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RESEARCH PAPER

Novel Nanostructured Lipid Carrier Co-Loaded with Doxorubicin and Docosahexaenoic Acid Demonstrates Enhanced *in Vitro* Activity and Overcomes Drug Resistance in MCF-7/Adr Cells

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ABSTRACT

Purpose To develop a nanostructured lipid carrier (NLC) co-loaded with doxorubicin and docosahexaenoic acid (DHA) and to evaluate its potential to overcome drug resistance and to increase antitumoral effect in MCF-7/Adr cancer cell line.

Methods The NLC was prepared by a hot homogenization method and characterized for size, zeta potential, entrapment efficiency (EE) and drug loading (DL). Drug release was evaluated by dialysis in complete DMEM, and NLC aggregation was assayed in the presence of serum. The cytotoxicity of formulations, doxorubicin uptake or penetration were evaluated in MCF-7 and MCF-7/Adr as monolayer or spheroid models.

Results The formulation had a size of about 80 nm, negative zeta potential, EE of 99%, DL of 31 mg/g, a controlled drug release in DMEM and no particles aggregation in presence of serum. The NLC loaded with doxorubicin and DHA showed the same activity as free drugs against MCF-7 but a stronger activity against MCF-7/Adr cells. In monolayer model, the doxorubicin uptake as free and encapsulated form was similar in MCF-7 but higher for the encapsulated drug in MCF-7/Adr, suggesting a bypassing of P-glycoprotein bomb efflux. For spheroids, the NLC loaded with doxorubicin and DHA showed a prominent cytotoxicity and a greater penetration of doxorubicin.

Conclusions These findings suggest that the co-encapsulation of doxorubicin and DHA in NLC enhances the cytotoxicity and overcomes the doxorubicin resistance in MCF-7/Adr.

KEY WORDS cancer therapy · docosahexaenoic acid · doxorubicin · nanostructured lipid carrier · resistance overcoming

ABREVIATIONS

DDS	Drug delivery system
DHA	Docosahexaenoic acid
DL	Drug loading
DOX	Doxorubicin
EE	Entrapment efficiency
EPR	Enhanced permeability and retention
NE	Nanoemulsion
NLC	Nanostructured lipid carrier
PO	Peanut oil
SLN	Solid lipid nanoparticles
TEA	Triethanolamine

INTRODUCTION

Doxorubicin is an anthracycline with broad-spectrum anticancer activity that is widely used in cancer therapy but has serious side effects highlighted by potentially fatal cardiotoxicity (1,2). Its low tumor penetration and limited distribution within solid tumors are the main causes of its failures as a therapeutic agent (3,4). Anthracycline-based combination chemotherapy regimens have shown improvement in activity compared to a single anthracycline, including combined doxorubicin or epirubicin with cyclophosphamide, doxorubicin with cyclophosphamide and fluorouracil, epirubicin with cyclophosphamide and fluorouracil. However, despite the greater activity, these regimens are also more toxic than single agent regimens (1,5).

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Among alternatives, the combination of docosahexaenoic acid (DHA) and doxorubicin has been described as increasing the sensitivity of tumors to chemotherapy compared to doxorubicin alone (6–8). DHA is a long-chain (C_{22}) polyunsaturated fatty acid (omega 3) that enhances oxidative stress and subsequent lipid peroxidation of tumor cells (9). Several studies have reported an increase in the sensitivity of mammary tumors in rodents to doxorubicin when accompanied by a prolonged supplementation with DHA (7,10) and others have verified that this is probably due to the increased level of oxidative stress, particularly in cancer cells (11). In addition, DHA attenuates drug resistance and improves anticancer drug efficacy in resistant cell lines (8,9). Recently, a phase II trial verified that a dietary DHA supplementation improved the outcome of chemotherapy in metastatic breast cancer patients (12). However, the varieties of drug administration schedules have complicated the management of the pharmacokinetic and pharmacodynamic profiles, and obtaining uniform temporal and spatial co-delivery remains challenging. Thus, the combination of drugs co-delivered in drug delivery systems (DDSs) has emerged as an attractive alternative (13).

DDS-based combination therapy offers many advantages, such as more synchronized, controlled pharmacokinetics of the drugs, improved drug solubility and bioavailability and the potential to bypass mechanisms of multidrug resistance. Overall, these combinations can result in improved drug efficacy with a single formulation (13,14). Among these DDSs, nanocarriers including liposomes, polymeric micelles and lipid nanoparticles have been described. A few DDSs have reached the market, e.g. liposomal doxorubicin. Such nanocarriers with sizes of 50–200 nm enhance the concentration of drugs in the tumor by a mechanism known as the "enhanced permeability and retention (EPR) effect" (15–17).

Lipid nanoparticles such as solid lipid nanoparticles (SLNs) and nanostructured lipid carrier (NLC) have gained much attention in recent years. SLNs are derived from nanoemulsions where the liquid lipid is replaced by a solid lipid. NLC represents an improved generation of SLNs that consists of solid lipid matrices with spatially distributed liquid lipids, resulting in a structure with imperfections in the crystal structure and can better accommodate the drug (19). NLC and SLN share advantages including biocompatibility and the possibility of production on an industrial scale, since they do not require the use of organic solvents and due to their similarity to parenteral nutrition manufacturing methods (18–20). However, the controlled drug release for NLC can be problematical depending of the solid/liquid lipid ratio (19,21). A disadvantage of SLN/NLC is the low entrapment of hydrophilic drugs. To increase the drug encapsulation, the formation of an ion pairing with a lipophilic counter-ion has been proposed as alternative (22–24).

This work aims to develop a new NLC co-loaded with doxorubicin and DHA triglyceride and to evaluate the physicochemical characteristics, drug release and the *in vitro* efficacy in sensitive and resistant human breast cancer cell lines in a cell monolayer model and resistant cells in a spheroid model.

MATERIALS AND METHODS

Materials

Doxorubicin hydrochloride (DOX) was purchased from ACIC Chemicals (Ontario, Canada). Doxorubicin liposome injection (Lipodox®) was purchased from Sun Pharma Global FZE (Gujarat, India). Triethanolamine (TEA), oleic acid (OA), ethylenediamine tetraacetic acid sodium (EDTA) were purchased from Sigma-Aldrich (Missouri, USA). Glyceryl behenate (Compritol 888 ATO®) was kindly provided by Gattefossé (Rhône-Alpes, France). Monooleate of sorbitan ethoxylated (Super refined™ polysorbate 80; Tween ™ 80), docosahexaenoic acid (DHA) as triglyceride (Incromega™ DHA 500TG) and peanut oil (PO) (Super Refined ™ Peanut Oil) were kindly provided by Croda Inc. (New Jersey, USA). Dulbecco's Modified Eagle Medium (DMEM), antibiotic stock solution (10,000 I.U. of penicillin + 10,000 μg/mL of streptomycin) and 0.25% Trypsin-EDTA were purchased from CellGro (Virginia, USA). Heat inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Georgia, USA). The Cell Titer Blue assay kit and CytoTox 96® Non-Radioactive Cytotoxicity Assay were purchased from Promega (Wisconsin, USA). All other chemicals were of analytical grade.

Preparation of Formulation

The formulation was prepared by the hot melting homogenization method using emulsification-ultrasound. The composition was very similar to the one previously described (24) but with some modifications. The DHA was used in a triglyceride form (Incromega® DHA 500 TG). The formation of the ion pairing to improve the doxorubicin encapsulation was obtained with oleic acid. Briefly, for a batch of 10 ml of formulation, the oily phase (OP) was prepared with 100 mg of Tween 80, 10 mg of oleic acid, 5 mg of doxorubicin, 6 mg of triethanolamine and 150 mg of matrix lipid composed of 110 mg of Compritol and 40 mg of DHA (0.4% w/v). The aqueous phase (AP) was composed of 4 mg of EDTA and purified water. About 8 mL of water was added at the beginning of preparation and at the end volume was completed to 10 mL. This formulation was termed NLC-DOX + DHA 0.4%. A NLC containing DHA at 0.8% (70 mg of Compritol and 80 mg of DHA; NLC-DOX + DHA 0.8%) and a nanoemulsion containing only DHA as lipid matrix (150 mg of DHA; NE-DOX + DHA 1.5%), were also prepared to evaluate the influence of the lipid liquid on doxorubicin release. Furthermore, a NLC loaded with DOX without DHA was prepared replacing the 0.4% of DHA by a liquid

lipid with a low amount of unsaturated lipids (peanut oil – PO) (NLC-DOX + PO 0.4%). To prepare them, firstly the OP and the AP were heated separately to 80°C. After OP melting at 80°C, the AP was gently dropped into the OP and homogenized using a glass rod for 1 min. This emulsion was immediately subjected to intense probe sonication (8 W) for 5 min, using a high intensity ultrasonic processor (Fisher F60 Sonic Dismembrator Fisher F-6; Fisher Scientific, Pennsylvania, USA). The pH was adjusted to 7.0 with 0.1 M HCl or 0.1 M NaOH and the volume was adjusted to 10 mL. The formulation was stored at 4°C, protected from light in a nitrogen atmosphere.

Characterization of Formulations

The mean particle diameter and zeta potential were measured by dynamic light scattering (DLS) and electrophoretic mobility, respectively, using the Zeta PLUS particle size analyzer (Brookhaven Instruments; Brookhaven, USA). All measurements were performed in triplicate.

The encapsulation efficiency (EE) and drug loading (DL) of doxorubicin in NLC were determined by an ultrafiltration method using centrifugal devices (Amicon® Ultra - 0.5 mL 100 k; Millipore, USA). To eliminate the binding of doxorubicin on the devices, a pretreating of the filters was performed as previously described (24). The devices were soaked in a passivating solution (Tween^{TM} 20, 5% w/v), maintained overnight at room temperature, and washed with distilled water prior to use.

The EE and DL were calculated using the following equations:

$$EE_{(\%)} = (C_T - C_{AP})/C_T \times 100$$
$$DL_{(mg/g)} = W_{DL}/W_{NP}$$

where C_T = total doxorubicin concentration in NLC, C_{AP} = doxorubicin concentration in aqueous phase (non-encapsulated), W_{DL} = mg of drug loaded in nanoparticles and W_{NP} = g of nanoparticles (lipids).

Briefly, the C_T, C_{AP} and W_{DL} were evaluated as follows. The C_T was analyzed by dissolving an aliquot of the NLC dispersion in a mixture of tetrahydrofuran (THF)/methanol (MeOH) 40:60 v/v, centrifugation for 10 min at 2,400 g and analysis of the supernatant by spectrophotometry at 480 nm (UV-mini 1240; Shimadzu, Japan). The C_{AP} was evaluated from an aliquot of the aqueous phase separated from the NLC dispersion by ultrafiltration (10 min at 2,400 g), dilution with THF/MeOH and analysis by UV-visible. The W_{DL} was derived using the calculated EE x mg total of doxorubicin added.

Serum Stability

Serum stability of the NLC was evaluated by incubation of the NLC with FBS 1:1 at 37°C with shaking (250 rpm) for up to 24 h. Aliquots were sampled, diluted in purified water and the measurement of zeta potential and size were performed using the Zeta Plus Analyzer.

Release of Doxorubicin from NLC

The release study was conducted by dialysis in DMEM containing 10% fetal bovine serum (FBS) (25). Dialysis tubes with a cutoff size of 100 KDa (Cellulose ester membrane; Spectrum Laboratories; Rancho Dominguez, USA) were filled with 3 mL of formulations diluted in media (1:2), sealed and incubated with 50 ml of the media for up to 48 h at 37°C, with continuous shaking at 250 rpm. A doxorubicin aqueous solution (0.5 mg/mL) was used as a control. At various time points, aliquots were withdrawn and doxorubicin concentration was measured with the Synergy HT Multi-Mode Microplate Reader (Biotek; Winnooski, USA) at 485/590 ex/em wavelengths. The values were plotted as cumulative percentage of drug release.

Cell Culture

MCF-7 were purchased from The American Type Culture Collection (ATCC; Manassas, USA) and MCF-7/Adr was obtained from The National Cancer Institute (Frederick, MD, USA). Cells were grown and maintained in DMEM, pH 7.4, supplemented with 10% (v/v) FBS and 1% of penicillin/streptomycin stock solution in a humidified, 5% (v/v) CO_2 atmosphere at 37°C.

Adherent Monolayer Cells

Cell Viability

Cell viability was assayed with the CellTiter-Blue® Cell Viability Assay kit (CTB) following the manufacturer's protocol. Cells were seeded in 96-well tissue culture plates at 5×10^3/well about 24 h before the treatment. The drug solutions were freshly prepared by dissolving doxorubicin in purified water and DHA in ethanol. We evaluated the cytotoxicity of the encapsulated drug compared to the free drug with doxorubicin concentrations between 0.5 and 16 μM and DHA at 3.5–112 μM. The cells were incubated with treatments for 24 h, washed thrice with complete DMEM, supplemented with 100 μL of DMEM and 20 μL of the CTB reagent. The incubation was performed for 2 h and the fluorescence intensity was measured using a Synergy HT Multi-Mode Microplate Reader at 530/590 nm excitation/emission wavelengths. The solvent controls were done.

Doxorubicin Uptake

The cellular uptake of doxorubicin was assessed by flow cytometry by using the natural fluorescence of the drug. Cells

were seeded in 12 well plates at a density of 1×10^5 cells/well 24 h before the treatment. The seeding medium was then removed and replaced by media containing 16 μM of doxorubicin. After 2 h incubation, the cells were washed thrice with PBS, trypsinized, centrifuged at 2000 g for 5 min, re-suspended in PBS pH 7.4 and analyzed using a BD FACSCalibur flow cytometer. The cells were gated using forward (FSC-H) *versus* side-scatter (SSC-H) to exclude debris and dead cells before analysis of 10,000 cell counts. The mean fluorescence intensity (MFI) of the cells was measured at 488/585 nm (FL2 channel) excitation/emission wavelengths.

Spheroids

Preparation of Spheroids

MCF-7/Adr were used to prepare spheroids of 400–500 μm diameter from 10,000 cells in 96 well plates by a liquid overlay method (26). Briefly, DMEM with 1.5% of agarose was added to each well and the cells were seeded. After this, the plates were centrifuged for 15 min at 1,500 RCF. Spheroid formation was monitored using a Nikon Eclipse E400 microscope (Nikon Inc., Melville, USA) at 10× magnification with a Spot Insight™ 3.2.0 camera with Spot Advanced™ software (Spot Imaging, Sterling Heights, MI).

Cytotoxicity

Cytotoxicity was measured with a Cytotox 96 Non-Radioactive Cytotoxicity kit (Promega; Fitchburg, USA) (26). Briefly, both the lactate dehydrogenase (LDH) released into the medium and the LDH from spheroids dissociated after incubation for 1 h at 37°C with 0.9% Triton®-X100 in DMEM media with 10% FBS were measured. The amount of LDH released into the medium after incubation with the treatments was calculated as a percentage in relation to the total (total LDH = medium LDH + lysate LDH).

Penetration of Doxorubicin

The penetration of doxorubicin throughout the spheroids was evaluated by confocal microscopy after 2 h of incubation with 100 μM of doxorubicin in complete DMEM using 488 nm and 535 nm long pass filters for doxorubicin excitation/emission, respectively. The spheroids were removed from the plate, washed with PBS and placed into Lab-Tek chambers (Fisher Scientific) prior to imaging. They were visualized at 10× magnification, and the distribution of doxorubicin throughout the spheroids was analyzed with a Zeiss LSM 700 confocal microscope using Z-stack imaging with 10 μm intervals and the LSM Image Browser software.

Data Analysis

Analyses of the cytotoxicity studies were carried out using a one-way ANOVA followed by a Newman-Keuls' test. Student's *t*-test was performed to compare data on the physico-chemical characteristics of the formulations. For all analyses, differences were considered significant when the *P* value was < 0.05.

RESULTS

NLC Characterization

Table I lists the characterization data of the formulations for size, zeta potential, entrapment efficiency and drug loading. The particle size was in the range of 76–86 nm with values of the polydispersivity index (PDI) lower than 0.22 suggesting monodispersion. The zeta potential was negative for all formulations and varied between −23 and −36 mV. The entrapment efficiency reached nearly 100%, and the drug loading was 31 mg/g. The formulations as NLC-DOX + DHA 0.8% and NE-DOX + DHA 1.5% showed results (data not shown) very similar to those of NLC-DOX + DHA 0.4% for all characteristics.

Drug Release

The results of the doxorubicin release assay performed by dialysis with complete DMEM are included in Fig. 1. The released doxorubicin from NLC-DOX + DHA 0.4% was 5 ± 1% in the first 30 min and gradually increased to 30 ± 1% 4 h, followed by a plateau. No further drug was released for up to 48 h. In contrast, NLC-DOX + DHA 0.8% and NE-DOX + DHA 1.5% allowed a doxorubicin release at 30 min of 17 ± 1% and 17 ± 2%, respectively, and increased to more than 75% within 4 h. Therefore, we verified a higher amount of doxorubicin released from NLC-DOX + DHA 0.8% and NE-DOX + DHA 1.5% for all evaluated points and a lower capacity to retain the drug than NLC-DOX + DHA 0.4%. Based on these data, the formulation NLC-DOX + DHA 0.4% was chosen for the subsequent studies. It is noteworthy that the free doxorubicin evaluated as a control and was more than 90% outside of the bag at 2 h, demonstrating a sink condition.

NLC Stability in Serum

Considering the intended use (intravenous administration), the stability of NLC-DOX + DHA 0.4% in serum was investigated to check for aggregation in the presence of proteins. The size, PDI and zeta potential are described in Table II. Their size was 68 nm after incubation with serum for up to

Table I Nanostructured Lipid Carrier (NLC) Formulations Characterization

Formulation	Size (nm)	PDI	Zeta potential (mV)	Doxorubicin EE (%)	Doxorubicin DL (mg/g)
Blank NLC	84 ± 8	0.22 ± 0.03	−32 ± 12	–	–
NLC-DOX + PO 0.4%	76 ± 10	0.20 ± 0.01	−40 ± 4	99 ± 0	31 ± 0
NLC-DOX + DHA 0.4%	86 ± 7	0.13 ± 0.04	−36 ± 9	99 ± 0	31 ± 0

N = 3; Mean ± SD

PDI Polydispersity index; *EE* encapsulation efficiency; *DL* drug loading; *Blank NLC* NLC with no drugs; *NLC-DOX + PO 0.4%* NLC loaded with doxorubicin and peanout oil 0.4%; *NLC-DOX + DHA 0.4%* NLC loaded with doxorubicin and DHA 0.4%

24 h. The variation of PDI was very small (in the range of 0.15 to 0.18). There was no significant difference in zeta potential (−32 to −37 mV). Thus, the NLC-DOX + DHA 0.4% was considered very stable after incubation with serum and suggests that this formulation will not aggregate after intravenous administration.

Adherent Monolayer Cells

Cytotoxicity

The results of cytotoxicity testing in monolayer culture are described in Fig. 2. For the sensitive MCF-7 (Fig. 2a), the higher cytotoxicity of NLC-DOX + DHA 0.4% was verified only at 16 μM of doxorubicin (equivalent to 112 μM of DHA). In these concentrations, the cell viability was 18 ± 1%, 17 ± 2%, 14 ± 0%, 12 ± 0% for free doxorubicin, free doxorubicin + DHA, NLC-DOX + PO 0.4% and NLC-DOX + DHA 0,4%, respectively. There was no significant difference between the groups at 0.5–8 μM of doxorubicin. The results were remarkable for the resistant MCF-7/Adr cell line, described in the Fig. 2b. Higher anticancer activity of NLC-based drug combination over co-treatment with both free drugs was apparent at 2 μM to 16 μM of doxorubicin. At 4 μM of doxorubicin (28 μM of DHA) the cell viability was 62 ± 8%, 63 ± 2%, 37 ± 2%, 30 ± 1% for free doxorubicin, free doxorubicin + DHA, NLC-DOX + PO 0.4% and NLC-DOX + DHA 0.4%, respectively. The highest cytotoxicity detected was at 16 μM of doxorubicin (112 μM of DHA) with 59 ± 2%, 61 ± 5%, 20 ± 1%, 7 ± 0% of viability for free doxorubicin, free doxorubicin + DHA, NLC-DOX + PO 0.4% and NLC-DOX + DHA 0.4%, respectively. There was no significant difference between free doxorubicin and free doxorubicin + DHA for all evaluated concentrations. The Blank NLC showed no significant cytotoxicity for both cell lines.

Doxorubicin Uptake

Doxorubicin uptake was evaluated by FACS (Fig. 3). The drug-sensitive MCF-7 cell line had similar fluorescence intensities for cells incubated with free doxorubicin and free doxorubicin + DHA. On the other hand, these preparations had an uptake higher than those observed for cells incubated with NLC-DOX + PO 0.4% and NLC-DOX + DHA 0.4%. In contrast, for the MCF-7/Adr resistant cell line, we observed a 2.6-fold higher fluorescence of the cells incubated with the

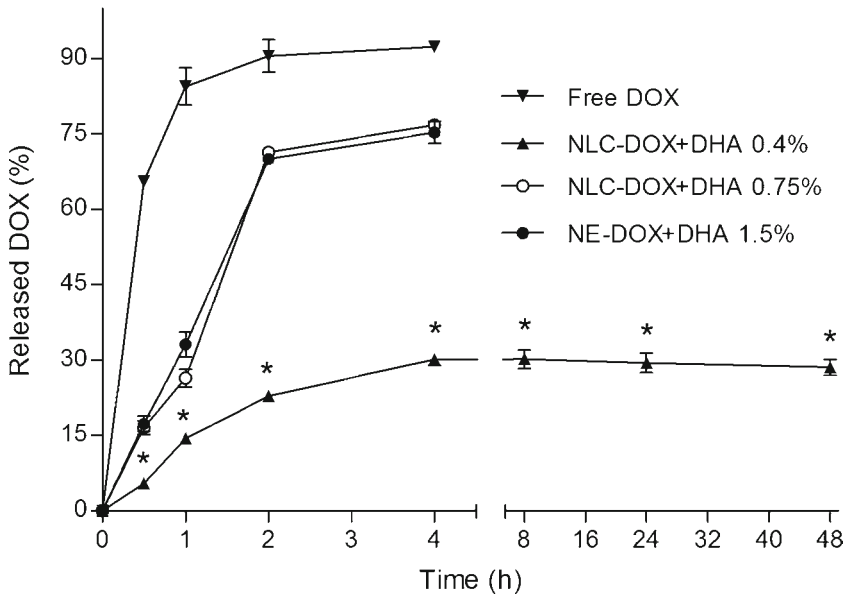

Fig. 1 Doxorubicin release in complete DMEM by dialysis using a cellulose ester membrane, 100 KDa MWCO. Formulations loaded doxorubicin or free doxorubicin were diluted in media and placed into dialysis tubes, incubated in 50 ml of media for up to 48 h at 37°C, with continuous shaking at 250 RPM. The released doxorubicin measured in the media was analyzed by fluorescence. *N* = 3; Mean ± SD; **P* < 0.05.

Table II Stability in Serum of the NLC Loaded with Doxorubicin and DHA 0.4%

Time (h)	Size (nm)	PDI	Zeta potential (mV)
0	68 ± 9	0.16 ± 0.01	−34 ± 6
4	70 ± 3	0.18 ± 0.01	−32 ± 8
8	70 ± 8	0.15 ± 0.00	−34 ± 7
24	70 ± 5	0.16 ± 0.01	−37 ± 4

N = 3; Averages ± SD

PDI Polydispersity index

NLC than the cells incubated with the free drugs. There was no difference between free doxorubicin and free doxorubicin + DHA. Neither was there between NLC-DOX + PO 0.4% and NLC-DOX + DHA 0.4%. No cell-associated fluorescence was detected with the blank formulation.

Spheroids

Cytotoxicity

The cytotoxicity of the formulations and free drugs in spheroids was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Fig. 4). Treatment of the spheroids with NLC-DOX + DHA 0.4% with doxorubicin at 100 μM + DHA at 700 μM resulted in a pronounced cytotoxicity with 93 ± 6% of released LDH. For NLC-DOX + PO 0.4%, Lipodox®, free doxorubicin + DHA and free doxorubicin, the release was 51 ± 13%, 44 ± 5%, 35 ± 10% and 25 ± 9%, respectively. In addition, after 24 h of incubation with NLC-DOX + DHA 0.4%, a complete destruction of the spheroid was observed microscopically, with many scattered lost cells (data not shown). It is noteworthy that the blank formulation and the cells treated only with media (control) produced no

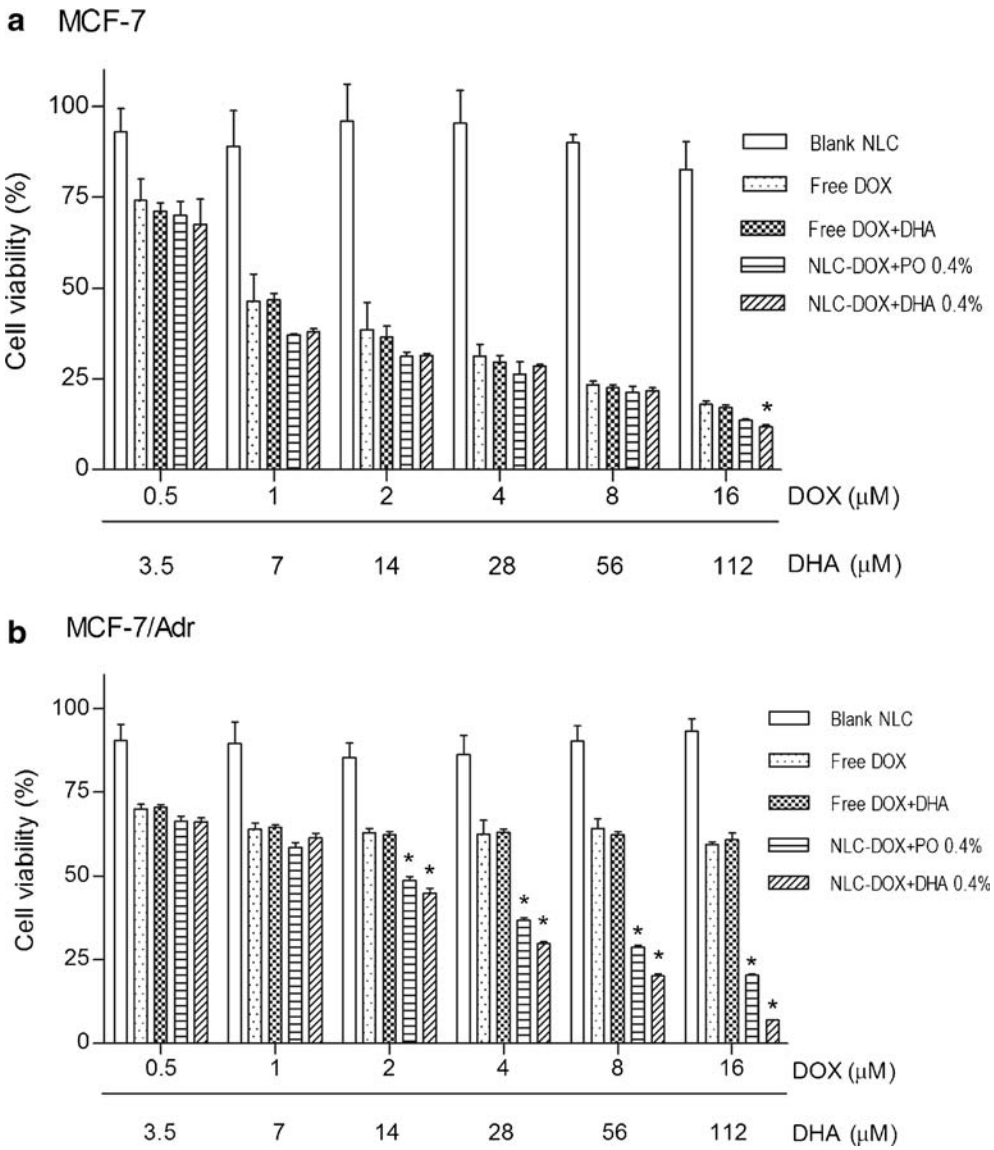

Fig. 2 Cytotoxicity of blank formulation (Blank NLC), free doxorubicin (Free DOX), free doxorubicin + DHA (Free DOX + DHA), doxorubicin + PO 0.4%-loaded NLC (NLC-DOX + PO 0.4%) and doxorubicin + DHA 0.4%-loaded NLC (NLC-DOX + DHA 0.4%) evaluated in (**a**) MCF-7 and (**b**) MCF-7/Adr after 24 h of incubation. *N* = 3; Mean ± SD. * *P* < 0.05 (comparing all the groups at the same concentration).

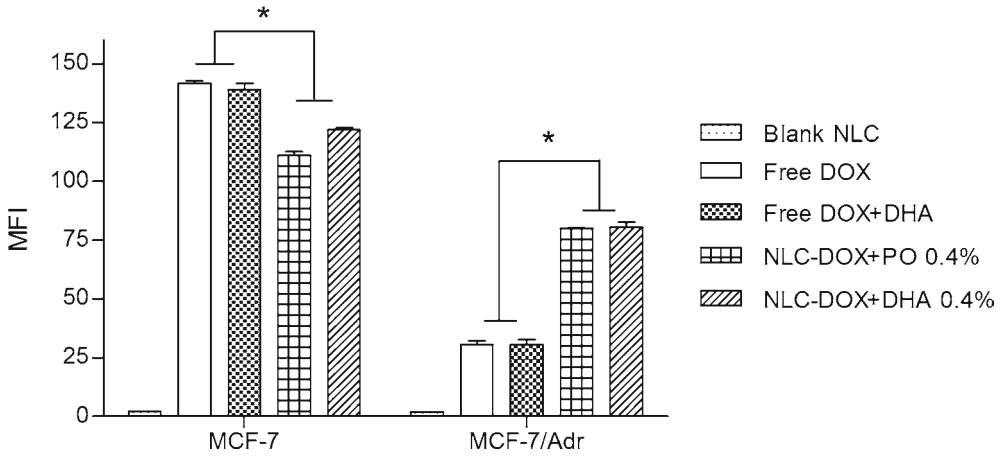

Fig. 3 Uptake of a blank nanostructured lipid carrier (Blank NLC), free doxorubicin (Free DOX), free doxorubicin + DHA (Free DOX + DHA), doxorubicin + PO 0.4%-loaded NLC (NLC-DOX + PO 0.4%) and doxorubicin + DHA 0.4%-loaded NLC (NLC-DOX + DHA 0.4%) in the drug sensitive and resistant cancer cell lines. The incubation time was 2 h with the equivalent of 16 μM of doxorubicin and the cells were analyzed for mean fluorescence intensity (MFI) by FACS. $N = 3$; Mean ± SD. *$P < 0.05$.

significant toxicity (less than 2% of released LDH) and no alteration of the spheroid structure.

Doxorubicin Penetration

To evaluate the potential of the formulations for penetration of avascular tumors, we analyzed the distribution of doxorubicin throughout spheroids by confocal microscopy (Fig. 5). In this figure, images represent increasing deeper layers from left to the right. A reduction of the fluorescence occurred with increased depth for all treatments. Differences in the fluorescence intensities between the groups can be organized for all analyzed layers as follows: NLC-DOX + DHA 0.4% > NLC-DOX + PO 0.4% > Lipodox® > Free doxorubicin + DHA (data not shown) = Free doxorubicin. Furthermore, at 70 μM depth (the deepest layer observed), the spheroids incubated with NLC-DOX + DHA 0.4% were notably more fluorescent in the inner core of the sectional cut than the others groups, indicating more penetration for this formulation. The spheroids incubated with the Blank NLC as well as with media only (controls) showed no fluorescence.

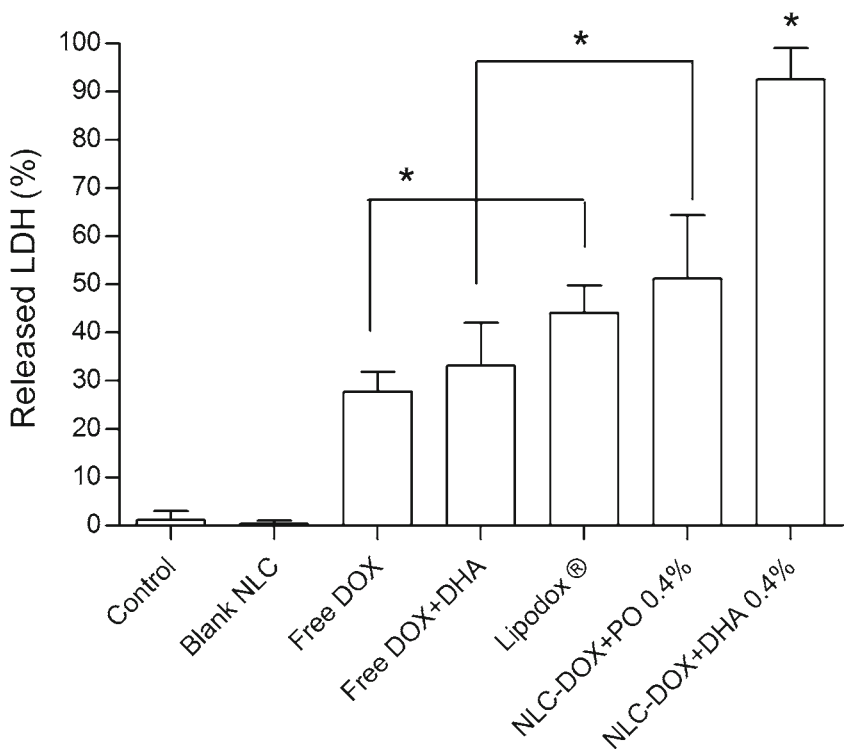

Fig. 4 Toxicity of medium (Control), the blank nanostructured lipid carrier (Blank NLC), free doxorubicin (Free DOX), free doxorubicin + DHA (Free DOX + DHA), liposomal doxorubicin (Lipodox®), doxorubicin + PO 0.4%-loaded NLC (NLC-DOX + PO 0.4%) and doxorubicin + DHA 0.4%-loaded NLC (NLC-DOX + DHA 0.4%) against MCF-7/Adr spheroids. The incubation was 48 h with the equivalent of 100 μM of doxorubicin. $N = 3$; Mean ± SD. *$P < 0.05$.

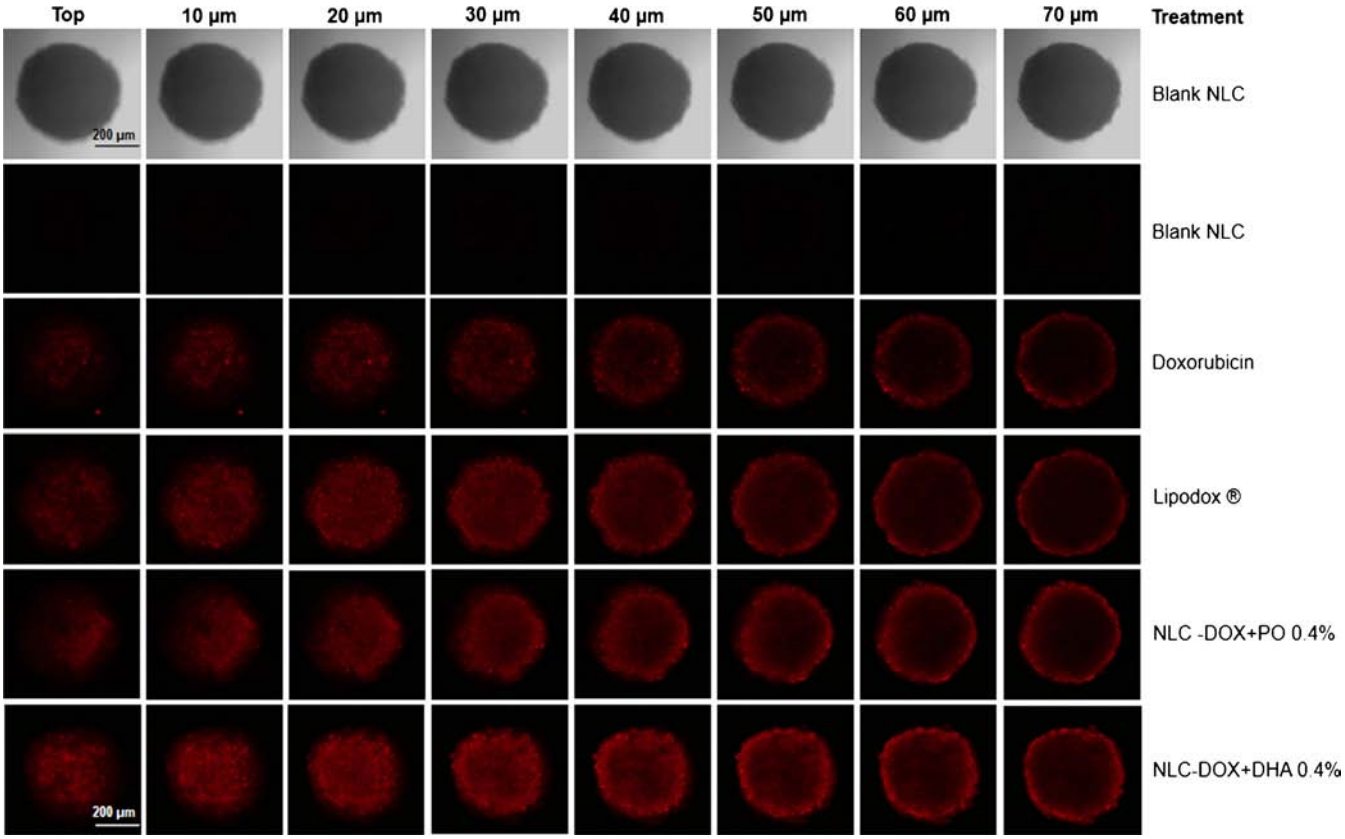

Fig. 5 Confocal images of MCF-7/Adr tumor spheroids after incubation for 2 h with different treatments. Z-stack images were obtained from the surface top towards the tumor spheroid equatorial plane at 10 μm intervals of thickness. The images are representative of duplicate samples.

DISCUSSION

Many efforts have been made to improve cancer treatment with combination therapies (1,5,27), and studies have described the increase of the doxorubicin activity through the combination with DHA (6–12). However, problems related to the route, formulation and schedule are still to be adequately addressed. Nanocarries have been proposed as a tool to overcome these limitations (13). In this work, we have proposed the co-encapsulation of doxorubicin and DHA in NLC a combination therapy of cancer.

Recently, NLC have been described as a promising drug delivery system for enhanced cancer therapy (28–30). Among the advantages of the NLC is the retention of the drug in the lipid core to promote the control of its release. Incorporation of up to 30% of liquid of the total lipid has been recommended for having a retained drug release (19,21). The NLC developed by loading doxorubicin and DHA at 0.4% w/v (corresponding to 30% liquid lipid) was evaluated for its release profile in comparison with formulations containing higher DHA concentrations. Our data clearly showed a prolonged controlled doxorubicin release for NLC-DOX+DHA 0.4% whereas the NLC-DOX+DHA 0.8% (50% liquid lipid) and NE-DOX+DHA 1.5% (100% liquid lipid) were unable to retain the drug in the matrices. However, a fast drug release (burst effect) of 30% was verified for NLC-DOX+DHA 0.4%. Similar burst effects have been described for SLN and NLC in many works as characteristic of these types of formulation (31,32). This may be attributed to some part of the ion pairing located at the interface of the nanoparticle (as represented in the scheme showing in Fig. 6), and would be quickly released by diffusion after incubation with media. We speculate that the 70% remaining not released up to 48 h has the potential to prolong the drug circulation, modify the pharmacokinetics, target the tumor and reduce the doxorubicin-induced toxicity compared with the free drug (33). The strategy of ionic pairing employed to prepare the formulations in this present work has been described as important for a higher drug release at a low pH (24). This may be of interest for a tumor site-specific release of the drug, since the tumor pH is slightly acidic (4,34). Doxorubicin has an anticancer mechanism of action that is cell cycle non-specific. Additionally, a sharp peak of concentration of the drug in a short period of time to prevent cell recovery and resistance is desirable (35). Therefore, NLC-DOX+DHA 0.4% was selected to further experiments, which included cytotoxicity assays with cancer cells. The cell viability in a MCF-7/Adr monolayer cell model showed a significantly higher cytotoxicity for the NLC-DOX+DHA 0.4%. This can be attributed to the co-encapsulation of doxorubicin and DHA in NLC since

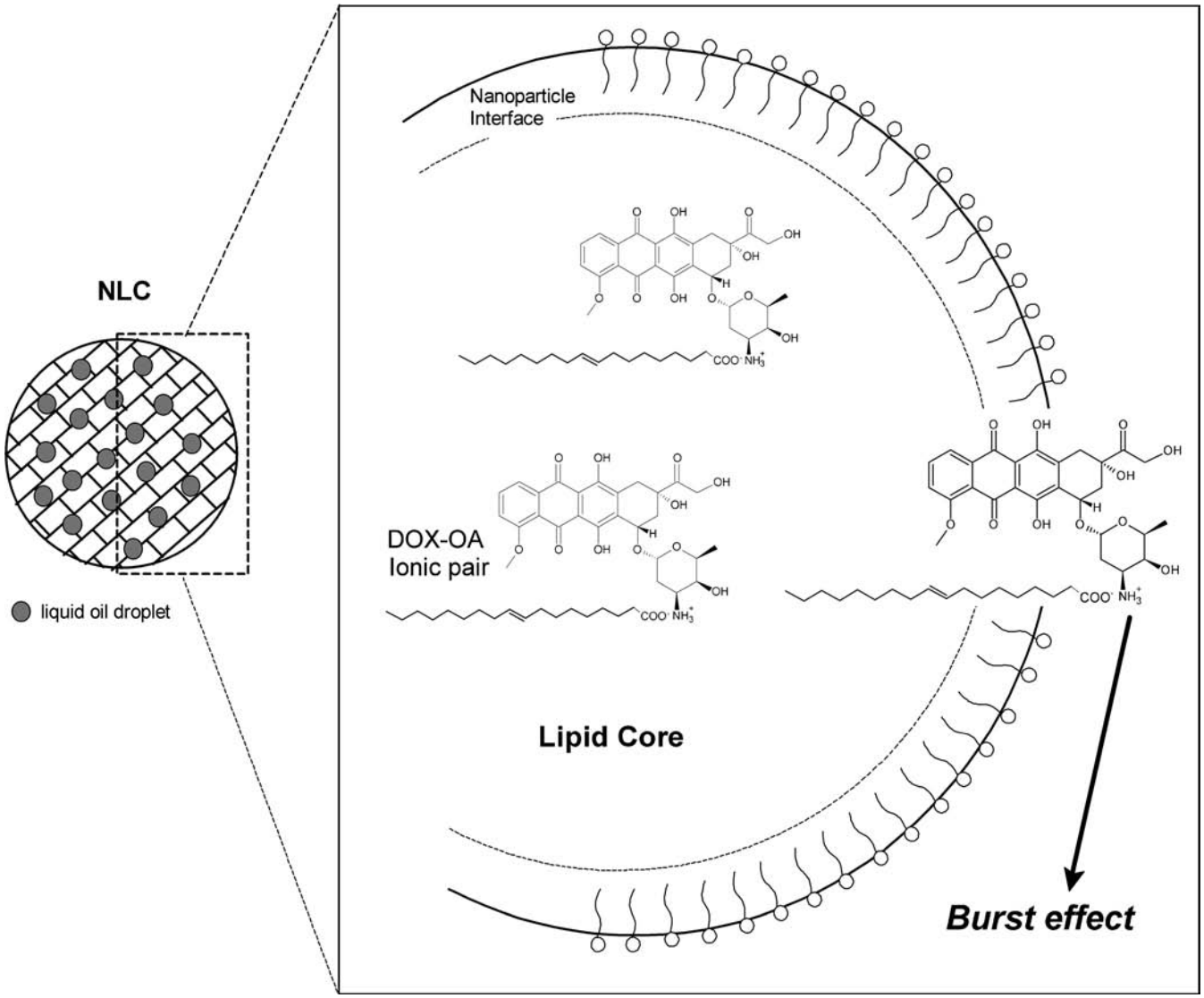

Fig. 6 Scheme of the NLC loaded with doxorubicin as an ionic pair with oleic acid (DOX-OA ionic pair) and DHA (represented by a liquid oil droplet). The burst effect seen in the release assay may be explained by presence of a fraction of the ionic pair located in the nanoparticle interface.

the combination of free doxorubicin and DHA did not show any improvement of the activity compared with free doxorubicin. The lack of benefit for the combination of free drugs may also be related to the time of incubation with cells. We have incubated for 24 h, reports of a longer incubation, about 7 days, to obtain an increase of the doxorubicin cytotoxicity by the combination with DHA have been made (9). The co-encapsulation of doxorubicin and DHA seems to have potentiated the effect of the combination in MCF-7/Adr. The importance of the DHA co-encaspulation can also been verified by the comparison of the cytotoxicity of NLC-DOX+DHA 0.4% and NLC-DOX+PO 0.4%. The latter formulation showed some improvement of the cytotoxicity compared with the free drugs and may be due only to the encapsulation of the doxorubicin. The NLC-DOX+PO 0.4% was less cytotoxic than NLC-DOX+DHA 0.4%. The oleic acid (the major compound of peanut oil) has very low anticancer activity compared with DHA (9). It is well know that the antitumor activity of DHA is greater than that observed for unsaturated fatty acids, such as oleic acid, and it is attributable to the higher level of unsaturation (9). In addition, it was reported that MCF-7/Adr is more sensitive to the doxorubicin+DHA combination than MCF-7 (9). Our doxorubicin uptake studies reinforce the hypothesis of a higher cytotoxicity due the co-encapsulation of drugs. The increased fluorescence verified for MCF-7/Adr cells incubated with either NLC-DOX+DHA 0.4% or NLC-DOX+PO 0.4% was very similar. The higher cytotoxicity of the former could not be attributed to higher doxorubicin internalization. In addition, it's noteworthy that the fluorescence of the cells incubated with these

formulations was higher than for those incubated with free drugs. It seems that both formulations bypassed P-glycoprotein efflux pump, since we verified its overexpression in this cell line (data not shown). Our uptake data are in accordance with previous works that indicated higher uptake in MCF-7/Adr of doxorubicin encapsulated in lipid nanoparticle than with the free drug (29,36).

The higher cytotoxicity of NLC-DOX+DHA 0.4% was also verified in the MCF-7/Adr spheroid model and these results are promising. Spheroids have been proposed as a tool for negative selection to evaluate drug candidates because most therapeutic approaches were found to be less active in this model than cell monolayer cultures (37). Nevertheless, they have also been described as a more relevant *in vitro* tumor model for use in assessment of drug combinations (38) and mimic *in vivo* tumors in many ways, including the architecture, gradients of pH and PO_2 and vascularization (37). Due to these characteristics, this model is also more suitable for drug penetration studies *in vitro* than to monolayered cell cultures (39). Our data showed a deeper doxorubicin penetration in tridimensional structure of spheroids with NLC-DOX+DHA 0.4% that seems to overcome the binding barrier. The presence of this barrier, characterized by a limited drug penetration of the tumor structure, was confirmed since the free doxorubicin was more confined to the periphery of the spheroids as previously reported (26,40). The penetration of doxorubicin with NLC-DOX+DHA 0.4% was higher than that observed with NLC-DOX+PO 0.4%, the commercial liposomal formulation (Lipodox®) and with free drugs. These finding also support the importance of the presence of DHA and its co-encapsulation with doxorubicin in NLC.

Thus, NLC-DOX+DHA 0.4% has potentially useful antitumor formulation characteristics. Its activity was evidently high in drug resistant MCF-7/Adr when compared with the two commercially available forms of doxorubicin (free hydrochloride salt and liposomal). We suggest that this newly developed nanocarrier for codelivery of doxorubicin/DHA with a potential for effective combination cancer therapy and overcoming drug resistance.

CONCLUSION

Our nanostructured lipid carrier (NLC) loaded with doxorubicin and DHA showed size, zeta potential and serum stability adequate for parental administration and a controlled release of doxorubicin. The *in vitro* antitumor activity of such NLC loaded with doxorubicin and DHA 0.4% in monolayer models was significant higher for MCF-7/Adr compared to the free drugs and the entrapped drug not combined with DHA. These findings in cell monolayers were supported by studies with spheroids that showed an enhanced penetration into the tumor structure. Thus, the association of doxorubicin and DHA and their co-encapsulation in NLC improved the cytotoxicity and overcame the drug resistance associated with P-glycoprotein activity in MCF-7/Adr. Thus, the NLC loaded with doxorubicin and DHA represents a promising alternative for combination cancer therapy.

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REFERENCES

1. Livi L, