Departamento de Farmacia y Tecnología Farmacéutica
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"LIPID NANOPARTICLES FOR PAEDIATRIC OSTEOSARCOMA TREATMENT"

Trabajo presentado por Yolanda González Fernández para obtener el Grado de Doctor

Fdo. Yolanda González Fernández

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A mis padres y hermanos

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ABBREVIATIONS

ANOVA	analysis of variance
ATCC®	American type culture collection
ATL	antitumour lipids
B cells	primary bone tumour cells
CI	combination index
CIS	cisplatin
CTC	circulating tumour cell
CTxB subunit	cholera toxin B subunit
DHFR	dihydrofolate reductase
DL	drug loading
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DOX	doxorubicin
DRI	dose-reduction index
DTC	disseminated tumour cells
EPR	enhanced permeability and retetntion
ET	edelfosine
ETO	etoposide
Fa	fraction of affected cells
GRAS	denerally recognized as safe
HD-MTX	high-dose methotrexate
IC_{50}	inhibitory concentration 50
IFO	ifosfamide
LEC	lecithin
LN	lipid nanoparticles
MDR	multidrug resistance
MICRO-CT	microcomputed tomography
MβCD	methyl β cyclodextrin
M cells	metastatic bone tumour cells
MWCO	molecular weight cut-off
NLC	nanostructured lipid carriers
N cells	hepatitis C virus

OS	osteosarcoma
PBS	phosphate-buffered saline
P-gp	P-glycoprotein
PSC	photon correlation spectroscopy
PVA	polyvinyl alcohol
RFC	reduced folate carrier
RNTI	Registro Nanocional de Tumores Infantiles
ROS	reactive oxygen species
RT-PCR	real time polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SLN	solid lipid nanoparticles
T80	tween 80
TC	taurocholic acid sodium salt hydrate
UHPLC MS/MS	ultrahigh performance liquid chromatography tandem mass spectrpmetry
v/v	volume/volume
w/v	weight/volume
$W_1/O/W_2$	water-in-oil-in-water (double emulsion)
W/O	oil-in-water (single emulsion)

INTRODUCTION

INTRODUCTION

The World Health Organization defines cancer as a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can invade adjoining parts of the body and spread to other organs (1). In 2017 the "American Cancer Society Statistics Centre" estimated that there will be 1,688,780 new cancer cases of cancer and 600,920 cancer deaths in the U.S. alone, that is to say, 3 new cases diagnosed and 1 death every minute (2).

Despite childhood and adolescent cancer being a rare disease, it is the second leading cause of death among children aged 1-14 and the fourth one for teens in developed countries (2). The "International Agency for Research on Cancer" recently disclosed, that the global occurrence of childhood cancer is higher than previously assessed with approximately 300,000 cancer diagnosed in adolescents and children under 19 worldwide (3). Particularly in Spain, the latest report of the "Registro Nacional de Tumores Infantiles" (RNTI) revealed that 1,100 children are diagnosed with cancer every year. Leukaemias, lymphomas, nervous system tumours and bone tumours are the most common type of cancer diagnosed in children aged 0-15, and bone tumours among adolescents aged 15-19 (4).

The great advances achieved in early diagnosis, treatment and surveillance during the last decades have led to encouraging statistics for certain types of cancers. For instance, the five-year survival rate reported by the RNTI for children with Hodgkin lymphoma is close to 95 % and slightly less striking, but still encouraging, are the data for children with leukaemia, non-hodgkin lymphoma, astrocytoma or rhabdomyosarcoma. Unfortunately, bone sarcomas, particularly osteosarcoma (OS), do not present this favourable prognosis. Patients with no detectable metastases at diagnosis present a five-year survival rate close to 70%; however, at the present time, there is no reliable therapeutic option to control the disease of patients with metastases (5).

OS is the most common malignant bone tumour diagnosed in the paediatric population. It presents a bimodal age distribution with a first peak of incidence in children and adolescent (more predominantly teens aged 11-14) and a second peak in adults more than 65 years old. Although its cell of origin is unclear, it is known that OS derives from the mesenchymal lineage. These cells are transformed and acquire a tumoral phenotype (5). Histologically, OS is characterized by the presence of malignant spindle cells producing extracellular bone matrix, named osteoid. Depending on which matrix-producing cells dominate, OSs are

classified into the conventional (osteoblastic, chondroblastic, or fibroblastic subtypes), small cell, telangiectatic, low grade central or surface OSs, conventional osteoblastic OS being the most diagnosed. Nonetheless, as to prognosis, no correlation has been found with the histological subtype (6).

OS usually appears in the metaphyseal growth plate of long bones such as femur, tibia, or less frequently humerus or pelvis, but it rapidly spreads to lungs. It is assumed that 80% of patients with OS present non-detectable micrometastases at diagnosis which, if not treated systemically, will evolve into established metastases in the lungs (7). For that reason, the treatment of choice for all types of OSs is based on a sequence of neo-adjuvant systemic chemotherapy followed by the resection of the primary tumour and resectable metastases and a new phase of post-operative chemotherapy. Factors like tumour location, age of appearance, date of diagnosis and first response to chemotherapy clearly affect the outcome of the disease (8).

In the latest 1970s high dose methotrexate (HD-MTX), doxorubicin (DOX) and cisplatin were included as the backbone drugs for the treatment of OS. With the introduction of this protocol a 70% long term relapse free survival was achieved for patients with localized disease. Nonetheless, this rate drastically drops to 20% for patients with metastases at diagnosis. In these cases ifosfamide, etoposide and new drugs in clinical trials are included in the post-operative phase as a second line chemotherapy(9).

ET is an alkyl-lysophospholipid which has been used as a bone-marrow purging agent prior to transplant in leukaemic patients (10). It is synthetic analogue of perifosine, which has shown efficacy against OS and paediatric solid tumours in several studies, some of them in clinical trials (16,17). At a clinical practice, the use of ET use is hampered by its low bioavailability and severe gastro toxicity after oral administration; and haemolytic activity when given intravenously (11). Despite these drawbacks, ET has shown *in vitro* and *in vivo* efficacy against a wide variety of cancers, namely leukaemia, lymphoma, breast cancer etc (12–15). Given the well-known anti-tumour efficacy of ET and the physicochemical similarities to perifosine, in the present, work we proposed ET as a novel anti-osteosarcoma agent. However, herein, to circumvent its toxicity, ET was encapsulated into LN for the oral delivery of the drug (18).

LN are colloidal carriers made of biocompatible and biodegradable lipids, solid at body temperature, that were launched on the market in the mid-1980s. Due to their lipid nature LN may overcome the solubility problems of certain drugs, increase their bioavailability and protect them from degradation. Some of the advantages of the use of LN as drug delivery systems include the excellent tolerability of the lipids used for their production, the avoidance of organic solvent in their manufacturing process, their easy production at industrial scale and the possibility of lyophilisation for long-term storage (19). Furthermore, due to the defective and leaky vasculature surrounding solid tumours, together with their poor lymphatic drainage, LN are accumulated and retained in the proximities of the tumour (EPR effect, figure 1). This passive targeting contributes to achieving higher concentrations of drug in the tumour area and to minimizing their undesirable effects (20).

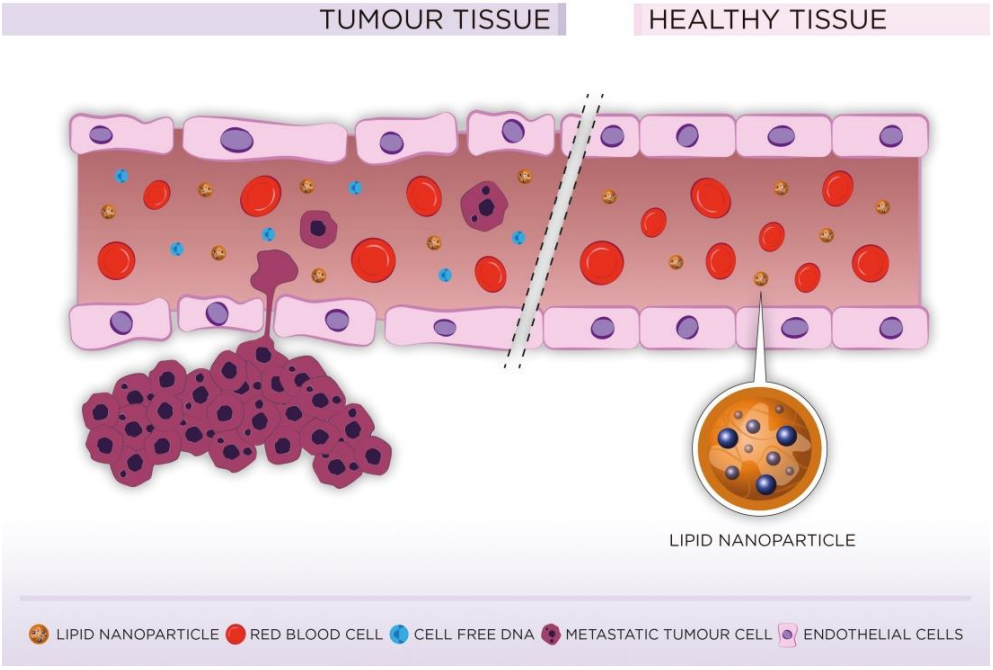

Figure 1: Enhanced Permeability and Retention Effect (EPR Effect) in tumour tissues. Due to the defective vasculature surrounding solid tumours, as well as their impaired lymphatic clearance from the interstitial space, LN can extravasate and remain retained while releasing their cargo to the tumour.

This described potential role of LN as drug delivery systems has been extensively explored for the encapsulation of first and second line anti-osteosarcoma agents as compiled in the review work included in Annex 1. However, only a limited number of studies have been carried out on OS cells.

Given the great interest that LN arouse as vehicles for drug delivery, together with the necessity of new therapeutic approaches for OS patients, the present project address the encapsulation of anti-osteosarcoma agents and a new potential drug for OS treatment.

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HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

Osteosarcoma (OS) is the primary bone tumour most frequent in the paediatric population. It usually appears in the limbs, although the presence of non-visible micrometastases in the lungs is assumed at the time of diagnose. The five-year survival-rate for patients with non-metastatic disease is 70%, however for those patients with metastases at diagnosis it drastically drops to 20-30%. The lack of effective treatments for OS patients makes mandatory the development of new therapeutic approaches mainly focused on the metastatic disease.

Against this background, nanomedicine has emerged as a promising alternative to conventional therapies for cancer treatment. Particularly lipid nanoparticles (LN) present several advantages over conventional therapies: for instance, they increase the bioavailability of the encapsulated drugs, they protect drugs from degradation, they avoid their indiscriminate distribution, and they can be orally administered.

On the basis of the foregoing, the initial hypothesis of the present work is:

The use of LN for the encapsulation of drugs currently used for OS treatment (methotrexate and doxorubicin) may circumvent their associated toxicity and increase their *in vivo* bioavailability and efficacy. Besides, the inclusion of new drugs for OS therapy, such as edelfosine, would offer alternative treatments for OS patients.

Based on this premise the main objective of the present work was to design, develop and optimize LN for the oral delivery of edelfosine, methotrexate and doxorubicin, and to evaluate their *in vitro* and *in vivo* efficacy against OS murine models.

To that end, the following partial objectives were addressed:

1. To design, develop and characterize LN for the encapsulation of methotrexate and doxorubicin (edelfosine LN were developed in a previous project).
2. To evaluate the efficacy of the developed LN against commercial and patient derived OS cells, and to elucidate if the combination of doxorubicin and edelfosine has synergistic effects on OS cells.
3. To evaluate the *in vivo* efficacy of the different treatments against two different OS orthotopic murine models.

CHAPTER 1

Lipid nanoparticles enhance the efficacy of chemotherapy in primary and metastatic human osteosarcoma cells

CHAPTER 1


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Lipid Nanoparticles Enhance the Efficacy of chemotherapy in Primary and Metastatic Human Osteosarcoma cells

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ABSTRACT

Osteosarcoma (OS) is the most frequent primary malignant bone tumor in the paediatric population. Current treatments are not able to slow the progression of the disease in patients with metastasis, and their survival rate is still 10-30 %. Therefore, new therapeutic approaches are required. In the last decades, lipid nanoparticles (LN) have been widely researched for cancer treatment. These systems are able to carry the cytostatic drug to the affected organs and control the release of their cargo in a prolonged manner.

The purpose of the present study was to evaluate edelfosine efficacy in various primary OS cell lines and a commercial cell line, and to compare its efficacy to that of methotrexate, a first-line agent traditionally employed in OS therapy. We also studied whether the encapsulation of both drugs in LN, edelfosine and methotrexate, affected their *in vitro* efficacy.

Methotrexate and edelfosine LN were successfully developed and characterized. Edelfosine showed higher efficacy treating the metastatic cells when compared to the primary cell lines due to its mechanism of entrance via lipid rafts. This efficacy was improved when edelfosine was administered in LN. Moreover, methotrexate showed higher efficacy in the cells that did not overexpress dihydrofolate reductase, the target enzyme of methotrexate.

Keywords: Osteosarcoma, chemotherapy, methotrexate, edelfosine, lipid nanoparticles, lipid rafts

1. INTRODUCTION

Although pediatric cancer has not traditionally been one of the most frequent causes of pediatric demises, the decreasing rates of mortality associated with other pathologies have placed cancer as the second leading cause of death following accidents [1]. In 2015, the American Cancer Society estimated that there were 10,380 new cases of cancer in children among 0-15 years in the USA alone. However, the most alarming fact is that cancer incidence rates in children and adolescents have continued to increase by about 0.6 % per year since 1975 [2]. Osteosarcoma (OS) is the most frequent primary malignant bone tumor during these first decades of life [1], and the second most common cause of cancer death among young people following leukemias [2].

OS is an aggressive tumor which initially appears in bones with high growth potential, such as the long bones of the extremities, but rapidly metastasizes to the lungs [3]. Like many other types of pediatric cancer, the main drawback of OS is the lack of specific symptoms, which usually results in late diagnosis. It is assumed that 80 % of the patients present silent micrometastases in the lungs at the time of diagnosis which, if not treated systemically, dramatically decrease the 5 year survival rates to 10-30 % [4]. Regarding the treatment, OS was considered a chemoresistant tumor until the 1960´s; back then, amputation was the only way to treat this cancer and the survival rates were extremely low [5]. Current protocols for OS are based on a combination of chemotherapy and surgical resection. High dose methotrexate (HD-MTX) has traditionally been a key drug in the therapy of this type of cancer. However, due to the high doses required to treat OS and the associated systemic toxicity, MTX has to be administered together with leucovorin to rescue normal cells. Doxorubicin, cisplatin and ifosfamide, are also employed as first line agents [6]. Despite the progress that current protocols have made for the outcome of the disease, patients with metastatic cancer still have a poor prognosis. Besides, childhood cancers are treated with even higher doses of antineoplastic agents than those in adults [7], which is also associated with more short and long-term side effects. The late effects of this aggressive treatment represent serious risks for the health and quality of life of children and young patients who will probably develop second tumors or suffer from specific morbidities associated with the chemotherapy. Great efforts should be made in order to improve current protocols for the treatment of the systemic disease and to make them safer and more effective.

In this context, nanomedicine and especially lipid nanoparticles (LN) have been proposed to mitigate the limitations of the current protocols for the treatment of several malignancies,

including cancer [8]. Nanocarriers can act as therapeutic vehicles for antineoplastic drugs with the advantage that, in the case of LN, they can be orally administered and therefore avoid the intravenous route [9,10]. Once in the systemic circulation ,due to the enhanced permeability and retention (EPR) effect or to an active targeting by surface modifications [11,12], these nanoscaled drugs can specifically be directed towards the desired site of action, achieving a sustained release of the drug in the affected area and increasing their bioavailability inside the tumor [13]. LN use should therefore lead to decreased systemic toxicity and lower doses of the antineoplastic drugs would be required for the treatment of the disease.

In addition to the progress made in nanotechnology, recent advances in the knowledge of the pharmacology of cancer at a molecular level have led to the development of new active agents able to specifically target cancer cells. That is the case with edelfosine (ET), an alkyl-lysophospholipid that has already shown efficacy against several types of cancer [13-16] and that could also be a safer alternative for the treatment of OS, since it has been previously demonstrated that ET selectively targets tumor cells, sparing the healthy tissues [17]. ET efficacy is the result of several mechanisms of action involving intrinsic and extrinsic apoptotic signaling pathways [15]. ET binds membrane lipid rafts causing the reorganization of transmembrane proteins that trigger several death signaling pathways (FAS/CD95 receptors). Besides, ET accumulates in the endoplasmic reticulum and/or mitochondria of the tumor cells leading to late apoptosis [18]. However, regardless of the promising preliminary results with this alkyl-lysophospholipid, when orally administered, ET presents gastrointestinal and hematologic toxicity that hinder its clinical use [19,20]. The encapsulation of ET into LN has overcome these limitations and increased the oral bioavailability and anti-tumor efficacy of the drug in several tumor models [13,16], while decreasing its toxicity [21].

The purpose of the present study was to evaluate ET efficacy in various commercial and patient-derived OS cell lines, and to compare its efficacy to that of MTX, a first-line agent traditionally employed in osteosarcoma therapy. We also studied whether the encapsulation of both drugs in LN, ET and MTX, affected their *in vitro* efficacy.

2. MATERIALS

2.1 Chemicals

ET was purchased from Apointech (Salamanca, Spain), MTX was kindly provided by Dr. A. Aldaz, from the Dpt. Of Pharmacy, University Clinic of Navarra (Pamplona, Spain) and Precirol® ATO 5 was gently provided by Gattefossé (Lyon, France). Tween® 80 (T80) was purchased from Roig Pharma (Barcelona, Spain), L-α-phosphatidylcholine from egg yolk (Lecithin, LEC), taurocholic acid sodium salt hydrate (Taurocholate, TC), Pluronic® F127 (PL), Polyvinyl alcohol (PVA) and D-(+)-trehalose dehydrate were obtained from Sigma-Aldrich. All reagents employed for mass spectroscopy were of gradient grade for liquid chromatography. Chloroform was obtained from Panreac (Madrid, Spain), methanol from Merck (Barcelona, Spain) and formic acid 99% from Fluka (Barcelona, Spain). Amicon® Ultra-15 10,000 MWCO filter devices were provided by Millipore (Cork, Ireland) and all reagents for cell culture were from Gibco®. Cholera toxin B-subunit-FITC (CTxB subunit-FITC) was purchased from Sigma-Aldrich.

3. METHODS

3.1 Preparation and characterization of lipid nanoparticles

3.1.1 Edelfosine and methotrexate lipid nanoparticle preparation

Edelfosine lipid nanoparticles (ET-LN) were prepared according to the hot homogenization method followed by high shear homogenization and ultrasonication, previously patented by our group with minor modifications [13]. Briefly, 30 mg of ET and 300 mg of Precirol® were melted 5° C above the lipid's melting point (60° C). Ten ml of the aqueous phase (T80 at 2% w/v) heated at the same temperature were added to the lipid phase and both phases were processed with the help of a Microson ultrasonic cell disruptor (NY, USA) for 4 min at 13 W. The emulsion formed was cooled in an ice bath to allow LN to solidify. LN suspension was centrifuged at 4,500 g, 30 min with Amicon® Ultra-15 10,000 MWCO filter devices and washed twice with water to remove the excess of non-incorporated drug and surfactant. Finally, the formulation was frozen (−80°C) and subsequently lyophilized using 450 mg of trehalose as cryoprotectant to obtain a nanoparticulated powder.

Regarding the methotrexate lipid nanoparticles (MTX-LN), these were also prepared following the method described above with minor modifications. The lipid phase consisted of 300 mg of Precirol®, but due to the partial hydrophilicity of MTX and its high melting point, it was necessary to add a solvent (150 µl of ammonia) to disperse the drug into the lipid phase. The aqueous phase (10 ml of T80, 2% w/v) heated at 60° C was added to the lipid phase containing the drug, processed with a Microson ultrasonic cell disruptor (NY, USA) for 4 min at 13 W and cooled to obtain the LN. The suspension was washed and freeze-dried as previously described.

3.1.2 Physicochemical characterization of edelfosine and methotrexate lipid nanoparticles

The LN were characterized in terms of size, polydispersity index (PDI), ζ potential and drug loading (DL). LN were diluted 60 fold in distilled water prior to evaluating their particle size and PDI by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, UK). Similarly, the ζ potential was measured using the same equipment combined with laser Doppler velocimetry. ET and MTX DL were studied by quantifying the amount of drug in 5 mg of LN after it had been extracted and dissolved with 900 µl of a chloroform: methanol mixture (1:3). Afterwards, the solution was centrifuged at 15,000 g for 10 min, and the supernatants analyzed by different techniques. In the case of ET, the amount of loaded drug was quantified by a previously validated ultra high-performance liquid chromatography tandem mass spectrometry method (UHPLC MS/MS) [22] and in the case of MTX by UV-visible spectroscopy at 373 nm with an Agilent UV-VIS spectrophotometer 8453. Each measurement was performed in triplicate and data were expressed as a mean value ± standard deviation.

Once characterized, MTX-LN were optimized varying the amount of drug and lipid matrix, the surfactant and the drug solvents used to prepare the formulation. The particle size and entrapment efficiencies were defined as dependent variables.

3.2 Cell culture

Several OS cell lines were used for the *in vitro* assays. A human immortalized and commercialized OS cell line, U-2 OS (ATCC® HTB96™), purchased from the American Type Culture Collection (Sigma-Aldrich), and four cell lines derived from two patients treated at the University Clinic of Navarra, coded as 595 and 588 and described elsewhere [23]. From

each patient two cell lines were obtained: the first one from the patient's primary chemonaïve bone tumor (named as "B" cell lines) and the second one from a lung metastatic implant (referred as "M" cell lines). All cell lines were maintained in alpha-MEM, supplemented with 10% of fetal bovine serum and 1% penicillin/streptomycin in an incubator at 37°C and 5% carbon dioxide.

