

Complete inhibition of extranodal dissemination of lymphoma by edelfosine-loaded lipid nanoparticles

Ander Estella-Hermoso de Mendoza, Miguel A. Campanero, Hugo Lana, Janny A. Villa-Pulgarin, Janis de la Iglesia-Vicente, Faustino Mollinedo and María J. Blanco-Prieto

Ander Estella-Hermoso de Mendoza, PhD, *Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, E-31008, Spain; Tel: (+34) 948425600 x6313, Fax: (+34) 948425649; email: aestella@alumni.unav.es*

Miguel A. Campanero, PhD, *Clinical Pharmacology Service, University of Navarra Clinic, E-31008, Spain; Tel: (+34) 948255400 x3233, Fax: (+34) 948296500; email: macampaner@unav.es*

Hugo Lana, laboratory technician, *Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, E-31008, Spain; Tel: (+34) 948425600 x6313, Fax: (+34) 948425649; email: hlana@unav.es*

Janny A. Villa-Pulgarín, PhD student, *Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, CSIC-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain; APOINTECH, Centro Hispano-Luso de Investigaciones Agrarias (CIALE), Parque Científico de la Universidad de Salamanca, C/ Río Duero 12, E-37185 Villamayor, Salamanca, Spain; Tel: (+34) 923294806; Fax: (+34) 923294795; email: jannyalexander@gmail.com*

Janis de la Iglesia-Vicente, laboratory technician, *Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, CSIC-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain; Tel: (+34) 923294806; Fax: (+34) 923294795; email: jiglesia@usal.es*

Faustino Mollinedo, PhD, Full Professor CSIC, *Instituto de Biología Molecular y Celular del Cáncer, Centro de Investigación del Cáncer, CSIC-Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain; Tel: (+34) 923294806; Fax: (+34) 923294795; email: fmollin@usal.es*

***María J. Blanco-Prieto, PhD,** *Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, E-31080 Pamplona, Spain, Tel: (+34) 948425600 x6519, Fax: (+34) 948425649, email: mjblanco@unav.es*

***Corresponding author.**

Summary

Lipid nanoparticles (LN) made of synthetic lipids Compritol[®] 888 ATO and Precirol[®] ATO 5 were developed, presenting an average size of 110.4 ± 2.1 nm and 103.1 ± 2.9 nm, for Compritol[®] and Precirol[®], respectively, and encapsulation efficiency above 85 % for both type of lipids. These LN decrease the hemolytic toxicity of the drug by 90 %. Pharmacokinetic and biodistribution profiles of the drug were studied after intravenous and oral administration of edelfosine-containing LN, providing an increase in relative oral bioavailability of 1500 % after a single oral administration of drug-loaded LN, maintaining edelfosine plasma levels over 7 days in contrast to a single oral administration of edelfosine solution, which presents a relative oral bioavailability of 10 %. Moreover, edelfosine-loaded LN showed a high accumulation of the drug in lymph nodes and resulted in slower tumor growth than the free drug in a murine lymphoma xenograft model, as well as potent extranodal dissemination inhibition.

Key Words: edelfosine, lipid nanoparticles, bioavailability, pharmacokinetics, biodistribution, lymphoma

1. Introduction

Edelfosine is considered the prototype of a promising class of antitumor agents, collectively known as alkyl-lysophospholipid analogues or antitumor ether lipids. These agents present the singular characteristic of not targeting the DNA, but affecting the cell membrane and the apoptotic machinery of the cancer cell [1]. Recent *in vitro* studies have shown that edelfosine is preferentially uptaken by tumoral cells, sparing normal cells [2].

However, edelfosine presents some drawbacks when administered intravenously, as dose-dependent hemolysis that hampers its administration at certain doses [3]; and gastrointestinal irritation when administered orally [4, 5]. In addition, edelfosine presents bioavailability values below 10 % after a single oral administration of 30 mg/kg; however, this bioavailability increased to 64 % after multiple oral administration of the same dose after six days [2].

Owing to the drawbacks of this molecule, there has been an attempt to design new drug delivery systems that can modify the absorption rate, selectively transport the drug to the target, modifying the drug distribution profile and extending the drug release time in order to improve drug bioavailability, and decrease its toxicity. Among the different lipid-made colloidal carriers, edelfosine was incorporated into liposomes [6] and lipid nanoparticles (LN) made of biocompatible lipids [7]. The liposomal formulation was able to prevent the hemolytic toxicity of the drug, but the main inconvenience found was its rapid clearance from plasma. Edelfosine-loaded liposomes showed both *in vivo* and *in vitro* activity against methylnitrosourea-induced tumors, and it was approximately 4 - 8 times less acutely toxic than free edelfosine. Edelfosine-

loaded LN developed by our group were considered another alternative to deliver the drug to the organism [7]. These carriers are colloidal transporters composed of a biocompatible and biodegradable lipid matrix. They combine advantages of liposomes, polymeric nanoparticles and emulsions, while diminishing possible drawbacks associated with them [8]. Lipids employed to form these lipid cores are biodegradable raw materials that are biocompatible: triglycerides (i.e. tristearin), partial glycerides (i.e. Compritol[®] 888 ATO and Precirol[®] ATO 5), fatty acids (i.e. stearic acid), steroids (i.e. cholesterol) or waxes (i.e. cetyl palmitate) [9]. The formulation methods are also diverse [9, 10]. However, most techniques employ organic solvents, which may imply regulatory and toxicity issues. Moreover, an improvement of LN over the liposomes is their physical and chemical long-term stability up to 12 - 24 months [11]. The freeze-drying process of LN has been shown to increase their physicochemical stability over long periods of time [12]. Besides, LN have attracted rising interest for their ability to overcome certain biological barriers, resulting in increased therapeutic efficacy of the encapsulated drug and increase in tumor accumulation [13].

Mantle cell lymphoma (MCL) is a B-cell malignancy that comprises about 7 % of all non-Hodgkin's lymphomas (NHLs), which is characterized clinically by extranodal disease in older male patients who present at an advanced stage [14]. Even though most patients initially gain a benefit from systemic treatments, the responses obtained are generally of limited duration. As a result, patients generally relapse with less responsive disease, showing a consistently bad outcome with a median overall survival from diagnosis of 43 months [15, 16]. Several approaches using more severe combination chemotherapy, namely

stem cell transplantation, have shown higher response rates and more lasting remission in selected patients, but the greater part of MCL patients are not candidates for such dose-intensive regimens [17, 18]. MCL has recently become an area of intense clinical research, and it appears that median overall survival may be improving, but MCL is still considered incurable with current treatments [19]. Although several cytotoxic combinations including cisplatin, gemcitabine, carmustine and other alkylating drugs have been employed, their inherent toxicity is considered the main drawback at the time of election [20].

In a previous study, we determined that edelfosine displays a biexponential pharmacokinetic behavior in mice, presenting no significant differences regardless of the mouse strain employed [2]. The tissue distribution of edelfosine in mice shows that the drug is widely scattered across different organs, although it is preferentially internalized by the tumor both *in vitro* and *in vivo*. The present work tries to point out how the biodistribution and pharmacokinetic profile of edelfosine is altered compared to that of the free drug, when it is encapsulated in LN. The efficacy of the chemotherapeutic potential of edelfosine loaded LN via the oral route in experimental murine lymphoma xenograft model was also evaluated.

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). Platelet Activating Factor

(PAF) and PBS (10 mM phosphate, 0.9 % NaCl) were obtained from Sigma-Aldrich (Madrid, Spain). Chloroform was purchased from Panreac (Madrid, Spain) and methanol was obtained from Merck (Barcelona, Spain). All other solvents were of analytical grade.

