Efficacy of edelfosine lipid nanoparticles in breast cancer cells

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ABSTRACT

Breast cancer is a heterogeneous group of neoplasms predominantly originating in the terminal duct lobular units. It represents the leading cause of cancer death in women and the survival frequencies for patients at advanced stages of the disease remain low. New treatment options need to be researched to improve these rates. The anti-tumor ether lipid edelfosine (ET) is the prototype of a novel generation of promising anticancer drugs. However it presents several drawbacks for its use in cancer therapy, including gastrointestinal and hemolytic toxicity and low oral bioavailability. To overcome these obstacles, ET was encapsulated in Precirol ATO 5 lipid nanoparticles (ET-LN), and its anti-tumor potential was *in vitro* tested in breast cancer. The formulated ET-LN were more effective in inhibiting cell proliferation and notably decreased cell viability, showing that the cytotoxic effect of ET was considerably enhanced when ET was encapsulated. In addition, ET and ET-LN were able to promote cell cycle arrest at G1 phase. Moreover, although both treatments provoked an apoptotic effect in a time-dependent manner, such anti-tumor effects were noticeably improved with ET-LN treatment. Therefore, our results indicate that encapsulating ET in LN played an essential role in improving the efficacy of the drug.

Keywords: Breast cancer, MCF7, lipid nanoparticles, edelfosine, alkylphospholipids.

Abbreviations

Edelfosine: ET

Alkylphospholipids: ALPs

Lipid nanoparticles: LN

Enhanced permeability and retention effect: EPR effect

Edelfosine-loaded lipid nanoparticles: ET-LN

Mantle cell lymphoma: MCL

Polydispersity index: PDI

Blank (unloaded) nanoparticles: B-LN

Propidium iodide: PI

Inhibitory concentration 50: IC_{50}

1. Introduction

Breast cancer is a heterogeneous group of neoplasms, predominantly originating in the terminal duct lobular units, regardless of histological type (Weigelt and Reis-Filho, 2009; Wellings and Jensen, 1973; Wellings et al., 1975). It represents the fifth most common cancer worldwide, the second most common cause of cancer death and the leading cause of cancer death in women (Siegel et al., 2013). The global burden of breast cancer exceeds all other cancers and the incidence rates of breast cancer are increasing. The ability of breast cancer cells to metastasize to distant organs makes this disease refractory and incurable and is the key factor in the treatment and prognosis of breast cancer (Lu and Kang, 2007). Current treatment approaches usually involve intrusive processes, chemotherapy to shrink any cancer present, surgery to then remove the tumor if possible, followed by more chemotherapy and radiation. However, the survival rates for patients at advanced stages of the disease remain low (Siegel et al., 2013). Consequently, new treatment options have to be studied to improve these rates. Current research areas include, on the one hand, the development of carriers that allow alternative dosing routes and reduce toxicity; and on the other, new therapeutic targets such as blood vessels fueling tumor growth and targeted therapeutics that are more specific in their activity (Brannon-Peppas and Blanchette, 2004).

The anti-tumor ether lipid edelfosine (ET-18-OCH3, ET) has been shown as an effective anti-tumor agent in different malignancies (Estella-Hermoso de Mendoza et al., 2009a; Mollinedo et al., 2010a; Na and Surh, 2008; Shafer and Williams, 2003). However, when it is administered in its free form, it presents several drawbacks such as dose-dependent hemolytic toxicity after intravenous administration (Ahmad et al., 1997), poor oral bioavailability and gastrointestinal irritation when administered orally (Estella-

Hermoso de Mendoza et al., 2009a; Estella-Hermoso de Mendoza et al., 2012; Houlihan et al., 1995; Munder and Westphal, 1990).

