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TITLE:

Encapsulation of cancer therapeutic agent dacarbazine using nanostructured lipid carrier

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SHORT ABSTRACT:

The most commonly used method for nanostructured lipid carrier (NLC) synthesis involves oil-in-water emulsion, homogenization and solidification. This was modified here by applying high shear dispersion after solidification to achieve a NLC with desirable size, improved drug encapsulation and drug loading efficiency as a potential carrier for dacarbazine delivery.

LONG ABSTRACT:

The only formula of dacarbazine (Dac) in clinical use is intravenous infusion, presenting a poor therapeutic profile due to the low dispersity of the drug in aqueous solution. To overcome this, a nanostructured lipid carrier (NLC) consisting of glyceryl palmitostearate and isopropyl myristate was developed to encapsulate Dac. NLCs with controlled size were achieved using high shear dispersion (HSD) following solidification of oil-in-water emulsion. The synthesis parameters, including surfactant concentration, the speed and time of HSD were optimized to achieve the smallest NLC with size, polydispersion index and zeta potential of 155±10 nm, 0.2±0.01, and -43.4±2 mV, respectively. The optimal parameters were also employed for Dac-loaded NLC preparation. The resultant NLC loaded with Dac possessed size, polydispersion index and zeta potential of 190±10 nm, 0.2±0.01, and -43.5±1.2 mV, respectively. The drug encapsulation efficiency and drug loading reached 98% and 14%, respectively. This is the first report on encapsulation of Dac using NLC, implying that NLC could be a new potential candidate as drug carrier to improve the therapeutic profile of Dac.

INTRODUCTION:

Dacarbazine (Dac) is an alkylating agent that exerts anti-tumor activity through nucleic acids methylation or direct DNA damage, leading to cell cycle arrest and cell death [1].

As a first line chemotherapeutic agent, Dac has been used alone or in combination with other chemotherapy drugs for treating various cancers [2-6]. It is the most active agent so far used in treating cutaneous and metastatic melanoma, which is the most aggressive form of skin cancer [3, 7, 8]. The response rate, however, is only 20% at best, and the therapeutic effects are often accompanied with severe systemic side effects.

In its natural form, Dac is hydrophilic and is unstable due to its photosensitivity [9]. The only available formula for clinical use currently is a sterile powder to be used in suspension for intravenous infusion [7, 8]. The low response rate and high systemic toxicity rate of the drug is largely attributable to its poor water solubility, therefore low availability at target site, and high distribution at non-target sites, which limits the maximum dose of the drug [10]. The rapid degradation and metabolism after intravenous admission together with the development of drug resistance limit the clinical application and therapeutic effect of the drug [11]. Therefore, there is an urgent need to develop alternative Dac formulations for treating malignant melanoma.

Colloidal systems containing liposomes, micelles or nanostructured particles have been intensively investigated for their use in drug delivery as reviewed by Marilene *et al.* [12]. Nanostructured particles as potential drug carriers have been attracting increasing attention in the last decade due to their ability to increase drug loading efficiency, control drug release, improve drug pharmacokinetics and biodistribution, and therefore reduce drug systemic toxicity [13]. Only a few nanoformulations, however, have been investigated so far for Dac delivery, showing protection of the drug from photo degeneration, increased drug solubility, and improved therapeutic effect [10, 14, , 15]. However these formulations suffered from low encapsulating efficiency while some also using synthetic polymer nanoparticles that are not cost effective.

Nanostructured lipid carriers (NLC), made of a mixture of solid and liquid lipids, have been developed for drug delivery [16, 17]. The drugs to be encapsulated are often soluble in both the liquid lipids and solid lipids phases [18], resulting in a high loading and controlled release [19]. This study aims to develop a new Dac formulation based on NLC-encapsulation using glyceryl palmitostearate and isopropyl myristate as lipids. The preparation involved oil-in-water emulsion, evaporation, solidification, and homogenization. The preparations have been characterized for NLC size, shape, ultrastructure, and dispersity, drug encapsulation efficiency and drug loading [20].

PROTOCOL:

1. Preparation of oil-in-water emulsion

1.1) Weigh glyceryl palmitostearate (120 mg), isopropyl myristate (60 mg), d-α-tocopheryl polyethylene glycol succinate (30 mg) and soybean lecithin (30 mg), and add them to 12.5 mL of organic solvents (6.25 mL acetone and 6.25 mL ethanol). Quickly dissolve the mixture at the temperature 70 ^{o}C (5 ^{o}C above the melting point of the solid lipid) in water bath.

