silico* screening, via development of a multi-stage SYBYL Surflex-Dock tool, and identify the *in vitro* implications of cofactor concentrations, potentially via artificial simulation of cell profiles;

- ✓ Consequent to the elevated potency of BPA's predominant metabolite, 4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene, investigation of metabolites generated in liver S9 fractions, may provide useful information regarding the 'true' hazards of chemical exposure. Incorporating predictions of metabolism to *in silico* (T.I.M.E.S. software) and *in vitro* (Liver S9 fractions) assays may inform hazard characterisation and risk assessment.

"Give me fruitful error anytime, full of seeds, bursting with its own corrections. You can keep your sterile truth for yourself"

Vilfredo Pareto

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APPENDICES

Appendix A List of Prioritised Endocrine Disrupting Chemicals

A chemical database (n=378) was curated from prioritisation lists; 166 chemicals from the EC candidate list and 241 from the DWI report, of which 37 were EC duplicates, in addition to 9 hormones. To enable crude sensitivity assessments of the *in silico* methods, published bioassay data for each of the 378 chemicals was retrieved from PubChem (Table 4.1). Table_A 1 details the mechanistic information of the chemical database; summarising the published high-throughput agonism and antagonism *in vitro* testing results. Collating information from ChemSpider, PubChem and ZINC12, Table_A 1 also summarises the classification, usage and assumed primary endocrine mode-of-action.

Table_A 1 Prioritised Potential EDC list used in *in silico* analysis of binding affinity

Table details chemical name, the class/categorisation, usage and exposure pathways and the binding information available from PubChem (http://pubchem.ncbi.nlm.nih.gov/#).

Chemical Name	Usage and Exposure Pathways	Toxicity	ED MoA
1-Naphthol	Metabolite of carbaryl and naphthalene insecticdes. Precursor for the pharmaceutical Nadolol. Used in production of azo-dyes. Biomarker for PAH exposure (Sreekanth *et al*, 2013).	1N is associated with decreased testosterone levels in adult men (Meeker *et al*., 2006)	
2-Naphthol/	Naphthalen-2-ol, used in production of dyes, pigments, antioxidants (for ubber, fats oils), insecticide, pharmacueticals and as a lubricant. Biomarker for PAH exposure (Sreekanth *et al*., 2013).	Active in ERα signalling pathway agonist qHTS assay, potency 43.65-61.65 μM (AID743077). Inactive in qHTS assay to detect AR signalling pathway antagonists (AID743054) and ERα antagonists (AID743091).	ER+
2,2'Biphenol	Biphenols are used as preservatives. Forms inter- and intra- molecular hydrogen bonds, used as a reagent in chemistry, biology and medicine (Sahnoun *et al*., 2006).	Cytotoxicity study in Hep-G2 cells identified IC_{50} of 200 μM (AID247905).	
2,2'-Dihydroxy-4,4'-dimethoxy benzophenon	Phenolic additive for light stabilisation for polymers.	Estrogenicity detected in a yeast hERα assay 2.5 x 10^{-5} relative to E2 (Miller *et al*., 2001). In qHTS studies: active inhibitor of BRCA1 expression in qHTS study (AID624202); active PPARγ signalling pathway antagonist (AID743199); active ERα signalling pathway agonist, 57.52 μM (AID743077); and, active AhR activator, 14.60 μM (AID743122).	ER+ PPARγ+ AhR+
2,3,4-Trichlorobiphenyl (PCB 33)	PCBs were used as dielectic, coolant fluids (in electric motors) and insulating fluids, usually sold as a mixture of congeners.		
2,4-D	Pesticide used to destroy unwanted vegetation, particularly weed, grasses and woody plants.	Irritant effects on the eye and gastrointestinal system; NOEL 500ppm; ADI: 0.01- 0.03 mg/kg.	
2,4-Dichlorophenoxybutyric acid (2,4-DB)/ 4-(2,4-Dichlorophenoxy) butyric acid	General-use pesticide. Selective systemic phenoxy herbicide used to control broadleaf weeds in alfalfa, peanuts, soybeans and other crops; the active metaboliet inhibtis growth at the tips of stems and roots.	Chronic to in dogs at 25 mg kg^{-1}/day, chronic toxicity in rats at 30 mg kg^{-1}/day (EPA, 1988).	AR+

2,4-Dinitrophenol	2,4-DNP is used in the manufacture of dyes and diaminophenol. A toxic dye, chemically related to trinitrophenol (picric acid), used in biochemical studies of oxidative processes where it uncouples oxidative phosphorylation. It is also used as a metabolic stimulant.	Active qHTS assay identifying AR signalling pathway antagonists, potency 54.7-61.1 μM (AID743042/AID743042).	AR+
2,5-Dihydroxybenzoic acid (2,5-DHBA)	Patented for cancer, ocular disease, actinic keratosis, arthritis and tissue reactive disease medical applications. Use as chemical toner.		
2-Benzylphenol		Inhibited 5-lipoxygenase ($IC_{50:}$ 63 μM) in rat basophilic leukemia (RBL-1) cell line (AID6790). Inconclusive results for interactions with prostaglandin G/H synthase (AID160712).	
2-Chlorobiphenyl (PCB 1)	Aroclor1232 (PCB mixture) has not been produced commercially in the US since 1977, but is environmental persistent and leaches out of landfills. Still detected in transforms and capacitators (HSDB).	Inhibition of human cytochrome P450 at 36 μM (AID241172), inhibition of human CYP2A6 at 35.97 μM (AID420671) and inhibition of mouse CYP2A5 at 13 μM (AID420670).	
2-Phenylphenol	Production and use in rubber chemicals, food packaging, an intermediate for dyes and food presevrative. Also used as pesticide and household disinfectant.	Active DSSTox Carcinogenic rat bioassay results (AID1208) and salmonella mutagenicity (AID1194). Acute toxicant in DSSTox EPA Fathead Minnow test (AID1188). Interactions with aldehyde dehydrogenases and mitochondrial membranes (AID1030/AID720637).	
3,4,5,6-Tetrabromo-o-cresol	A preservative in cosmetics, active ingredient in deodorants and disinfectants, and a fungicide in textile materials.	Active in NCI Yeast Anticancer Drug Screen for *rad50, mlh1 rad18, bub3, sgs1 mgt1, mec2-1* and *cln2 rad14* strains (AID155/175/167/161/165).	
3-Chlorobiphenyl (PCB 2)	Widely used in agriculture and industry for several decades, as with other PCBs commercially restricted.		
3-Methyl cholanthrene	Carcinogen widely used in experimental studies of cancer, produced by burning organic compounds at high temperatures. Derivative of benz[a]anthracene. 3-Methylcholanthrene builds up in the prostate due to cholesterol breakdown, and is implicated in prostate cancer (Malins *et al*., 2004).	In qHTS syudy, activated the AhR signalling pathway, 0.402 μM (AID:743122). Antagonist of AR signalling pathway in MDA cell line, 0.173 μM (AID:743054), ERα signalling pathway antagonism at 0.358 μM (AID:743091) and agonism at 6.178 μM (AID:743075). Agonist of thyroid hormone receptor beta signalling at 44.67 μM (AID:588545).	AR+ ER+ AhR+ TR+
3-t-Butyl-4-hydroxy anisole	The primary use of BHA is an antioxidant and preservative in food (E number E320), food packaging, animal feed, cosmetics, rubber and petroleum products. BHA is also used in pharmaceuticals, such as isotretinoin, lovastatin and simvastatin, and pesticides (HSDB, ***http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~lBGY6Z:1***).	Acitve in mitochondrial membrane disrupter assays (AID:720637), rat bioassay (AID:1208) and mouse bioassay (AID:1199) carcinogenicity studies. Activator of the Pregnane X receptor signalling pathway, 50.12 μM (AID:720659).	PXR+
4,4'-(1,3-Dimethylbutylidene) diphenol	Colour former, developer and reagent for high-performance polymer research. Structurally related to Diethylstilbestrol, BPA and Dienestrol.	Active in qHTS assay for AR signalling pathway antagonists, potency randing from 10.58 μM (AID:743063), to 61.2 μM (AID:743042). Active in qHTS assay for ERα signalling pathway agonists, 97.7 μM (AID:743075) and antagonists, 0.062 μM - 0.31 μM (AID:743069). Active PPARgamma antagonist (AID:743199).	AR+ ER+ PPARγ
4,4'-Biphenol	Phenolic derivative of biphenyl, structurally related to 3,3'-dichlorobenzidine and Paraquat.	Active in qHTS AR signalling pathway antagonist assay, 11.028 μM (AID:743035). ERα signalling pathway agonist, 36.25 μM (AID:743077) and 27.7 μM (AID:743075). Active PPARgamma antagonist (AID:743199).	AR+ ER+ PPARγ+

4,4-Dihydroxy benzophenon	Main use as a UV-light stabiliser found in cosmetics, plastics, films, adhesives and coatings, optical fibre and printed circuit boards. It is the precursor to certain polycarbonate polymers (Parker *et al*., 2002). Structurally similar to Pterosupin, a drug isolated from the Pterocarpus marsupium bark and heartwood, believed to lower blood sugar activity (Mukherjee *et al*., 2006).	Binds to sterol 14α-demethylase (CYP51). DHBP represents a benzophenone scaffold binding in the CYP51 active site via a type 1 mechanism, which means that the chemical may interfere with sterol biosynthesis (Eddine *et al*., 2008).	
4,4'-Ethylidene bisphenol/ Bisphenol E	Colour former, developer and reagent for high-performance polymer research. Structurally related to Diethylstilbestrol, BPA and Dienestrol.		
4-Benzylphenol	Used as a germicide, antiseptic and preservative.	Active in qHTS ERα signalling pathway agonist assay, 50.88 μM (AID:743077) and 57.06 μM (AID:743075).	ER+
4-Chloro-3-methylphenol	Also known as chlorocresol, used as a disinfectant and preservative.	Active in DSSTox (KIERBL) EPA Estrogen Receptor Ki Binding Study (Laws *et al*. 2006). Active in qHTS assay for TR signalling pathway agonists, 27.3 μM (AID:743066).	ER+ TR+
4-Chloroaniline	4-Chloroaniline's used as an intermediate for the synthesis of dyes, pharmaceuticals and agricultural chemicals. It is a precursor to the widely used antimicrobial and bacteriocide chlorhexidine and is used in the manufacture of pyraclostrobin, anilofos, monolinuron and chlorphthalim pesticides. Degradation product of phenylurea herbicide.	Active in qHTS assay for PPARgamma antagonists (AID:743199), ERα signalling pathway antagonists, 9.14 μM (AID:743078) and 9.94 μM (AID:743069), active TR signalling pathway antagonist, 27.78 μM (AID:743065).	PPARγ+ ER+ TR+
4-Chlorobiphenyl (PCB 3)	Widely used in agriculture and industry for several decades, as with other PCBs commercially restricted.	Inhibition of mouse CYP2A5, IC_{50} 13.002 μM (AID:420670). Inhibitory of mouse cytochrome P450 2A5, IC_{50} 18 μM (AID:241174). Inhibitory concentration against recombinant human cytochrome P450 1A2, IC50 49 μM (AID:241334).	
4-Chloroxylenol/ 4-Chloro-3,5-diethylphenol	Chloroxylenol's used as an antibacterial, germicide, antiseptic and in mildew prevention, commonly used in adhesives, emulsions, paints and wash tanks. It is also commonly used in antibacterial soaps and household antiseptics, such as Dettol liquid and cream	Not believed to be significantly toxic to humans and other mammals (***http://www.epa.gov/oppsrrd1/REDs/factsheets/3045fact.pdf***).	
4-Hydroxy-4'-chlorobiphenyl/ 4-Chloro-4'-hydroxybiphenyl	Also known as 4-Chloro-4'-biphenylol. Biphenyl are used as the building blocks of liquid crystals and functional materials. Metabolite of 4-Chloro and 4,4'-Dichloro Biphenyl in rats (Safe *et al*., 1974).	Active DSSTox (NCTRER) National Center for Toxicological Research Estrogen Receptor Binding Database (AID:1204) (Fang *et al*., 2001).	ER+
4-Hydroxypropiophenone	Intermediate in the formation of liquid crystals. Patented for use in pharmaceuticals and pesticides. Drug name, Paroxypropione, under pituitary gonadotropic hormone inhibitor (anti-gonadotropin) therapeutic class.	Activator of calcium-activated chloride channels (AID:623877).	
4'-Isoburyl acetophenone	Structurally related to Ibuprofen; a degradation product of Ibuprofen in tablets.		
4-iso-Pentylphenol	On EC priority list of substances. Data lacking.		
4-Nitrophenol	4-NP is used in the manufacture of methyl and ethyl parathion, acetominophen, dyestuffs and leather treatment agents. In addition, it is the degradation product and impurity of parathion, the insecticide.	Active in qHTS assay to identify AR signalling pathway agonists, potency 0.001μM (AID:743053).	AR+

4-Nitrotoluene	4-Nitrotoluene's used in the manufacture of azo and sulfur dye intermediates and explosives.	Evidence of toxicity and carcinogenicity in mice (NTP, 2002). Active in DSSTox carcinogenicity potency database (AID:1189) and rat bioassay (AID:1208). Ihhibitor of acetylcholinesterase, IC_{50} 0.1 μM (AID:32248).	
4-Nonylphenol/ 4-[(2R)-2,6-Dimethylheptyl] phenol	Alkylphenol used in the preparation of lubricating oil additives, resins, plasticisers, surface active agents, stabilisers, petroleum demulsifiers, fungicides, rubber antioxidants and as a starting material for the production of phenolic resins.	Active in qHTS ERα signalling pathway agonist, 12.20 μM and 6.93 μM (AID:743079). DRUGMATRIX: Active ERα radioligand binding. IC_{50} 0.377 μM (AID:625258). Inhibitory concentration against recombinant rat AR expressed in *E. coli* using R1881 and Testosterone, IC_{50} 11.48 μM (AID:255211). Cytotoxic in MultiTox-Fluor Cytotoxicity Assay - LYMP1-003-Dead cells, 39.81 μM (AID:961). Active in qHTS assay for inhibitors of aldehyde dehydrogenase 1 (ALDH1A1) (AID:1030).	ER+ AR+
4-Phenylphenol	4-phenylphenol is used in the manufacture of dyes and resins, rubber chemicals, fungicide and nonionogenic emulsifiers. Also formed through the photodegradation of 4-chlorobiphenol.	Active in qHTS assay for ER signalling pathway agonists, 0.44 μM - 21.58 μM (AID:743075), and 8.52 μM in the BG1 cell line (AID:743079). DSSTox (NCTRER) Estrogen Receptor Binding database (AID:1204). Active in qHTS AR signalling pathway antagonist assay, 27.31 μM (AID:743042).	ER+ AR+
4-sec-Pentylphenol/ Pentylcyclohexanol	Also known as 4-Pentylcyclohexanol.	EC_{50} was 1.68 μM in human ER MCF-7 cell assay and 0.91 μM in rat ER CV-1 cell assay (Sun *et al*., 2007).	ER+
4-t-Butylphenol	Also known as 4-(2-Methyl-2-propanyl)phenol.	Active in DSSTox (NCTRER) Estrogen Receptor Binding Database (AID:1204). Estrogenic activity in human MCF-7 cells assessed as cell proliferation after 7 days by WST-8 assay (AID:721698).	ER+
4-t-Octylphenol	Alkylphenol. The Surface-Active Agents modify interfacial tension of water, usually substances that have one lipophilic and one hydrophilic group in the molecule; includes soaps, detergents, emulsifiers, dispersing and wetting agents and several groups of antiseptics.	Active AR signalling pathway antagonist in qHTS assay, 15.09 μM (AID:743035). Displaced 5αDihydrotestosterone from human sex hormone binding globulin, 2.14 μM Kd (AID:318680). Active ER signalling pathway agonist, 0.86 μM and 1.19 μM (AID:743079). Antagonist of ER signalling at 33.49 μM (AID:743080) and 33.49 μM in BG1 cell line (AID:743091). On DSSTox (NCTRER) Estrogen Receptor Binding Database (AID:1204).	ER+ AR+
4-Vinylguaiacol (4-VG)	Aromatic substance used as a flavouring agent, responsible for the natural aroma of buckwheat (Janes *et al*., 2008).		
4-Vinylphenol (4-VP)	4-Vinylphenol is a phenolic compound produced by the spoilage yeast Brettanomyces, and is found in wine and beer.		
5-Aminosalicylic acid	5-Amino-2-hydroxybenzoic acid, also known as Mesalamine, structurally related to salicylates, Mesalamine is used as an anti-inflammatory agent in the treatment of inflammatory bowel disease, Crohn's disease and ulcerative colitis (Sandborn *et al.*, 2007). Mesalamine is also used in the manufacture of light-sensitive paper and, azo and sulfur dyes.	Inhibitor of Prostaglandin G/H synthase 1 (Chen *et al*., 2002) and inhibitor of Prostaglandin G/H synthase 2 (Cipolla *et al.*, 20002). PPARγ signalling agonist (Desreumaux *et al*., 2006). Inhibitor of Nuclear Factor Kappa-B Kinase subunit beta (NF-Kappa-B) (Bantel *et al.*, 2000). Inhibitor of Arachidonate 5-lipoxygenase (Nielsen *et al*., 1987).	PPARγ+
6-Bromo-2-naphthol	Aromatic polycyclic hydrocarbons, Naphthalenes.	Active in NCI Yeast Anticancer Drug Screen for rad50, mlh1 rad18, bub3, sgs1 mgt1, mec2-1 and cln2 rad14 strains (AID157/175/161/165/167).	

9-cis-Retinoic acid	9-cis-retinoic acid is a form of vitamin A, used as a regulator of gene expression used as a antineoplastic agent.	Dissociation constant for retinoid X receptor alpha (RXR), 0.0015 μM (AID:254256). qHTS ERα signalling pathway antagonist in the BG1 cell line, 0.179 μM (AID:743091). Thyroid Receptor signalling pathway agonist, 1.535 μM (AID:743066). PPARγ signalling agonist, 3.21 μM (AID:743140).	RXR+ ER+ TR+ PPARγ
Acebutolol	A cardioselective beta-1 adrenergic antagonist with little effect on the bronchial receptors. The drug has stabilising and quinidine-like effects on cardiac rhythm, as well as weak inherent sympathomimetic action.	Beta-1 adrenergic receptor partial agonist (Chen *et al*., 2002), Beta-2 adrenergic receptor partial agonist (Fraysse *et al*., 2005). Cytochrome P450 2D6 inhibitor (Preissner *et al*., 2010).	
Acephate	Acephate is an organophosphate foliar insecticide, used for control of aphids in vegetables and in horticulture.	Acephate is considered to be a fetotoxin (can poisin the fetus) (Briggs, 1992).	
Acetaminophen (Paracetemol)	Acetaminophen is used as an analgesic and in the manufacture of azo dyes and photographic chemicals. Antipyretic derivative of acetanilide with weak anti-inflammatory properties and is used as a common analgesic, but may cause liver, blood cell and kidney damage.		
Acetochlor	Chloroacetanilide herbicide developed by Monsanto Company and Zeneca, which works by inhibition of geranylgeranyl pyrophosphate (GGPP) cyclisation enzymes and elongase inhibition (Arregui *et al*., 2010).	Active in qHTS assay to identifiy small molecule antagonists of the AR signalling pathway, 21.9 μM (AID:743035) and 54.48 μM (AID:743042), results consistently replicated. qHTS ERα signalling pathway antagonist, 24.54 μM (AID:743069) and agonism, 0.71 μM (AID:588514).	AR+ ER+
Acipimox	Hypolipidemic agent, used to lower levels of certain lipids in the blood and to treat hyperlipidemias. Acipimox inhibits the production of triglycerides by the liver and secretion of VLDL, indirectly leading to a reduction in LDL and increase in HDL.	Active at GRP109a in CHO cells, indicated by inhibition of forkolin-induced cAMP generation, IC_{50} 5.3 μM (AID:281255). IC_{50} 39 μM required for inhibition of free fatty acid liberation in Canine myocardial lipase assay (AID:143599). Inactive in ER and AR qHTS assays.	
Alachlor	Alachlor is a chloroacetanilide herbicide used to control the growth of broad-leafed weeds and grasses in crops, by inhibiting elongase and geranylferanyl pyrophosphate (GGPP) crystalisation enzymes. Mixes well with other herbicides, and is found in mixed formulations with atrazine, glyphosate, trifluralin and imazaquin.	Activity in qHTS ERα agonist signalling pathway assay, 0.159 μM (AID:588514), agonist of the antioxidant response element, 4.19 μM (AID:743219). qHTS AR signalling pathway antagonist: 26.66 μM (AID:743035), 26.6 μM (AID:743063) and 38.9 μM (AID:743035); in the MDA cell line, potencies were slightly lower, 61.13 μM (AID:743042). qHTS TR signalling pathway antagonist, 54.5 μM (AID:743065).	ER+ AR+ TR+
Aldicarb	Carbamate insecticide effective against thrips, aphids, spider mites, and lygus, fleahoppers, by inhibiting cholinesterase, which prevents the breakdown of acetylcholine in the synapse. Aldicarb is the active substance in the pesticide Temik.	Active in qHTS agonsts of AhR signalling pathway, 39.8 μM (AID:651777) and PPARγ signalling pathway, 39.81 μM (AID:588537). Agonist of the Antioxidant Response Element (ARE), 27.31 μM (AID:651741).	AhR+ PPARγ+
Amitriptyline	Tricyclic antidepressant with anticholinergic and sedative properties. It appears to prevent the re-uptake of norepinephrine and serotonin at nerve terminals, thus potentiating the action of these neurotransmitters. Amitriptyline also appears to antagonise cholinergic and alpha-1 adrenergic responses to bioactive amines.	0.00005 μM IC_{50} for inhibitory activity against Alpha-1 adrenergic receptor (AID:36031).	

Amitraz	Non-systemic acaricide and insecticide with alpha-adrenergic agonist activity, octopamine receptor interaction and inhibition of monoamine oxiase and prostaglandin synthesis, leading to insect death.	qHTS PPARδ signalling pathway antagonist, 24.53 μM (AID:743213). Activator of the Aryl Hydrocarbon Receptor signalling, 41.82 μM (AID:743122). Antagonist of PPARγ signalling, 43.83 μM (AID:743218). Antagonist of AR signalling pathway, 43.83 μM (AID:743033). Thyroid Receptor signalling antagonist, 48.51 μM (AID:743064). Glucocorticoid Receptor signalling antagonist, 49.19 μM (AID:720693).	PPARδ + AhR+ PPARγ+ AR+ TR+ GR+
Amitrol/ 3-amino-1,2,4-triazole	A non-selective post-emergence triazole, translocated herbicide, used to control annual grasses and broad-leaves and aquatic weeds. 3-AT is a competitive inhibitor of imidazoleglycerol-phosphate dehydratase, the product of the HIS3 gene. Suspected as a carcinogen, irreversible inhibitor of catalase and thus impairs activity of peroxisomes.	Active in qHTS assay to identify ERα signalling pathway agonists, 61.1μM (AID:743079).	ER+
Amoxicillin	Broad spectrum semi-synthetic antibiotic, used to treat gram positive and gram negative bacterial infections.		
Amphetamine	L-amphetamine is a powerful central nervous system stimulant and sympathomimetic which blocks the uptake of adrenergics and dopamine, stimulates monoamine release and inhibits monoamine oxidase.		
Ampicillin	Broad-spectrum semi-synthetic penicillin derivative.		
Androstanedione	Also know as Androstane-3,17-dione. Endogenous 19-carbon steroid hormone produced in the adrenal glands and the gonads as an intermediate in androgen (testosterone) and estrogen (estrone and estradiol) biosynthesis.	Androstenedione acts as a partial agonist of the androgen receptor, however, in the presence of stronger agonists (i.e. tesosterone or DHT), it can antagonise (Chen *et al.*, 2004).	AR+
Androsterone	The metabolite of testosterone of androstenedione with a 3-alpha-hydroxyl group and without the double bond. The 3-beta hydroxyl isomer is epiandrosterone. Can be converted to diydrotestosterone (DHT) from 17-hydroxyprogesterone, bypassing conventional intermediates, such as androstenedione and testosterone	Potency approximately 1/7 of testosterone, but can displace DHT from human sex hormone binding globulin, 0.71 μM (AID:318680). IC_{50} 40.74 μM antagonistic activity in recombinant rat AR expressed in *E. coli* using R1881 (AID:255211).	AR+
Anisole	Aromatic compound with the smell of anise seed; many of its derivatives are found in natural and artifical fragrances.	Inhibition of human CA4 using 4NPA as substrate for 3 minutes by Lineweaver burk plot analysis (AID:667535).	
Anthracene	Anthracene is a component of coal tar, as a natural component of incomplete fossil fuel combustion. Also, anthracene is used in the product of red dye alizarin.	Active qHTS activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 27.31 μM (AID:743085).	AhR+
Aspirin	Prototypical analgesic used in the treatment of mild to moderate pain, with anti-inflammatory and antipyretic properties. Acts as an inhibitor of cyclooxygenase which results in the inhibition of the biosynthesis of prostaglandins. Aspirin also inhibits platelet aggregation and is used in the prevention of arterial and venous thrombosis.	COX-1 inhibition.	
Atenolol	A cardioselective beta-1 adrenergic blocker, possessing properties and potency similar to propranolol, but without a negative inotropic effect, used primarily in the treatment of cardiovascular disease.	*In vitro* inhibitory activity against beta-1 adrenergic receptor, measured by inhibition of positive chronotropic effect of isoproterenolin, 0.024 μM (AID:39943).	

Atorvastatin	Atorvastatin is a statin used for lowering blood cholesterol, marketed by Pfizer under the trade name Lipitor. Atorvastatin is a competitive inhibitor of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the rate determining enzyme in cholesterol biosynthesis via the mevalonate pathway.	qHTS assay to identify small molecule antagonists of AR signalling pathway, 10.64 µM (AID:743063). TR signalling pathway antagonist in qHTS assay in rat, 57.11 µM (AID:743065).	AR+ TR+
Atrazine	Selective triazine herbicide used to control grass and broadleaf weeds in crops such as sorghum, maize, sugarcane, lupins and pine.	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 68.5 µM (AID:743085).	AhR+
Azithromycin/ Sumamed	A semi-synthetic macrolide antibiotic structurally related to erythromycin. It has been used in the treatment of mycobacterium avium intracellulare infections, toxoplasmosis and cryptosporidiosis.	qHTS assay to identify antagonists of TR signalling pathway (ratTRbeta), 36.13 µM (AID:743065) and cell viabilitiy screen 29.85 µM (AID:743064).	TR+
Bendroflume thiazide	Bendroflumethiazide is a thiazide diuretic used to treat hypertension; works by inhibiting sodium reabsorption at the beginning of the distal convoluted tube. Similar to hydrochlorothiazide, it has been used in the treatment of familial hyperkalemia, hypertension, edema and urinary tract disorders.		
Benomyl	Benomyl is a systemic benzimidazole fungicide that is selectively toxic to microorganisms and invertebrates, commonly used on stone fruit. Benomyl interacts with tubulin, affecting polymerisation of microtubules.	Activator of the human Aryl Hydrocarbon Receptor (AhR) signalling pathway, 6.08 µM (AID:743085). qHTS rat TR signalling pathway antagonist, 7.66 µM (AID:743065), 8.31 µM (AID:743067). Rat pregnane X receptor (PXR) signalling pathway antagonist, 11.2 µM (AID:651751). Human ERα signalling pathway agonist, 27.31 µM (AID:743079). Human AR signalling antagonist, 38.71 µM (AID:743033) and 61.34 µM (AID:743035).	AhR+ TR+ PXR+ ER+ AR+
Bentazone	Bentazone is a selective thiadiazine herbicide that only damages plants unable to metabolise the chemical, and is considered safe for use on alfalfa, beans, corn, peanuts, peas, pepper, rice and soybeans.		
Benz(a) anthracene/ Tetraphene	Polycyclic aromatic hydrocarbon that occurs in coal tar. Primarily the product of incomplete combustion, including vegetation and food products.	qHTS activator of human Aryl Hydrocarbon Receptor (AhR) signalling pathways, 4.86 µM (AID:743122). Agonist of the ERα signalling pathway in the BG1 cell line, 19.33 µM (AID:743079). Activator of rat pregane X receptor (PXR) signalling pathway, 28.18 µM (AID:651751). Agonist of AR signalling pathway in MDA cell line, 68.59 µM (AID:743040).	AhR+ ER+ PXR+ AR+
Benzo[a]pyrene/ 7,12-Dimethyl benz[a]anthracene	An environmental pollutant, as a component of coal tar, incomplete combustion and tobacco smoke, benzo(a)pyrene is a potent mutagen and carcinogen.	qHTS activator of human AR signalling pathway in the MDA cell line, 0.22 µM (AID:743054) and ERα signalling in the BG1 cell line, 0.85 µM (AID:743091). Activator of the AhR signalling pathway, 1.24 µM (AID:743085). Agonist of PPARdelta signalling pathway, 2.41 µM (AID:743227) and PPARγ, 4.28 µM (AID:743140). Agonist of the glucocorticoid receptor (GR) signalling pathway, 2.81 µM (AID:720719).	AR+ ER+ AhR+ PPARδ

Benzophenone	Benzophenone is an organic compound, derivatives of which (oxybenzone and dioxybenzone) are used in sunscreen. In addition, benzophenone can be used as a photo initiator in UV-curing applications such as inks, imaging and clear coatings in the printing industry, as it prevents UV damage of scents and colours in products.	qHTS agonist of the human AR in the MDA cell line, 26.6 μM (AID:743040). ERα agonist in BG1 cell line, 27.31 μM (AID:743079).	AR+ ER+
Benzotriazole	Benzotriazole's is used as a corrosion inhibitor, photographic restrainer, UV absorber and chemical intermediate.	Agonist of the vitamin D receptor (VDR) signalling pathway, 38.9 μM (AID:743224). Active in qHTS asssay for PPARdelta signalling pathway antagonists, 38.9 μM (AID:743213).	PPARδ +
(-) Benzoylecgonine	Benzoylecgonine is the major metabolite of cocaine, formed by the hydrolysis of cocaine in the liver, catalysed by carboxylesterases, it is excreted in the urine of cocaine users after processing in the liver.	Relative binding affinity against human antibody 2E2, compared to [H_3]cocaine, Ki 0.043 μM (AID:127360).	
Benzylparaben/ Benzyl-4-hydroxybenzoate	Parabens are widely used as preservatives in cosmetics and pharmaceuticals.	qHTS ERα signalling pathway agonist in the BG1 cell line, 10.59 μM (AID:743079). PPARγ signalling pathway antagonist, 43.6 μM (AID:743191). Antagonist of the TR signalling pathway in qHTS assay, 74.1 μM (AID:743067). DSSTox (KIERBL) EPA ER Ki Binding study database (AID:1576).	ER+ PPARγ+ TR+
Betaxolol	A cardioselective beta-1 adrenergic antagonist with no partial agonist activity, used in the treatment of hypertension and glaucoma.	Interacts with cytochrome P450 1A2 (Preissner *et al.*, 2010).	
Bezafibrate (BZF)	An antilipemic agent that lowers cholesterol and triglycerisdes by decreasing low density lipoproteins and increasing high density lipoproteins.	Agonist of PPARγ in HepG2 cells transactivation assay, EC_{50} 1.05 μM (AID:696365) and CV1 cells, EC_{50} 3 μM (AID:320687). Agonist activity of human PPARδ in transcriptional activation assay, EC_{50} 20 μM (AID:156469).	PPARδ + PPARγ+
Bifenthrin	Pyrethroid insecticide used against the red imported fire ant, by influencing the invertebrate nervous system.	Significant differences in estrogenic potential were observed between the two enantiomers in the *in vitro* human breast carcinoma MCF-7 cell proliferation assay (i.e. E-Scrren), the relative proliferative effect rations of 1S-cis-BF and 1R-cis-BF were 74.2% and 20.9%, respectively. The data suggest that enantioselectivity should be considered in acute and chronic endocrine studies (Wang *et al.*, 2007).	ER+
Bisoprolol	A cardioselective beta-1 adrenergic blocker, bisoprolol is used in the management of hypertension and angina pectoris.		

