In vitro and *in vivo* efficacy of edelfosine-loaded lipid nanoparticles against glioma

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Abstract

Edelfosine is the prototype molecule of a family of anticancer drugs collectively known as synthetic alkyl-lysophospholipids. This drug holds promise as a selective antitumor agent, and a number of preclinical assays are in progress. In this study, we observe the accumulation of edelfosine in brain tissue after its oral administration in Compritol® and Precirol® lipid nanoparticles (LN). The high accumulation of edelfosine in brain was due to the inhibition of P-glycoprotein by Tween® 80, as verified using a P-glycoprotein drug interaction assay. Moreover, these LN were tested *in vitro* against the C6 glioma cell line, which was later employed to establish an *in vivo* xenograft mouse model of glioma. *In vitro* studies revealed that edelfosine-loaded LN induced an antiproliferative effect in C6 glioma cell line. In addition, *in vivo* oral administration of drug-loaded LN in NMRI nude mice bearing a C6 glioma xenograft tumor induced a highly significant reduction in tumor growth ($p<0.01$) fourteen days after the beginning of the treatment. Our results showed that Tween® 80 coated Compritol® and Precirol® LN can effectively inhibit the growth of C6 glioma cells *in vitro* and suggest that edelfosine-loaded LN represent an attractive option for the enhancement of antitumor activity on brain tumors *in vivo*.

Key Words: edelfosine, glioma, lipid nanoparticles

1. Introduction

Brain targeting has posed a great challenge throughout the history of therapeutics. Drug delivery to the brain is rigorously controlled by the blood brain barrier (BBB), which represents an overwhelming obstacle for many essential drugs including cytotoxics, antibiotics or central nervous system (CNS) active drugs [1]. The BBB is the homeostatic mechanism of defense of the brain against toxic molecules and pathogens. This exclusive membrane is formed by special endothelial cells sealed with tight junctions, which hamper the way into the brain from the bloodstream of many compounds that might be of therapeutic value in the treatment of a great assortment of pathologies [2, 3]. These issues, along with poor knowledge regarding the physiology of the CNS, have been the main limiting factors in the development of effective drugs and appropriate drug delivery systems (DDS) for brain targeting [1, 4-7]. In order for a drug to reach a target within the brain, some drawbacks that hinder its way must be taken into account. Among other factors, the drug must overcome the weak permeability of the BBB separating the blood vessels from the cerebral parenchyma [8] or the BBB active drug efflux transporters that are widely present in the cerebral endothelium and play an important role in the efflux mechanism of a wide variety of drugs [9].

Gliomas are the most common primary brain tumors [10], glioblastoma multiforme being the most aggressive subtype. The prognosis for patients with glioblastoma is poor, with a median survival of less than a year. The failure of chemotherapy in glioma patients is often due to the development of resistance by tumor cells and the difficulty of ensuring that drugs cross the BBB. Multidrug resistance (MDR) effect is due to the overexpression of membrane-bound proteins that efflux drugs from the cells, resulting in decreased intracellular drug concentration [11]. These drug efflux proteins are expressed not only in the BBB but also in brain glioma cells [12, 13]. Therefore, these proteins restrain the transport of the drug across the BBB, and decrease intracellular drug concentrations inside the brain gliomas via efflux.

Lipid nanoparticles (LN) have arisen in the last ten years as promising colloidal systems for the delivery of drugs to several diseases of the central nervous system, including tumors, as they are able to bypass the drawbacks mentioned above [14-16].

In a previous study we showed that the alkyl-lysophospholipidic antitumor drug edelfosine presents a wide distribution through the organism after multiple oral administration of the drug in solution, presenting a higher affinity for tumor tissue as well as kidney and intestine, and showing very low absorption after single oral administrations [17]. Conversely, after a single oral administration of edelfosine-loaded LN, the drug was absorbed in the gastrointestinal tract and had high concentrations in plasma (Estella-Hermoso de Mendoza *et al.*, submitted). Unlike different polymeric or liposomal systems, these LN are obtained by a solvent-free process and are physico-chemically stable [18]. Besides, the Tween® 80 included within the LN shell inhibits the P-gp efflux pump [19, 20].

The objective of the present study was to evaluate the possibility of brain targeting with LN after their administration by the oral route and to assess their efficacy, both *in vitro* against a rat glioma cell line and *in vivo* in a glioma-bearing xenograft mouse animal model.