3.3 Cell proliferation and viability assays

Cell proliferation and viability after the treatment with free and encapsulated drugs were studied by MTS included in the CellTiter 96 ® Aqueous One Solution Cell Proliferation Assay (Promega, Madrid. Spain) according to the manufacturer's recommendation. U-2 OS cells and patient-derived cell lines were seeded in a 96-well plate at a cell density of 500 cells per well in the case of 595/588B cells and 200 cells per well in the case of 595/588M and U-2 OS. Cells were incubated during 96 hours to allow primary cells to adhere and multiply. Afterwards, cell media was removed and replaced with increasing concentrations of free ET, ET-LN, free MTX and MTX-LN. After 72 h of treatment, 15 µl of MTS reagent solution were added to each well. The mixture was incubated for 4 h and the produced formazan was measured at 490 nm in a Sunrise Basic Tecan cell plate reader (Austria) with the Magellan 6 software. Blank-LN (LN without drug) were also tested as controls. All experiments were repeated 3 times.

3.4 Semiquantitative Real-time Polymerase Chain Reaction assays: Reduced folate carrier (RFC1) and dihydrofolate reductase (DHFR) Expression Analysis

With the aim of studying the expression of two of the genes involved in the mechanism of action of MTX by real time-PCR assays, 18 osteosarcoma cell lines were used, including the 4 patient-derived previously described cell lines. Six cell lines were obtained from bone primary tumor biopsies "B", 6 from metastases "M" and 6 from paired bone normal tissue, named as "N" cell lines. All samples were obtained from the University Clinic of Navarra. Results were jointly analyzed, however those related with RFC1 and DHFR expression in 595B/M and 588B/M were highlighted.

Total RNA was isolated from confluent cultures using Trizol RNA (2 µg). After treatment with DNase I, RNA was reverse transcribed using SuperScript II reverse transcriptase and random primers.

Semiquantitative analysis of DHFR/RFC mRNA expression in the tumor samples ("B" vs "M") was carried out by real-time polymerase chain reaction (RT-PCR) with the ABI PRISM 7300 Sequence Detector and the software Sequence Detector version 1.4 (Perkin-Elmer/Applied Biosystems, Foster City, California). RFC and DHFR expression of B and M cells was referred to that of normal bone samples (N samples) extracted from the same patients at tumor resection. The semiquantitative DHFR/RFC mRNA levels were expressed relative to glyceraldehyde-3-phosphatedehydrogenase mRNA (GAPDH), and determined by TaqMan assays (Hs99999905-m1, Life Technologies). Relative levels of expression were determined by the $2^{-\Delta\Delta Ct}$ method and every assay was performed in triplicate.

3.5 Visualization of lipid rafts by Cholera Toxin B Subunit labeling

Lipid rafts were visualized by labeling the cells with CTxB subunit-FITC. Briefly, patient-derived cell lines (595B/M and 588B/M) were seeded in glass inserts settled in a 24-well plate at a cell density of 6,000 cells per well for "M" cells and 10,000 cells per well for "B" cells, and allowed to grow for 4 days. After that period of time, medium was removed and cells were washed with PBS and fixed with 4% (v/v) formaldehyde for 15 min. Subsequently, cells were washed twice with PBS containing 10% of BSA for 5 min and then incubated 1 h with the labeled CTxB subunit-FITC at a concentration of 8 μg/mL. Nuclei were stained using 1:1000 DAPI. In order to avoid the internalization of the toxin, all the process was performed in ice. Cells lacking the marker were employed as negative controls. Samples were visualized by fluorescence microscopy.

3.6 Edelfosine uptake in osteosarcoma cells and involvement of lipid rafts

To study the influence of lipid rafts in the uptake of ET, 63,000 metastatic cells (595/588M) were grown in 6 mm cell culture dishes during 72 h. Afterwards, cells were treated with 5 mM of methyl-β-cyclodextrin (MβCD, cholesterol depleting agent) during 60 min in order to disrupt the lipid rafts microdomains. After the incubation time, cells were washed with PBS and exposed to 10 μM of ET during 5 h. Then, cells were washed with ice-cold PBS and lysed with methanol for their analysis. ET content of the cells was quantified by a validated UHPLC-MS/MS method [22] and the amount of internalized drug was referred to the amount of proteins in the sample quantified by the Lowry method. All experiments were repeated 4 times.

In order to clarify ET and ET-LN uptake by OS cells, primary tumor-derived cell lines were cultured into 6 mm cell culture dishes at a cell density of 100,000 (B) and 63,000 (M) cells per dish. After 96 h, cells were treated with 10 μM of free ET and encapsulated ET for 24 h. The amount of internalized drug was quantified as previously described. All experiments were repeated 4 times

3.7 Statistical analysis

Data analysis and graphical presentations were done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Comparison of means between groups was performed by Mann-Whitney U-test. The statistical significance level was defined as $p < 0.05$.

4. RESULTS AND DISCUSSION

4.1 Lipid nanoparticles physicochemical characterization

The ET-LN prepared by the method previously optimized in our group [13] presented a mean diameter of 136.96 ± 10.13 nm, an optimal size for oral and intravenous administration [24,25], and a PDI value around 0.2, which denotes a homogeneous size distribution of the nanoparticles [26]. Moreover, LN presented high negative ζ potential values (-20.3 ± 5.77 mV) indicating the stability of the suspension [25]. Regarding the DL, LN encapsulated 25.31 ± 3.06 μg of ET per mg of formulation.

Concerning MTX, LN showed sizes of 113.10 ± 5.89 nm, PDI values less than 0.3, high negative ζ potentials (-46 ± 0.66 mV) and a DL of 12.13 ± 0.35 μg of MTX per mg of particles. However, as OS treatment requires HD- MTX [6], the formulation was optimized in order to increase the amount of MTX entrapped by the particles (Fig. 1).

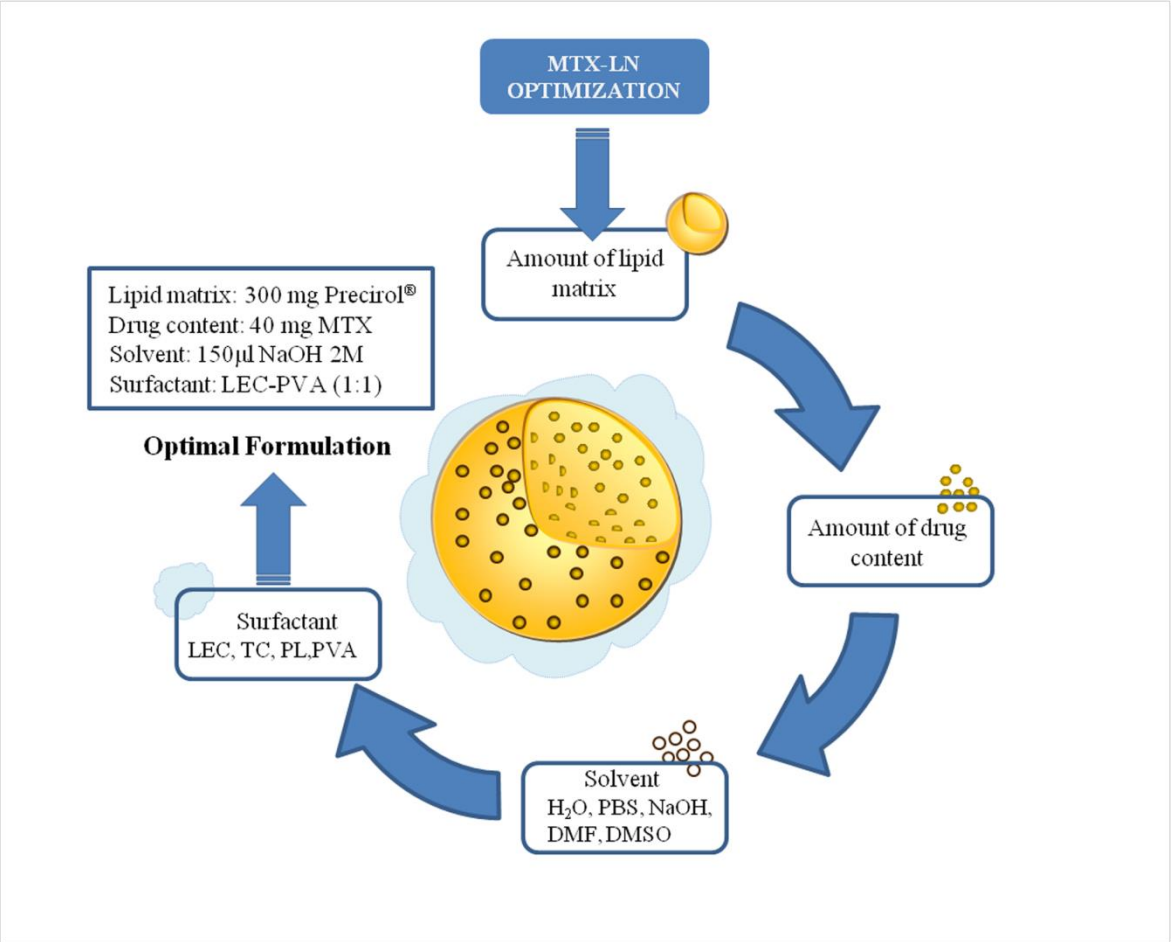

Figure 1. Methotrexate lipid nanoparticles (MTX-LN) optimization process. Abbreviations; PBS: phosphate-buffered saline, DMF: dimethylformamide, DMSO: dimethyl sulfoxide, T80: Tween 80®, LEC: lecithin, TC: taurocholate, PL: pluronic, PVA: polyvinyl alcohol.

As a first approach, we tried to increase the DL by decreasing the amount of lipid matrix or increasing the amount of drug incorporated in the formulation. Both of these changes negatively affected the encapsulation values (data not shown). Then, the influence of the solvent employed to dissolve MTX in the lipid matrix was studied. For this purpose, LN were prepared using water, PBS, sodium hydroxide, dimethylformamide and dimethyl sulfoxide as solubilizing agents. MTX resulted to be more soluble, and therefore better incorporated into the formulation in 150 µl of ammonia and sodium hydroxide; consequently, they were employed as the solubilizing agents during the following steps. The next variable analyzed was the surfactant employed to emulsify the lipid and aqueous phase (Fig. 2). LEC, TC, PL, PVA and T80 (at a final concentration of 2% w/v) or combinations of these, gave rise to particle sizes from 100 to 300 nm. Smaller particle sizes were obtained with PL and TC when compared to LEC and PVA, however, the content of MTX was found lower or even minimum with the first surfactants.

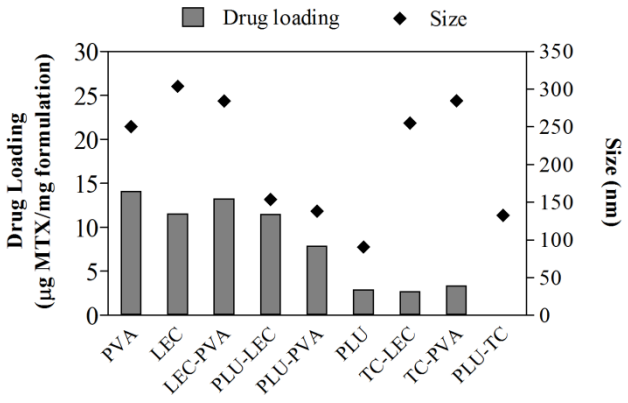

Figure 2. Surfactant influence on particle properties. Abbreviations; LEC: lecithin, TC: taurocholate, PL: pluronic, PVA: polyvinyl alcohol.

Maximum drug incorporation loading was achieved by LEC and PVA, obtaining a DL around 15 μg of MTX per mg of LN in both cases. For this reason LEC and PVA were mixed in proportion 1:1 to form the aqueous final phase. This surfactant combination gave rise to particle sizes and DL similar to the ones obtained by the individual tensoactives. When ammonia was replaced by sodium hydroxide (1 or 2 M) the amount of drug loaded by the LEC-PVA formulation increased from 15 to 20 μg of MTX per mg of LN, and made possible to incorporate 40 mg of MTX into the formulation. Finally, the optimized formulation of MTX-LN was the one composed of 300 mg of Precirol®, 10 ml of LEC-PVA (1:1), 40 mg of MTX previously dissolved in 150 μl of sodium hydroxide (2 M) and 450 mg of trehalose. LN presented a diameter of 273 ± 12.39 nm, a PDI value of 0.2, a ζ potential of -42.1 ± 6.50 mV and DL of 25.49 ± 0.63 μg of MTX per mg of particles. On the other hand, previous studies of our group have demonstrated the suitability of T80 as stabilizer agent for lipid nanoparticles [13]. For that reason PVA was replaced by T80 in the optimized LEC-PVA formulation. The particle size was reduced to 122 nm and DL also decreased from 25 to 20 μg of MTX per mg of particles. LEC-PVA MTX-LN were finally chosen for the *in vitro* studies due to their high DL and adequate size.

4.2 *In vitro* proliferation assays

After LN development, several commercial and OS primary tumor-derived cell lines were exposed to free and encapsulated ET and MTX in order to evaluate their anti-tumor efficacy. Cytotoxicity of the treatments was assessed by comparing their inhibitory concentration 50 (IC_{50}) values, so that cell lines with higher IC_{50} were considered more chemoresistant to the exposed drug solution than those with lower values.

Table 1 summarizes the results obtained for ET and MTX in the different cell lines studied. The first aspect that might be underlined is that while MTX activity was measured in the millimolar range (IC_{50} values from 3.95 to 25.56 mM), ET, having a similar molecular weight to MTX, was 100 times more effective, being active in the micromolar range (IC_{50} values from 15.04 to 65.94 μM). This indicates that, while in clinical practice MTX is administered at high doses (10-12 g/m^2) in OS patients, ET could be administered in very low doses. This aspect, together with the described selectivity of ET towards tumor cells, positions ET as a promising alternative in OS therapy.

Table I. IC_{50} values of edelfosine (ET) and methotrexate (MTX) in osteosarcoma commercial and primary tumor-derived cell lines

Cell line	Free-ET (μM)	ET-LN (μM)*	Free-MTX (mM)
595B	37.42 ± 10.23	23.35 ± 6.35	3.95 ± 0.40
595M	15.04 ± 4.26	3.66 ± 1.40	13.71 ± 4.07
588B	65.94 ± 8.41	26.17 ± 2.37	25.53 ± 3.37
588M	11.09 ± 4.58	12.70 ± 0.88	5.75 ± 2.50
U-2 OS (ATCC® HTB96™)	17.99 ± 6.57	6.15 ± 2.47	14.23± 3.55

*Equivalent blank-LN showed > 90% viability in the concentration range studied

Focusing on ET efficacy in the patient-derived cell lines, it was observed that ET was more effective at treating the metastatic cells (595M/588M) than their primary counterparts (595B/588B), with IC_{50} values even six-fold those of 588B cells *vs* 588M cells. This means that metastatic tumor cells are more chemosensitive to the free drug than the primary tumor cell lines. Many researchers have reported that metastatic cells, due to their need to proliferate and migrate through the bloodstream, present in their membranes more cholesterol than primary cells. The cholesterol is organized into membrane lipid microdomains named lipid rafts [27-29]. Taking into consideration the high affinity of ET for lipid rafts, their higher presence in metastatic cells could give rise to a higher activation of extrinsic apoptotic pathways by ET, as reflected by the lower IC_{50} values. Moreover, it has also been described that ET can enter the cell by raft dependent endocytosis. Therefore, the higher presence of these lipid microdomains in metastatic cells could favor a higher accumulation of the drug, thus contributing to cell death [30]. Interestingly, when ET was loaded into LN all treatments became more, or at least, as effective as the free-ET solution, probably as a result of the higher internalization of the encapsulated drug. This confirmed that the encapsulation strategy could improve the potential of ET as a new anti-osteosarcoma agent. These results

are in agreement with those previously published by other authors who also reported enhanced cytotoxicity of ET against tumor cells when loaded into LN [14,15].

Regarding MTX efficacy in the patient-derived cell lines (Table 1), it was observed that in 595 cell lines, MTX was more effective at treating the primary tumor cells ("B") than the metastatic ones ("M"). These results could be supported by the described resistance to MTX that OS cells acquire as metastases develop [31,32]. However, this tendency of tumor cells to become more resistant to MTX was not observed in the cell lines derived from patient 588. Therefore, innate resistance of the primary tumor might confer chemoresistance on the antineoplastic drug that is overcome in the metastatic tumor, where the drug appears to be more effective. These data perfectly reflect one of the main challenges of cancer. Each OS tumor develops after unique malignant genetic and epigenetic transformations that confer different outcomes. In this context, cancer research aspires to understand these genetic characteristics in the primary and metastatic tumors that could contribute to more personalized therapies according to individual tumor genetic patterns. Finally, MTX-LN were also tested *in vitro*, but the experimental conditions were not optimal to evaluate their efficacy. Owing to the high doses of drug required to treat OS cells and the static condition of the *in vitro* assays, MTX-LN and non-loaded-LN precipitated and asphyxiated the cells. Therefore, it was not possible to predict whether the toxicity was produced by the encapsulated drug or the lipid vehicle.

Free drugs, ET and MTX, and loaded LN were also tested in the commercial cell line U-2 OS for comparison. As Table 1 shows, IC_{50} values were similar to those obtained with the patient-derived cell lines.

4.3 Reduced Folate Carrier (RFC1) and Dihydrofolate Reductase (DHFR) expression analysis in osteosarcoma clinical samples

MTX is a folic acid analog that enters the cells via folate carriers (RFC). Once inside, it blocks *de novo* purine synthesis through the competitive inhibition of the enzyme dihydrofolate reductase (DHFR), leading to the alteration of DNA replication [32,40]. RFC is present in considerable quantities in most cells, but OS is characterized by the decreased expression of these carriers [41]. Consequently, in order to overcome this impaired transport, MTX is given at high doses together with an antidote (leucovorin) to avoid the associated side effects. HD-MTX enters the cells by passive diffusion in a dose-dependent manner. However, leucovorin, which shares the same carriers as folic acid and MTX, cannot enter the cells by passive

diffusion unless it is administered at high doses. Leucovorin is thus able to conserve the healthy cells (which do not have altered folate receptors), without protecting the OS cells. With this approach, normal cells are protected from the toxicity but cancer cells are not [6]. Apart from the low expression of the carriers, increased expression of DHFR has also been described as one of the main resistance mechanisms of OS cells to MTX [31]. For this reason, we studied the expression of RFC and DHFR in 18 OS samples, including cell lines derived from normal bone tissue, primary bone tumors and lung metastases.

Regarding DHFR expression, it has been described that tumor cells overexpress DHFR when the primary tumor metastasizes [31]. However, in the patient-derived cell lines, individual effects could be observed (**Fig. 3**). While patient 595 seemed to acquire the aforesaid MTX-resistance in the paired metastases, patient 588 appeared to present innate resistance to the drug, as both the primary tumor and the paired normal bone tissue presented high levels of DHFR. These results are consistent with the efficacy results measured in terms of IC_{50} in the previous section. MTX was more effective at treating primary cells in patient 595, in contrast to the patient-derived cell lines 588 where MTX turned out to be more effective in metastatic cells. Therefore, although the response of a given tumor to a chemotherapeutic agent cannot be simply understood and simplified to a single gene trait, studying these genes, either expression or specific SNP genotypes, might allow us to determine critical points involved in drug efficacy and help developing effective therapies to overcome this resistance.

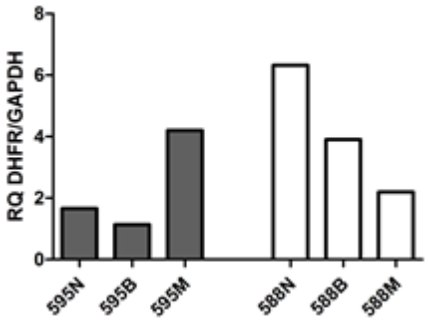

Figure 3. RQ values of DHFR expression normalized with GAPDH in cell lines derived from patients 595 and 588 in their normal tissues (N), osteosarcoma primary tumors (B) and metastatic tissues (M).

Finally, with regard to RFC, we could not detect significant expression in any of the samples studied (data not shown), which is in good agreement with the clinical requirement of HD-MTX to treat OS patients. In this sense, the encapsulation of the drug into LN could avoid the

need for transporters to go into the cell, and would therefore overcome this innate resistance to MTX efficacy.

4.4 Plasma membrane distribution of lipid rafts detected by the binding of cholera toxin-B subunit

To further clarify whether ET was more effective against metastatic (595/588M) cells due to their higher lipid rafts content, as it has been postulated, the presence of these domains in the patient-derived cell lines was studied. Lipid rafts are low-density and detergent resistant membrane microdomains, enriched in sterols and sphingolipids, which are implicated in the regulation of signaling circuits, cell survival, proliferation and migration [33]. It has been reported that several solid tumors accumulate cholesterol on the plasma membrane of their cells, and this overexpression is correlated with the progression of the disease [29,33,34]. Furthermore, several authors suggest that increased levels of cholesterol in the plasma membrane of the cells favor the gathering of sphingolipids, proteins and cholesterol into more organized and dynamic structures, promoting the lipid raft platform formation [35]. Therefore, raised levels of cholesterol and thus elevated levels of lipid rafts are related to advanced stages of disease, and could be implicated in the metastatic process of OS cells. In order to visualize these lipid microdomains, lipid rafts were labeled with the cholera toxin B subunit that binds pentavalently to the glucosphingolipid GM1 ganglioside, which is present in large quantities in lipid rafts, and causes the formation of clusters of five GM1 molecules [36].

As Fig.4 shows, 588M cells (Fig. 4A) and 588B cells (Fig. 4B) were observed to acquire intense green fluorescence which indicates the presence of GM1-CTxB assemblies, defined as lipid rafts.

When comparing mestastasic (M) cells *vs* primary (B) cells, it could be noticed that almost every M cell presented patches of lipid rafts, but not all B cells presented labeling of GM1-CTxB. Several authors have already reported that, in order to promote cell invasion, metastatic cancer cells present in their plasma membrane some protusions called invadopodia which are rich in lipid rafts [37]. This higher presence of lipid rafts in M cells might therefore be responsible for the chemosensitivity of metastatic cells to the drug, due to a greater activation of the extrinsic apoptosis pathways once cells are exposed to the ET activity on the one hand, and to a higher internalization of the drug on the other.

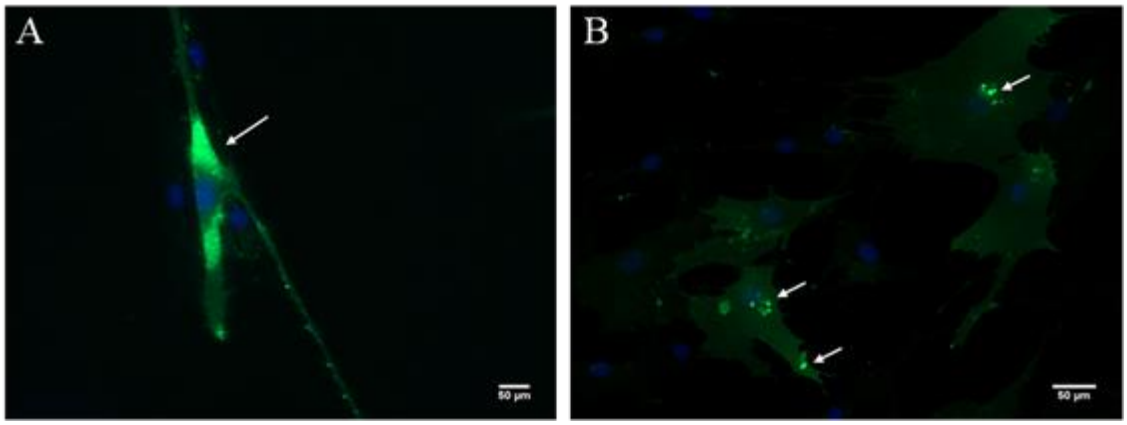

Figure 4. Cell surface expression of GM1 visualized by fluorescence microscopy in the 588 cells. The white arrows indicate GM1 staining by CTxB subunit-FITC. A) M cells showing big patches of label. B) B cell lipid rafts stained as discreet spots of label. Nuclei were stained with DAPI in blue.

4.5 Edelfosine and edelfosine lipid nanoparticles internalization in the patient-derived cell lines

As discussed above, it seems clear that the higher presence of lipid rafts in metastatic cells contributes to the higher efficacy of ET in these cells. Alteration of these lipid rafts by the binding of a ligand (for instance, ET) can directly activate multiple apoptotic pathways such as Fas-FasL pathway [38], leading to cell death. However, ET can also be internalized by OS cells and induce a rapid apoptotic response, targeting the mitochondria and endoplasmic reticulum [18]. In that sense, ET efficacy in mestastasic cells could also be due to its higher internalization mediated by lipid rafts [30].

To gain further insight into ET endocytosis in metastatic cells, lipid rafts of metastatic cells were disrupted by the cholesterol depletion agent methyl-β-cyclodextrin (MβCD), as described in the literature [14,39]. After the removal of cholesterol, 595/588M cells were exposed to ET for 5 h and afterwards the amount of internalized drug was quantified. As shown in Fig. 5, ET uptake was considerably reduced when lipid rafts were altered, confirming the key role of lipid rafts in the uptake of ET by metastatic cells. This implication of lipid rafts in ET uptake has also been previously described for other types of cancers such as leukemia, lymphoma and multiple myeloma [14,30].

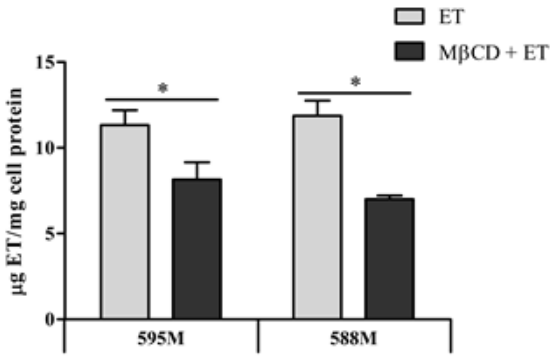

Figure 5. Edelfosine (ET) internalization in metastatic osteosarcoma cell lines after lipid raft disruption with methyl-β-cyclodextrin (MβCD). *p value < 0.05 by Mann-Whitney U-test.

In view of the higher efficacy of ET when loaded into LN, we studied the internalization of both ET and ET-LN. For this purpose, metastatic and patient-derived primary OS cells were cultured with 10 μM of free and encapsulated ET for 24 h and the amount of internalized drug was quantified by a validated UHPLC-MS/MS method. As Fig. 6 shows, either free or encapsulated, ET accumulated to a greater extent in metastatic cells than in OS primary cell lines ("B"), which correlates with the *in vitro* efficacy studies, in which metastatic cells were shown to be more sensitive to ET than primary cells. Moreover, it is important to highlight that when ET was loaded into LN, its uptake increased in all cell lines. In that regard, the higher inhibitory activity of ET-LN in OS cells could be due to their higher internalization. This result is in agreement with those for other cancer cell lines in which the encapsulation of ET in LN improved its internalization and boosted the cytotoxic activity of the drug [14].

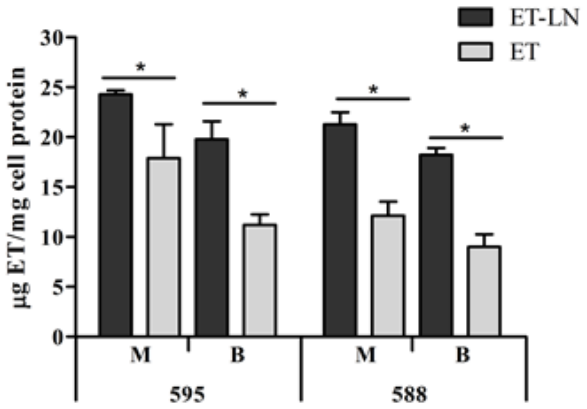

Figure 6. Edelfosine (ET) and edelfosine lipid nanoparticle (ET-LN) uptake by patient-derived cell lines. *p value < 0.05 by Mann-Whitney U-test.

5. CONCLUSION

Our findings lead us to conclude that ET could be a new potential agent against OS. ET has shown a higher efficacy against metastatic cancer cells, which was partly influenced by their higher lipid rafts content. Moreover, encapsulation of ET in LN considerably improved the efficacy of the drug against OS cells, favored by their higher uptake in primary and metastatic OS cells. Regarding MTX, innate resistance of OS cells towards the drug has been observed. In order to overcome these limitations, LN with high MTX loading were developed and optimized. Future studies will elucidate whether the encapsulation of MTX in LN might make it possible to overcome these resistance mechanisms.

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CHAPTER 2

Doxorubicin and edelfosine lipid nanoparticles are effective acting synergistically against drug-resistant osteosarcoma cancer cells

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Doxorubicin and edelfosine lipid nanoparticles are effective acting synergistically against drug-resistant osteosarcoma cancer cells

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ABSTRACT

Despite the great advances that have been made in osteosarcoma therapy during recent decades, recurrence and metastases are still the most common outcome of the primary disease. Current treatments include drugs such as doxorubicin (DOX) that produce an effective response during the initial exposure of tumor cells but sometimes induce drug resistance within a few cycles of chemotherapy. New therapeutic strategies are therefore needed to overcome this resistance. To this end, DOX was loaded into lipid nanoparticles (LN) and its efficacy was evaluated in commercial and patient-derived metastatic osteosarcoma cell lines. DOX efficacy was heavily influenced by passage number in metastatic cells, in which an overexpression of P-gp was observed. Notably, DOX-LN overcame the resistance associated with cell passage and improved DOX efficacy fivefold. Moreover, when DOX was co-administered with either free or encapsulated edelfosine (ET), a synergistic effect was observed. This higher efficacy of the combined treatment was found to be at least partially due to an increase in caspase-dependent cell death. The combination of DOX and ET is thus likely to be effective against osteosarcoma.

Keywords: Edelfosine, Doxorubicin, Lipid nanoparticles, Osteosarcoma, Synergy, Metastases

Abbreviations: **DOX:** doxorubicin; **CI:** Combination index; **DRI:** drug reduction index; **ET:** Edelfosine; **Fa:** Fraction of affected cells; **IC_{50}:** inhibitory concentration 50; **LN:** lipid nanoparticles; **MDR:** Multidrug resistance; **P-gp:** P-glycoprotein; **ROS:** Reactive oxygen species; **RT-PCR:** real-time polymerase chain reaction; **UHPLC-MS/MS:** ultra-high performance liquid chromatography tandem mass spectrometry

Highlights

*Doxorubicin has been successfully encapsulated into lipid nanoparticles by the hot melting homogenization method

*Doxorubicin accumulation and efficacy is impaired in patient-derived metastatic osteosarcoma cells as passage number in culture increases

*Encapsulation into lipid nanoparticles overcomes P-gp-related doxorubicin resistance in late passage metastatic osteosarcoma cells

*Doxorubicin and edelfosine lipid nanoparticles act synergistically against osteosarcoma cells potentiating caspase-dependent cell death

1. INTRODUCTION

Osteosarcoma is the most common type of pediatric bone cancer. It originates from mesenchymal cells and is preferentially localized in the large bones with high growth potential, such as femur or tibia. Primary osteosarcoma tends to spread to the lungs from the earliest stage, giving a poor outcome to the patients at the time of diagnosis [1]. For this reason, osteosarcoma is aggressively treated with systemic neoadjuvant chemotherapy followed by surgical resection of the primary tumor and a new phase of post-operatory chemotherapy. In this protocol, doxorubicin (DOX) is, together with high-dose methotrexate and cisplatin, a first line agent and a key antitumoral drug [2,3].

DOX is a chemotherapeutic anthracycline which is widely used alone or in combination for the treatment of osteosarcoma and a wide variety of solid and hematologic tumors such as leukemia, Hodgkin's lymphoma or breast cancer [4]. However, despite being one of the most effective antineoplastic agents on the market, its efficacy is hampered by the development of multidrug resistance (MDR), which contributes to the recurrence or progression of the disease in almost one third of patients with localized osteosarcoma [5]. In this regard, ATP-binding cassette transporters such as P-glycoprotein (P-gp) are one of the main agents responsible for the efflux of the drugs from the cells [6]. Anthracyclines are substrates of P-gp and, thereby, their expression has a major role in the acquired resistance of tumors to DOX and chemotherapy failure. This fact, together with the acute and multidirectional toxicity of the drug, hinders the clinical use of DOX.

With a view to overcoming these limitations, several strategies have been extensively studied. On the one hand, the inclusion of the drug in delivery systems has reduced its systemic toxicity by targeting the tumor cells more specifically, and has avoided the drug efflux through P-gp efflux pumps [1]. On the other hand, combinations of DOX with other anti-cancer agents such as the alkyl lysophospholipid perifosine, or its analog edelfosine (ET) have shown synergistic effects against osteosarcoma and Ewing's sarcoma cells respectively [7,8], which could decrease the required doses of DOX *in vivo*.

ET is a synthetic alkyl-lysophospholipid analog that has been shown to be active against several cancer cell lines including osteosarcoma [9-11]. Its mechanism of action involves the plasma membrane of the cells, specifically the lipid microdomains of the plasma membrane or lipid rafts, triggering the extrinsic apoptotic pathway of cell death [12]. Besides, ET-induced apoptosis is also mediated through the endoplasmic reticulum and mitochondria [7,13]. The different intracellular targets of DOX and ET, nucleus and membrane, together

with their potent antitumor efficacy when administered separately, make these two drugs suitable candidates for a multiple targeting co-administration regimen. This therapeutic approach could be employed in the treatment of aggressive and resistant cancers such as osteosarcoma. Moreover, DOX and ET inclusion in drug delivery systems could prevent the systemic toxicity induced by both drugs and favor the accumulation of the drugs inside the tumor cells.

In the present study we investigated the potential of DOX lipid nanoparticles (DOX-LN) for the treatment of osteosarcoma and tested whether their *in vitro* combination with ET could boost the efficacy of both drugs. We also focused on whether their entrapment in LN could preserve their effectiveness against primary-derived and commercial osteosarcoma cell lines.

2. MATERIAL AND METHODS

2.1 Materials

Doxorubicin hydrochloride was purchased from Sigma-Aldrich (Madrid, Spain), ET was obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland), and Precirol® ATO 5 was kindly provided by Gattefosse (Lyon, France). Tween® 80 was purchased from Roig Pharma (Barcelona, Spain), and other reactives for nanoparticle formulation were supplied by Sigma-Aldrich (Madrid, Spain). Amicon® Ultra-15 10,000 MWCO filter devices were provided by Millipore (Cork, Ireland) and all reagents employed for mass spectroscopy were of gradient grade for liquid chromatography and were obtained from Merck (Barcelona, Spain). DAPI was obtained from Invitrogen (Madrid, Spain), fluorescence mounting medium was obtained from Dako (Barcelona, Spain) and all reagents for cell culture were from Gibco®.

2.2 Preparation of DOX-LN

2.2.1. Double water-in-oil-in water ($W_1/O/W_2$) emulsion solvent evaporation method

For the production of DOX-LN by the double emulsion method DOX (1 mg) was disolved in the internal water phase, which consisted of 200 μL of an acidified 0.5% (w/v) taurocholate solution. This water phase was added to 2 mL of an ethyl acetate:dichloromethane solution

(1:1 v/v) containing 100 mg of Precirol® ATO 5 and 10 mg of lecithin and mixed by ultrasonication at 13 W for 30 seconds on an ice bath. The obtained W/O nanoemulsion was rapidly dispersed in 4 mL of a 2% (w/v) Tween® 80 solution and sonicated again at 13 W for 30 seconds. Finally, the double $W_1/O/W_2$ emulsion was poured onto 10 mL of a 2% Tween® 80 solution and stirred for 2 h to completely evaporate the organic solvent and cause the solidification of LN. In order to remove the excess of surfactant and the non-incorporated drug, the LN suspension was washed three times by centrifugation (30 minutes, 4500 g) using Amicon Ultra-15 10,000 MWCO centrifugal filters. Finally, trehalose (150% w/w of lipid) was added as cryoprotectant to the LN suspension, which was kept at -80 °C and freeze-dried to obtain a stable powder.

2.2.2. Single oil-in water (O/W) emulsion solvent evaporation method

To prepare nanoparticles by the single emulsion method 1 mg of DOX was dissolved in 1 mL of a dichloromethane: triethanolamine solution (1000:1, v/v) under magnetic stirring, overnight and mixed with 1 mL of ethyl acetate containing 100 mg of Precirol® ATO 5 and 10 mg of lecithin. This organic phase was poured onto 4 mL of a 2% Tween® 80 aqueous solution and processed with the ultrasonic cell disruptor for 1 minute on an ice bath at a power of 13 W. The resulting O/W emulsion was dispersed in 10 mL of a 2% Tween® 80 solution and stirred until the complete evaporation of the organic solvent was achieved. LN were washed and freeze-dried as described above.

2.2.3. Hot melting homogenization method

DOX-LN were prepared according to the hot melting homogenization method previously published by Mussi *et al* [14], but with slight modifications. Briefly, the lipid phase consisting of 150 mg of Precirol® ATO 5, 1 mg of DOX and 6 mg of triethanolamine was melted 5° C above the lipid melting point. Just before adding the aqueous phase to the formulation, 12 µL of oleic acid were added to the lipid phase. Then, 10 ml of Tween® 80 2% with 4 mg of EDTA, preheated at the same temperature, were added to the melted lipid phase and the mixture was processed with the help of the ultrasonic device for 4 minutes at 13 W. Finally, LN were obtained by cooling the emulsion in ice. LN were washed and lyophilized as described above.

2.3 Characterization of DOX-LN

The nanoparticles developed were first characterized in terms of size, polydispersity index (PDI) and ζ potential. For this purpose, LN were diluted 60-fold in double distilled water in

order to ensure that the light scattering intensity was within the sensitivity range of the instrument. The average particle size and PDI were analyzed by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, UK) and ζ potential was determined by the study of the surface charge through particle mobility in an electric field in the same instrument. The entrapment of DOX was analyzed by fluorimetry in a Tecan GENios microplate reader (Tecan Group Ltd, Maennedorf, Switzerland) (excitation wavelength 485 nm, emission wavelength 580 nm) after it was extracted in DMSO and diluted in methanol.

2.4 Cell lines and culture conditions

The cell lines used throughout the study were the human immortalized osteosarcoma cell line U-2 OS (ATCC® HTB96™), purchased from the American Type Culture Collection (Sigma-Aldrich), and the patient-derived 595M cell line, obtained from a lung metastatic implant of an osteosarcoma patient treated at the University Clinic of Navarra [15]. U-2 OS cell line and patient-derived cells were maintained in McCoy's 5A medium and alpha-MEM medium, respectively, supplemented with 10% of fetal bovine serum and 1% penicillin/streptomycin in an incubator at 37 ºC and a humidified atmosphere with 5% carbon dioxide.

2.5 Cytotoxicity studies

The antitumor activity of the formulations developed was tested using the MTS included in the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madrid, Spain) according to the manufacturer's recommendations. Briefly, U-2 OS and patient-derived cells were plated on 96-well plates at a density of 1700 cells per well 24 h before the addition of different concentrations of free and encapsulated DOX in triplicate wells. After 72 h of incubation with the drugs the medium was withdrawn and 100 µL of complete medium containing 15 µL of MTS were added to each well. Absorbance was measured 4 h later in a microplate reader (iEMS reader MF, Labsystem, Helsinki, Finland) at a test wavelength of 492 nm with the reference wavelength set at 690 nm. The concentration of drug required to inhibit cell growth by 50% (IC_{50}) was estimated by fitting the dose–response curve to a sigmoidal function using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

2.6 DOX accumulation in osteosarcoma cells

For DOX cellular accumulation studies, cells (1.4 x 10^4 U-2 OS cells and 3.5 x 10^3 595M cells per cm^2 in 5 cm diameter cell culture dishes) were incubated with 100 nM of either free or encapsulated DOX in complete cell culture medium. At predetermined time intervals, culture dishes were transferred onto crushed ice, medium was discarded and cells were washed four times with ice-cold PBS to remove the non-internalized drug. Cells were then collected by scraping them into methanol to lysate the cells and dissolve the LN. DOX cell content was quantified by UHPLC-MS/MS using an Acquity UPLC™ system (Waters Corp., Milford, USA) coupled to an Acquity™ TQD (Triple Quadrupole Detector) mass spectrometer. Cell-associated drug content was expressed with reference to the corresponding total protein content of the samples, measured in parallel using the Folin-Ciocalteau/biuret method [16].

For fluorescence microscopy studies, 2 x 10^4 cells per well were placed on 24-well plates with a cover glass on the surface of the wells and after 24 h of incubation cells were treated for 8 h with 100 nM of DOX and DOX-LN. Samples were then fixed with p-formaldehyde 4% (300 μL, 5 min) and stained with DAPI (1:1000) for 5 min. Finally, cover glasses were extracted and placed on microscope slides with fluorescence mounting media and examined on a fluorescence microscope (Zeiss, 120 Libra).

2.7 P-gp expression studies

To evaluate the expression of P-gp (coded by ABCB1 gene) in the osteosarcoma cell lines, 595M cells were cultured *in vitro* during several weeks and the expression of P-gp was evaluated in early and late passage cells.

RNA was extracted from 595M cells using TRIzol Reagent (Sigma Aldrich). After treatment with DNase I, RNA was reverse transcribed using SuperScript II reverse transcriptase and random primers. Finally, semiquantitative analysis of ABCB1 mRNA expression was carried out by real-time polymerase chain reaction (RT-PCR) with the ABI PRISM 7300 Sequence Detector and Sequence Detector version 1.4 software (PerkineElmer/ Applied Biosystems, Foster City, California).

The semiquantitative ABCB1 mRNA levels were expressed relative to glyceraldehyde-3-phosphatedehydrogenase mRNA (GAPDH, Hs02758991_g1), and determined by TaqMan assays (Hs00184500_m1, Life Technologies). Relative levels of expression were determined by the $2^{-\Delta\Delta Ct}$ method and every assay was performed in triplicate.

2.8 Drug combination analysis

For drug interaction analysis free and encapsulated DOX were combined with ET and ET-LN respectively, at a constant combination ratio of equipotent concentrations [(IC_{50})DOX:(IC_{50})ET]. ET-LN used for this study were prepared as previously described [9] and presented a mean diameter of 136.96 ± 10.13 nm and a drug loading of 25.31 ± 3.06 μg of ET per mg of formulation.

Cell viability was analyzed after 72 h of incubation with each treatment alone and in combination by the MTS assay described above and the effect of drug interactions was quantified by determining the CI (combination index) according to the Chou-Talalay method [17]. CI < 1, CI = 1 and CI > 1 indicate synergy, additivity and antagonism, respectively. Moreover, the dose-reduction index (DRI) of the drugs was also calculated as a measure of the fold-decrease in dose for each drug in a synergistic combination to achieve a given effect compared with the dose of each drug alone required to produce the same effect.

2.9 Caspase 3/7 activity

The activity of caspase-3/7 was measured by Caspase-Glo-3/7 assay kit (Promega Corp.,USA) following the manufacturer's instructions. Briefly, U-2 OS cells were seeded at a density of 1700 cell per well in 96-well white plates. After 24 h of cell adherence, cells were treated for 72 h with free or encapsulated DOX (20 nM) and ET (10 μM), alone or in combination. Caspase activity was evaluated by adding the assay reagent to each well (1:1) and measuring the luminescence signal in a Tecan GENios microplate reader (Tecan Group Ltd, Maennedorf, Switzerland) after 30 minutes of incubation. All samples were measured in triplicate and luminescence intensity was normalized to cell viability.

2.10 Statistical analysis

Statistical analysis and graphical representations were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). Statistical comparison between different groups was performed using the Mann-Whitney U test. All quantitative data were reported as mean ± standard deviation and statistical significance levels were defined as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

3. RESULTS

3.1 Characterization of DOX-LN produced by different methods

DOX-LN were prepared following three methods, the particle size, ζ potential, PDI and drug loading being defined as dependent variables for the selection of the final formulation. As shown in Table I, DOX-LN prepared by the single emulsion solvent evaporation method presented the largest particle sizes (above 350 nm) and the lowest drug loading, as well as a negative ζ potential due to the use of tween 80 as surfactant. In contrast, LN prepared by the double emulsion technique and hot melting homogenization procedure were able to incorporate 4 times more drug and presented particle sizes below 200 nm. Between these two methods, the hot melting homogenization method produced the smallest LN, with particle sizes smaller than 100 nm, and presented the advantage of not requiring organic solvents in the formulation process. This one was therefore selected for further studies.

Table I: Physicochemical characteristics of unloaded and doxorubicin-loaded lipid nanoparticles developed by different methods.

Formulation method	Formulation type	Size (nm)	PDI	ζ Potential (mV)	DL (μg/mg)
$W_1/O/W_2$ emulsion solvent evaporation	*DOX-LN*	191.4± 38.7	0.398±0.143	-25.8±0.7	2.74±0.65
	unloaded LN	211.8±14.3	0.225±0.029	-28.7±1.3	-
O/W emulsion solvent evaporation	*DOX-LN*	259.35±18.5	0.238±0.005	-19.8±0.7	2.06±0.14
	unloaded LN	343.3±18.3	0.256±0.001	-16.9±0.7	-
Hot melting homogenization	*DOX-LN*	95.4±12.9	0.194±0.009	-27.8±3.6	2.57±0.80
	unloaded LN	74.7±3.05	0.212±0.019	-28.4±4.8	-

DL: drug loading (μg of doxorubicin/mg of lipid nanoparticles formulation); DOX: doxorubicin; LN: lipid nanoparticles; PDI: polydispersity index.

3.2 DOX and DOX-LN induce loss of cell viability in osteosarcoma cells

After LN development, the biological activity of DOX and DOX-LN was studied in the commercial osteosarcoma cell line U-2 OS and the patient-derived metastatic osteosarcoma cell line 595M by comparing their IC_{50} values (Table II).

Table II: IC_{50} values of doxorubicin in solution and doxorubicin-loaded lipid nanoparticles on different osteosarcoma cell lines after 72 h of treatment.

Cell line	Doxorubicin (nM)	Doxorubicin lipid nanoparticles (nM)*
595M (early passages)	55.50 ± 11.65	109.00 ± 3.61
595M (late passages)	255.00 ± 51.96	107.33 ± 15.37
U-2 OS (ATCC® HTB96™)	56.33 ± 9.02	47.00 ± 2.65

*Equivalent non-loaded lipid nanoparticles showed ≥ 90 % cell viability

MTS studies revealed that despite the inherent sensitivity of U-2 OS cells to DOX, the encapsulation of the drug in LN slightly improved the efficacy of the free drug, reducing its IC_{50} by 15%, from 56 to 47 nM.

Regarding 595M cells, due to their rapid growth and the delayed diagnosis of osteosarcoma that favors the progression of the metastatic *foci*, the cytotoxic activity of DOX was studied in cells with different passage numbers. First, as the passage number in culture increases, cells became more resistant to DOX, with IC_{50} values 5 times higher for 595M cells in late passages (above 20) than those obtained in earlier passages (below 10). Interestingly, this phenomenon was not observed when cells were treated with DOX-LN, where IC_{50} values around 100 nM were obtained. Thus, although DOX-LN did not improve the efficacy of the free drug in low passage cells, the activity of DOX-LN was not influenced by the passage number and they were 2.4 times more effective than DOX in inhibiting the growth of high passage metastatic cells, which indicates that nanoparticles may overcome some of the intrinsic resistance mechanisms acquired by metastatic tumor cells in late stages.

3.3 Evaluation of DOX cellular accumulation

In order to determine if there was a relationship between the observed cytotoxicity and drug concentrations in the cells, the accumulation of the different DOX treatments (100 nM of free DOX and 100 nM of DOX-LN) in osteosarcoma cells was monitored for 72 h and analyzed by UHPLC-MS/MS and fluorescence microscopy (Figure 1).

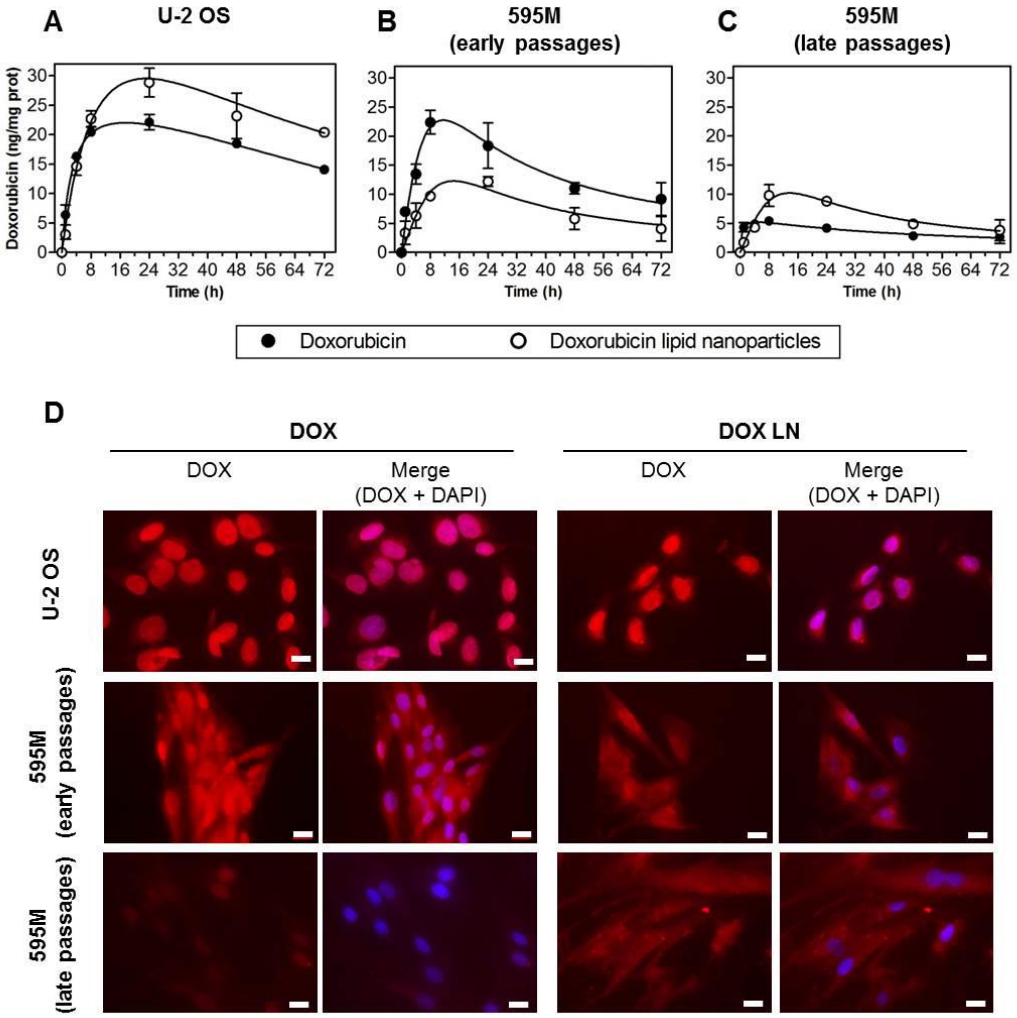

Figure 1: Doxorubicin accumulation in osteosarcoma cell lines. Accumulation kinetics of 100 nM of doxorubicin (DOX) and doxorubicin-lipid nanoparticles (DOX-LN) in (A) U-2 OS cells, (B) 595M cells in early passages and (C) 595M cells in late passages over 72 h. Data are expressed as mean ± standard deviation of ng of internalized drug per mg of cell protein. (D) Fluorescence images (40 X) of osteosarcoma cells cultured with 100 mM of DOX and DOX-LN for 8 h. Scale bar corresponds to 20 μm.

Figure 1 shows the time-dependent accumulation pattern of DOX, with an initial phase of rapid accumulation for 8-24 h followed by a progressive decrease in cell associated drug. This decrease could be attributed to drug dilution per cell as the consequence of cell division or to an imbalance between drug uptake and metabolism/efflux.

In the case of U-2 OS cells (Figure 1A), the uptake rate was similar in both free and encapsulated DOX up to 8 h, achieving higher intracellular DOX levels at later time points when the drug was loaded into LN. Concerning, 595M cells, cells in early passages presented a maximum DOX concentration close to that obtained in U-2 OS. However, DOX accumulation

was lower with ascending passage number; in particular, there was a fourfold reduction in the accumulation of free DOX in cells in late passages compared to those in early passages (Figures 1B and C). Interestingly, when DOX was administered in its encapsulated form, 595M cells presented essentially the same accumulation profile regardless of the passage number, which indicates that LN overcome the resistance mechanism acquired by 595M against DOX as the passage number increases. To leverage the inherent fluorescence of DOX, cells were also analyzed by fluorescent microscopy in order to monitor the localization of the drug within the cells. Fluorescence images confirmed the different accumulation patterns obtained by UHPLC-MS/MS, showing the lowest fluorescence intensity for late-passage 595M cells treated with DOX, intermediate intensity for 595M cells treated with DOX-LN and the highest fluorescence for early-passage 595M cells treated with DOX and U-2 OS cells. Moreover, images showed that red fluorescence was all over the cell but especially in the nucleus, where DOX exerts its action (Figure 1D).

These findings are in concordance with the decrease in the cytotoxic activity observed for the free drug with high passaging and its maintenance when the drug was encapsulated. In fact, a close correlation was detected between the maximum intracellular DOX levels achieved in the different cells and the IC_{50} value for each one ($p=0.0167$, Spearman's $rho=-0.9429$).

3.4 The effect of DOX and cell passage on P-gp expression in metastatic osteosarcoma cells

To further evaluate if the reduction in the intracellular accumulation of DOX and the consequent progressive loss of DOX efficacy with ascending passage number in 595M cells was at least partially related to an increase in the expression of drug efflux pumps, expression analysis of ABCB1 was performed.

595M cells in passage #9 had a 2.8-fold increase in ABCB1 mRNA levels compared to the cells in passage #3. This difference in the drug resistance associated to the gene expression progressively increased throughout 30 passages, achieving a 3.8-fold increase in the relative expression of the gene in passage #30 compared to cells in passage #3.

3.5 DOX and ET act synergistically against osteosarcoma cells

In order to evaluate whether the antitumor effect of ET in osteosarcoma cells (reported in previous studies [9]) was synergistic with that of DOX demonstrated in the present work,

osteosarcoma cells were exposed to the individual or combined drugs at a constant equipotent ratio.

The combination treatments turned out to be synergistic in U-2 OS and 595M cells, showing CI values below 1 in all cases. Moreover, according to the DRI values shown in Table III, when combining DOX and ET, the concentration of DOX required to inhibit 50% of osteosarcoma cells could be reduced by 2.22 to 2.77 fold and 2.14 to 2.49 fold in the case of free and encapsulated drugs, respectively. These results suggest that combination therapy could be useful to reduce the DOX dose required to maintain treatment efficacy while reducing its toxicity and establish ET as a potential candidate in therapy for osteosarcoma.

Table III: Synergy analysis for doxorubicin and edelfosine combination therapy at the IC_{50} in the different osteosarcoma cell lines.

Cell line	Treatment	Drug ratio DOX (nM):ET (μM)	DRI $_{doxorubicin}$	DRI $_{edelfosine}$	CI
U-2 OS	*DOX*	2:1	2.77 ± 0.27	2.82 ± 0.16	0.72 ± 0.02
	DOX-LN	3:1	2.14 ± 0.36	2.79 ± 0.25	0.84 ± 0.11
595M (early passages)	*DOX*	4:1	2.47 ± 0.11	2.18 ± 0.08	0.86 ± 0.02
	DOX-LN	5:1	2.45 ± 0.36	2.99 ± 0.42	0.75 ± 0.08
595M (late passages)	*DOX*	20:1	2.22 ± 0.35	3.25 ± 0.94	0.78 ± 0.11
	DOX-LN	4:1	2.49 ± 0.26	3.10 ± 0.46	0.73 ± 0.02

CI: combination index, DOX: doxorubicin, DRI: drug reduction index, ET: edelfosine.

Additionally, as drug-drug interactions can change as a function of concentration or activity, the dose-response curves obtained for individual and combined drugs were also examined to determine the type of interactions at different cell inhibition levels (Figure 2). Regarding free drugs, the Fa-CI plots, depicting CI values *vs* Fa (fraction of inhibited viability) showed the occurrence of synergistic effects between DOX and ET at low and medium effect levels (Fa 0.25 and Fa 0.5) in U-2 OS cells, but lost this effect as anti-proliferative activity increases (Figure 2A). In contrast, the combination responded differently in 595M cells at early passage numbers, where the synergistic effect was observed only at medium and high dose levels (Fa 0.5, Fa 0.75 and Fa 0.9) (Figure 2B). Finally, in 595M cells at late passage numbers, a synergistic interaction was achieved at all the cell inhibition rates (Figure 2C). It is noteworthy that the combined treatment of DOX-LN and ET-LN maintained its synergy at the different drug effect levels studied in all the cell lines. These results have important clinical implications as no antagonistic interactions were observed for this combination in any of the conditions studied.

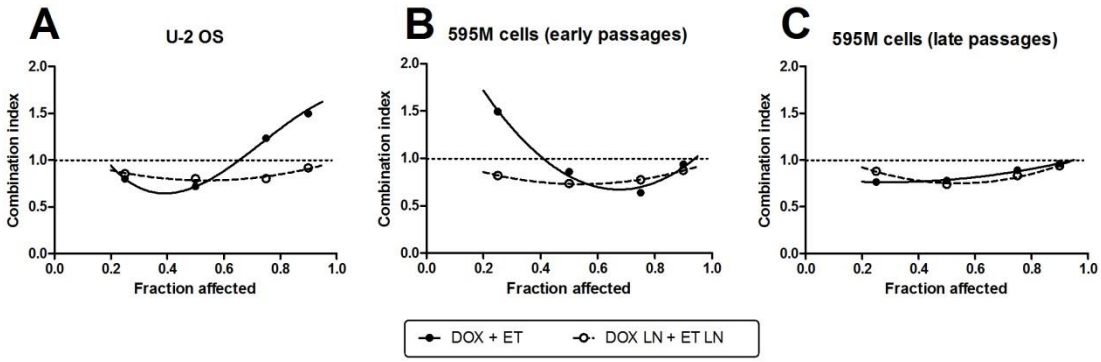

Figure 2: Combination index (CI) analysis. Graphical representation of CI for doxorubicin (DOX) and edelfosine (ET), either free or encapsulated into lipid nanoparticles (LN), in U-2 OS cells (A), 595M cells in early passages (B) and 595M cells in late passages (C) at different fractions of dead cells (Fa). The calculated CI values for the experimental Fa for the two-drug combination relative to the individual drugs are shown. CI values below 1 indicate synergy.

3.6 DOX and ET co-treatment boosts caspase 3/7 dependent cell death

To further evaluate the mechanism of cell death, the effects of DOX, ET and the combination treatments on cell apoptosis were evaluated using the caspase-3/7 activity assay. For this purpose, the commercial U-2 OS cell line was selected as a model of osteosarcoma. The activity of caspases 3/7 was significantly increased by all treatments at 48 h and more intensely at 72 h. At this time, exposure to free ET and DOX alone resulted in a 2.2- to 2.7-fold increase of caspase activity relative to the control cells and this activity was enhanced 1.9 to 3.4 times in the case of the encapsulated drugs. Interestingly, the combined treatments significantly increased caspase activity not only in comparison to the control cells, 4.3-fold in the case of free drugs and 6.1-fold for encapsulated drugs, but also as regards to the individual drugs alone (Figure 3). These results suggest that cell death caused by the co-administration of DOX and ET is at least in part mediated by caspase activation.

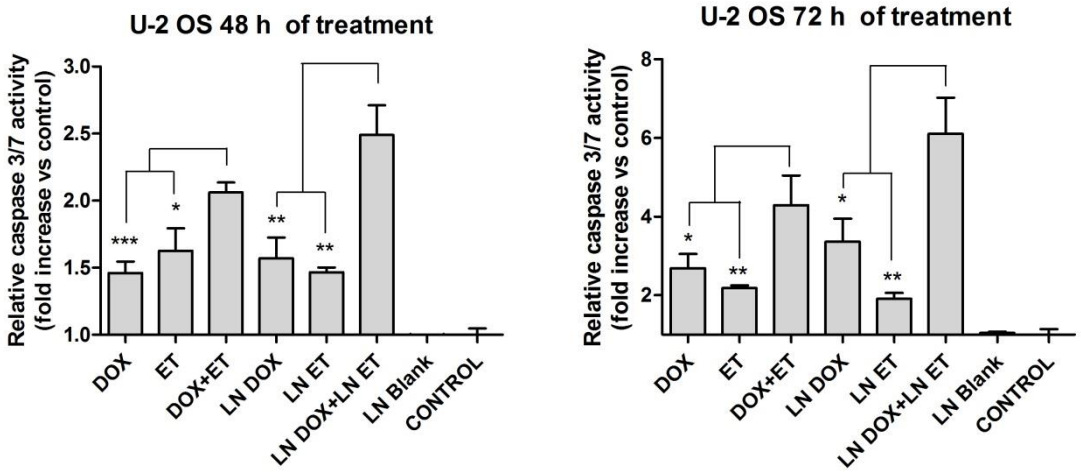

Figure 3: Doxorubicin (DOX) and edelfosine (ET) promote caspase-dependent apoptosis in osteosarcoma cells. Caspase 3/7 activities were measured using the Caspase-Glo 3/7 luminescence assay after incubation for 48 and 72 h with the different treatments and relative caspase activities were normalized to cell viability and to control cells.

4. DISCUSSION

DOX is a first line agent traditionally employed in osteosarcoma therapy. However, despite the great efficacy of this anthracycline against a wide variety of cancers, its indiscriminate distribution (mainly to the heart) and consequent overall toxicity limit its clinical use. In that context, in order to increase the effectiveness of the drug but decrease the associated side effects, the entrapment of DOX in LN offers a potential solution.

DOX is a cationic anthracycline highly insoluble in lipid phases. For that reason, several strategies were employed to encapsulate the drug into LN such as including the drug in the internal aqueous phase of a multiple emulsion or creating an ion pair with an anionic counterpart as previously described by Mussi *et al* [14,18]. With these strategies, DOX-LN were prepared following 3 different methods i) double emulsion solvent evaporation method, ii) single emulsion solvent evaporation method, and iii) hot emulsion technique.

DOX-LN produced by the double emulsion method presented a size of around 200 nm, an accepted size for oral absorption and tumor cell uptake [19]; however, their PDI value was above 0.3, meaning that the formulation was highly heterogeneous in size. In contrast, DOX-LN produced by the hot emulsion technique presented an average size of 95 nm and a well-accepted PDI. This size is considered the most suitable size for oral delivery, as suggested by

Huipeng Li et al [20] and, therefore, because of the smaller size and the avoidance of solvents in their formulation, DOX-LN produced by the hot emulsion technique were selected for the rest of the assays.

The efficacy of DOX-LN against osteosarcoma cells was then evaluated. Cytotoxicity studies revealed that DOX-LN were as effective as DOX in the commercial U-2 OS cell line, even though they require cell internalization and intracellular hydrolysis before exerting any cytotoxic effect. The similar efficacy of free and encapsulated DOX in U-2 OS could be associated with the comparable profile of internalization of both treatments during the first few hours. On the other hand, and more importantly, a strong influence of the passage number in DOX efficacy and cellular accumulation was observed in the patient-derived metastatic osteosarcoma cell line 595M. Cells with high passage number, which probably better reflects the clinical situation present on late diagnosis, accumulated 4 times less free DOX and were 5 times more resistant to the drug than cells at low passage number. Acquisition of drug-resistance is considered one of the major causes of anticancer chemotherapy failure [21]. In the case of anthracyclines such as DOX, some of the most frequent mechanisms that contribute to the development of acquired resistances are: i) decreased intracellular concentration of the drug due to the expression of many membrane efflux pumps or increased drug metabolism, ii) failure of the cellular apoptotic mechanisms and ii) reduced concentration/activity or mutation of the target enzyme topoisomerase II [22]. However, the fact that the drug incorporated in LN was equally accumulated in cells with high and low passage number and, at the same time, the fact that LN did not show this loss of efficacy with increasing cell passage numbers suggested that the main mechanism leading to the acquired resistance would be that associated with efflux pumps and not with the action of the drug itself. PCR studies confirmed a progressive increase in P-gp expression in 595M cells as passage number increased, with almost a fourfold increase in P-gp expression from passage 3 to 30. These findings strongly correlated with the decreased DOX uptake and subsequent loss of efficacy of the drug in late passage 595M cells. Importantly, neither the DOX efficacy nor its cellular accumulation were affected by passage numbers in metastatic cell lines when the drug was loaded in LN. This fact was attributed to the Tween® 80 used for the formulation of LN, as we have previously demonstrated that this surfactant reduces the activity of P-gp pump in tumor cells, reverting P-gp related MDR [10].

Once the efficacy of DOX-LN was confirmed, the possibility of enhancing DOX activity by combining it with ET was studied. DOX and ET co-administration, either in their free or encapsulated forms, showed a synergistic activity in all the osteosarcoma cell lines tested.

Moreover, this synergy was correlated with an increase in Caspase 3/7 activity, meaning that this effect was at least partially due to boosting of the caspase-mediated cell death. Boosting of the cytotoxic activity between ET and DOX has also been observed by other authors in Ewing´s sarcoma cells [7], which was attributed to their different mechanisms of action. While DOX exerts its action by the nuclear inhibition of DNA synthesis and repair [4], ET targets the membrane of the cells, endoplasmic reticulum and mitochondria [23]. A potentiation of the cytotoxic effect of DOX and other alkyl lysophospholipids such as perifosine has also been reported in osteosarcoma [24] and multiple myeloma cells [25]. In these studies, it was observed that low doses of perifosine sensitized the cell to the action of DOX probably by blocking Akt activation and the pro-survival mTOR signal transduction pathway. This pathway is frequently overexpressed in tumor cells, such as osteosarcoma, and contributes to chemoresistance of cancer cells [26,27]. Inhibition of the Akt signaling could lead to a decreased apoptotic threshold and to the induction of apoptosis, favoring the cytotoxic activity of other antitumor drugs. ET has also shown a lipid raft-mediated inhibition of the Akt pathway [28], and so, the same mechanism of cell sensitization might occur with this drug. Moreover, DOX and ET have been shown to boost the Fas/CD95-mediated apoptotic pathway by different mechanisms. DOX has been reported to upregulate Fas/CD95 expression dramatically [29], and ET activates Fas/CD95 intracellularly by death receptor recruitment in lipid rafts [30]. Thus, Fas/CD95 upregulation and subsequent activation could explain, at least in part, the above boosting antitumor effect detected here when both drugs DOX and ET are administered together. To sum up, our data suggest that the combination of DOX and ET is likely to improve the efficacy of the treatment of osteosarcoma.

5. CONCLUSION

DOX was successfully encapsulated in LN by the hot emulsion technique. The use of LN can reverse the acquired resistance of osteosarcoma cells to DOX, partly caused by the upregulation of P-gp in metastatic cells, by enhancing the drug cellular uptake and therefore its efficacy. Moreover, DOX acted synergistically with ET, in either their free or encapsulated forms, which implies that a future combination of these drugs may not only provide more effective treatment for osteosarcoma but also make it possible to reduce the individual drug doses required.

ACKNOWLEDGEMENTS

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CHAPTER 3

Therapeutic benefits of oral edelfosine lipid nanoparticles in pre-clinical osteosarcoma models.

CHAPTER 3

Therapeutic benefits of oral edelfosine lipid nanoparticles in pre-clinical osteosarcoma models.

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ABSTRACT

Purpose: The objective of the present study was to evaluate the *in vitro* and *in vivo* efficacy of edelfosine (ET), edelfosine loaded lipid nanoparticles (ET-LN) and doxorubicin (DOX) against osteosarcoma (OS) cells. The final objective was to establish the clinical relevance of ET-LN in metastatic OS animal models.

Experimental Design: The *in vitro* efficacy of the drugs was evaluated in HOS OS cells by means of viability assays, clonogenic assays and drug uptake assessment. Subsequently, the *in vivo* efficacy of the drugs was studied in two orthotopic murine models of human HOS and 143B OS.

Results: ET and ET-LN decreased the growth of HOS cells *in vitro* in a time and dose-dependent manner. However, both compounds did not affect the ability of tumour cells to form colonies *in vitro*. Interestingly, the uptake of ET and ET-LN was decreased when OS cells were pre-treated with DOX. *In vivo* investigations revealed that ET and ET-LN slowed down the primary tumour growth in both OS models. However, the combination of both drugs did not show any additional antitumour effect. In addition, ET-LN inhibited the metastases in an orthotopic OS model.

Conclusion: The data reported here show that orally administered ET-LN have an outstanding effect against primary and metastatic OS tumours and provide the proof of concept and rationale for further evaluation of these nanosystems to improve OS patient outcome.

Keywords: Lipid nanoparticles, osteosarcoma, edelfosine, orthotopic tumour, lung metastases, nanomedicine

Running title: Regression of osteosarcoma lung metastases using edelfosine lipid nanoparticles

1. INTRODUCTION

In 2017, the American Cancer Society estimates there will be 10,270 new cases of childhood and adolescent cancers, among which osteosarcoma (OS) leads the list of the most deadly, only preceded by leukemia in adolescents and leukemia and brain tumours in the pediatric population (1). Despite the encouraging prognosis for patients with localized OS achieved during the last decades, the five-year survival rate drastically drops to 15-30% for patients with pulmonary disease (2). Tumour resection together with chemotherapy allows for the successful control of localized OS; however the management of metastatic disease poses one of the main challenges for researchers and clinicians.

Nanomedicine is defined as the science and technology of diagnosing, treating and preventing disease using molecular tools and molecular knowledge of the human body (3). One of these molecular tools refers to nanoparticles that act as nanometric vehicles for drug delivery. Nanomedicine is considered as one of the most promising antimetastatic strategies for several reasons. First, drug delivery systems can be passively directed to the primary tumour due to the well-known enhanced permeability and retention effect. Moreover the surface of the nanocarriers can be modified for the active targeting of the primary focus. This targeting ability of nanoparticles allows for a higher dose of drug to reach the tumour area whereas indiscriminate toxicity is minimized. Second, nanoparticles can act directly on invasive cancer cells or re-educate the tumour microenvironment to avoid the outbreak of the metastatic cascade. Then, nanomedicines allow for the identification and treatment of circulating tumour cells (CTCs), averting the dissemination of metastatic potential cells. Finally nanomedicines can act in the metastatic host-tissue and prevent the colonization of a distal organ by tumour cells (4–6) (Fig. 1).

In the specific case of OS, different strategies, most of them based on the prolonged and sustained delivery of genes and drugs from drug delivery platforms, are being explored to restrain the progress of the disease and improve the overall survival of OS patients (7,8). Some of these strategies are based on reinforcing the immune response against metastatic cells with T cells or natural killer cells (9,10) inducing the activation of alveolar macrophages (11), characterizing the host-tissue tumour microenvironment to target pulmonary metastases or targeting surface antigens expressed in OS circulating cells (12-15).

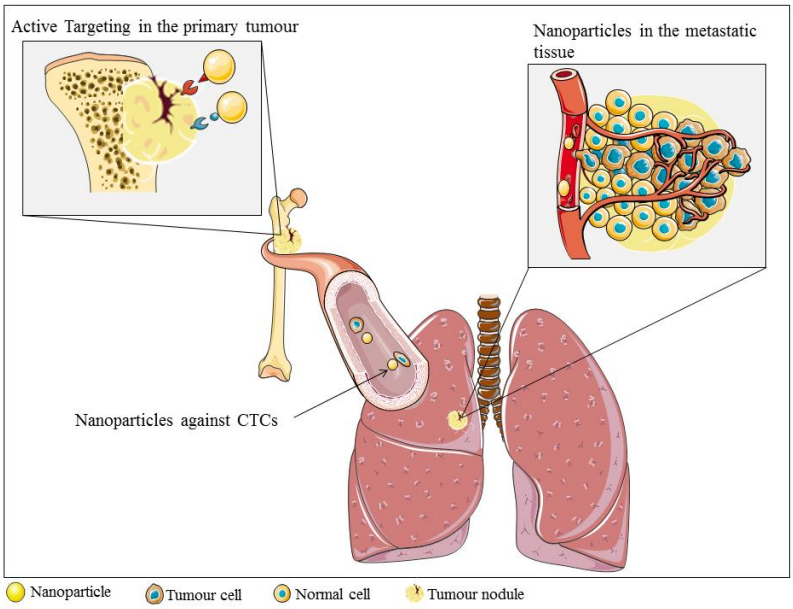

Figure 1: Nanomedicines against primary and metastatic osteosarcoma.

Previous findings from our group highlighted the *in vitro* efficacy of alkyl-lysophospholipid edelfosine (ET) and edelfosine encapsulated in lipid nanoparticles (ET-LN) against metastatic-patient derived OS cell lines (16). In addition, a synergistic effect of ET-LN and doxorubicin (DOX) against OS cells was also reported in *in vitro* experiments (17). With these precedents, in the present study we evaluated the efficacy of edelfosine in solution, ET-LN and its combination with commercial DOX in two OS orthotopic murine models established with HOS and 143B OS cells. The effect of the different treatments against the primary tumour and the metastatic disease was evaluated.

2. MATERIAL AND METHODS

2.1 Materials

Doxorubicin-hydrochloride was purchased from Sigma Aldrich (Madrid, Spain), edelfosine (ET) was obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland), and Precirol® ATO 5 was kindly provided by Gattefosse (Lyon, France). Tween® 80 was purchased from Roig Pharma (Barcelona, Spain), and other reagents for nanoparticle formulation were supplied by Sigma Aldrich (Madrid, Spain). Amicon® Ultra-15 10,000 MWCO filter devices

were provided by Millipore (Cork, Ireland) and all reagents employed for mass spectroscopy were of gradient grade for liquid chromatography and were obtained from Merck (Barcelona, Spain). For the *in vivo* assays commercial DOX (Farmiblastina®; DOX plus excipients) was obtained from the Clínica Universidad de Navarra (Spain). Ki67 monoclonal antibody from Leica Biosystems; reference NCL-L-Ki67-MM1 (Barcelona, Spain).

2.2 Preparation and characterization of edelfosine lipid nanoparticles

Edelfosine lipid nanoparticles (ET-LN) were prepared following the hot homogenization and ultrasonication method previously described by our group (18). Briefly, 30 mg of ET and 300 mg of Precirol® were melted 5 °C above the lipid's melting point (60 °C). Ten ml of the aqueous phase (Tween® 80 at 2% w/v) heated at the same temperature were added to the lipid phase and both phases were processed with the help of a Microson ultrasonic cell disruptor (NY, USA) for 4 min at 13 W. The formed emulsion was cooled in an ice bath to allow LN to solidify. LN suspension was centrifuged at 4,500 g, 30 min with Amicon® Ultra-15 10,000 MWCO filter devices and washed twice with water to remove the excess of non-incorporated drug and surfactant. Finally, the formulation was lyophilized using trehalose as cryoprotectant.

The nanoparticles were characterized in terms of size, polydispersity index (PDI) and ζ potential. For this purpose, LN were diluted 60 fold in double distilled water. The average particle size, PDI and ζ potential were analyzed by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, UK). ET entrapment into lipid nanoparticles was quantified by a previously validated ultra-high-performance liquid chromatography tandem mass spectrometry method (UHPLC MS/MS) (19).

2.3 Cell culture

HOS-MNNG (HOS) and 143B cell lines were purchased from the American Type Culture Collection and were used for the *in vitro* and *in vivo* assays. 143B and HOS cells were maintained in α-MEM, 10% of fetal bovine serum and D-MEM 10% fetal bovine serum respectively in an incubator at 37 °C and 5% carbon dioxide.

2.4 Cell viability assays

The cytotoxic activity of doxorubicin, ET and ET-LN against OS cells was evaluated via the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS). Briefly, 800 HOS cells/well were plated in 96 well-plates and allowed to attach. 24 h later, cells were exposed to increasing concentrations of drugs for 48 and 72 h. At that time, MTS reagent was added and the absorbance at 490 nm was recorded after 4 h. The half maximal inhibitory concentration values (IC_{50}) were calculated after adjusting the data to log (inhibitor) vs. response curve in GraphPad Prism Software and data were expressed as mean ± SD.

2.5 Clonogenic assays

To study the survival and colony formation ability of OS cells exposed to the IC50 dose of the afore-mentioned treatments, HOS cells were plated at a cell density of $1x10^5$ cells/well in a 6-well plate. Adherent cells were then treated for 72 h with ET, ET-LN, DOX or the combination of DOX with ET or ET-LN. Then, cell growth was determined by trypan blue exclusion assay and $1x10^3$ of resistant living cells of each condition were seeded in 6-wells plates. Resistant cells were cultured for 6-7 days and macroscopic colonies were counted at the end point with Crystal Violet (Merck). Experiments were repeated at least 4 times and results expressed as mean ± SEM.

2.6 Edelfosine uptake assay

$20x10^3$ HOS cells were seeded in 6 well-plates, allowed to attach and grow for 48 h. Subsequently, cells were exposed to a previously calculated non-lethal dose of DOX (5 nM) for 72 h followed by 1.25 μM of ET and ET-LN. 72 h later, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with methanol for their analysis. At the same time $50x10^3$ HOS cells were seeded in 6 well-plates. 24 h later cells were treated with 1.25 μM of ET and ET-LN during 72 h and washed and lysed for their analysis. ET content of the cells was quantified by a validated UHPLC-MS/MS method (19) and the amount of internalized drug was normalized to the amount of proteins in the sample quantified by the Lowry method (20). All experiments were repeated 4 times and results were expressed as mean ± SEM.

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2.7 Animal experiments

For both experiments 5-week-old female athymic nude mice were purchased from Harlan Laboratory (UK, Spain) and procedures involving animal handling and care were approved by the Animal Care and Ethics Committee of the University of Navarra (084-14) and the Home office in UK [PPL: 70/8967, Establishment license n°: 50/2509]. Mice were acclimatized for at least one week prior to experimental manipulation.

HOS -induced model of OS

HOS cultured cells were harvested and diluted in PBS to a final concentration of 500 x 10^3 cells per 10 µl. For the intratibial implantation of tumour cells, 10 µl of the cell suspension were injected through the medullar cavity of the tibia. Six days after, mice were treated with ET (per oral, 30 mg/kg, three times/week), ET-LN (same treatment regimen than ET), commercial DOX (intravenous, 2 mg/kg x 3 consecutive days every 21 days), the combination of commercial DOX and ET-LN or water as control. Mice weight was measured and tumour size was monitored twice a week using a caliper. Tumour volume was calculated from two diameters according to the formula [0.5 x a^2 x b]. In the equation, "a" represents the shorter (width) and "b" the longer dimension (depth) of the observed measures. Data were expressed as average of increase of tumour size (Δ tumour size: volume of tumour leg – volume of healthy leg) ± SEM.

At the time of necropsy, tibiae were preserved in 4% paraformaldehyde for microcomputed tomography imaging (micro-CT). Micro-CT analyses were carried out using a Skyscan 1172 x-ray-computed microtomography scanner (Brucker microCT, UK) equipped with an x-ray tube (voltage, 49kV; current, 200uA) and a 0.5-mm aluminium filter. Pixel size was set to 5.86 µm and scanning initiated from the top of the proximal tibia as previously described (21). To determine the effect of the different treatments on the tumour cell proliferation, Ki67 immunohistochemistry was carried out on 3 mm-thick deparaffinized sections of the tibiae. Immunostaining was performed overnight, at 4 °C, in a dilution 1:100 with PBS. Image J® software was used to analyze the results. Results were expressed as mean ± SEM.

143B-induced model of OS

The efficacy of ET and ET-LN (with the same dose and therapeutic regimen previously described) was analyzed in a 143B OS-induced model. Briefly, 200 x 10^3 tumour cells/10 µl PBS were inoculated with a Hamilton syringe and a rotating-like movement in the medullary

cavity of the tibia. The efficacy of the drugs was assessed monitoring the primary tumour growth with a digital caliper and by means of micro-CT images of the tumour-bearing legs. Histological visualization of the recovered lungs in order to evaluate the presence of microscopic metastases was also assessed.

2.8 Statistical analyses

In vivo data were analyzed using GraphPad Prism Software and 2 way-ANOVA followed by multiple comparisons test. Statistical significance was set at $P \leq 0.05$. For the immunohistochemistry analysis ANOVA statistic test was also applied.

3. RESULTS

Edelfosine-lipid nanoparticles, edelfosine and doxorubicin inhibit osteosarcoma cell proliferation

Prior to the *in vitro* efficacy assays, ET-LN were characterized. ET-LN presented a size of 124 ± 12 nm and a mean polydispersity index of 0.16 . The surface charge of the batch of particles used for the studies was -14.5 mV and the drug loading around 30 μg ET/mg of formulation. *In vitro*, ET-LN, ET and DOX inhibited HOS cells proliferation in a dose- and time-dependent manner (Table I). At 72 h, ET-LN induced the same cytotoxic activity against the two cell lines compared to the non-encapsulated drug, demonstrating the total release of the drug from the lipid matrix.

Table I: IC_{50}'s 48 and 72 h after the exposure of HOS cells to edelfosine (ET), edelfosine lipid nanoparticles (ET-LN) and doxorubicin (DOX) (n=3, mean ± SD).

		Edelfosine (μM)	Edelfosine lipid nanoparticles (μM)	Doxorubicin (nM)
48 h	**HOS-GFP**	6.2 ± 2.1	8.57 ± 3.45	232.6 ± 64.93
	HOS-MNNG	10.6 ± 4.42	21.37 ± 4.42	297 ± 94.06
72 h	**HOS-GFP**	2.72 ± 0.54	2.53 ± 0.83	55.58 ± 27.37
	HOS-MNNG	5.50 ± 1.39	3.95 ± 0.93	69.12 ± 53.56

Edelfosine-lipid nanoparticles and edelfosine do not alter the ability of osteosarcoma cells to form colonies

Regarding the clonogenic assays (Fig 2), HOS cells were treated with single agents or combination of ET or ET-LN and DOX at their corresponding IC_{50} doses [ET either free or encapsulated (2.5 μM); DOX (50 nM)]. As evident from figure 2A and 2B, OS cells exposed to ET and ET-LN were able to form colonies in a similar quantity to OS cells pre-treated with empty nanoparticles or maintained in culture without drug (control). On the contrary, OS cells plated after the exposure to DOX were not able to form colonies during the six days of culture. Finally, cells treated with the co-administration regimen lost the clonogenic ability of non-treated OS cells in a similar way to those treated with DOX alone.

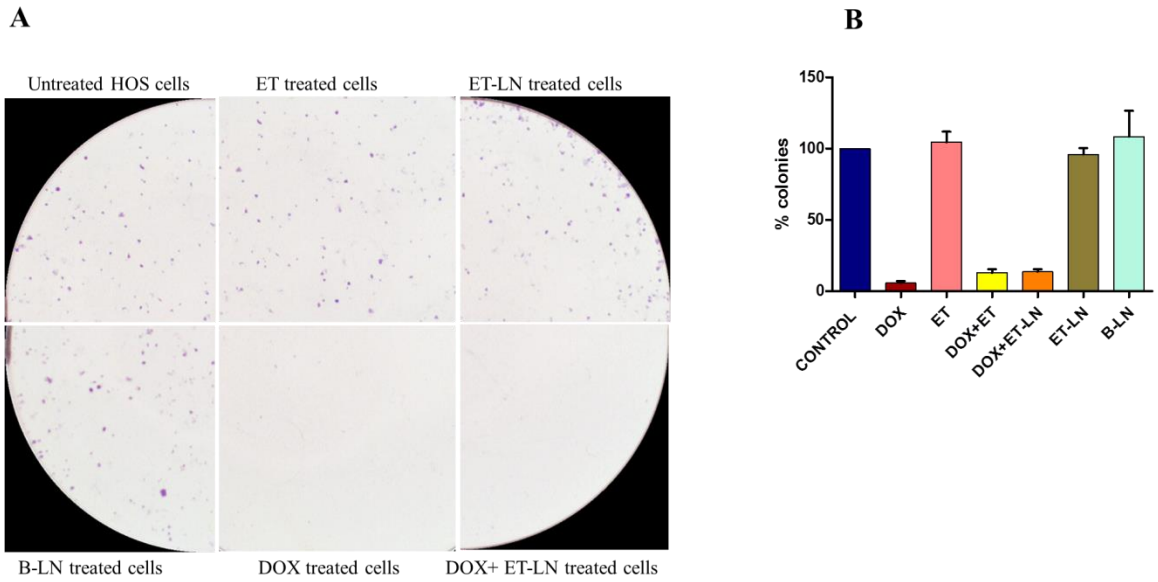

Figure 2: Edelfosine (ET) and edelfosine lipid nanoparticles (ET-LN) did not inhibit the colony formation ability of HOS cells. OS cells were treated for 72 h with ET, ET-LN, B-LN (empty LN), DOX (doxorubicin) or the combination of DOX with ET or ET-LN at their IC_{50} dose. 1x103 resistant living cells of each condition were cultured for 6-7 days and macroscopic colonies were counted at the end point with Crystal Violet. A) macroscopic colonies B) Histrogram representing percentage of colonies vs treatment (n= 4, mean + SEM).

The exposure of osteosarcoma cells to doxorubicin decreases the uptake of edelfosine in solution or edelfosine loaded into lipid nanoparticles

To determine whether the exposure of OS cells to DOX prior to an ET treatment affected the drug uptake, the amount of internalized ET in HOS cells with and without a pre-treatment

with DOX was quantified by an UHPLC/MS-MS method and normalized to the amount of protein quantified by the Lowry method. As depicted in figure 3, the pre-treatment of OS cells with 5 nM DOX reduced by half the uptake of ET and up to three times the uptake of ET-LN.