Bisphenol A	As a biphenol, it has two hydroxyphenyl functionalities. BPA is used as a plasticiser and in epoxy resins, commonly found in consumer goods. World production capacity of BPA was 1 millions tons in the 80's and more than 2.2 million tons in 2009 (Fiege *et al*., 2000).	qHTS assay in the BG1 cell line for ER signalling agonists, 0.22 µM (AID:743079). Lipoxygenase 15-LO enzyme inhibition, IC_{50} 1.30 µM (AID:625146). Serotonin (5-hydroxytryptamine) 5-HT6 radioligand binding, IC_{50} 5.42 µM (AID:625221). Antagonist of human PPARγ signalling pathway (AID:743199). Antagonist of ER signalling pathway, 8.71 µM (AID:743069). Activator of human PXR signalling pathway, 19.95 µM (AID:720659). Agonist of PPARδ signalling pathway, 22.27 µM (AID:743211). Antagonist in qHTS stufy of glucocorticoid receptor signalling pathway, 24.26 µM (AID:720692). Activator of the Aryl Hydrocarbon Receptor (AhR), 30.64 µM (AID:743219). qHTS AR antagonist, 39.96 µM (AID:743063). Antagonist of the TR signalling pathway, 61.13 µM (AID:743065).	ER+ PPARγ+ AR+ AhR+ PPARδ + PXR+
Bisphenol B/ 2,2-Bis(4-hydroxyphenyl) butane	As a biphenol, it has two hydroxyphenyl functionalities. Bisphenols are used as plasticisers and in epoxy resins and commonly found in consumer goods.	qHTS Agonist of human ERα signalling pathway, 0.14 µM (AID:743077) and antagonism at 0.31 µM (AID:743069). DSSTox (NCTRER) Estrogen Receptor Binding Database (AID:1204). Antagonist of PPARδ signalling pathway, 19.49 µM (AID:743215). PPARγ antagonist, 30.87 µM (AID:743191). Human AR antagonist, 21.85 µM (AID:743035). PXR activator, 35.48 µM (AID:720659). Antagonist of glucocorticoid receptor (GR) signalling pathway, 43.64 µM (AID:720692). TR antagonists in the MDA cell line, 63.46 µM (AID:743065).	ER+ PPARδ + PPARγ+ PXR+ TR+ AR+
Bisphenol F/ 4,4'-Dihydroxydiphenyl methane	Bis(4-hydroxyphenyl)methane, the bisphenol has two hydroxyphenyl funcitonalities.	Active in qHTS ER agonist signalling pathway, 2.45 µM (AID:743079), 15.61 µM (AID:743075). DSSTox (NCTRER) estrogen receptor binding database.	ER+
Bitertanol	Triazole, azole heterocyclic compound.	Active in qHTS assay for human pregnane X receptor (PXR) signalling pathway activators, 10 µM (AID:720659) and agonist or antioxidant response element (ARE) signalling pathway, 68.59 µM (AID:651741).	PXR+
Bromacil	First registered for use as a pesticide in 1961, it works by interfering with photosynthesis. As a substituted uracil, bromacil is used as a broad spectrum herbicide for bush control and non-cropland areas, in addition to citrus fruit and pineapples.	qHTS activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 21.67 µM (AID:743085) and 21.67 µM (AID:743122).	AhR+
Bromoform	Bromoform is a trihalomethane, brominated organic solvent. Natural production of bromoform by phytoplankton and seaweed is believed to be a predominant environmental source.	Carcinogenic	
Bromoxynil/ 3,5-dibromo-4-hydroxybenzonitrile	Nitrile herbicide used to control post-emergent annual broadlead weeds, and in particular cereal, corn, soghum, onions, flax and on non-cropland.	qHTS human AR signalling pathway agonist, 0.769 µM (AID:743040). Retinoid X Receptor (RXR) agonist, 15.84 µM (AID:588544). AhR activator, 21.67 µM (AID:743122). PPARα signalling pathway agonist, 25.12 µM (AID:651778). Human PPARγ antagonist, 54.89 µM (AID:743191). ERα signalling pathway antagonist, 52.46 µM (AID:743080). Thyroid Receptor antagonist in qHTS assay, 52.46 µM (AID:43065/AID:743067).	AR+ RXR+ AhR+ PPARα + PPARγ+ ER+
Butylated hydroxytoluene (BHT)/ 2,6-Di-tert-9-methylphenol	A lipophillic di-tert-butylphenol with antioxidant properties, primarily used as a food additive (E321), but also as an antioxidant additive in cosmetics, pharmaceuticals, rubber, electrical transformer oil and embalming fluid.	Retinoid X Receptor qHTS agonist, 17.78 µM (AID:588544). Antagonist of the rat Thyroid receptor (TR) signalling pathway, 30.64 µM (AID:743065). qHTS human ER signalling pathway antagonist in the BG1 cell line, 34.45 µM (AID:743080).	PXR+ TR+ ER+

Butylbenzyl phthalate (BBP)/ Benzylbutyl phthalate	The phthalate BBP is an ester of phthalic acid, benzyl alcohol and n-butanol, commonly used as a plasticiser for PVC and cellulose resins.	Agonists of ER signalling, 6.31 μM (AID:588514) and 10.87 μM in the BG1 luc cell line (AID:743079). Aryl Hydrocarbon Receptor (AhR) signalling pathway, 7.70 μM (AID:743085). Agonist of the PPARδ signalling pathway, 19.49 μM (AID:743211). qHTS antagonist of the human AR signalling pathway, 21.70 μM (AID:743035).	ER+ AhR+ PPARδ + AR+
Butylparaben/ Butyl-4-hydroxy benzoate	Butylparaben is used as a preservative in topical antibiotics or corticosteroid preparations, and as a food preservative.	qHTS ER signalling pathway agonst in BG1 cell line, 5.52 μM (AID:743079). Inhibitor of cytochrome P450 2C19, 6.31 μM (AID:899). Antagonist of ER signalling pathway, 44.20 μM (AID:743069). Antagonist of the AR signalilng pathway, 45.35 μM (AID:743063).	ER+ AR+
Caffeine	A naturally occuring methylxanthine which stimulates the central nervous system, increasing alertness and agitation. Caffeine relaxes smooth muscle, and stimulates cardiac muscles and is used as a pharmacological agent. Caffeine inhibits the cyclic nucleotide phosphodiesterases, antagonises the adenosine receptors and modulates intracellular calcium handling.	Antagonistic activity against human adenosine A2B receptor, Ki 10.4 μM (AID:494495). qHTS assay aromatase inhibitors, 55.15 μM (AID:743084) and antagonist of AR signalling pathway in MDA cell line, 49.15 μM (AID:743042).	AR+
Captopril	Potent and specific inhibitor of peptidyl-dipeptidase A, blocking the conversion of angiotensin I to angiotensin II, a vasoconstrictor and important regulator of arterial blood pressure. Captopril is used in the treatment of hypertension and congestive heart failure. Captopril suppresses the renin-angiotensin system and inhibits pressure responses to exogenous angiotensin.	Inhibitory concentration against angiotensin I converting enzyme, 0.023 μM (AID:254756).	
Carazolol	Carazolol is an anagonist and partial inverse agonist of the beta-adrenergic receptor (beta-blocker).	Dissociation constant against beta-adrenergic receptor, Kd 0.0001 μM (AID:41147).	
Carbamate/ Carbamic acid	Carbamates are organic compounds derived from carbamic acid, which are functional groups that are inter-related structurally and often interconverted chemically.		
Carbamazapine	Carbamazepine (CBZ) is an anticonvulsant and mood stabilising drug used in the treatment of epilepsy, bipolar disorder and trigeminal neuralgia.	Active in qHTS assay to identify agonists of the AR signalling pathway, 4.42 μM (AID:743053) and 11.88 μM in the MDA cell line (AID:743040)	AR+
Carbaryl	A carbamate insecticide and potent anticholinesterase agent which reversibly inhibits cholinesterase. Carbaryl is branded under the name Sevin by the Bayer company, but is referred to as Carbaril when used for veterinary applications.	Active in qHTS assay to identify agonists of the AR signalling pathway in the MDA cell line, 29.85 μM (AID:743040). Inhibitor of HADH2 (Hydroxyacyl-coenzyme A dehydrogenase, Type II), 39.81 μM (AID:886). Activator of the Aryl Hydrocarbon Receptor (AhR), 27.31 μM (AID:743085).	AR+ AhR+
Carbendazim	Widely used broad spectrum benzimidazole fungicide; casting worm control agent in turf. Metabolite of the fungicide benomyl, listed collectively on EU pesticides database (http://ec.europa.eu/sanco_pesticides/public/?event=substance.resultat&s=1).	qHTS antagonist of the Vitamin D Receptor (VDR) signalling pathway (AID:743242). Agonist of the p53 signalling pathway, 14.22 μM (AID:720552). Activator of the Aryl Hydrocarbon Receptor (AhR), 10.87 μM (AID:743085).	AhR+

Carbofuran	Carbofuran is a carbamate pesticide used as a systematic insecticide, acaricide and nematocide by inhibiting cholinesterase. Carbofuran is used to control insects on field crops such as potatoes, corn and soybeans.	Active in qHTS ERα agonist singalling pathway in the BG1 cell line, 24.33 μM (AID:743079).	ER+
Carisoprodol	A centrally acting skeletal muscle relaxant, used as an adjuct in the symptomatic treatment of musculoskeletal conditions associated with painful muscle spasm	Inhibitor of HP1-beta Chromodomain Interactions with Methylated histone tails, 0.006 μM (AID:488953).	
Cashmeran	Cashmeran is a synthetic compound used in perfume for its unique musky, powdery odour. Also known as 6,7-dihydro-1,1,2,3,3-pentamethyl-4-(5H)indanone.		
Cefuroxime	Broad-spectrum second generation cephalosporin antibiotic, resistant to beta-lactamse, discovered by GlaxoSmithKline (Zinacef).		
Celestolide	Also known as acetyl tert-butyl dimethylindan, which is a substance, extract or preparation for diffusing or imparting an agreeable or attractive smell (perfume). As a synthetic musk, the aromachemical emulates the scent of natural musk.	Active in qHTS assay to identiy aromatase inhibitors, 27.31 μM (AID:743083), antagonist of TR signalling pathway, 43.28 μM (AID:743064) and PPARδ, 43.64 μM (AID:743211).	TR+ PPARδ +
Celiprolol	A cardioselective beta-1 adrenergic antagonist that has symopathomimetic activity, used in the management of angina pectoris and hypertension.		
Chlofentezine	An arcaricide belonging to the tetrazine chemical group which acts as an ovicide, used to control mites on apples, pears, stone fruit, nuts, ornamentals and almonds.	qHTS agonist of ERα signalling pathway in BG1 cell line, 1.53 μM (AID:743079) and Aryl Hydrocarbon Receptor (AhR), 20.68 μM (AID:743122). Activator of the human pregnane X receptor (PXR) signalling pathway, 11.22 μM (AID:720659).	ER+ AhR+ PXR+
Chloramphenicol	Chloramphenicol is a broad-spectrum bacteriostatic antimicrobial isolated from Streptomyces venequelae, which act by interfering with bacterial protein synthesis.	Inhibitor of human tyrosyl-DNA phosphodiesterase 1 (TDP1), 6.51 μM (AID:686978). qHTS inhibitor of aldehyde dehydrogenase 1 (ALDH1A1), 14.13 μM (AID:1030).	
Chlorazepate	Benzodiazepine derivative with anxiolytic, anticonvulsant, sedative, hypnotic and skeletal muscle relaxant properties. Used in the treatment of anxiety. Modulator of the GABA receptors.		
Chlordimeform	An acaricide used against many organophosphate and carbamate resistant pests, which acts as an uncoupling agent and monoamine oxidase inhibitor. Target species include ticks and Lepidoptera insects.	Cytotoxicity study in chicken DT40 cell line, 61.13 μM (AID:743012).	
Chlorfenvinphos	An organophosphorus cholinesterase inhibitor, trade name was Dermaton®/Birlane® and was used to control flies.	qHTS rat Pregnane X Receptor (PXR) signalling pathway activator, 12.59 μM (AID:651751), and activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 50.12 μM (AID:651777).	PXR+ AhR+
Chloridazon/ 5-Amino-4-chloro-2-phenylpyridazin-3-(2H)-one	Also known as Pyrazon. Chloridazon is a selective pyridazine-derived herbicide, which inhibits photosynthesis and the Hill reaction, used in beet cultivation.	Active in uHTS fluorescent assay for identification of inhibitors of hexokinase domain containing I (HKDC1) (AID:493160).	
Chloroform/ Trichloromethyl	Chloroform is a commonly used laboratory trihalomethane solvent, used in the manufacture of PTFE, previously used as an anesthetic.	Active in qHTS assay to identiy AR signaling pathway antagonists, 48.6 μM (AID:743033), and inhibitor of RGS12 GoLoco motif Activity (red fluorophore) (AID:880).	AR+

Chlorotetracycline	A tetracycline with a 7-chloro substitution, in veterinary medicine chlortetracycline is commonly used to treat conjunctivitis in cats.		
Chlorpyrifos	Organophosphate insecticide, which acts on the nervous system of insects by inhibiting acetylcholinesterase.	binding of Cytochrome P450 3A4, 5.01 µM (AID:884). Activator of the Aryl Hydrocarbon Receptor (AhR) signaling pathway, 9.69 µM (AID:743122). Antagonist of the PPARγ signalling pathway, 24.61 µM (AID:743194). Thyroid receptor antagonist, 27.36 µM (AID:743064) and androgen receptor (AR) antagonist, 49.09 µM (AID:743033).	AhR+ PPARγ+ TR+ AR+
Chrysene	Also known as Benzo[a]phenanthrene. Polycyclic aromatic hydrocarbon, consisting of four fused benzene rings, that occurs in coal tar. Primarily the product of incomplete combustion.	qHTS rat Pregnane X Receptor (PXR) signalling pathway activator, 1.995 µM (AID:651741), and activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 4.42 µM (AID:743122) and 11.22 µM (AID:651777).	PXR+ AhR+
Cimetidine	A histamine H2-receptor antagonist that inhibits stomach acid produciton, used in the treatment of heartburn and peptic ulcers.		
Ciprofloxacin	A broad-spectrum antimicrobial carboxyfluoroquinoline used in the treatment of respiratory, urinary tract, gastrointestinal and abdominal infections.	Inhibition of human CYP2C19 (Cytochrome P450 2C19) (AID:678712).	
Clarithromycin	A semi-synthetic macrolide antibiotic structurally related to erythromycin. It inhibits protein synthesis in bacteria by reversibly binding to the 50S ribosomal subunits. This inhibits the translocation of aminoacyl transfer-RNA and prevents peptide chain elongation.	Inhibition of human CYP2C9 (Cytochrome P450 2C9) (AID:668268).	
Clenbuterol	A substituted phenylaminoethanol that has beta-2 adrenomimetic properties at very low doses, used as a bronchodilator in asthma.	Adrenergic beta2 agonist, 0.013 µM (AID:395059).	
Clofibrate	A fibric acid derivative used in the treatment of hyperlipoproteinemia type III and severe Hypertriglyceridemia. It increases lipoprotein lipase activity, promoting conversion of VLDL to LDL.	ERα signalling pathway agonist in qHTS assay, 26.6 µM (AID:743079). PPARα agonist (AID:742844). Inhibition of human CYP2C19 (AID:678712).	ER+ PPARα +
Clotrimazole	An imidazole derivative with broad-spectrum antimycotic activity. Clotrimazole inhibits biosynthesis of the fungal cell membrane component, ergostol, increasing membrane permeability and disruption of enzyme systems bound to the membrane.	Antagonist of PPARγ signalling pathway, 11.88 µM (AID:743191) and antagonist of ER signaling pathway, 6.44 µM (AID:743069). Inhibitor of human Cytochrome P450 17A1 activity, 0.082 µM (AID:53377), among others.	PPARγ+ ER+
Cocaine	An alkaloid ester extracted from the leaves of plants including coca, functioning as a local anaesthetic and vasoconstrictor. Potent central nervous sytems effects, involving multiple mechanisms on catecholaminergic neurons.	Inhibitor of Sodium-dependent serotonin transporter and sodium-dependent noradrenaline transporter.	
Codeine	Also known as 3-methylmorphine, codeine is a naturally occurring methylated morphine, used for its analgesic, antitussive, antidiarrheal, antihypertensive, anxioltic, antidepressant, sedative and hypnotic properties.	Inhibition of sterorspecific [H_3]-naltrexone (10e-9 M) binding towards opiate receptor, 0.01 µM (AID:145933). qHTS agonist of the AR signaling pathway, 61.44 µM (AID:743040).	AR+

Cortisol	The main glucocorticoid secreted by the adrenal cortex, also used in the treatment of inflammation, allergy, collagen diseases, asthma, adrenocortical deficiency, shock, and some neoplastic conditions.	qHTS assay for agonists of the Human AR signalling pathway, 0.0103 μM (AID:743053) and glucocorticoid receptor (GR) signalling pathway, 0.039 μM (AID:720719). Agonist of the ERα signalling pathway in the BG1 cell line, 3.75 μM (AID:743079).	AR+ GR+
Cortisone	A naturally occuring glucocorticoid, which though inactive in itself, is converted to hydrocortisone in the liver. Used as an anti-inflammatory agent and in adrenal insufficiency.	Agonist of AR signaling pathway in qHTS assay, 0.014 μM (AID:743036), and glucocorticoid receptor (GR) signaling pathway, 6.92 μM (AID:720692).	AR+ GR+
Coumestrol	A phytochemical, coumestrol is a daidzein derivative, occuring naturally in forage crops. Detected in clover, legumes, soybeans, brussel sprouts and spinach.	Human ERα binding affinity, IC_{50} 0.002 μM (AID:70514) and ERα agonist in qhTS assay, 0.057 μM (AID:743079). Inhibitor of human Glutaminase (GLS), 7.94 μM (AID:624170). Aryl Hydrocarbon Receptor (AhR) signaling pathway, 46.5 μM (AID:743122).	ER+ AhR+
Cyanazine	Cyanazine is the active triazine substance in an effective herbicide, registered as a Plant Protection Product.	qHTS activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 61.01 μM (AID:743085).	AhR+
Cyclophosphamide	Precursor of an alkylating nitrogen mustard antineoplastic and immunosuppressive agent that is activated in the liver, previously used in the treatment of lymphoma and leukemia.	Inhibition of Cytochrome P450 enzymes (AID:678712).	
Cyhalothrin	A pyrethroid insecticide that mimics the structure and insecticidal properties of pyrethrum.		
Cypermethrin	A synthetic pyrethroid used as an insecticide in large-scale commercial agriculture and consumer goods.	Inhibitor of Aldehyde Dehydrogenase 1 (ALDH1A1), 25.12 μM (AID:1030). qHTS activator of Human PXR, 35.48 μM (AID:720659).	PXR+
Cyproconazole	Broad-spectrum fungicide.	qHTS aromatase inhibitor, 24.19 μM (AID:743083). Activator of rat Pregnane X Receptor (rPXR) signalling pathway, 31.62 μM (AID:651751).	PXR+
Daidzein	Isoflavone present in a number of plants and herbs.	qHTS antagonist of human ERα signalling pathway, 0.0015 μM (AID:743075). Binding affinity against human ERβ, IC_{50} 0.303 μM (AID:70514) and diaplacement of estradiol from human recombinant ERβ , IC_{50} 1.2 μM (AID:361464). Activator of the Aryl Hydrocarbon Receptor (AhR) signaling pathway, 4.86 μM (AID:743085). Glucocorticoid Receptor antagonism, 4.89 μM (AID:720692). Activator of PXR signaling pathway, 44.67 μM (AID:720659).	ER+ AhR+ GR+ PXR+
DDT	Dichlorodiphenyltrichloroethane (DDT) is an organochloride insecticide, resistant to destruction by light and oxidation. These unusual properties have led to residues in water, soil and foodstuffs, despite its ban.	Agonist of the human ERα signalling pathway in qHTS assay, 4.36 μM (AID:743075), and in the BG1 cell line, 6.16 μM (AID:743079). Agonist of the vitamin D receptor (VDR), 13.8 μM (AID:743224). Antagonist of the Glucocorticoid Receptor (GR) signalling pathway, 16.93 μM (AID:720692). Antagonist of the PPARγ pathway, 17.37 μM (AID:743194) and PPARδ at 49.32 μM (AID:743211). Thyroid Receptor (TR) antagonism in qHTS assay at 18.83 μM (AID:743064). Human PXR activator at 39.8 μM (AID:720659). Antagonist of the human AR signalling pathway at 43.64 μM (AID:743035).	ER+ GR+ PPARγ+ TR+ PXR+ AR+
Dehydroabietic Acid	Diterpene component, that is a component of resin acid, used in soap manufacture.		

Dehydrotestosterone	Also known as Boldenone, dehydrotestosterone is an anabolic steroid developed for veterinary use, mostly for horses - stimulating anabolism and inhibiting catabolism, stimulating muscle mass, strength and power.	Androgen Receptor agonist (Merlanti *et al.*, 2007).	AR+
Deltamethrin	Pyrethroid ester insecticide, preventing spread of diseases carried by tick-infested prairie dogs, rodents and burrowing animals. Also eliminates spiders, fleas, ticks, carpenter ants, carpenter bees, cockroaches and bed-bugs.	human Pregnane X Receptor (PXR) signalling pathway activator, 11.22 µM (AID:720659) and TR signalling pathway antagonist, 71.27 µM (AID:743067).	PXR+ TR+
Demeclocycline	Tetracycline antibiotic derived from *Streptomyces aureofaciens*. Demeclocycline excreted slower than tetracycline, increasing the half life of the drug.		
Demeton-s-methyl	Demeton-s-methyl is a flammable organothiophosphate acaricide and an aliphatic organothiophosphate insecticide.	Active in qHTS assay for agonists of the antioxidant response element (ARE) signalling pathway, 54.48 µM (AID:651741).	
Desethylatrazine (DEA)/ 6-Chloro-2N-(propan-2-yl)-1,3,5-triazine-2,4-diamine	Desethylatrazine is the degradation product of atrazine, a widely used herbicide.		
DET/ N,N-Diethyl-2-(1H-Indol-3-yl) ethanamine	DET, also known as diethyltryptamine, is a psychedelic drug, active orally (50-100 mg) without the MAO inhibitors, lasting 2-4 hours, believed to work via serotonin receptor agonism. DET is a substituted tryptamine, structurally similar to DMT and dipropyltryptamine (DPT).		
Dexamethasone	An anti-inflammatory 9-fluoro-glucocorticoid	Activity at human Glucocorticoid receptor in CV1 cells, ED_{50} 0.0001 µM (AID:330359), GR agonistic activity IC_{50} 0.00051 µM (AID:626146). Androgen Receptor signalling pathway agonist in MDA cell line, 0.0078 µM (AID:743040). qHTS rat pregnane X receptor signalling pathway activator, 6.31 µM (AID:651751). DRUGMATRIX: Progesterone radioligand binding, IC_{50} 23.29 µM (AID:625172) and Androgen AR radioligand binding , IC_{50} 24.56 µM (AID:625228).	GR+ AR+ PXR+ PR+
Di-(2-ethylhexyl)phthalate (DEHP)/ Bis(2-ethylhexyl) phthalate	An ester of phthalic acid, DEHP is an odourless liquid plasticiser for resins and elastomers. DEHP is the most common class of phthalate plasticisers, accounting for 54% of the market share (***http://www.ceresana.com/en/market-studies/additives/plasticizers/***)	Activator of the rat pregnane X receptor signalling pathway, 11.22 µM (AID:651751). Human Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 24.34 µM (AID:743085).	AhR+ PXR+
Diatrizoic acid	A commonly used X-ray contrast medium used for gastrointestinal studies, angiography and urography.		
Diazepan	A benzodiazepine with anticonvulsant, anxiolytic, sedative, muscle relaxant and amnesic properties, actions of which are mediated by enhancement of gamma-aminobutyric acid activity.	Active in qHTS Human ERα signalling pathway antagonist in the BG1 cell line, 23.71 µM (AID:743080) and antagonist of the AR signalling pathway, 15.72 µM (AID:743035). Antagonist of the Glucocorticoid receptor (GR) signalling pathway, 13.45 µM (AID:720692). Thyroid Receptor signalling pathway antagonist, 69.63 µM (AID:743067).	ER+ AR+ GR+ TR+

Diazinon	A cholinesterase inhibitor that is used as an organothiophosphorus insecticide, used to control cockroaches, silverfish, ants and fleas.	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 6.86 μM (AID:743085). Antagonist of the Thyroid Receptor signalling pathway, 17.23 μM (AID:743064) and aromatase inhibitor, 17.23 μM (AID:743083). Antagonist of the Glucocorticoid receptor signalling pathway, 43.64 μM (AID:720693). PPARδ signalling pathway agonist, 54.94 μM (AID:743211).	AhR+ TR+ GR+ PPARδ +
Dibenzo(a,h)anthracene/ Dibenz[a,h] anthracene	Also known as 1,2,5,6-dibenzanthracene.	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 0.085 μM (AID:743085). Agonist of ER signalling pathway, 7.69 μM (AID:743077) and thyroid hormone receptor beta agonist, 39.81 μM (AID:588545).	AhR+ ER+ TR+
Dibromochloromethane/ Chlorodibromomethane	Dibromochloromethane is a trihalomethane compound formerly used as a flame retardant and laboratory reagent. Dibromochloromethane is also formulated during drinking water chlorination processes and by ocean algae in small quantities.	qHTS antagonist of the androgen receptor (AR) signalling pathway in the MDA cell line, 60.08 μM (AID:743042).	AR+
Dichlorobromomethane	Bromodichloromethane is a trihalomethane previousoly used as a flame retardant, solvent for fats and mineral separation. Also occurs in municipally-treated drinking water as a by product of chlorination.	qHTS agonist of the human AR signalling pathway, 21.95 μM (AID:743036).	AR+
Dicyclohexylphthalate (DCPH)	Dicyclohexylphthalate is used as a plasticiser for nitrocellulose, ethyl cellulose, chlorinated rubber, polyvinyl acetate, polyvinyl chloride and other polymers.	Antagonist of the Thyroid Receptor (TR) signalling pathway, 22.86 μM (AID:743064). qHTS agonist of the PPARδ signalling pathway, 46.01 μM (AID:743211). Aromatase inhibitor, 72.3 μM (AID:743084).	TR+ PPARδ +
Dichlorvos	An organophosphorus insecticide that inhibits acetylcholinesterase.	qHTS agonist of the AR signalling pathway, 4.95 μM (AID:743036) and an antagonist of ERα signalling pathway, 10.68 μM (AID:743069). Human Pregnane X receptor (PXR) signalling pathway activator, 39.81 μM (AID:720659). Antagonist of PPARγ signalling pathway (AID:743199).	AR+ ER+ PXR+ PPARγ+
Diclofenac	A non-steroidal anti-inflammatory agent (NSAID) with antipyretic and analgesic actions. Diclofenac is used to treat pain, inflammatory disorders and dysmenorrhea.	*In vitro* inhibitory activity against human whole blood Prostaglandin G/H synthase 2, IC_{50} 0.05 μM (AID:162494).	
Dicofol	Dicofol is an organochlorine insecticide, structurally related to DDT, used to control the red spider mite.	Antagonist of the PPARγ signalling pathway, 15.48 μM (AID:743194). Antagonist of the Androgen Receptor (AR) signalling pathway, 48.97 μM (AID:743033). Aromatase inhibitor, 61.13 μM (AID:743084).	PPARγ+ AR+
Dieldrin	An organochlorine insecticide, used to control locusts, tropical disease vectors and non-food seed and plant treatment.	DRUGMATRIX: Androgen AR radioligand binding (Mibolerone), IC_{50} 6.89 μM (AID:625228). Aromatase inhibitor, 17.22 μM (AID:743083). Antagonist of the thyroid receptor signalling pathway, 30.64 μM (AID:743064). Activator of human PXR signalling pathway, 39.81 μM (AID:720659).	AR+ TR+ PXR+
Diethylphthalate	DEP is a phthalate ester used as a plasticiser, detergent base and to bind cosmetics and fragrances.	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 62.19 μM (AID:743085).	AhR+

Diethylstilbestrol (DES)	A synthetic nonsteroidal estrogen used in the treatment of menopausal and postmenopausal disorders. DES was given to cattle as a growth supplement and misguidingly given to pregnant women to reduce the risk of birth complications.	*In vitro* agonist of ERα transcriptional activation in MCF-7 cells at 10 pM (EC_{50} 7e-0.6) (AID:102438). Activation of Estrogen Response element in HeLa cells stably transfected with human ERα, EC_{50} 2e-0.5 μM (AID:70505). Displacement of 0.5nM E2 from human ER , Ki 0.00049 μM (AID:70002). DRUGMATRIX Progesterone radioligand binding, IC_{50} 4.68 μM (AID:625172) and Glucocorticoid Radioligand binding, IC_{50} 10.6 μM (AID:625263). qHTS TR signaling pathway antagonist, 13.69 μM (AID:743065). Inhibitor of recombinant rat AR in *E. coli* using R1881, IC_{50} 14.125 μM (AID:255211), human AR antagonism confirmed in qHTS assay in MDA cell line, 27.72 μM (AID:743054).	ER+ PR+ TR+ AR+
Diethyltoluamide (DEET)/ N,N-Diethyl-3-methhylbenzamide	DEET is used as a topical insect repellent, providing protection against mosquitos, ticks and fleas.	qHTS assay ERα signalling pathway agonist, 5.31 μM (AID:743079). Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 60.51 μM (AID:743085).	ER+ AhR+
Difenoconazole	Difenoconazole is a broad spectrum fungicide used to control Aschomycetes, basidomycetes and deuteromycetes families, acting as a seed treatment, foliar spray and systemic fungicide.	Antagonist of ERα signalling pathway in the BG1 cell line, 11.29 μM (AID:743091). Thyroid receptor signalling pathway, 12.19 μM (AID:743065). qHTS androgen receptor antagonist in the MDA cell line, 13.69 μM (AID:743042). Aromatase inhibitor, 30.64 μM (AID:743083). Small molecule antagonist of the retinoid X receptor signalling, 39.81 μM (AID:588546). Antagonist of the glucocorticoid receptor signalling pathway, 43.64 μM (AID:720692). PPARδ signalling pathway agonist/antagonist 48.97 μM (AID:743211/743194).	ER+ TR+ AR+ RXR+ GR+ PPARδ+
Diflubenzuron	An insect growth regulator which interferes with the formation of the insectcuticle, effective in the control of mosquitoes and flies.	Human Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 0.086 μM (AID:743085). qHTS ERα signalling pathway agonist in the BG1 cell line, 4.33 μM (AID:743079). Retinoid X Receptor signalling agonist, 15.85 μM (AID:588544).	AhR+ ER+ RXR+
Digoxigenin	Digoxigenin is a steroid found in the flowers and leaves of the *Digitalis* species, which as a hapten, has many molecular biology applications. Digoxigenin is used as an all-purpose immunotag. The aglycon of digoxin.	Antagonist of Thyroid Stimulating Hormone Receptor (AID:504810).	
Dihydrotestosterone/ Stanolone	Also known as Androstanolone and Stanolone, dihydrotestosterone is a potent androgenic metabolite of testosterone produced by the action of the enzyme 3-Oxo-5α-steroid 4-deyhydrogenase.	AR agonist in mouse C2C12 cells, EC_{50} 5e-05 μM (AID:569780), activity in human Saos2 cells, IC_{50} 5e-05 (AID:319592). Displacement of DHT from human sex hormone binding globulin, Kd 0.000182 μM (AID:318680) and binding to human AR in CV1 cells, Ki 0.0002 μM (AID:290231). qHTS TR signalling pathway antagonist, 0.0014 μM (AID:743065). Agonist of ERα signaling pathway in BG1 cell line, 0.42 μM (AID:743079). Displacement of progesterone from rabbit PR, IC_{50} 0.44 μM (AID:578353). DRUGMATRIX: Progesterone radioligand binding, IC_{50} 1.25 μM (AID:625172). Angatonist of the Glucocorticoid Receptor (GR) signaling pathway, 1.396 μM (AID:743077). Agonist of Retinoid X Receptor alpha signalling, 7.94 μM (AID:588544). Antagonist of the thyroid receptor (TR) signalling pathway, 26.6 μM (AID:743067).	AR+ ER+ GR+ PR+ RXR+ TR+

Diisobutylphthalate (DIBP)	Prepared by the esterification of isobutanol and phthalic anhydride, DIBP, is a heat and light stable plasticiser. The concentration of mono-isobutyl phthalate (MIBP), a metabolite of DIBP steadily increased between 1999-2008 in American's urine (***http://www.cdc.gov/exposurereport/***).	qHTS ERα agonist in the BG1 cell line, 0.0027 μM (AID:743079). Active in aromatase inhibitor qHTS assay, 61.13 μM (AID:743083).	ER+
Diisodecylphthalate	Diisodecyl phthalate is used as an all purpose plasticiser for polyvinyl chloride.	qHTS antagonist of the Androgen receptor (AR) signalling pathway, 9.77 μM (AID:743063).	AR+
Diisononylphthalate	Diisononylphthalate (DINP) is a plasticiser used in food contact materials, typically consisting of various isononyl esters of phthalic acid.		
Dilantin/ Phenytoin	Also known as Phenytoin, Dilantin is an anticonvulsant, used in a wide variety of seizures, which also acts as an anti-arrhythmic and muscle relaxant.	qHTS human pregnane X receptor signalling pathway activator, 35.48 μM (AID:720659).	PXR+
Diltiazem	Diltiazem is a benxothiazepine, nondihydropyridine calcium channel blocker, used in the treatment of hypertension, angina pectoris and some types of arrhythmia.	Antagonist of thyroid receptor (TR) signalling pathway, 23.71 μM (AID:743064).	TR+
Dimethoate	An organothiophosphorus cholinesterase inhibitor that is used as a systemic and contact insecticide.	qHTS Aryl Hydrocarbon Receptor (AhR) signalling pathway (AID:743122).	AhR+
Dimethylformamide (DMFA)/ N,N-Diethylformamide	Dimethylformamide is an organic solvent with a low evaporation rate, which is used in the productio of acrylic fibres and plastics.	qHTS retinoid X receptor signalling agonist, 14.13 μM (AID:588544).	RXR+
Di-n-butylphthalate (DBP)	A plasticiser used in most plastics, which is also used in adhesives and printing inks.	qHTS ERα signalling pathway agonist in the BG1 cell line, 30.64 μM (AID:743079).	ER+
Diphenyl/ Biphenyl	Organic compound used as the starting material for the production of polychlorinated biphenyls (PCBs), but also used as an intermediate for the production of emulsifiers, optical brighteners, crop protection products and plastics.	qHTS agonist of the retinoid X receptor (RXR) signalling pathway, 35.48 μM (AID:588544).	RXR+
Diuron	Also known as DCMU or 3-(3,4-dichlorophenyl)-1,1-dimethylurea, Diuron is a herbicide introduced by Bayer that inhibits photosynthesis.	qHTS Aryl Hydrocarbon Receptor (AhR) signalling pathway, 22.82 μM (AID:743122).	AhR+
Domperidone	A specific dopamine receptor blocker, increasing the speed of gastrointestinal peristalsis and causes prolactin release.	Antagonise of the thyroid receptor signalling pathway, 26.60 μM (AID:743064).	TR+
Doxazosin	A quinazoline selective alpha-1-adrenergic blocker, used to treat high blood pressure and urinary retention associated with benign prostatic hyperplasia. Rlated to prazosin.		
Enalapril	Angiotensin-converting enzyme inhibitor used to treat hypertension.		
Endosulfan	A polychlorinated compound used as an insecticide, by repetitive nerve discharges increasing the temperature. Endosulfan is a neurotoxicant.	qHTS ERα signalling agonist, 1.41 μM (AID:588514), confirmed in the BG1 cell line, 1.935 μM (AID:743079). IC_{50} 23.44 μM against recombinant rat AR in *E. coli* (AID:255211). PPARγ signalling pathway antagonist, 24.53 μM (AID:743194) and PPARδ agonist, 24.53 μM (AID:743211). Aromatase inhibitor, 27.33 μM (AID:743080). Thyroid Receptor antagonist, 30.61 μM (AID:743064). Glucocorticoid Receptor signalling pathway antagonist, 48.94 μM (AID:720693).	ER+ AR+ TR+ PPARγ+ PPARδ + GR+

Epiandrosterone	A metabolite of testosterone and androstenedione with a 3-alpha hydroxyl group without the double bond. The 3-beta hydroxyl isomer of epiandrosterone.	Binds to Androgen Receptor (Stobaugh *et al.*, 1990).	AR+
Epichlorohydrin (-R)	Epichlorohydrin is organochlorine epoxide used in the production of glycerol, plastics, epoxy glues and resins, and elastomers.		
Epichlorohydrin (-S)	A chlorinated epoxy compound used as an industrial solvent, strong skin irritant and carcinogen.		
Epoxiconazole	Epoxiconazole is an azole fungicide, developed to protect cereals, wheat, barley, rye and triticale, soybeans, banana, rice, coffee, turnips and beats.	Aryl Hydrocarbon Receptor signalling pathway activator, 23.34 µM (AID:743085). Thyroid Receptor (TR) antagonist, 30.64 µM (AID:743065). Antagonist of the Androgen Receptor (AR) signalling pathway, 48.96 µM (AID:743035) and 66.01 µM in the MDA cell line (AID:743054). Estrogen Receptor (ERα) signalling pathway antagonist, 66.59 µM in the BG1 cell line (AID:743080). Aromatase inhibitor (AID:743139).	AhR+ TR+ AR+ ER+
Erythromycin	A bacteriostatic antibiotic macrolide produced by Streptomyces erythreus, which inhibits protein synthesis by binding to 50S ribosomal subunits, which inhibits peptidyl transferase activity and interferes with translocation of amino acids during translation and protein assembly.	Active in qHTS estrogen receptor alpha (ERα) signalling pathway agonist assay in the BG1 cell line, 9.44 µM (AID:743079). However, was inactive in 17 of 18 studies.	ER+
Esfenvalerate/ Fenvalerate	Also known as the S-enantiomer of Fenvalerate, esfenvalerate is a synthetic pyrethroid insecticide.	qHTS rat pregnane X receptor (rPXR) signalling pathway activator, 14.13 µM (AID:651751) and human PXR activator at 31.62 µM (AID:720659).	PXR+
Ethofenoprox/ Etofenprox	Ethofenoprox is a pyrethroid insectice, used in veterinary medicines.		
Ethylparaben/ Ethyl-4-hydroxy benzoate	Ethylparaben (ethyl-p-hydroxybenzoate) is an antifungal preservative, used as a food additive (E number E214). Sodium ethyl para-hydroxybenzoate, the sodium salt of ethylparaben, has the same uses and is given the E number E215. Also used to preserve pharmaceuticals and cosmetic products.	qHTS human estrogen receptor alpha (ERα) signalling pathway agonist in the BG1 cell line, 23.71 µM (AID:743079). DSSTox (NCTRER) National Center for Toxicological Research Estrogen Receptor Binding Database (AID:1204).	ER+
Etofenamate	Used in the treatment of joint and muscular pain, Etofenamate is a non-steroidal anti-inflammatory drug.		
Etofibrate	Etofibrate is a amphipathic carboxylic acid, used to treat metabolic disorders, such as hypercholesterolemia, and is a hypolipidemic agent.		
Etridiazole/ Imidazole	Also known as Terrazole, Etridiazole is a fungicide used to control *Pythium* and *Phytophthora* root rot and stem rot, commonly used on turf and nursing ornamentals. It is also used in barley, bean, corn, cotton, peanut, pea, sorghum, soybean, safflower and wheat production.		

