The Mechanism of Cryptolepine-Induced Cell Death

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Abstract: The objective of the present study was to use morphological and biochemical approaches to characterize the mode of CLP-induced cell death. Using a differential staining technique, a Chinese Hamster fibroblast cell line (V79 cells) and a human lymphoblastoid cell line (MCL-5) showed morphology consistent with apoptosis after treatment with CLP. In contrast, HepG2, a human hepatoma cell line showed morphology that was more like necrosis after treatment with CLP. Using annexin V staining for apoptotic cells, MCL-5 cells showed a three fold increase in apoptosis within 6 h. Although we observed only a marginal increase in BAX protein expression, cytochrome c was released into the cytosol of CLP-treated MCL-5 cells. Furthermore, procaspase-3 was processed into the active caspase-3 (17 kDa). Consistent with the caspase-3 activation, PARP was cleaved to the typical 85 kDa fragment confirming apoptosis as the mode of cell death in CLP-treated MCL-5 cells. However, there was no evidence of increased BAX expression, cytochrome c release, caspase activation or PARP cleavage in CLP-treated HepG2 cells. This observation together with the morphology of CLP-treated HepG2 cells indicates that in contrast to MCL-5 cells, the CLP-mediated demise of HepG2 cells is not apoptotic.

Keywords: Cryptolepine, cell death, human lymphoblastoid MCL-5 cells, hepatoma HepG2 cells, apoptosis, necrosis

INTRODUCTION

Cryptolepine (CLP) is a cytotoxic (Dassonneville et al., 2000; Ansah and Gooderham, 2002) anti-malarial agent (Kirby et al., 1995; Wright et al., 2001) obtained as the major alkaloid of the West African anti-malarial herbal Cryptolepis sanguinolenta (Dwuma-Badu et al., 1978; Tacik et al., 1991) and is a candidate anti-tumour agent. A DNA intercalator (Lisgarten et al., 2002), the cell-kill activity of CLP is established, but the mechanisms by which it causes the demise of a variety of mammalian cell lines in vitro (Bonjean et al., 1998; Dassonneville et al., 2000; Ansah and Gooderham, 2002) remains poorly understood. Two modes of cell death with distinguishable morphological and biochemical features are well described, apoptosis and necrosis (Wyllie et al., 1980). Apoptosis is associated with cell shrinkage, chromatin condensation and internucleosomal DNA fragmentation (Kerr, 1971; Wyllie et al., 1980). In contrast, necrosis is accompanied by a massive increase in cell volume, eventual rupture of the cell membrane and spilling of the cellular contents into the tissue surroundings (Gores et al., 1990). We observed previously by flow cytometry that treatment of Chinese hamster V79 cells with CLP, led to an increase in the sub-G1 population (Ansah and Gooderham, 2002), indicative of cell death. Though sub-G1 populations are generally considered apoptotic (Darzynkiewicz et al., 1992), they can be necrotic. Further, these two types of cell death

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can occur simultaneously in culture (Ankarerona et al., 1995; Shimizu et al., 1996). The potent
cytotoxicity of CLP coupled with its ability to inhibit clonal expansion of several mammalian tumour
cell lines in vitro (Ansah and Gooderham, 2002) supports the potential of CLP as a cancer
chemotherapeutic agent. We have therefore used morphological and biochemical approaches to
investigate the possible mode of CLP-induced cell death in vitro with the objective of exploring further,
the anti-cancer potential of this anti-malarial plant alkaloid.

MATERIALS AND METHODS

Chemicals

Cryptolepine (CLP) (formula weight 233, purity >99%) was a kind donation from Dr. J. Addae-
Kyereme (Wright et al., 2001), formerly of the Department of Pharmaceutical Chemistry, College of
Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Diff-Quick®
staining kit was purchased from Dade Behring AG, Switzerland. Fluorescein isothiocyanate-Annexin V
FITC-Annexin V staining kit was obtained from Alexis Corporation, Nottingham, United Kingdom.
Western blotting reagents and all other chemicals unless otherwise indicated were obtained from Sigma
Chemical Co. (Poole, England). Primary anti-bodies were purchased from Santa Cruz Biotechnology
(Santa Cruz, CA) and secondary anti-bodies from Sigma Chemical Co. (Poole, England).