2. Materials and Methods

2.1 Chemicals

Edelfosine was from APOINTECH (Salamanca, Spain). Compritol® 888 ATO and Precirol® ATO 5 were a gift from Gattefossé (Lyon, France). Tween® 80 was obtained from Roig Farma (Barcelona, Spain). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide) (MTT), Platelet Activating Factor (PAF) and PBS (10 mM phosphate, 0.9 % NaCl) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chloroform was purchased from Panreac (Madrid, Spain) and methanol was obtained from Merck (Barcelona, Spain). All solvents employed for the analysis were of analytical grade. C6 (rat glioma) cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Dubelcco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), trypsin–EDTA and penicillin–streptomycin mixtures were from Gibco® BRL (Carlsbad, CA, USA). Ultra-purified water was used throughout and all other chemicals were of analytical grade.

2.2 Preparation and characterization of LN incorporating edelfosine

LN were prepared by the hot homogenization method consisting of high shear homogenization and ultrasonication. Briefly, the lipid phase comprised 300 mg of either Compritol® 888 ATO or Precirol® ATO 5 along with 15 mg of edelfosine, while the aqueous phase consisted of 10 mL of a 2 % Tween® 80 aqueous solution. The aqueous phase was heated at 75 ºC, in the case of Compritol® 888 ATO, or 60 ºC, in the case of Precirol® ATO 5 (5 ºC above the melting point of the lipid) and added to the melted lipid phase at the same temperature. This temperature was maintained during the complete process of the nanoparticle formulation. The mixture was dispersed with the help of a Microson™ ultrasonic cell disruptor (NY, USA) for 1 minute at an effective power of 10 W. The preformed emulsion was then homogenized in an Ultraturrax® (IKA-Werke, Germany) for 1 minute at 24,000 rpm and sonicated again with the Microson™ ultrasonic cell disruptor (NY, USA) for 1 minute at 10 W. The nanoparticle suspension was cooled in an ice bath and washed twice with filtered water by diafiltration with Amicon Ultra–15 filters of 10,000 dalton molecular weight cut-off membrane (Millipore®, Cork, Ireland) (4,000 × *g*, 30 min) to remove the excess of surfactant and non-incorporated drug. Nanoparticles were then resuspended in 10 % trehalose solution and kept at -80 ºC for freeze-drying. These frozen LN suspensions were introduced into the freeze-dryer, kept at -50 ºC. Once inside the freeze-drier, a slow temperature rising process was performed up to 5 ºC, for the sublimation of the solvent. From this point on, temperature was raised to room temperature to evaporate the residual molecules of water. The whole process takes place at reduced pressure ($12 \cdot 10^{-3}$ mbar).

Particle size and distribution of the nanoparticles were measured by photon correlation spectroscopy (PCS) using a Zetasizer Nano (Malvern Instruments, UK). Each sample was diluted with distilled water until the appropriate concentration of particles was achieved to avoid multiscattering events. The homogenous suspension obtained was examined to determine the mean volume diameter, size distribution and polydispersity. All measurements were done in triplicate. The surface charge was determined by measuring the zeta potential of LN based on the Smoluchowski equation, using the same equipment.

Edelfosine loading was quantified by an ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method previously validated [21]. A sample of 10 mg of lyophilized nanoparticles was dissolved in 1 ml of chloroform and 10 µL of the internal standard PAF (0.2 mg/mL) were then spiked to the samples. Finally, 3 mL of methanol were added to the mixture. This solution was vortex mixed for 1 min at room temperature and centrifuged at 20,000 × *g* for 10 min. Eventually, 2 µL aliquots of the supernatant were injected into the chromatographic system.

2.3 P-glycoprotein inhibition experiments

To test the ability of the LN to modulate ATPase activity of membrane located P-gp [22], drug loaded LN made of both lipids were tested and compared to drug loaded LN prepared without Tween® 80 with the P-gp drug interaction assay kit (SPIbio®, Massy, France). Briefly, this assay is based on the study of modulation of basal or induced ATPase activity from enriched P-gp membrane vesicle preparation. P-gp ATPase activity is measured by a spectrophotometric method based on continuous monitoring of ADP formation, in the vesicle suspension medium.

LN without Tween® 80 were prepared like the regular LN, replacing the 10 mL of a 2 % Tween® 80 aqueous solution by 10 mL of water.