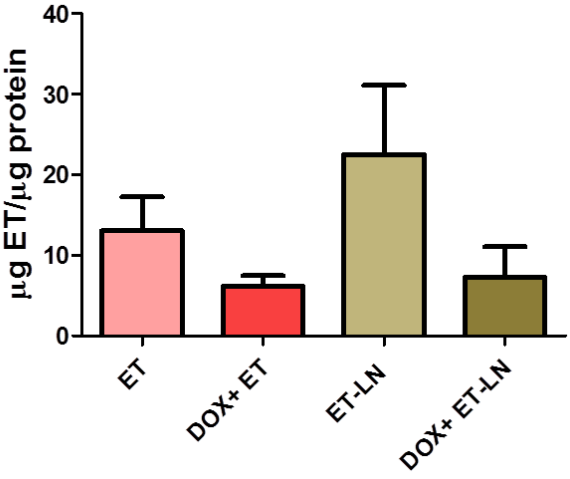

Figure 3: Edelfosine (ET) and edelfosine lipid nanoparticles (ET-LN) internalization was decreased after the exposure to doxorubicin (DOX). HOS cells were exposed to 5 nM DOX for 72 hours followed by 1.25 µM of ET and ET-LN. 72 h later cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with methanol for their analysis. ET content of the cells was quantified by UHPLC-MS/MS and normalized to the amount of proteins in the sample quantified by the Lowry method. The graph displays the µg of ET internalized in each condition per mg of protein (n=4, mean + SEM).

Edelfosine-lipid nanoparticles and edelfosine delay osteosarcoma primary tumour growth in a HOS orthotopic model and preserve the bone microarchitecture

The therapeutic potential of ET, ET-LN, DOX and the combination of DOX with ET-LN was evaluated in an orthotopic OS tumour model. At day six after tumour cell inoculation mice were randomly assigned to their corresponding group of drug, dose and treatment regimen. No apparent sign of toxicity (e.g. loss of body weight) was observed during the course of the experiment.

Figure 4A illustrates the growth of the primary tumour during the course of the study. As plotted in the graph, DOX and the combination of DOX with ET-LN did not show any significant anti-tumour effect compared to the control group. On the contrary, ET and ET-LN considerably delayed the appearance and growth of the primary tumour.

As expected, micro-CT images revealed a marked ectopic bone formation (pink mark) and tumour-associated osteolysis (yellow mark) in the non-treated mice (fig. 4B). These lesions were similar or even more aggressive in the combined and DOX treated group. On the contrary, ET treated mice showed some regions with apparently less bone density (white mark), but mice treated with ET and ET-LN presented smaller lytic lesions and less ectopic bone formation than the rest of the group. Interestingly, we did not observe any impact on healthy bones. Neither the trabecular nor the cortical bone was affected by the treatments.

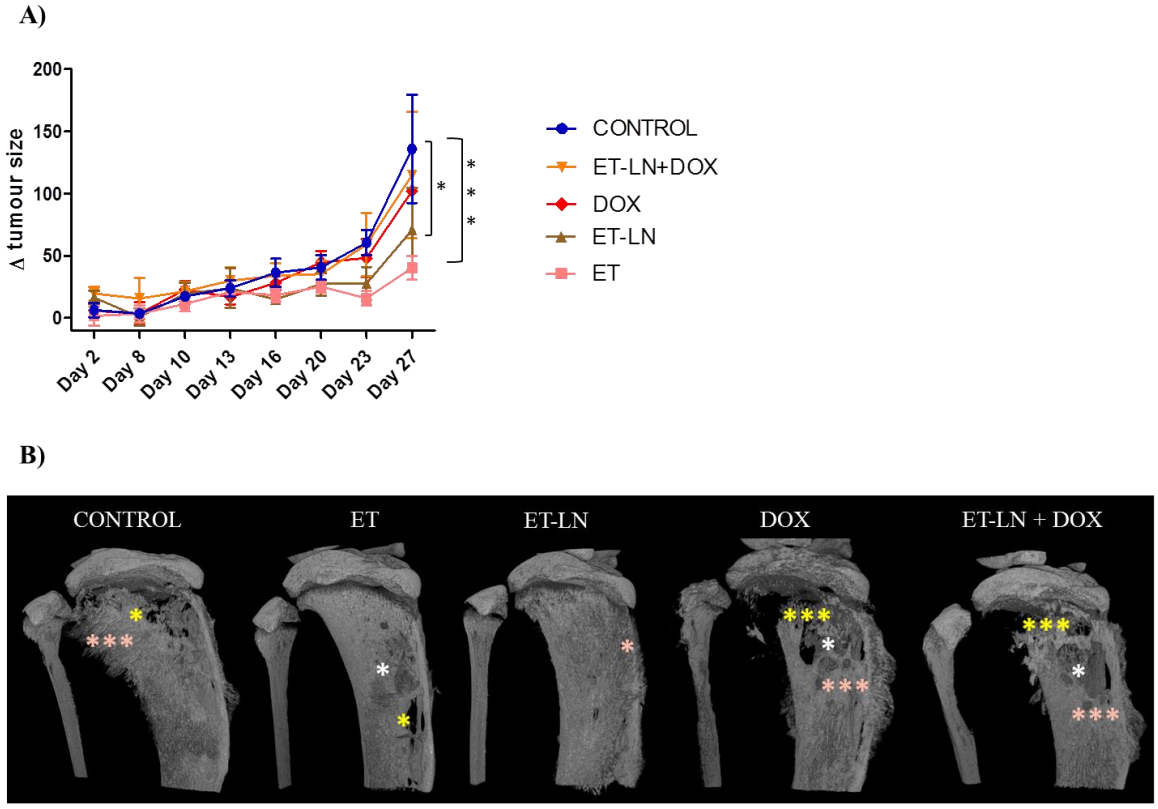

Figure 4: Edelfosine (ET) and edelfosine lipid nanoparticles (ET-LN) exhibited an anti-tumour effect in an HOS-osteosarcoma induced model. A) Effect of ET (per oral, 30mg/kg, three times/week), ET-LN (per oral, 30mg/kg, three times/week), doxorubicin (DOX, intravenous, 2 mg/kg x 3 consecutive days every 21 days) and their combination in primary OS tumour growth (n=5, mean + SEM). *: $p \leq 0.05$, ***: $p \leq 0.001$. B) Representative micro-CT images of the tumour-bearing tibia for each treatment group. Pink asterisks: ectopic bone formation, yellow asterisks: osteolytic regions and white asterisks: regions with reduced bone density.

To determine the potential impact of the various treatments on OS cells *in vivo*, Ki67 immunostaining was performed to assess the proliferative index (Figure 5). ET and ET-LN treated mice presented considerably fewer proliferating tumor cells (ANOVA, multiple

comparison Dunnett's test. $p<0.05$) than untreated mice or those treated with DOX and the combination of DOX with ET-LN.

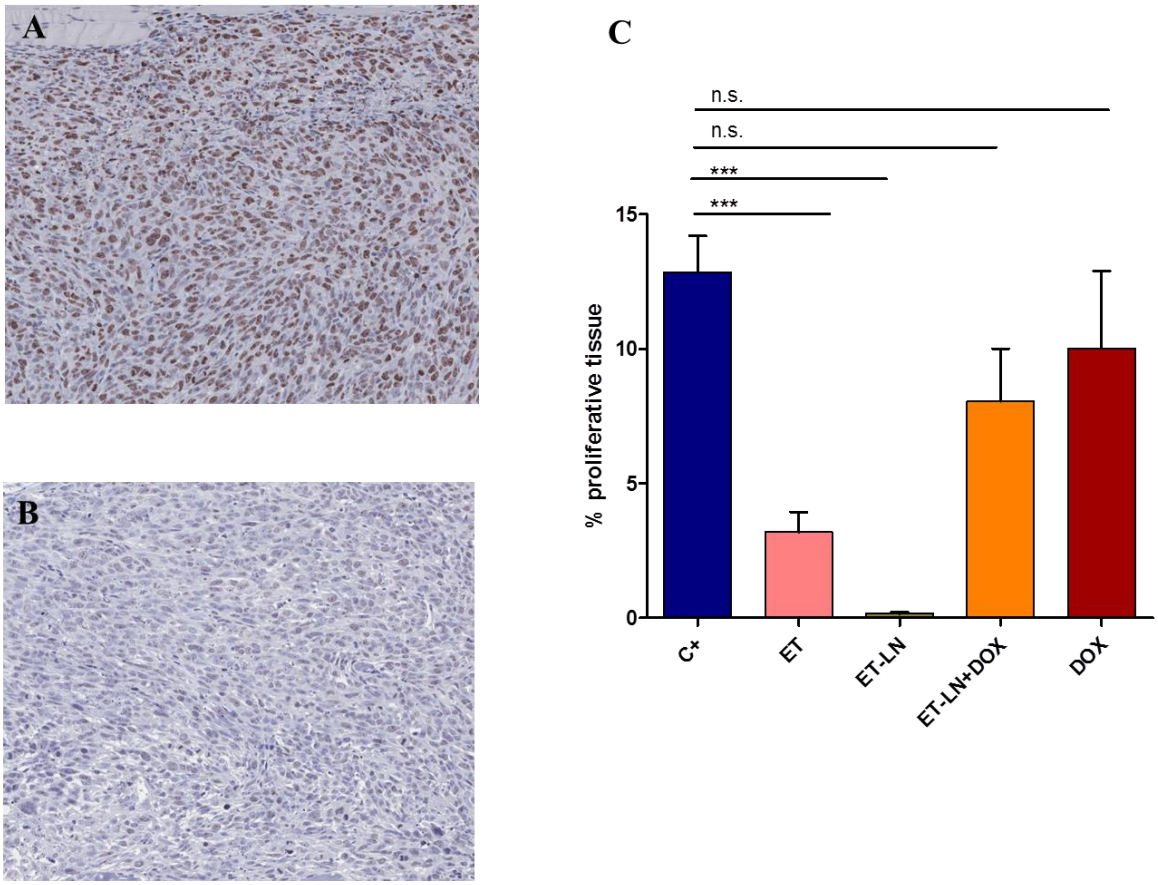

Figure 5: Edelfosine lipid nanoparticles (ET-LN) decreased the number of tumour proliferating cells. Ki67 immunohistochemistry of the treated tibiae was carried out on 3 mm-thick deparaffinized sections. Positive immunostaining was quantified using Image J® software. ET, edelfosine. ET-LN, edelfosine lipid nanoparticles; DOX doxorrubicin. A) representative image of an untreated tibia B) representative image of an ET-LN treated tibia C) Histogram representing the percentage of stained (proliferative) tissue vs treatmet (n=5, mean + SEM).

Edelfosine-lipid nanoparticles inhibited the primary tumour growth of 143B-orthotopic osteosarcoma mice and caused the regression of lung metastases.

The therapeutic benefit of ET and ET-LN was also assessed in a 143B orthotopic OS model. As illustrated in Figure 6, both treatments were equally effective 20 days after tumour cells implantation. However after three weeks, mice treated with ET presented an exponential tumour growth whereas the tumours of those mice treated with ET-LN displayed a significantly much slower growth. At the end point of the experiment ET significantly delayed

the primary tumour growth compared to the non-treated mice ($p<0.001$). Nevertheless, when the drug was administered loaded in lipid nanoparticles an almost complete regression of the primary tumour was observed, with a median increase of tumour size 3.3 times lower than in the control group ($p<0.001$).

The presence of microscopic metastases in the lungs was further examined by histology. Eight out of nine mice in the control group developed multiple lung metastases, similar to mice treated with ET. Interestingly, only 1/10 mice treated with ET-LN was found to have a single metastatic nodule, evidencing the huge potential of ET-LN as anti-metastatic agent against OS.

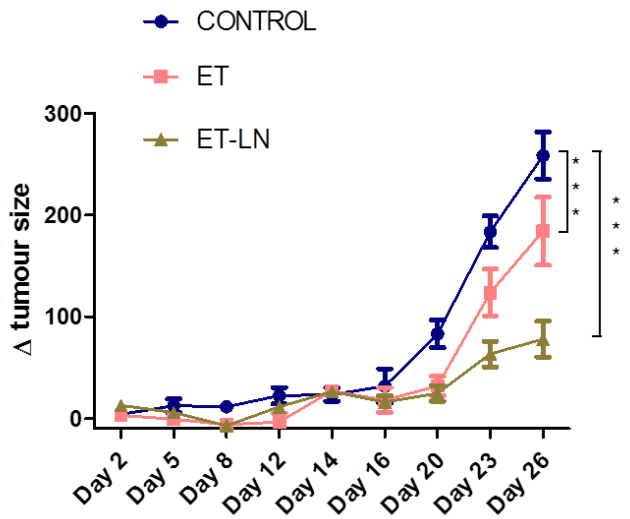

Figure 6. Edelfosine (ET) and edelfosine lipid nanoparticles (ET-LN) exhibited an anti-tumour effect in a 143B-osteosarcoma induced model. Effect of edelfosine (ET) and edelfosine-lipid nanoparticles (ET-LN) in primary OS tumour growth induced by 143B cells. ***: $P \leq 0.001$, (n=9, mean + SEM)

4. DISCUSSION

The great advances in therapeutic protocols for OS over the last thirty years have considerably improved the five-year survival rate for patients with a localized tumour (22). This survival improvement among non-metastatic patients has led to the establishment of a standard of care that is insufficient for patients with metastases, considering their poor outcome. Focusing on those patients, new therapeutic protocols including different drugs or therapeutic strategies are mandatory. Given our previous findings, ET holds promise for the

treatment of OS. This alkyl-lysophospholipid showed an apparent selectivity to cancer cells, and especially metastatic cells, due to their higher content in lipid rafts that mediate the drug uptake (16). However, orally administered ET presents a severe gastrointestinal toxicity that hampers its clinical use (23). For that reason we encapsulated ET into lipid nanoparticles and observed that its cytotoxic activity was maintained *in vitro* (16). Moreover, when OS cells were treated with ET and DOX a synergistic effect in their cytotoxic activity was observed (17). In this line, in the present study the efficacy of ET, ET-LN, DOX and the combination of DOX and ET-LN was evaluated in two orthotopic OS murine models (HOS and 143B).

First, the cytotoxic activity of DOX, ET and ET-LN was confirmed in two HOS-derived OS cell lines: HOS and HOS-MNNG. Table I reflects the time and dose-dependent efficacy of DOX, ET and ET-LN against OS cells. The IC_{50} values of all the compounds at 72h were comparable to those obtained previously for the OS-metastatic 595M patient-derived cell line and the U2-OS cell line, with values in the micromolar range for ET and ET-LN and in the nanomolar range for DOX (16,17). Furthermore, the cytotoxic activity of ET was maintained when the drug was encapsulated into lipid nanoparticles, meaning that at 72 h the entire drug loaded into the particles was released and internalized by the cells and exerted exactly the same cytotoxic activity as ET in solution. Since not only the direct cytotoxicity but the long-term toxicity ought to be taken into account when evaluating a new drug, clonogenic assays were performed with the different treatments.

ET, either free or encapsulated, did not inhibit the clonogenic ability of OS cells. Similar observations were previously reported by Lohmeyerl and Workman in other cancer cell types (24). These authors showed that antitumour lipids (ATL) such as ET can act as cytostatic or cytotoxic agents depending on the administered dose. Concentrations less than 5 μM in the human promyelocytic cell line HL-60 (which present a similar sensitivity to ET than OS cells) caused an accumulation of cells in M phase, that is to say, a cytostatic effect. However, with concentrations higher than 5 μM the cytotoxic effects described for ATL were observed. As suggested, the cytostatic effects of ATL are due to the rapid equilibrium established between the drug in the plasma membrane of the cells and the proteins of the serum. In our experiment, OS cells were treated with 2.5 μM of ET (IC_{50} dose for HOS cells); consequently, ET may have been eliminated from the cell membrane, and not have exerted its anti-tumour action. On the other hand, cells treated with DOX or the combination of DOX with ET-LN, were unable to form colonies after being exposed to the drugs. The intercalation of DOX in the nuclear DNA may have avoided the proliferation of the resistant cells living after the exposure to the drug, and so in this case DOX did behave as an anti-clonogenic agent.

Once the *in vitro* cytotoxic effects of ET, DOX and ET-LN against HOS cells were known, their *in vivo* efficacy against an OS murine model induced by the same cell line was assessed. As depicted in figure 4A, only those animals treated with ET and ET-LN presented a significant decrease in the final tumour volume compared to the untreated mice. In contrast, DOX and DOX plus ET-LN did not show any antitumour effect. Regarding the apparent loss of efficacy of ET-LN when combined with DOX, the pharmacodynamic interactions of these two compounds administered at the same time might result in the observed antagonism *in vivo*.

To gain further insight into these possible pharmacodynamic interactions *in vitro*, the influence of DOX exposure to OS cells on ET uptake was evaluated. Although previous findings of our group revealed a synergistic effect *in vitro* of DOX and ET combined at their IC_{50} dose (17), as seen in figure 3, when OS cells were pre-treated with a non-lethal dose of DOX, the amount of ET internalized by the cells was substantially diminished. It is evident that the drug dosages determine the synergy and drug uptake in OS cells; however, the influence of drug sequence administration might play a pivotal role, as was previously suggested by Neville-Webbe et al (25). In order to have a maximal apoptosis ratio in breast cancer cells, these authors concluded that a drug sequence of DOX and zoledronic acid with a 24 h interval was necessary. These results were confirmed in a spontaneously occurring mammary tumour model (26). In the light of these precedents a different sequencing of the drugs in our experiment could have improved the efficacy of the combined treatment.

All these results extracted from the continuous monitoring of the primary tumour growth were confirmed at the end of the experiment by micro-CT analyses of the tibiae. As shown in figure 4B, ET and ET-LN treatments preserved the integrity of the tibiae to a greater extent than DOX and DOX plus ET-LN, confirming the suitability of these treatments against primary OS. Moreover, a marked proliferation of tumour cells was observed in the tibiae of mice treated with DOX and DOX plus ET-LN whereas the percentage of proliferative tissue in mice treated with ET and ET-LN was substantially smaller (fig 5).

Subsequently, and due to the well-known metastatic properties of 143B OS cells, the efficacy of ET and ET-LN (the two treatments that were effective in the previous mouse model) was assessed in an orthotopic model of OS induced by the intratibial inoculation of 143B cells. As depicted in Fig. 6, both treatments successfully slowed the progression of the disease. Beside, mice treated with ET-LN presented minimal tumour growth during the course of the experiment with a five-fold reduction in tumour volume at the end-point. The anti-metastatic activity of the two treatment modalities could be evaluated given the high efficiency of 143B

cells to spontaneously disseminate from the primary tumour to lungs (8/9 untreated mice presented metastases). ET in solution did not show any anti-metastatic effect, with only 2 mice out of 10 being free from metastases. On the contrary, all the mice except for one treated with ET-LN were free from metastases at the end point of the experiment. As mentioned in the introduction section, one of the main advantages of the use of nanomedicines for cancer treatment is the ability of these nanocarriers to identify and battle CTCs. This, together with their ability to act on invasive cancer cells from the primary tumours and/or to modify the metastatic host-tissue could have led to this markedly antimetastatic effect of ET loaded into lipid nanoparticles.

5. CONCLUSION

The results compiled in the present article led the authors to propose ET, and more specifically, ET encapsulated into LN, as a potent alternative to conventional treatments for metastatic osteosarcoma patients. ET-LN showed an immediate cytotoxicity against HOS cells. However, ET-LN showed no long-term toxicity evaluated by mean of the clonogenic assays, probably due to incorrect selection of the dose used to treat the cells. *In vivo*, ET and ET-LN were able to slow the progression of the primary tumour growth in HOS and 143B orthotopic OS models. Besides, ET-LN successfully avoided the metastatic spread of 143B OS cells from the primary tumour to lungs.

ACKNOWLEDGEMENTS

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GENERAL DISCUSSION

GENERAL DISCUSSION

Osteosarcoma (OS) is the most common primary bone malignancy diagnosed in the paediatric population. It usually appears during the first or second decade of life in the metaphyseal area of long bones. Despite this apparently localized origin, it is assumed that 80% of patients present microscopic metastasis in the lungs at the time of diagnosis that, if not treated systemically, will evolve to macroscopic ones (1). For this reason current treatment for OS therapy involves primary tumour resection and multiple cycles of systemic chemotherapy. Due to the lack of specific treatments for patients with early metastasis, the five-year survival rate in these patients is close to 30% (2). Within this context new therapeutic options including new drugs and drug delivery strategies are being explored, and some of them implemented, for the treatment of the disease (3).

Nanomedicine,has emerged as one of the most promising anti-cancer strategies. Since the early approval in 1995 of the doxorubicin-liposomal formulation Doxil®/Caelyx (Jansenn)for Kaposi's sarcoma, the "Food and Drug Administration" (FDA) has approved more than half a hundred new nanomedicines and almost 80 are currently in clinical trials (4). The rise of this technology applied to cancer is due to several reasons. First, drug delivery systems provide protection to the loaded compounds. Then they target the affected areas and therefore reduce the systemic toxicity of the drugs, and in consequence, they enhance the efficacy of the drugs. Moreover, particularly lipid nanoparticles (LN) can be orally administrable, enhancing the oral bioavailability of certain compounds and enhancing patient welfare (3). Prostate cancer, ovarian cancer, lymphoma or leukaemia are some of the indications for the drug delivery systems approved by the FDA; and one of the latest incorporation to this list has OS with the liposomal-muramyl tripeptide phosphatidylethanolamine (L-MTP-PE)(**Annex 1**, section 5) (3,4).

Owing to the aforesaid advantages of drug delivery, in the present project we have focused on two main strategies: i) the use of new drugs, such as edelfosine (ET), or new combinations of drugs (ET and doxorubicin, DOX) for OS treatment and ii) the inclusion of anti-osteosarcoma agents in drug delivery systems (more specifically LN) to target cancer cells. These results have been described in three main chapters. The first one describes the preparation, characterization and *in vitro* evaluation of methotrexate-lipid nanoparticles (MTX-LN) and edelfosine-lipid nanoparticles (ET-LN). The second chapter involves the preparation, characterization and *in vitro* evaluation of doxorubicin-lipid nanoparticles (DOX-LN), and in the third chapter the *in vivo* efficacy of the compounds is assessed.