Cell Lines and Conditions

V79-MZ, a Chinese hamster lung fibroblast cell line was propagated in Dulbecco’s Modified
Eagle’s Medium (DMEM) (Invitrogen, Paisley, Scotland, UK) with 10% FBS and HepG2, a human
hepatoma cell line was cultured in Minimum Essential Medium (MEM) supplemented with 10% Foetal Calf Serum (FCS). MCL-5, a human lymphoblastoid cell line transfected with two plasmids
encoding multiple cytochrome P450s (CYP1A2, 2A6, 2E1, 3A4) and epoxide hydroxylation genes and
also has inducible constitutive CYP1A1 (Crespi et al., 1991) was obtained from Gentest (Woburn,
MA, USA). These were grown in Gentest RPMI 1640 media (without histidine and with 2 mM
histidinol for plasmid selection), supplemented with 9% horse serum. Additionally, all media contained
2 mM L-glutamine and 100 units of penicillin/streptomycin. MCL-5 cells were grown to a density of
10⁶ cells mL⁻¹ and sub-cultured to 2.5×10⁶ cells mL⁻¹ every 48 h. Hygromycin B (100 μg mL⁻¹) was
added at each sub-culturing for plasmid maintenance. All cell lines were routinely maintained at 37°C
in a humidified atmosphere of 5% CO₂/95% air. All experiments were repeated at least three times.

Cytological Examination of CLP-Treated Cells

The Diff-Quick® staining kit (Dade Behring AG), was used to study qualitatively the morphology
of the treated cells. Exponentially growing V79, HepG2 and MCL-5 cells cultured and maintained as
described were seeded at 2×10⁵ cells mL⁻¹ in 5 mL of culture medium in 25 cm² tissue culture flasks
overnight before treatment with vehicle (culture medium) or CLP for a period of 24 h. Treated cells
were then cytopspun directly on to microscopic slides at 450 rpm for 5 min. The slides were air-dried
and stained with the Diff-Quick® staining kit according to the manufacturer’s instructions. Briefly, the
slides were dipped 5–1 see each in the fixative and the two staining solutions, rinsed in Weisss Buffer
pH 7.2 (Merck) and allowed to dry overnight. The slides were then mounted with DPX and the
morphology of the cells examined using a Zeiss light microscope at 1000x magnification under oil
immersion. Apoptotic cells were defined as containing one or more characteristic darkly stained nuclear
fragments.

FITC-Annexin V/PI Double Staining

Double staining for FITC-Annexin V binding and for cellular DNA by PI was performed
according to the method of Vermes et al. (1995) using an Annexin V staining kit (Alexis Corporation)

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according to the manufacturer's instructions. Briefly, two million (2 × 10⁶) MCL-5 cells were incubated overnight in 5 mL culture medium in 25 cm² tissue culture flasks before treatment with 2.5 μM CLP or vehicle for up to 6 h. Cells centrifuged at (200 × g) were washed with cold PBS, resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and cell density adjusted to 2.5 × 10⁵ cells mL⁻¹. Cells were then double-stained with 5 μL Annexin-FITC and 10 μL of 20 μg mL⁻¹ Propidium Iodide (PI). Samples were stored on ice until data acquisition with a Becton Dickinson FACScan flow cytometer using the Cell Quest software (Becton Dickinson). FITC fluorescence was measured at 514 nm and DNA-PI complexes at 590 nm. Ten thousand (10,000) cells were analyzed for each treatment.