2.2. Preparation of LN incorporating edelfosine

LN were prepared by the hot homogenization method followed by high shear homogenization and ultrasonication. The lipid phase consisted of either Compritol[®] 888 ATO or Precirol[®] ATO 5 along with edelfosine, while the aqueous phase consisted of a 2 % (w/v) Tween[®] 80 aqueous solution. The aqueous phase was heated at about 5 °C above the melting point of the lipid and added to the melted lipid phase at the same temperature. The mixture was dispersed with the help of a Microson[™] ultrasonic cell disruptor (NY, USA) for 1 minute. The preformed emulsion was then homogenised in an Ultraturrax[®] (IKA-Werke, Germany) for 1 minute and sonicated again with the Microson[™] ultrasonic cell disruptor (NY, USA) for 1 minute. 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) to remove the excess of surfactant. Nanoparticles were then resuspended in PBS for animal administration or in 10 % trehalose solution for freeze-drying.

2.3. Characterization of edelfosine loaded LN

2.3.1. Particle size and zeta potential

The average particle size and polydispersity index of edelfosine loaded LN were determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano (Malvern Instruments, UK). Each sample was diluted 30 fold in distilled water until the appropriate concentration of particles was achieved to avoid multiscattering events. The obtained monodisperse suspension was examined to determine the volume, mean diameter, size distribution and polydispersity and repeated three times for each sample. Similarly, the zeta potential was measured using the same equipment with a combination of Laser Doppler (LD) electrophoresis [21, 22]. Samples were diluted with distilled water (pH 5.5) and each experiment was performed in triplicate. All data are expressed as a mean value \pm standard deviation.

2.3.2 Loading capacity

Edelfosine was quantified by an ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method that had been previously validated [23]. The drug was extracted from a sample of 10 mg of lyophilized nanoparticles, to which 1 ml of chloroform was added in order to dissolve them. 10 μ L of the internal standard PAF (0.2 mg/mL) were then spiked to the samples. 3 mL of methanol were added to the mixture, and after vortex mixing for 1 min at room temperature and centrifuging at 20,000 \times g for 10 min, 2 μ L aliquots of the supernatant were injected into the chromatographic system.

2.4. Hemolysis experiments

Erythrocytes from fresh human blood were separated from plasma by centrifugation (4,000 x g, 7 min) and washed three times with PBS. 4 mL of the washed erythrocyte suspension were diluted to 100 mL with PBS. 1.5 mL of this suspension were treated with 0.5 mL of tested samples: free edelfosine (10 µg/mL), edelfosine loaded Compritol[®] and Precirol[®] nanoparticles (10 µg/mL) and drug free Compritol[®] and Precirol[®] nanoparticles. Absorbance was measured in an Agilent 8453 UV-visible spectrophotometer (Agilent, Palo Alto, CA, USA) at 540 nm 1 h after the treatment.

2.5. Animal experiments

Animal handling was conducted in compliance with the regulations of the Ethical Committee of the University of Navarra as well as with the European Community Council Directive Ref. 86/609/EEC. For pharmacokinetic studies, BALB/c mice (20 g) were obtained from Harlan Interfauna Ibérica S.L. (Barcelona, Spain). For efficacy studies, SCID mice (Janvier, Genest St Isle, France) were employed. Animals received a standard diet and water *ad libitum*, except for the animals that received the oral doses, which were fasted for 24 hours prior to administration.

2.5.1. Pharmacokinetic and biodistribution studies after intravenous administration

An i.v. single dose of edelfosine-loaded LN (50 mg/kg) was administered to BALB/c ($n=8$ per group) mice via the tail vein. Group 1 received Compritol[®] 888 ATO LN and group 2, Precirol[®] ATO 5 LN. At various time points after administration (0, 1, 2, 5, 8, 24 and 31 h for Compritol[®] group and 0, 1, 2, 5, 8, 24, 48, 72, 96, 120, 144 and 168 h for Precirol[®] group), blood was collected in EDTA surface-coated tubes and then centrifuged at $2,000 \times g$ for 10 min (4 °C) to separate the plasma (100 μ L). Then, animals were sacrificed and spleen, liver, lungs, kidneys, heart, stomach and intestine were collected and weighed. Tissues were homogenised in 1 mL of PBS pH=7.4 using a Mini-bead Beater (BioSpect Products, Inc., Bartelsville, Oklahoma, USA) and centrifuged at $10,000 \times g$ for 10 min. Both plasma and tissue supernatants were collected and stored at -80 °C until UHPLC-MS/MS analysis was performed.

2.5.2. Pharmacokinetic and biodistribution studies after oral administration

Three BALB/c mice groups ($n=8$ per group) were treated with a single oral administration of free edelfosine (50 mg/kg) and edelfosine-loaded LN (edelfosine concentration of 50 mg/kg). Group 1 was treated with an oral administration of free edelfosine; group 2 received Compritol[®] 888 ATO LN and group 3, Precirol[®] ATO 5 LN. At various time points after the administration (0, 1, 2, 5, 8, 24, 48, 72, 96, 120, 144 and 168 h for free drug and Compritol[®] groups and 0, 1, 2, 5, 8, 24, 48, 72, 96, 120, 144, 168, 192 and 216 h for Precirol[®] group), blood was collected in EDTA surface-coated tubes and then centrifuged at $2,000 \times g$ for 10 min (4 °C) to collect plasma (100 μ L). After

sacrifice by cervical dislocation, tissues were collected, weighed and processed as explained above.

2.5.3. Lymphatic absorption studies

BALB/c mice (n=8 per group) received oral doses of free edelfosine (group 1), Compritol LN (group 2) and Precirol LN (group 3) (edelfosine concentration of 50 mg/kg). 24 hours later, an oily emulsion (milk) was orally administered 1 hour prior to sacrifice, in order to make the lymph ducts and nodes more visible, animals were sacrificed and mesenteric lymph nodes were spotted close to the ascending colon, extracted with the aid of dissection forceps. Then, they were processed like the rest of the organs and analyzed by UHPLC-MS/MS.

2.5.4. Efficacy studies

Cell culture studies

The human mantle-cell lymphoma cell line JVM-2 (DSMZ, Germany) was grown in RPMI-1640 containing 10 % heat-inactivated fetal calf serum (GIBCO/BRL, Carlsbad, CA, USA), 2 mM of L-glutamine, 100 µg/ml streptomycin sulphate (Sigma) and 100 U/ml penicillin (Sigma), at 37 °C in a humidified atmosphere of air containing 5 % CO₂.

Animal studies

Eight-week SCID mice were subcutaneously inoculated into the lower dorsum with 1×10^7 JVM-2 cells in 100 µL of PBS and 100 µL of Matrigel

basement membrane matrix (Becton Dickinson, San Jose, CA). Animals received a standard diet and water ad libitum. When tumors were palpable, mice were randomly assigned to the treatment groups. Six groups of mice ($n=8$ per group) were treated orally: 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, equivalent to a 30 mg/kg edelfosine dose). The treatments were administered by oral gavage every four days. The experiment ended when control group tumors reached a volume of $5.0\pm 0.5\text{ cm}^3$. At this point, animals were sacrificed and tumors were collected for the determination of their volume and weight. Axillary, inguinal and mesenteric lymph nodes were also extracted from MCL-bearing mice treated with either free or vectorized edelfosine and macroscopically analyzed.

2.6. Data analysis

Pharmacokinetic analysis was performed with plasma samples obtained from experiments with all mice. All these plasma concentration data were analyzed by non-compartmental and compartmental analysis using WinNonlin Professional Edition Version 2.1 (Pharsight, Mountain View, CA, USA). The area under the plasma concentration vs. time curve (AUC) was determined using the log-linear trapezoidal rule with extrapolation to infinitum and

normalized against the dose. The CL value is the volume of plasma completely cleared of a specific compound per unit time by the organism; it was calculated by dividing the dose by AUC. The maximum plasma concentration (C_{max}) was calculated from the plasma concentration-time curve and normalized against the dose. Oral bioavailability (F) was determined by ratio of the dose-normalized AUC following oral and i.v. administration. V_{ss} is the volume of fluid that would be required to contain the amount of drug in the body if it were uniformly distributed at a concentration equal to that in the plasma. The $t_{1/2}$ value refers to the time taken for plasma concentration to fall by 50 %, and it was determined using the following formula: $t_{1/2} = \ln(2) \cdot V_{ss} / CL$.