Owing to the drawbacks of this molecule, new drug delivery systems have been designed (Estella-Hermoso de Mendoza et al., 2012). Lipid nanoparticles (LN) are colloidal transporters composed of a biocompatible and biodegradable lipid matrix. They are passively targeted at the tumor tissue due to the well-known enhanced permeability and retention effect (EPR), resulting in an increased concentration of drug in tumor cells and in lower side effects (Peer et al., 2007; Torchilin, 2011). Besides, LN can be administered orally and are mainly absorbed via the lymphatic system, circumventing first-pass hepatic metabolism, and thereby opening a new window in the treatment of cancer metastases (Estella-Hermoso de Mendoza et al., 2012; Lasa-Saracibar et al., 2012). Moreover, LN may modify the entrance mechanism of the ET into cancer cells and this might overcome the resistance that some cell lines show to the free drug, (Wagner et al., 1998). In this context, the potential of ET-LN in overcoming the resistance of cancer cells has recently been proved in leukemic cell lines (Lasa-Saracibar et al., 2013).

The purpose of this study was to develop LN loaded with ET to enhance its therapeutic activity against breast cancer cells. We formulated ET-LN and we characterized their physicochemical properties. The cytotoxicity of ET-LN, their effects in cell cycle and the cell death induction mechanisms in breast cancer were also investigated.

2. Material and Methods

2.1. Chemicals

ET was from Apointech (Salamanca, Spain). Precirol ATO 5 was a gift from Gattefossé (Lyon, France). Tween® 80 was obtained from Roig Farma (Barcelona, Spain). Phosphate-buffered saline (PBS; 10 mM phosphate, 0.9% NaCl), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Trypan Blue, RNase and Propidium iodide (PI) were obtained from Sigma-Aldrich (Madrid, Spain). Chloroform was purchased from Panreac (Madrid, Spain) and methanol was obtained from Merck (Barcelona, Spain). Ultra-purified water was used throughout and all other chemicals were of analytical grade.

2.2. Cell Culture

MCF7 breast cancer cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Dubelcco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), trypsin–EDTA and penicillin–streptomycin mixtures were purchased from Gibco® BRL (Carlsbad, CA, USA).

MCF7 breast cancer cell line was grown in DMEM supplemented with 50 U/mL penicillin, 50 U/mL streptomycin and 10% FBS at 37˚C in a humidified incubator supplemented with 5% carbon dioxide.

2.3. Preparation of LN

ET-LN formulations were prepared by the hot homogenization method followed by high shear homogenization and ultrasonication as previously described (Estella-Hermoso de Mendoza et al., 2012). The lipid phase consisted of 300 mg of Precirol ATO 5 with 30

mg ET, while the aqueous phase consisted of 10 mL of a 2% (w/v) Tween® 80 aqueous solution. The nanoparticle suspension obtained was subsequently cooled in an ice bath and washed twice with filtered water by diafiltration with Amicon Ultra-15 filters of 10,000 Da molecular weight cut-off membranes (Millipore®, Cork, Ireland) to remove the excess of surfactant and non-incorporated drug. LN were then resuspended in 3% trehalose (75% of the Precirol ATO 5 weigh) and the suspension was kept at -80ºC and freeze-dried to preserve the formulation for further studies. Blank (unloaded) nanoparticles (B-LN) were formulated as empty control of LN for *in vitro* experiments using an identical procedure.

2.4. Nanoparticle characterization

2.4.1. Particle size and Zeta potential

Nanoparticle size and polydispersity index (PDI) of LN were determined in triplicate by photon correlation spectroscopy and zeta potential by laser doppler anemometry, using a Zetasizer Nano (Malvern, UK). Each sample was diluted 30-fold in distilled water until the appropriate concentration of particles was achieved to avoid multiscattering events. Similarly, the zeta potential was measured using the same equipment with a combination of laser doppler electrophoresis (Clogston and Patri, 2011; Kaszuba et al., 2010). Each experiment was performed in triplicate. All data are expressed as a mean value ± standard deviation.