Immunoblotting

For immunoblotting, 5 × 10⁶ exponentially growing cells in 10 mL culture medium in 75 cm² tissue culture flasks treated with CLP or vehicle (culture medium) were harvested by centrifuging at 200 x g for 10 min. Cell pellets were lysed by rapid freeze/thaw and sonication for 30 sec with 500 μL PBS solution containing a protease inhibitor cocktail (2 μg mL⁻¹) (Sigma Chemical Co. Ltd., Poole, England). Protein concentration of the samples was determined by the Bradford method using a BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Cell lysates were diluted two fold with sample buffer (0.5 M Tris-HCl, 10% w/v SDS, 0.5% bromophenol blue, deionised water and 50 μL β-mercaptoethanol) and heated at 95°C for 5 min. Proteins were electrophoretically resolved at (200 V) in a running buffer (25 mM Tris, 192 mM glycine, 1% SDS, pH 8.3). Fractionated proteins were then transferred on to Hybond-C nitrocellulose membranes for 90 min at 450 mA in a transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). Uniform protein transfer was checked by Ponceau red staining before blocking the membranes with 5% non-fat milk powder in PBST (25 mM phosphate buffered saline, containing 0.1% Tween-20) for 1 h. Following three consecutive washings with PBST each of 10 min duration, the blots were incubated with the primary antibody in 5% non-fat milk overnight at 4°C. After washing the blot three times (15 min each with PBST), the blots were incubated with appropriate dilutions of horseradish peroxidase-conjugated secondary antibody (HRP-conjugated secondary antibody). Proteins were then visualized using an enhanced chemiluminescence (ECL) detection kit (Pierce, IL) according to the manufacturer's instructions. Equal protein loading for each lane was confirmed using an antibody to β-actin. All blotting experiments were repeated at least once using lysates from separately treated cultures.

RESULTS

Morphology of CLP-Treated Cells

V79 cells examined by flow cytometry with propidium iodide staining showed a sub-G1 (hypodiploid) peak, after exposure to CLP (Ansalh and Goodeham, 2002), which is generally considered to be indicative of apoptosis (Darzynkiewicz et al., 1992). However, this observation does not discount necrotic cell death. As a first step to investigate the possible mode of CLP-induced cell death, treated cells were stained with Diff-Quick® and the morphology of the cells examined by light microscopy. Treated V79 cells showed membrane blebbing, condensed chromatin and fragmented nuclei, typical morphology of apoptotic cells (Fig. 1, panel B). Similarly, the morphology of MCL-5 cells would suggest that the treated cells underwent apoptosis (Fig. 1, panel D). In contrast, HepG2 cells appeared to have necrotic morphology (Fig. 1, panel F), with little evidence of apoptotic cell death. Based on the morphology of treated cells, this data suggests that the mode of CLP-induced cell death may well be cell-type dependent, being predominantly apoptotic in V79 and MCL-5 cells but necrotic in HepG2 cells.
Fig. 1: Morphology of V79, MCL-5 and HepG2 cells treated with CLP for 24 h. Cells were harvested immediately after treatment and stained with the Diff-Quick* stain as described in the methods. Slides were examined for morphological changes under oil immersion using a light microscope. Panel A, C and E are vehicle-treated controls for V79, MCL-5 and HepG2 and panels B, D and F represent V79, MCL-5 and HepG2 treated with 2.5 μM CLP for 24 h, respectively. Examples of apoptotic cells (AP) and necrotic cells (*) are shown. The magnification has been increased in panels B, D and F to aid interpretation.

Annexin V Staining

The cytological studies suggested that MCL-5 cells exposed to CLP die predominantly by apoptosis. Annexin V staining (Vermes et al., 1995) was therefore used for the confirmation of apoptosis in this cell line. MCL-5 cells were treated with or without 2.5 μM CLP for up to 6 h and stained with Annexin-V-PI as described in the methods. Lower left quadrants of the cytograms (Fig. 2) showed viable An−/PI− cells. Lower right quadrants showed early apoptotic cells with intact
plasma membrane integrity (An"/PI"). Upper right quadrants showed cells, which have lost their plasma membrane integrity and were therefore An"/PI" and upper left quadrants represented necrotic cells, which were PI" only.

Consistent with the cytological findings, CLP induced a time-dependent increase in the lower right quadrant (An"/PI") cell population (Fig. 2), which represent apoptotic cells. The percentage of Annexin-V stained cells increased from 4.2 to 13.8% over the 6 h period (Fig. 2). No cells were detected in the upper right quadrant of the cytograms (PI'/An", late apoptotic cells), almost certainly due to the short treatment times. At time 0, 4.3% of the total cell population was detected in the lower right quadrant (An" only cells) suggesting that under normal conditions of cell culture, approximately 4% of the cell population is in the process of apoptosis. It is important to note the lack of PI" only cells in the upper left quadrant, which would represent the necrotic cell population. The detection of An" cells (apoptotic) in CLP-treated MCL-5 cells was consistent with the cytological findings.