In **Chapter 1** and aiming to reduce the clinical doses of MTX needed to be effective and the need for leucovorin to rescue non-tumour cells (**Annex 1** section 4.2.1.), MTX-LN were developed.

LN were prepared by the hot emulsion and ultrasonication method, an easy and time saving process that requires the solubilisation of the drug in the lipid phase for the LN production. The first MTX-LN formulation developed presented an average size of 113 nm, acceptable PDI (less than 0.3) and a highly negative ζ potential. However, the amount of drug loaded per mg of particle, that is to say the drug payload, was limited to 12 μg MTX/mg of lyophilized LN. This payload was insufficient (given the low sensitivity of OS cells to MTX (5)) to treat OS cells and therefore several parameters of the initial formulation were optimized, namely: the amount of lipid and drug content, the solvent employed to dissolve the drug in the lipid phase and the surfactant. Despite the increased size obtained in the optimization process (LN presented an average size of 273 nm and PDI 0.2), the drug payload was doubled and the organic solvent used to dissolve MTX was replaced by a non-organic one, NaOH.

Once MTX-LN were prepared and optimized, the *in vitro* efficacy of MTX and MTX-LN against OS ATCC® commercial and primary (B) and metastatic (M) patient-derived OS cells was assessed (Department of Paediatrics, Clínica Universidad de Navarra).

As for MTX and the cells derived from patient 595, the primary cell line (cells derived from the biopsy of the primary tumour) was more sensitive to the cytotoxic action of the drug than the metastatic cell line (higher IC_{50} value for 595M than for 595B cells). However, those cells derived from patient 588 responded inversely, the drug being more effective against the metastatic cell line.

To clarify the genetic and epigenetic transformations responsible for the different behaviour of the drugs in individual OS patients was not the main goal of our work. However, the literature has described some of the genetic transformations that influence drug bioavailability. These are i) the overexpression of dihydrofolate reductase (DHFR), target enzyme of MTX and ii) the impaired transport of the drug due to the decreased expression of folate receptor carriers (RFC).

In order to study the expression of the genes involved in MTX resistance, semiquantitative real-time PCR was performed. Concerning DHFR expression, patient 595 presented higher levels of the enzyme in metastatic cells than in primary ones. This increased level of the target enzyme was correlated with the decreased efficacy of MTX in metastatic *vs* primary

cells. In line with this result but with the opposite effect, patient 588 appeared to present innate resistances to the drug as both the primary tumour and the normal bone tissue overexpressed DHFR. For this patient the metastatic process entailed a decreased expression of DHFR. This result was consistent with the IC_{50} values for patient 588, with lower IC_{50}'s (and therefore higher efficacy) for metastatic than primary cells. Concerning RFC, a pool of 18 OS cells samples was analysed; however, and in agreement with clinical practice, we could not detect the gene expression in any of the samples.

Brief, although the response of a given tumour cannot be simplified to the expression of a couple of genes, it is evident that these genes may contribute to the drug-resistance acquired by tumour cells. Besides, any attempt to dig deeper into tumour behaviour may lead to great progress in the search for personalized medicine.

Although it was already well-known, confirming the absence of RFC in our patient-derived cell lines corroborated the suitability of MTX encapsulation for OS treatment, since the drug in LN would be internalized by an endocytic process independent of transporters (6). Therefore, once MTX was formulated in LN we moved on to the evaluation of the cytotoxic effect of the loaded drug. Unfortunately, the efficacy of MTX-LN could not be successfully evaluated. Due to the high doses of drug required to treat OS cells and the static conditions of conventional protocols in 96-well plates, MTX-LN precipitated and asphyxiated the cells, hampering the evaluation of the cytotoxic activity of the loaded compound.

Aiming to overcome the limitations of traditional assays, Mitxelena-Iribarren *et al* developed a microfluidic device optimal for cell culture under dynamic conditions (**Annex 2,** here figure 1A-B). This micro device allowed us to keep the LN in suspension thanks to the recirculation of cell media and to evaluate their cytotoxic effect. As seen in figure 1C, the cells attached to the bottom of the device were not affected by the recirculation of cell media and grew normally at the flow rate studied. However, if cells were exposed to 11 μM of non-loaded LN, a slight variation in cell growth was observed, probably due to increased stress produced by the LN to the cells. Cells were barely affected by the circulation of 11 μM MTX (as previously discussed, the IC_{50} dose for OS cells was in the millimolar and not micromolar range). On the contrary, when OS cells were exposed to the same dose of MTX-LN cell growth was inhibited and cell death was observed.

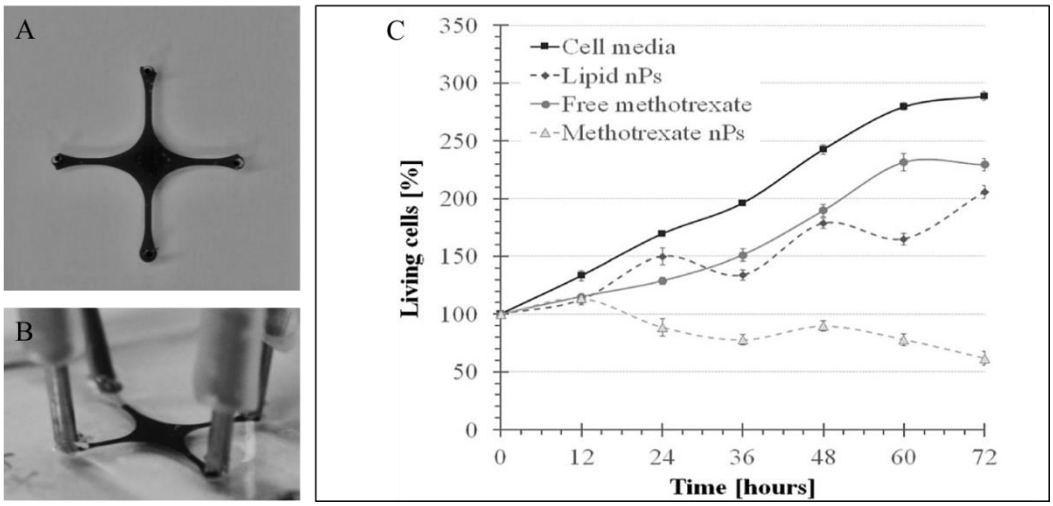

Figure 1: Microfluidic device for the evaluation of the cytotoxic activity of MTX-LN. A) Optimized microfluidic platform. B) Real glass microfluidic platform with the chosen final geometry filled with ink and with the corresponding connectors. C) Percentage of living cells when exposed to the different treatments. (In this image nanoparticles are referred to as nPs).

As previously described, low doses of MTX cannot enter OS cells due to the impaired transport via RFC. However when the drug is loaded into LN a different endocytic uptake non-mediated by RFC may occur, leading to the observed anti-tumour effects.

Concerning ET efficacy against the patient-derived cells we observed that OS cells were more sensitive to the action of the alkyl-lysophospholipid than MTX, with IC_{50} values in the micromolar and not millimolar range. These different drug sensitivity profiles of OS cells could indicate that ET could be administered at lower doses to be as effective as MTX, which is a major advantage in clinical practice. Moreover, ET efficacy was greater in 595 and 588 patient-derived metastatic cells compared to primary ones, confirming the suitability of ET for patients with metastatic disease. Our next step was therefore, to explore why ET was more effective in metastatic cells.

Lipid rafts are membrane micro-domains implicated in the regulations of signalling circuits, cell survival, proliferation and migration (7). Many authors have reported that metastatic cells present in their membranes more cholesterol due to their need for migration. This cholesterol is organized in membrane lipid micro-domains, named lipid rafts (8–11). At the same time, it is known from the literature that ET enters the cell via lipid rafts, triggering the extrinsic apoptotic pathway of cell death (12–14). Consequently, we hypothesized that ET may have been more effective in metastatic cells due to a higher internalization via lipid rafts.

In order to confirm our hypothesis, lipid microdomains were visualized by the labelling with cholera-toxin-B subunit (Sigma Aldrich). As expected, metastatic cells presented numerous patches of lipid rafts while only scarcely perceptible spots of labelled rafts were seen in primary cells. Besides, disrupting these membrane microdomains with methyl-β-cyclodextrin, a cholesterol depleting agent, the internalization of ET was decreased in metastatic cells, confirming the key role of lipid rafts in the drug uptake. This implication of lipid rafts in ET uptake has also been previously described for other types of cancers such as leukaemia, lymphoma and multiple myeloma (15,16).

Finally, when ET was loaded in LN all the treatments became more effective than, or at least, as effective as ET in solution against metastatic and primary-patient derived OS cells. Drug uptake studies revealed that when the drug was loaded into LN it was internalized to a greater extent than the free drug, boosting the cytotoxic activity of the alkyl-lysophospholipid. This result is in agreement with those for other cancer cell lines in which the encapsulation of ET in LN improved its internalization and boosted the cytotoxic activity of the drug (15).

Our findings in chapter 1 encouraged us to keep working with ET and ET-LN as potent candidates for OS treatment; however, due to the low sensitivity of OS cells to MTX, we dismissed working *in vitro* with this folate analogue.

Our second key drug to encapsulate was the cationic anthracycline DOX. DOX is a chemotherapeutic agent widely used for the treatment of several types of cancer like breast cancer, leukaemias, Hodgkin's lymphoma, etc. (17). As for OS, it is an essential component of most regimens, but its use is limited by its severe cardiotoxicity (18). In order to minimize the clinical doses of drug used for OS treatment and its associated side effects, several strategies were explored in **chapter 2**. On the one hand, with the goal of minimizing the drug's side effects, DOX was encapsulated into LN and its efficacy was evaluated against OS commercial and patient-derived cell lines. On the other hand, based on published synergisms of DOX with ET or its analogue persifosine in Ewing Sarcoma and OS cells (19,20), whether the *in vitro* combination of DOX and ET could boost the efficacy of the drugs was also evaluated.

DOX-LN were prepared by three different methods, namely, a double emulsion method, single emulsion solvent evaporation method and the hot melting homogenization technique. The particles produced by the last method were the smallest (around 100 nm) and together with those produced by the single emulsion method, encapsulated the largest amount of drug.

Nevertheless, due to the avoidance of organic solvent more appropriate for paediatric patients' safety, the hot melting homogenization procedure was chosen for the LN preparation. DOX-LN presented an average size of 95 nm, PDI of 0.19 and a drug payload of 2.57 μg of DOX/mg of lyophilized particle.

After LN were developed, their biological activity, together with that of free DOX, was evaluated against OS commercial and patient-derived cells 595M. Furthermore, due to the rapid growth and delayed diagnosis of OS metastases, the cytotoxic activity of DOX against 595M cells was studied in different passage number, namely, late passage cells (above 20) and early passage cells (bellow 10).

Focusing on metastatic patient-derived cell lines, a reduction of DOX activity was observed after 10 to 15 cellular passages, with increasing IC_{50} values from 55 to 255 nM. Moreover, when studying the internalization profiles of free DOX in early and late 595M passage cells, a correlation between the aforementioned efficacy and the drug's internalization was observed. This reduction in the intracellular drug concentration and the cytotoxic effect of the anthracycline is widely known in the clinics as one of the defence mechanisms acquired by cancer cells to favour the progression of the disease (21). However, when the drug was loaded into LN, these resistance mechanisms were not observed. The IC_{50} value for 595M early and late passage cells remained constant independently of the cellular passage, as well as the drug's internalization quantified by UHPLC-MS/MS and observed by fluorescent microscopy.

Taking all this together, the resistance mechanism of OS metastatic cells that seemed to be responsible for the loss of efficacy of free DOX was successfully reverted when the drug was loaded in LN. Besides, although the encapsulation did not improve the efficacy of the free drug in early metastatic cells (IC_{50} of DOX in early metastatic cells: 55 nM *vs* 109 for DOX-LN), it did increase the efficacy of DOX when OS cells become resistant (IC_{50} of DOX for late metastatic cells: 255 nM *v*s 107 for DOX-LN), which is more likely to be the case in clinical practice.

One of the most common resistance mechanisms acquired by cancer cells is the overexpression of membrane efflux pumps such as the multidrug resistant P-glycoprotein (P-gp). These molecules decrease the intracellular concentrations of the chemotherapeutics to sub-toxic levels decreasing the chance of providing successful treatments against the disease (21). To further evaluate if the overexpression of P-gp was partially responsible for the

decreased efficacy of DOX as passage number increased, real time semiquantitative PCR analyses of ABCB1 gen were assessed.

The results obtained revealed an almost four-fold increase in P-gp expression from passage 3 to 30. This fact strongly confirmed our hypothesis that metastatic cells were becoming resistant to the non-loaded anthracycline as passage number increased. Importantly, when DOX was encapsulated into LN this overexpression did not affect the drug's uptake and efficacy. This fact was attributed to the surfactant employed for the LN preparation. As described by other authors, Tween® 80 is a potent modulator of P-gp activity (22,23). Its mechanism of action is still controversial. Several authors have reported that the non-ionic surfactant increases the plasma bilayer fluidity preventing the interaction of drugs and efflux pumps while others report the creation of a hydrogel bone with the protein that hampers the recognition of the substrates by P-gp (23,24). Whatever its mechanism of action may be, we could observe how the encapsulation of the drug in LN containing Tween® 80 was able to revert the acquired resistance of late passage metastatic cells, increasing the drug's uptake and therefore, its efficacy.

Once the improved efficacy of the drug when loaded into LN was known, we evaluated whether its combination was synergistic with ET. Previous findings in the bibliography revealed that perifosine (ET analogue) enhanced DOX-induced anti-osteosarcoma activity (20). Furthermore, Bonilla *et al* reported synergistic effects of ET and DOX against Ewing's sarcoma cells (19). Within that context and following the Chou-Talalay method, drug combination studies against 595M-patient derived cells were performed. Brief, cells were exposed to individual and combined drugs, free and encapsulated into LN, at equipotent ratios [$(IC_{50})_{DOX}$: $(IC_{50})_{ET}$] and cell viability data were processed with CompuSyn Software for their analysis. Combination Index (CI) values given by the software revealed that for either early or late metastatic cells, the combination of DOX and ET free or encapsulated, was synergistic.

Subsequently, and to gain further insights into cell death mechanism, the expression of caspases 3 and 7 was studied. DOX and ET administered free or encapsulated in LN, alone or in combination, were able to promote caspase-dependent apoptosis. Interestingly when the drugs were combined, the caspase level increased compared with when they were given alone, suggesting that cell death was partially mediated by caspase activation.

Several theories could explain the synergies observed between these drug combinations. On the one hand, as suggested by other authors, their different cellular targets and mechanisms

of action could boost the efficacy of the drugs alone (19). While DOX exerts its action through the nucleus (17), ET targets the membrane lipid rafts of the cells, endoplasmic reticulum and mitochondria (25). On the other hand, the synergistic effects observed between the drugs could be due to the increased sensitivity of OS cells to DOX, due to the sensitizing action of ET. As is well known, OS cells overexpress the pro-survival Akt-mTOR signalling pathway (26). ET, similar to other alkyl-lysophospholipids, is able to trigger a lipid-raft-mediated Akt-mTOR inhibition that would decrease the apoptotic threshold of OS cells, making them more sensible to the action of DOX (25,27). Finally, DOX has been reported to upregulate the expression of Fas/CD95 receptor (28). At the same time, ET's main mechanism of action involves the activation of Fas/CD95 by its recruitment into lipid rafts, which activates the extrinsic apoptotic pathway (29). Therefore, the upregulation of the receptors and their recruitment into lipid micro-domains could explain, at least in part, the boost of efficacy observed when combining the two drugs.

All the previously commented results concerning the encapsulation and efficacy of DOX and ET, led us to propose DOX-LN as well as the co-treatment of DOX and ET as a new therapy for OS. For that reason, in chapter 3, we evaluated the anti-osteosarcoma efficacy of the treatments in OS murine models.

Prior to the efficacy evaluation of DOX-LN in the OS murine models, a pilot study of the pharmacokinetic characteristics of the loaded drug was performed (**Annex 3**). Following the Body Surface Area (BSA) formulae for the *in vivo* extrapolation of clinical doses to animals (30,31), mice with OS should be treated with 7.5 mg DOX/kg of weight during three consecutive days every 3 weeks (figure 2 for the calculations).

After these calculations, a pilot pharmacokinetic study of a single dose of commercial DOX (farmiblastina®) and DOX-LN 7.5 mg/kg was performed. A C_{max} of 2180 ± 848 ng DOX/mL of plasma was obtained 5 minutes after the single intravenous administration of the commercial drug to healthy nude mice. Most notably, mice receiving the same dose of DOX loaded in LN by the oral route did not present any plasma level of drug.

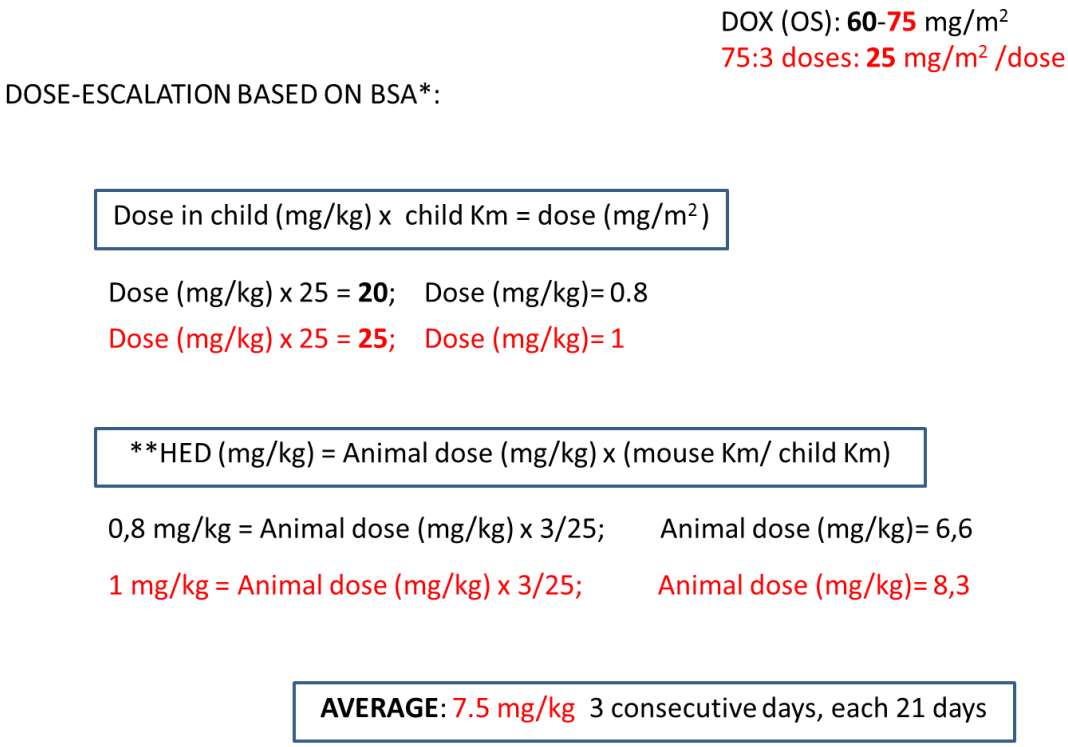

Figure 2: Dose Escalation from human child to mice**.** Highlighted in red are the data used if considered 75 mg/m^2 as the therapeutic dose and in black if considered 60 mg/m^2. Finally, an average of the highest and lowest therapeutic dose is performed (38,39).

This last result led us to hypothesize that the whole drug may have been released from the lipid matrix and due to the numerous efflux pumps of the intestinal tract, may have been eliminated without entering the bloodstream. To clarify this fact, *in vitro* release studies of DOX from the LN were carried out (**Annex 3**).

As displayed in figure 3, DOX diffused from the dialysis membrane in 48h, with approximately 50% of drug traffic over the first 8 hours and a more progressive diffusion in the remaining time. However, when DOX was loaded into LN only 11% of drug was released from the lipid matrix during the first 8 h and 30% at 48 h.

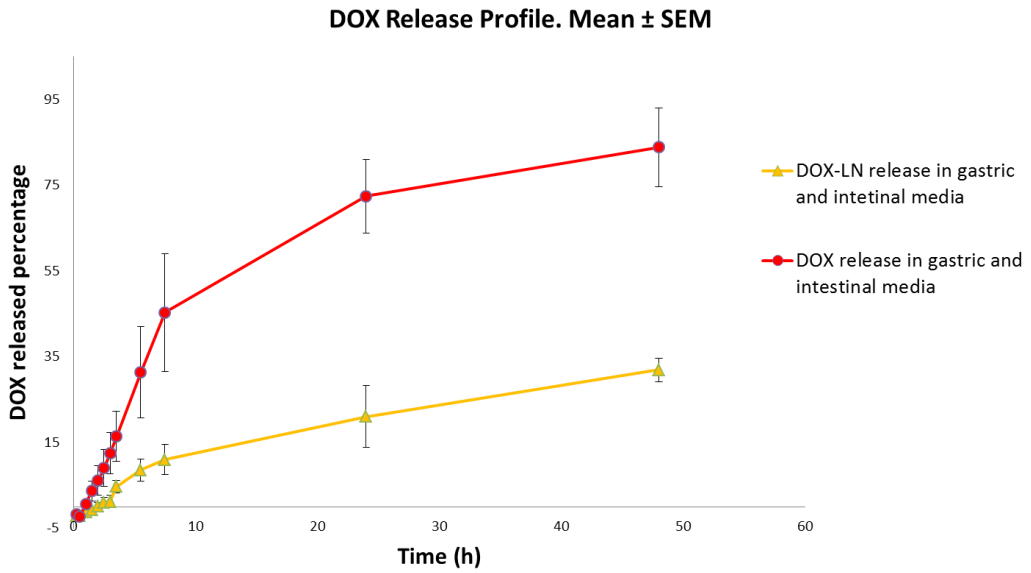

Figure 3: Release profile of DOX in solution and DOX-LN in gastric and intestinal fluid.