2.4 Cell growth inhibition studies

The cytotoxicity of edelfosine-loaded LN against C6 rat glioma cells was measured by MTT assay. Tumor cells were maintained in culture in DMEM supplemented with 10 % heat-inactivated FBS, 100 units/mL penicillin and 100 mg/mL streptomycin, at 37 ºC in a humid atmosphere containing 5 % CO_2. The cells were seeded in a 96-well plate at a density of 2000 cells per well, and incubated for 24 h. The medium was then replaced with increasing concentrations of free edelfosine, drug-loaded LN (edelfosine concentrations of 0.1, 1, 5, 10, 15, 20, 25, 30, 40, 50 and 70 μg/ml) and drug-free LN (lipid concentration of 0.5 mg/ml, corresponding to an edelfosine concentration of 70 μg/mL). Incubation was continued as above for 72 h. All media were then removed and 100 μL of MTT solution (0.5 mg/mL in DMEM) were added to the wells. The cells were incubated for 3 h. MTT was removed and DMSO was added to dissolve the formazan crystals [23]. The optical density at 570 nm was determined using a BioRad microplate reader. Untreated cells were taken as control with 100 % viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. Triton X-100 1 % was used as positive control of cytotoxicity. All experiments were performed in triplicate.

2.5 Animal studies

All animal studies had previous approval from the Animal Care and Ethics Committee of the University of Navarra and the Université Catholique de Louvain. For biodistribution studies, BALB/c mice (20 g) were obtained from Harlan Interfauna Ibérica S.L. (Barcelona, Spain). For efficacy studies, NMRI mice (Janvier, Genest St Isle, France) were employed. Animals received a standard diet and water *ad libitum*. Freeze dried formulations were resuspended in PBS by 1 minute vortexing (JP Selecta, Barcelona, Spain) followed by ultrasound bath (JP Selecta, Barcelona, Spain) for 5 minutes prior to administration.

2.5.1 Biodistribution studies after oral administration

A single oral administration of edelfosine-loaded Compritol® or Precirol® LN was given to two BALB/c mice groups (edelfosine concentration of 50 mg/kg, n=8 per group). The endpoint of the experiment was taken as the day after the concentration of edelfosine in plasma reached 0.5 μg/mL (168 h for Compritol® and 216 h for Precirol® group). At this time point, blood was collected in EDTA surface-coated tubes and then centrifuged at 2,000 × g for 10 min (4 ºC) to collect plasma (100 μL). After sacrifice by cervical dislocation, brain, liver, kidneys and intestine were collected and weighed. Tissues were homogenized in 1 mL of PBS pH=7.4 using a Mini-bead Beater (BioSpect Products, Inc., Bartelsville, Oklahoma, USA) and centrifuged at 10,000 × g for 10 min.

All plasma and tissue supernatants were then collected and stored at -80 ºC until UHPLC-MS/MS analysis was performed [21].

2.5.2 In vivo tumor growth inhibition study

Eight-week NMRI mice were subcutaneously inoculated into the back right flank with 1 x 10^5 C6 cells in 100 µL of DMEM. The effect of the different treatments on the growth of C6 was assessed by the measurement of the diameter of the tumors with an electronic caliper every three days using the following equation: $V=D \cdot d^2/2$, where D and d correspond to the longest and shortest diameter of the tumor, respectively. Treatments were initiated when tumors reached 6.0 ± 0.5 mm in diameter. Mice were then divided into six groups (9 mice per group): group 1: PBS; group 2: edelfosine solution (30 mg/kg; dissolved in PBS); group 3: edelfosine-loaded Compritol® 888 ATO nanoparticles (edelfosine concentration of 30 mg/kg); group 4: edelfosine-loaded Precirol® ATO 5 nanoparticles (edelfosine concentration of 30 mg/kg); group 5: blank Compritol® 888 ATO nanoparticles (10 mg/mL lipid concentration); and group 6: blank Precirol® ATO 5 nanoparticles (10 mg/mL lipid concentration). The treatments were administered by oral gavage every three days. Mice were sacrificed when their tumor diameter reached 18.0 ± 0.5 mm (30-fold increase in size).

