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Oral edelfosine lipid nanoparticles caused the regression of lung metastases in pre-clinical osteosarcoma animal models

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ABSTRACT

Osteosarcoma (OS) is the most frequent paediatric bone cancer, responsible for 9% of all cancer-related deaths in children. In this paper, a new strategy based on delivering edelfosine (ET) in lipid nanoparticles (LN) was explored in order to target the primary tumor and eliminate metastases. The *in vitro* and *in vivo* efficacy of the free drug, drug loaded into lipid nanoparticles (ET-LN) and doxorubicin (DOX) against osteosarcoma (OS) cells was analysed. ET and ET-LN decreased the growth of OS cells *in vitro* in a time and dose-dependent manner. Interestingly, the uptake of ET and ET-LN was lower when OS cells were pre-treated with DOX. *In vivo* investigations revealed that ET and ET-LN slowed down the primary tumour growth in two OS models. However, the combination of both drugs showed no additional antitumour effect. Importantly, ET-LN successfully prevented the metastatic spread of OS cells from the primary tumour to the lungs. On the whole, the ET-LN is a promising candidate for OS chemotherapy.

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

1. INTRODUCTION

In 2017, the American Cancer Society estimated there would be 10,270 new cases of childhood and adolescent cancers, among which osteosarcoma (OS) leads the list of the most deadly, only preceded by leukaemia in adolescents and leukaemia and brain tumours in the paediatric population [1]. Despite the encouraging prognosis for patients with localised 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 successful control of localised 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 to be 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. The ability to target nanoparticles allows us to transport a higher dose of drug into the tumour area, while indiscriminate toxicity is minimised. Second, nanoparticles can act directly on invasive cancer cells or re-educate the tumour microenvironment to avoid the outbreak of the metastatic cascade [4-6].

As for OS, different strategies, mostly 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] by inducing the activation of alveolar macrophages [11], characterising the host-tissue tumour microenvironment to target pulmonary metastases, or targeting surface antigens expressed in OS circulating cells [12-16].

Previous work by our group has demonstrated the efficacy *in vitro* of alkyllysophospholipid edelfosine (ET) and edelfosine encapsulated in lipid nanoparticles (ET-LN) against metastatic-patient derived OS cell lines [17]. In addition, ET-LN and doxorubicin (DOX) have been reported to exercise a synergistic effect against OS cells [18]. The present study investigates the therapeutic efficacy of orally administered ET, ET-LN and its combination with DOX in two OS orthotopic murine models induced by HOS and 143B human OS cells. The effect of the different treatments against the primary tumour and metastatic disease is assessed.

The results show that orally administered ET loaded LN have an outstanding effect against primary OS tumours. Importantly, the nanosystems developed successfully prevented the metastatic spread of OS cells from the primary tumour to the lungs. On the whole, the ET-LN is a promising candidate for OS chemotherapy.

2. MATERIALS AND METHODS

2.1. Materials

Doxorubicin-hydrochloride (DOX) 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. 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 DOX (Farmiblastina®;) was obtained from the University Hospital of Navarra (Pamplona, Spain). Ki67 monoclonal antibody was obtained from Leica Biosystems (Barcelona, Spain).

2.2. Preparation and characterisation of edelfosine lipid nanoparticles

ET-LN were prepared following the hot homogenisation and ultrasonication method as previously described [19]. 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 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 surfactant and the non-incorporated drug. Finally, the formulation was lyophilised using trehalose as cryoprotectant.

The hydrodynamic size, polydispersity index (PDI), and ζ -potential of the LN were determined by dynamic light scattering (DLS) technique using a Nano ZetaSizer (Malvern Instruments, UK). The measurements were performed at a fixed scattering angle of 90° and at an equilibrated temperature of 25°C. Each sample was adequately diluted with distilled water prior to measurement, and three measurements were performed for each sample.

