Cytotoxicity of solid lipid nanoparticles and nanostructured lipid carriers containing the local anesthetic dibucaine designed for topical application

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Abstract. Dibucaine (DBC) is powerful long-lasting local anesthetic, but it is also considered fairly toxic to the CNS. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have attracted attention as carriers for drug delivery. The aim of this study was to develop and to evaluate the cytotoxic activity of DBC-loaded SLN and NLC against 3T3 fibroblast and HaCat keratinocyte cells. The SLN and NLC had myristyl myristate and Liponate®GC as their lipid matrices, respectively, plus a surfactant. SLN and NLC were characterized in terms in their diameter, size distribution, surface charge and DBC encapsulation efficiency. The particle size of SLN and NLC were around 234.33 and 166.62 nm, respectively. Negative surface charges were observed for both nanoparticles, which decreased in the presence of the anesthetic. Encapsulation efficiency reached 76% and 90%, respectively, in SLN and NLC. DBC alone was found to be toxic to 3T3 and HaCat cells in culture. However, NLC and SLN loaded DBC decreased its intrinsic cytotoxic effect against 3T3 and HaCat cells. In conclusion, encapsulation of DBC in SLN and NLC decreased the in vitro toxicity of the local anesthetic, indicating the potential of these nanocarriers for clinical applications.

1. Introduction

Pain management continues to be one of the greatest challenges in medicine. Local anesthetics (LA) are widely used to reversibly block neural transmission [1, 2] and they differ from general anesthetics that cause loss of consciousness and inhibition of sensory and autonomic reflexes [3]. LA are amphiphilic medicines for which potency and toxicity are highly correlated with the molecule’s hydrophobicity [4, 5].

Dibucaine (2-butoxy-N-(2-diethylaminoethyl) quinoline-4-carboxamide) belongs to the amino-amide class of local anesthetic. The DBC chemical structure (Figure 1) differs from those of the LA commonly used in clinical practice, due to its large quinolinic ring, to which a butyl ether group is attached [6]; this rigid ring imposes restrictions on the molecule, and modulates its interaction with model membranes [6-8].

Figure 1: Chemical structure of dibucaine (deprotonated form).

Dibucaine is one of the most powerful long-lasting local anesthetics, being 15 times more potent than procaine [9]. However, it is also considered to be fairly toxic to the CNS and
its use is limited to surface anesthesia, mainly in the form of creams applied to the skin or mucous membranes. The symptoms of DBC overdoses can include convulsions, hypoxia, acidosis, bradycardia, arrhythmia, and cardiac arrest [10-12]. These problems have led us to investigate suitable delivery systems for the encapsulation of dibucaine.

Drug encapsulation in nanocarriers has been extensively studied for the transport of bioactive agents of low bioavailability and/or high toxicity [13-19]. Regarding local anesthetics, macrocyclic carriers (e.g. cyclodextrins), polymers (nanospheres, nanocapsules), and lipid-based carriers such as liposomes [19] and solid lipid nanoparticles [20] have provided effective drug delivery systems with prolonged anesthesia and reduced toxicity.

Solid lipid nanoparticles (SLN) have been used for the encapsulation of different active agents [20-27]. This type of carrier offers the same benefits as traditional ones such as liposomes and polymeric nanoparticles, without their disadvantages (such as physical instability, scale up problems, leakage of the active agent, and, in some cases, cytotoxicity). SLN are attractive to the pharmaceutical and cosmetic industries because they are stable, versatile, use safe excipients, and are readily scalable [21, 28-30].

Nanostructured lipid carriers (NLC) are considered to be the second generation of lipid-based particles (SLN being the first generation). They present at least one liquid lipid at room temperature to form their inner core. Recently, Gallarate & Battaglia [31] classified these structures as nanocapsules for having this liquid core and an outer envelope. Although both types of lipid nanoparticles, SLN and NLC, are able to encapsulate high amounts of hydrophobic drugs, NLC have not shown the limitations of SLN, regarding drug expulsion during storage caused by the crystalline rearrangements of the solid lipid [32, 33].