2.7. Statistical analysis

The presence of differences in tissue/plasma ratios and pharmacokinetic parameters was measured by the Mann Whitney test for double comparisons using Social Package of Statistical Sciences (SPSS). Student's t test was used for measuring differences in efficacy and tumor dissemination inhibition studies. A value of $p < 0.05$ was considered to be statistically significant for all statistical tests.

3. Results and discussion

3.1. Particle size, size distribution and zeta potential

The peroral route is the most preferred route of administration, but this path is limited for many substances as they present poor oral bioavailability due to

biopharmaceutical (low solubility, low permeability, and/or instability in gastrointestinal environment) and pharmacokinetic (extensive first pass metabolism and/or rapid clearance) drawbacks in their delivery approach. Therefore, the development of delivery systems that would be able to overcome these drawbacks is essential to ensure the effectiveness of such molecules. Edelfosine-loaded LN were produced by a solvent-free hot homogenization method followed by high shear homogenization and ultrasonication, and freeze-dried. The physical-chemical characteristics of the developed nanoparticles are compiled in Table I.

Table I. Average size, PDI, zeta potential, encapsulation efficiency (EE) and drug loading of edelfosine-loaded LN ($n=20$) prepared by the warm microemulsion formation followed by high shear homogenization and ultrasonication method.

LN	Size (nm)	PDI	ζ Potential (mV)	%EE	Drug loading (μg edelfosine/mg form.)
Drug-free Compritol [®] LN	130.6 \pm 3.1	0.275 \pm 0.021	-28.6 \pm 2.1	---	---
Drug-loaded Compritol [®] LN	110.4 \pm 2.1	0.261 \pm 0.050	-21.2 \pm 1.5	84.68 \pm 7.18	17.57 \pm 1.97
Drug-free Precirol [®] LN	117.7 \pm 2.4	0.243 \pm 0.026	-29.1 \pm 1.7	---	---
Drug-loaded Precirol [®] LN	103.1 \pm 2.9	0.231 \pm 0.012	-22.4 \pm 2.0	82.62 \pm 5.73	13.95 \pm 0.79

All data are expressed as mean value \pm standard deviation. Drug-free LN presented diameters of 130.6 ± 3.1 and 117.7 ± 2.4 for Compritol[®] and Precirol[®] LN, respectively. The average diameter of edelfosine-loaded Compritol[®] LN was 110.4 ± 2.1 nm, while drug-loaded Precirol[®] LN presented a mean diameter of 103.1 ± 2.9 nm, suggesting that edelfosine might be responsible for the reduction in size of LN, as it is a surfactant structured molecule. These particles present a smaller size than those prepared by the emulsion formation and solvent evaporation method previously developed [7]. This method therefore provides smaller particles, which are organic solvent-free, in a shorter formulation time. PDI was below 0.3 in all cases indicating that the LN were monodisperse. These particles present an appropriate size for their oral administration, since it has been widely demonstrated that sizes below 300 nm are suitable for intestinal transport to the thoracic duct [24-27].

Zeta (ζ) potential can make a prediction about the stability of colloid dispersions. A high ζ potential ($>|30|$ mV) can provide an electric repulsion to avoid the aggregation of particles, as they are considered strongly ionic [21, 28]. In our case, the ζ -potential values measured in double-distilled water were negative. The mean ζ potentials of edelfosine-loaded and drug-free Compritol[®] LN were -21.2 ± 1.5 mV and -28.6 ± 2.1 mV, respectively. Precirol[®] LN showed similar values of -22.4 ± 2.0 mV and -29.1 ± 1.7 mV for edelfosine-loaded and drug-free Precirol[®] LN, respectively. The incorporation of edelfosine slightly modified the zeta potential of the LN, supporting the idea of existence of drug in the surface of the LN along with the Tween[®] 80. This negative surface charge could be due to the presence of oleic acid traces in Tween[®] 80 on the particle surface, forming a denser surfactant film, and thus eliciting increased

electrophoretic mobility. Besides, the steric impediment of Tween[®] 80 might be another effect which would increase the stability of colloidal dispersions [29].

3.2. *Loading capacity*

It is well known that the crystalline state in the LN structure leads to faster drug expulsion. However, lattice defects of the lipid structure offer space to accommodate the drugs [30]. As a result, the structure of less ordered arrangement in the nanoparticles should be beneficial to the drug loading capacity, as in the case of the particles developed in this work.

The entrapment efficiency of edelfosine in the LN prepared by the warm microemulsion followed by high shear homogenization and ultrasonication was similar for nanoparticles prepared with both types of lipids (Table I). Nanoparticles formulated using Compritol[®] encapsulated 84.68 ± 7.18 % of edelfosine (equivalent to 17.57 ± 1.97 μg edelfosine/mg formulation), while Precirol[®] LN encapsulated 82.62 ± 5.73 % of the drug (13.95 ± 0.79 μg edelfosine/mg formulation). This high encapsulation efficiency is likely to be due to the partially amorphous state of the lipids in the formulation, which allows more edelfosine to be incorporated among lipid chains [7].

3.3. *Hemolytic experiments*

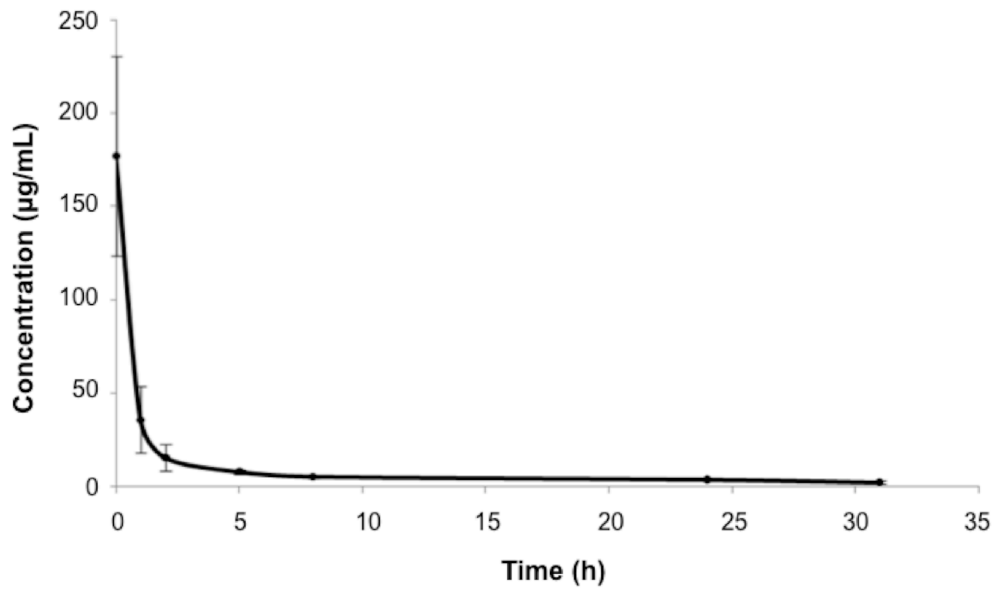
Edelfosine presents hemolytic toxicity due to its amphipathic structure [31]. Therefore, a hemolytic assay was performed to assess whether LN reduced the hemolytic effect of edelfosine. Results clearly showed that LN

protected red blood cells from hemolysis caused by edelfosine, because while the free drug was 100 % hemolytic at 10 µg/mL, both drug-loaded and unloaded LN only presented 9.88 and 12.02 % of hemolysis at the same drug concentration, for Compritol® and Precirol® respectively. This means that the low hemolysis produced was due to the LN composition itself, more concretely to the Tween® 80 present on the surface of the LN, rather than to the effect of the drug. In previous studies, Kötting et al. determined that in an *in vitro* study a 50% of spontaneous hemolysis was caused by a concentration of 2 µmol/L of edelfosine within 2 minutes [31]. In an *in vivo* study Ahmad et al. revealed that approximately 5% of circulating red blood cells hemolysed in 30 minutes after a single i.v. dose of 50 mg/kg [3]. As a result, LN showed a significant decrease in hemolytic toxicity of edelfosine.