2.4.2. Encapsulation efficiency and loading capacity

Encapsulated ET was quantified by a previously validated ultra-high performance liquid chromatography-tandem mass spectrometry method as previously validated (Estella-Hermoso de Mendoza et al., 2009b). The drug was extracted from a sample of 5 mg of

lyophilized LN, to which 1 mL of chloroform was added in order to dissolve them and subsequently 3 ml of methanol were added to the mixture. After vortex mixing for 1 min at room temperature and centrifuging at 20,000 × g for 10 min, 2 μl aliquots of the supernatant were injected into the chromatographic system.

2.5. Cytotoxicity studies

The cytotoxic potential of ET-LN was evaluated with the MTT assay. 21000 cells/cm^2 were grown in 96-well plates in the presence of increasing amounts of ET or equivalent concentrations of ET-LN for 72 h. B-LN were also tested as control. Then, MTT solution was added directly to the culture media at a final concentration of 0.5 mg/mL and then incubated for 3 h at 37ºC. Afterwards, MTT containing medium was removed from all wells and the remaining cells containing formazan crystals were dissolved in DMSO. Optical density was determined with a BioRad microplate reader at 570 nm after background correction at 690 nm. Average cell viability of treated cells was expressed as a percentage of the absorbance of control cells. Untreated cells were taken as control with 100% viability and cells grown in presence of 10% DMSO were used as positive control of cytotoxicity. All experiments were performed in triplicate.

Cell number and viability, as denoted by Trypan Blue exclusion, were calculated by cell counting with a Bright-Line Hemacytometer (Sigma-Aldrich, Madrid, Spain). Cell counts were performed in triplicate.

Images of the morphological changes induced by ET and LN were obtained using a Nikon Eclipse TS100 microscope.

2.6. Cell cycle analysis

For cell cycle evaluation, 21000 cells/cm^2 were seeded in 6-well plates and incubated with 20 μg/mL of ET or equivalent concentrations of ET-LN. B-LN were also tested as control. Cells were trypsinized, collected by centrifugation, washed with PBS and fixed with 70% ethanol at 4ºC for 1 h. Next, cells were incubated with 0.45 units/mL RNase and stained with 10 μg/mL of propidium bromide. Cell fluorescence was detected on a FACSCalibur flow cytometer (BD Biosciences, Madrid, Spain) and analyzed with CellQuest Pro (BD Biosciences) and FlowJo data analysis software package (TreeStar, USA). All experiments were performed in triplicate after 48 and 72 h of incubation with the corresponding treatments.

2.7. Assessment of apoptosis by Annexin-V FITC staining

For evaluation of apoptosis in MCF7 cells after ET and LN treatments, 21000 cells/cm^2 were seeded in 6-well plates and incubated with 20 μg/mL of ET or equivalent concentrations of ET-LN. B-LN were also tested as control. After 24, 48 or 72 h of incubation, cells were collected and washed twice with PBS and subsequently labeled. Two different approaches were used for the assessment of apoptosis induction of MCF7 cells.

On the one hand, apoptosis was determined by flow cytometry as the percentage of cells in the sub-G1 region (hypodiploidy) in cell cycle analysis as previously described (Gajate et al., 2000).

On the other hand, an Annexin V-FLUOS Apoptosis Detection Kit (Roche Molecular Biochemicals, Barcelona, Spain) was used according to the manufacturer's protocol. The percentage of cells that underwent apoptosis was quantified by flow cytometry on a

FACSCalibur flow cytometer using CellQuest software and analyzed with FlowJo software. All experiments were performed in triplicate.

2.8. Statistical Analysis

Data are presented as a mean of three or more independent experiments, with error bars indicating the standard deviation. The inhibitory concentration 50 (IC_{50}) values were calculated with GraphPad Prism software using the sigmoidal dose-response function (variable slope). Statistical comparisons were performed by analysis of variance, and further post-hoc testing was conducted using the statistical software GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Non-parametric statistics (Kruskal-Wallis test followed by Dunn's Post Hoc test) were used for the analysis of Sub-G1 data. Cell viability, cell cycle and apoptosis statistical analyses were performed with two-way Anova and Bonferroni post hoc tests. Groups that are significantly different are indicated in the figures as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