CLP-Induced Expression of Apoptotic Proteins in MCL-5 Cells
To further explore the mode of cell death in the MCL-5 cells, immunoblotting was performed on key proteins known to be involved in the execution of the apoptotic process. CLP induced a clear
Fig. 3: Effect of CLP on apoptosis associated proteins in MCL-5 cells. For the determination of p53 and BAX, total cellular proteins were resolved with 12.5% SDS-PAGE, blotted on to nitrocellulose membrane and probed with a rabbit polyclonal antibody against p53 and BAX, respectively. For cytochrome c, cytosolic fractions of treated cells were prepared as described by Liu et al. (1996). Proteins were resolved by 15% SDS-PAGE, blotted on to nitrocellulose membrane and probed with a mouse monoclonal antibody against cytochrome c.

increase in p53 protein expression in MCL-5 cells in a dose-dependent manner (Fig. 3). The BAX protein, a member of the BCL-2 family, has pro-apoptotic effects and BAX is a p53 target gene. Yet examination of both dose (0-5 μM) and temporal (0-24 h) effects of CLP treatment on expression of BAX protein indicated that changes were at best only marginal (Fig. 3), suggesting that BAX induction by p53 may not play a major role in CLP-induced apoptosis in MCL-5 cells. However, there was clear evidence of increased expression of cytosolic cytochrome c in a dose-dependent manner (Fig. 3) except at 5.0 μM. The lack of cytochrome c release in cells treated with 5.0 μM CLP (the highest concentration tested) could be due to excessive cell death leading to the loss of plasma membrane integrity. Increased membrane permeability can lead to leakage of cytosolic cytochrome c into the culture medium. Indeed, Ashida et al. (2001) showed a similar trend for cytochrome c release, where levels were much lower at later time points following treatment of hepatocytes with 3-amino-1, 4-dimethyl-5H-pyridole [4,3-b] indole (Trp-P-1).

We also examined the processing of pro-caspase-3 using a polyclonal antibody that recognizes both pro-caspase-3 and active caspase-3. Both pro-caspase-3 (32 kDa) and the active caspase-3 (17 kDa) were detected in CLP-treated MCL-5 cells (Fig. 4, panel A), suggesting that CLP-induced apoptosis involves caspase-3 processing. Consistent with the caspase-3 activation, PARP was cleaved to the typical 85 kDa fragment (Fig. 4, panel B) confirming the involvement of apoptosis in CLP-treated MCL-5 cells. The substantial conversion of full-length PARP to fragment at high dose CLP (5.0 μM) (Fig. 4, panel B) is in agreement with increased levels of active caspase-3 (17 kDa) at high dose CLP (Fig. 4, panel A) and suggests advanced apoptosis, which is compatible with the absence of cytosolic cytochrome c, possibly due to cell leakage at high dose treatments.

Because caspase-8 can directly activate caspase-3 (Stennicke et al., 1998) and can also act through the mitochondria to effect the release of cytochrome c into the cytosol (Kuwara et al., 1998), we assessed the levels of caspase-8 in this cell line. However, the presence of caspase-8 was not detected (data not shown), suggesting that the caspase-3 activation and cytochrome c release were likely to be independent of caspase-8.
Fig. 4: Effect of CLP treatment on Pro-caspase-3 and PARP in MCL-5 and HepG2 cells. Pro-caspase-3 processing in MCL-5 cells (A) and HepG2 cells (C) treated with CLP (0-5.0 μM) for 24 h. PARP cleavage in MCL-5 cells (B) treated with CLP (0-5.0 μM) for 24 h. For the pro-caspase determination, cell lysates were subjected to 12.5% SDS-PAGE followed by blotting with an anti-caspase-3 antibody that recognizes both pro-caspase-3 and the active 17 kDa cleaved product. Arrows show full-length 32 kDa pro-caspase-3 and the active 17 kDa caspase-3 fragment. For PARP detection in MCL-5 cells, cell lysates were subjected to 12.5% SDS-PAGE followed by blotting on to nitrocellulose membranes. PARP was detected by a mouse anti-PARP monoclonal antibody that recognizes both whole length (116 kDa) and cleaved PARP (85 kDa).