Exifone	An antiplatelet drug	qHTS activator of the Human Aryl Hydrocarbon Receptor (AhR) signalling pathway, 20.48 μM (AID:743122). Androgen Receptor signalling pathway antagonist, 24.54 μM (AID:743035). Aromatase inhibitor, 34.27 μM (AID:743084). qHTS Thyroid Receptor (TR) signalling pathway, 43.15 μM (AID:743056). ERα signalling antagonist in the BG1 cell line, 61.13 μM (AID:743081).	AhR+ AR+ TR+ ER+
Famotidine	Famotidine is a competitive histamine H2-receptor antagonist, inhibiting gastric secretion and used in the treatment of ulcers.		
Fenarimol	Fenarimol inhibits fungal biosynthesis, and is used to protect against rusts, blackspot and mildew.	qHTS ERα signalling agonist in BG1 cell line, 9.69 μM (AID:743079). Androgen Receptor signalling antagonist, 24.54 μM (AID:743035). Thyroid Receptor signalling pathway antagonist, 36.34 μM (AID:743014). Glucocorticoid Receptor signalling pathway antagonist, 48.96 μM (AID:720692).	ER+ AR+ TR+ GR+
Fenitrothion	Dimethyl o-(3-methyl-4-nitrophenol) phosphorothioate or Fenitrothion is an organophosphate insecticide and acaricide used in greenhouses.	qHTS Human AR signalling pathway antagonist, 7.76 μM (AID:743063). Activator of the Aryl Hydrocarbon Receptor signalling pathway, 21.69 μM (AID:743085). Aromatase inhibitor (AID:743129).	AR+ AhR+
Fenofibrate	Antilipemic fibrate drug used to reduce cholesterol and triglycerides in the blood.	PPARα signalling pathway agonist, EC_{50} 30 μM (AID:91237). Binding affinity at PPARγ, EC_{50} 41 μM (AID:317698). Androgen Receptor signalling antagonist in the MDA cell line, 48.56 μM (AID:743042). ERα signalling, 61.13 μM (AID:743091). Thyroid Receptor signalling antagonist, 61.13 μM (AID:743065).	PPARα + PPARγ+ AR+ ER+ TR+
Fenoprofen (FNP)	Fenoprofen is pharmacologically similar to Aspirin, functioning as an anti-inflammatory analgesic, typically used to relieve the symptoms of rheumatoid arthritis, osteoarthritis and pain. Inhibits cyclooxygenase.		
Fenoterol	Fenoterol is a sympathomimetic beta-2-adrenergic agonist, used in the treatment of asthma as a bronchodilator and tocolytic.		
Fenothrin	Fenothrin (Phenothrin) is a synthetic pyrethroid insecticide, used to control lice, fleas and ticks.		
Fenoxycarb	Fenoxycarb is a carbamate insecticide, which mimics juvenile hormones, preventing insects from reaching maturity.	qHTS PPARγ signalling pathway antagonist, 38.90 μM (AID:743191). Estrogen Receptor (ERα) signalling pathway antagonist, 38.90 μM (AID:743069) and 43.64 μM (AID:743078). Pregnane X Receptor (PXR) signalling activator, 50.12 μM (AID:720659). Antagonist of AR signalling in the MDA cell line, 54.47 μM (AID:743042).	PPARγ+ ER+ PXR+ AR+
Fentiazac	Non steroidal anti-inflammatory analgesic used in muscular and joint pain.	Agonist of human PPARγ signalling pathway, 1.51 μM (AID:743140). Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 29.85 μM (AID:743122).	PPARγ+ AhR+
Fenvalerate	Insecticide used to control insects in food, feed and cotton products, and flies and ticks in barns and stables.	Agonist of Estrogen Receptor (ERα) signalling pathway in the BG1 cell line, 13.69 μM (AID:743079). Thyroid Receptor signalling antagonist, 17.47 μM (AID:743067). Antagonist of the AR signalling pathway, 50.89 μM (AID:743063).	ER+ TR+ AR+

Ferulic acid	A phenolic compound in asafoetida, from fennel (*Ferula communis*), Ferulic acid has antibacterial and antioxidant properties. Pharmacological actions include, anti-inflammatory, anticoagulant, antihypertensive and cholagogues and choleretic actions. Found widely in soil humus.		
Fipronil	Broad-spectrum insecticide that disrupts the central nervous system via GABA receptors.	Antagonist of the AR signalling pathway in the MDA cell line, 1.14 µM (AID:743054). ERα signalling pathway antagonist in the BG1 cell line, 7.50 µM (AID:743080). Pregnane X Receptor signalling pathway activator, 12.59 µM (AID:720659). Aromatase inhibitor, 19.45 µM (AID:743083). Thyroid Receptor signalling antagonist, 26.60 µM (AID:743065). PPARδ signalling pathway antagonist, 43.90 µM (AID:743213) and PPARγ at 49.26 µM (AID:743194).	AR+ ER+ PXR+ TR+ PPARδ + PPARγ+
Flumethasone	Flumethasone is an anti-inflammatory glucocorticoid in veterinary practices, structurally related to dexamethasone.	qHTS Glucocorticoid Receptor (GR) signalling pathway agonist, 0.0016 µM (AID:720719). Androgen receptor signalling pathway agonist in the MDA cell line, 0.0025 µM (AID:743040).	GR+ AR+
Fluorene	Product of incomplete combustions. Fluorene is extractable from coal tar, but can also be synthesied from the dehydrogenation of diphenylmethane.	qHTS Thyroid Receptor (TR) signalling pathway antagonist, 66.01 µM (AID:743067).	TR+
Fluoxetine	Commonly known as Prozac, fluoxetine is a selective serotonin reuptake inhibitor (SSRI), used as an antidepressant, which is prescribed in the treatment of depression, obsessive-compulsive disorder, bulimia nervosa, panic disorder and premenstrual dysphoric disorder.	qHTS Peroxisome proliferator-activated receptor delta (PPARδ) signalling antagonist, 9.52 µM (AID:743213). Antagonist of the thyroid receptor (TR) signalling pathway, 11.88 µM (AID:743065). Aryl Hydrocarbon Receptor (AhR) signalling activator, 23.7 µM (AID:743086). Antagonist of the AR signalling pathway in the MDA cell line, 27.82 µM (AID:743041).	PPARδ + TR+ AhR+ AR+
Flurbiprofen	An anti-inflammatory analgesic and antipyretic, Flurbiprofen inhibits carbonic anhydrase and is used in peridontal disease.	qHTS agonist of the Estrogen Receptor (ERα) signalling pathway in the BG1 cell line, 4.52 µM (AID:743079). Agonist of the PPARγ signalling pathway (AID:743140).	ER+ PPARγ+
Fluticasone	Fluticasone is a synthetic glucocorticoid agonist, used as an anti-allergic, anti-inflammatory, bronchodilator and dermatologic agent.	Androstane	GR+
Flutriafol	Flutriafol is a pesticide used to control fungal disease on wheat, barley and canola.	Aromatase inhibitor (AID:743139).	
Fluvalinate	As a pyrethroid insecticide, Fluvalinate is used to control varroa mites in honey bea colonies.		
Formononetin	Formononetin is phytochemical present in leguminous plants and Fabaceae (beans and soy).	qHTS agonist of the ERα signalling in the BG1 cell line, 1.54 µM (AID:743079). Activator of the Aryl Hydrocarbon Receptor (AhR), 5.05 µM (AID:743122).	ER+ AhR+
Furosemide	Furosemide is a benzoic-sulfonamide-furan diuretic used in the treatment of congestive heart failure and edema.	Estrogen Receptor (ERα) signalling pathway agonist, 14.96 µM (AID:743079). Androgen Receptor (AR) signalling antagonist, 26.83 µM (AID:743063).	ER+ AR+
Gabapentin	Also known as Neurontin, Gabapentin is used in the treatment of epilepsy and neuropathic pain.		
Galaxilide	Also known as Galoxolide, Galaxolide is a polycyclic aromatic synthetic musk used in soaps, cosmetics and detergents.	Agonist of ERα signalling pathway in qHTS assay, 11.22 µM (AID:588514).	ER+

Gemfibrozil (GFB)	Gemfibrozil is a lipid-regulating agent that decreases serum triglycerides; incrases HDL subfraction HDL2 and HDL3, in addition to apolipoproteins A-I and A-II.	Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 48.90 µM (AID:743085).	AhR+
Genistein	An isoflavonoid compound that inhibits protein-tyrosine kinase and topoisomerase-II, and has been identified as an antineoplastic and antitumor agent.	Displacement of [H_3]E2 from human ERβ in 293T cells, IC_{50} 0.0013 µM (AID:257300). Inhibition of human ERα LBD, IC_{50} 0.0097 µM (AID:292714). Cytotoxicity against human MCF-7 cells after 48 hours by MTT assay, IC_{50} 1 µM (AID:517124). Aryl Hydrocarbon Receptor signalling pathway activator, 3.35 µM (AID:743085). Thyroid Receptor antagonist, 11.88 µM (AID:743065). PPARγ signalling pathway antagonist, 17.37 µM (AID:743191). Human Pregnane X receptor (PXR) signalling, 19.95 µM (AID:720659). Androgen Receptor (AR) signalling pathway antagonist, 24.13 µM (AID:743063). Retinoid X Receptor signalling antagonist, 35.48 µM (AID:588546). Antagonist of the glucocorticoid receptor (GR) signalling pathway, 54.02 µM (AID:720693).	ER+ AhR+ TR+ PPARγ+ PXR+ RXR+ AR+ GR+
Glufosinate	Glufosinate interferes with the biosynthetic pathway of glutamine, and is used in non-selective herbicides and pre-harvest crop desiccation.		
Glycitein	As an O-methylated isoflavone, glycitein is a phytestrogen present in soy products.	Activity in human ER expressed in transfenic Arabidopsis plant at 5 µM by pER8-GFP reporter assay (AID:402363).	ER+
Glyphosate	Systemic broad-spectrum herbicide, used on broadleaf weeds and grasses.		
Hexachlorobenzene (HCB)	An agricultural fungicide and seed treatment.		
Hydrochlorothiazide	A thiazide diuretic used in the treatment of edema, hypertension, diabetes insipidus and hypoparathyroidism.	Peroxisome proliferator-activated receptor delta (PPARδ) signalling agonist, 38.32 µM (AID:743213).	PPARδ +
Hydrocinnamic acid/ 3-Phenylpropanoic acid	Hydrocinnamic acid is used as a preservative, prolonging the life of frozen foods, spices, fragrances and medicides.		
Hydroxyhydro quinone/ 1,2,3-Benzenetriol	Also known as hydroxyquinol is a benzenetriol biodegradation product of *Bradyrhizobium japonicum.*		
Ibuprofen	Ibuprofen is an anti-inflammatory analgesic used in the management of rheumatism and arthritis.	Inhibition activity against recombinant human Prostaglandin G/H synthatse 2, IC_{50} 0.1 µM (AID:162632). Active in qHTS assay for ERα signalling pathway agonists, 47.6 µM (AID:743079).	ER+
Ifosfamide	Immunosuppressive alkylating agent, trade name Mitoxana® is a chemotherapeutic agent used to treat sarcoma, testicular cancer and some lymphomas.		

Imazalil/ Enilconazole	Also known as Enilconazole, Imazalil is fungicide widely used in the post-harvest treatment of bananas and citrus fruits.	Human aromatase inhibitor, 0.387 μM (AID:743139). Aryl hydrocarbon receptor (AhR) signalling pathway activator, 8.66 μM (AID:743122). Pregnane X receptor signalling pathway activator, 15.85 μM (AID:651751). Glucocorticoid receptor signalling pathway antagonist, 21.94 μM (AID:720693). PPARδ signalling pathway antagonist, 24.54 μM (AID:743215). Thyroid Receptor signalling pathway antagonist, 30.64 μM (AID:743065). Estrogen Receptor antagonist in the BG1 cell line, 48.56 μM (AID:743091). Androgen receptor antagonist, 49.12 μM (AID:743033). Aromatase inhibitor (AID:743139).	AhR+ PXR+ PPARδ + TR+ ER+ AR+
Indapamide	Benzamide-sulfonamide-indole diuretic that inhibts sodium chloride symporters. Indapamide is used in the treatment of hypertension.		
Indenol(1,2,3-cd)pyrene	Polycyclic aromatic hydrocarbon that occurs in coal tar. Primarily the product of incomplete combustion, including vegetation and food products.		
Indomethacin (IDM)	Used to reduce fever, pain, stiffness and swelling, indomethacin inhibits cyclooxygenases enzymes necessary for the formation of autacoids, such as prostaglandins. Indomethacin is a non-steroidal anti-inflammatory agent which also inhibts the motility of polymorphonuclear leukocytes.	PPARγ signalling pathway agonist, 1.96 μM (AID:743094).	PPARγ+
Iohexol	low-chemotoxic non-ionic water soluble contrast agent used in myelography, arthrography, nephroangiography, anteriography and radiographic procedures.		
Iopamidol	Non-ionic water soluble contrast agent used in myelography, arthrography, nephroangiography, anteriography and radiographic procedures.	Active in qHTS Androgen Receptor (AR) signalling pathway agonist assay, in the MDA cell line, 10.12 μM (AID:743040). Glucocorticoid Receptor signalling pathway agonist, 7.23 μM (AID:720719).	AR+ GR+
Iopromide	Marketed under Ultravist, Iopromide is a non-ionic water soluble contrast agent.		
Iothalamic acid	Contrast medium.		
Ioxynil/ 4-Hydroxy-3,5-diiodobenzonitrile	Also known as Bentrol, nitrile substituted active ingredient in Plant Protection Product, used in combination with a number of herbicides.	Agonist of PPARα signalling pathway, 15.85 μM (AID:651778). qHTS antagonist of the Thyroid Receptor signalling pathway, 21.69 μM (AID:743065). ER signalling pathway antagonist, 27.54 μM (AID:743078). Androgen Receptor signalling pathway antagonist, 43.64 μM (AID:743033). Aromatase inhibitor (AID:743139).	PPARα + TR+ ER+ AR+
Iprodione	Marketed by Bayer CropScience, Iprodione is used to control Botrytis bunch rot, brown rot, Sclerotinia and other fungal diseases in plants.	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 4.86 μM (AID:743085). PPARγ antagonist, 44.67 μM (AID:588537).	AhR+ PPARγ+
Irgarol	Trade name Cybutryne, Biocide algaecide triazine that inhibits photosynthesis and is used in marine antifouling agent	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 0.49 μM (AID:743122). Androgen Receptor (AR) signalling pathway antagonist, 48.97 μM (AID:743033). PPARδ agonist, 48.97 μM (AID:743211).	AhR+ AR+ PPARδ +
Isoproturon	Isoproturon is a herbicide that disrupts photosynthesis and is applied to soil to control weeds.	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 61.07 μM (AID:743122).	AhR+

Kaempferol	Kaempferol and its glucoside is a natural flavonoid found in tea, broccoli, witch-hazel, grapefruit, kale, beans, endive, leek and a number of other plant sources.	Inhibition of Aryl Hydrocarbon Receptor (AhR), IC_{50} 0.028 µM (AID:311070). qHTS agonist of the estrogen receptor (ERα) signalling pathway, 7.94 µM (AID:588514), and 9.65 µM in the BG1-cell line (AID:743079). Antagonism of the Androgen Receptor in MDA-kb2 cells, assessed as inhibition of DHT-induced luciferase activity, IC_{50} 9.7 µM (AID:429199). Antagonist of PPARγ signalling pathway, 29.47 µM (AID:743191). qHTS activator of the human PXR signalling pathway, 44.67 µM (AID:720659). Antagonist of the thyroid receptor (TR) signalling pathway, 60.88 µM (AID:743065). Inhibition of PPARγ-meidated adipocyte differentiation in mouse 3T3L1 cells (AID:517389). Antagonist of the Glucocorticoid receptor signalling pathway (AID:720725).	AhR+ ER+ AR+ PPARγ+ PXR+ TR+ GR+
Ketoconazol	Broad-spectrum antifungal agent, typically used in immunosupressed patients, at high doses for long periods of time.		
Ketoprofen (KTP)	Non-steroidal anti-inflammatory drug, with analgesic and antipyretic properties similar to ibuprofen, used in the treatment of rheumatoid arthritis and osteoarthritis.		
Ketorolac	Non-steroidal anti-inflammatory (NSAID) pyrrolizine carboxylic acid derivative, related to indomethacin, used as an analgesic.		
Lansoprazole	A 2,2,2-trifluoroethoxypyridyl derivative of timoprazole used in the treatment of stomach ulcers, inhibits an ATPase found in gastric parietal cells.	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 4.22 µM (AID:743085). Antagonist of the PPARγ signalling pathway (AID:743199).	AhR+ PPARγ+
Levonorgestrel	A synthetic progestational hormone with actions similar to those of progesterone, it is used for contraception, control of menstrual disorders and treatmetn of endometriosis.	Progesterone Receptor agonist (AID:742449). qHTS agonist of the AR signalling pathway, 0.001 µM (AID:743053). Displacement of [3H]5α-Dihydrotestosterone from human Sex Hormone Binding Globulin, Kd 0.00123 µM (AID:318680). Agonism of AR in MDA cell line, 0.0047 µM (AID:743040). Antagonism of AR signalling pathway, 0.017 µM (AID:743035). Agonism of ERα Signalling pathway in BG1 cell line, 0.039 µM (AID:743079). qHTS Glucocorticoid Receptor signalling pathway antagonist (AID:720725).	AR+ PR+ ER+ PR+
Lincomycin	A *Streptomyces lincolnesis* antibiotic used in the treatment of staphylococcal, streptococcal and bacteroides fragilis infections.		
Lindane	Organochlorine insecticide, also known as hexachlorocyclohexane and Gammaxene, used as a pediculicide and scabicide. Lindane is approved for use as a second-line topical treatment therapy for *Pediculosis capitis* (head lice) by the FDA.	qHTS agonist of the Estrogen Receptor (ERα) signalling pathway, 0.195 µM (AID:743075), and in the BG1 cell line, 3.86 µM (AID:743079). Thyroid Receptor (TR) signalling pathway antagonist, 33.49 µM (AID:743067). Glucocorticoid Receptor (GR) signalling pathway agonist, 39.68 µM (AID:720691). qHTS activator of the human PXR signalling pathway, 44.67 µM (AID:720659). PPARδ signalling pathway agonist, 52.87 µM (AID:743227). Activator of the Aryl Hydrocarbon Receptor (AhR), 61.69 µM (AID:743085).	ER+ TR+ GR+ PXR+ PPARδ + AhR+

Linuron	Also known as Afalon, Lorox and Linurex, Linuron is a pre- and post-emergence herbicide.	qHTS activator of the AhR signalling pathway, 8.64 μM (AID:743085). Antagonist of the AR signalling pathway, 34.82 μM (AID:743063). Thyroid Receptor (TR) signalling pathway, 61.13 μM (AID:743067).	AhR+ AR+ TR+
Loratadine	Second-generation histamine H1 receptor antagonist used in the treatment of allergic rhinitis and urticaria.	Thyroid Receptor signalling pathway antagonist, 9.69 μM (AID:743065). Agonist of the PPARδ signalling pathway, 10.59 μM (AID:743211). Antagonist of the PPARγ signalling pathway, 13.33 μM (AID:743191). Antagonist of the AR signalling pathway, 17.37 μM (AID:743035). Glucocorticoid Receptor (GR) signalling pathway antagonist, 23.92 μM (AID:720692). ERα signalling pathway antagonist, 22.58 μM (AID:743078).	TR+ PPARδ + PPARγ+ AR+ GR+ ER+
Malathion	Alphatic organophorphate (organophosphate parasympathomimetic) broad-spectrum insecticide, used commercially and domestically, that binds to cholinesterase.	Activator fo the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 32.97 μM (AID:743122).	AhR+
Maneb/ Amobam	Complexed manganese derivative of ethylenebisdithiocarbamate used as an agricultural fungicide. Maneb has been shown to affect glucocorticoid metabolism by interfering with the conversion of cortisol to cortisone, via the 11β-hydroxysteroid dehydrogenase type-2 enzyme.		
MCPA/ 4-Chloro-2-methylphenoxy) acetic acid	2-Methyl-4-chlorophenoxyacetic acid (MCPA) is a powerful selective phenoxy herbicide (weed killer).		
Meclocycline/ Samil	Meclocycline (INN), also known as Meclociclina and Meclocyclinum, INN is a tetracycline antibiotic, used topically to treat skin infections. INN is not taken orally as it may cause systemic liver and kidney damage.		
Meclofenamic acid/ Meclofenamate	Branded as Meclomen, Meclofenamic acid is NSAID agent with antipyretic and antigranulation activity, while inhibiting prostaglandin biosynthesis, typically used for joint, muscular pain, arthritis and dysmenorrhea.		
Mecoprop/ 2-(4-Chloro-2-methylphenoxy)propanoic acid	Also known as methylchlorophenoxypropionic acid or MCPP, Mecoprop is a broad-spectrum herbicide, primarily used to control broadleaf weeds.		
Mefenamic acid	Non-steroidal anti-inflammatory cyclooxygenase inhibitor analgesic with antipyretic properties, used in the treatment of menstrual pain.	qHTS antagonist of the Thyroid Receptor (TR) signalling pathway, 21.13 μM (AID:743065) and antagonist of the Glucocorticoid Receptor (GR) signalling pathway, 48.97 μM (AID:720692). Antagonist of the PPARγ signalling pathway (AID:743199).	TR+ GR+ PPARγ+
Mestranol	As a 3-methyl ether prodrug of ethinylestradiol, Mestraol has to be demethylated in the liver to be biologically active, and is a component of some oral contraceptives.	qHTS agonist of ERα signalling pathway in BG1 cell line, 0.0008 μM (AID:743079), Drugmatrix ER IC_{50} 0.018 μM (AID:625258). Androgen Receptor (AR) signalling pathway antagonist, 3.34 μM (AID:743063) and Drugmatrix AR IC_{50} 10.68 μM (AID:625228). Glucocorticoid Receptor (GR) signalling pathway antagonist (AID:720725).	ER+ AR+ GR+

Methoxychlor	Synthetic organochlorine insecticide used to protect crops, ornamentals, livestock and pets.	qHTS human ERα signalling pathway agonst in BG1 cell line, 6.08 μM (AID:743079). Antagonist of the PPARγ signalling pathway, 13.60 μM (AID:743194). Androgen Receptor (AR) signalling pathway antagonist, 17.48 μM (AID:743063). Thyroid Receptor (TR) signalling pathway, 21.56 μM (AID:743064). Glucocorticoid Receptor (GR) signalling pathway, 24.39 μM (AID:720692). Pregnane X Receptor activation, 28.18 μM (AID:651751).	ER+ PPARγ+ AR+ TR+ GR+ PXR+
Methylester/ Methyl-2-amino-4,5-dimethylthiophene-3-carboxylate	Also known as methyl 2-amino-4,5-dimethylthiophene-3-carboxylate, classified as a PPP regulated product and chemical reagent.		
Methylparaben	Methylparaben, or Methyl 4-hydroxybenzoate, is the methyl ester of p-hydroxybenzoic acid, naturally found in fruits, which is commonly artificially added to cosmetics, personal care products and food items (E number E218).	qHTS activator of the Aryl Hydrocarbon Receptor signalling pathway, 69.29 μM (AID:743085). Listed in the DSSTox (NCTRER) National Center for Toxicological Research Estrogen Receptor Binding Database (AID:1204).	AhR+ ER+
Metolachlor	A derivative of aniline, metolachlor is an organic herbicide.	Androgen Receptor signalling pathway antagonist, 44.40 μM (AID:743063) and in the MDA cell line, 51.34 μM (AID:743054). PPARγ signalling pathway antagonist (AID:743199).	AR+ PPARγ+
Metoprolol	Selective adrenergic β1 receptor blocker used in the treatment of cardiovascular diseases, such as angina pectoris, hypertension and cardiac arrhythmias.		
Metribuzin	Also known as 4-amino-6-tert-butyl-3-(methylthio)-as-triazin-5 (4H)-one, Metribuzin is a pre- and post- emergence herbicide, that inhibits photosynthesis and is commonly used on soy beans, potatoes, tomatoes and sugar cane.	qHTS agonist of the retinoid X receptor (RXR) signalling pathway, 39.81 μM (AID:588544).	RXR+
Metronidazole	Nitroimidazole antibiotic used against the anaerobic bacteria and protozoa, associated with amebiasis, vaginitis, trichomonas infections, giardiasis and treponemal infections. Highlighted as a potential radiation-sensitising agent.	qHTS PPARγ signalling pathway antagonist, 48.97 μM (AID:743194).	PPARγ+
Mevinphos	Organophosphate cholinesterase inhibitor insecticide.		
Mirex	Banned organochlorine insecticide, previously used to control fire ants.		
Molinate	Also known as Ethyl N, N'-Hexamethylenethiolocarbamate, Molinate is a pre- and post- emergent systemic thiocarbamate herbicide, used to cotnrol *Echinochloa sp*. And *Diplachne fusca*. WHO assigned a drinking water quality guideline of 6 μg/L.		
Mono-2-ethylhexylphthalate (MEHP)/ 2-(((2-ethyl)hexyl)oxy)carbonyl benzoic acid	Plasticiser and hydrolysed product of DEHP.	qHTS PPARγ signalling pathway agonist, 3.34 μM (AID:743140) and PPARα at 28.18 μM (AID:651778). Human PXR signalling pathway activator, 14.13 μM (AID:720659).	PPARγ+ PPARα + PXR+
Mono-n-butylphthalate	Plasticiser	Developmental toxicity in F1 Wistar rats has been reported at 500 mg kg^{-1}/day maternal exposure on days 7-15 (Ema *et al.*, 1995).	

Musk ketone	Also known as white musks, are synthetic aromachemicals used in perfumery.	qHTS Retinoid X receptor signalling agonist, 28.18 μM (AID:588544). Estrogen Receptor alpha (ERα) sgnalling pathway antagonist in the BG1 cell line, 30.64 μM (AID:743080). PXR signalling pathway activator, 44.67 μM (AID:720659).	RXR+ ER+ PXR+
Mycobutanil	Also known as Systhane, myclobutanil is a triazole systemic fungicide, that inhibits ergosterol biosynthesis.	qHTS aromatase inhibitor, 24.19 μM (AID:743083). Activator of rat Pregnane X Receptor (rPXR) signalling pathway, 31.62 μM (AID:651751). Antagonist of the ERα signalling pathway, 43.10 μM (AID:743091).	PXR+ ER+
2-Acetylamino fluorene/ N-(9N)-Fluoren-2-yl acetamide	Also known as n-2-fluorenylacetamide, 2AAF is an aromatic hepatic carcinogen.	Estrogen Receptor (ERα) signalling pathway agonist, 11.04 μM (AID:743079). Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 34.90 μM (AID:743122). Glucocorticoid Receptor (GR) signalling pathway (AID:720725).	ER+ AhR+ GR+
Nadolol	Non-selective beta-adrenergic antagonist used in the treatment of cardiovascular disease for arrhythmias, angina pectoris and hypertension. The preparation is a mixture of stereoisomers.		
Naproxen	An anti-inflammatory, antipyretic, used in the treatment of rheumatoid arthritis, muscoskeletal disorders, dysmenorrhea and acute gout.	*In vitro* inhibition of prostaglandin G/H synthase in rat neutrophils (AID:160881).	
Naphthalene	Simplest polycyclic aromatic hydrocarbon used in the manufacture of phthalic anhydride and plastics. Product of wood, fossil fuel and crude oil incomplete combustion.		
Naringenin	Flavonone found in grapefruit, oranges and tomatoes, with antioxidant properties.	qHTS Cytochrome P450 interactions (AID:884).	
n-Butylbenzene	N-butylbenzene is used in organic synthesis.		
Nimesulide	COX-2 selective non-steroidal anti-inflammatory drug (NSAID) used in the treatment of osteoarthritis and dysmenorrhoea.	qHTS antagonist of ERα signalling pathway in BG1 cell line, 61.13 μM (AID:743081) and PPARγ signalling pathway (AID:743199).	ER+ PPARγ+
Nitroso piperidine	N-Nitrosopiperidine is a disinfection byproduct of water treatment.	DSS Carcinogenic Potency Database Rat Bioassay Results (AID1208).	
N-Nitroso dimethylamine	NMDA is a industrial-by-product of several industrial processes, present in foodstuffs at trace concentrations.		
Norethindrone	A synthetic progestational hormone with action similar to progesterone, functioning as an inhibitor of ovulation in the contraceptive pill. Norethindrone has also been used to treat amenorrhea, functional uterine bleeding and endometriosis.	Dissociation constant for progesterone receptor, Kd 0.0004 μM (AID:162459) and for the rat uterine estrogen receptor alpha, Kd 0.00063 μM (AID:69387). Modulation of hPR-B in co-transfected CV-1 cells, EC_{50} 0.0022 μM (AID:161792). Agonist of the Androgen receptor signaling pathway, 0.0022 μM (AID:743053). IC_{50} against recombinant rat AR in *E. coli* using R1881, 0.12 μM (AID:255211). DRUGMATRIX: Glucocorticoid radioligand binding, 0.397 μM (AID:625263). Thyroid Receptor signaling pathway antagonist, 6.86 μM (AID:743065).	PR+ ER+ AR+ GR+ TR+
Norfloxacin	Synthetic fluoroquinolone broad-spectrum antibacterial agent, which inhibits bacterial DNA gyrase.		
Nortriptyline	Antidepressive metabolic breakdown product of Amitriptyline, used in the treatment of depression and dysthymia.	qHTS cytochrome P450 binding (AID:891).	
***o,p*-DDD**	Known as Mitotane, DDD is a derivative of dichlorodiphenyldichloroethane (DDT) which inhibits the cells of the adrenal cortex and is used in the treatment of adrenal tumours.	qHTS small molecule agonist of ERα signalling pathway in BG1 cell line, 2.37 μM (AID:743079). Antagonists of TR signalling pathway, 13.33μM (AID:743064). Glucocorticoid Receptor (GR) signalling pathway antagonist, 18.99 μM (AID:720692).	ER+ TR+ GR+ AR+ PPARδ + PPARγ+

		Activator of PXR signalling pathway, 31.62 µM (AID:720659). AR signalling pathway antagonist, 38.9 µM (AID:743033). PPARδ signalling pathway agonist, PPARγ antagonist (AID743194).	
o,p-DDE/ 2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene	2,2-(2-chlorophenyl-4'-chlorophenyl)-1,1-dichloroethene.	Inhibitory concentration against recombinant AR expressed in *E. coli* against R1881, IC_{50} 20.42 µM (IAD:255211). DSSTox (NCTRER) Estrogen Receptor Binding Database (AID:1204).	AR+ ER+
o,p-DDT	Dichlorodiphenyltrichlroethane is a organochlorine widely used as an insecticide, prior to its ban in 1972.	qHTS ERα signalling pathway agonist, 0.696 µM (AID:743075) and PPARδ agonist, 14.37 µM (AID:743211) and antagonism of PPARγ, 16.13 µM (AID:743194). TR signalling pathway antagonist, 17.95 µM (AID:743065).	ER+ PPARδ + PPARγ+ TR+
Octyl-4-Methoxycinnamate (OMC)/ 4-Hydroxy-3,5-diidobenzonitrile	Octylmethoxycinnamate (OMC)/ Ethylhexyl methoxycinnamate is a UV-B filter used in sunscreens and lip balms.	Schlumpf *et al*. (2001) detected *in vitro* estrogenicity.	ER+
17α-Ethinylestradiol (EE2)	Semi-synthetic alkylated estradiol with high oral bioavailability, used in contraceptive pills.	qHTS ERα signalling pathway agonist, 0.00077 µM (AID:743077), binding affinity to ERα IC_{50} 0.008 µM (AID:478658) and ERβ IC_{50} 0.0081 µM (AID:265000). Displacement of 5α-dihydrotestosterone from human sex hormone binding globulin, 0.155 µM Kd (AID318680). AR signalling pathway, 0.687 µM (AID:743035). Progesterone radioligand binding, 1.067 µM (AID:625172). Glucocorticoid radioligand binding, 1.71 µM (AID:625228). Thyroid receptor signalling pathway, 9.689 µM (AID:743065). PXR signalling pathway, 12.59 µM (AID:720659). PPARδ antagonist (AID:743226).	ER+ AR+ PR+ GR+ TR+ PXR+ PPARδ +
17β-Estradiol (E2)	An aromatised C18 steroid with hydroxyl group at the 3-beta and 17-beta position, and is the most potent endogenous mammalian estrogenic steroid. E2 is the most predominant circulating hormone during reproductive years.	Agonist of human ERα signaling pathway, 0.00052 µM (AID:743077). Antagonist of the Androgen receptor (AR), 0.06 µM (AID:743063) and agonist at 2.23 µM (AID:743036). Antagonsit of Glucocorticoid Receptor signalling, 7.795 µM (AID:588533). Thyroid receptor signaling pathway antagonist, 23.71 µM (AID:743065). Antagonist of PPARγ signaling pathway, 39.8 µM (AID:588537).	ER+ PPAR+ AR+ TR+ GR+
17β-Eestriol (E3)	Hydroxylated metabolite of E2, produced in large quantities during pregnancy.	qHTS ERα signalling pathway agonist in the BG1 cell line, 0.0007 µM (AID:743079), ERα radioligand binding IC_{50} 0.00169 µM (AID:625258). Displacement of 5α-dihydrotestosterone from human sex hormone binding globulin, Kd 0.234 µM (AID:318680). qHTS AR signalling pathway antagonist, 13.8 µM (AID:743063). GR signalling pathway antagonist, 38.89 µM (AID:720692). Thyroid Receptor signalling pathway antagonist, 61.13 µM (AID:743067).	ER+ AR+ GR+ TR+
Estrone (E1)	Mammalian estrogen converted from androstenedione or testosterone via estradiol, produced primarily by the ovaries and adipose tissue.	Growth response in MCF-7 cells, 7.72e-05 µM (AID:103554). EC_{50} in STTA ERα, 0.0007 µM (AID:70186) and 0.0008 µM in the BG1 cell line (AID:743079). Displacement of 5α-dihydrotestosterone from human sex hormone binding globulin, Kd 0.0066 µM (AID:318680). qHTS assay antagonist of AR signalling pathway, 0.054 µM (AID:743033). Thyroid Receptor signalling pathway, 0.1508 µM (AID:743064). GR signalling pathway antagonist, 3.89 µM (AID:720692). Progesterone radioligand binding, IC_{50} 16.098 µM (AID:625172). PXR signalling pathway activator, 39.8 µM (AID:720659).	ER+ AR+ TR+ GR+ PR+ PXR+
Ofloxacin	Synthetic fluoroquinolone antibacterial agent, which inhibits DNA gyrase.		