ET entrapment into LN was quantified by a previously validated ultra-high-performance liquid chromatography tandem mass spectrometry method (UHPLC MS/MS) [20].

2.3. Cell culture

HOS-MNNG (HOS) and 143B cell lines were obtained from the American Type Culture Collection. 143B and HOS cells were maintained in α -MEM and DMEM respectively with 10% foetal bovine serum supplemented medium. Cells were cultured in a humidified 5% CO₂/air atmosphere at 37°C.

2.4. Cell viability assays

The cytotoxic activity of DOX, ET and ET-LN against OS cells was evaluated via the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Spain). Briefly, 800 HOS cells/well were plated in 96 well-plates. 24 h later, cells were exposed to increasing concentrations of drug for 48 and 72 h. MTS reagent was then added and the absorbance at 490 nm was recorded after 4h incubation. 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 $\pm$ SD.

2.5. Clonogenic assays

In order to study the survival and colony formation ability of OS cells exposed to the IC_{50} dose of the afore-mentioned treatments, HOS cells were plated at a cell density of 1×10^5 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 1×10^3 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 $\pm$ SEM.

2.6. Edelfosine uptake assay

20×10^3 HOS cells were seeded in 6 well-plates, and 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 μ M 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, 50×10^3 HOS cells were seeded in 6 well-plates. 24 h later cells were treated with 1.25 μ M of ET and ET-LN for 72 h. Intracellular ET content was quantified by a validated UHPLC-MS/MS method [20] and the amount of internalised drug was normalised to the

proportion of proteins in the sample quantified by the Lowry method [21]. All experiments were repeated four times and results were expressed as mean $\pm$ SEM.

2.7. Animal experiments

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

HOS OS model

HOS cultured cells were harvested and diluted in PBS to a final concentration of 500×10^3 cells per 10 μ l. For the intratibial implantation of tumour cells, 10 μ l of the cell suspension were injected through the medullar cavity of the tibia. Six days later, 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 PBS as control. The weight of the mice was measured and tumour size was monitored twice a week using a calliper. Tumour volume was calculated from two diameters according to the formula $[0.5 \times a^2 \times b]$. 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) $\pm$ 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 (dilution of the primary antibody: 1/100) was carried out on 3 μm -thick deparaffinised sections of the tibiae. Immunostaining was performed overnight, at 4 °C. Image J® software was used to analyse the results. Results were expressed as mean $\pm$ SEM.

143B OS model

The efficacy of ET and ET-LN (with the same dose and therapeutic regimen previously described) was analysed in a 143B OS-induced model. Briefly, 200 x 10³ 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 by monitoring the primary tumour growth with a digital calliper and by means of micro-CT images of the tumour-bearing legs. Histological visualisation of the recovered lungs in order to evaluate the presence of microscopic metastases was also assessed.

3. Statistical analyses

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

3. RESULTS

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

Prior to the *in vitro* efficacy assays, ET-LN were characterised (Figure 1). The size and size distribution of the nanoparticles is very important for oral absorption and tumour cell uptake. The size distribution of the nanoparticles is shown in Figure 1A. The size of the ET-LN was very uniform with an average size of 124 ± 12 nm (Figure 1B), and was associated with a low polydispersity index of 0.16 (Figure 1B). The surface charge was -14.5 mV and the drug loading around 30 μ g ET/mg of formulation (Figure 1B). *In vitro*, ET-LN, ET and DOX inhibited HOS cell proliferation in a dose- and time-dependent manner. IC₅₀ ET, ET-LN and DOX were 6.2 ± 0.54 , 2.53 ± 0.83 and 55.58 ± 27.37 respectively after 48h of drug exposure (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.

3.2. 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 IC₅₀ doses [ET either free or encapsulated (2.5 μ M); DOX (50 nM)]. As shown in Figure 2A and 2B, OS cells exposed to ET and ET-LN were able to form colonies in a similar manner to OS cells pre-treated with empty nanoparticles or maintained in culture without drug (Control). On the

other hand, 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.