2.6 Statistical analysis

Differences in P-gp inhibition studies were measured by Mann Whitney test, while mean values of the tumor sizes of all groups were analyzed by Student's *t* test using Social Package of Statistical Sciences (SPSS). IC_{50} values were calculated with GraphPad Prism 5 software using the sigmoidal dose-response function with variable Hill slope. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1 Characterization of edelfosine loaded lipid nanoparticles

The nanoparticles developed showed moderate edelfosine loading (18.5 and 15 µg edelfosine/mg formulation for Compritol® and Precirol®, respectively, corresponding to an encapsulation efficiency of 85 %). The average diameter was 111.2 ± 3.1 nm (Compritol® LN) and 105.5 ± 2.5 nm (Precirol® LN), with a PDI below 0.3 in all cases, indicating that LN were homogeneous in size (Table I).

Table I. Physicochemical characteristics of the developed LN (mean ± SD).

LN	Size (nm)	PDI	ζ Potential (mV)	%EE	Drug loading (µg edelfosine/mg form.)
Drug-free Compritol® LN	132.8 ± 5.5	0.269 ± 0.033	-30.3 ± 1.9	--	--
Drug-loaded Compritol® LN	111.2 ± 3.1	0.271 ± 0.020	-20.6 ± 2.1	85.53 ± 6.92	18.49 ± 2.77
Drug-free Precirol® LN	118.9 ± 2.3	0.246 ± 0.029	-30.1 ± 1.1	--	--
Drug-loaded Precirol® LN	105.4 ± 2.5	0.227 ± 0.015	-21.6 ± 3.1	84.62 ± 4.98	15.31 ± 3.29

Figure 1 represents the size distribution of representative formulations of both Compritol® and Precirol® LN. Zeta potential was negative in all cases, around -21 mV for both types of drug-loaded LN, while drug-free LN presented zeta potential values of -30 mV, meaning that some drug was located on the surface of the nanoparticles, being responsible for the decrease in the zeta potential by leaving less room for Tween® 80 to accommodate on the surface of the LN.

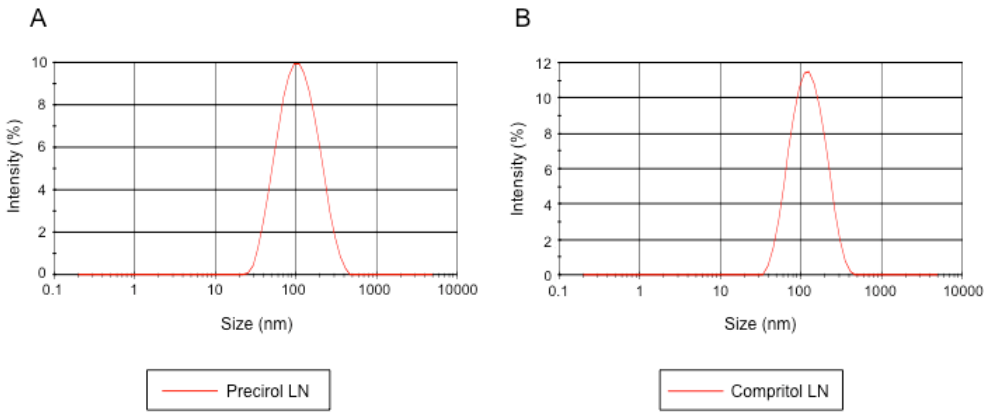

Figure 1. Representative size distribution of edelfosine-loaded A) Precirol® and B) Compritol® LN formulations.

3.2 Biodistribution study

In a previous study, the biodistribution patterns of orally administered free edelfosine revealed that the drug was widely scattered in many organs, but only a small amount was found in the brain [17]. However, when edelfosine is orally administered in LN, the biodistribution pattern is altered and accumulation of drug in brain is increased. This change in the biodistribution pattern supports the existence of intact LN in plasma, which later will allow the drug cross the blood brain barrier. Besides, it has been described by Olbrich and Müller [24] that some stabilizers, such as Poloxamer 407 or Tween 80, distinctly slow down the degradation velocity of the LN, as they provide sterically stabilizing layers of different thicknesses which hamper the anchoring of lipases/colipases and consequently, decrease the degradation velocity. This gives time for the LN to be absorbed. Figure 2 shows how, after the oral administration of edelfosine-loaded Compritol® and Precirol® LN (168 h and 216 h, respectively), edelfosine presents high tissue/plasma ratios of 4.5 and 12.4 for Precirol® and Compritol® LN, respectively, indicating higher accumulation in mouse brain when edelfosine is administered encapsulated in LN. These values are much higher compared to those when edelfosine was administered in solution (tissue/plasma ratio < 0.5) [17].

