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Original Article

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

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A B S T R A C T

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.

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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.

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.

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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.

Material and methods

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®.

Preparation of DOX-LN

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 s 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 s. 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 min, 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.

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 min 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.

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 min at 13 W. Finally, LN were obtained by cooling the emulsion in ice. LN were washed and lyophilized as described above.

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.

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 °C and a humidified atmosphere with 5% carbon dioxide.

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).

DOX accumulation in osteosarcoma cells

For DOX cellular accumulation studies, cells (1.4×10^4 U-2 OS cells and 3.5×10^3 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, 20,000 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).

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