In contrast to V79 and MCL-5 cells, HepG2 cells showed necrotic morphology (Fig. 1, panel F). Consistent with this observation, there was no evidence of CLP-induced cleavage of pro-caspase-3 (Fig. 4, panel C) supporting the notion that apoptosis was probably not involved in CLP-induced cell death in HepG2 cells.

DISCUSSION

The ability of CLP to induce cell-death in a variety of cell lines is now well established (Bonjean et al., 1998; Dassonneville et al., 2000; Anshah and Gooderham, 2002). To date however, knowledge of the mode of CLP-induced cell-death is rather limited. We were prompted (i) by our previous observation of wide-spectrum cytotoxicity to different cell lines by CLP (Anshah and Gooderham, 2002), (ii) the ability of different cell lines exposed to the same insult to exhibit different modes of cell death and (iii) the proposition of an anti-cancer potential for CLP, to explore further the mode and possible mechanisms of CLP-induced cell death.

We selected three cell lines (V79, MCL-5 and HepG2), which we had previously shown susceptibility to CLP toxicity. In the first instance, cells exposed to CLP were examined for
morphological changes using the Diff-Quick staining kit, with the aim of establishing whether changes typical of apoptotic or necrotic cell death (Kent et al., 1972; Wyllie et al., 1980) were evident. The results showed that the agents appeared to induce morphological changes characteristic of apoptosis in V79 and MCL-5 cells but not in HepG2 cells. To further confirm apoptosis as the mode of cell death in MCL-5 cells, annexin V staining was performed. Annexin V binds to externalized phosphatidylserine (PS) and is considered a characteristic feature of cells undergoing apoptosis (Vermes et al., 1995; Khodarev et al., 1998). The presence of annexin-V-positive cells confirmed that CLP induces apoptosis in MCL-5 cells.

Because CLP induced an increase in p53 protein levels in MCL-5 cells, we reasoned that CLP-induced apoptosis might occur through increased transcription of p53 target genes such as BAX, a key protein in the mitochondrial apoptotic programme. The change in BAX expression was marginal, suggesting that BAX may not be an important driver of CLP-induced apoptosis in MCL-5 cells, yet cytochrome c release into the cytoplasm was evident in a dose-dependent manner after 24 h treatment with CLP. These findings suggest that the cytochrome c release into the cytosol might not be wholly dependent on BAX expression. Indeed other apoptotic targets of p53 including p53AIP1 (Oda et al., 2000a), Noxa (Oda et al., 2000b) and PUMA (Nakano and Vousden, 2001; Yu et al., 2001) could contribute to the release of cytochrome c from the mitochondria. p53 may also mediate mitochondria apoptotic signaling through the elevation of ROS (Polyak et al., 1997). From our previous studies, CLP-induced cell death occurred in different cell types irrespective of p53 status and from our current observations, we suggest that mechanisms other than transcription of p53 target gene products could be involved in CLP-induced cell death. In support of our suggestion it has been shown that p53-induced apoptosis does not require transcriptional activation, since p53 mediated apoptosis initiated by DNA damage can occur in the presence of actinomycin D or cycloheximide, which block RNA or protein synthesis (Caelles et al., 1994).

Oxidative stress is known to stimulate the opening of the mitochondrial permeability transition pore (MPT) (Halestrap et al., 1997), alter calcium homeostasis and induce the permeability of the transition pore leading to a dissipation of the membrane potential (Pollia et al., 1996). Subsequently, there is mitochondria swelling and disruption of the outer mitochondria membrane, leading to the release of AIF and cytochrome c into the cytoplasm, which eventually trigger apoptosis through caspase-9, an effector caspase (Desagher and Martinou, 2000). Cytochrome c release into the cytosol is known to occur during both apoptosis and necrosis (Samali et al., 1999). Release of cytochrome c in the present study suggests that mitochondria could be a primary target for CLP induced cell death.