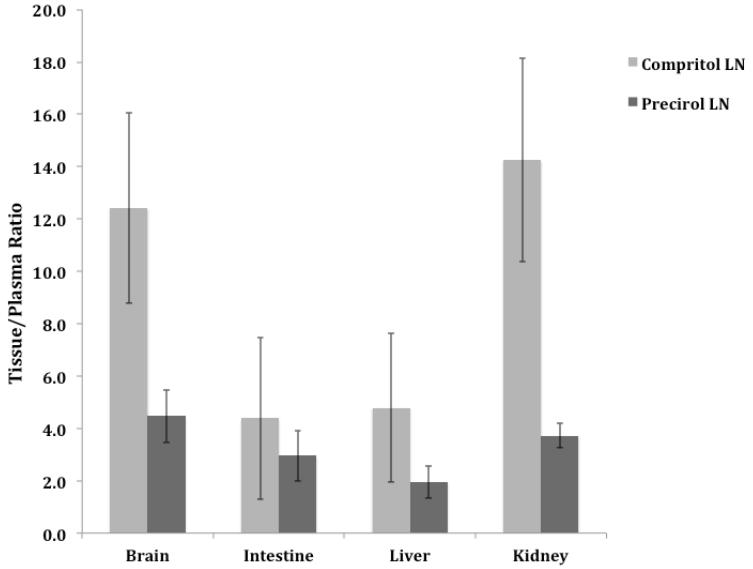

Figure 2. Tissue/plasma concentration ratios of edelfosine after a single oral dose of edelfosine-loaded Compritol® and Precirol® LN to BALB/c mice (*n*=8 per group, mean ± SD).

3.3 In vitro studies

The accumulation of drug in brain tissue when administered into LN is probably due to the inhibition of P-gp by the nanoparticles. To confirm this, a P-gp drug interaction *in vitro* assay was performed, in which Compritol® and Precirol® LN were tested against P-gp containing membrane vesicles. The P-gp inhibition in an *in vitro* test system was observed to be particle size and concentration independent. Figure 3 shows how Tween® 80 coated Precirol® and Compritol® LN (150 µg/mL of edelfosine) formulations were found to be more efficient in inhibiting P-gp, significantly decreasing P-gp relative activity compared to that of the baseline activity. On the other hand, LN prepared without Tween® 80 (150 µg/mL of edelfosine) did not decrease the relative activity of P-gp. Furthermore, lower LN concentrations (15 µg/mL and 1.5 µg/mL of edelfosine loaded LN) were observed still to be able to lower the P-gp activity. All these data confirm that Tween® 80 is an essential component in LN for inhibition of the P-gp.