3.4. *Pharmacokinetic characterization and biodistribution after intravenous administration*

Figures 1A and 1B show the concentration of edelfosine in mouse plasma plotted against time after a single i.v. administration of edelfosine-loaded LN (concentration ranging 30 - 60 mg/kg) to BALB/c mice. Dose-normalized pharmacokinetic analysis of edelfosine in blood plasma showed a C_{max} of approximately 0.3 µg/mL for both types of LN, showing no statistical differences between them. All obtained pharmacokinetic parameters were dose-normalized and are listed in Table II.

A



B

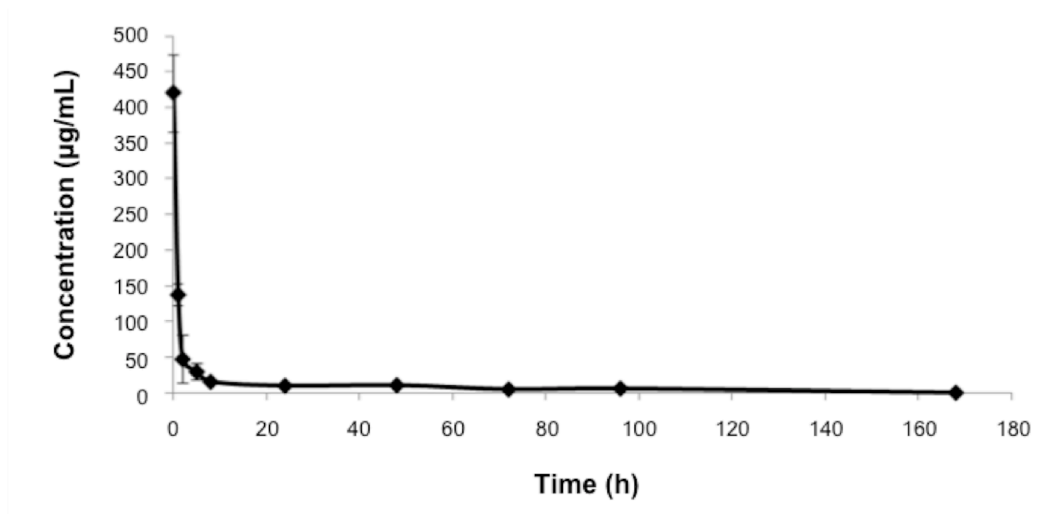


Figure 1. Time-concentration curve data of edelfosine after a single intravenous administration of edelfosine loaded (A) Compritol® and (B) Precirol® LN to BALB/c mice ($n=8$, mean \pm S.D.)

Table II. Comparison of pharmacokinetic parameters of edelfosine after intravenous administration of edelfosine loaded LN (50 mg/kg bw, $n=8$ per group, mean \pm SD). No statistical differences were found among parameters $p>0.05$.

Parameters	Compritol® 888 ATO LN i.v. administration	Precirol® ATO 5 LN i.v. administration
$t_{1/2\alpha}$ (h)	0.395 \pm 0.124	0.505 \pm 0.151
$t_{1/2\beta}$ (h)	16.970 \pm 5.775	23.718 \pm 17.743
C_{max} / D ($\mu\text{g}/\text{mL}/\mu\text{g}$)	0.290 \pm 0.087	0.341 \pm 0.044
CL (L/h/kg)	0.105 \pm 0.021	0.065 \pm 0.023
V_{ss} (L/kg)	1.668 \pm 0.730	1.313 \pm 0.838
MRT (h)	17.154 \pm 6.517	26.096 \pm 20.598
AUC_{inf} / D ($\mu\text{g}/\text{mL}/\mu\text{g}$)	0.573 \pm 0.053	0.894 \pm 0.399

Plasma concentration-time data of edelfosine in LN were well described by bi-exponential functions following i.v. administration of both types of LN. The distribution half-lives ($t_{1/2\alpha}$) of the two formulations were around 0.4 h, while the elimination half-lives ($t_{1/2\beta}$) were 17 h for Compritol® LN and 23 h for Precirol® LN, suggesting a much slower elimination of these last nanoparticles. These parameters are higher than those of the edelfosine-loaded liposomes described by Bhamra *et al.* (13.1 h) [6], indicating that these LN circulate in plasma for a longer period of time.

The rest of the pharmacokinetic parameters showed no statistical differences between the two types of LN. The mean systemic CL and V_{ss} values for edelfosine-loaded LN were around 0.08 L/h/kg and 1.5 L/kg, respectively. When edelfosine was loaded into liposomes [6], the V_{ss} was 0.203 L/kg, 7 times

lower than this value, suggesting that LN allow a broader distribution of the drug in the body. There was little variability in most of the values of the pharmacokinetic parameters, indicating a well-controlled and reproducible study, except for the elimination phase half-life value. AUC values were between 0.6 and 0.9 $\mu\text{g}\cdot\text{h}/\text{mL}/\mu\text{g}$, similar to those obtained in a previous research work [2] after the i.v. administration of edelfosine solution, indicating that the i.v. administration of edelfosine in LN presents an absolute bioavailability of 100 %.

Figure 2 depicts the scattering of the drug through the mouse body after i.v. administration of edelfosine-loaded LN, expressed as tissue/plasma ratios.

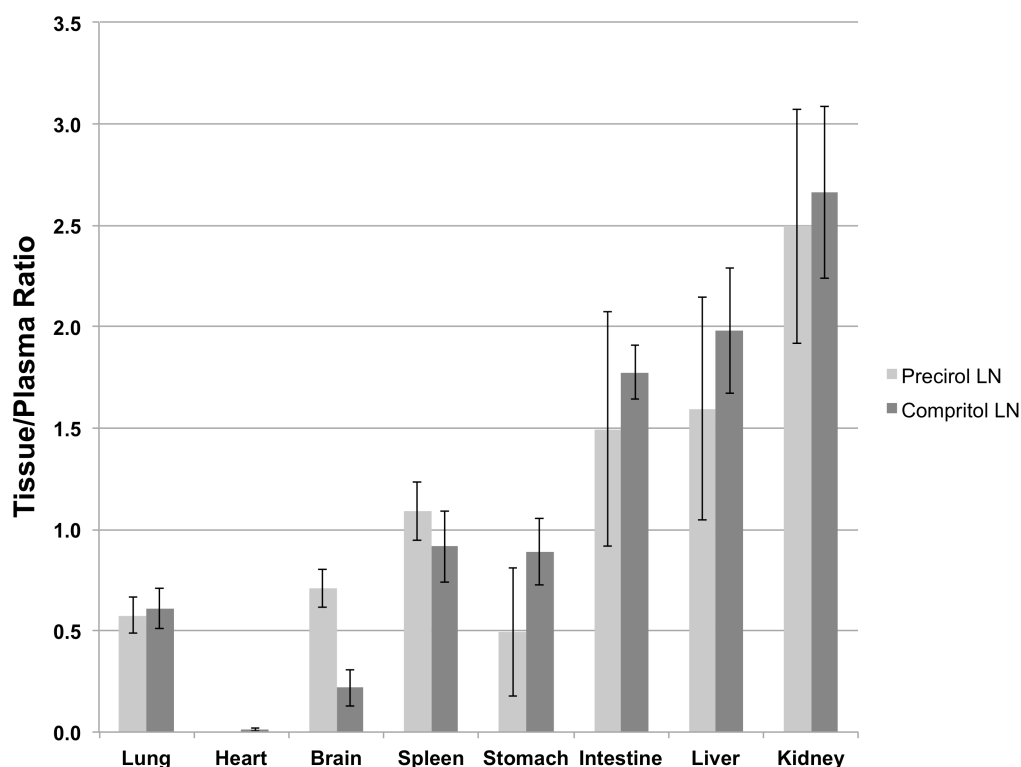


Figure 2. Tissue/plasma concentration ratios of edelfosine after a single intravenous dose of edelfosine-loaded Compritol[®] and Precirol[®] LN to BALB/c mice ($n=8$)

Whichever the type of nanoparticle employed, the highest accumulations of the drug were achieved in kidney, intestine and liver, followed by spleen, stomach and lung, with no statistical differences between the ratios.