2-Hydroxyhippuric acid	Also known as Salicyluric acid (salicylurate), glycine conjugate of salicylic acid, excreted in the urine.		
Olanzapine	Antipsychotic drug used in the treatment of schizophrenia and bipolar disorder.	Thyroid Receptor signalling pathway antagonist, 13.33 μM (AID:743064).	TR+
Omethoate	Also known as dimethoxon, Omethoate is a systemic organophosphorous insecticide and acaricide used to control insects and mites.		
Oxazepam	Benzodiazepine used in the treatment of anxiety, insomnia and symptoms of alcohol withdrawal.	qHTS ERα agonist signalling pathway in BG1 cell line, 0.668 μM (AID:743079). AR signalling pathway agonist in the MDA cell line, 6.16 μM (AID:743040) and antagonist at 27.53 μM (AID:743054). GR signalling pathway antagonist, 39.24 μM (AID:720692). TR signalling pathway antagonist, 54.92 μM (AID:743065). PPARγ signalling pathway antagonist (AID:743199).	ER+ AR+ GR+ TR+ PPARγ+
Oxydemeton-methyl	Also known as methyl demeton, Oxydemeton-methyl is an organothiophosphate acaricide/insecticide.		
Oxytetracycline	Broad spectrum tetracycline antibiotic.	qHTS Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 61.13 μM (AID:743085).	AhR+
Palmitic acid	A common saturated fatty acid found in olive oil, palm oil and body lipids.	qHTS ERα signalling pathway agonist in the BG1 cell line, 0.668 μM (AID:743079).	ER+
Papaverine	Alkaloid found in opium, which acts as a smooth muscle relaxant. Papaverine is used in the treatment of visceral spasm, vasospasm and erectile dysfunction.		
Paraquat	Poisonous dipyridilium compound used as a herbicide.		
Parathion	Cholinesterase inhibitor used as an acaricide and insecticide.	Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 10.58 μM (AID:743085). PXR signalling pathway activator, 11.22 μM (AID:720659).	AhR+ PXR+
Paroxetine	Serotonin uptake inhibitor effective in the treatment of depression.	qHTS AR signalling pathway antagonist, 8.485 μM (AID:743033). PPARδ signalling pathway antagonist, 8.485 μM (AID:743213). Thyroid Receptor signalling pathway, 11.88 μM (AID:743064).	AR+ PPARδ + TR+
***p*-Coumaric acid/ 4-Hydroxycinnamic acid**	p-coumaric acid is a major component of lignin, detected in peanuts, navy beans, tomatoes, carrots and garlic.		
Penconazole	Heterocyclic azole fungicide	qHTS Glucocorticoid Receptor signalling pathway antagonist, 3.089 μM (AID:720692). Antagonist of AR signalling pathway, 43.64 μM (AID:743035). ERα signalling pathway antagonist, 52.87 μM (AID:743078). Thyroid Receptor signalling pathway antagonist, 61.13 μM (AID:743065).	GR+ AR+ ER+ TR+
Pendimethalin	Dinitroaniline premergence and postemergence herbicide, which inhibits broadleaf weed cell division and elongation.	qHTS ERα signalling pathway agonist in the BG1 cell line, 7.688 μM (AID:743079).	ER+
Pentachlorobenzene/ 1,2,3,4,5-Pentachloro benzene	Chlorinated aromatic hydrocarbon byproduct, with no large scale direct industrial applications.		
Pentachloro phenol	Insecticide, herbicide and preservative that is a widespread environmental contaminant; exposure via contaminated air, groundwater, drinking water, food and dermal contact with soils or products treated with the chemical.	qHTS Aryl Hydrocarbon Receptor signalling pathway activator, 3.36 μM (AID:743085). PPARγ signalling pathway antagonist, 3.398 μM (AID:743191). TR signalling pathway antagonist, 8.79 μM (AID:743065). ERα signalling pathway antagonist, 10.74 μM (AID:743078).	AhR+ PPARγ+ TR+ ER+
Perchloroethylene/ Tetrachloroethylene	Also known as tetrachloroethylene.		
Perfluorodecanoic acid (PFDA)	Synthetic perfluorinated carboxylic acid, used as a surfactant and polymerisation agent.	qHTS ERα signalling pathway antagonist, 15.485 μM (AID:743069) and agonist of antioxidant response element signalling pathway, 24.34 μM (AID:743202).	ER+
Permethrin	Pyrethroid insecticide used against lice and scabies.	Activator of the Aryl Hydrocarbon Receptor (AhR) signalling pathway, 13.65 μM	AhR+ PXR+

		(AID:743085) and rat PXR signalling pathway, 19.95 μM (AID:651751).	
Phenanthrene	Polycyclic aromatic hydrocarbon found in cigarette smoke and coal tar. Phenanthrene is also the molecular backbone of the pharmaceutical morphinan, among other psychoactive chemicals.		
Phenol phthaleine	Acid-base indicator, which is colourless in acids, but pinky red in alkaline solution, with cathartic properties on ingestion.	Agonist of ERα signalling pathway, 3.37 μM (AID:743075) and TR signalling pathway antagonist, 13.33 μM (AID:743065). Aryl Hydrocarbon Receptor (AhR) Signalling pathway activator, 20.72 μM (AID:743122). AR signalling pathway antagonist, 26.83 μM (AID:743063). PXR signalling pathway activator, 31.62 μM (AID:720659). GR signalling pathway, 35.48 μM (AID:588533). PPARγ signalling antagonist, 39.81 μM (AID:588537) and PPARδ, 54.59 μM (AID:743213).	ER+ TR+ AhR+ AR+ PXR+ GR+ PPARγ+ PPARδ +
Phenothrin	Synthetic pyrethroid used in the treatment of headlice.		
Phloretin	Dihydrochalcone phenol found in apple tree leaves and the Manchurian apricot. Phloretin inhibits the active transport of glucose into cells by SGLT1 and SGLT2, leading to reduced glucose absorption in the small intestine and the inhibition of renal glucose reabsorption.	qHTS ERα signalling agonist, 4.96 μM (AID:743079). AR signalling antagonist, 48.20 μM (AID:743063), and activator of the Aryl Hydrocarbon Receptor, 49.65 μM (AID:743085). Thyroid Receptor signalling antagonist, 55.70 μM (AID:743065). Antagonist of PPARγ signalling pathway (AID:743199).	ER+ AR+ AhR+ TR+ PPARγ+
Picloram/ 4-Amino-2,5,6-trichloropyridine-2-carboxylic acid	Picolinic acid derivative used as a systemic herbicide for woody and broad-leaf plant control.	qHTS Aryl Hydrocarbon Receptor signalling pathway activator, 32.72 μM (AID:743122).	AhR+
Pindolol	Lipophilic beta-adrenergic antagonist, with non-cardioselective and sympathomimetic actions.	qHTS activator of the Aryl Hydrocarbon Receptor signalling pathway, 29.85 μM (AID:743085).	AhR+
Piperonyl butoxide	Piperonly butoxide is an insecticide synergist, particularly for pyrethroids such as rotenone.	qHTS activator of the AhR signalling pathway, 20.31 μM (AID:743122). Thyroid Receptor signalling pathway, 21.95 μM (AID:743064). Androgen Receptor signalling pathway antagonist in the MDA cell line, 31.01 μM (AID:743042). PXR signalling activator, 44.67 μM (AID:720659).	AhR+ TR+ AR+ PXR+
Piroxicam	Cyclooxygenase inhibiting, non-steroidal anti-inflammatory agent used in the treatment of rheumatoid arthritis, osteoarthritis and muscoskeletal disorders, dysmenorrhea and postoperative pain.	Aryl Hydrocarbon Receptor signalling pathway, 32.13 μM (AID:743122).	AhR+
Pravastatin	Competitive inhibitor of HMG CoA reductase extracted from cultures of *Nocardia autotrophica*.		
Prednisone	Synthetic anti-inflammatory glucocorticoid derived from cortisone used as an immunosuppressant drug to treat inglammatory diseases.	qHTS Androgen Receptor signalling antagonist, 0.035 μM (AID:743063). Antagonist of GR signalling, 2.46 μM (AID:720692). qHTS ERα signalling agonist in the BG1 cell line, 54.48 μM (AID:743079).	AR+ GR+ ER+
Prednisolone	Glucocorticoid with corticosteroid properties used to treat inflammatory and auto-immune conditions. Prednisolone is the active metabolite of prednisone.	Displacement of FITC-dexamethasone from GR in fluorescence polarisation asssy, ki 0.0015 μM (AID:351941). qHTS androgen receptor signalling pathway agonist in the MDA cell line, 0.037 μM (AID:743040).	GR+ AR+
Primidone	Antiepileptic agent structurally related to barbituates.	qHTS AR signalling agonist, 17.78 μM (AID:588515).	AR+
Prochloraz	Industrial fungicide	qHTS AhR signalling pathway activator, 0.545 μM (AID:743085). Activator of rat PXR signalling pathway, 15.85 μM (AID:651751). Androgen Receptor signalling antagonist, 19.91 μM (AID:743035). Thyroid Receptor signalling antagonist, 27.89 μM (AID:743065). PPARδ signalling, 44.57 μM (AID:743211). ERα signalling antagonist, 61.13 μM	AhR+ PXR+ AR+ TR+ PPARδ + ER+ GR+

		(AID:743080). Antagonist of GR signalling pathway (AID:720725).	
Prodiamine	Dinitroaniline herbicide registered for selective, pre-emergent control of broadleaf and grass weeds. Also known as Marathon.	qHTS Thyroid Receptor antagonist, 13.64 μM (AID:743064). Rat PXR signalling pathway activator, 15.85 μM (AID:651751). PPARδ signalling pathway agonist, 19.45 μM (AID:743194). Estrogen Receptor-α signalling, 31.62 μM (AID:588513). qHTS antagonist of the AR signalling pathway, 38.8 μM (AID:743033). Aryl Hydrocarbon Receptor signalling activator, 60.94 μM (AID:743086). Glucocorticoid Receptor signalling pathway, 48.85 μM (AID:720693).	TR+ PXR+ PPARδ + ER+ AR+ AhR+ GR+
Progesterone	Progestational steroid secreted primarily by the corpus luteum and placenta, progestesterone is required for implantation, pregnancy maintenance and the development of mammary tissue. Converted from pregnenolone, is an intermediate in steroid hormone and corticosteroid biosynthesis.	Activation of progesterone receptor in human T47D cells by PRE-tagged luciferase, 0.001 μM (AID:469431). Agonistic activity of PR, 0.0005 μM (AID:339656). qHTS AR agonist signalling, 0.0251 μM (AID:588515). Displacement of dexamethasone from GR, Ki 0.0305 μM (AID:74227). Antagonist ER activity in CV-1 cells, IC_{50} 10 μM (AID:162110). Antagonist of the TR, 16.15 μM (AID:743067). PPARγ signalling antagonist, 43.396 μM (AID:743194) and PPARδ, 48.69 μM (AID:743211).	PR+ AR+ ER+ AR+ GR+ TR+ PPARγ+ PPARδ +
Prometryn	Triazine selective pre- and post-emergence herbicide.	Pregnane X Receptor signalling pathway activator, 14.13 μM (AID:720659).	PXR+
Pronamide/ N-(3,4-dichlorophenyl) propanamide	Herbicide to control grasses and weeds, also called propyzamide and 3,5-dichloro-N-(1,1-dimethylpropynyl)benzamide	qHTS PXR signalling activator, 11.22 μM (AID:720659). Antagonist of the AR signalling pathway, 44.05 μM (AID:743063).	PXR+ AR+
Propanil	Chlorinated anilide herbicide.	Antagonist of AR signalling pathway, 20.67 μM (AID:743063). Antagonist of TR signalling pathway, 49.55 μM (AID:743054). ERα signalling antagonist in the BG1 cell line, 54.48 μM (AID:743080). Aryl Hydrocarbon Receptor signalling pathway, 54.48 μM (AID:743085).	AR+ TR+ ER+ AhR+
Propranolol	Sympatholytic non-selective beta-blocker used in the treatment of myocardial infarction, arrhythmia, angina pectoris, hypertension, hyperthyroidism, migraine, pheochromocytoma and anxiety.	qHTS PPARδ signalling agonist, 24.54 μM (AID:743211). ERα signalling pathway agonist in the BG1 cell line, 30.64 μM (AID:743079). Thyroid receptor signalling, 30.64 μM (AID:743064). PPARγ signalling antagonist, 55.499 μM (AID:743191).	PPARδ + PPARγ+ TR+ ER+
Propazine	Poorly soluble in water, herbicide.	qHTS pregnane X receptor signalling pathway activator, 35.48 μM (AID:720659) and AhR signalling activator, 68.59 μM (AID:743085).	PXR+ AhR+
Propiconazole	Triazole fungicide known as DMI, or demethylation inhibiting fungicide due to its MoA.	qHTS pregnane X receptor signalling activator, 22.38 μM (AID:720659). Activate AhR signalling, 24.33 μM (AID:743085). ERα signalling antagonist in the BG1 cell line, 27.8 μM (AID:743080). Thyroid Receptor signalling, 30.63 μM (AID:743064). AR signalling pathway, 43.64 μM (AID:743035).	PXR+ AhR+ TR+ ER+ AR+
Propoxur	A carbamate non-systemic insecticide, which inactivates acetylcholinesterase.		
Propylparaben/ Propyl-4-hydroxybenzoate	Naturally occurring n-Propyl ester of p-hydroxybenzoic acid, synthesised for use in cosmetics, pharmaceuticals and food. E number E216.	qHTS ERα signalling agonist, 3.55 μM (AID:588514) and AR signalling antagonist, 25.11 μM (AID:588516). PPARγ signalling antagonist, 35.48 μM (AID:588537).	ER+ AR+ PPARγ+
Pyrene	Polycyclic aromatic hydrocarbon formed during incomplete combustion of organics.		
Quercetin	Flavonol found in fruits, vegetables, leaves and grains with antioxidant properties.	qHTS ERα signalling agonist, 10.96 μM (AID:743077). Aryl Hydrocarbon Receptor signalling activator, 12.19 μM (AID:743085). TR signalling antagonist, 30.88 μM (AID:743064). Androgen Receptor signalling antagonist in the MDA cell line, 38.88 μM (AID:743054). Glucocorticoid Receptor signalling antagonist, 48.96 μM (AID:720692).	ER+ AhR+ TR+ AR+ GR+
Quinalphos	Organthiophosphate pesticide	qHTS Aryl Hydrocarbon Receptor signalling	AhR+ ER+

		activator, 1.22 μM (AID:743085). ERα signalling agonist, 15.35 μM (AID:743079). Androgen Receptor signalling antagonist, 48.97 μM (AID:743063).	AR+
Ramipril	Angiotensin-converting enzyme (ACE) inhibitor, converted to ramiprilat in the liver, to treat high blood pressure and congestive heard failure.		
Ranitidine	Histamine H2-receptor antagonist that inhibits stomach acid production, used in the treatment of gastrointestinal ulcers.		
Resbenzophenone/ 2,4-Dihydroxy benzophenone	Also known as 2,4-dihydroxybenzophenone, benzophenone-1, and Benzoresocrinol, Benzophenones are used in paints, plastics, packaging, inks and coatings to prevent UV colour and scent damage.	qHTS Estrogen Receptor –α signalling agonist in the BG1 cell line, 9.876 μM (AID:743079). Androgen Receptor (AR) signalling antagonist, 25.02 μM (AID:743033). Thryoid Receptor signalling agonist, 30.64 μM (AID:743066). PPARδ signalling antagonist, 35.34 μM (AID:743213).	ER+ AR+ TR+ PPARδ +
Resmethrin	Pyrethroid insecticide used to control adult mosquito populations.	qHTS activator of PXR signalling, 44.67 μM (AID:720659). Androgen Receptor signalling antagonist, 49.05 μM (AID:743035). Glucocorticoid Receptor (GR) signalling antagonist, 55.035 μM (AID:720692). PPARγ signalling pathway antagonist (AID:743199).	PXR+ AR+ GR+ PPARγ+
Resorcinol	Dihydroxybenzene with antifungal, antibacterial properties, used in the treatment of ringworm, eczema, psoriasis, seborrheic, dermatitis, acne rosaceae.		
Retene	Also known as methyl isopropyl phenanthrene or 1-methyl-7-isopropyl phenanthrene, retene is a polycyclic aromatic hydrocarbon present in coal tar.		
Retinol	Vitamin A is a retinol derivative which plays a role in metabolic functioning.	ERα signalling pathway agonist in the BG1 cell line, 0.944 μM (AID:743079). Pregnane X Receptor (PXR) signalling activator, 2.82 μM (AID:720659). qHTS GR signalling antagonist, 55.63 μM (AID:720692). Androgen Receptor signalling antagonist, 61.88 μM (AID:743042). TR signalling antagonist, 61.88 μM (AID:743065). Antagonist of the PPARγ signalling pathway (AID:743199).	ER+ PXR+ GR+ AR+ TR+ PPARγ+
Roxithromycin	Semi-synthetic macrolide antibiotic used in the treatment of respiratory, urinary and genital tract infections.		
Salbutamol/ Albuterol	Also known as Albuterol, salbutamol is a short-acting beta-2 adrenergic agonist used in the treatment of asthma.		
Salicylic acid	Active metabolite of Aspirin. Obtained from willow and wintergreen bark, salicylic acid is a monohydroxybenzoic acid plant hormone, which is also used as an anti-acne treatment.		
Sertraline	Selective serotonin inhibitor used in the treatment of depression.		
Simazine	Triazine herbicide used to contral broad-leaf weeds and grasses.		
Simvastatin	Hypolipidemic drug used to control hypercholesterolemia, derivative of lovastatin, may interfere with steroid hormone production.	Androgen Receptor signalling pathway antagonist, 5.71 μM (AID:743063) and agonist 10.12 μM (AID:743040). Glucocorticoid Receptor signalling, 7.23 μM (AID:720719). ERα signalling agonist in the BG1 cell line, 11.36 μM (AID:743079). PPARδ signalling pathway antagonist, 16.93 μM (AID:743213) and agonist at 29.85 μM (AID:743211). Thyroid Receptor signalling antagonist, 38.57 μM (AID:743064).	GR+ AR+ ER+ PPARδ + TR+
Sotalol	Beta-adrenergic receptor antagonist used in the treatment of arrhythmias.	Androgen Receptor signalling agonist in the MDA cell line, 33.49 μM (AID:743040).	AR+
Stearic acid	Saturated fatty acid with IUPAC name octadecanoic acid, present in many animal and vegetable oils.	ERα signalling agonist in the BG1 cell line, 0.611 μM (AID:743079).	ER+

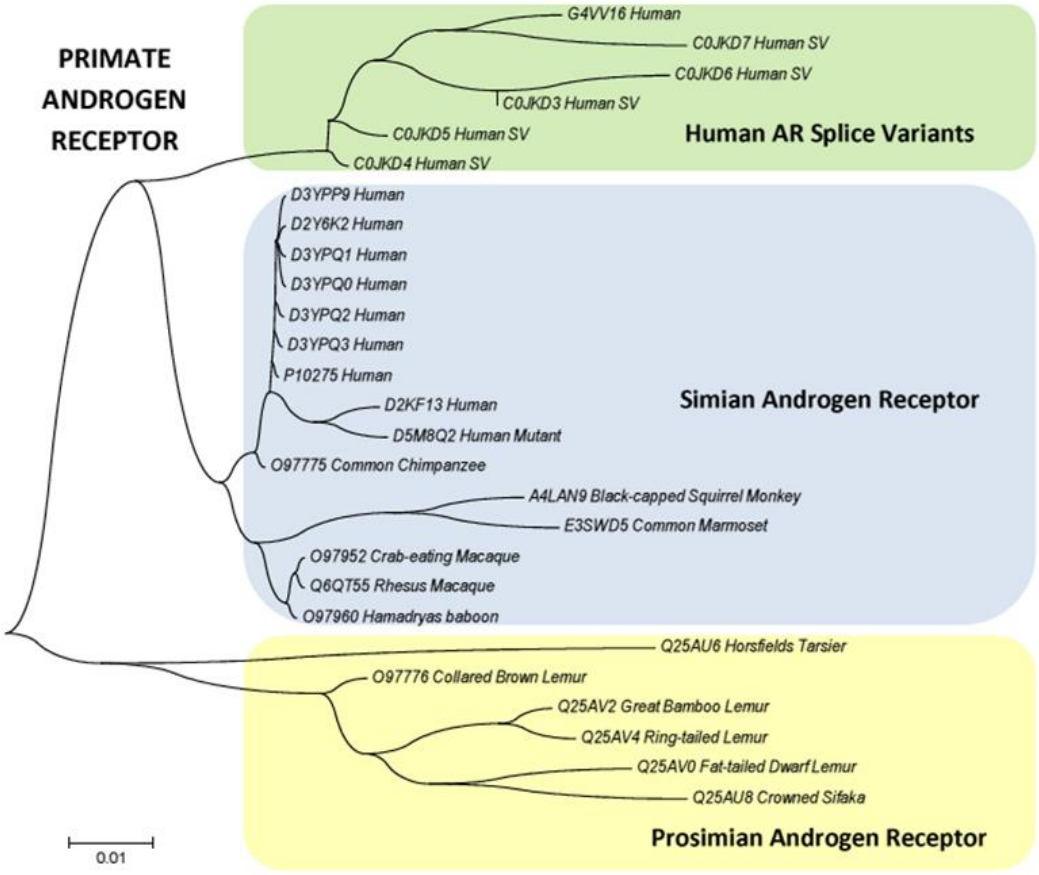
Styrene	Colourless, aromatic compound used to make rubbes, polymers and copolymers and polystyrene plastics.		
Sulfadimethoxine	A sulphanilamide used as an anti-infective agent.		
Sulfamethoxazole	Sulfonamide bacteriostatic antibiotic, which acts by interfering with folic acid synthesis in susceptible bacteria.		
Sulfapyridine	Antibacterial used to treat skin diseases, with potential toxicity due to crystalisation in the bladder or urethra.		
Sulfasalazine	Drug used in the management of inflammatory bowel disease.	Aryl Hydrocarbon Receptor (AhR) signalling pathway activator, 27.39 µM (AID:743086). ERα signalling antagonist in the BG1 cell line, 34.48 µM (AID:743081).	AhR+ ER+
Tamoxifen	Selective Estrogen Receptor Modulator, with tissue specific activities. Tamoxifen acts as an anti-estrogen in the mammary tissue, but an estrogen (stimulating agent) in cholesterol metabolism, bone density and cell proliferation in the endometrium.	Effective dose for estradiol against proflieration of MCF-7 cells, EC_{50} 0.00011 µM (AID:102435). Antagonistic activity of ERβ LBD expressed in yeast, IC_{50} 1.66 µM (AID:482348). Antagonist of TR signalling, 8.63 µM (AID:743064). qHTS AR signalling antagonist, 13.89 µM (AID:743033). PPARγ signalling antagonist, 21.87 µM (AID:743194). Antagonist of GR signalling, 24.54 µM (AID:720693). PPARδ signalling antagonist, 24.70 µM (AID:743194).	ER+ TR+ AR+ PPARγ+ PPARδ +
Tebuconazole	Triazole fungicide used to treat plant pathogenic fungi.	Activator of the rat PXR signalling pathway, 25.12 µM (AID:651751). ERα signalling antagonist in the BG1 cell line, 29.277 µM (AID:743091). Glucocorticoid Receptor signalling antagonist, 43.64 µM (AID:720692). Androgen Receptor signalling antagonise, 48.97 µM (AID:743035). Thyroid Receptor signalling pathway antagonist, 54.48 µM (AID:743063).	PXR+ ER+ GR+ AR+ TR+
Terbutalin	Also known as terbutaline, Terbutalin is a selective beta-2 adrenergic agonist used a bronchodilator and tocolytic.		
Terbutryn	Pesticide to destroy unwanted vegetation.	AhR signalling pathway activator, 3.85 µM (AID:743085). Androgen receptor signalling antagonist, 21.87 µM (AID:743035). PPARδ antagonist signalling, 38.86 µM (AID:743213). Antagonist thyroid Receptor signalling, 67.78 µM (AID:743067).	AhR+ AR+ PPARδ + TR+
Terbutylazine	Selective chlorotriazine herbicide, structurally related to atrazine and simazine.	qHTS ERα agonistic signalling in the BG1 cell line, 2.43 µM (AID:743079). Human GR signalling antagonist (AID:720725).	ER+ GR+
Tetrachlorvinphos	Organophosphate cholinesterast inhibitor used as an insecticide.	qHTS PPARγ signalling antagonist, 4.89 µM (AID:743191). ERα signalling antagonist, 16.72 µM (AID:743078). AR signalling pathway antagonist, 21.87 µM (AID:743035). GR signalling pathway antagonist, 24.54 µM (AID:720692). qHTS human PXR signalling, 25.12 µM (AID:720659). Thyroid Receptor signalling antagonist, 27.3 µM (AID:743065).	PPARγ+ ER+ AR+ GR+ TR+ PXR+
Tetracyclin	Also known as Tetracycline. A naphthacene antibiotic that inhibits amino acyl TRNA binding during protein synthesis.	HTS Estrogen Receptor-α coactivator binding inhibitor (AID629).	ER+
Tetra hydronaphthol-2/ 1,2,3,4-Tetrahydro napthalen-2-ol	Also known as Tetralol.		
Thiram	Dithiocarbamate ectoparasiticide used to prevent fungal disease in seeds and crops. Also used in rubber processing industry.	Thyroid Receptor signalling antagonist, 0.0154 µM (AID:743065). PPARδ signalling pathway agonist, 0.0309 µM (AID:743213). Glucocorticoid Receptor signalling agonist, 0.0608 µM (AID:720691). Androgen Receptor signalling antagonist in the MDA cell line, 0.0611 µM (AID:743042). PPARγ signalling antagonist, 0.0765 µM (AID:743194). AhR signalling pathway activator, 0.1536 µM	TR+ PPARδ + GR+ AhR+ PPARγ+ AhR+

		(AID:743086).	
Thyroxine	Tyrosine-based hormone from the thyroid gland. Thyroxine is released from thyroglobulin by proteolysis and secreted into the blood. Thyroxine is peripherally deiodinated to form triiodothyronine which exerts a braod spectrum stimulatory effect on metabolism.	qHTS TR signalling pathway agonist, 0.0084 μM (AID:743066). Activator of the AhR signalling pathway, 15.45 μM (AID:743122). PPARγ signalling antagonist, 39.81 μM (AID:588537). Antagonist of the GR signalling pathway (AID:720725).	TR+ AhR+ PPARγ+ GR+
Timolol	Non-selective beta-adrenergic receptor antagonist used in the treatment of glaucoma, heart attacks and hypertension.		
Tolfenamic acid	Non-steroidal anti-inflammatory drug used to treat migraines.	Thyroid Receptor signalling pathway antagonist, 8.41 μM (AID:743064). PPARδ signalling agonist (AID:743227) and PPARγ signalling (AID:743199). Glucocorticoid Receptor signalling antagonist (AID:720725).	TR+ PPARδ + PPARγ+ GR+
Tonalide/ 1-(3,5,5,6,8,8-hexamethyl-5,6,7,8-tetrahydronaphthalene-2-yl) ethanone	Also known as acetyl methyl tetramethyl tetralin and Tonalid. Tonalide is a synthetic musk, which emulates the aroma of natural musk.	qHTS TR signalling antagonist, 21.69 μM (AID:743064).	TR+
Toxaphene	Toxaphene is a mixture of at least 177 C10 polychloro derivatives, used as an insecticide, but a believed carcinogen.	qHTS ERα signalling agonist in the BG1 cell line, 3.81 μM (AID:743079). Antagonist of Thyroid Receptor signalling, 5.96 μM (AID:743064). Glucocorticoid Receptor signalling antagonist, 13.45 μM (AID:720693). Androgen Receptor signalling antagonist, 17.18 μM (AID:743035). PPARγ signalling antagonist, 21.62 μM (AID:743194). PPARδ signalling agonist, 34.27 μM (AID:743211).	ER+ TR+ GR+ AR+ PPARγ+ PPARδ +
Tramadol	A narcotic analgesic used for severe pain, acting vai opioid receptors, it may be habituating.		
Tris(2-butoxyethyl)phosphate	Flame retardant.		
Triadimefon	Triadimefon is used to control fungal disease in fruit and non-food sites.	Activator of the AhR signalling pathway, 35.68 μM (AID:743122). ERα signalling agonist, 39.81 μM (AID:588514). Pregnane X Receptor signalling activator, 44.67 μM (AID:720659).	AhR+ ER+ PXR+
Triadimenol	Triadimenol is used as a seed treatment on barley, corn, cotton, oats, rye, sorghum and wheat.	Pregnane X Receptor signalling activator, 6.31 μM (AID:720659). Aryl Hydrocarbon Receptor signalling activator, 21.69 μM (AID:743122). Antagonist of AR signalling in the MDA cell line, 61.13 μM (AID:743042).	PXR+ AhR+ AR+
Triamcinolone	Glucocorticoid taken orally, via injection of inhalation or as a topical ointment, for the treatment of eczema, psoriasis, arthritis, allergies, ulceratives colitis and lupus among other ailments.	qHTS Androgen Receptor signalling agonist in the MDA cell line, 0.0273 μM (AID:743040). Glucocorticoid Receptor signalling agonist, 0.032 μM (AID:720719). qHTS ERα signalling agonist in the BG1 cell line, 4.73 μM (AID:743079).	AR+ GR+ ER+
Triamcinolone acetonide	Anti-inflammatory glucocorticoid used in the treatment of skin disorders.	Glucocorticoid Receptor signalling agonist, 0.00187 μM (AID:720719). qHTS AR signalling pathway agonist in the MDA cell line, 0.0022 μM (AID:743040). Estrogen Receptor α signalling pathway agonist, 2.371 μM (AID:743079). Activator of the AhR signalling pathway, 69.01 μM (AID:743085).	GR+ AR+ ER+ AhR+
Tributylphosphate	Solvent and plasticiser for cellulose esters.	qHTS Estrogen Receptor-α signalling pathway agonist in the BG1 cell line, 15.35 μM (AID:743079).	ER+
Trichlorfon	Irreversible organophosphate acetylcolinesterase inhibitor, used to control flies and roaches, and in the treatment of schistosmiasis.		
Trichlorobenzene	Used in industry as a solvent and organic intermediate.		

Triclosan	Diphenyl ether derivative used in cosmetics and toilet soaps as an antiseptic – bacteriostatic and fungistatic action.	PPARδ signalling pathway antagonist, 4.47 μM (AID:743213) and PPARγ antagonist, 6.30 μM (AID:743194). Thyroid Receptor signalling antagonist, 8.83 μM (AID:743065). ERα signalling pathway antagonist, 11.22 μM (AID:743074). Antagonist of the AR signalling pathway, 12.59 μM (AID:743033). Activator of the AhR signalling pathway, 19.78 μM (AID:743086). Glucocorticoid Receptor signalling antagonist, 22.39 μM (AID:720693).	PPARγ+ PPARδ + TR+ ER+ AR+ AhR+ GR+
Trifluralin	Microtubule-disrupting pre-emergence herbicide, used to control annual grass and broadleaf weed species.		
Trimethoprim	Pyrimidine inhibitor of dihydrofolate reductase, trimethoprim is an antibacterial related to pyrimethamine.		
Triiodothyronine (T3)/ Liothyronine	T3 is a thyroid hormone secreted from the thyroid gland.	Displacement of [^{125}I]T3 from human TRα receptor, Kd 5.8e-05 μM (AID:323174). Inhibitory activity against [125I]T3 binding to human TRβ receptor, Kd 8e-05 μM (AID:213185). qHTS assay to identify TR signalling pathway agonists, 0.0021 μM (AID:743066). Aryl Hydrocarbon Receptor (AhR) signalling activator, 3.349 μM (AID:743122). ERα signalling pathway, 9.51 μM (AID:743075). Glucocorticoid Receptor signalling antagonist (AID:720725).	TR+ AhR+ ER+ GR+
Valsartan	Angiotensin-receptor blocker used to treat cardiac conditions, such as hypertension, isolated systolic hypertension, left ventricular hypertrophy and diabetic nephropathy.		
Vinclozolin	Dicarboximide fungicide used to control blights, rots and moulds in vineyards and on fruits and vegetables (raspberries, kiwi, lettuce, snap beans and onions).	qHTS Androgen Receptor signalling antagonist, 9.69 μM (AID:743054) and inhibitor of aromatase, 54.15 μM (AID:743083). PPARδ signalling pathway antagonist, 54.63 μM (AID:743213).	AR+ PPARδ +
Vinyl acetate	Organic precursor (monomer) to polyvinyl acetate, worldwide production millions of tonnes per annum.		
Warfarin	Anticoagulant used in the prevention of thrombosis and thromboembolism, by inhibiting the synthesis of vitamin K-dependent coagulation factors.	qHTS PPARγ signalling pathway agonist, 9.08 μM (AID:743140).	PPARγ+
β-Sitosterol	Phytosterol (plant sterol), structurally related to cholesterol, found ubiquitously in the plant kingdom.		

Appendix B Supplementary Phylogeny Results

Assuming protein sequence is indicative of protein function, phylogeny highlights the potential variability in function consequent to evolutionary divergence. Figure_Apx 1 shows the phylogeny of primate androgen receptors, highlighting inter- and intra-species variance within clades.



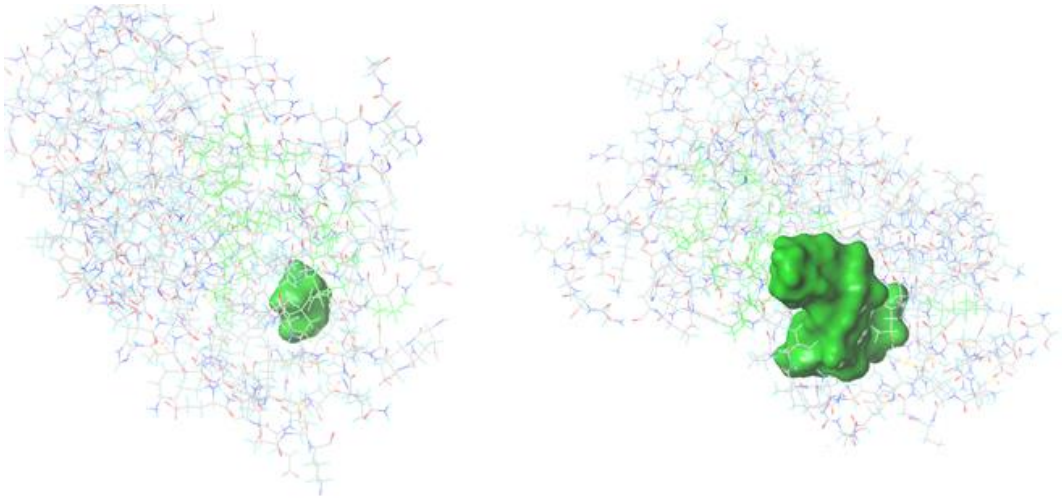
Figure_Apx 1 Phylogeny of Primate Androgen Receptors

Androgen receptor sequence homology reflects the taxonomic order, species and family, highlighting the infraorders human, simian and prosimian. The bootstrapped consensus phenogram, on a scale of 0.01, representing 1% sequence difference, presents a Σbranch length of 0.439, suggesting limited phylogenetic divergence within primates. However, the sequences of a number of human androgen receptor splice variants emphasise potential intraspecies variability. In order of appearance, the UniProtKB protein identifier and organism name (*Latin*) are: G4VV16 Human (*Homo Sapiens*) Androgen Receptor (AR) Isoform 8; C0JKD7 Human AR Splice Variant (SV) 6; C0JKD6 Human AR SV 5; C0JKD3 Human AR SV 3; C0JKD5 Human AR SV 4b; C0JKD4 Human AR SV 4; D3YPP9 Human AR Isoform 1 (ISO 1) Transcript Variant (TV) 1; D2Y6K2 Human AR ISO 1 TV1; D3YPQ1Human AR ISO 1 TV1; D3YPQ0 Human AR ISO 1 TV1; D3YPQ2 Human AR ISO 1 TV1; D3YPQ3 Human AR ISO 1 TV1; P10275 Human Androgen Receptor (Nuclear receptor subfamily 3 group C member 4); D2KF13 Human AR Variant (5-7); D5M8Q2 Human Mutant AR ISO 1 TV1; O97775 Common Chimpanzee (*Pan Troglodytes*) AR; A4LAN9 Black-capped Squirrel Monkey (*Saimiri boliviensis*) AR; E3SWD5 Common Marmoset (*Callithrix Jacchus*) AR; O97952 Crab-eating Macaque (*Macaca fascicularis*) AR; Q6QT55 Rhesus Macaque (*Macaca mulatta*) AR; O97960 Hamadryas baboon (*Papio hamadryas*) AR; Q25AU6 Horsfield's Tarsier (*Tarsius bancanus*) AR; O97776 Collared Brown Lemur (*Eulemur fulvus collaris*) AR; Q25AV2 Great Bamboo Lemur (*Hapalemur simus*) AR; Q25AV4 Ring-tailed Lemur (*Lemur catta*) AR; Q25AV0 Fat-tailed Dwarf Lemur (*Cheirogaleus medius*) AR; Q25AU8 Crowned Sifake (*Propithecus deckenii coronatus*) AR.

Appendix C SYBYL *in silico* Molecular Modelling of NR

Advances in computer-aided drug design has led to a variety of *in silico* molecular dynamics-based docking programs to predict ligand-receptor interactions and docking. For the purpose of this study, Tripos ® SYBYL software for macromolecular modelling, simulation and virtual screening was used. Supplementary information to Section 4 'Endocrine Activity in Silico; is presented herein. Figure_Apx 2 details Bloat parameters, Figure_Apx 3 shows coregulatory protein preparation and Figure_Apx 4 shows the Surflex-Dock results browser for coregulatory binding.