3.3. 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 internalised ET in HOS cells with and without a pre-treatment with DOX was quantified by an UHPLC/MS-MS method and normalised to the amount of protein quantified by the Lowry method. As illustrated in Figure 3, the pre-treatment of OS cells with 5 nM DOX reduced the uptake of ET by half and the uptake of ET-LN up to three times ($p < 0.05$).

3.4. Edelfosine-lipid nanoparticles and edelfosine delay primary osteosarcoma 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 were evaluated in an orthotopic OS tumour model. Six days 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 showed no significant effect on the tumor volume compared to the control group. However, after 27 days of treatment, the tumour volumes were significantly decreased by around 71% ($p < 0.001$) and 57% ($p < 0.05$) in the presence of ET-LN and ET compared to the control group respectively (Figure 4A).

As expected, micro-CT images revealed a marked ectopic bone formation (arrows) and tumour-associated osteolysis (asterisk) in the non-treated mice (Figure 4B). These lesions were similar or even more aggressive in the combined and DOX treated groups. By contrast, ET and ET-LN treated groups showed less ectopic bone formation than the control and DOX groups (Figure 4B). Interestingly, we observed no impact on healthy bones. Neither the trabecular nor the cortical bone was affected by the treatments. 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 tumour 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. MicroCT analysis of contralateral legs demonstrated that none of the treatments assessed affected bone remodelling parameters (Supplementary Figure 1).

3.5. Edelfosine-lipid nanoparticles inhibit the primary tumour growth of 143B-orthotopic osteosarcoma mice and cause 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 cell inoculation and significantly delayed the initiation of tumour formation. However, three weeks after cell inoculation, mice treated with ET presented an exponential tumour growth whereas ET-LN significantly slowed down the tumour growth compared to control and ET groups (Figure 6A). At the end point of the experiment, ET-LN reduced significantly the tumor volumes by around 72% compared to the non-treated mice ($p<0.001$) (Figure 6A). At day 26, non-encapsulated ET reduced the tumor volume around only 30% ($p<0.001$). The presence of microscopic metastases in the lungs was further examined by histology (Figure 6B). 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 ($p<0.01$).

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 localised 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 [22]. 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 [17]. 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* [17]. Moreover, when U2OS and 595M OS cells were treated with ET and DOX, a synergistic effect in their cytotoxic activity was observed [18]. Anthracyclines such as doxorubicin are antibiotics that form complexes with DNA and inhibit topo-isomerase II, leading to cancer cell death. Edelfosine does not target the DNA but rather binds to membrane lipid rafts inducing the reorganization of transmembrane proteins that indirectly trigger several death signalling pathways. These two very different modes of action can underlie and explain the difference of IC₅₀ between both drugs. Building on this, in the present study we evaluated the efficacy of ET, ET-LN, DOX and the combination of DOX and ET-LN 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 IC₅₀ 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 [17,18]. 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 internalised 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, whether free or encapsulated, did not inhibit the clonogenic ability of OS cells. Similar observations had previously been 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 (IC₅₀ 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 prevented 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 showed no 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 IC₅₀ dose [18], as seen in Figure 3, when OS cells were pre-treated with a non-lethal dose of DOX, the amount of ET internalised by the cells was substantially diminished. The cytotoxic activity of DOX is based on its direct interaction with DNA. Recently, Alves et al gave evidence of a new mechanism of action involving plasma membrane [25]. These authors demonstrated by Brewster angle microscopy that DOX interplays with the lipid monolayer, resulting in alterations of monolayers' shape. ET binds to the membrane lipid rafts, consequently such alterations of cell membrane induced by DOX may explain the lower uptake of ET by OS cells after DOX pre-treatment. 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 [26]. 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 [27]. In the light of these precedents a different sequencing of the drugs in our experiment might 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 (Figure 5).

Subsequently, and owing 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 Figure 6 shows, both treatments successfully slowed the progression of the disease. Furthermore, 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 other hand, 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 combat 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 lead 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 shows immediate cytotoxicity against HOS cells. However, ET-LN here has no long-term toxicity evaluated by means of clonogenic assays, probably due to incorrect selection of the dose used to treat the cells. *In vivo*, ET and ET-LN are able to slow the progression of the primary tumour growth in HOS and 143B orthotopic OS models. Moreover, ET-LN successfully prevents the metastatic spread of 143B OS cells from the primary tumour to lungs.

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Table 1: IC₅₀ for human MNNG-HOS osteosarcoma cells after 48 and 72h of treatment

Time	Edelfosine (μM)	Edelfosine lipid nanoparticles (μM)	DOX (nM)
48 h	6.2 ± 2.1	8.57 ± 3.45	232.6 ± 64.93
72 h	2.72 ± 0.54	2.53 ± 0.83	55.58 ± 27.37

FIGURE LEGENDS

Figure 1: Edelfosine lipid nanoparticles (ET-LN) characterization. (A) ET-LN morphology observed by transmission electron microscopy; (B) Distribution of particle size analysed by photon correlation spectroscopy using a Zetasizer Nano ZS; (C) PDI and ζ potential were determined by photon correlation spectroscopy and ET entrapment into lipid nanoparticles was quantified by ultra-high-performance liquid chromatography tandem mass spectrometry method.

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 (2.5 μ M), ET-LN (2.5 μ M), B-LN (empty LN, 2.5 μ M), DOX (doxorubicin, 50 nM) or the combination of DOX with ET or ET-LN.. 1×10^3 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) Histogram representing percentage of colonies vs treatment (n= 4, mean $\pm$ SEM). * p < 0.05.

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 μ M 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 $\pm$ SEM).

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, 30 mg/kg, three times/week), ET-LN (per oral, 30 mg/kg, three times/week), DOX (doxorubicin, intravenous, 2 mg/kg x 3 consecutive days every 21 days), their combination and PBS as control in primary OS tumour growth ($n=5$, mean $\pm$ 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.

Figure 5: Edelfosine lipid nanoparticles (ET-LN) decreased the number of tumour proliferating cells *in vivo*. HOS cells were inoculated in intrabial site of Nude mice. Six days after cell injection, mice were treated with ET (per oral, 30mg/kg, three times/week), ET-LN (per oral, 30mg/kg, three times/week), DOX (doxorubicin, intravenous, 2 mg/kg x 3 consecutive days every 21 days), their combination and PBS as control. Ki67 immunohistochemistry of the treated tibiae was carried out on 3 mm-thick deparaffinized sections. Positive immunostaining was quantified using Image J software. **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* treatment (n=5, mean $\pm$ SEM). ***: $P \leq 0.001$.

Figure 6: Edelfosine (ET) and edelfosine lipid nanoparticles (ET-LN) exhibited an anti-tumour effect in a 143B-osteosarcoma induced model. 143B-osteosarcoma cells were inoculated in intratibial site of Nude mice. Six days after cell injection, mice were treated with ET (per oral, 30mg/kg, three times/week), ET-LN (per oral, 30mg/kg, three times/week), DOX (doxorubicin, intravenous, 2 mg/kg x 3 consecutive days every 21 days), their combination and PBS as control. **A)** Effect of edelfosine (ET) and edelfosine-lipid nanoparticles (ET-LN) in primary OS tumour growth induced by 143B cells. **B)** After 26 days of treatment with ET and E-LN compared to the control group, macroscopic lung metastases were enumerated. ***: $P \leq 0.001$, (n=9, mean + SEM).

Figure 1

Figure 2

A

Control

ET

ET-LN

B-LN

DOX

DOX +
ET-LN

B

Figure 3

Figure 4

A

B

Figure 5

Figure 6