1.2) Add either 125, 250 or 375 mg of Poloxamer188 in 12.5 mL of ddH_2O to achieve 1-3% (respectively) of Poloxamer 188 solution, which is subject to heating at the same temperature as above.

1.3) Add the aqueous phase solution from step 1.2 to the oil phase solution from step 1.1 dropwise to form emulsions under magnetic stirring at 400 rpm . Stir the emulsion at 400 rpm for another 4 h to allow evaporation of the organic solvents.

2. Solidification and homogenization

2.1) Leave the emulsion in a cold room (4 ^{o}C) for 2 h to solidify/crystallize.

2.2) To obtain NLC, subject the emulsion to high sheer dispersion (HSD) with a homogenizer at 10000-15000 rpm for 10- 40 min.

3 Optimization of the NLC preparation

3.1) Take samples from step 2.2 with a surfactant concentration of 1, 2 and 3% undergoing HSD at speeds of 10000, 15000 and 20000 rpm, respectively, and time intervals of 10, 20, 30 and 40 min, respectively,

3.2) Examine the samples for particle size (PS), poly dispersion index (PDI), morphology and ultrastructure [20].

Note: The parameters that produce particles with the smallest size (155 nm) and PDI (0.2) value are determined as optimal.

4. Preparation of Dac-loaded NLC (NLC-Dac)

4.1) Prepare oil phase solution as described in step 1.1 with the addition of Dac (70 mg) before dissolving the mixture at 70 ^{o}C in water bath.

4.2) Prepare an aqueous phase solution as described in step 1.2 with 1% surfactant, and add this solution to that prepared in step 4.1 dropwise to form an emulsion under magnetic stirring at 400 rpm. Stir the emulsion for further 4 h to evaporate the organic solvents.

4.3) Leave the emulsion in a cold room (4 ^{o}C) for 2 h to solidify/crystallize as described in step 2, and finally, subject the emulsion to high sheer dispersion (HSD) using the optimal parameters determined in step 3.

REPRESENTATIVE RESULTS:

The preparations of the NLC and NLC-Dac using glyceryl palmitostearate and isopropyl myristate with different parameters were characterized for PS, PDI, morphology and ultrastructure [20]. The PS and PDI of the NLCs were surfactant concentration, HSD speed and duration dependent. As judged by PS and PDI of the NLCs, the best results were achieved with 1% of surfactant and a sheer dispersion speed of 15000 rpm for 30 min (Figure 1 A, B and C), which therefore wwew selected as the optimal parameters for NLC preparation in this study.

[place Figure 1 here]

The optimal parameters were used for NLC-Dac preparation. The smallest size achieved was 150±10 nm for NLC (Figure 2 A) and 190±10 nm for NLC-Dac (Figure 2 B), both with PDI of 0.2±0.001, indicating a good uniformity.

[place Figure 2 here]

Both NLC and NLC-Dac showed a spherical shape as observed under TEM (Figure 3).

[place Figure 3 here]

The uploading and encapsulation of Dac in NLC is indicated by the size and structure changes as seen in Figures 2 and 3 where NLC-Dac shows a larger size and altered internal structures as compared with NLC. The basic structure of NLC comprises a surfactant layer, a liquid lipid matrix and solid lipid crystals (Figure 3 A). NLC-Dac also exhibited the basic structure as a NLC but with expanded surfactant layer, liquid lipid matrix and solid lipid crystals, together with an extra substructure inside the solid lipid crystals (Figure 3 B), indicative of drug loading and encapsulation. The solid lipid crystals seen in NLC appeared denser than that in NLC-Dac, indicating that the solid lipid is less crystallized in NLC-Dac.

The drug encapsulation efficiency (EF) and drug loading (DL) percentage were derived from the following equations:
$EE\% = W_1 - W_2/W_1 \times 100 = 98.5\%$
$DL\% = W_1 - W_2/W_3 \times 100 = 14\%$
where W_1 amount of Dac added in the NLC, W_2 amount of un-entrapped Dac, W_3 amount of the lipids added [20].

Figure 1: Optimization of parameters used in NLC preparation. The optimal surfactant concentration and the speed and time of HSD were determined according to their effect on PS and PDI. A. Effect of surfactant concentration on PS; B. Effect of HSD speed and time on PS; C. Effect of HSD speed and time on PDI. This Figure has been modified from [20] . The data are presented as mean value of 3 replicates ± standard deviation (mean ±SD).

Figure 2: DLS measurement of NLC. A. The optimal size distribution of plain NLC; B. the optimal size distribution of NLC-Dac.