ROS generation has been associated with the toxicity of a number of non-oxidative inducers of apoptosis including glucocorticoids and topoisomerase II (topo II) inhibitors, where redox-sensitive transcription factors or diminution of antioxidant defense enzymes might be involved (Wolfe et al., 1994). The morphological changes associated with apoptosis require energy in the form of ATP (Leist et al., 1997; Lee and Shaeter, 1999). Oxidative stress leads to a rapid depletion of cellular ATP and can therefore inhibit apoptotic processes by converting cell death from apoptosis to necrosis (Lee and Shaeter, 1999). ROS generation may even lead to the inactivation of caspases (Hampton and Orru, 1997), which are essential to the apoptotic programme. Consistent with this we have previously reported ROS generation to be associated with CLP induced cell death in HepG2 cells (Ansah and Gooderham, 2005).

Our present findings suggest that in MCL-5 cells, cytochrome c release from the mitochondria could be due to the transcription of p53 target genes other than BAX. Cytochrome c release leads to the activation of a number of aspartate-specific cysteine proteases (Nunez et al., 1998). In particular, caspase-3 is considered essential to the execution of the apoptotic programme (Woo et al., 1998). The cleavage of pro-caspase-3 in the present study to the active 17 kDa product confirms that CLP induces apoptosis in MCL-5 cells. CLP is reported to be a topo II inhibitor (Bonjean et al., 1998) and this finding is consistent with previous reports of caspase-3 involvement in the apoptosis of HL-60 cells.
by topo II inhibitors such as camptothecin and etoposide (Shimizu and Pommier, 1997). PARP, an enzyme involved in DNA repair, is a substrate for caspase-3 (Kidd, 1998) and our experiments with MCL-5 cells showed PARP (116 kDa) was cleaved to the characteristic 85 kDa fragment upon treatment with CLP. The execution of the apoptotic programme is characterized by morphological and biochemical changes in the cell (Kerr et al., 1972). Many of the morphological changes associated with apoptosis such as chromatin condensation, cell shrinkage and membrane blebbing, as observed in MCL-5 cells treated with CLP in the present study, are orchestrated by activation of caspases (Cohen, 1997; Porter and Janicke, 1999). The morphology of CLP-treated MCL-5 cells as observed in the present study is therefore consistent with the biochemical findings of caspase activation and annexin-V-positive cells and together confirms apoptosis induction by CLP in MCL-5 cells.

Downstream caspases such as caspase-3 are activated not only by the cytochrome c-APAF-1-caspase-9 pathway, but also by the FAS-caspase-8 pathway (Sternick et al., 1998). Further, FAS/APO1 is a p53 target gene (Owen-Schaub et al., 1995). However, caspase-8 was not detected in our model using Western blotting and therefore may not be involved in the activation of caspase-3, suggesting that CLP-induced apoptosis in MCL-5 cells may not involve the Death-receptor pathway.

HepG2 cells treated with CLP showed a morphology more consistent with necrosis, but apoptosis and necrosis can occur simultaneously in tissues or cell cultures exposed to the same insult (Ankarcrona et al., 1995; Shimizu et al., 1996). To confirm necrosis as the major mode of cell death in HepG2 cells and to rule out the possible involvement of apoptosis, caspase-3 activation was assessed but we found no evidence for its involvement and this observation together with the morphology studies suggest that in contrast to MCL-5 cells, HepG2 cells treated with CLP die by necrosis. Our previous finding that CLP induced ROS in HepG2 cells (Ansah and Gooderham, 2005) and the propensity of ROS to favour necrotic cell death, is compatible with these observations.

The ability of CLP to induce acute toxicity and inhibit clonal expansion (Ansah and Gooderham, 2002) clearly supports CLP as an anti-cancer candidate. Our current observations that the mode of CLP-induced cell death depends on the cell type suggests a selectivity in its toxicity. Understanding the factors that contribute to CLP-mediated toxicity will be important in targeting the agent.

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REFERENCES