Encapsulation of dibucaine in SLN and NLC should render the drug available in higher doses at the site of administration, prolonging anesthesia and reducing its systemic toxicity due to controlled sustained release. In order to better understand the potential of such formulations, we prepared SLN and NLC containing dibucaine and characterized them in terms of their encapsulation efficiencies, particle sizes, zeta potentials and cytotoxicities against 3T3 fibroblasts and HaCat keratinocytes, in culture.

2. Material and methods

2.1. Materials

Dibucaine (DBC) and poloxamer 188 (Pluronic F68) were supplied by Sigma (USA), myristyl myristate (MM) was provided by Croda (Brazil) and Liponate® GC was from Lipo do Brasil Ltda (Brazil). Acetonitrile (J.T. Baker, USA), triethylamine (Vetec, Brazil), and orthophosphoric acid (Ecibra, Brazil) were of HPLC grade. Deionized water (18 MΩ) was obtained from an Elga USF Maxima Ultra Pure Water equipment. The solutions used in HPLC were filtered through 0.22 μm nylon membranes (Millipore, Belford, USA). All other chemicals and solvents used were of analytical grade.

2.2. Preparation of the solid lipid nanoparticles (SLN)

SLN were prepared by the hot emulsion technique. The solid lipid, myristyl myristate, was heated 10°C above its melting point (39°C, according to the manufacturer’s MSDS). To obtain the anesthetic-loaded formulations, dibucaine was solubilized into this oily phase after complete fusion. After that, this phase was carefully added (3 min.) to a hot aqueous solution of Pluronic F68 (0.02 mmol/L) under high speed agitation (10,000 rpm) using a Turrax blender (IKA Werke Staufen, Germany) up to a final myristyl miristate concentration of 47 mmol/L. This pre-emulsion was then homogenized at 600 bar - 3 cycles - in a Panda homogenizer (NiroSoavi, Italy). The Panda homogenizer and the sample’s milieu were insulated to keep the temperature above the melting point of the lipid. Finally, the SLN were cooled to room temperature, and conditioned in glass flasks at 4°C [34], as shown in Figure 2.
2.2. Preparation of the nanostrutured lipid carriers (NLC)
NLC were prepared using the same methodology described for preparing SLN (Figure 2) with modifications in the lipid components: in addition to myristyl myristate, Liponate® GC (a mixture of triglycerides of caprylic and capric acids that is liquid at room temperature) was used to form the oily phase. This oily phase was added to 0.06 mmol/L Pluronic F68 at 49°C. The final concentration of myristyl myristate and Liponate® GC in the formulation were 47 mmol/L and 0.5% w/w (referring to the total myristyl myristate lipid), respectively.

2.3. Particle size, polydispersity and zeta potential
The size distribution and average particle size (z-average) of the SLN and NLC particles were measured by photon correlation spectroscopy (PCS) in a Zetasizer Nano ZS (Malvern Instruments, UK), at 25°C with 10 mm path length polystyrene cuvettes. The samples were diluted in Milli-Q water prior to the analysis (sample/ Milli-Q water ratio of 1:100, v/v). The zeta potential was measured in a solution of 0.1 mmol/L of sodium chloride. The size, polydispersity, and zeta potential of the SLN and NLC samples (with and without DBC) were observed one day after preparation.

2.4. Transmission electron microscopy (TEM)
The nanoparticle morphology was analyzed using a Zeiss LEO-906, 60 kV transmission electron microscope. A drop of each nanoparticle sample was placed in a 200 mesh copper grid and a drop of a 2% aqueous uranyl solution added; excess volumes were removed with filter paper. Samples were incubated for 4 hours to dry at room temperature prior to TEM analysis.

2.5 High performance liquid chromatography (HPLC)
Dibucaine was quantified at 247 nm, using a Varian ProStar HPLC fitted with a PS 325 UV-Vis detector, a PS 210 solvent delivery module, and an automatic injector. Galaxy Workstation software was used for data collection and processing. The chromatographic conditions were optimized using a Merck reversed phase C18 column (LiChroCART 100 RP-18, 5 µm, 110 Å, 125 mm x 4 mm). The mobile phase consisted of a mixture of acetonitrile and 0.04 mol/L triethylamine phosphate buffer (55:45 v/v), pH 2.3. The flow rate was 1.0 mL/min, the detection and the injection volumes were 30 µL. Peak areas were measured. The analyses were
conducted at 35°C [35]. Quantification of free DBC employed a calibration curve \((Y=−0.52868+ 58.52676 \times X, r=0.999)\) obtained in the 1.5-30.0 µg/mL concentration range.

2.6 Encapsulation efficiency
The encapsulation efficiency (%EE) of dibucaine into the nanoparticles (SLN, NLC) was determined according to equation 1 [29]. The samples were diluted 2:100 v:v in Milli-Q water and transferred to a 10 kDa cellulose filtration unit (Millex, Millipore) and centrifuged (4000 g, 20 min). Free dibucaine in the filtered solution was quantified by HPLC (section 2.5).

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\% EE = \frac{\text{DBC}_{\text{bound}}}{\text{DBC}_{\text{total}}} \times 100
\]

(Eq 1)

Where, DBC\text{bound} is the amount of DBC entrapped in the SLN or NLCs and DBC\text{total} is the DBC added to the formulation, prior to phase separation. DBC\text{bound} = DBC\text{total} - [free DBC], detected by HPLC.

2.7 In vitro cytotoxicity
The cytotoxicity tests were performed using mouse 3T3 fibroblasts and human keratinocytes (HaCaT) cultured cells. MTT dye (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to assess cell viability, according to the capacity of the cells to reduce MTT to its formazan derivative. This reduction is catalyzed mainly by mitochondrial and cytoplasmic dehydrogenases, so that any alteration in mitochondrial function can be detected in the ability of the cell to reduce MTT to the purple formazan dye. The mouse embryo BALB/c 3T3 fibroblast cell line was obtained from the National Institutes of Health (Baltimore, USA) and the immortalized human keratinocytes, HaCaT, from the Academic Medical Center, Amsterdam University.

The cells were cultivated in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic. The cells were plated out onto 96-well plates, at a density of 1x10^4 cells/mL, and incubated at 37°C under a humid atmosphere with 3.5% CO₂. The medium was modified using different concentrations of dibucaine (0.02-0.30 mM, free of encapsulated) or non-DBC loaded nanoparticles (SLN or NLC, 0.53-4.20 mM).

In order to improve the solubility of free DBC 0.1% v:v of dimethyl sulfoxide was added to its water solution. The treatment medium was removed after 2 hours of incubation, and replaced by a medium without serum but containing 0.5 mg/mL MTT. After incubation for 3 hours at 37°C, the medium was carefully removed, and 100 µL of ethanol was added to solubilize the formazan derivative. The plates were agitated for 10 min. and the absorbance corresponding to each well was measured at 570 nm, using a BIO-TEK ELx 800 spectrophotometer. The values were expressed as the percent MTT reduction, relatively to a sample of control cells, not exposed to the tested agents [36]. Averages (± standard deviations) were calculated from six separate measurements.

3. Results and discussion
3.1. Production and characterization of nanoparticles
SLN and NLC were prepared with myristyl myristate (MM) and myristyl myristate/Liponate® GC, respectively, and Pluronic F-68. High pressure homogenization was the technique employed to prepare the nanoparticles. The mean diameters (Z-averages), polydispersity indices (PI), and zeta potentials of the SLN and NLC obtained are presented in Table 1. The diameters of the SLN and NLC were 188.02 ± 7.07 and 167 ± 14 nm, respectively. Although, in both cases, incorporation of dibucaine slightly increased the nanoparticle size, statistical analysis (unpaired t-test) revealed no significant differences (p > 0.05) between the formulations, with and without DBC. Moreover, as shown in Table 1, the type of nanoparticle had no significant effect on the particle features such as size, PI and zeta potential (p > 0.05). In all formulation the polydispersity index was smaller than 0.25, which is within the range expected for homogeneously dispersed colloids [23]. The measured zeta potentials were
negative (ca. 30 mV) and, upon addition of dibucaine, became more positive (~15-18 mV), for both SLN and NLC formulations. This result was taken as an indication that at least a fraction of the DBC molecules resides at the nanoparticle surfaces, affecting their surface charge distribution. All formulations exhibited high encapsulation efficiencies, as expected from the high lipophilicity of DBC, which favors its interaction with lipids [6]. The encapsulation efficiency of NLC was significantly higher than that observed with SLN (Table 1).

Table 1. Size (Z-Averages), polydispersity indices (PIs) and zeta potentials of SLN and NLC, prepared by high pressure homogenization, with and without DBC. The encapsulation efficiency (EE%) of such nanoparticles for the anesthetic is also given (pH 8.2).

| Sample     | Z-Averages (nm) | PI     | zeta potential (mV) | EE%       |
|------------|-----------------|--------|---------------------|-----------|
| SLN        | 188.02± 7.07a   | 0.15± 0.02a | -26.91± 7.72a      |           |
| SLN DBC    | 234.33± 42.87b  | 0.23± 0.06b | -18.47± 2.55b       | 76.58 ± 7.88c** |
| NLC        | 166.62± 14.96   | 0.12± 0.01 | -28.83± 4.41        |           |
| NLC DBC    | 177.39± 15.87   | 0.20± 0.08 | -14.89± 2.78        | 90.54± 0.95 |

(Mean ± SD, n=3) Z-Averages, PI and zeta potential: a. SLN vs NLC, b. SLN DBC vs NLC DBC. EE%: c. SLN DBC vs NLC DBC, (unpaired t test, **p < 0.01 and ns (not significant), p >0.05).

TEM images of SLN and NLC containing DBC (Figure 3 A, B) revealed spherical structures, with delineated surfaces and homogenous size distribution. The nanoparticle sizes in TEM micrographs are in good agreement with those determined by photon correlation spectroscopy.

![TEM images of SLN and NLC containing DBC](image)

Figure 3. Morphology of the nanoparticles containing dibucaine. (A) SLN DBC, (B) NLC DBC. The images in the right (60,000 x magnification) and the images in the left (100,000 x magnification) give details of one chosen particle.

3.2 Cytotoxicity tests
The cytotoxicity of the nanoparticles and their DBC formulations on two distinct cell lines (3T3 fibroblasts and HaCaT) were tested (Figure 4). Under the experimental conditions (0.5 - 4.2 mmol/L lipid concentration), SLN and NLC can be considered safe, since they did not affect the survival of either cell lines (~100% cell viability) (Figure 4 A).

Free dibucaine (Figure 4B) decreased the viability of 3T3 and HaCat cells: the concentration required to reduce cell viability by 50 %, IC50 = 0.25 mmol/L and 0.24 mmol/L for 3T3 fibroblasts and HaCaT, respectively. The cytotoxic effect of DBC has also been observed with other anesthetic agents [37] and seem to be related to the partitioning of the amphiphile molecule inside the biomembrane [38] leading to membrane destabilization [7].

Encapsulation of DBC into the nanoparticles significantly decreased its intrinsic cytotoxic effect (p > 0.001, ANOVA and Turkey-Kramer test), as shown in Figure 4B. The protection was even more evident when NLC were used in comparison to SLN (p < 0.05, ANOVA).

Figure 4. Cytotoxicity tests: cell viability of 3T3 fibroblasts (left) and HaCat cells (right) (mean ± SD, n = 6) treated with: (A) SLN and NLC; (B) free DBC and DBC-loaded SLN or NLC. a. free DBC vs SLN DBC, b. free DBC vs NLC DBC and c. SLN DBC vs NLC DBC. ANOVA and Turkey-Kramer test (ns: no significant, p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.001.

4. Conclusions and perspectives
Solid lipid nanoparticles and nanostructured lipid carriers containing dibucaine, were prepared by the high pressure homogenization technique. The high pressure process is attractive to the pharmaceutical industry, since it avoids scalability problems, and the equipment required can be easily acquired at low cost. A high DBC encapsulation efficiency (90.54±0.95%) was achieved with NLC. The nanoparticles presented high encapsulation efficiency and reduced the intrinsic toxic effect of the anesthetic against 3T3 fibroblasts and HaCat keratinocyte cells. Overall, these results show that encapsulation of dibucaine in NLC and SLN promotes sustained release of the anesthetic, while the in vitro cytotoxicity decrease supports the potential of this type of drug delivery system for future clinical (dermal anesthesia) applications. In vivo studies are being carried in our laboratory to test the antinociceptive effects of both drug delivery systems.

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