Figure 3: TEM imaging of NLC and NLC-Dac. Both NLC and NLC-Dac showed a spherical shape. A. Basic NLC structure comprises a surfactant layer (solid black arrow), liquid lipid matrix (white solid arrow), and solid lipid crystals (dotted white arrows); B. NLC-Dac also exhibited the basic structure as seen in NLC but the surfactant layer, liquid lipid matrix and solid lipid crystals appeared expanded; an extra substructure could be seen inside the solid lipid crystals (indicated by dotted black arrows), indicative of drug loading. Bar scale: 50 nm, Magnification: x 55000. This Figure has been modified from [20].

DISCUSSION:

Lipid-based nanostructured particles have been utilized to provide a highly lipophilic carrier for delivery of hydrophobic drugs. A NLC is the second generation of solid lipid nanostructured carrier, which are solid at room and body temperature. The incorporation of a solid lipid into a liquid lipid in a NLC results in a less perfect crystallization, thus increasing the drug loading efficiency and also reducing the expulsion of encapsulated drugs during storage.

For NLC synthesis, the most commonly used method involves oil-in-water emulsion, homogenization and solidification/crystallization [21, 22]. The homogenization allows NLCs to disperse thoroughly in an aqueous phase, whilst the solidification at low temperature allows the inner oil phase to crystallize. Different homogenization methods have been reported including magnetic stirring, ultrasonication, and HSD that are used before and/or during solidification [23 24].

In this study, the commonly used method was initially followed for NLC preparation. As the result was unsatisfactory, the method was modified such that HSD was applied after solidification. This modification proved highly effective in particle generation, PS and PDI control, while also making the NLC synthesis simpler, compared with previous reports on NLC preparation using the same lipids [23 25]. It was worth noting that the length of the evaporation (protocol 1.3) and solidification (protocol 2.1) is very critical as too long or too short a time would have negative effects on the generation of NLC.

This study suggests that NLC particles and their aggregation were formed during solidification. The HSD could disrupt the aggregation, which was possibly due to hydrophobic interaction between NLCs, and also stabilize the particles by thoroughly remixing them with surfactant. The synthesis procedure was optimized such that the NLC and NLC-Dac were produced with a size 155 ±10 nm and 190 ± 10 nm, respectively, and a PDI of 0.2±0.01. The high level of uniformity with particle sizes and the small PDI values indicates that a sufficient dispersion energy was achieved and is well distributed within the solution for disruption of particle aggregates. It has been suggested that particles of 100–200 nm are not prone to uptake by non-targeted cells, including mononuclear phagocytic system, thus having a long blood circulation time *in vivo* [26 27], whilst a PDI of more than 0.5 is an indication of particle aggregation [28]; the lower the PDI value, the higher the size homogeneity between the particles [29]. Further increase of the HSD speed and time above the optimal point could result in further PS reduction, and consequently an increase in interactions between small particles as well as re-aggregation. The difference in size and structure between NLC and NLC-Dac suggests that the Dac loading and encapsulation was successful. The drug binding to the outer layer of the NLC and encapsulation inside lipid matrices provide the potential for prolonged drug release, that could involve drug release firstly from the outer layer, followed by the release from the liquid lipid matrix and then from the solid lipid crystals of the NLC [30].

Currently four nanoformulations have been attempted for delivery of Dac as a single agent. The latest formulation reported was designed for duel encapsulation of Dac and vitamin A [32]. However these formulations suffered from a low encapsulation efficiency and/or relatively complex synthesis procedures. This is the first report for encapsulation of Dac with a NLC, proving advantageous over other encapsulations reported previously. NLC-Dac is easy to make and presents higher drug encapsulation and drug loading efficiency [20]. The NLC-Dac showed nearly 50% of drug released within the first 2 h whilst the remaining released slowly for up to 30 h [20]. The early release could be due to the binding of the drug with surfactant layer on the surface of the NLC, indicating that this formulation may not be ideal to replace the formulation currently in clinical use. However the drug in NLC-Dac appeared more stable compared with the nanoemulsion reported previously [10]. In addition, lipid based vehicles have been proposed for treating cutaneous melanoma and epidermoid carcinoma through topical drug delivery [32 10], indicating that the NLC-Dac developed in this study could also be potentially beneficial for topical application where early drug release would not potentially lead to severe systemic toxicity.

Due to the collective limitations with the available drug delivery carriers developed so far, further research is needed to develop more advanced nanomaterials for Dac delivery for targeted cancer treatment.

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DISCLOSURES:

The authors have nothing to disclose.

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