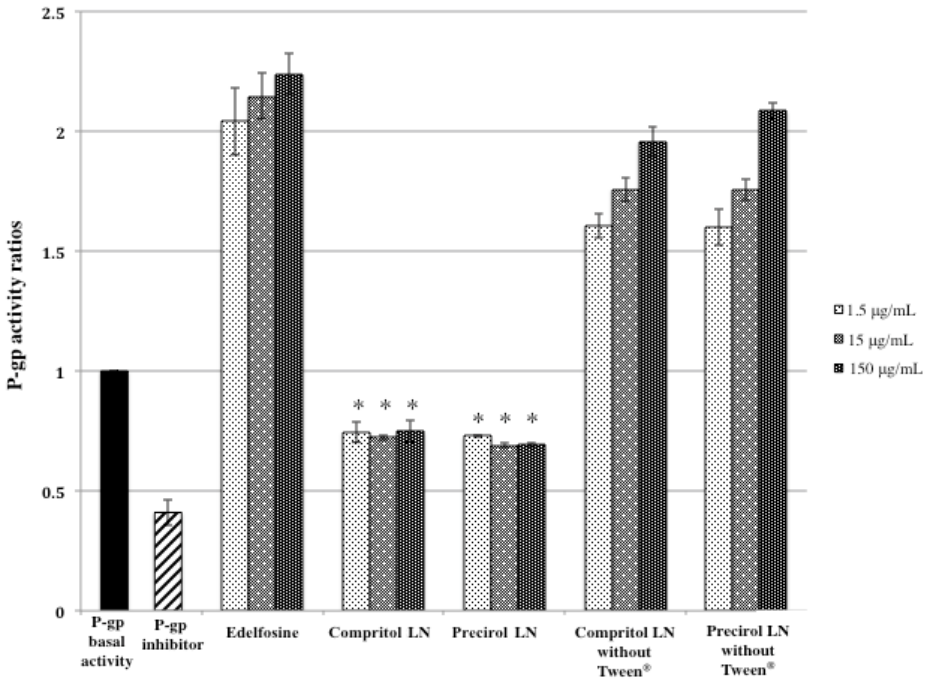

Figure 3. P-gp inhibition experiments with the SPIbio® test system for the different LN formulations and concentrations of edelfosine (1.5, 15 and 150 μg/mL). Comparable standards are given by the baseline activity of the test system with additional results from P-gp inhibitor vinblastine. The baseline activity of the test system itself was taken as 1.0 value. (n=4, mean ± SD) *p<0.05 levels by Mann Whitney's U test compared with baseline ATPase activity of P-gp.

Several studies have recently demonstrated that pharmaceutical excipients which are largely used as inert vehicles in drug formulations, like Cremophor-EL, Triton X-100 and Tween® 80 are emerging as a special class of P-gp inhibitors [25]. Some of these may even disrupt the function of P-gp and thus enhance the absorption of the drugs that are substrates of P-gp across different barriers. On the other hand, the mechanism by which nanoparticles cross the BBB is still uncertain but depending on the LN composition, the P-gp efflux pump inhibition can be conferred to the LN. For instance, Tween® 80 produces changes of membrane fluidity in the cells [26]. More concretely, it contains oxyethylene groups that may change the fluidity of the polar head group regions of cell membranes [27]. The disturbance of this environment might cause changes in secondary and tertiary structure of P-gp, leading to the loss of its function. Besides, the effect of Tween® 80 may also result in a negative regulation of the ATPase activity of the P-gp [9, 14].

In addition to this, another accepted way by which Tween® 80 coated LN are able to cross the BBB implies the adsorption of apolipoproteins (especially ApoE) onto the surface of the LN, so that after being recognized by the low-density lipoprotein (LDL) receptors present in the BBB, they are internalized [28-30]. The adsorption of apolipoproteins onto the nanoparticle surface, along with the solubilization of endothelial cell membrane lipids and membrane fluidization due to surfactant effects of polysorbates, is thought to be responsible for LN uptake in the brain after their adhesion to the endothelial cells of the blood brain barrier [28, 30]. After confirming that LN are able to get through the BBB by means of P-gp inhibition and assuming ApoE adsorption, we aimed to assess the cytotoxicity of edelfosine-loaded LN, and their tumor cell killing activity was determined against the C6 rat glioma cell line by MTT assay. Cytotoxicity was assessed by the comparison of IC_{50} values of free edelfosine, edelfosine-loaded LN and drug-free LN. The results of the cytotoxicity studies indicate that edelfosine-loaded Compritol® and Precirol® LN significantly reduced IC_{50} values in a P-gp overexpressing glioma cell line over free drug. Free edelfosine showed a very high IC_{50} value of 27.5 μg/mL (55 μM), suggesting resistance of this cell line to the drug after 72 h (Figure 4) and, indeed, the disruption of tumor cell membranes, is due to a detergent effect of the molecule, rather than apoptotic, as edelfosine presents an amphiphilic structure [31, 32].