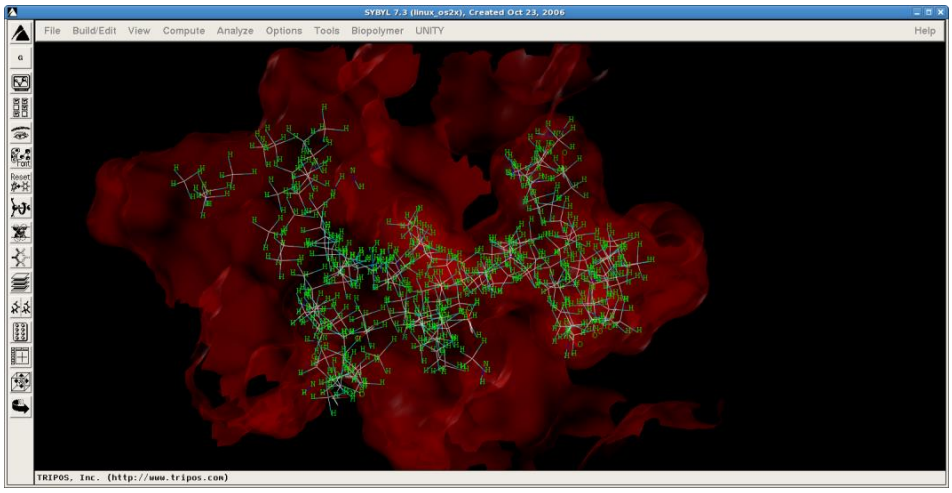
SYBYL Software Parameters



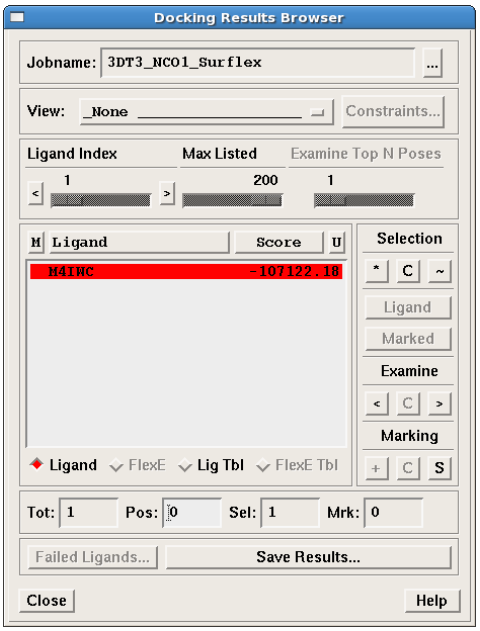
Figure_Apx 2 SYBYL Surflex-Dock Protomol Bloat Parameter

Consisting of molecular probes, such as CH_4, C=O and N-H, protomol's provide an object-orientated framework for molecular dynamics and virtual screening. SYBYL's Surflex-Dock can generate protomols through the ligand, specific residues or using the solvation method, default values are set at 0.5 Threshold and 0 Bloat. Bloat inflates the protomol to include nearby crevices (scale 0 to 1 (A)). Superimposed onto ligand binding domain stick models, the left side protomol was created with bloat of 0, while the right shows the same protomol, generated with bloat of 1. Increasing the bloat reduces the specificity of the binding pocket.

Molecular Modelling of Coregulators

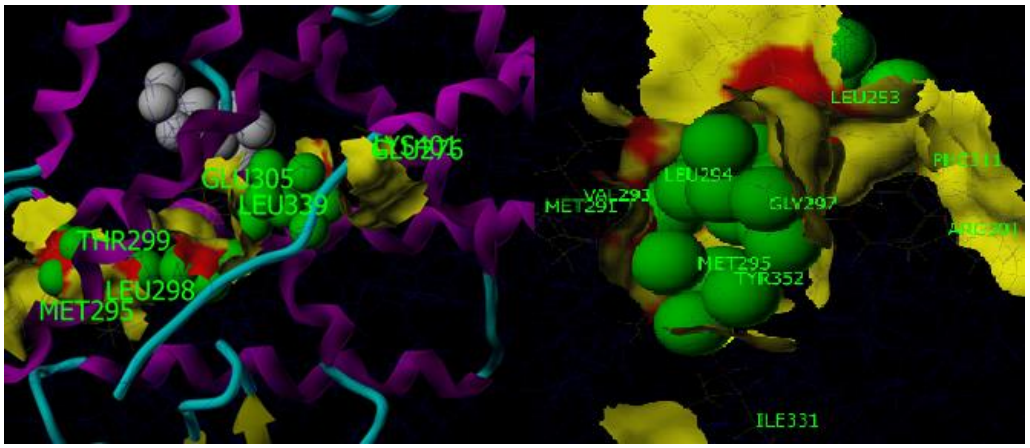


Figure_Apx 3 SYBYL Surflex-Dock LXXXLL Cofactor (NCO1) Protein Preparation
SYBYL Surflex-Dock software browser screen-print during protein preparation of LXXXLL containing coregulatory molecule.



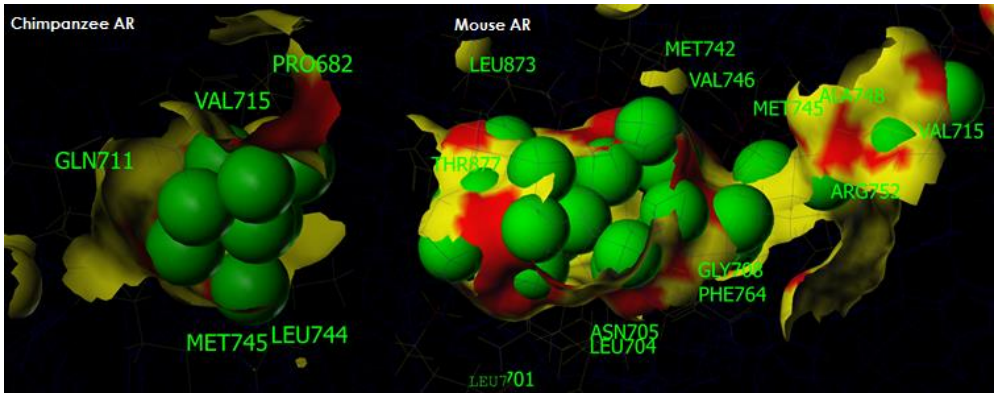
Figure_Apx 4 SYBYL Surflex-Dock Cofactor Binding Experiment Results Browser
Coregulatory molecules have been demonstrated to play a vital role in ligand-dependent nuclear receptor transactivation. SYBYL Surflex-Dock software generated protomols of specific residues, on ligand-bound receptor complexes, to screen coregulatory molecules with LXXXLL motifs. The Surflex-Dock binding methods, which generate a hypothetical binding region, did not effectively identify the coregulatory-NR affinity, generating a positive score (i.e. energetically unfavourable). The docking results browser shows the score (-107122 –log(K_d)) of NCO1 coactivator binding to a protomol generated from residues Ile358, Val376, Leu379, Glu380 and Met543 (Shiau *et al.*, 1998). Surflex-Dock, while appropriate for binding cavities ('pockets'), does not appear to be appropriate for surface interactions.

SYBYL SiteID Solvation Method Ligand Binding Pockets



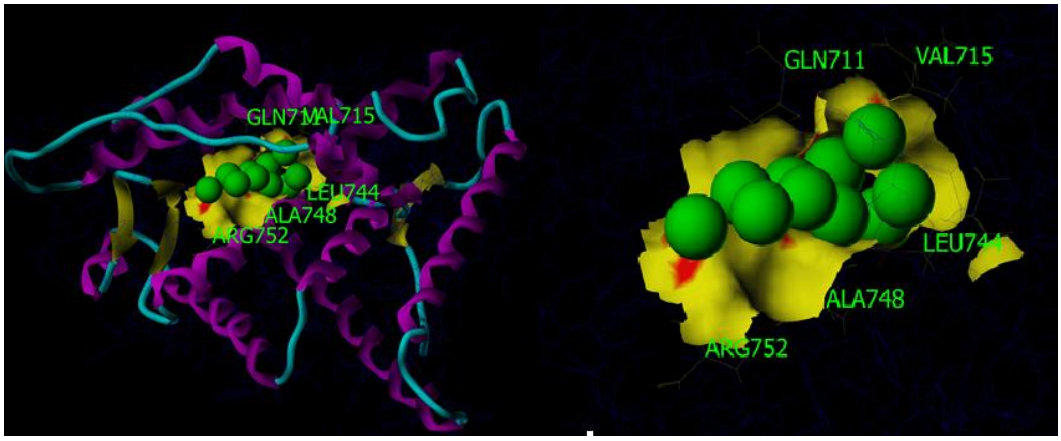
Figure_Apx 5 Human and Rat Estrogen Receptor-β (ERβ) Ligand Binding Pockets Identified with Solvation Method in SYBYL SiteID

The Human ERβ (1L2J), rendered with α-helices and β-sheet secondary structure features, was bound to tetrahydrochrysene-2,8-diol, an antagonist, and presents a 'clamped' conformation. Residues identified within 8Å of the solvents include Lys401, Leu339, Glu305, Thr299, Leu298, Glu276 and Met295. The rat ERβ (1HJ1) was bound to the ICI164,384 antiestrogen. Residues identifies within 8Å of the solvent spheres include Ile331, Tyr352, Phe311, Arg301, Gly297, Met295, Val293, Met291, Leu254 and Leu253. SYBYL identified the reside regions associated with binding for both human and rat ERβ.



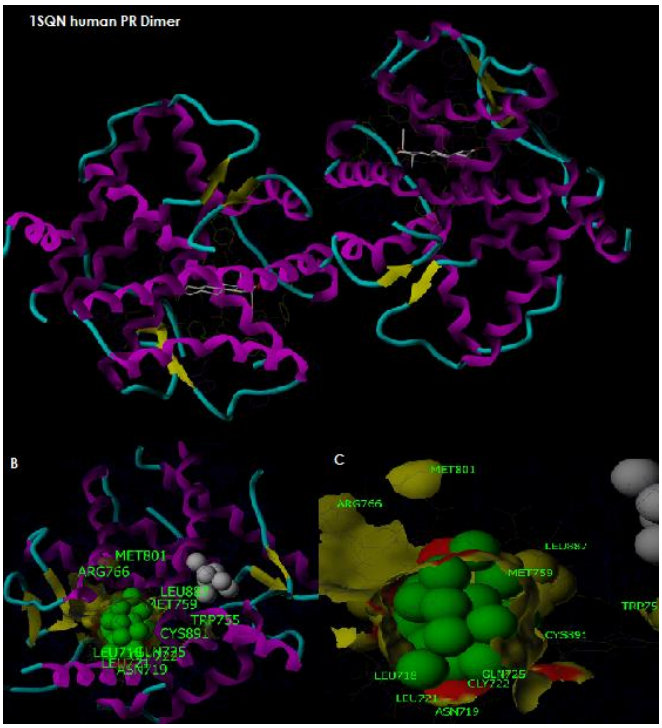
Figure_Apx 6 Chimpanzee (1T7T) and Mouse (2PQY) Androgen Receptor Ligand Binding Pockets Identified with Solvation Method in SYBYL SiteID

Image of SYBYL SiteID MOLCAD outputs for Androgen Receptors (AR); Chimpanzee (*Pan troglodytes*) AR structure was complexed with 5α-dihydrotestosterone, Mouse (*Mus musculus*) AR was complexed with 5α-dihydrotesterone and an allosteric regulator 4-(4-hydroxy-3-ido-phenoxy)-3,5-diiodophenyl)-acetic acid. Residues within 8Å of the solvent spheres in $_{chimp}$AR include: Pro682, Gln711, Val715, Leu744 and Met745. In $_{mouse}$AR, the LBP visually appeared to be a completely different shape. However identifying: Leu701, Leu704, Asn705, Gly708, Val715, Met742, Met745, Val746, Ala748, Arg752, Phe764 and Leu873, both $_{mouse}$AR and $_{chimp}$AR identified the LBP region (residues 675-900).



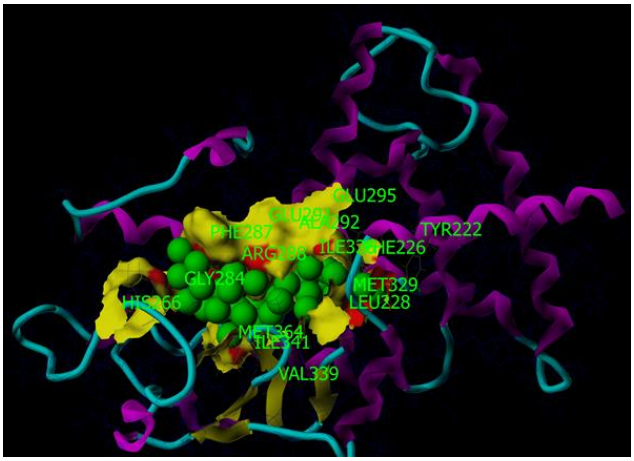
Figure_Apx 7 The Rat Androgen Receptor Ligand Binding Pocket Identified in SYBYL SiteID using Solvation Method

Figure shows a ribbon representation of the rat (*R. norvegicus*) Androgen Receptor (AR), with the MOLCAD surface and SiteID solvent spheres superimposed, is also enlarged to the right. The X-ray crystallography structure used to model the protein was complexed to an n-aryl-oxazolidin 2-imine inhibitor LGB (2-chloro-4-{[(1R,3Z,7S,7aS)-7-hydroxy-1-(trifluoromethyl)tetrahydro-1H-pyrrolo[1,2-c][1,3]oxazol-3-ylidene]amino}-3-methylbenzonitrile), explaining the αH12 clamped conformation. Residues within 8Å of solvent spheres in $_{rat}$AR LBP: Gln711, Val715, Leu744, Ala748 and Arg752. The LGB ligand, has been demonstrated to bind and block AR with an EC_{50} of 0.2 nM (Nirschl *et al*., 2009).



Figure_Apx 8 Human Progesterone Receptor (hPR) 1SQN Dimer and Monomer Ligand Binding Pockets Identified with Solvation Method in SYBYL SiteID

The Human PR (1SQN) dimer rendered with α-helices and β-sheet secondary structure features complexed with Norethindrone. B (rendered) and C (MOLCAD shading) show residues identified within 5Å of the solvents: Leu718, Asn719, Leu721, Gly722, Gln725, Trp755, Met759, Arg766, Met801, Leu887, Cys891 (steroid binding residues 700-900). The white spheres in B, suggest another, smaller, hydrophobic pocket; possible allosteric ligand binding or coregulatory binding.

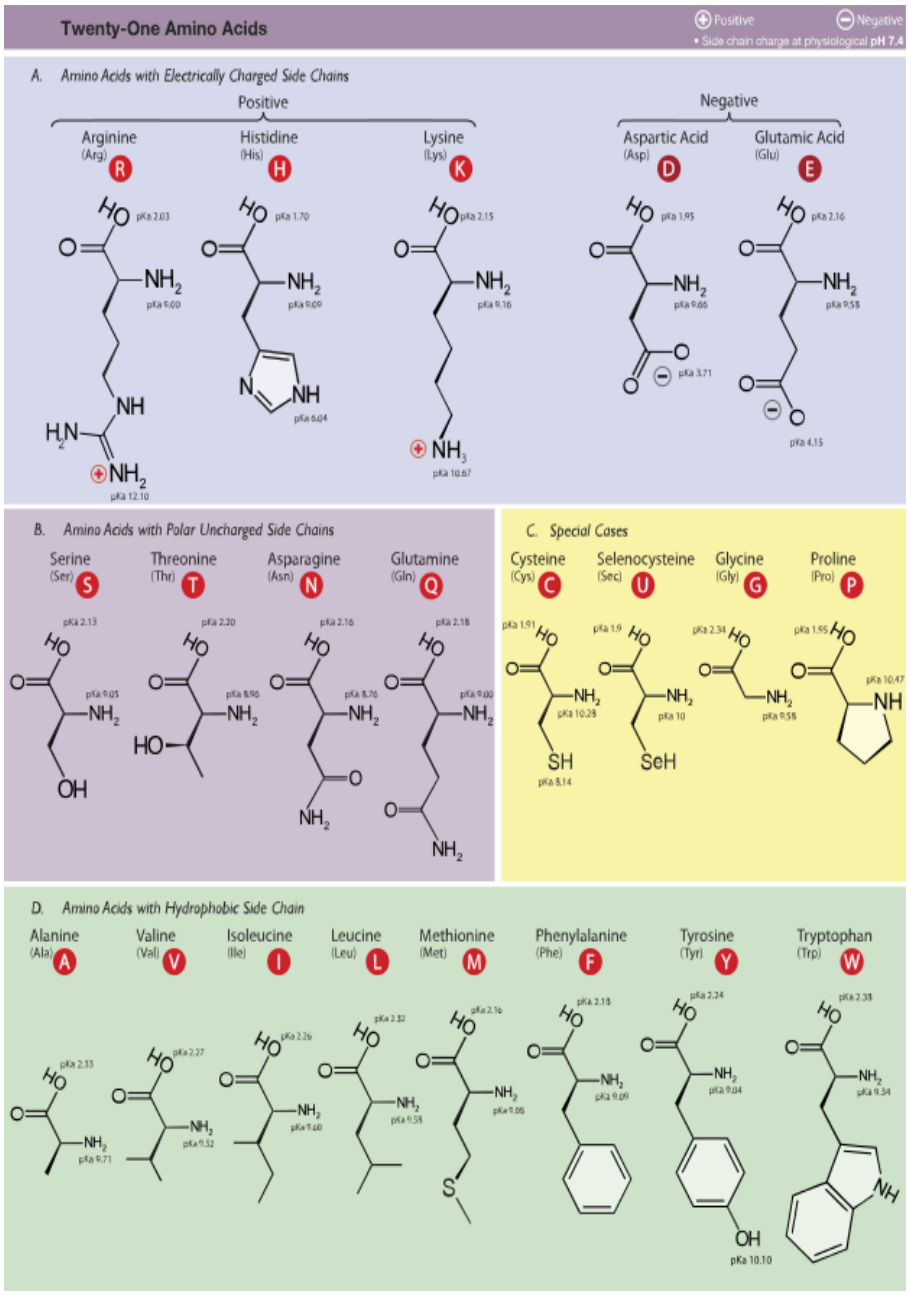


Figure_Apx 9 Human Peroxisome Proliferator Activated Receptor-γ (1PRG) Ligand Binding Pocket Identified with Solvation Method in SYBYL SiteID

The Human PPARγ (1PRG), rendered with α-helices and β-sheet secondary structure features and MOLCAD shading around the SiteID LBP presented, was bound to rosiglitazone during X-ray crystallography. Residues modelled (207-476) in contact with solvent spheres: Tyr222, Phe226, Leu228, His266, Gly284, Phe287, Glu291, Ala292, Glu295, Met329, Val339, Ile341 and Met364, correctly identifying the entry opening and binding site region (Nolte *et al.*, 1998).

Appendix D Amino Acid Properties

Amino acids, also termed residues in the context of proteins, consist of an amine (-NH2) and carboxylic acid (-COOH) functional group, in addition to a central side-chain specific to each amino acid. Figure_Apx 10 shows the common classifications of the 21 amino acids reported in Eukaryotes.



Figure_Apx 10 The Twenty-one Amino Acid's Found in Eukaryotes

Appendix E SYBYL Surflex Dock Potential EDC Receptor Binding Score Data

Surflex-Dock enables flexible molecular docking, incorporating small-molecule force fields which evaluate Cartesian coordinates, constrained by ligand energetics (Jain, 2007), supporting dynamic ring flexibility and optimisation of docked ligand poses. The Surflex-Dock results generated for *in silico* modelled ER, AR. PR and PPARγ are presented in Table_Apx 2. The score for each ligand of the chemical database (n=378) represents the highest calculated -log(Kd) value achieved from 10 poses, irrespective of crash score.

Table_Apx 2 Virtual Screening of Potential-EDC's against *in silico* Nuclear Receptor models (ERα/β, AR, PR and PPARγ) in SYBYL Surflex-Dock

All protomols were generated with a 0.5 Threshold and 0 Bloat. Results show S = score in –log(Kd) and C = crash (degree of inappropriate binding, scores nearing 0 preferable). For each compound (column 1), MoA reported in the literature are summarised in column 3. Colour coding is reference to concordance of *in silico* predictions with the literature: True positives are in green, assumed positives (on the basis of mechanistic studies in other vertebrates) are shaded light green. False negatives are redy-orange, presumed false negatives (on the basis of mechanistic studies in other vertebrates) are shaded pink. Unfavourable binders are shaded light-orange/tan. The results presented were replicated on three separate occasions, however, due to increased chemical database minimisation, the results were the same on each day (Standard Deviation = 0).

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
TRICLOSAN	Antibacterial/ Cosmetic	PPARγ PPARδ TR ER AR, AhR, GR	3.45	-1.39	3.07	-0.81	3.16	-0.84	3.6	-0.18	2.41	-1.79	3.3	-0.58	2.35	-1.37	2.64	-0.32	3.74	-1.46
(-)TETRA CYCLINE	Antibiotic	ER	2.07	-8.47	-10.6	-21.1	-2.11	-8.85	0.14	-10.4	-1.61	-11.3	-10.1	-21.3	-2.44	-11.5	-0.32	-9.31	4.84	-2.05
CEFUROXIME SODIUM	Antibiotic	-	6.22	-1.18	3.45	-4.78	3.99	-2.36	4.63	-4.16	0.24	-8.32	-1.19	-10.8	1.37	-5.96	4.21	-5.04	5.95	-4.25
CHLOR AMPHENICOL	Antibiotic	-	3.27	-0.72	2.86	-2.69	4.68	-1.14	4.26	-1.83	2.52	-2.6	1.46	-3.53	3.79	-2.12	3.43	-1.87	5.2	-1.34
CHLORO TETRACYCLINE	Antibiotic	-	-0.21	-7.11	-5.55	-15.3	-0.87	-6.94	2.17	-6.55	-2.06	-10.7	-7.11	-17.2	1.97	-6.84	3.51	-7.42	3.65	-1.6
CIPROFLOXACIN	Antibiotic	-	5.45	-4.34	5.12	-3.31	4.76	-4.01	7.42	-3.62	3.55	-4.33	4.79	-4.9	5.58	-2.7	4.67	-3.44	4.72	-1.55
CLARITHRO MYCIN	Antibiotic	-	-40.6	-57.5	-107	-126	-50.8	-66	-92.7	-110	-135	-153	-136	-157	-94.1	-113	-106	-126	1.25	-6.55
DEMECLO CYCLINE	Antibiotic	-	-0.27	-5.01	-1.41	-10.7	0.91	-4.46	-0.52	-7.65	-2.64	-11.1	-13.2	-21.8	0.62	-8.76	-0.91	-10.8	4.12	-1.37
ERYTHROMYCIN	Antibiotic	ER	-29.3	-41.7	-106	-126	-12.3	-23	-91.5	-110	-101	-120	-125	-144	-77.8	-95.5	-75.8	-96.4	6.57	-3.15
KETOCONAZOLE	Antibiotic	-	6.04	-2.85	-4.42	-15.2	5.96	-1.15	-6.94	-19.1	-14.3	-26.8	-16.3	-27.5	-4.15	-15.6	-10.4	-22.4	8.95	-3.35
LINCOMYCIN	Antibiotic	-	5.68	-5.65	-2.23	-13	6.09	-3.22	6.32	-7.37	-2.14	-12.9	-3.82	-16	3.18	-8.77	4.64	-7.86	9.65	-4.87
OXYTETRA CYCLINE	Antibiotic	AhR	0.33	-8.57	-6.44	-16.2	-1.21	-8.47	2.91	-7.37	-0.83	-12.1	-7.05	-17.9	-0.48	-8.98	2.56	-8.28	4.3	-4.9
SAMIL	Antibiotic	-	3.12	-7.08	0.14	-11.8	3.1	-4.02	-0.17	-9.08	-3.02	-13.5	-4.19	-17.1	1.64	-8.59	2.87	-7.6	4.84	-3.85
SULFADI METHOXINE	Antibiotic	-	8.07	-2.04	3.75	-3.29	3.1	-3.36	5.34	-1.21	1.92	-4.98	3.74	-3.88	3.84	-2.15	5.01	-1.58	5.22	-1.13
SULFA METHOXAZOLE	Antibiotic	-	4.29	-2.51	3.95	-1.48	4.85	-0.6	4.23	-1.14	3.33	-0.93	3.62	-1.48	4.58	-1.06	3.66	-1.48	3.49	-0.8
SULFAPYRIDINE	Antibiotic	-	6.29	-1.04	5.64	-0.53	4.87	-1.42	6.12	-1.46	4.44	-0.73	4.72	-1.25	3.07	-1.35	4.06	-1.21	4.3	-0.8
TRIMETHOPRIM	Antibiotic	-	7.53	-2.06	5.33	-2.24	5.24	-1.55	6.95	-1.24	4.89	-2.59	4.06	-4.1	4.94	-1.89	5.08	-1.47	6.35	-1.3
2-HYDROXY BENZOIC ACID	Antibiotic/ Fungicide/ Cosmetic	-	2.84	-0.24	3.18	-0.44	2.79	-0.39	3.46	-0.47	3.06	-0.88	2.26	-0.14	2.62	-0.18	3.75	-0.34	3.82	-1.45
ROXITHRO MYCIN	Antibiotic/ Pharmaceutical	-	-49.4	-60.1	-128	-148	-43.6	-54.9	-127	-145	-153	-172	-164	-186	-118	-138	-117	-137	-0.07	-8.49

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
(R)-EPICHLORO HYDRIN	Chemical Reagent	-	1.48	-0.08	1.61	-0.13	1.57	-0.27	2.31	-0.25	2.44	-0.24	2.18	-0.3	2.36	-0.34	2.45	-0.53	2.68	-0.13
1,2,3,4 5-PENTACHLORO BENZENE	Chemical Reagent	-	1.47	-0.28	0.72	-0.46	1.09	-0.09	1.63	-0.25	0.99	-0.62	2.21	-0.44	0.86	-0.46	1.05	-0.16	1.52	-0.67
1,2,3 4-TETRA HYDRONAPHTH ALEN-2-OL	Chemical Reagent	-	3.8	-0.6	4.36	-0.8	4.22	-0.9	4.58	-1.35	4.18	-0.73	3.95	-1.73	3.71	-0.6	4.13	-0.36	3.99	-0.88
1-NAPHTHOL	Chemical Reagent	-	3.88	-0.2	3.62	-0.8	3.84	-0.49	3.81	-0.14	3.63	-0.99	4.38	-0.67	3.15	-0.83	3.61	-0.64	3.9	-0.49
1H-BENZO TRIAZOLE	Chemical Reagent	PPARδ	3.64	-0.29	2.58	-0.13	3.34	-0.34	2.78	-0.25	3.16	-0.44	2.56	-0.11	3.24	-0.99	3.89	-0.25	4.12	-0.07
2,2'-DIHYDROXY-4 4-DIMETHOXY BENZOPHENONE	Chemical Reagent	PPAR, ER, AhR	5.24	-1.31	3.9	-2.57	4.74	-1.88	5.73	-0.71	4.12	-1.58	5.14	-1.65	4.52	-1.66	4.86	-0.45	4.8	-1.73
2 4-DINITRO PHENOL	Chemical Reagent	AR	3.52	-0.42	2.24	-0.57	4.09	-0.41	2.67	-0.39	1.45	-0.6	3.34	-0.62	2.67	-0.35	1.99	-0.4	4.53	-0.09
2-BENZYL PHENOL	Chemical Reagent	-	3.46	-0.38	4.61	-2.19	4.71	-1.57	3.44	-0.35	4.56	-0.33	4.83	-0.87	3.78	-0.52	4.5	-0.75	4.27	-0.77
4-NITRO TOLUENE	Chemical Reagent	-	3.35	-0.71	3.75	-0.53	2.74	-0.48	3.29	-0.78	1.86	-0.83	2.89	-0.28	3.08	-0.45	2.5	-0.43	4.12	-1.15
4-PENTYL CYCLOHEXANOL	Chemical Reagent	ER	6.02	-1.86	6.58	-0.81	7.37	-1.03	4.83	-0.9	4.97	-0.56	6.02	-0.74	5.06	-1.56	5.73	-1.36	6.69	-1.4
4-PHENYL PHENOL	Chemical Reagent	ER, AR	4.03	-0.76	3.5	-0.79	3.47	-0.59	2.92	-0.49	4.2	-0.39	4.49	-1.26	3.19	-0.29	3.17	-0.48	3.22	-0.29
4-TERT-BUTYL PHENOL	Chemical Reagent	ER	3.54	-0.53	3.92	-0.68	3.75	-0.58	5.86	-2.17	4.03	-0.83	3.37	-0.8	3.89	-0.55	4.1	-0.48	3.64	-0.58
4-TERT-OCTYL PHENOL	Chemical Reagent	ER, AR	6.14	-0.64	5.89	-0.86	5.84	-1.29	5.54	-2.21	5.72	-2.01	5.64	-2.2	5.13	-0.63	5.57	-0.58	5.07	-0.86
4-NONYL PHENOL	Chemical Reagent	ER, AR	6.36	-0.81	6.55	-0.67	6.79	-0.97	7.45	-1.18	6.46	-0.81	6.41	-2.84	7.13	-0.78	7.33	-0.79	6.04	-2.98
ANISOLE	Chemical Reagent	-	3.54	-0.5	3.25	-0.22	3.18	-0.39	4.02	-0.26	3.17	-0.24	3	-0.21	3.41	-0.58	2.98	-0.37	3.71	-2.27
BENZOPHENONE	Chemical Reagent	AR, ER	3.75	-0.53	4	-1	3.3	-1.4	3.33	-0.95	3.47	-0.34	4.05	-0.44	3.13	-0.34	3.7	-0.48	3.78	-0.87
BIPHENYL	Chemical Reagent	RXR	3.71	-0.33	3.89	-0.86	3.11	-0.41	4.56	-0.36	2.91	-0.31	3.7	-1.13	2.55	-0.26	2.99	-0.35	2.5	-0.53
BROMOFORM	Chemical Reagent	-	2.88	-0.13	2.79	-0.07	2.7	-0.09	2.72	-0.11	2.51	-0.14	2.7	-0.61	2.77	-0.17	2.83	-0.13	2.73	-0.05
CARBAMIC ACID	Chemical Reagent	-	1.72	-0.06	1.69	-0.06	1.95	-0.07	2.05	-0.13	2.14	-0.17	0.98	-0.13	1.59	-0.03	2.96	-0.33	3.36	-0.36
DEHYDRO ABIETIC ACID	Chemical Reagent	-	4.64	-3.94	4.02	-4.3	0.26	-8.12	1.08	-6.05	-1.42	-8.78	-0.83	-10.5	3.1	-4.77	5.14	-2.56	4.24	-1.98
N N-DIMETHYL FORMAMIDE	Chemical Reagent	RXR	3.2	-0.91	2.31	-0.29	2.21	-0.23	2.01	-0.28	4.11	-1.2	5.15	-1.55	3.67	-0.56	2.62	-0.12	3.24	-0.37
N-(9H-FLUOREN-2-YL) ACETAMIDE	Chemical Reagent	ER, AhR, GR	3.99	-0.4	4.29	-1.38	3.61	-0.61	4.08	-0.89	4.8	-0.74	5.54	-0.51	3.27	-0.4	3.59	-0.56	3.78	-0.43
NAPHTHALEN-2-OL	Chemical Reagent	ER	3.75	-1.11	3.7	-0.76	3.75	-0.87	4.21	-0.42	3.2	-0.59	2.9	-0.86	3.39	-0.45	3.59	-0.74	4.17	-1.29
PHENOL PHTHALEIN	Chemical Reagent	ER, TR, AhR, AR, PXR, GR, PPARγ, PPARδ	4.96	-2.37	5.01	-3.65	4.18	-3.07	0	-6.92	-2.6	-9.64	-3	-10.1	-0.01	-4.89	-0.09	-6.58	3.13	-1.7
(S)-EPICHLORO HYDRIN	Chemical Reagent	-	1.73	-0.08	1.67	-0.19	2.31	-0.27	2.07	-0.36	2.23	-0.13	2.06	-0.32	1.72	-0.68	2.7	-0.18	2.83	-0.09
CHLOROFORM	Chemical Reagent	AR	2.52	-0.16	2.34	-0.06	2.64	-0.16	2.35	-0.3	2.55	-0.13	2.83	-0.24	2.39	-0.11	2.8	-0.1	2.5	-0.15
4-NITROPHENOL	Chemical Reagent	AR	3.44	-0.31	3.07	-0.17	2.68	-0.21	2.29	-0.33	2.89	-1.12	2.14	-0.53	2.63	-1.24	3.72	-0.46	4.6	-0.16
2 5-DIHYDROXY BENZOIC ACID	Chemical Reagent/ Pharmaceutical	-	3.43	-0.24	3	-0.6	2.43	-0.79	2.97	-0.54	3.14	-0.82	2.76	-0.28	2.66	-0.54	3.33	-0.74	3.8	-0.18
2 5-DIHYDROXY BENZOIC ACID	Chemical Reagent/ Pharmaceutical	-	3.44	-0.2	3.06	-0.51	2.35	-0.79	3.04	-0.75	2.45	-0.89	2.92	-0.67	2.93	-0.42	3.59	-0.94	3.74	-0.18
METHYL-2-AMINO-4 5-DIMETHYLTHIOP HENE-3-CARBOXYLATE	Chemical Reagent/ PPP	-	3.84	-0.23	3.51	-0.92	4.18	-0.35	4	-0.36	3.02	-0.82	4.61	-0.33	4.81	-0.94	3.09	-0.77	3.7	-1.13
2 2'-BIPHENOL	Chemical Reagent/ Preservative	-	4	-0.78	4.41	-1.23	3.16	-0.9	2.74	-0.61	4.49	-0.59	3.63	-0.87	3.53	-0.3	3.73	-1.62	3.61	-1.22