3. Results and discussion

ET is an anti-tumor drug belonging to the ALPs family that has been employed for the treatment of various malignancies with promising results (Estella-Hermoso de Mendoza et al., 2009a; Mollinedo et al., 2010a; Na and Surh, 2008; Shafer and Williams, 2003). However, due to its nature, ET presents several drawbacks that have to be overcome by protecting the molecule. Nanotechnology applications in medicine have successfully enhanced the therapeutic efficacy of many anti-cancer drugs. In fact, the nanoencapsulation approach of ET has yielded promising results (Estella-Hermoso de Mendoza et al., 2012; Lasa-Saracibar et al., 2013), demonstrating that the vehiculization strategy is essential to ensure the effectiveness of ET (Estella-Hermoso

de Mendoza et al., 2012). Such hopeful results prompted us to develop ET-loaded LN for studying its *in vitro* anti-tumor effect compared to the free drug in breast cancer.

3.1. Nanoparticle characterization

ET-loaded LNs were produced by a solvent-free hot homogenization method followed by high shear homogenization and ultrasonication, and were freeze-dried afterwards. LN were characterized in terms of size, zeta potential, encapsulation efficiency and drug loading. As shown in Table 1, we were able to obtain submicron-sized LN with homogeneous diameter. All data are expressed as mean value ± standard deviation. Both drug-free LN and ET-LN mean diameters were below 150 nm, indicating that LN developed are suitable for both gastrointestinal and intravenous administration without entailing embolism risks (Charman and Stella, 1992; Estella-Hermoso de Mendoza et al., 2009c; Varshosaz et al., 2010; Zimmermann et al., 2000). Furthermore, the incorporation of edelfosine into LN proved to significantly decrease the hemolytic toxicity of the free drug in a previous work published by our group (Estella-Hermoso de Mendoza et al., 2012). PDI was below 0.3, proving that the developed particles were monodisperse (Anton et al., 2008; Estella-Hermoso de Mendoza et al., 2008). Zeta potential of LN, a parameter that indicates the stability of colloid dispersions, was not affected by the incorporation of edelfosine (-28.46 for B-LN and -27.07 mV for ET-LN). In general, zeta potential values of -30 mV are enough for suitable stabilization (Estella-Hermoso de Mendoza et al., 2009c). Therefore, in our case the zeta-potential values obtained indicate that the formulations presented a good physical stability and that the LN generated would not induce cell membrane instability (Estella-Hermoso de Mendoza et al., 2009c; Müller RH, 1996). The LN obtained showed good encapsulation efficiency of 58.80%, presenting a drug loading of 30 μg ET per mg of formulation.

3.2. ET anti-tumor effect is enhanced when drug is encapsulated in LN

As the encapsulation procedure may modify the anti-tumor potential of ET, we analyzed the effectiveness of the ET-LN in MCF7 breast cancer cells. In order to evaluate the cytotoxic potential of the encapsulated drug, MCF7 cells were cultured for 72 h in the presence of growing concentrations of ET or equivalent concentrations of ET-LN. B-LN were also tested as control. Cytotoxicity was assessed by the comparison of IC_{50} values. The optimized ET-LN were effective in inhibiting the proliferation of MCF7 breast cancer cell line at an IC_{50} concentration of 12.9 (± 2.23) μg/mL, whereas the corresponding IC_{50} values of the free drug and B-LN were 17.8 (± 4.06) and 28.7 (± 8.08) μg/mL respectively (Fig. 1).

The efficacy of the drug may also be assessed by visualizing changes in the appearance and size of cells after being incubated with the different treatments. Optical inspection of the MCF7 cell cultures by light microscopy revealed morphological changes in MCF7 after the treatment with either ET or ET-LN. Compared with untreated controls and with cells incubated with B-LN, treated cell cultures showed expression of intracellular vesicles (indicated with arrows in Fig. 2A) and presented distorted and detached rounded cells that had shrunk. These signs are indicative of cell death (Kroemer et al., 2009). In fact, the percentage of viable cells was dramatically diminished in ET-LN-treated cultures in comparison to cells treated with B-LN. Moreover, the observed decrease was significantly higher than that of the cells treated with the free drug, confirming that the encapsulation strategy increased the cytotoxic potential of ET (Fig. 2B). This effect was time-dependent and reached statistical significance after 72 h of incubation.