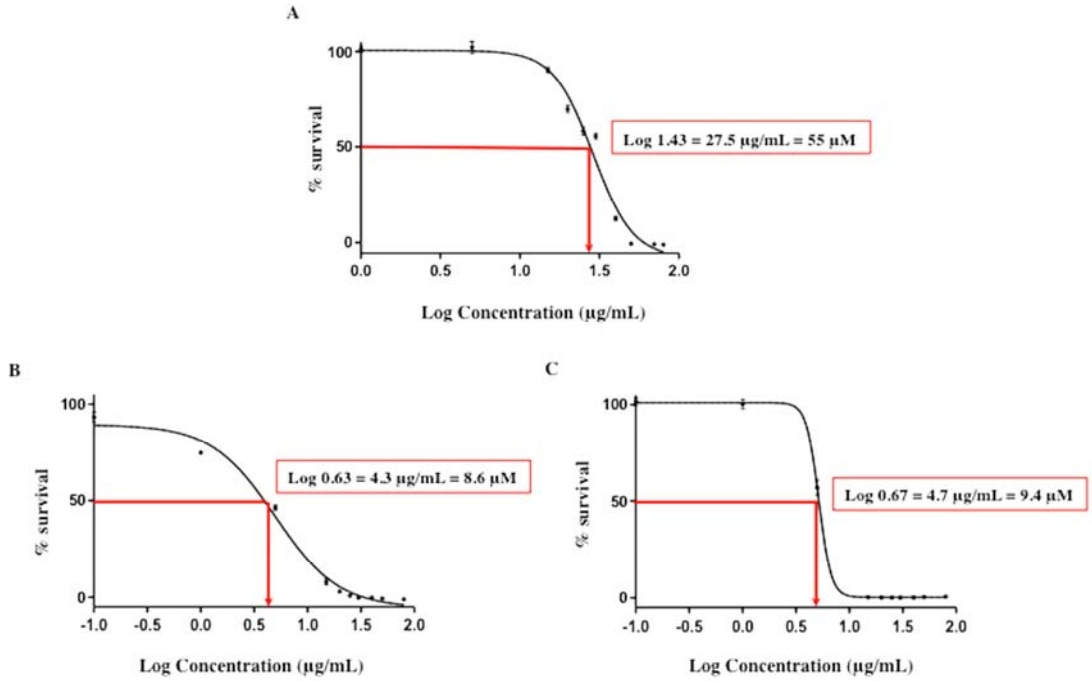

Figure 4. Viability study of C6 rat glioma cell line 72 h after the treatment with increasing concentrations of (A) free edelfosine and drug loaded (B) Compritol® and (C) Precirol® LN (*n*=6, mean log Concentration ± SD).

This low drug effectiveness is mainly ascribed to enhanced active efflux of the drug out of the cell, for which P-gp is responsible [33]. However, when edelfosine was incorporated into either Compritol® or Precirol® LN, the IC_{50} value 72 h after the treatment decreased dramatically to 4.3 µg/mL (8.6 µM) and 4.7 µg/mL (9.4 µM), respectively. The intense decrease in the IC_{50} value is probably due to the small size of LN, making possible an intracellular uptake, and the P-gp inhibiting properties already described, since Tween® 80 can inhibit P-gp related drug transport [19, 20]. Thus, the adjuvant effect of Tween® 80 seems to be imperative for high intracellular drug concentration maintenance. Edelfosine-free LN vehicles did not cause cytotoxicity against C6 cell line since the percentage of cell survival was above 85% (data not shown). The current study therefore suggests that there might be at least two major reasons for enhanced cytotoxicity of edelfosine-loaded LN in P-gp-mediated resistant cells: (a) a possible increased extent of drug uptake by endocytosis of nanoparticles, which helps to partially bypass P-gp; and (b) a decreased efflux rate of drug through inhibition of P-gp function caused by Tween® 80, a component of nanoparticles. They both increase intracellular drug concentrations, which is the key to overcoming transporter-mediated resistance [19].

3.4 In vivo tumor growth inhibition study

After the *in vitro* effectiveness of the LN had been confirmed, we evaluated their efficacy in an *in vivo* xenograft C6 rat glioma bearing mouse model. For that reason, xenograft C6 glioma bearing mice were treated with oral administrations of 30 mg/kg of edelfosine solution and edelfosine-loaded Compritol® and Precirol® LN every 3 days for 14 days. Tumor growth was followed as a function of time after treatment (Figure 5). In addition, changes in mean body weight were determined as a measure of drug induced toxicity. Regardless of the type of LN used, the therapeutic activity of the

edelfosine-loaded LN formulations was significantly higher than the edelfosine solution administered at the same dose. Treatments did not show visible toxicity, as a decrease in body weight was not observed in any group (data not shown).