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
3-METHYL CHOLANTHRENE	Combustion Product	AR, ER, AhR, TR	4.21	-1.88	5.65	-2.27	5.35	-1.68	4.89	-1.98	3.73	-3.52	3.12	-4.97	4.76	-2.28	5.12	-1.31	4.54	-0.27
7 12-DIMETHYL BENZ[A] ANTHRACENE	Combustion Product	AR, ER, AhR, PPARδ	5.03	-1.05	5.88	-0.77	5.58	-0.77	5.56	-2.74	4.31	-2.22	2.41	-6.11	2.81	-1.75	4.82	-0.82	4.82	-1.09
DIBENZ[A H] ANTHRACENE	Combustion Product	AhR, ER, TR	3.62	-1.05	2.88	-5.06	4.47	-1.17	6.08	-1.78	3.11	-3.97	2.8	-4.77	2.9	-3.92	2.55	-3.7	3.85	-0.62
FLUORENE	Combustion Product	TR	3.9	-0.25	3.44	-0.52	3.44	-0.18	3.97	-0.47	3.07	-1.11	4.11	-0.37	3.18	-0.34	3.34	-0.47	4.13	-0.46
INDENO(1,2 3-CD)PYRENE	Combustion Product	-	5.14	-2.63	4.54	-0.91	5.12	-0.81	2.78	-2.97	2.56	-3.83	-0.5	-7.08	4.66	-2.07	4.8	-1.36	3.64	-2.85
PHENANTHRENE	Combustion Product	-	3.81	-0.33	3.95	-0.43	3.89	-0.42	4.25	-0.39	3.68	-0.3	4.22	-1.42	2.89	-0.42	3.96	-0.22	3.03	-0.38
PYRENE	Combustion Product	-	4.79	-0.4	4.33	-0.51	3.84	-0.5	4.36	-0.9	3.41	-1.03	2.69	-2.78	3.4	-1.79	4.19	-0.4	2.78	-2.31
RETENE	Combustion Product	-	3.61	-0.91	4.6	-1.95	4.77	-1.58	6.51	-0.91	5.21	-1.17	4.41	-3.02	4.99	-0.98	5.15	-0.45	5.36	-1.56
TETRAPHENE	Combustion Product	AhR, ER, PXR, AR	4.87	-0.75	4.09	-0.83	4.3	-0.59	4.85	-1.46	4.66	-0.13	3.94	-2.13	4.74	-0.78	5.05	-0.32	4.12	-1.38
ANTHRACENE	Combustion Product/ Reagent	AhR	4.12	-0.2	4.15	-0.77	3.73	-0.48	4.39	-0.5	2.96	-0.35	3.74	-0.51	2.92	-0.76	3.11	-0.33	3.49	-0.23
NAPHTHALENE	Combustion Product/ Reagent	-	2.77	-0.32	3.29	-0.25	3.45	-1.17	4.17	-0.17	2.87	-0.36	4.38	-0.57	3.11	-0.76	3.86	-0.5	3.31	-0.23
CHRYSENE	Combustion Production	PXR, AhR	4.54	-0.58	3.62	-1.25	4.11	-0.55	5.77	-0.59	4.23	-0.59	4.03	-2.07	3.79	-0.96	4.43	-1.44	3.92	-0.55
CASHMERAN	Cosmetic	-	4.2	-0.58	4.35	-1.54	4.34	-0.96	3.19	-3.11	3.37	-1.33	3.02	-5	3.96	-1.36	3.7	-1.64	4.51	-0.99
CELESTOLIDE	Cosmetic	TR, PPARδ	4.95	-2.62	2.22	-4.13	3.61	-2.86	0.36	-6.74	-1.94	-8.31	-0.15	-8.02	2.32	-4	1.43	-5.39	4.16	-1.58
GALAXOLIDE	Cosmetic	ER	3.36	-3.31	4.47	-3.28	4.76	-2.34	2.05	-5	4.59	-2.62	-0.73	-8.96	5.27	-1.75	4.13	-2.66	4.01	-1.51
PROPYL-4-HYDROXY BENZOATE	Cosmetic/ Food Additive	ER, AR, PPARγ	4.24	-1.5	5.02	-0.41	4.65	-0.28	3.67	-0.49	3.7	-0.64	4.22	-0.98	3.56	-0.38	4.12	-0.36	3.35	-0.86
2 4-DIHYDROXY BENZOPHENONE	Cosmetic/ Industrial Chemical	ER, AR, TR, PPARδ	4.34	-0.48	4.02	-1.68	4.71	-0.64	4.37	-0.79	4.69	-0.48	4.03	-0.47	3.95	-0.36	4.18	-0.38	4.26	-1.53
MUSK KETONE	Cosmetic/ Musk	RXR, ER, PXR	4.35	-2.92	3.89	-3.33	3.94	-2.01	-0.83	-6.49	3.12	-2.03	1.04	-6.31	0.64	-3.83	3.42	-2.24	3	-2.09
4-METHOXY CINNAMIC ACID	Cosmetic/ UV Filter	ER	6.09	-1.44	4.26	-4.74	5.51	-3.12	6.55	-3.94	2.04	-6.87	5.05	-6.66	6.87	-2.45	6.64	-2.81	6.3	-1.68
DICHLORO BROMO METHANE	Flame Retardant/ Reagent	AR	2.36	-0.3	2.53	-0.07	2.47	-0.33	2.57	-0.3	2.79	-0.1	2.96	-0.21	2.51	-0.06	2.84	-0.21	2.82	-0.14
TRIS(2-BUTOXYETHYL) PHOSPHATE	Flame Retardant/ Reagent	-	10.4	-3.58	1.19	-11.6	6.79	-3.02	6.47	-6.04	3.76	-9.83	1.44	-14.6	3.32	-7.41	6.58	-6.32	9.21	-2.43
2-METHOXY-4-VINYLPHENOL	Food Additive/ Component	-	3.48	-1.41	3.83	-0.62	4.03	-0.6	3.55	-0.36	3.95	-0.79	3.79	-1.27	3.79	-1.04	4.05	-0.34	4.93	-1.61
4-VINYLPHENOL	Food Additive/ Component	-	3.5	-0.48	3.15	-0.19	3.9	-0.45	3.07	-0.48	2.56	-0.8	3.28	-0.64	3.48	-0.32	3.2	-0.16	5.41	-0.13
CAFFEINE	Food Additive/ Component/ Pharmaceutical	AR	3	-0.91	3.35	-0.28	3.99	-0.65	4.05	-1.3	3.3	-0.94	3.95	-1.09	2.05	-0.25	3.3	-0.44	3.41	-0.25
2 6-DI-TERT-BUTYL-4-METHYLPHENOL	Food Additive/ Industrial Chemical	PXR, TR, ER	4.61	-1.92	2.85	-2.87	4.81	-1.66	2.49	-4.63	5.14	-1.92	1.56	-6.45	6.35	-1.18	6.25	-1.82	4.54	-1.65
BUTYLATED HYDROXY ANISOLE	Food Additive/ Pesticide/ Antioxidant	PXR	3.41	-0.76	4.41	-1.28	5.51	-0.74	4.33	-1.8	4.28	-0.53	4.22	-2.4	4.54	-0.71	3.87	-1.52	3.88	-0.67
3-PHENYL PROPANOIC ACID	Food Additive/ Preservative	-	3.8	-1.06	4	-0.91	3.62	-0.26	4.55	-0.62	3.93	-0.54	4.36	-0.69	4.57	-1.44	4.27	-0.66	4.77	-0.45
MANEB	Fungicide	-	2.6	-0.77	2.26	-1.36	2.87	-1.24	2.47	-1.07	2.02	-0.37	2.46	-1.04	2.55	-0.25	2.6	-1.41	2.45	-0.51
BENOMYL	Fungicide	AR, TR, PXR, ER, AR	3.2	-1.73	3.76	-2.14	4.54	-1.91	2.87	-2.66	4.59	-2.6	3.41	-4.43	3.91	-1.35	2.38	-3.14	3.41	-2.17
CARBENDAZIM	Fungicide	AhR	3	-0.42	1.87	-0.12	2.97	-1.13	3.16	-0.87	2.19	-0.43	3.23	-0.47	3.64	-1.95	2.29	-1.13	3	-0.67

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
DIFENOCONAZOLE	Fungicide	ER, TR, AR, RXR, GR, PPARδ	5.65	-1.64	-0.49	-9.43	6.19	-1.55	4.85	-4.23	-1.05	-9.34	0.31	-10.5	3.65	-5.23	3.12	-4.75	5.96	-1.93
ENILCONAZOLE	Fungicide	AhR, PXR, PPARδ, TR, ER, AR	5.98	-1.62	6.08	-1.25	5.59	-1.46	5.35	-1.63	5.11	-2.03	5.46	-1.33	4.14		5.06	-1.59	5.63	-1.79
EPOXICONAZOLE	Fungicide	AhR, TR, AR, ER	5.29	-1.24	2.93	-3.94	3.76	-2.91	3.95	-3.97	3.71	-2.57	1.45	-6.26	4.09	-1.03	4.91	-1.44	5.09	-0.82
FENARIMOL	Fungicide	ER, AR, TR, GR	5.35	-1.41	0.87	-4.46	3.86	-0.93	2.95	-2.59	-0.29	-5.78	-1.57	-6.43	-0.57	-5.15	0.6	-4.15	3.35	-1.57
MYCLOBUTANIL	Fungicide	PXR, ER	6.1	-0.96	4.35	-1.72	5.26	-2.01	5.35	-2.15	4.97	-1.61	5.85	-2.31	4.46	-1.74	4.19	-2.9	5.2	-1.21
PENCONAZOLE	Fungicide	GR, AR, ER, TR	4.96	-1.61	4.33	-1.65	5.23	-1.98	4.93	-1.28	4.24	-1.39	2.91	-2.62	4.88	-2.06	5.63	-1.87	4.86	-2.51
PROCHLORAZ	Fungicide	AhR, PXR, AR, TR, PPARδ, ER, GR	4.69	-1.92	2.89	-5.02	3.86	-2.7	3.9	-2.96	1.84	-4.2	0.39	-6.15	4.14	-2.78	3.72	-3.5	6.15	-1.18
PROPICONAZOLE	Fungicide	PXR, AhR, TR, ER, AR	5.93	-2.31	3.63	-4.02	5.22	-3.07	5.35	-2.31	4.49	-2.32	3.36	-4.48	3.78	-2.48	4.47	-2.74	4.34	-3.55
TEBUCONAZOLE	Fungicide	PXR, ER, GR, AR, TR	4.73	-1.42	5.22	-2.81	4.36	-2.91	3.63	-4.3	4.85	-1.68	3.25	-4.34	4.6	-2.42	4.78	-2.4	6.23	-2.2
THIRAM	Fungicide	TR, GR, PPARγ, PPARδ, AhR, AR	2.96	-1.17	3.32	-1.61	3.78	-1.31	1.5	-4.21	2.94	-1.03	3.53	-1.95	2.96	-0.5	3.72	-1.89	2.9	-1.8
VINCLOZOLIN	Fungicide	AR, PPARδ	2.66	-1.76	1.8	-2.35	2.66	-1.69	4.13	-0.7	2.58	-1.21	4.18	-1.74	2.49	-1.37	2.35	-0.96	5.13	-0.64
RESORCINOL	Fungicide/ Pharmaceutical	-	4.4	-0.36	2.84	-0.29	3.23	-0.38	2.92	-0.37	3.02	-0.41	3.63	-0.28	2.82	-0.43	3.16	-0.3	2.65	-0.76
4-BENZYL PHENOL	Germicide/ Antiseptic/ Preservative	ER	4.28	-0.51	4.88	-0.58	4.17	-0.45	3.39	-1.24	3.65	-0.62	4.39	-0.81	3.78	-1.01	3.8	-0.8	4.38	-0.86
4-CHLORO-3 5-DIMETHYL PHENOL	Germicide/ Antiseptic/ Preservative	-	4.1	-0.24	4.32	-0.66	4.18	-0.66	4.55	-0.17	4.32	-0.52	3.89	-0.24	4.52	-0.36	5.32	-0.37	4.44	-1.8
2 3-D	Herbicide	-	3.63	-0.78	3.19	-0.71	3.59	-0.54	3.93	-1.57	3.7	-0.44	2.64	-0.73	4.05	-1.47	3.71	-0.44	5.66	-0.79
3 5-DIBROMO-4-HYDROXY BENZONITRILE	Herbicide	AR, RXR, AhR, PPARα, PPARγ, ER	1.95	-0.32	1.21	-0.58	1.46	-0.11	3.29	-0.49	1.43	-0.39	1.77	-1.03	1.3	-0.42	0.84	-1.01	3.05	-0.74
PRONAMIDE	Herbicide	PXR, AR	2.04	-1.69	1.86	-2.62	1.89	-2.36	2.97	-0.54	3.44	-0.79	2.81	-1.7	3.24	-0.66	1.96	-1.05	2.66	-0.52
3-AMINO-1,2 4-TRIAZOLE	Herbicide	ER	3.49	-0.25	2.98	-0.2	3.09	-0.31	2.83	-0.14	2.04	-0.3	2.25	-0.17	2.35	-0.19	2.69	-0.83	2.72	-0.06
4-(2 4-DICHLORO PHENOXY) BUTANOICACID	Herbicide	AR	3.74	-0.43	3.87	-0.9	3.81	-0.47	4.68	-2.23	4.72	-0.96	4.47	-0.46	5.11	-0.99	4.42	-0.81	5.21	-0.72
4-AMINO-2,5 6-TRICHLORO PYRIDINE-2-CARBOXYLIC ACID	Herbicide	AhR	2.26	-0.44	2.59	-0.22	2.3	-0.93	3.04	-0.21	1.86	-0.27	3.02	-0.91	2.55	-1.06	2.2	-0.24	3.5	-0.84
ACETOCHLOR	Herbicide	AR, ER	5.4	-1.03	3.45	-3.36	4.27	-2.54	3.22	-3.5	2.66	-3.3	3.02	-5.03	3.51	-2.66	5.09	-1.4	4.65	-2.24
ALACHLOR	Herbicide	ER, AR, TR	4.5	-1.02	3.33	-3.9	4.02	-1.76	0.7	-4.58	2.96	-3.86	1.92	-5.28	3.51	-1.05	4.06	-1.92	3.86	-3.41
ATRAZINE	Herbicide	AhR	3.76	-0.83	3.57	-1.04	3.84	-0.66	4.26	-1.18	3.51	-1.09	3.53	-2.25	3.69	-1.3	4.69	-0.77	3.91	-1.14
BENTAZON	Herbicide	-	3.38	-0.55	2.98	-2.11	3.88	-0.83	2.6	-2.62	3.91	-0.89	3.24	-1.07	4.04	-0.51	2.05	-3.38	3.49	-1.02
BROMACIL	Herbicide	AhR	3.17	-0.7	2.85	-1.03	4.2	-0.77	3.87	-0.63	3.58	-0.41	3.39	-0.72	3.23	-0.5	3.64	-1.3	4.11	-0.67
DIURON	Herbicide	AhR	1.71	-0.88	2.05	-0.76	2.21	-2.09	3.7	-0.48	2.41	-0.29	3.95	-0.26	2.62	-0.87	2.82	-0.4	2.63	-0.59
GLUFOSINATE AMMONIUM	Herbicide	-	3.99	-1.02	3.89	-0.78	4	-1.19	4.09	-0.86	3.37	-0.57	3.66	-1.9	3.72	-1.29	4.01	-0.98	6.13	-1.46
GLYPHOSATE	Herbicide	-	2.55	-0.4	3.73	-0.92	2.75	-1.21	3.62	-0.88	3.56	-0.39	3.1	-0.81	3.02	-1.09	3.54	-0.42	3.84	-0.57

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
N-(3 4-DI CHLOROPHENYL) PROPANAMIDE	Herbicide	PXR, AR	3.81	-1.06	1.9	-0.33	3.21	-0.93	3.13	-0.6	2.32	-0.45	3.47	-0.46	2.09	-0.58	3.04	-1.55	3.15	-0.83
PARAQUAT	Herbicide	-	3.4	-1.32	4.53	-0.83	4.8	-0.72	4.95	-0.24	4.61	-0.48	5.31	-0.3	3.05	-0.6	3.89	-0.81	3.55	-0.44
PENDIMETHALIN	Herbicide	ER	6.07	-1.65	3.22	-4.41	5.46	-2.01	3.8	-2.31	1.44	-3.34	3.86	-3.69	4.99	-1.75	3.65	-1.95	6.2	-3.5
PROMETRYN	Herbicide	PXR	3.94	-1.94	4.64	-1.7	3.91	-0.46	3.19	-4.05	3.99	-1.79	2.13	-5.63	3.56	-2.39	4.12	-1.99	4.26	-1.4
PROPAZINE	Herbicide	PXR, AhR	2.82	-1.22	3.73	-1.28	4.98	-0.69	4.6	-1.86	3.33	-1.58	3.5	-3.12	3.69	-1.65	4.62	-1.01	3.27	-2.28
SIMAZINE	Herbicide	-	2.92	-1.17	2.79	-0.96	3.35	-2.01	4.3	-1	2.52	-1.01	4.46	-1.09	2.99	-0.68	3.22	-0.63	3.26	-0.57
TERBUTRYN	Herbicide	AhR, AR, PPARδ, TR	3.63	-2	4.75	-1.73	4.96	-0.76	4.26	-1.49	4.97	-1.7	3.98	-3.28	4.05	-2.37	4.83	-0.68	3.44	-1.05
TERBUTYLAZINE	Herbicide	ER, GR	6.66	-2.89	7.02	-2.51	5.76	-2.25	6.49	-2.76	6.7	-1.85	5.47	-4.94	6.53	-1.94	6.5	-1.58	7.52	-1.79
TRIFLURALIN	Herbicide	-	6.34	-1.39	-0.58	-7.82	5.43	-1.57	0.73	-5.7	3.02	-3.47	1.64	-4.84	3.37	-2.63	4.71	-1.63	7.06	-3.29
1,2 3-TRICHLORO BENZENE	Industrial Chemical	-	1	-0.12	1.64	-0.22	1.86	-1.05	2.83	-0.17	1.36	-0.79	2.48	-0.13	1.77	-0.31	1.72	-0.18	1.51	-1.16
BISPHENOL B	Industrial Chemical	ER, AR, TR, PXR, PPARγ, PPARδ	5.17	-1.26	4.06	-2.33	4.36	-2.58	4.05	-1.76	3.04	-2.35	2.89	-5.82	4.54	-2.07	6.16	-0.98	5.47	-1.24
2,3 4-TRICHLORO BIPHENYL	Industrial Chemical	-	3.28	-0.97	3.46	-1.08	2.88	-0.84	3.39	-0.4	3.02	-0.98	4.18	-0.38	2.3	-0.94	3.4	-0.4	2.91	-0.43
2-(((2-ETHYLHEXYL)OXY)CARBONYL) BENZOIC ACID	Industrial Chemical	PPARγ, PPARα, RXR	7.6	-2.32	6.11	-3.12	7.71	-1.77	6.09	-2.19	7.56	-1.66	4.89	-3.45	5.69	-1.24	5.97	-1.79	6.13	-1.6
2-CHLORO BIPHENYL	Industrial Chemical	-	3.57	-0.98	3.34	-0.55	3.26	-1.36	3.39	-0.86	3.15	-1.14	3.26	-0.38	2.73	-0.38	3.1	-0.36	3.07	-0.81
3-CHLORO BIPHENYL	Industrial Chemical	-	3.6	-0.78	3.96	-0.45	3.34	-0.27	3	-0.7	2.93	-0.92	3.84	-0.37	2.53	-0.48	2.82	-0.77	3.23	-0.48
4 4'-BIPHENOL	Industrial Chemical	AR, ER, PPARγ	3.27	-0.96	4.8	-0.7	5.46	-0.76	4.41	-0.87	5.46	-0.7	4.9	-1.53	4.72	-0.57	3.96	-0.55	3.62	-1.87
BISPHENOL F	Industrial Chemical	ER	4.28	-1.01	4.08	-1.46	4.87	-0.65	3.63	-1.14	4.04	-0.81	4.92	-1.42	4.07	-0.73	4.91	-0.4	4.32	-0.83
4-CHLORO-4'-HYDROXY BIPHENYL	Industrial Chemical	ER	4.7	-0.68	4.14	-0.49	4.32	-0.55	3.71	-0.32	3.61	-0.24	4.02	-0.46	3.7	-0.47	3.36	-0.51	3.69	-0.6
4-CHLORO BIPHENYL	Industrial Chemical	-	3.09	-0.22	2.57	-0.29	3.07	-0.3	2.24	-0.63	2.89	-0.45	3.52	-0.62	1.55	-1.4	2.85	-0.66	2.8	-0.85
4-ISOPENTYL PHENOL	Industrial Chemical	-	4.14	-0.63	4.57	-1.12	4.77	-0.57	4.51	-1.12	4.47	-0.23	5.18	-1.38	4.71	-0.51	4.14	-0.83	5.31	-0.47
6-BROMO-2-NAPHTHOL	Industrial Chemical	-	3.52	-0.65	3.1	-0.84	3.52	-0.34	3.14	-0.1	3.71	-0.49	3.5	-1.15	3.33	-0.53	2.6	-0.34	2.87	-0.17
BENZYLBUTYL PHTHALATE	Industrial Chemical	ER, AhR, PPARδ, AR	7	-0.7	6.84	-2.18	5.56	-1.45	3.6	-3.92	6.96	-1.84	3.64	-6.04	5.25	-1.54	5.03	-1.38	7.11	-2.27
BIS(2-ETHYLHEXYL) PHTHALATE	Industrial Chemical	AhR, PXR	8.76	-2.99	0.02	-13.4	6.35	-4.99	0.34	-11.7	-1.5	-14.8	-10.6	-23.6	-2.84	-12.7	4.47	-8.01	8.29	-0.76
BISPHENOL A	Industrial Chemical	ER, PPARγ, AR, AhR, PPARδ, PXR	4.88	-0.62	5.76	-0.63	5.21	-0.82	4.97	-0.87	3.11	-1.66	3.65	-3.17	3.46	-1.66	4.39	-0.98	4.8	-0.91
BISPHENOL E	Industrial Chemical	-	3.93	-1.32	4.48	-0.53	5.3	-0.66	4.41	-1.48	3.19	-2.53	4.08	-2.96	3.01	-1.61	4.76	-2.09	4.87	-1.03
BUTYLBENZENE	Industrial Chemical	-	3.62	-0.67	4.13	-0.53	3.4	-0.48	3.57	-0.98	3.34	-0.68	4.08	-0.53	3.59	-1.27	4.05	-1.4	3.57	-0.22
CHLORO DIBROMO METHANE	Industrial Chemical	AR	2.52	-0.07	2.6	-0.03	2.56	-0.47	2.77	-0.27	3.05	-0.11	2.59	-0.4	2.51	-0.18	2.9	-0.19	2.99	-0.13
DIBUTYL PHTHALATE	Industrial Chemical	ER	7.15	-2.02	4.35	-4.21	6.55	-1.74	5.49	-3.72	5.9	-1.74	5.96	-3.9	5.68	-1.58	7.3	-1.55	6.22	-0.9
DICYCLOHEXYL PHTHALATE	Industrial Chemical	TR, PPARδ	6.61	-2.03	4.12	-6.2	5.93	-1.78	-0.75	-9.04	-0.21	-9.56	-6.68	-16.8	0.6	-6.88	2.13	-6.87	6.82	-1.46
DIETHYL PHTHALATE	Industrial Chemical	AhR	4.13	-1.98	4.59	-0.69	5.24	-0.98	3.95	-1.37	3.7	-0.85	3.98	-1.99	4	-0.81	3.99	-1.27	4.41	-0.66
DIISOBUTYL PHTHALATE	Industrial Chemical	ER	5.81	-1.42	5.02	-2.86	6.62	-1.32	3.95	-5.64	4.36	-2.72	3.99	-5.1	4.76	-2.06	6.01	-2.67	6.43	-1.11

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
DIISODECYL PHTHALATE	Industrial Chemical	AR	8.13	-3.4	-0.59	-15.2	5.84	-5.97	-6.38	-21.4	-18.1	-31.6	-19.1	-35.2	-1.49	-13.8	-0.74	-16	8.42	-4.93
DIISONONYL PHTHALATE	Industrial Chemical	-	11	-3.48	-5.6	-19.8	7.17	-4.08	5.47	-8.98	-6.84	-17.9	-16	-29.3	0.29	-13.7	2.52	-11.4	9.62	-1.09
MONOBUTYL PHTHALATE	Industrial Chemical	-	6.24	-0.73	5.6	-1.25	5.19	-1.55	4.96	-0.93	6.19	-0.77	5.55	-0.66	4.15	-0.59	6.72	-1.17	5.77	-0.38
N-NITROSO DIMETHYL AMINE	Industrial Chemical	-	2.05	-0.12	2.49	-0.15	2.17	-0.24	2.58	-0.42	2.55	-0.37	2.75	-0.31	3.06	-0.21	2.63	-0.34	3.18	-0.22
N-NITROSO PIPERIDINE	Industrial Chemical	-	3.04	-0.77	3.43	-0.63	3.16	-0.26	3.57	-0.74	3.24	-0.3	2.4	-0.55	3.6	-0.51	3.66	-0.74	4.6	-0.74
PERFLUORO DECANOIC ACID	Industrial Chemical	ER	4.17	-1.27	3.9	-1.75	5.34	-1.17	3.89	-1.75	5.5	-1.83	4.34	-3.41	5.58	-1.34	5.11	-1.19	6.05	-1.95
STYRENE	Industrial Chemical	-	3.2	-0.63	3.18	-0.22	2.41	-0.4	3.36	-0.43	3.18	-0.65	3.46	-0.12	3.32	-0.67	2.97	-0.17	3.18	-0.57
TETRACHLORO ETHYLENE	Industrial Chemical	-	0.5	-0.17	0.47	-0.18	0.63	-0.22	0.75	-0.03	0.36	-0.18	0.83	-0.04	0.25	-0.07	0.35	-0.05	0.67	-0.17
TRIBUTYL PHOSPHATE	Industrial Chemical	ER	6.69	-1.67	6.93	-1.79	5.91	-2.07	6.13	-2.28	7.28	-1.74	6.81	-2.85	7.11	-1.51	6.72	-1.32	6.17	-0.97
VINYLACETATE	Industrial Chemical	-	2.86	-0.3	2.95	-0.85	3.02	-0.24	3.04	-1.19	2.48	-0.14	3.13	-0.24	3.27	-0.46	3.07	-0.41	4.47	-0.38
DDE	Insecticide	AR, ER	2.4	-2.12	1.31	-2.88	2.57	-0.5	2	-2.09	-1.36	-4.34	1.2	-2.19	0.81	-2.08	2.61	-0.88	3.96	-0.9
ACEPHATE	Insecticide	-	4.33	-0.6	1.97	-0.67	3.24	-0.53	3.24	-0.56	2.11	-1	3.53	-1.31	2.57	-1.24	3.56	-0.55	3.42	-1.66
BIFENTHRIN	Insecticide	ER	4.48	-5.19	0.02	-10.3	1.91	-2.86	-4.37	-15.3	-1.57	-12	-11.6	-20.9	1.07	-8.16	5.49	-4.99	6.33	-2.34
CHLOR FENVINFOS	Insecticide	PXR, AhR	6.11	-1.28	3.89	-2.56	5.54	-1.16	4.57	-2.01	3.36	-2.99	1.55	-5.36	3.21	-2.16	4.43	-1.43	3.97	-1.02
DDD	Insecticide	ER, TR, GR, PPARδ, PPARδ, AR	3.7	-0.89	2.41	-2.15	2.69	-1.39	3.19	-1.38	0.1	-3.32	0.98	-2.81	1.75	-1.7	3.02	-0.94	3.76	-1.35
DDT	Insecticide	ER, GR, PPARγ, TR, PXR, AR	2.78	-0.87	1.55	-2.72	1.66	-2.27	1.27	-2.95	-0.5	-3.64	-2.3	-6.76	-0.21	-3.41	2.46	-1.4	3.41	-1.29
DELTAMETHRIN	Insecticide	PXR, TR	5.74	-1.61	-4.21	-12.8	3.32	-4.96	0.33	-9.22	-7.43	-15.2	-5.33	-15.6	-4.39	-12.6	0.18	-8.31	5.53	-1.92
DEMETON-S-METHYL	Insecticide	-	4.46	-1.05	4.2	-1.35	5.06	-0.86	4.01	-1.28	4.09	-0.61	4.7	-1.79	3.68	-2	4.75	-1.66	4.67	-1.35
DIAZINON	Insecticide	AhR, TR, GR, PPARδ	6.12	-1.6	5.17	-3.72	6.54	-1.94	6.24	-1.81	4.16	-3.28	4.46	-4.41	6.14	-1.28	6.56	-2.1	5.54	-3.72
DICHLORVOS	Insecticide	AR, ER, PXR, PPARγ	2.65	-0.57	2.06	-0.65	3	-0.64	2.78	-1.49	1.99	-0.8	2.86	-0.76	2.29	-1.1	4.07	-0.71	3.85	-1.13
DICOFOL	Insecticide	PPARγ, AR	1.25	-4.17	0.51	-3.68	2.23	-2.74	0.28	-3.58	-0.58	-4.63	-1.48	-5.86	-0.31	-2.89	2.05	-1.76	2.25	-1.32
DIELDRIN	Insecticide	AR, TR, PXR	0.57	-3.63	0.11	-3.96	-2.02	-5.1	-7.51	-10.6	-1.95	-4.74	-8.43	-13.4	-1.19	-4.88	0.68	-2.64	0.3	-4.62
DIMETHOATE	Insecticide	AhR	4.68	-0.67	3.06	-0.79	5.13	-1.08	3.47	-1.91	3.11	-0.79	2.34	-2.34	2.95	-1.34	3.32	-2.35	4.74	-0.87
ETOFENPROX	Insecticide	-	6.94	-1.61	2.06	-8.11	5.34	-1.65	0.75	-8.75	1.69	-9.3	-10.4	-21.4	0.85	-8.14	3.93	-6.9	8.23	-1.53
FENITROTHION	Insecticide	AR, AhR	4.62	-1.38	5.04	-1.36	5.67	-0.78	4.37	-1.42	3.86	-1.25	5.51	-1.4	4.36	-0.88	3.54	-1.74	4.47	-1.71
FENOXYCARB	Insecticide	PPARγ, AR, ER, PXR	6.34	-1.45	4.71	-2.79	5.78	-2.06	7.92	-1.67	5.73	-1.8	6.86	-3.18	7.41	-1.73	6.33	-3.41	5.17	-1.17
FENVALERATE	Insecticide	PXR, ER, TR, AR	4.83	-3.48	-4.09	-15.6	5.17	-3.58	3.78	-7.39	-6.75	-17	-17	-25.6	-0.83	-10.2	-2.61	-13.7	6.79	-2.59
FIPRONIL	Insecticide	AR, ER, PXR, TR, PPARδ, PPARγ	0.29	-3.78	2.95	-3.76	0.78	-3.28	3.13	-3.8	0.82	-4.25	1.38	-4.56	2.43	-2.11	1.92	-2.67	2.12	-2.37
FLUVALINATE	Insecticide	-	5.25	-3.45	-3.52	-16.7	7.92	-4.35	-7.57	-19.8	-3.59	-15.9	-18.6	-30.4	-4.11	-15.2	0.44	-11.8	6.51	-2.25
LINDANE	Insecticide	ER, TR, GR, PXR, PPARδ, AhR	1.79	-0.66	2.02	-0.7	1.42	-0.83	1.19	-2.14	1.03	-2.15	0.95	-2.44	1.03	-0.74	1.35	-0.49	1.99	-0.4
MALATHION	Insecticide	AhR	5.48	-1.48	4.73	-2.46	4.81	-4.24	5.89	-2.17	5.31	-2.86	3.89	-4.57	3.81	-2.05	5.59	-1.8	5.28	-2.98

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
MEVINPHOS	Insecticide	-	4.44	-0.73	4.14	-0.96	4.63	-0.88	5.13	-1.05	4.3	-0.82	4.52	-1.53	4.55	-0.98	5.21	-0.95	6.69	-1.15
MIREX	Insecticide	-	-6.25	-8.58	-7.7	-9.73	-4.53	-7.45	-13.3	-17.8	-9.33	-12.1	-18.3	-20.9	-7.83	-10.6	-4.27	-6.35	-1.18	-0.45
OMETHOATE	Insecticide	-	4.01	-0.77	4.56	-0.76	4.14	-0.67	4.44	-1	3.25	-1.02	3.87	-0.86	3.92	-1.67	4.92	-1.27	4.03	-0.7
OXYDEMETON-METHYL	Insecticide	-	4.79	-0.98	4.35	-1.18	4.88	-0.72	4.49	-1.31	3.82	-1.7	4.84	-1.28	3.79	-1.63	3.91	-0.99	5.56	-1.32
PARATHION	Insecticide	AhR, PXR	4.17	-0.59	5.76	-2.17	4.62	-1.61	4.36	-1.3	3.68	-1.82	4.6	-2.37	4.49	-1.64	3.71	-1.26	5.36	-1.19
PERMETHRIN	Insecticide	AhR, PXR	3.11	-7.21	-0.54	-9.62	4.71	-3.72	-0.32	-10.3	-6.09	-12.6	-4.38	-14.1	0.54	-7.49	4.3	-6.04	5.93	-1.29
PROPOXUR	Insecticide	-	4.01	-0.61	5.23	-0.44	4.76	-0.47	2.94	-2.02	4.12	-0.7	3.81	-1.95	4.15	-2.7	3.57	-0.74	4.43	-0.87
RESMETHRIN	Insecticide	PXR, AR, GR, PPARγ	6.5	-4.86	1.42	-10.1	5.97	-3.69	6.01	-5.35	-3.04	-13.8	-3.87	-13.8	1.32	-8.08	3.64	-6.89	7.18	-1.82
TETRACHLORVINPHOS	Insecticide	ER, AR, GR, TR, PPARγ, PXR	4.05	-1.31	3.17	-2.35	3.81	-1.91	3.19	-2.31	3.08	-1.81	2.29	-3.46	2.46	-1.62	3.72	-1.89	3.42	-1.45
TOXAPHENE	Insecticide	ER, TR, GR, AR, PPARδ, PPARγ	0.68	-3.37	-2.58	-6.13	-1.75	-5.37	-1.98	-8.26	-1	-5.42	-7.53	-11.9	-2.47	-6.07	0.79	-1.98	0.53	-3.16
TRICHLORFON	Insecticide	-	2.74	-1.4	2.67	-0.8	2.62	-0.77	2.27	-1.38	1.57	-1.58	0.85	-2	1.95	-1.19	3.23	-0.41	2.72	-0.75
PIPERONYL BUTOXIDE	Insecticide	AhR, TR, AR, PXR	7.43	-2.33	5.34	-5.9	7.36	-2.43	8.85	-2.59	3.26	-5.81	5.22	-7.97	9.13	-2.17	8.05	-2.9	8.36	-1.28
TONALIDE	Musk Ketone	TR	2.77	-2.86	5.86	-2.48	3.78	-2.97	0.27	-6.32	2.27	-5.15	-1.57	-12.3	2.96	-3.91	3.97	-4.33	4.45	-2.61
RETINOL	Natural Compound	ER, PXR, GR, AR, TR, PPARγ	6.64	-2.49	5.97	-4.94	6.04	-3.79	8.16	-3.63	6.04	-6.4	4.55	-8.4	6.46	-3.12	6.94	-4.25	7.43	-1.63
STEARIC ACID	Natural Compound	ER	7.59	-2.19	6.77	-3.41	8.51	-2.37	8.62	-2.65	8.34	-3.61	3.7	-10.2	9.32	-4.08	9.02	-3.68	9.29	-1.54
ANDRO STANEDIONE	Natural Hormone	AR	3.47	-2.63	3.49	-4.6	3.45	-4.01	2.76	-4.61	7.74	-0.95	0.48	-8.71	6.99	-1.11	5.31	-2.22	3.77	-5.81
ANDRO STERONE	Natural Hormone	AR	4.1	-4.36	1.9	-6.1	2.45	-6.16	2.86	-4.89	7.44	-1.64	1.58	-8.71	3.12	-3.46	5.51	-4.39	3.3	-1.18
DEHYDRO TESTOSTERONE	Natural Hormone	AR	5.06	-0.91	1.26	-5.62	5.05	-1.97	3.84	-4.81	7.14	-0.86	-3.2	-11.9	6.14	-1.43	5.85	-1.92	3.54	-2.43
CORTISONE	Natural Hormone	AR. GR	4.58	-3.91	0.92	-8.72	3.95	-4.9	-0.7	-10.6	5.27	-4.44	-3.38	-14.4	5.54	-4.68	7.89	-3.33	4.51	-0.84
EPI ANDROSTERONE	Natural Hormone	AR	3.52	-4.68	2.44	-6	4.13	-5.36	4.04	-4.46	7.94	-1.46	2.83	-7.85	6.76	-1.18	4.22	-2.72	4.39	-0.87
ESTRADIOL	Natural Hormone	ER, AR, TR, GR, PPARγ	5.09	-1.21	5.47	-3.85	5.19	-1.82	4.42	-3.39	4.49	-2.84	-0.13	-7.94	4.32	-2.73	5.21	-0.66	4.28	-1.27
ESTRIOL	Natural Hormone	ER, AR, GR, TR	6.47	-0.99	5.78	-2.15	7.91	-1.18	5.37	-3.01	7.49	-1.08	4.34	-5.28	6.53	-1.78	6.18	-1.35	2.81	-2.11
ESTRONE	Natural Hormone	ER, AR, TR, GR, PR, PXR	6.2	-0.75	5.55	-1.86	8.13	-0.85	4.69	-2.9	4.87	-1.5	3.78	-4.41	5.84	-1.7	6.21	-0.95	5.22	-1.16
L-THYROXINE	Natural Hormone	TR, AhR, PPARγ, GR	-0.62	-7.93	-6.6	-13.6	0.94	-6.39	-3.02	-10.3	-2.53	-8.67	-8.27	-14.8	-4.1	-10.9	-0.48	-9.44	3.59	-3.57
PROGESTERONE	Natural Hormone	PR, AR, ER, GR, TR, PPARγ, PPARδ	4.1	-3.55	3.59	-6.91	3.55	-4.91	2.05	-6.41	5.99	-3.54	-2.13	-12.3	6.56	-2.96	8.35	-2.37	3.86	-0.85
STANOLONE	Natural Hormone	AR, ER, GR, PR, RXR, TR	4.08	-2.67	3.5	-5.51	4.44	-3.91	3.58	-5.94	9.38	-0.57	-0.1	-9.16	8.18	-0.97	7.15	-2.22	4.52	-1.46
LIOTHYRONINE	Natural Hormone/ Pharmaceutical	TR, AhR, ER, GR	1.17	-6.93	-0.01	-6.75	-2.55	-9.07	-0.95	-7.1	-4.81	-11.3	-0.88	-9.61	0.96	-7.04	0.42	-6.08	5.15	-2.38
BUTYL-4-HYDROXY BENZOATE	Paraben/ Antiseptic	ER, AR	4.56	-1.13	4.93	-0.56	4.69	-0.88	3.88	-0.63	5.17	-0.6	4.33	-0.73	5.1	-0.47	4.07	-0.13	4.75	-0.91
ETHYL-4-HYDROXY BENZOATE	Paraben/ Antiseptic	ER	3.95	-0.73	3.89	-0.27	3.76	-0.9	2.9	-0.84	3.17	-0.92	3.17	-0.82	3.2	-0.19	4.36	-0.58	4.59	-0.19
METHYL-4-HYDROXY BENZOATE	Paraben/ Antiseptic	AhR, ER	3.57	-1.42	3.8	-0.72	3.34	-0.44	3.38	-0.55	3.05	-0.31	3.41	-0.48	3.18	-0.3	3.32	-0.62	3.48	-0.85
BENZYL-4-HYDROXY BENZOATE	Paraben/ Bactericidal/ Fungicidal	ER, PPARγ, TR	4.76	-0.87	4.17	-1.04	4.2	-0.53	4.28	-0.43	4.64	-0.34	5.3	-0.66	4.31	-0.53	4.47	-0.61	2.8	-0.43

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
(4-CHLORO-2-METHYLPHENOXY)ACETIC ACID	Pesticide	-	4.35	-0.7	3.79	-0.63	4.11	-0.63	5.66	-1.36	4.1	-0.47	3.5	-0.32	5.2	-1.46	3.77	-0.74	6.97	-0.94
2-(4-CHLORO-2-METHYLPHENOXY)PROPANOIC) ACID	Pesticide	-	3.96	-0.78	3.93	-1.23	4.21	-0.4	5	-1.64	3.99	-1.17	3.17	-0.59	4.29	-0.62	4.87	-0.72	5.98	-0.82
4-HYDROXY-3 5-DIIODOBENZONITRILE	Pesticide	AhR, PPARγ	1.98	-0.52	0.71	-0.31	1.55	-0.13	2.69	-1.16	-0.17	-1.82	1.27	-0.84	0.77	-0.44	0.52	-0.34	3.01	-0.12
5-AMINO-4-CHLORO-2-PHENYLPYRIDAZIN-3(2H)-ONE	Pesticide	-	2.79	-0.22	4.04	-1.29	3.82	-0.53	0.84	-3.55	2.91	-0.42	3.98	-0.69	3.09	-0.47	3.06	-0.14	3.85	-0.43
6-CHLORO-2-N-(PROPAN-2-YL)-1,3,5-TRIAZINE-2 4-DIAMINE	Pesticide	-	3.25	-1.19	3.59	-0.27	3.08	-0.34	4.22	-0.42	2.22	-0.63	3.81	-0.34	2.51	-0.5	2.34	-0.86	3.7	-1
ALDICARB	Pesticide	AhR, PPARγ	2.52	-1.54	4.39	-0.85	3.9	-0.54	2.58	-1.92	3.23	-0.47	3.01	-1.6	3.59	-0.74	3.16	-0.46	4.47	-1.67
AMITRAZ	Pesticide	PPARδ, PPARγ, AhR, AR, TR, GR	5.6	-3.12	-1.61	-10.2	6.17	-4.5	3.85	-6.03	0.46	-10.1	0.74	-8.47	3.39	-5.45	4.63	-3.17	5.87	-1.17
BITERTANOL	Pesticide	PXR	5.41	-1.69	1.56	-7.25	6.22	-1.5	4.63	-5.67	4.03	-6.13	-1.16	-11.3	2.45	-5.25	6.01	-2.9	6.87	-1.43
CARBARYL	Pesticide	AR, AhR	3.84	-0.68	3.42	-0.57	4.78	-0.28	3.39	-2.73	2.59	-0.72	4.84	-0.46	3.08	-1.01	4.72	-0.38	3.75	-0.8
CARBOFURAN	Pesticide	ER	3.83	-0.97	4.16	-0.89	4.36	-1.33	4.53	-1.96	3.2	-2.23	3.61	-2.52	3.69	-1.01	4.56	-0.9	4.61	-0.58
CHLORDIMEFORM	Pesticide	-	3.74	-1.26	3.58	-0.46	4.76	-0.91	4.13	-1.08	4.44	-0.82	4.94	-0.14	3.77	-0.51	4.05	-0.63	4.12	-0.88
CHLORPYRIFOS	Pesticide	AhR, PPARγ, TR, AR	5.03	-1.21	4.39	-1.23	3.36	-1.22	3.03	-2.37	3.69	-2.09	2.31	-3.4	3.14	-1.15	3.61	-2.35	4.72	-1.01
CLOFENTEZINE	Pesticide	ER, AhR, PXR	3.34	-0.98	3.3	-2.3	3.29	-0.98	3.02	-1.79	2.76	-2.21	2.7	-2.6	3.49	-0.92	4.13	-1.36	3.74	-0.84
CYANAZINE	Pesticide	AhR	3.79	-0.85	2.75	-2.08	3.24	-1	3.63	-1.38	2.65	-1.17	3.12	-2.64	2.6	-1.33	4.42	-1.27	3.04	-0.98
CYHALOTHRIN	Pesticide	-	4.58	-4.98	-5.03	-13.3	3.4	-4.17	-0.19	-9.47	-3.71	-14.3	-13.7	-23.3	-2.83	-12	-1.86	-11.1	4.95	-2.87
CYPERMETHRIN	Pesticide	PXR	5.39	-3.56	-4.68	-13.3	4.71	-2.26	-0.66	-11	-4.05	-14.6	-4.81	-16.1	-0.34	-11.1	2.61	-7.79	5.71	-3.42
CYPROCONAZOLE	Pesticide	PXR	3.43	-1.35	3.97	-3.97	4.04	-2.19	5.68	-2.31	2.6	-2.86	2.39	-5.49	5.24	-1.69	4.79	-1.2	5.69	-1.11
DIFLUBENZURON	Pesticide	AhR, ER, RXR	3	-1.04	0.79	-3.84	3.24	-0.84	3.12	-2.55	1.9	-2.39	0.57	-4.18	2.85	-1.92	3.2	-2.14	4.62	-0.65
ENDOSULFAN	Pesticide	ER, AR, TR, PPARγ, PPARδ, GR	-0.55	-3.19	-0.81	-4.81	1.48	-2.63	0.05	-3.11	-2.14	-4.85	-7.95	-11.6	-0.83	-3.77	0.07	-3.1	0.53	-3.81
FLUTRIAFOL	Pesticide	-	4.26	-1.17	4.42	-0.79	4.8	-0.98	4.03	-1.81	3.75	-1.46	1.35	-5.16	5.28	-2.18	4.63	-0.84	4.65	-1.07
HEXACHLOROBENZENE	Pesticide	-	0.78	-0.36	0.46	-0.36	0.87	-0.17	1.3	-0.9	1.04	-0.54	1.27	-1.69	0.54	-0.7	1.01	-0.51	0.38	-0.22
IMIDAZOLE	Pesticide	-	1.89	-0.11	1.91	-0.26	1.86	-0.08	2.07	-0.34	1.51	-0.38	2.18	-0.28	2.48	-0.16	2.47	-0.23	3.29	-0.02
IPRODIONE	Pesticide	AhR, PPARγ	-0.62	-4.95	-1.5	-6.04	-0.61	-5.48	2.98	-3.34	0.31	-4.7	-1.78	-7.68	1.65	-2.47	0.78	-5.18	3.79	-0.78
IRGAROL	Pesticide	AhR, AR, PPARδ	3.89	-1.46	3.48	-3.13	4.85	-1.54	3.52	-2.74	1.75	-3.38	3.93	-3.55	3.37	-2.66	4.63	-1.4	5.03	-0.9
ISOPROTURON	Pesticide	AhR	3.98	-0.83	4.47	-2	3.65	-1.23	4.69	-0.64	4.53	-1.43	4.82	-3.07	4.03	-1.09	3.5	-0.73	2.8	-0.74
LINURON	Pesticide	AhR, AR, TR	2.57	-1.58	3.27	-0.3	2.83	-0.93	3.4	-0.64	2.92	-0.43	3.78	-0.47	2.13	-0.29	2.76	-0.45	3.3	-1.42
METHOXYCHLOR	Pesticide	ER, AR, TR, GR, PXR, PPARγ	4.17	-1.95	1.87	-6.05	3.11	-1.22	2.8	-5.04	-3.68	-9.43	-1.24	-7.91	2.8	-3.94	3.49	-2.46	4.84	-2.14
METOLACHLOR	Pesticide	AR, PPARγ	5.93	-1.52	2.19	-5.28	4.29	-1.61	1.51	-6.41	2.5	-4.02	2.07	-6.6	3.48	-2.77	5.27	-3.28	3.29	-3.37
METRIBUZIN	Pesticide	RXR	3.39	-0.99	3.43	-1.23	3.25	-0.87	3.66	-2.45	4.59	-0.72	4.69	-0.79	3.9	-0.64	3.77	-1.37	4.01	-1.5
MOLINATE	Pesticide	-	4.05	-1.14	4.4	-1.1	4.6	-0.7	4.39	-2.63	3.73	-1.63	4.53	-1.37	3.64	-1.09	4.51	-1.06	3.25	-0.51
N N-DIETHYL-3-METHYL BENZAMIDE	Pesticide	ER, AhR	4.09	-0.57	4.24	-1.28	4.05	-0.9	3.34	-1.58	2.54	-0.32	2.53	-0.39	3.54	-0.58	3.96	-1.04	4.25	-2
PHENOTHRIN	Pesticide	-	6.7	-0.7	-0.34	-11	5.68	-3.56	-0.31	-12.6	0.26	-9.79	-16.1	-25.4	-0.29	-8.51	4.9	-4.1	6.77	-0.95

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
PRODIAMINE	Pesticide	TR, PXR, PPARδ, ER, AR, AhR, GR	5.43	-2.41	0.65	-6.84	5.26	-3.22	1.2	-5.55	3.27	-3.46	0.62	-6.78	2.43	-2.52	5.18	-0.91	5.32	-1.48
QUINALPHOS	Pesticide	AhR, AR, ER	5.38	-1.41	5.07	-1.83	4.18	-1.73	5.01	-0.92	5	-0.97	4.04	-3.23	4.52	-1.33	5.71	-1.78	4.81	-1.3
TRIADIMEFON	Pesticide	AhR, ER, PXR	4.55	-1.12	4.14	-0.61	4.76	-0.77	5.22	-2.47	3.81	-1.42	2.97	-4.11	3.87	-1.8	3.94	-1.49	4.67	-0.81
TRIADIMENOL	Pesticide	PXR, AhR, AR	4.43	-0.9	4.73	-0.9	4.49	-2.07	4.41	-2.63	5.36	-1.52	3.5	-3.12	3.67	-1.86	4.24	-2.44	5.67	-1.35
PENTACHLORO PHENOL	Pesticide/ Disinfectant	AhR, PPARγ, TR, ER	0.83	-2.8	0.97	-0.32	1.91	-0.63	1.8	-0.42	0.98	-1.38	1.49	-1.27	1.14	-0.5	2.02	-0.2	1.78	-0.26
4-CHLORO ANILINE	Pesticide/ Reagent	PPARγ, ER, TR	3.23	-0.17	3.09	-0.28	2.78	-0.28	2.57	-0.53	2.17	-0.68	2.55	-0.14	1.81	-0.19	2.45	-0.22	3.02	-0.17
4-NITROPHENOL	Pesticide/ Reagent	AR	3.48	-0.19	3.1	-0.18	2.72	-0.38	2.26	-0.33	2.97	-1.12	2.43	-0.38	3.57	-0.31	3.74	-0.43	3.66	-0.95
(-)BENZOYLECGONINE	Pharmaceutical	-	5.68	-0.67	5.41	-2.14	5.34	-1.4	4.95	-0.97	5.2	-0.66	4.66	-1.53	4.24	-1.21	4.59	-1.22	7.53	-0.84
2-HYDROXY HIPPURIC ACID	Pharmaceutical	-	4.2	-1.06	4.23	-1.76	3.24	-0.49	2.83	-0.7	2.98	-0.72	3.29	-0.89	4.64	-1	3.81	-1	5.07	-0.82
9-CIS-RETINOIC ACID	Pharmaceutical	RXR, ER, TR, PPARγ	6.65	-2.66	4.74	-4.87	6.5	-3.59	3.14	-5.86	3.99	-6.04	1.31	-9.38	5.35	-3.6	4.9	-6.43	7.3	-3.1
TRIAMCINOLONE	Pharmaceutical	AR, ER, GR	2.33	-7.02	-2	-11.7	1.03	-7.63	-4.77	-11.9	2.11	-7.81	-5.12	-16.8	-1.26	-8.53	3.84	-4.67	2.71	-4.09
ACEBUTOLOL HYDRO CHLORIDE	Pharmaceutical	-	6.35	-2.55	1.1	-6.91	5.52	-2.91	6.71	-4.63	4.85	-4.94	-1.11	-11.2	5.57	-4.62	6.37	-3.11	7.46	-1.97
AMITRIPTYLINE HYDRO CHLORIDE	Pharmaceutical	-	5.92	-2.14	5.21	-5.28	6.16	-2.89	4.37	-3.81	4.89	-3.36	2.67	-7.08	3.89	-3.46	6.76	-2.79	6.37	-0.46
AMOXICILLIN TRIHYDRATE	Pharmaceutical	-	5.57	-3.04	3.62	-6.48	5.44	-4.66	6.2	-4.31	7.68	-5.36	1.78	-8.86	7.95	-2.49	6.96	-3.79	5.88	-0.9
AMPICILLIN	Pharmaceutical	-	5.76	-3.2	5.55	-4.35	6.75	-2.8	3.5	-3.66	4.4	-3.81	1.85	-6.42	4.53	-1.98	7.45	-1.51	5.72	-1.44
ASPIRIN	Pharmaceutical	-	3.95	-1.3	4.56	-0.81	3.9	-0.84	3.68	-1.35	2.83	-0.56	2.73	-0.29	3.28	-1.18	4.19	-1.06	4.8	-0.32
BENDROFLUMETHIAZIDE	Pharmaceutical	-	5.17	-2.8	2.69	-5.53	4.78	-4.05	4.77	-2.96	3.83	-4.52	-0.96	-10.4	5.81	-3.08	3.42	-4.7	6.13	-1.18
BETAXOLOL	Pharmaceutical	-	7.27	-3.51	6.59	-4.53	7.26	-1.87	6.69	-3.49	7.34	-3.44	5.43	-5.15	8.17	-2.71	7.03	-4.17	6.55	-1.78
BEZAFIBRATE	Pharmaceutical	PPARδ, PPARγ	2.01	-5.11	3.5	-6.33	6.05	-2.54	0.84	-5.92	0.67	-5.68	-5.37	-13	3.16	-5.41	1.83	-3.45	6.33	-1.86
BISOPROLOL FUMARATE	Pharmaceutical	-	6.49	-2.56	6.61	-5.26	7.72	-2.6	8.29	-3.8	7.61	-3.82	5.39	-6	7.86	-2.64	8.6	-4.81	9.1	-1.89
CAPTOPRIL	Pharmaceutical	-	3.94	-0.75	3.98	-0.55	3.66	-0.68	3.49	-1.2	3.9	-4.87	4.13	-2.35	3.97	-1.29	4.64	-1.97	3.74	-0.79
CARAZOLOL	Pharmaceutical	-	6.75	-3.16	4.03	-5.32	5.98	-3.33	6.01	-2.8	4.29	-4.37	3.39	-7.63	5.34	-2.62	7.16	-2.77	6.34	-1.61
CARBAMAZEPINE	Pharmaceutical	AR	4.85	-0.48	3.47	-1.65	5.09	-1.78	5.02	-0.99	4.79	-2.5	2.81	-3.8	5.02	-1.17	5.08	-1.33	5.45	-1.01
CARISOPRODOL	Pharmaceutical	-	5.95	-2.58	4.49	-3.77	4.62	-1.86	6.72	-1.85	6.12	-1.77	5.25	-3.54	5.14	-1.52	6.57	-1.02	6.56	-1.78
CELIPROLOL HYDRO CHLORIDE	Pharmaceutical	-	4.49	-2.81	-1.83	-14.2	4.99	-3.07	5.14	-6.48	-5.12	-16.9	-5.99	-16.9	1.49	-8.67	-0.34	-10.2	6.06	-1.79
CHLORAZEPATE	Pharmaceutical	-	5.52	-0.56	1.83	-4.04	5.18	-1.17	0.46	-4.88	0.45	-4	-1.51	-7.72	0.79	-5.42	0.49	-4.55	4.21	-1.44
CIMETIDINE	Pharmaceutical	-	5.77	-2.25	5.64	-2	4.45	-0.96	6.57	-0.73	5.82	-0.85	4.93	-3.16	5.57	-1.92	4.54	-1	4.67	-1.34
CLENBUTEROL HYDRO CHLORIDE	Pharmaceutical	-	4.2	-1.82	3.93	-2.91	5.64	-1.01	4.31	-2.66	3.78	-1.44	2.75	-3.08	4.25	-1.36	4.77	-1.3	4.18	-0.71
CLOFIBRATE	Pharmaceutical	ER, PPARα	4.1	-0.94	3.59	-1.01	3.83	-2.49	5.24	-0.73	4.25	-0.99	4.32	-1.4	3.99	-0.68	4.52	-1.17	4.54	-0.58
CLOTRIMAZOLE	Pharmaceutical	ER, PPARγ	5.23	-3.84	-6.84	-14	-2.88	-8.78	-6.14	-13.6	-5.25	-12.4	-12.4	-19.6	-4.26	-10.4	-2.89	-9.93	1.56	-5.64
DEXAMETHASONE	Pharmaceutical	GR, AR, PXR, PR	4	-4.22	-3.91	-13.6	-0.06	-8.44	-2.82	-10.9	5.67	-4.92	-6.1	-16.5	3.54	-5.31	8.24	-3.5	4.25	-2.63
DIATRIZOIC ACID	Pharmaceutical	AhR, PXR	0.68	-3.88	-0.81	-5.64	1.26	-3.94	-4.56	-9.89	-5.88	-9.42	-8.85	-12.2	-1.41	-5.24	-2.71	-7.12	1.57	-1.69
DIAZEPAM	Pharmaceutical	ER, AR, GR, TR	5.88	-1.52	2.15	-5.14	5.24	-0.53	-0.15	-4.39	1.12	-3.84	0	-6.4	1.81	-2.46	2.21	-4.84	4.06	-1.34
DICLOFENAC SODIUM	Pharmaceutical	-	5.27	-1.52	4.41	-2.76	4.33	-1.48	1.95	-3.84	3.24	-2.56	1.61	-3.32	3.41	-2.64	3.59	-1.31	3.14	-2
DIGOXIGENIN	Pharmaceutical	-	-4.91	-13	-10.4	-20.4	-5.14	-12.6	-8.03	-17.4	-5.26	-16	-16.2	-25.6	0.98	-9.42	-4.34	-11.1	2.77	-1.36

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
DILTIAZEM HYDRO CHLORIDE	Pharmaceutical	TR	4.22	-5.31	-3.67	-14.5	2.24	-6.25	1.15	-11.1	-5.31	-15.7	-3.78	-16.4	-0.36	-11	1.8	-7.68	6.67	-2.53
DOMPERIDONE	Pharmaceutical	TR	7.96	-3.3	2.2	-8.9	5.14	-1.65	2.02	-7.39	0.74	-9.9	-6.94	-16.5	1.89	-7.18	4.6	-6.42	7.81	-3.62
DOXAZOSIN MESYLATE	Pharmaceutical	-	4.31	-3.33	-6.95	-18.1	4.95	-1.29	-6.42	-16.4	-10.6	-20.6	-20.4	-30	0.77	-9.45	-11.5	-22	6.5	-1.99
ENALAPRIL	Pharmaceutical	-	7.68	-3.08	3.39	-6.89	7.82	-2.27	7.24	-4.36	5.61	-5.93	-0.17	-14	4.17	-6.69	9.3	-3.34	7.36	-1.37
ETOFENAMATE	Pharmaceutical	-	6.73	-1.77	4.55	-4.85	6.15	-2.52	4.98	-5.28	3.09	-3.75	1.4	-8.43	7.01	-2.56	5.41	-2.42	6.83	-1.61
ETOFIBRATE	Pharmaceutical	-	5.62	-1.34	4.06	-4.82	6.84	-1.92	4.65	-3.84	5.17	-3.57	-1.24	-9.01	4.2	-2.35	4.82	-2.61	6.51	-1.7
FAMOTIDINE	Pharmaceutical	-	6.57	-2.51	4.49	-2.05	5.58	-1.5	4.85	-2.58	3.03	-3.24	2.78	-4.18	5.37	-1.11	5.75	-1.15	6.54	-1.12
FENOFIBRATE	Pharmaceutical	PPARγ, PPARα, AR, ER, TR	1.48	-4.13	-0.43	-8.9	5.42	-2.06	5.41	-4.62	1.69	-5.98	-1.88	-10.1	0.65	-8.17	7.11	-3.05	5.96	-1.15
FENOPROFEN	Pharmaceutical	-	5.43	-0.56	4.6	-2.19	4.53	-1.31	4.42	-1.9	4.97	-1.44	4.21	-2.27	5.1	-2.33	5.03	-1.14	5.84	-0.91
FENOTEROL HYDROBROMIDE	Pharmaceutical	-	6.36	-2.04	5.01	-4.8	6.45	-1.78	7.11	-2.01	6.58	-2.97	6.17	-4.49	6.8	-2.48	6.7	-1.82	7.26	-1.17
FENTIAZAC	Pharmaceutical	PPARγ, AhR	3.74	-1.37	-0.94	-6.53	4.1	-1.05	2.91	-3.72	2.71	-2.91	-0.03	-6.32	-1.15	-4.07	0.94	-4.26	5.16	-1.78
FLUOXETINE HYDRO CHLORIDE	Pharmaceutical	PPARδ, TR, AhR, AR	6.25	-1.38	6.87	-1.8	5.26	-2.8	6.94	-1.5	5.27	-2.22	4.78	-3.94	5.7	-2.37	6.06	-1.76	6.89	-1.55
FLURBIPROFEN	Pharmaceutical	ER, PPARγ	4.81	-0.58	4.55	-1.53	3.9	-1.54	5.59	-1.39	3.98	-0.91	3.92	-1.83	4.31	-0.93	4.7	-0.83	3.97	-1.05
FLUTICASONE PROPIONATE	Pharmaceutical	GR	-0.51	-11.3	-21	-30.9	-8.26	-20.9	-11.6	-23.1	-9.57	-20.8	-19.3	-30.2	-8.89	-19	-1.73	-12.6	0.67	-7.8
FUROSEMIDE	Pharmaceutical	ER, AR	4.72	-2.36	4.41	-1.36	4.87	-1.93	4.14	-1.38	3.37	-2.79	2.51	-3.99	4.71	-1.61	3.84	-0.9	5.44	-1.77
GABAPENTIN	Pharmaceutical	-	4.38	-0.34	4.85	-0.85	4.54	-0.43	4.04	-0.61	6.42	-0.82	3.19	-2.11	5.3	-0.94	5.58	-1.16	5.88	-1.66
GEMFIBROZIL	Pharmaceutical	AhR	5.64	-1.41	5.77	-2.04	7.86	-2.26	7.7	-1.34	5.76	-2.27	5.66	-2.73	6.47	-0.99	6.91	-1.45	6.55	-1.56
HYDROCHLORO THIAZIDE	Pharmaceutical	PPARδ	2.9	-0.47	3.65	-1.26	3.43	-0.86	2.73	-0.53	2.3	-1.06	3.41	-3.27	1.74	-1.76	2.83	-1.33	5.26	-0.61
IBUPROFEN	Pharmaceutical	ER	4.82	-0.18	5.77	-1.31	4.61	-1.14	4.9	-0.49	4.46	-0.51	4.69	-1.33	5	-0.59	5.72	-0.73	5.32	-0.78
IFOSFAMIDE	Pharmaceutical	-	4.45	-0.77	3.64	-1.56	4.16	-0.77	4.34	-0.91	4.41	-1.12	4.19	-1.54	3.56	-1.08	4.82	-1.22	5.11	-1.72
INDAPAMIDE	Pharmaceutical	-	2.82	-6.39	2.15	-6.54	4.01	-1.64	4.6	-4.41	-0.52	-8.86	1.76	-6.24	0.69	-5.4	2.92	-6.06	5.08	-1.44
INDOMETHACIN	Pharmaceutical	PPARγ	4.49	-2.88	1.68	-7.59	5.23	-1.72	2.41	-4.53	1.44	-6.53	0.1	-8.8	3.17	-4.07	2.34	-4.49	4.89	-3.15
IOHEXOL	Pharmaceutical	-	1.33	-7.5	-18.2	-28.8	-2.16	-10.6	-10.6	-20.2	-18.1	-25.4	-15.6	-27.3	-12.5	-19.7	-10.1	-19.3	3.45	-5.28
IOMAPIDOL	Pharmaceutical	AR, GR	-2.81	-12.5	-16.6	-27.7	-7.94	-15.6	-14.5	-25.2	-21	-29.9	-16.1	-24.9	-11.4	-20.3	-14.4	-22.4	3.02	-4.23
IOPROMIDE	Pharmaceutical	-	-1.04	-11.4	-17.7	-28.4	-0.78	-5.48	-10.8	-23.1	-16	-26.5	-19.4	-28.8	-5.26	-18.6	-11.6	-22.3	3.2	-5.83
IOTHALAMIC ACID	Pharmaceutical	-	-1.23	-5.21	-1.49	-6.01	1.1	-4.4	-4.49	-9.91	-3.14	-7.98	-7.88	-13.6	-2.22	-5.21	-3.22	-6.46	1.28	-2.26
KETOPROFEN	Pharmaceutical	-	5.31	-1.45	5.45	-1.14	5.3	-0.27	4.93	-2.76	5.22	-1.01	3.9	-2.81	4.87	-2.11	5.77	-0.8	5.42	-1.65
KETOROLAC TROMETHAMINE	Pharmaceutical	-	4.64	-1.16	5.1	-1.03	7.19	-0.91	5	-1.22	5.23	-1.52	5.42	-1.71	5.2	-0.96	6.03	-1.32	6.49	-0.96
L-AMPHETAMINE	Pharmaceutical	-	3.77	-0.28	4	-1.76	3.96	-0.62	3.05	-0.47	4.65	-0.96	4.61	-0.53	3.56	-0.55	4.11	-0.57	4.29	-0.54
LANSOPRAZOLE	Pharmaceutical	AhR, PPARγ	5.64	-1.62	5.22	-2.9	6.84	-1.35	6.13	-1.69	3.43	-4.38	0	-8	4.13	-2.83	5.78	-1.77	5.8	-1.75
LORATADINE	Pharmaceutical	TR, PPARδ, PPARγ, AR, GR, ER	4.86	-2.61	-2.33	-13.4	0.04	-7.78	-0.02	-9.51	-9.97	-19	-12.1	-22.1	1.14	-8.94	-2.76	-12.2	6.04	-1.52
MECLO FENAMATE SODIUM	Pharmaceutical	-	4.98	-1.16	3.06	-2.27	4.54	-1.38	2.43	-2.18	3.54	-1.35	2.5	-3.41	2.33	-1.4	2.9	-0.6	3.49	-1.21
MEFENAMIC ACID	Pharmaceutical	TR, GR, PPARγ	5.54	-1.16	5.07	-1.27	3.98	-2.07	4.24	-0.95	4.55	-1.55	4	-2.05	4.75	-1.79	4.82	-2.04	4.27	-2.57
MESTRANOL	Pharmaceutical	ER, AR, GR	4.31	-3.25	-0.44	-7.66	3.87	-5.69	2.78	-6.26	2.8	-6.11	2	-8.32	4.35	-4.83	2.8	-5.46	2.97	-1.38
METHYLTRIENO LONE (R1881)	Synthetic Hormone	AR	4.72	-1.83	2.73	-5.79	6.66	-1.96	5.28	-4.38	6.63	-1.35	6.12	-4.4	6.79	-1.17	6.9	-1.01	3.99	-0.99
N N-DIETHYL-2-(1H-INDOL-3-YL)ETHAN AMINE	Pharmaceutical	-	5.52	-1.24	6.3	-0.84	6.18	-0.76	6.44	-1.59	6.09	-1.52	5.95	-1.86	5.11	-0.71	6.53	-0.78	6.18	-1.12
NADOLOL	Pharmaceutical	-	5.92	-4.61	4.96	-4.99	6.56	-1.27	4.66	-5.91	7.09	-2.98	3.46	-7.62	7.15	-2.76	7.51	-2.66	7.39	-2.22
NAPROXEN	Pharmaceutical	-	5.16	-0.8	4.54	-0.88	4.2	-0.87	5.19	-1.47	3.65	-0.72	4.71	-1.66	3.62	-1.46	6.03	-1.01	3.8	-0.52

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
NIMESULIDE	Pharmaceutical	ER, PPARγ	4.89	-0.99	4.07	-2.25	4.92	-0.65	3.06	-1.65	1.21	-2.79	1.47	-4.29	3.19	-0.61	3.23	-1.27	5.8	-1.09
NORFLOXACIN	Pharmaceutical	-	4.6	-3.76	6.06	-2.9	4.19	-2.2	8.01	-3.48	4.61	-4.48	5.93	-3.83	6.24	-1.99	5.24	-2.05	4.39	-0.87
NORTRIPTYLINE HYDRO CHLORIDE	Pharmaceutical	-	5.03	-2.77	5.02	-3.32	5.81	-1.05	4.37	-4.71	4.66	-3.34	3.72	-6.27	4.22	-2.41	5.29	-1.47	6.01	-1.05
OFLOXACIN	Pharmaceutical	-	4.21	-5.08	3.78	-6.38	4.63	-1.19	5.99	-4.56	5.87	-3.91	5.04	-5.66	2.96	-5.54	4.25	-3.37	5.61	-2.23
OLANZAPINE	Pharmaceutical	TR	4.31	-3.61	1.12	-7.06	4.22	-2	-2.17	-8.24	-2.69	-10.4	-0.27	-7.33	1.69	-4.61	1.15	-5.8	5.44	-0.74
OXAZEPAM	Pharmaceutical	ER, AR, GR, TR, PPARγ	5.91	-1	2.31	-4.01	4.31	-1.47	2.59	-2.29	0.37	-4.75	-0.83	-5.89	1.58	-1.55	2.68	-2.23	4.2	-1.49
PAPAVERINE HYDRO CHLORIDE	Pharmaceutical	-	6.4	-2.57	3.07	-6.98	3.97	-5.3	5.83	-4.67	-0.53	-7.89	-0.99	-11.3	6.79	-1.84	4.6	-5.39	7.26	-2.73
PAROXETINE HYDRO CHLORIDE	Pharmaceutical	AR, PPARδ, TR	7.97	-1.88	5	-4.47	7.07	-2.46	6.53	-2.82	4.47	-3.46	4.88	-4.36	6.46	-2.46	6.78	-1.78	7.7	-1.56
PHENYTOIN SODIUM	Pharmaceutical	PXR	4.65	-2.31	1.98	-4.24	4.44	-1.77	4.87	-0.7	1.69	-1.7	1.08	-4.63	2.07	-3.28	2.7	-2.28	5.51	-1.68
PINDOLOL	Pharmaceutical	AhR	6.72	-1.49	7.69	-1.45	5.98	-1.86	6.43	-1.73	6.12	-2.22	7.29	-2	7.28	-0.92	6.29	-1.41	6.35	-2.6
PIROXICAM	Pharmaceutical	AhR	4.21	-2.79	5.34	-1.56	4.23	-0.88	4.43	-1.43	4.95	-3.85	2.66	-4.78	3.7	-4.11	2.77	-3.05	3.33	-1.6
PRAVASTATIN SODIUM	Pharmaceutical	-	6.02	-3.63	0.44	-13.5	6.46	-7.54	4.58	-8.45	-0.88	-13.3	-8.75	-21	2.5	-10	3.17	-11.2	7.81	-4.45
PREDNISOLONE	Pharmaceutical	AR, GR	3.91	-3.29	-3.8	-11.7	1.54	-7.66	-0.17	-8.06	5.68	-4.17	-2.67	-12.6	4.13	-4.73	7.52	-2.82	2.39	-3.04
PREDNISONE	Pharmaceutical	AR, ER, GR	4.34	-2.8	-0.84	-7.87	1.65	-5.63	-3.38	-10.6	5.27	-3.37	-6.6	-16	4.67	-4.39	5.26	-3.25	3.07	-2.09
PRIMIDONE	Pharmaceutical	AR	4.15	-1.06	3.05	-2.1	4.25	-2.18	4.09	-1.12	3.15	-1.41	3.09	-2.63	2.68	-0.6	4.74	-1.25	3.47	-0.95
PROPRANOLOL HYDRO CHLORIDE	Pharmaceutical	PPARδ, PPARγ, ER, TR	6.55	-1.75	6.84	-2.17	5.96	-1.87	6.26	-3.2	6.81	-1.99	7.98	-2.48	6.69	-0.77	7.26	-1.34	5.68	-1.74
RAMIPRIL	Pharmaceutical	-	4.64	-6.96	-1.66	-14.1	5.12	-4.54	2.81	-10.8	1.97	-10.8	-4.35	-17.9	4.63	-7.35	7.42	-6.19	6.66	-1.94
RANITIDINE	Pharmaceutical	-	5.78	-2.24	6.86	-1.93	6.94	-1.06	6.67	-2.48	6.81	-2.67	3.79	-5.54	7.09	-2.18	7.6	-2.3	7.76	-1.23
SERTRALINE HYDRO CHLORIDE	Pharmaceutical	-	5.72	-1.76	2.33	-5.16	5.98	-1	3.85	-4.19	2.32	-4.56	2.28	-6.25	2.83	-3.25	3.67	-2.89	4.63	-0.79
SIMVASTATIN	Pharmaceutical	GR, AR, ER, PPARδ, TR	5.99	-8.02	-0.86	-15.4	4.01	-9.12	0.54	-14.4	-3.4	-17.7	-11.4	-25.4	-0.11	-12.6	3.52	-10.5	7.3	-1.8
SOTALOL HYDRO CHLORIDE	Pharmaceutical	AR	3.53	-1.41	4.65	-2.5	3.68	-3.04	6.55	-1.41	5.13	-3.3	5.59	-3.19	5.77	-1.36	6	-1.47	5.77	-0.98
SULFASALAZINE	Pharmaceutical	AhR, ER	-7.48	-12.1	-4.67	-9.65	1.49	-1.11	-0.32	-5.83	-18.8	-24.5	-12.3	-17.8	-3.74	-9.72	-8.83	-16	4.23	-1.24
SUMAMED	Pharmaceutical	TR	-31.9	-44.5	-118	-139	-17.2	-33.9	-83.1	-105	-94.9	-116	-115	-136	-90.4	-111	-85.3	-104	4.6	-3.6
TAMOXIFEN CITRATE	Pharmaceutical	ER, AR, TR, PPARδ, PPARγ	4.96	-5.75	-0.67	-13.3	2.84	-8.4	-3.69	-15.1	-1.63	-11.8	-12.8	-25	0.18	-10.7	6.28	-6.05	6.83	-3.38
TERBUTALINE HEMISULFATE	Pharmaceutical	-	5.8	-0.96	4.44	-1.2	5.28	-1.6	5.25	-1.33	5.67	-1.49	5.29	-1.79	5.64	-2.74	5.57	-1.87	7.77	-0.88
TIMOLOL MALEATE	Pharmaceutical	-	5.38	-2.83	4.59	-3.83	5.24	-1.56	6.86	-3.36	5.99	-3.33	3.97	-5.97	6.45	-2.59	6.12	-3.34	6.3	-1.31
TOLFENAMIC ACID	Pharmaceutical	TR, GR, PPARγ, PPARδ	4.96	-1.3	3.7	-1.54	4.03	-2.05	2	-1.6	3.74	-1.01	3.76	-1.58	2.02	-0.85	3.35	-0.81	2.72	-1.6
TRAMADOL HYDRO CHLORIDE	Pharmaceutical	-	4.28	-2.16	6.8	-2	5.4	-1.16	4.89	-4.49	3.37	-4.8	2.35	-6.62	4.83	-1.79	4.25	-2.79	5.45	-1.34
TRIAMCINOLONE ACETONIDE	Pharmaceutical	GR, AR, ER, AhR	1.81	-8.86	-8.04	-19.5	-6.51	-12.8	-4.7	-14.7	-3.32	-14.5	-18.5	-29.3	-5.83	-13.9	3.61	-7.64	3.15	-4.07
VALSARTAN	Pharmaceutical	-	3.7	-4.94	-3.29	-15	4.15	-4.85	-0.83	-11.7	0.37	-12.6	-11.4	-23.3	-4.38	-15.3	5.13	-6.85	6.9	-2.87
WARFARIN	Pharmaceutical	PPARγ	5.9	-1.45	5.09	-2.16	5.7	-1.28	5.21	-1.74	4.98	-3.23	4	-4.35	5.55	-1.71	4.35	-1.86	4.4	-2.65
ACIPIMOX	Pharmaceutical	-	2.53	-1.36	3.22	-0.89	3.09	-0.46	2.75	-0.13	2.3	-0.95	2.06	-0.3	1.95	-0.94	2.64	-0.39	4	-0.24
ALBUTEROL	Pharmaceutical	-	6.33	-1.79	5.9	-1.49	5.1	-1.83	8.36	-1.51	5.21	-1.67	5.84	-1.97	5.46	-1.25	5.76	-2.14	6.63	-1.06
ATORVASTATIN CALCIUM	Pharmaceutical	AR, TR	-1.76	-16.4	-22.6	-38	3.26	-5.82	-27.6	-43.6	-28	-41.5	-26	-40.1	-22.2	-34.5	-18.9	-30.6	9.01	-2.14
METOPROLOL TARTRATE	Pharmaceutical	-	6.72	-4.13	6.3	-2.75	6.57	-1.09	6.85	-3.44	6.7	-2.53	8.87	-4.15	8.22	-2.51	7.37	-1.76	6.94	-1.78
CYCLOPHOSPHAMIDE	Pharmaceutical/ Chemical Reagent	-	3.19	-1.53	3.87	-1.21	2.7	-2.28	4.12	-1.97	3.87	-2	3.76	-2.44	2.73	-1.36	3.85	-0.87	3.08	-1.38

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
4'-HYDROXY PROPIOPHENONE	Pharmaceutical/ Reagent	-	3.55	-0.44	3.88	-0.79	5.59	-1.47	3.81	-0.45	3.27	-0.73	3.97	-0.58	3.56	-0.27	4.01	-0.46	4.69	-1.46
4-ISOBUTYL ACETOPHENONE	Pharmaceutical/ Reagent	-	4.58	-0.78	4.25	-1.58	4.54	-0.65	4.38	-0.98	5.38	-0.84	4.78	-0.32	3.72	-0.85	3.92	-1.15	5.43	-0.88
5-AMINO-2-HYDROXY BENZOIC ACID	Pharmaceutical/ Reagent	PPARγ	3.85	-0.53	3.37	-0.38	4.09	-0.63	3.67	-0.11	3.05	-0.61	4	-0.64	3.29	-0.48	3.29	-0.4	3.12	-0.51
ACET AMINOPHEN	Pharmaceutical/ Reagent	-	3.79	-0.37	3.03	-0.61	3.91	-0.69	2.98	-0.47	3.22	-0.37	3.28	-1.07	3.01	-0.79	3.58	-0.85	3.43	-1.37
BENZOYLEC GONINE	Pharmaceutical/ Recreational Drug	-	5.11	-1.15	1.79	-4.96	4.7	-1.44	4.09	-2.74	4.88	-2.35	1.66	-5.82	4.79	-1.91	4.84	-2.26	5.57	-3.11
COCAINE	Pharmaceutical/ Recreational Drug	-	5.41	-0.82	2.88	-4.53	5.56	-2.06	3.27	-3.68	4.93	-3.52	1.95	-6.84	5.55	-2.85	5.79	-2.51	5	-1.97
CODEINE	Pharmaceutical/ Recreational Drug	AR	3.39	-3.1	-2.04	-7.6	2.78	-2.69	5.35	-3.12	2.43	-6.09	-2.97	-10.6	2.31	-4.42	4.62	-3.3	3.9	-0.5
FLUMETHASONE	Pharmaceutical/ Veterinary M	GR, AR	3.39	-5	-2.32	-12.3	0.4	-7.75	-4.24	-12.5	5.28	-5.18	-7.97	-18.2	4.06	-6.18	7.94	-3.72	4.4	-2.8
METRONID AZOLE	Pharmaceutical/ Antibiotic	PPARγ	4.16	-0.84	2.73	-0.2	3.85	-0.38	3.83	-0.67	3.02	-0.52	3.43	-0.94	4.12	-0.41	3.91	-0.83	4.57	-0.35
ATENOLOL	Pharmacuetical	-	6.69	-1.49	6.27	-3.06	5.56	-2.27	5.94	-2.26	6.6	-1.98	5.95	-4.96	7.94	-2.08	7.2	-1.21	6.89	-1.33
1,2 4-BENZENE TRIOL	Phytochemical	-	3.47	-0.34	2.67	-0.69	2.87	-0.35	2.86	-0.73	2.91	-0.41	3.54	-0.48	2.64	-0.53	2.83	-0.57	2.74	-0.3
4-HYDROXY CINNAMIC ACID	Phytochemical	-	3.73	-0.42	2.98	-0.47	3.95	-0.57	3.3	-0.54	3.59	-0.7	4.3	-0.4	3.06	-0.93	3.91	-1.03	2.81	-0.29
BETA-SITOSTEROL	Phytochemical	-	-0.97	-10.2	-17.2	-29.4	-0.65	-8.96	-17.4	-30.7	-17.4	-29.7	-23.4	-36.1	-8.5	-20.6	-11	-22.5	5.64	-5.82
COUMESTROL	Phytochemical	ER, AhR	5.83	-0.9	5.5	-0.47	6.54	-0.4	5.67	-0.77	4.65	-0.88	4.77	-1.65	4.17	-0.36	4.03	-1.16	4.28	-1.64
DAIDZEIN	Phytochemical	ER, AhR, GR, PXR	5.4	-1.69	5.99	-0.85	6.28	-0.8	4.6	-1.16	5.56	-0.68	5.81	-0.84	4.61	-0.63	4.39	-0.84	4.21	-0.55
FERULIC ACID	Phytochemical	-	3.94	-0.46	3.41	-0.42	4.27	-0.87	4.23	-0.57	3.2	-0.33	3.51	-0.55	3.27	-0.62	3.37	-0.9	3.75	-1.2
FORMONONETIN	Phytochemical	ER, AhR	3.06	-1.83	4.33	-2.52	5.02	-1.67	5.4	-2.03	4.96	-1.47	4.35	-2.72	4.18	-1.28	4.95	-1.82	5.08	-0.86
GENISTEIN	Phytochemical	ER, AhR, TR, PPARγ, PXR, RXR, AR, GR	5.9	-1.93	5.76	-1.32	5.09	-1.64	4.35	-1.64	4.63	-0.78	5.94	-1.2	4.68	-0.39	3.99	-1.2	5.07	-1.61
GLYCITEIN	Phytochemical	ER	5.91	-2.39	4.92	-2.5	5.75	-2.67	6.52	-2.71	5.09	-2.4	4.84	-2.15	5.29	-1.3	6.18	-1.8	4.31	-0.69
KAEMPFEROL	Phytochemical	AhR, ER, AR, PPARγ, PXR, TR, GR	6.96	-0.68	5.82	-0.62	5.32	-0.89	5.22	-1.33	4.69	-1.45	5.15	-1.74	3.99	-0.51	4.19	-0.3	4.56	-0.89
NARINGENIN	Phytochemical	-	7.17	-0.84	6.63	-1.62	6.39	-1.68	4.89	-0.73	5.11	-1.01	4.63	-1.46	5.42	-0.86	5.35	-1.75	5.01	-1.83
PALMITIC ACID	Phytochemical	ER	6.95	-1.5	8.16	-2.18	8.18	-1.92	8.38	-1.82	8.07	-1.62	6.5	-5.58	9.44	-2.66	7.63	-2.18	8.47	-0.94
PHLORETIN	Phytochemical	ER, AR, AhR, TR, PPARγ	5.34	-1.45	5.42	-2.11	6.4	-1.56	6.45	-1.03	5.96	-0.93	6.65	-1.86	5.7	-1.41	5.42	-1.31	5.28	-0.83
QUERCETIN	Phytochemical	ER, AhR, TR, AR, GR	7.13	-0.78	6.15	-1.19	4.61	-1.6	7.61	-1.27	4.15	-1.46	4.75	-1.81	4.15	-0.76	4.91	-1.71	5.08	-1.12
2-PHENYL PHENOL	Plasticiser/ Preservative/ Pesticide	-	4.59	-0.8	4.22	-1.27	3.84	-0.71	3.12	-0.46	4.32	-0.68	3.72	-1.08	3.07	-0.43	3.34	-0.5	3.57	-1.2
4,4'-(1 3-DIMETHYLBUTY LIDENE) DIPHENOL	Plasticiser/ Toner/ Reagent	AR, ER, PPARγ	4.56	-2.79	4.15	-4.66	4.52	-0.98	6.44	-1.97	3.4	-4.53	-1.07	-8.37	3.76	-2.51	4.87	-2.51	4.95	-1.43
4-CHLORO-3-METHYLPHENOL	Preservative/ Disinfectant	ER, TR	3.49	-0.71	4.01	-0.45	3.48	-0.28	2.83	-0.78	4.25	-0.24	3.1	-0.28	4.35	-0.19	4.86	-0.23	5.34	-0.5
3,4,5 6-TETRABROMO-O-CRESOL	Preservative/ Fungicide	-	1.05	-0.75	1.78	-0.51	2.41	-1.06	1.43	-1.45	2.7	-0.62	2.58	-2.24	0.58	-0.65	3.66	-0.61	2.07	-0.47
DIETHYLSTILBE STROL	Synthetic Hormone/ Pharmaceutical	ER, PR, TR, AR	5.71	-3.87	6.08	-2.13	6.35	-2.46	3.76	-2.73	5.85	-1.56	4.32	-5.54	6.26	-1.14	5.19	-2.7	5.56	-2.43

Chemical Name	Chemical Category	Mode of Action	3DT3 hERα		1X7J hERβ		1HJ1 ratERβ		3V49 hAR		1T7T chAR		21HQ ratAR		2QPY mAR		1SQN hPR		1PRG hPPARγ	
			S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
ETHINYL ESTRADIOL	Synthetic Hormone/ Pharmaceutical	ER, AR, PR, GR, TR, PXR, PPARδ	6.04	-1.34	2.81	-5.17	6.35	-2.99	4.64	-3.98	5.51	-3.65	2.7	-7.32	6.3	-2.78	7.14	-1.98	2.8	-1.27
LEVON ORGESTREL	Synthetic Hormone/ Pharmaceutical	AR, PR, ER, GR	4.71	-3.91	1.06	-8.04	5.4	-5.11	5.55	-4.34	5.01	-4.54	2.49	-9.42	4.44	-4.11	8.27	-1.85	3.88	-4.92
NOR ETHINDRONE	Synthetic Hormone/ Pharmaceutical	PR, ER, AR, GR, TR	3.88	-2.94	1.04	-6.53	3.98	-4.44	5.12	-3.55	6.44	-3.43	2.16	-8.25	4.83	-2.82	6.61	-1.38	2.78	-2.04
4 4'-DIHYDROXY BENZOPHENONE	UV Stabiliser/ Cosmetic	-	3.97	-0.72	4.44	-1.08	3.84	-0.83	4.08	-1.2	5.54	-0.6	4.2	-1.73	4.01	-0.53	4.5	-0.86	4.33	-0.86
BIOCHANIN A			4.8	-2.39	3.78	-3.41	4.95	-1.84	3.67	-2.17	4.67	-3.05	3.66	-3.18	5.41	-1.12	5.02	-0.68	4.33	-1.02
HYDRO CORTISONE			5.09	-3.65	-3.09	-11.2	2.59	-5.91	-2.91	-10.6	4.93	-4.96	-3.89	-14.3	4.98	-4.99	7.6	-3.63	3.94	-2.16
METHOMYL			2.9	-0.59	3.3	-0.71	3.8	-0.82	3.9	-0.72	2.13	-0.4	2.83	-0.54	3.53	-0.72	2.76	-1.75	3.98	-1.72

Appendix F *In vitro* Testing Plate Layout

Assays were 'upscaled' in the presence of intraplate variation, to minimise error. Data replicates presented in the main body of text were colour coded according to assay microtitre plate format; highlighting slight differences in method.

96-Well Plate Layout

The plate layouts of *in vitro* ER and AR Agonism transactivation assays (HeLa9903 and HeLa4-11, respectively) included 1nM Testosterone positive controls (n=12 'green'), DMSO vehicle controls (n=6 'red') and 3 replicates of 7 concentrations. Test substance (TS) concentration varied, however, low concentrations were always plated on the left (column 3), while the highest concentrations were in column 9. Figure_Apx 11 shows the Costar® black clear bottom 96-well plate layout. Cells were plated in outside wells to minimise evapouration effects.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa
B	1 x 104 HeLa	PC (1nM)	TS 1pM	TS 10pM	TS 100pM	TS 1nM	TS 10nM	TS 100nM	TS 1µM	PC (1nM)	VC (0.1% DMSO)	1 x 104 HeLa
C	1 x 104 HeLa	PC (1nM)	↓	↓	↓	↓	↓	↓	↓	PC (1nM)	VC (0.1% DMSO)	1 x 104 HeLa
D	1 x 104 HeLa	PC (1nM)	↓	↓	↓	↓	↓	↓	↓	PC (1nM)	VC (0.1% DMSO)	1 x 104 HeLa
E	1 x 104 HeLa	VC (0.1% DMSO)	TS2 1pM	TS2 10pM	TS2 100pM	TS2 1nM	TS2 10nM	TS2 100nM	TS2 1µM	PC (1nM)	PC (1nM)	1 x 104 HeLa
F	1 x 104 HeLa	VC (0.1% DMSO)	↓	↓	↓	↓	↓	↓	↓	PC (1nM)	PC (1nM)	1 x 104 HeLa
G	1 x 104 HeLa	VC (0.1% DMSO)	↓	↓	↓	↓	↓	↓	↓	PC (1nM)	PC (1nM)	1 x 104 HeLa
H	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa	1 x 104 HeLa

Figure_Apx 11 96-Well Plate Layout for STTA Assays in HeLa4-11 and HeLa9903 TA Agonism Assays

For antagonism STTA assays in the HeLa4-11 cell line, the positive control was 1fM Testosterone, which was plated in all wells, excluding the DMSO vehicle controls ('red'). Additionally, three of the positive control wells (Column 10, Rows E, F, & G) were also exposed to the moderate antagonist Flutamide, with the aim of controlling for assay sensitivity – reduction in transactivation consequent to $10x10^{-5}$ M Flutamide exposure. Z' values were calculated using columns 2 and 11.

24-Well Plate Layout

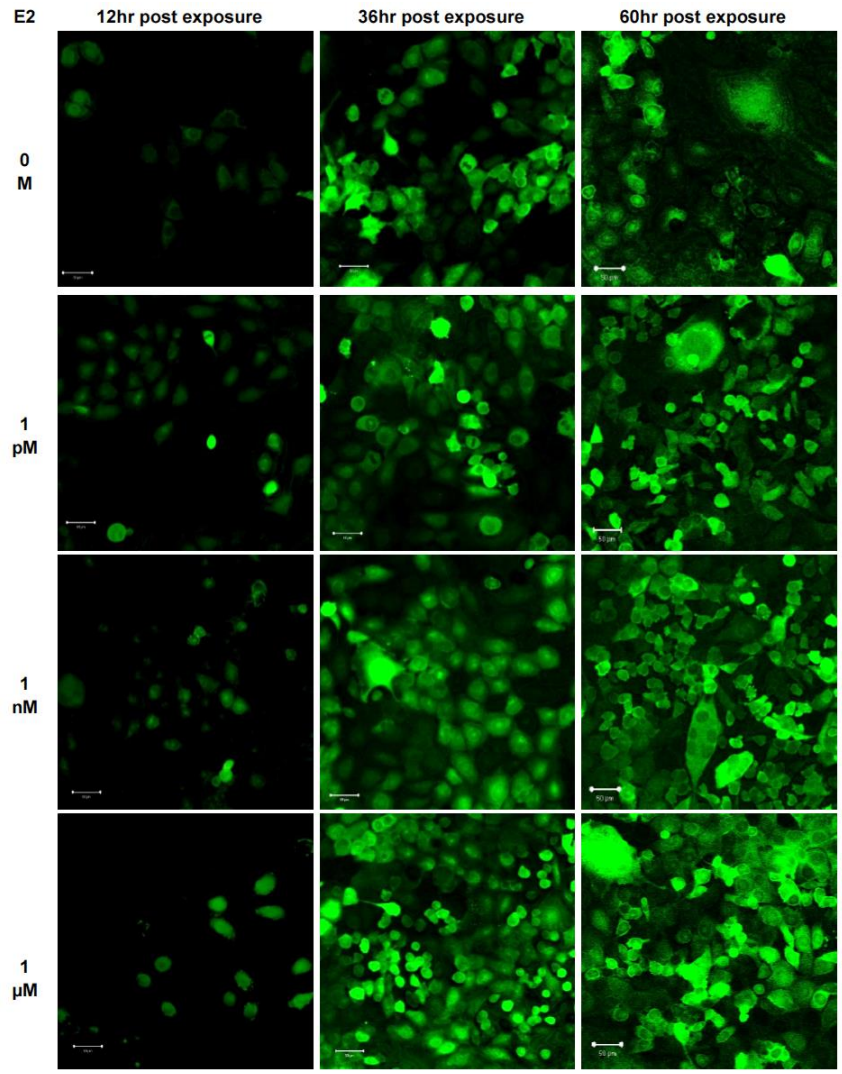
	1	2	3	4	5	6
A	DMSO 1 x 104 HeLa	DMSO 1 x 104 HeLa	DMSO 1 x 104 HeLa	0.1nM 1 x 104 HeLa	0.1nM 1 x 104 HeLa	0.1nM 1 x 104 HeLa
B	0.1pM 1 x 104 HeLa	0.1pM 1 x 104 HeLa	0.1pM 1 x 104 HeLa	0.1nM 1 x 104 HeLa	0.1nM 1 x 104 HeLa	0.1nM 1 x 104 HeLa
C	1pM 1 x 104 HeLa	1pM 1 x 104 HeLa	1pM 1 x 104 HeLa	10nM 1 x 104 HeLa	10nM 1 x 104 HeLa	10M 1 x 104 HeLa
D	10pM 1 x 104 HeLa	10pM 1 x 104 HeLa	10pM 1 x 104 HeLa	1µM 1 x 104 HeLa	1µM 1 x 104 HeLa	1µM 1 x 104 HeLa

Figure_Apx 12 24-Well Plate Layout for 'Upscaled' HeLa9903 Assays and Transient Transfection Assays.

Figure_Apx 12 shows the 24-well plate format for transactivation assays, for both HeLa9903 cell STTA and HEK293 and HepG2 cells transient-transfection transactivation assays. However, for the latter, to account for potentially low transactivation, a higher concentration of $5x10^4$/well was adopted.

Appendix G Alternative *in vitro* Methods

Pilot MCF-7 proliferation assays were conducted, modifying the methods detailed by Oden *et al*. (1998). MCF-7-GFP cells stably express Green Fluorescent Protein (GFP), enabling crude predictions of cell growth via non-invasive fluorescent readings and/or confocal microscopy. One-week old live MCF-7-GFP cells were suspended in 10% DCC-FBS, 2% Glutamide EMEM and plated at 1x104 cells per well, and attached for 24 hours prior to chemical exposure. Figure_Apx 13 shows the confocal microscopy of MCF-7-GFP cells in response to 17β-Estradiol (E2).

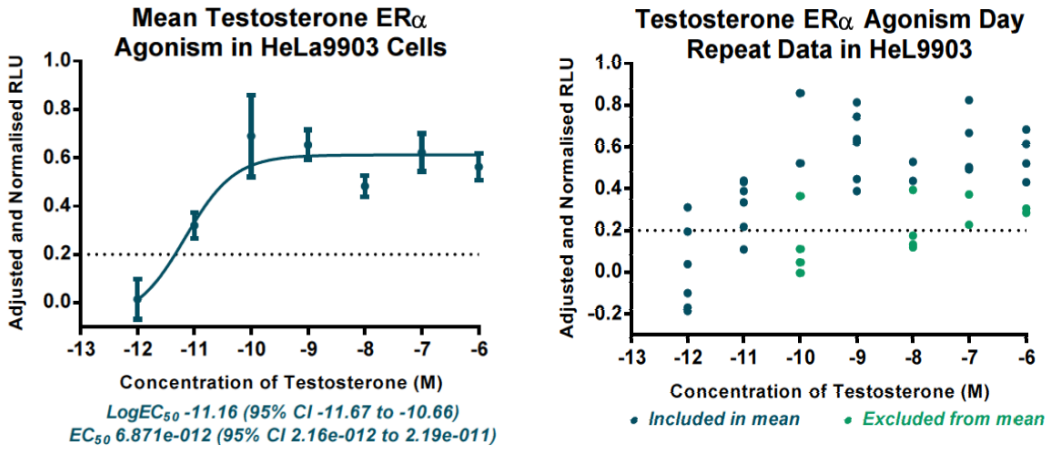


Figure_Apx 13 Confocal Microscopy of MCF-7 GFP Cell E2 Proliferation Assay
LSM510 ZEISS Model Confocal Microscope, 488nM (excitation) laser; 505-530nM narrow channel 12; maximum transparency mode. The images collected of the MCF-7-GFP cells, appeared to reflect estrogenic growth proliferation in response to E2, however, these observations were not observed in Presto®Blue cell viability assays run in parallel. Interestingly, many of the cells present altered morphology (EMT).

Appendix H Supplementary *in vitro* STTA Results

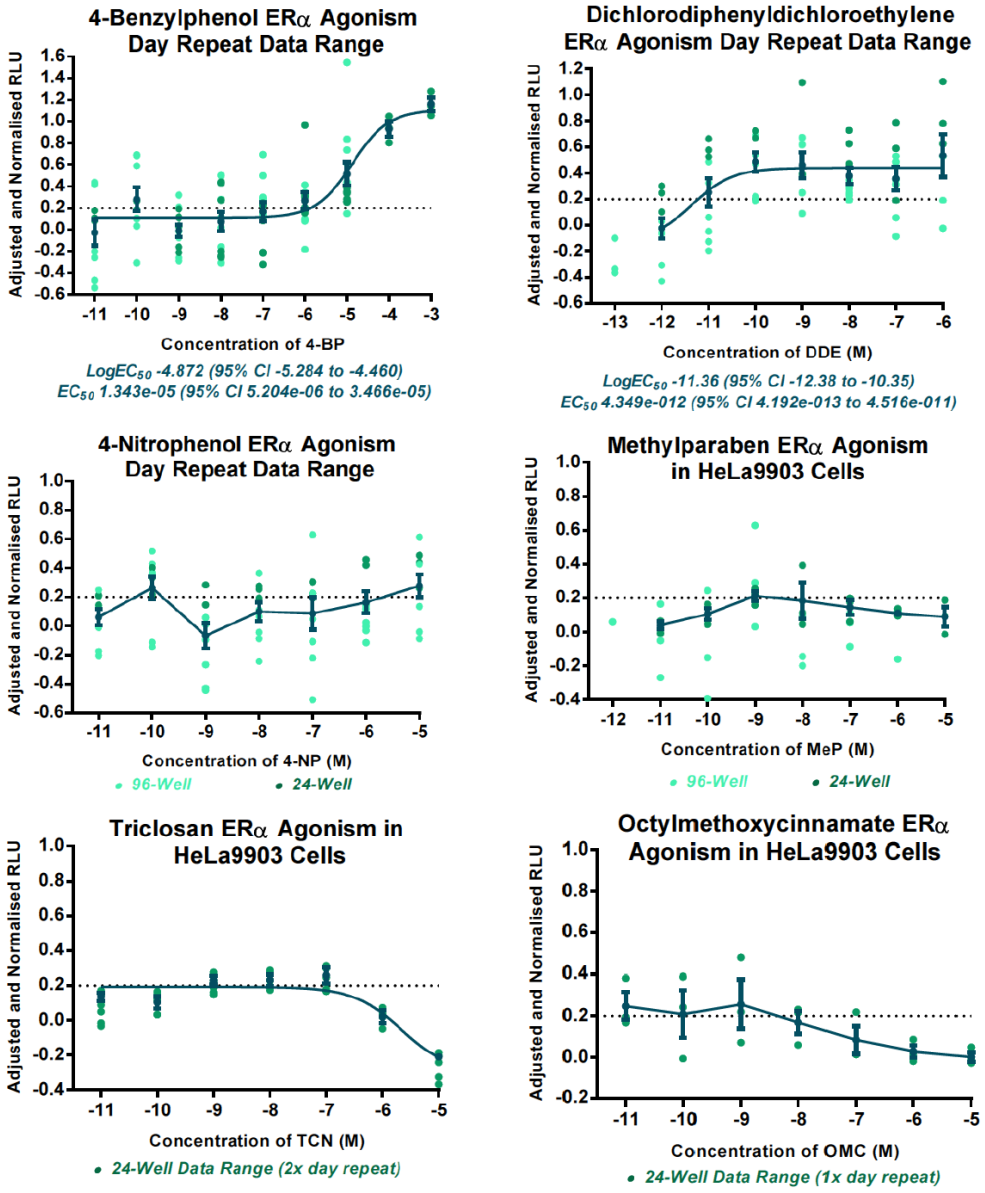
This section presents the graphs of the *in vitro* screening of potential-EDCs (Section 5.3) in Stably Transfected Transcriptional Activation Assays (ER and AR). All graphs were drawn with GraphPad Prism®.

Stably Transfected ERα Transactivation Assays for ER Agonism



Figure_Apx 14 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to Testosterone

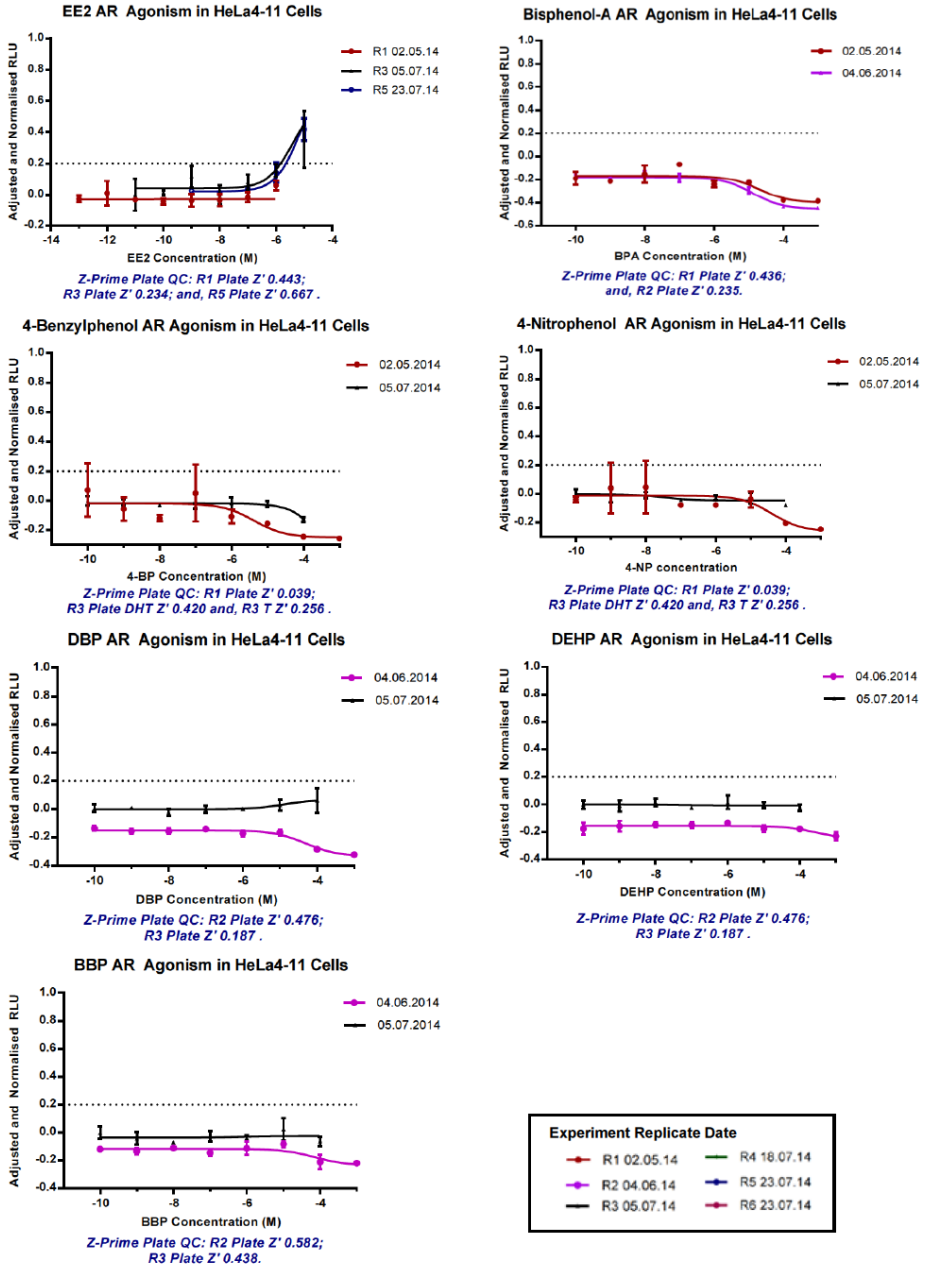
Graphs show the adjusted and normalised relative light units (RLU) emitted consequent to 40-48hr exposre to the androgen receptor agonist, Testosterone. Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM E2 (maximal agonistic response). Cell's were plaed in 24-wells (1000µL) with $1x10^5$ HeLa9903 cells per well; testosterone was solvated in 100% DMSO and administered as a 1µL volumes. GraphPad Prism® Log(agonist) vs. response (three parameters) was used to calculate the line of best fit (right), which is presented as the mean and standard error of the mean (SEM). $LogEC_{50}$ and EC_{50} with 95% Confidence Intervals (95% CI) were calculated from this line of best fit. Testosterone bottom -0.0749 (95% CI -0.2846 to 0.1348) and top 0.612 (95% CI 0.532 to 0.692), R square = 0.722 (n=29). The dotted line (y=0.2) highlights 20% 1nM E2 normalised RLU, as a threshold for categorising positive, negative and/or inadequate data.



Figure_Apx 15 Estrogen Receptor-α (ERα) Transactivation in HeLa9903 Cells Exposed to Anthropogenic Chemicals (4-BP, 4-NP, DDE, TCN, MeP and OMC)

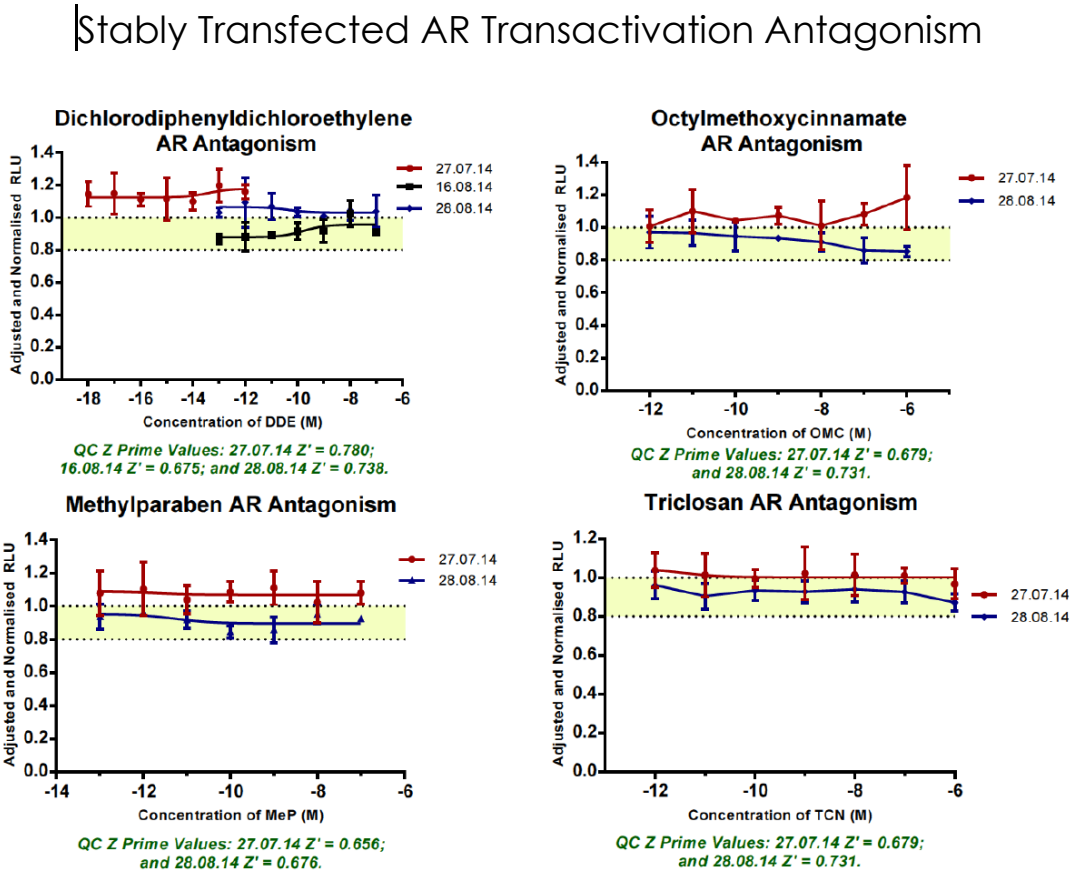
Graphs show the adjusted and normalised relative light units (RLU) emitted consequent to 40-48hr exposre to either 4-Benzylphenol (4-BP), 4-Nitrophenol (4-NP), Dichlorodiphenyldichloroethylene (DDE), Methylparaben (MeP), Triclosan (TCN) or Octylmethoxycinnamate (OMC). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM E2 (maximal agonistic response). Cell concentration was $1x10^4$/well in 96-well (200µL) and $1x10^5$/well in 24-well (1000µL); test chemicals were solvated in 100% DMSO and administered in 0.2µL and 1µL volumes, respectively. Lines of best fit were drawn from the mean and standard error of the mean (SEM). 96-well and 24-well experiment replicates are shown as a scatter plot behind (dark and light teal, respectively). Where applicable (4-BP and DDE) GraphPad Prism® Log(agonist) vs. response (three parameters) software was used to calculate the $LogEC_{50}$ and EC_{50} with 95% Confidence Intervals (95% CI). 4-BP bottom 0.110 (95% CI 0.0295 to 0.187) and top 1.110 (95% CI 0.813 to 1.407), R square = 0.44 (n=84). DDE bottom -0.1313 (95% CI -0.517 to 0.254) and top 0.4401 (95% CI 0.351 to 0.530), R square = 0.27 (n=60). The dotted line (y=0.2) highlights 20% 1nM E2 normalised RLU, as a threshold for categorising positive, negative and/or inadequate data.

Stably Transfected AR Transactivation Assay for AR Agonism



Figure_Apx 16 No Observed Agonism in HeLa4-11 Cells Consequent to 24h Exposure

Drawn using GraphPad Prism® Log(agonist) vs. Response (three parameters) function, graphs show the relative light units (RLU) emitted consequent to exposure to either 17α-Ethinylestradiol (EE2), Bisphenol A (BPA), 4-Benzylphenol (4-BP), 4-Nitrophenol (4-NP), Dibutylphthalate (DBP), Diethylhexylphthalate (DEHP) or Butylbenzylphthalate (BBP). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by the RLU of 1nM testosterone (maximal agonistic response). Error shown is equivalent to the standard deviation, highlighting the distribution of the data over the different testing days. The dotted line (y=0.2) highlights 20% luciferase induction of the positive control (1nM testosterone ~ maximal response), as a threshold for categorising positive, negative and/or inadequate data. Quality assurance data (Z-prime) for each 96-well plate are stated in blue. Presented data strongly suggest that BPA, 4-BP, 4-NP, DBP, DEHP and BBP are not androgen agonists in the HeLa4-11 cell line at the tested concentrations. Slight luciferase induction at 1e-05 M EE2 was reported, however, this coincided with cytotoxicity (see Figure 5.1).



Figure_Apx 17 Antagonism of Androgen Receptor Transactivation in HeLa4-11 Cells Exposed to Consumer Products (DDE, OMC, MeP and TCN) for 24 Hours

Graphs, drawn with GraphPad Prism® Log(inhibitor) vs. Response (three parameters), show the adjusted and normalised relative light units (RLU) emitted during luciferase assays, consequent to coexposure of testosterone (1fM) and either Dichlorodiphenyldichloroethylene (DDE), Octylmethoxycinnamate (OMC), Methylparaben (MeP) or Triclosan (TCN). Results were normalised by subtracting vehicle control (DMSO) RLU and adjusted by dividing by 1fM testosterone RLU (maximal agonistic response).Error bars show the standard deviation, indicating the distribution of data over the different testing days. Quality assurance data (Z-prime) for each 96-well plate are stated in green. DDE, MeP, OMC and TCN were not identified as antagonists of androgen receptor transactivation in any of the assays, all of which passed quality checks Z' >0.65.