Hence the developed ET-LN formulation maintained the anti-tumor activity of ET; moreover, ET-LN were 1.37 times more effective in inducing cell death in MCF7 cells than the free drug, and produced a remarkable decrease in cell viability.

Our results are in accordance with previously reported work, in which the cytotoxic effect of ET against other tumor types was found to be significantly improved when encapsulated in LN (Estella-Hermoso de Mendoza et al., 2009b; Estella-Hermoso de Mendoza et al., 2011; Lasa-Saracibar et al., 2013), thereby giving additional support to the usefulness of encapsulating the ET in LN.

3.3. Cell cycle arrest and induction of apoptotic mechanisms by ET and ET-LN in MCF7 breast cancer cells

The aforementioned decrease in cell viability may be caused by an abnormality in the cell cycle or in the cell growth. Therefore, MCF7 cells were stained with PI and subsequently cell cycle status was examined by flow cytometry (Fig. 3A). In cells grown either with ET or ET-LN, a cell accumulation in G1 stage, with a concomitant reduction of cells in S phase, was detected (Fig. 3B). It has been reported that ET-treated cells may accumulate in the G0/G1 and G2/M phases of the cell cycle, due to an inhibition of cytokinesis (Mollinedo et al., 2004; Pushkareva et al., 1999), although the cell cycle blockage induced by ET has more commonly been detected at the G2/M stage (Lasa-Saracibar et al., 2013; Mollinedo et al., 2004; Nieto-Miguel et al., 2007). This suggests that ET may provoke cell cycle arrest by influencing diverse molecular pathways, and the phase where the cell cycle is blocked would depend on the cell signaling routes characteristic of each cancer cell type.

On the other hand, the aforementioned cell cycle arrest may reflect defective cell growth or an induction of cell death mechanisms caused by the effects that ET exerts in tumor cells. Moreover, the disturbance of phospholipid metabolism and the interference in the membrane interactions with signaling molecules caused by ET may induce cellular stress, deregulation of apoptotic pathways, growth inhibition and cell cycle arrest and apoptosis (Mollinedo et al., 2010b; Van Blitterswijk and Verheij, 2008; Van der Luit et

al., 2007). In addition, ET has been described to induce apoptosis through the aggregation of Fas/CD95 in clustered rafts (Gajate et al., 2009; Gajate and Mollinedo, 2007). Accordingly, we analyzed the apoptotic status of treated-MCF7 cultures. Firstly, the percentage of cells in the sub-G1 region (hypodiploidy) in cell cycle analysis was determined by staining the cells with PI. As shown in Fig. 4A, the sub-G1 subset of MCF7 cells exhibited a strong increase in cells treated with either ET or ET-LN after 72 h of treatment. Along the lines of previous reported analyses of sub-G1 of ET-LN in several cancer malignancies (Mollinedo et al., 2010b), our data indicates that MCF7 undergoes apoptosis in the presence of ET in the culture media. However, the extensive DNA fragmentation and loss of DNA fragments is not exclusive to apoptotic death. Besides, the sub-G1 peak may represent apoptotic cells, as well as nuclear fragments, clusters of chromosomes, micronuclei or nuclei with normal DNA content but altered chromatin structure and diminished accessibility of fluorochrome to DNA (Darzynkiewicz et al., 2001; Riccardi and Nicoletti, 2006).

