International Journal of Pharmaceutics 454 (2013) 720–726

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Efficacy of edelfosine lipid nanoparticles in breast cancer cells

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ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form 27 March 2013
Accepted 15 April 2013
Available online 30 April 2013

Keywords:
Breast cancer
MCF7
Lipid nanoparticles
Edelfosine
Alkylphospholipids

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.

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, 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

Abbreviations: ET, edelfosine; ALPs, alkylphospholipids; LN, lipid nanoparticles; EPR effect, enhanced permeability and retention effect; ET-LN, edelfosine-loaded lipid nanoparticles; MCL, mantle cell lymphoma; PDI, polydispersity index; B-LN, blank (unloaded) nanoparticles; PI, propidium iodide; IC_{50}, inhibitory concentration 50.

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http://dx.doi.org/10.1016/j.ijpharm.2013.04.068

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). Ultrapurified 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 −80 °C 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. 21,000 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 37 °C. 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, 21,000 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 4 °C for 1 h. Next, cells were incubated with 0.45 U/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, 21,000 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