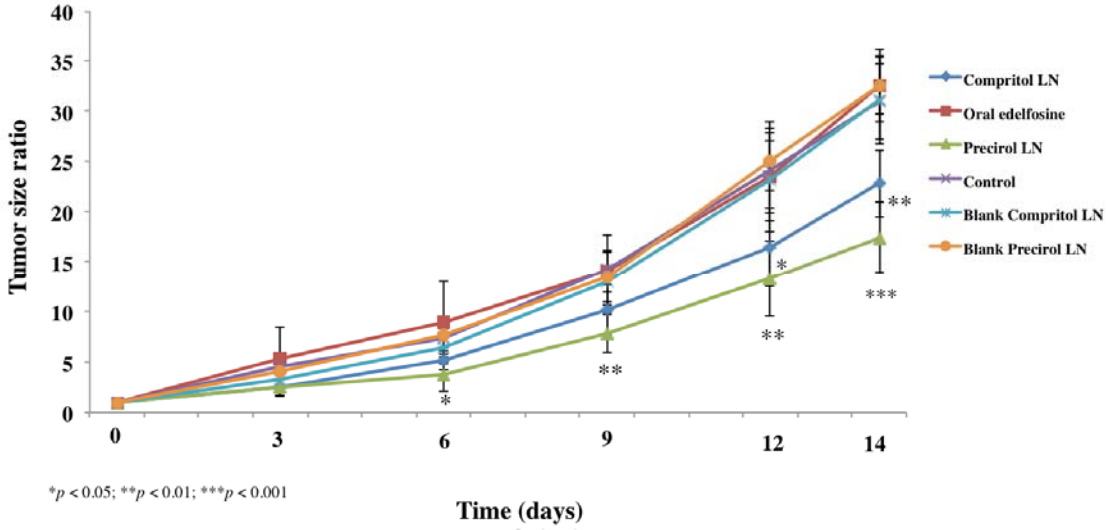

Figure 5. Evolution of tumor growth of xenograft C6 rat glioma implanted in mice, expressed as fold-increase ratio compared to tumor initial size, after treatments with PBS, free edelfosine (30 mg/kg bw), drug loaded LN (30 mg/kg bw) and drug-unloaded LN every three days over the oral route (n=9 per group, mean ± SD). Statistical analysis by Student's t test.

Regarding efficacy, significant differences were observed in tumor regression at day 6, after the third oral dose of edelfosine-loaded LN (Figure 5), compared to the oral administration of the edelfosine solution and the drug-unloaded LN, which did not have any effect on the tumor growth. The tumor burden of edelfosine-loaded LN treated mice was significantly smaller compared with control (PBS), free drug or drug-unloaded LN treated mice. The hypothesis for the mechanism of action of these LN against this xenograft glioma can be attributed to two main issues. First, the inhibition of the P-gp by Tween® 80 by the oxyethylene groups that it contains in its molecular structure, which may alter the fluidity of the membrane leading to P-gp disfunction [27]; and second, the release of the drug from the nanoparticles once these are internalized in the cell. The combination of these two effects could be responsible for the high intracellular accumulation of the drug in the tumor, preventing its removal from the cytosol by efflux pump proteins (P-gp).As a result, the consequent increase in drug concentration would permit the onset of tumor cell death.

4. Conclusions

In this article, a high accumulation of edelfosine was found in brain tissue when administered in LN, suggesting that these nanocarriers might be useful for the delivery of edelfosine to the brain. Moreover, *in vitro* studies against C6 cell line showed that edelfosine loaded Compritol® and Precirol® LN were able to revert the resistance of the cell to the drug, due to the inhibition of P-gp by Tween® 80. The oral administration of edelfosine-loaded LN to mice every three days decreased tumor growth significantly, when compared to the administration of the same dose of the free drug.

In conclusion, these nanocarriers show promise as a potential effective therapeutic agent in order to treat neoplastic diseases located in brain tissue.

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Figure Legends

Figure 1. Representative size distribution of edelfosine-loaded A) Precirol® and B) Compritol® LN formulations.

Figure 2. Tissue/plasma concentration ratios of edelfosine after a single oral dose of edelfosine-loaded Compritol® and Precirol® LN to BALB/c mice (n=8 per group, mean ± SD).

Figure 3. P-gp inhibition experiments with the SPIbio® test system for the different LN formulations and concentrations of edelfosine (1.5, 15 and 150 μg/mL). Comparable standards are given by the baseline activity of the test system with additional results from P-gp inhibitor vinblastine. The baseline activity of the test system itself was taken as 1.0 value. (n=4, mean ± SD) *p<0.05 levels by Mann Whitney's U test compared with baseline ATPase activity of P-gp.

Figure 4. Viability study of C6 rat glioma cell line 72 h after the treatment with increasing concentrations of (A) free edelfosine and drug loaded (B) Compritol® and (C) Precirol® LN (n=6, mean log Concentration ± SD).

Figure 5. Evolution of tumor growth of C6 rat glioma implanted in mice, expressed as fold-increase ratio compared to tumor initial size, after treatments with PBS, free edelfosine (30 mg/kg bw), drug loaded LN (30 mg/kg bw) and drug-unloaded LN every three days over the oral route (n=9 per group, mean ± SD). Statistical analysis by Student's t test.