3.5. Pharmacokinetic characterization and biodistribution after oral administration

Pharmacokinetic studies were performed after a single oral administration of 50 mg/kg of edelfosine-loaded in LN. This oral dose was well tolerated by the mice and no hemolytic side effects or body weight loss was observed (data not shown). Figure 3 shows the concentration of edelfosine in mouse plasma plotted against time after a single oral administration of edelfosine-loaded LN to BALB/c mice.

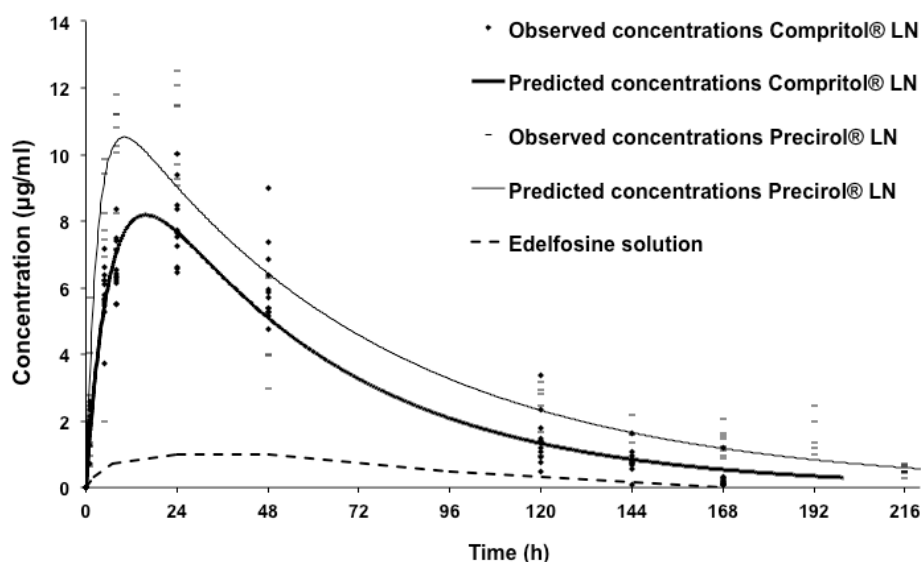


Figure 3. Time-plasma concentration curve data of edelfosine obtained with the WinNonLin program after a single oral administration of edelfosine solution and edelfosine loaded Compritol® and Precirol® LN to mice ($n=8$ per group)

The endpoint of the experiment was the day after the concentration of edelfosine in plasma reached 0.5 µg/mL. It can be observed that unlike edelfosine solution, drug-loaded LN enhanced the absorption of the drug maintaining detectable concentrations for over seven days. As a result, the maintenance times in plasma achieved with the developed LN were much longer than those observed by different studies recently published with the same Compritol® lipid, which did not last longer than 24 h [27, 32].

All dose-normalized pharmacokinetic parameters obtained after the administration of edelfosine in LN are summarized in Table III.

Table III. Comparison of pharmacokinetic parameters of edelfosine after oral administration of edelfosine loaded LN (50 mg/kg bw, *n*=8 per group, mean ± SD). Asterisks indicate significantly different values between the two types of LN, *p*<0.05 (*) and *p*<0.01 (**) by Mann-Whitney test.

Parameters	Compritol® 888 ATO LN oral administration	Precirol® ATO 5 LN oral administration
$t_{1/2}$ (h)	37.652 ± 12.187	45.221 ± 14.564
$t_{1/2Ka}$ (h)	4.938 ± 1.818	4.045 ± 1.873
C_{max} / D (µg/mL/µg)	0.007 ± 0.001	0.012 ± 0.006**
T_{max} (h)	15.890 ± 2.839	14.663 ± 4.150
CL (L/h/kg)	0.045 ± 0.009	0.034 ± 0.009*
V_{ss} (L/kg)	2.343 ± 0.483	2.425 ± 1.274
MRT (h)	39.155 ± 7.656	52.919 ± 4.068*
AUC_{inf} / D (µg/mL/µg)	0.635 ± 0.103	0.813 ± 0.155*

The maximum concentration (C_{max}/dose) after the administration was $0.007 \pm 0.001 \mu\text{g}/\text{mL}/\mu\text{g}$ and $0.012 \pm 0.006 \mu\text{g}/\text{mL}/\mu\text{g}$ for Compritol[®] and Precirol[®] LN, respectively, after 15 h, which is the time at which peak concentration is reached (T_{max}). In previous studies, we hypothesized that the interaction of edelfosine with P-glycoprotein (P-gp) could be the explanation for the lack of gastrointestinal absorption of the drug [2]. However, the Tween[®] 80 present in the composition of the LN shows an inhibition effect of P-gp [33, 34]. Therefore, both types of edelfosine-loaded LN crossed the gastrointestinal barrier and prolonged the release of the drug for over one week. Different authors had also observed this effect, after they administered different drugs via the oral route [26, 35-38]. The $\text{AUC}_{(0-24)}$ values after the oral administration of edelfosine in LN were much higher (15-fold) than those after oral administration of edelfosine in solution. The oral bioavailability of edelfosine 24 h after the oral administration of 30 mg/kg of edelfosine in solution was proved to be less than 10 % [2]. Owing to the expected slow release of edelfosine from the LN, it was decided to test a higher dose (50 mg/kg, a dose at which edelfosine still presents linear pharmacokinetics, data not shown) than that used for previous studies, to determine the pharmacokinetic and biodistribution profile of edelfosine-loaded in LN. As a result, the encapsulation of edelfosine in LN provided an increase in relative oral bioavailability of 1500 %. It is interesting to note that even if multiple administration of free edelfosine was able to increase the bioavailability of the drug up to 64 %, it presents the drawback of causing gastrointestinal irritation [4, 5]. On the other hand, the oral administration of LN greatly

increased the bioavailability of the drug with just a single dose avoiding the GI toxicity.

The mean systemic *CL* for orally administered edelfosine-loaded LN presented statistically significant differences. The value was 0.045 L/h/kg for Compritol® LN and 0.034 L/h/kg for Precirol® LN. These values represent half the value of that of intravenously administered LN, presenting statistical differences ($p < 0.05$). This decrease in the *CL* values is responsible for the longer permanence of the particles in plasma. V_{ss} presented a value of 2.4 L/kg, doubling the value of that of intravenously administered particles. This appears to suggest that there is higher tissue distribution of the drug when LN are administered orally, compared to an i.v. administration of same particles.

Mean residence time (MRT) of orally administered edelfosine-loaded LN was 39 and 53 h for Compritol® and Precirol® LN, respectively, which is approximately three times the value of edelfosine solution administered orally (14 h) and twice that of intravenously administered drug-loaded LN (17 and 26 h, for Compritol® and Precirol®, respectively). These observations suggest a partial indirect absorption of edelfosine-loaded LN after oral administration. It has been described in the literature that the possible mechanisms of the gastrointestinal uptake of colloidal carriers include 3 pathways: an intracellular uptake, a paracellular uptake and an uptake via the M-cells and the Peyer's patches [39]. In fact, different theories have been proposed to study the uptake of lipophilic drugs using nanoparticles. The main uptake has been shown to happen either via isolated lymphoid follicles or by Peyer's patches after oral administration, as reported by Florence et al. [40]. Still, in the last decade some papers have reported that both systemic exposure of a lipophilic drug and

lymphatic transport are enhanced after coadministration with lipidic vehicles, and variations in the composition of the lipid formulation may lead not only to the promotion of significant changes in drug transport via the lymphatic system but may also bring about changes in systemic plasma levels [41].