It is evident when comparing the release profiles of the drug in solution and the drug in LN, that the encapsulation of DOX into LN clearly favours the controlled release of the drug. However, the fact that in the presence of lipases the drug was not released from the LN may indicate that the ion pair created between triethanolamine/oleic acid and DOX for the encapsulation of the polar drug in LN was inadequate for drug release. Contrary to our speculations, the drug may not have been released from the matrix *in vivo*, or its structure may have been chemically modified so that we were not able to detect the drug by our UHPLC-MS/MS developed method. Any of these theories could explain why no detectable level of DOX was found in the plasma of DOX-LN administered mice.

All these unexpected results hampered the pre-clinical evaluation of DOX-LN *in vivo*. However, we successfully evaluated the anti-osteosarcoma potential of ET, ET-LN, commercial DOX (farmiblastina®) and the combination of ET-LN with commercial DOX in two OS murine models induced by the orthotopic implantation of HOS and 143B OS cells. Notably, the experimental model used in this study facilitates reproducing the local tumour growth of the disease and the metastatic pathways seen in paediatric OS patients (32).

The results concerning the *in vivo* experiments were compiled in **chapter 3.**

Prior to the *in vivo* efficacy evaluation of the drugs, the cytotoxic activity of ET, ET-LN and DOX against HOS-osteosarcoma cells was evaluated. Free ET, ET-LN and DOX successfully inhibited the growth of HOS cells in a concentration and time-dependent manner. However,

at this time, we not only evaluated the direct cytotoxic activity of the compounds but also their long-term toxicity through the clonogenic assays. To that end, HOS cells were treated during 72 h with the IC_{50} dose of the drugs alone and in combination. The ability of the remaining viable cells to form colonies during 6 days was subsequently analysed. Despite the great direct efficacy against OS cells that ET and ET-LN had previously shown, none of the tested drugs had an anti-clonogenic effect. , On the other hand DOX and the combinations of DOX with ET-LN and ET prevented the survival and subsequent proliferation of HOS cells. This lack of anti-clonogenic efficacy of ET and ET-LN was also reported on leukaemic cells by Lohmeyerl and Workman (33). Authors suggested that at low concentrations ET may interchange between the plasma membrane of the cells and the proteins of the cell media avoiding the drug's membrane-dependent effect (33). However, as DOX's mechanism of action focuses on the nuclear DNA, its effect may remain after the drug's withdrawal and prevent the formation of cellular clones.

After the *in vitro* characterization of the drugs' effects, their *in vivo* behaviour against two OS murine models was assessed. In the first place, the anti-tumour activity of ET, ET-LN, commercial DOX and the combination of DOX plus ET-LN was evaluated against an orthotopic murine model of OS induced by HOS cells. After three weeks of treatment only ET and ET-LN were able to slow down the progression of the primary tumour. Neither DOX, nor the combination of DOX with ET-LN were able to delay the progress of the disease. At the end point of the experiment the bone structure observed by micro-CT of those mice treated with ET and ET-LN was preserved more than in the other groups. Fewer osteolytic regions and fewer proliferating areas (Ectopic bone) were observed. Moreover, immunohistochemistry assays of Ki67 antigen revealed that ET and ET-LN treated tibiae presented fewer proliferative tumour cells than the untreated tibiae or those treated with DOX or the combined treatment.

Regarding DOX, a sub-therapeutic dosage of the drug may be responsible for the aforesaid inefficacy. Following the calculations shown in figure 2, we should have administered to the mice 7.5 mg/kg of weight, for 3 consecutive days every 21 days. Nevertheless, a total of 6 mg/kg was the maximum administrable and non-lethal dose for athymic nude mice. For that reason, mice were treated in the experiment with 2 mg/kg of weight, 3 consecutive days every 21 days, which had no effect on OS tumours. As for the combined treatment, because ET-LN alone showed a significant anti-tumour effect, an antagonism between DOX and ET-LN may be responsible for the observed effect. In order to clarify if the interaction between the two drugs took place at a membrane level impairing the internalization of any of the drugs,

the amount of internalized ET in the presence or absence of DOX was quantified *in vitro*. Our results indicated that the pre-treatment of HOS cells with a non-lethal dose of DOX and the subsequent treatment with an effective dose of ET reduced the internalization of the latter. All of this led us to hypothesize that a sequential administration of DOX and ET-LN might have been more effective than the co-treatment, as authors like Nebille-Webbe *et al* previously suggested (34).

We next reproduced the experiment in an orthotopic model of OS induced by the injection of the well-known metastatic 143B cells. Only the treatments that were effective against the HOS-induced-primary tumour were analysed, that is to say, ET and ET-LN.

Similar to the results obtained in the first *in vivo* experiment, ET and ET-LN were also effective in decreasing the primary tumour growth of 143B-injected animals. Both treatments were equally effective 20 days after tumour cell implantation. However after three weeks, mice treated with ET presented an exponential tumour growth whereas the tumours of those mice treated with ET-LN displayed significantly slower growth. Moreover, at the end point of the experiment, the average tumour growth of those mice treated with ET-LN was five times lower than that in untreated mice.

More importantly, whereas 90% of the untreated mice and 80% of the ET-treated ones developed the metastatic disease, only one out of the nine mice treated with ET-LN presented a small metastatic nodule in the lungs. Therefore, ET-LN did not only considerably delay the progression of the primary tumour growth, but the treatment with ET-LN did prevent the dissemination of OS cells from the primary tumour to the lungs. This fact was one of the main goals proposed at the beginning of the work.

As a global conclusion, in the present study we successfully developed MTX and DOX-LN. These, together with the previously developed ET-LN, were effective *in vitro* against numerous commercial and patient-derived OS tumour cells. The encapsulation of the drugs in LN favoured their cellular internalization, partially due to the inhibition of the well-known efflux pump P-gp. Moreover, the combined *in vitro* administration of DOX and ET, free and encapsulated in LN, showed promising synergistic effects against OS cells. *In vivo*, ET and ET-LN were effective at decreasing the growth of primary OS tumours. However, only the treatment with ET-LN inhibited the dissemination from the primary tumour to the lungs. To sum up, ET-LN has shown promising results against primary and metastatic OS

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CONCLUSIONS

CONCLUSIONES

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The main objective of this work was to develop and optimize lipid nanoparticles for the oral delivery of edelfosine, methotrexate and doxorubicin and to evaluate their *in vitro* and *in vivo* efficacy against osteosarcoma (OS) murine models. As a result of all the work carried out throughout the present PhD, the following can be concluded:

1. Edelfosine, methotrexate and doxorubicin were successfully loaded into lipid nanoparticles by the hot melting homogenization and ultrasonication technique. In the case of the hydrophilic anthracycline doxorubicin, an ion pair with oleic acid and triethanolamine was used to increase the solubility of the drug in the lipid matrix. The developed nanoparticles presented a size and PDI optimal for oral delivery.

2. Edelfosine, free or loaded into lipid nanoparticles, was effective at inhibiting the growth of OS commercial and patient-derived tumour cells. Notably, edelfosine was more effective against metastatic-patient derived OS cells than primary patient-derived cells. The higher efficacy of edelfosine in metastatic cells was partially due to their higher lipid raft content, which favours the drug's uptake. Moreover, the encapsulation of edelfosine in lipid nanoparticles increased the internalization of the drug in primary and metastatic patient-derived OS cells.

3. OS cells were less sensitive to the action of methotrexate than edelfosine. Furthermore, primary and metastatic-patient-derived OS cells showed mechanisms of resistance to the chemotherapeutic agent, such as overexpression of the target enzyme dihydrofolate-reductase and the impairment of reduced folate carriers.

4. The *in vitro* efficacy of methotrexate lipid nanoparticles could not be successfully evaluated by means of conventional cytotoxic protocols. A microfluidic platform was developed to circumvent the limitations of the conventional techniques. Methotrexate lipid nanoparticles showed more efficacy when inhibiting OS cells growth than the free drug.

5. Doxorubicin, free and encapsulated, was effective at inhibiting the growth of commercial and metastatic-patient derived OS cells. However, the efficacy of the free drug decreased as cell passage number increased. This loss of efficacy was correlated with an over-expression of the P-glycoprotein with cell passage.

6. Doxorubicin and edelfosine, both free and encapsulated, showed synergistic effects *in vitro* against commercial and metastatic-patient derived OS cells. Moreover, an increased level in the effector caspases 3 and 7 was observed.

7. A pilot pharmacokinetic study of a single dose of orally administered doxorubicin lipid nanoparticles in athymic nude healthy mice did not show any plasmatic level of the loaded drug after its administration. Release studies of doxorubicin loaded lipid nanoparticles revealed a minimal drug release from the lipid matrix in gastric and intestinal fluids containing pepsin and pancreatin.

8. In an orthotopic murine model of OS induced by the intratibial injection of HOS cells, free and encapsulated edelfosine were able to decrease the tumour size at the end point of the experiment. Commercial doxorubicin and the combination of doxorubicin with edelfosine lipid nanoparticles were not able to slow the progression of the primary tumour when compared to the tumour of non-treated mice.

9. In an orthotopic murine model of OS induced by the injection of 143B cells, edelfosine was effective at slowing down the progression of the primary tumour. Besides, when edelfosine was loaded in lipid nanoparticles its *in vivo* efficacy significantly increased. Notably, only the treatment with edelfosine lipid nanoparticles prevented the dissemination of the primary tumour cells to the lungs.

CONCLUSIONES

El objetivo principal del presente trabajo fue desarrollar y optimizar nanopartículas lipídicas para la administración oral de edelfosina, metotrexato y doxorrubicina; y evaluar su eficacia *in vitro* e *in vivo* frente a modelos de osteosarcoma (OS) murinos. Las conclusiones de este trabajo son las siguientes:

1. La edelfosina, el metotrexato y la doxorrubicina fueron encapsulados en nanopartículas lipídicas por la técnica de homogeneización en caliente seguido de ultrasonidos. En el caso de la antraciclina polar doxorrubicina, con el objetivo de incrementar la solubilidad del fármaco en la matrix lipídica, se realizó un par iónico con ácido oleico y trietanolamina. Las nanopartículas desarrolladas presentaron un tamaño e índice de polidispersión adecuados para la administración oral.

2. La edelfosina, libre o encapsulada en nanopartículas lipídicas, inhibió el crecimiento de células de OS comerciales y de células derivadas de paciente. En particular, la edelfosina fue más eficaz frente a las células metastásicas derivadas de paciente que frente a las líneas primarias. La mayor eficacia de edelfosina frente a células metastásicas fue debido, en parte, a su mayor contenido en balsas lipídicas, lo que favorece su internalización celular. Además, la encapsulación de edelfosina en nanopartículas lipídicas aumentó la internalización del fármaco tanto en líneas primarias como metastásicas derivadas de paciente.

3. Las células de OS fueron menos sensibles a la acción del metotrexato que a la acción de edelfosina. Además, tanto las líneas primarias como metastásicas derivadas de paciente mostraron mecanismos de resistencia a fármacos antitumorales como son, la sobreexpresión de la enzima diana del fármaco dihidrofolato reductasa o la alteración de los receptores de folato reducidos.

4. La eficacia *in vitro* de las nanopartículas lipídicas de metotrexato no pudo ser evaluada mediante los ensayos citotóxicos convencionales. Para solventar esta limitación, se desarrolló un dispositivo de microfluídica. En este dispositivo, las nanopartículas de metotrexato mostraron mayor eficacia que el fármaco libre inhibiendo el crecimiento de células de OS.

5. La doxorrubicina, libre o encapsulada en nanopartículas lipídicas, fue eficaz inhibiendo el crecimiento de células de OS comerciales y derivadas de paciente. Sin embargo, la eficacia del fármaco libre disminuyó al aumentar el pase de las células. Esta pérdida de eficacia fue correlacionada con un aumento de la expresión de la glicoproteína-P con el cultivo.

6. La doxorrubicina y edelfosina, libre y encapsulada, mostraron efectos sinérgicos *in vitro* frente a células de OS comerciales y derivadas de paciente. Además, se observó un aumento en los niveles de las caspasas 3 y 7.

7. Un estudio farmacocinético piloto, en ratones atímicos desnudos sanos, a los cuales se les adminitró una dosis única de nanopartículas lipídicas de doxorrubicina, no mostró niveles plasmáticos de fármaco tras la administración oral. Los estudios de liberación de doxorrubicina encapsulada revelaron una liberación de fármaco mínima desde la matriz lipídica en medios gástrico e intestinales con pepsina y pancreatina.

8. En un modelo murino, ortotópico de OS inducido por la inyección intratibial de células HOS, tanto la edelfosina libre como la encapsulada en nanopartículas lipídicas disminuyó el tamaño tumoral a tiempo final. La doxorrubicina comercial y la combinación de doxorrubicina con nanopartículas de edelfosina, no disminuyeron la progresión del tumor primario en comparación con el tumor de los ratones no tratados.

9. En un modelo ortotópico de OS inducido por la inyección de células 143B, la edelfosina disminuyó la progresión del tumor primario. Además, cuando el fármaco se administró encapsulado en nanopartículas lipídicas, su eficacia *in vivo* aumentó significativamente. Es de destacar que sólo el tratamiento con edelfosina encapsulada en nanopartículas lipídicas evitó la diseminación de las células del tumor primario a los pulmones.

ANNEX 1

Antitumoral-Lipid-Based Nanoparticles:

A Platform for Future Application in Osteosarcoma Therapy

Antitumoral-Lipid-Based Nanoparticles: A Platform for Future Application in Osteosarcoma Therapy

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González-Fernández Y, Imbuluzqueta E, Patiño-García A, Blanco-Prieto MJ. Antitumoral-Lipid-Based Nanoparticles: A Platform for Future Application in Osteosarcoma Therapy. Current Pharmaceutical Design, 2015, 21 (2015), 6104-6124.

ANNEX 2

EFFECTIVENESS OF NANOENCAPSULATED METHOTREXATE AGAINST OSTEOSARCOMA CELLS: *IN VITRO* CYTOTOXICITY UNDER DYNAMIC CONDITIONS

Mitxelena-Iribarren O, Hisey CL, Errazquin-Irigoyen M, González-Fernández Y, Imbuluzqueta E, Mujika M, Blanco-Prieto MJ, Arana S. Effectiveness of nanoencapsulated methotrexate against osteosarcoma cells: in vitro cytotoxicity under dynamic conditions. Biomed Microdevices, 2017, 19(2):35. https://doi.org/10.1007/s10544-017-0177-0

ANNEX 3

DOXORUBICIN PHARMACOKINETIC STUDIES AND DRUG RELEASE ASSAYS

PILOT PHARMACOKINETIC STUDY OF COMMERCIAL DOXORUBICIN AND DOXORUBICIN-LIPID NANOPARTICLES

A pilot pharmacokinetic study of a single dose of doxorubicin (DOX) and doxorubicin lipid nanoparticles (DOX-LN) 7.5 mg/kg was performed (in the case of free DOX, commercial farmiblastina®, DOX + excipients, was employed). To that aim, healthy athymic-nude mice were kept with free access to water and food *ad libitum* up to the time of administration. Drugs were administered either by the oral (DOX-LN) or parenteral route (farmiblastina®). At predetermined times, around 250 µL of blood samples were collected into refrigerated EDTA-tubes from the lateral saphenous vein. EDTA-tubes were centrifuged twice for plasma isolation and maintained at -80 °C until UHPLC/MS-MS analysis.

As showed in figure 1, a C_{max} of 2180 ± 848 ng DOX/mL of plasma was obtained 5 minutes after the single intravenous administration of the drug to healthy nude mice. Most notably, mice receiving the same dose of DOX loaded in LN by the oral route did not present any plasma level of drug (data not showed).

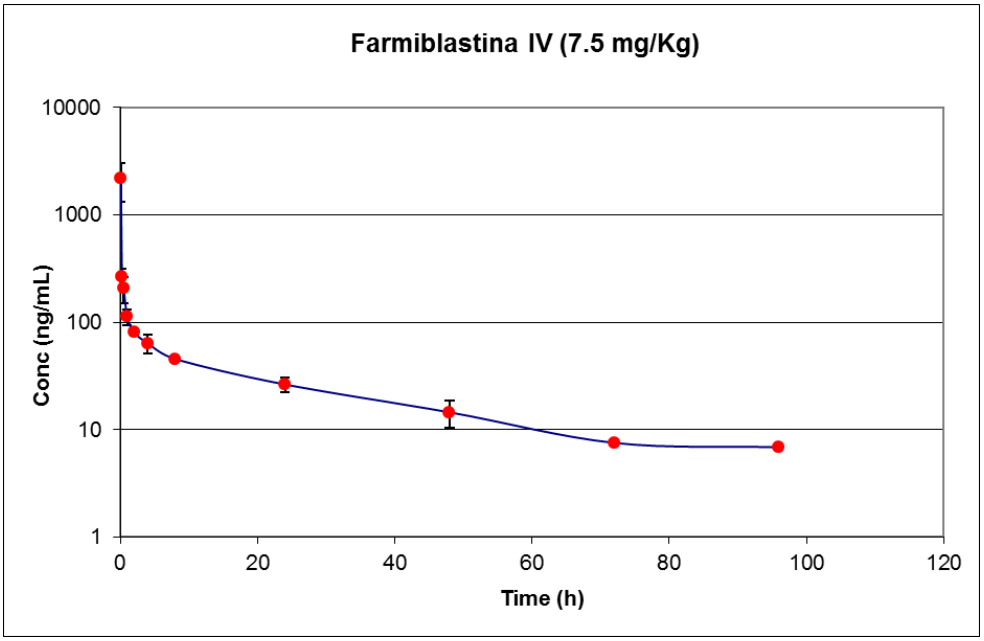

Figure 1: Pharmacokinetic profile of a single dose of 7.5 mg/Kg of commercial DOX (Farmiblastina®)

RELEASE ASSAYS OF DOXORUBICIN AND DOXORUBICIN LIPID NANOPARTICLES IN SIMULATED GASTRIC AND INTESTINAL MEDIUM

In order to clarify if the absence of DOX in the plasma of the mice was due, for instance, to the release of the drug prior to the entrance to the bloodstream, *in vitro* release studies of DOX from the lipid nanoparticles (LN) were carried out.

Brief, a solution of 16mg of DOX-LN in water and 87.2 µg DOX/mL water (corresponding amount of drug loaded in 16 mg of DOX-LN) was placed in a dialysis membrane of Float-A-Lyzer devices (spectrum labs). The final pore size of the membrane (molecular weight cut-off 8-10 KD) was chosen after testing that LN did not pass along the membrane. The dialysis bags containing DOX and DOX-LN were immerse into falcons containing 20 mL of gastric fluid including pepsin during 3 h; and intestinal fluid with pancreatin for 48 h (gastric and simulated intestinal fluid were prepared according to the european pharmacopoeia, 2017). The whole setup was placed on a magnetic stirrer under continuous agitation of 500 rpm and at 37°C. At predetermined times, 1 mL of dissolution media was collected and assayed spectrophotometrically for drug content at an excitation/emission wavelengths of 485/580 nm. The mL of medium drawn out was replenished with the same volume of fresh medium in order to keep sink conditions. At the end of the experiment, the percentage of drug release was plotted over time.

As displayed in figure 2, DOX diffused from the dialysis membrane in 48h, with approximately 50% of drug traffic over the first 8 hours and a more progressive diffusion in the remaining time. When DOX was loaded into LN only an 11% of drug was released from the lipid matrix during the first 8 h and a 30% at 48 h.

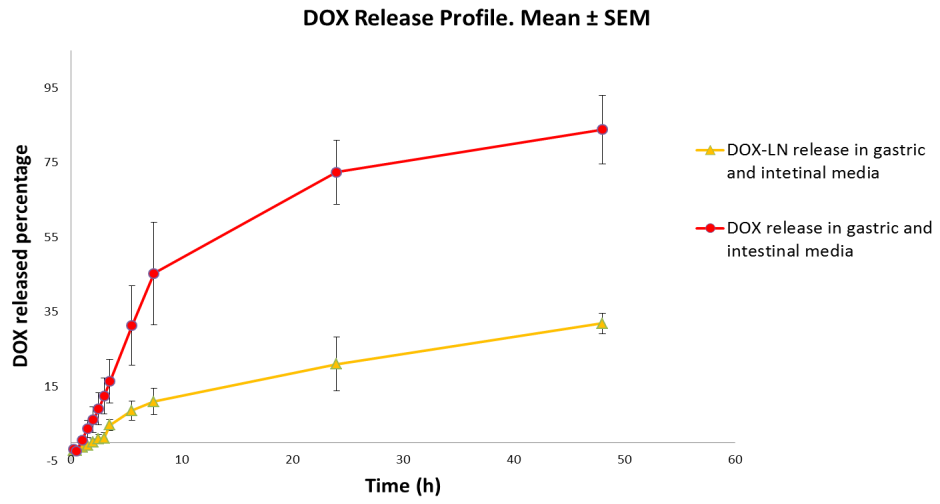

Figure 2: Release profile of DOX in solution and DOX-LN in gastric and intestinal fluid.

It is evident when comparing the release profiles of the drug in solution and the drug in LN, that the encapsulation of DOX into LN clearly favours the controlled release of the drug. However, the fact that in the presence of lipases the drug was not released from the LN may indicate that the ion pair created between triethanolamine/oleic acid and DOX for the encapsulation of the polar drug in LN was inadequate for the drug release.