The aforementioned effect may dim the DNA degradation caused by apoptosis. Consequently, to further characterize this cell death mechanism, cells were treated in identical conditions, stained with annexin V-FITC and PI dyes and subsequently analyzed by flow cytometry to determine the percentage of viable, early apoptotic, and late apoptotic/necrotic sub-populations in MCF7-treated cultures (Fig. 4B and C). As is shown in Fig. 4C, the early apoptosis and the late apoptosis/necrosis/death cell subsets were increased in treated cells in comparison to their corresponding control. This induction was detected even with lower concentrations of the treatments (data not shown), and was statistically significant after 48 and 72 h of incubation with 20 μg/mL of ET or equivalent concentrations of ET-LN. Interestingly, the induction of cell death was stronger in ET-LN than in cells grown in the presence of the free drug, compared to their equivalent B-LN controls. In particular, the cell late apoptosis/necrosis/death subset was

highly increased in cells grown in the presence of 20 μg/mL of ET-LN for 72 h. The results are in agreement with those recently reported by Lasa-Saracíbar *et al* in leukemic cells when treated with the free drug and ET-LN (Lasa-Saracibar et al., 2013). Accordingly, our results show that both ET and ET-LN displayed a pro-apoptotic activity against MCF7 breast cancer cells in a time- and dose-dependent manner. This effect was probably caused by the characteristic controlled drug release of LN. In addition, there was a substantial increment in the population of cells that underwent apoptosis in the presence of ET-LN in comparison to the free drug treatment.

The results presented indicate that the cytotoxic effect of ET on MCF7 was considerably enhanced when the drug was entrapped in LN. Moreover, although ET was able to promote both an apoptotic and a cytostatic effect in MCF7, such anti-tumor effects were considerably enhanced in ET-LN-treated cells.

4. Conclusions

The data presented in this paper provide evidence that entrapping ET in LN entails an improvement in its efficacy when treating MCF7 breast cancer cells, leading to a moderate cell cycle arrest. Besides, ET-LN induced apoptosis in a time-and dose-dependent manner to a greater extent in comparison with the treatment using the free drug, suggesting that encapsulating ET in LN is essential to improve the efficacy of the drug. Molecular cell death mechanisms must be studied in greater depth in order to further characterize the induction process by which ET-LN exert their anti-tumor effect.

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Conflict of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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FIGURE CAPTIONS

Fig. 1. MTT assay was used to determine the dose-response curve in MCF7 cells at 72 h of incubation with different doses of free edelfosine (ET), unloaded (B-LN) or drug-loaded (ET-LN). IC_{50} values were calculated using the sigmoidal dose-response function. DMSO-treated cells were included as positive control of cell death.

Fig. 2. Cytotoxic effects in MCF-7 cells after treatment with 20 μg/mL of edelfosine (ET) or equivalent concentrations ET-loaded nanoparticles (ET-LN). B-LN were also tested as control. A) Light microscope images revealed that at 48 h treated groups showed unattached and distorted cells and the expression of intracellular vesicles (indicated with arrows). B) Trypan blue assay was used to determine cell viability of treated cells at 48 and 72 h. Untreated (WT) cells were included as control. *** $p<0.001$ vs. corresponding ET group by two-way ANOVA (Bonferroni post-test).

Fig. 3. Free and nanoencapsulated drug effect in cell cycle after 48 and 72 h of incubation. Cells were treated with medium, blank-LN, free ET and drug-loaded LN at a dose equivalent to 20 μg/mL of edelfosine (ET). (A) Cell cytometry images of treated cells. (B) Quantification of cell cycle peaks.

*$p<0.05$; **$p<0.01$; vs. control by two-way ANOVA (Bonferroni post-test).

Fig. 4. Apoptosis analysis of MCF7 after incubation with medium (WT), blank-LN (B-LN), free edelfosine (ET) and drug-loaded LN (ET-LN) at a dose equivalent of 20 μg/mL of edelfosine. Apoptosis was firstly assessed with quantification of DNA content in cells and determination of sub-G1 cell subset (A) and subsequently analyzed with flow cytometry (B).

*p<0.05; vs control with Kruskall-Wallis test (Dunns post-hoc test) for sub-G1 analysis, *p<0.05 **p<0.01; ***p<0.001 vs. B-LN control by two-way ANOVA (Bonferroni post-test).