The avoidance of the presystemic metabolism in the liver (first-pass effect) is one of the most important advantages that lymphatic absorption of a drug may provide via the portal route after gastrointestinal administration. Besides, lymphatic targeting may also be of interest to enhance oral absorption of macromolecules, in order to achieve different goals, as it may bring about an improvement in lymphatic anti-cancer therapy. In order to confirm this lymphatic absorption, another *in vivo* experiment was performed in BALB/c mice, in which mesenteric lymph nodes were extracted and analyzed 24 h after the oral administration of LN and free edelfosine. Free drug treated animals showed drug amounts below the limit of quantitation of the chromatographic technique, while Compritol LN treated mice presented a mean concentration of 18.6 µg/g lymph node, while Precirol LN treated ones had 16.4 µg/g lymph node, confirming a high lymphatic absorption of the LN. Our results are in accordance with a study in which another cytotoxic drug (methotrexate) was encapsulated into Compritol[®] LN and orally administered [27]. In this study, a periodic lymphatic concentration of methotrexate following oral administration of LN-based formulations was determined by mesenteric duct cannulation and collection of samples. This study revealed that the formulation based on Compritol[®] 888 ATO could noticeably improve the oral bioavailability of the drug, presumably following LN constituting lipid digestion and co-absorption through lymphatic transport and route.

The tissue distribution expressed as edelfosine concentration after the administration of a drug dose of 50 mg/kg to mice is shown in Figure 4.

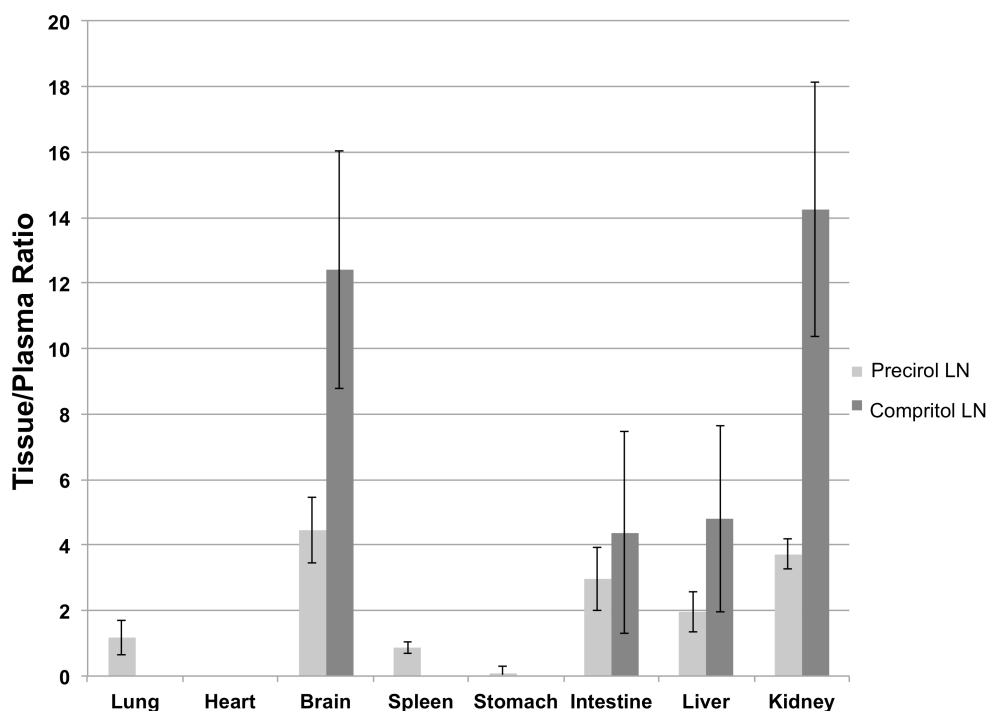


Figure 4. Tissue/plasma concentration ratios of edelfosine after a single oral dose of edelfosine-loaded Compritol[®] and Precirol[®] LN to BALB/c mice ($n=8$ per group, mean \pm SD).

Compritol[®] LN treated animals were sacrificed 168 h post-administration, while mice treated with Precirol[®] LN were sacrificed 216 h post-administration, because it was at these time points when the edelfosine concentration in plasma was below 0.5 $\mu\text{g/mL}$. It was observed that edelfosine was mainly scattered through the major drug clearance tissues liver and kidney, along with the intestine, the typical organ for phospholipid elimination. This accumulation is probably due to the previously mentioned P-gp inhibiting properties of Tween[®] 80, as these organs present high expression of P-gp [42]

3.6. *In vivo* efficacy of edelfosine-loaded LN

Previous studies have provided evidence for the uptake and transport of LN in the lymph and, to a lesser extent, in the blood [43]. Therefore, due to the ability of the developed LN to be absorbed by the lymphatic system, we hypothesized that LN could be able to treat lymphatic diseases, and in particular, lymphomas.

It is known that the therapy for lymphatic neoplasms is comprised of different drugs that always involve several hematological side effects [20]. Edelfosine lacks those negative secondary effects because it causes no mielotoxicity [1]. Besides, this drug has already been effective in *in vitro* experiments against JVM-2 mantle-cell lymphoma cell line [44]. However, we have to bear in mind that edelfosine presents low bioavailability [2] and may cause hemolysis at high concentrations [31]. In this study, LN with a suitable size for oral administration have been developed; these LN increase the bioavailability and avoid hemolysis, so an *in vivo* experiment was designed with MCL-bearing mice to evaluate the efficacy of the drug-loaded LN (Figure 5). It is remarkable that a daily administration of 30 mg/kg edelfosine was mandatory to diminish the tumor burden of MCL bearing mice as much as Compritol[®] and Precirol[®] LN did after the treatment every four days (Figure 5A). Besides, the treatment of mice with Compritol[®] LN every four days reduced the tumor weight more efficiently than the daily administration of edelfosine solution (Figure 5B).

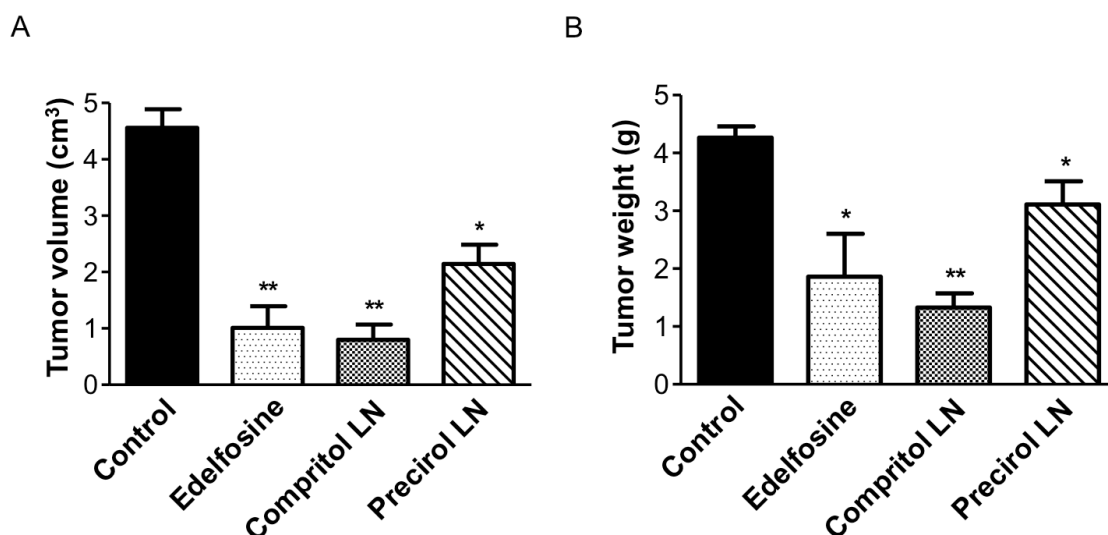


Figure 5. Differences in A) volume and B) weight of implanted mantle-cell lymphoma xenograft tumors 20 days after a treatment of daily PBS (control), daily edelfosine solution (30 mg/kg bw) and Compritol® and Precirol® LN every four days (30 mg/kg bw) ($n=8$ per group, mean \pm SD). * $p<0.05$; ** $p<0.01$ by Student's t test

From these results it can be concluded that the oral administration of edelfosine-loaded LN every four days was as effective against a xenograft model of lymphoma as the daily oral administration of the free drug. Regarding tumor weight, both types of nanoparticles reduced the weight of the tumor significantly, compared to the control. These data show that edelfosine-loaded LN present a potential anti-lymphoma effect and could be tested as treatment against lymphomas. Besides, these drug-loaded nanoparticulate systems do not present the side effects that other antineoplastic drugs do [16, 45].

Due to the confirmation that edelfosine-loaded LN circulate through lymph nodes, by the present study as well as by other researchers [32], we also wanted to assess the ability of these delivery systems to inhibit extranodal dissemination of MCL cells. For that reason, axillary, inguinal and mesenteric lymph nodes were also extracted from MCL-bearing mice treated with either

free or vectorized edelfosine and macroscopically analyzed. As can be seen in Figure 6, all the lymphatic metastases were not completely removed after the treatment with a daily oral administration of edelfosine solution to mice, and a certain degree of extranodal dissemination could still be observed.

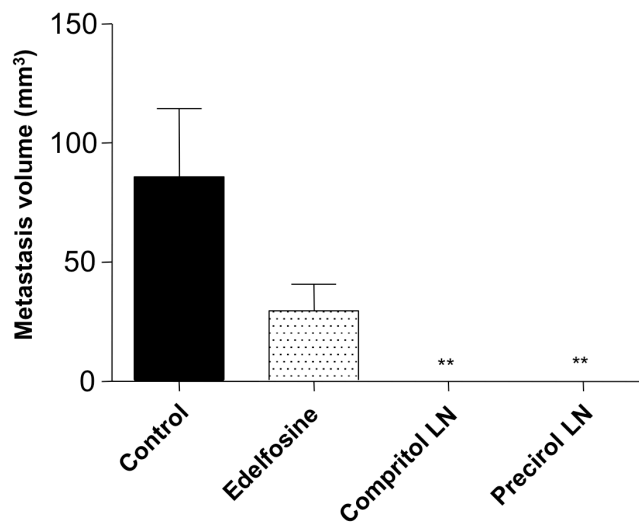


Figure 6. Representation of the antimetastatic efficacy of LN, expressed as the measurement of the volume of metastatic nodes, 20 days after a treatment of daily PBS (control), daily edelfosine solution (30 mg/kg bw) and Compritol[®] and Precirol[®] LN every four days (30 mg/kg bw) ($n=8$ per group, mean \pm SD). ** $p<0.01$ by Student's t test

However, the administration of edelfosine-loaded LN every four days completely eradicated the metastasization process, leaving no sign of extranodal dissemination. Lu *et al.* obtained similar results using Compritol[®] LN against breast cancer and its metastases after a local injection; however, they did not achieve complete eradication of the extranodal dissemination [46]. This complete inhibition of the metastases can be mainly attributed to the lymphatic absorption and accumulation of LN in the thoracic duct, which acts as a

reservoir, and from which LN are continuously released throughout lymph nodes. This outcome is of great interest in the treatment of different types of lymphomas, especially via the oral route, as no orally administered treatments have been described yet [47, 48]; therefore, our results concerning edelfosine-loaded LN as being effective against both MCL and its lymph node metastases shows a strong potential to benefit the improvement of clinical efficiency.

4. Conclusions

The data presented above give evidence that it was possible to produce LN endowed with high encapsulation efficiency and with a well-determined size distribution. Moreover, the incorporation of edelfosine in LN greatly improves the oral bioavailability of the drug. These LN that are uptaken by the lymphatic system offer a selective accumulation in lymph, showing effectiveness against mantle-cell lymphoma, and an important antimetastatic effect. All the results in this work show great promise for the oral treatment of lymphomas and the eradication of extranodal dissemination, as no such treatment is yet available in clinical practice.

Future perspective

The development of LN that target the extranodal dissemination of hematological disorders opens a promising path in the treatment of these diseases. New therapies using drug loaded LN will be designed to focus and increase the therapeutic efficacy of drugs against diseases, while decreasing their secondary effects. Moreover, the oral administration of these delivery systems will increase the patients' compliance.

Acknowledgements

Caja Navarra Foundation, Ibercaja, University of Navarra (FUN), the Government of Navarra, Department of Health (ref: 63/09, "Ortiz de Landázuri" fellowship), the Spanish Ministry of Science and Innovation (SAF2007-61261, SAF2008-02251, SAF2010-15547, PCT-090100-2007-27 and RD06/0020/1037 from "Red Temática de Investigación Cooperativa en Cáncer", Instituto de Salud Carlos III, cofunded by the "Fondo Europeo de Desarrollo Regional" of the European Union), Junta de Castilla y León (GR15-Experimental Therapeutics and Translational Oncology Program, Biomedicine Project 2009). Ander Estella-Hermoso de Mendoza is supported by the research grant from the Department of Education of the Basque Government (BFI06.37).

Executive Summary

- Edelfosine loaded lipid nanoparticles increased oral bioavailability in 1500%.
- Lipid nanoparticles were absorbed through the lymphatic system.
- Drug loaded lipid nanoparticles inhibited 100% of extranodal dissemination in mantle cell lymphoma bearing mice.

References

1. Gajate C, Mollinedo F: Biological activities, mechanisms of action and biomedical prospect of the antitumor ether phospholipid ET-18-OCH₃ (edelfosine), a proapoptotic agent in tumor cells. *Curr. Drug. Metab.* 3(5), 491-525 (2002).
2. Estella-Hermoso De Mendoza A, Campanero Ma, De La Iglesia-Vicente J, Gajate C, Mollinedo F, Blanco-Prieto Mj: Antitumor alkyl ether lipid edelfosine: tissue distribution and pharmacokinetic behavior in healthy and tumor-bearing immunosuppressed mice. *Clin. Cancer Res.* 15(3), 858-864 (2009). Reference of **considerable interest** as it shows pharmacokinetic parameters of free edelfosine.
3. Ahmad I, Filep Jj, Franklin Jc *et al.*: Enhanced therapeutic effects of liposome-associated 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine. *Cancer Res.* 57(10), 1915-1921 (1997).
4. Houlihan Wj, Lohmeyer M, Workman P, Cheon Sh: Phospholipid antitumor agents. *Med. Res. Rev.* 15(3), 157-223 (1995).
5. Munder Pg, Westphal O: Antitumoral and other biomedical activities of synthetic ether lysophospholipids. *Chem. Immunol.* 49, 206-235 (1990).
6. Bhamra R, Bolcsak Le, Ahmad I *et al.*: Activity, pharmacokinetics and tissue distribution of TLC ELL-12 (liposomal antitumor ether lipid) in rats with transplantable, s.c. methylnitrosourea-induced tumors. *Anticancer Drugs.* 14(6), 481-486 (2003).
7. Estella-Hermoso De Mendoza A, Rayo M, Mollinedo F, Blanco-Prieto Mj: Lipid nanoparticles for alkyl lysophospholipid edelfosine encapsulation:

- Development and in vitro characterization. *Eur. J. Pharm. Biopharm.* 68(2), 207-213 (2008).
8. Estella-Hermoso De Mendoza A, Campanero Ma, Mollinedo F, Blanco-Prieto Mj: Lipid nanomedicines for anticancer drug therapy. *J. Biomed. Nanotechnol.* 5, 1-21 (2009). Reference of **interest** to review the state-of-the-art of lipid nanoparticles for anticancer therapy.
 9. Mehnert W, Mader K: Solid lipid nanoparticles: production, characterization and applications. *Adv. Drug Deliv. Rev.* 47(2-3), 165-196 (2001).
 10. Hu Fq, Jiang Sp, Du Yz, Yuan H, Ye Yq, Zeng S: Preparation and characterization of stearic acid nanostructured lipid carriers by solvent diffusion method in an aqueous system. *Colloids Surf. B Biointerfaces.* 45(3-4), 167-173 (2005).
 11. Siekmann B, Westesen K: Sub-micron sized parenteral carrier systems based on solid lipids. *Pharm. Pharmacol. Lett.* 1, 123 (1992).
 12. Cavalli R, Caputo O, Carlotti Me, Trotta M, Scarnecchia C, Gasco Mr: Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. *Int. J. Pharm.* 148, 47-54 (1997).
 13. Chen Db, Yang Tz, Lu WI, Zhang Q: In vitro and in vivo study of two types of long-circulating solid lipid nanoparticles containing paclitaxel. *Chem. Pharm. Bull. (Tokyo)* 49(11), 1444-1447 (2001).
 14. Bertoni F, Rinaldi A, Zucca E, Cavalli F: Update on the molecular biology of mantle cell lymphoma. *Hematol Oncol* 24(1), 22-27 (2006).

15. Andersen Ns, Pedersen Lb, Laurell A *et al.*: Pre-emptive treatment with rituximab of molecular relapse after autologous stem cell transplantation in mantle cell lymphoma. *J Clin Oncol* 27(26), 4365-4370 (2009).
16. Diefenbach Cs, O'Connor Oa: Mantle cell lymphoma in relapse: the role of emerging new drugs. *Curr Opin Oncol* 22(5), 419-423 (2010).
17. Dreger P, Rieger M, Seyfarth B *et al.*: Rituximab-augmented myeloablation for first-line autologous stem cell transplantation for mantle cell lymphoma: effects on molecular response and clinical outcome. *Haematologica* 92(1), 42-49 (2007).
18. Weigert O, Unterhalt M, Hiddemann W, Dreyling M: Current management of mantle cell lymphoma. *Drugs* 67(12), 1689-1702 (2007).
19. Williams Me, Densmore Jj: Biology and therapy of mantle cell lymphoma. *Curr Opin Oncol* 17(5), 425-431 (2005).
20. Park Bb, Kim Ws, Eom Hs *et al.*: Salvage therapy with gemcitabine, ifosfamide, dexamethasone, and oxaliplatin (GIDOX) for B-cell non-Hodgkin's lymphoma: a consortium for improving survival of lymphoma (CISL) trial. *Invest New Drugs* 29(1), 154-160 (2011).
21. Clogston Jd, Patri Ak: Zeta potential measurement. *Methods Mol Biol* 697, 63-70 (2011).
22. Kaszuba M, Corbett J, Watson Fm, Jones A: High-concentration zeta potential measurements using light-scattering techniques. *Philos Transact A Math Phys Eng Sci* 368(1927), 4439-4451 (2010).
23. Estella-Hermoso De Mendoza A, Campanero Ma, Mollinedo F, Blanco-Prieto Mj: Comparative study of A HPLC–MS assay versus an UHPLC–MS/MS for anti-tumoral alkyl lysophospholipid edelfosine determination

- in both biological samples and in lipid nanoparticulate systems. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 877, 4035-4041 (2009).
24. Charman Wn, Stella Vj: Estimating the maximum potential for intestinal lymphatic transport of lipophilic drug molecules. *Int J Pharm* 34, 175-178 (1986).
 25. Shah M, Chuttani K, Mishra Ak, Pathak K: Oral solid compritol 888 ATO nanosuspension of simvastatin: optimization and biodistribution studies. *Drug Dev Ind Pharm* 37(5), 526-537 (2011).
 26. Varshosaz J, Minayian M, Moazen E: Enhancement of oral bioavailability of pentoxifylline by solid lipid nanoparticles. *J Liposome Res* 20(2), 115-123 (2010).
 27. Paliwal R, Rai S, Vaidya B *et al.*: Effect of lipid core material on characteristics of solid lipid nanoparticles designed for oral lymphatic delivery. *Nanomedicine: Nanotechnology, Biology and Medicine* 5(2), 184-191 (2009). Reference of **considerable interest** to understand the lymphatic delivery of lipid nanoparticles.
 28. Levy My, Schutze W, Fuhrer C, Benita S: Characterization of diazepam submicron emulsion interface: role of oleic acid. *J. Microencapsul.* 11(1), 79-92 (1994).
 29. Teeranachaideekul V, Muller Rh, Junyaprasert Vb: Encapsulation of ascorbyl palmitate in nanostructured lipid carriers (NLC)--effects of formulation parameters on physicochemical stability. *Int J Pharm* 340(1-2), 198-206 (2007).

30. Hou D, Xie C, Huang K, Zhu C: The production and characteristics of solid lipid nanoparticles (SLNs). *Biomaterials* 24(10), 1781-1785 (2003).
31. Kotting J, Marschner Nw, Neumuller W, Unger C, Eibl H: Hexadecylphosphocholine and octadecyl-methyl-glycero-3-phosphocholine: a comparison of hemolytic activity, serum binding and tissue distribution. *Prog. Exp. Tumor Res.* 34, 131-142 (1992).
32. Aji Alex Mr, Chacko Aj, Jose S, Souto Eb: Lopinavir loaded solid lipid nanoparticles (SLN) for intestinal lymphatic targeting. *Eur J Pharm Sci* 42(1-2), 11-18 (2011).
33. Constantinides Pp, Wasan Km: Lipid formulation strategies for enhancing intestinal transport and absorption of P-glycoprotein (P-gp) substrate drugs: in vitro/in vivo case studies. *J Pharm Sci* 96(2), 235-248 (2007).
34. Rege Bd, Kao Jp, Polli Je: Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers. *Eur J Pharm Sci* 16(4-5), 237-246 (2002).
35. Hu L, Jia H, Luo Z, Liu C, Xing Q: Improvement of digoxin oral absorption in rabbits by incorporation into solid lipid nanoparticles. *Pharmazie* 65(2), 110-113 (2010).
36. Hu L, Xing Q, Meng J, Shang C: Preparation and enhanced oral bioavailability of cryptotanshinone-loaded solid lipid nanoparticles. *AAPS PharmSciTech* 11(2), 582-587 (2010).
37. Tsai Mj, Huang Yb, Wu Pc *et al.*: Oral apomorphine delivery from solid lipid nanoparticles with different monostearate emulsifiers: Pharmacokinetic and behavioral evaluations. *J Pharm Sci* 100(2), 547-557 (2011).

38. Rawat Mk, Jain A, Singh S: In vivo and cytotoxicity evaluation of repaglinide-loaded binary solid lipid nanoparticles after oral administration to rats. *J Pharm Sci*, (2011).
39. Kreuter J: Peroral administration of nanoparticles. *Adv. Drug Deliv. Rev.* 7(1), 71-86 (1991).
40. Florence At, Hillery Am, Hussain N, Jani Pu: Factors affecting the oral uptake and translocation of polystyrene nanoparticles: histological and analytical evidence. *J Drug Target* 3(1), 65-70 (1995).
41. Caliph Sm, Charman Wn, Porter Cj: Effect of short-, medium-, and long-chain fatty acid-based vehicles on the absolute oral bioavailability and intestinal lymphatic transport of halofantrine and assessment of mass balance in lymph-cannulated and non-cannulated rats. *J Pharm Sci* 89(8), 1073-1084 (2000).
42. Varma Mv, Ashokraj Y, Dey Cs, Panchagnula R: P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. *Pharmacol Res* 48(4), 347-359 (2003).
43. Bargoni A, Cavalli R, Caputo O, Fundaro A, Gasco Mr, Zara Gp: Solid lipid nanoparticles in lymph and plasma after duodenal administration to rats. *Pharm. Res.* 15(5), 745-750 (1998).
44. Mollinedo F, De La Iglesia-Vicente J, Gajate C *et al.*: In vitro and In vivo selective antitumor activity of Edelfosine against mantle cell lymphoma and chronic lymphocytic leukemia involving lipid rafts. *Clin Cancer Res* 16(7), 2046-2054 (2010).
45. Brody J, Advani R: Treatment of mantle cell lymphoma: current approach and future directions. *Crit Rev Oncol Hematol* 58(3), 257-265 (2006).

46. Lu B, Xiong Sb, Yang H, Yin Xd, Chao Rb: Solid lipid nanoparticles of mitoxantrone for local injection against breast cancer and its lymph node metastases. *Eur. J. Pharm. Sci.* 28(1-2), 86-95 (2006).
47. Weigert O, Unterhalt M, Hiddemann W, Dreyling M: Mantle cell lymphoma: state-of-the-art management and future perspective. *Leuk Lymphoma* 50(12), 1937-1950 (2009).
48. Ogura M: Current treatment strategy and new agents in mantle cell lymphoma. *Int J Hematol* 92(1), 25-32 (2010).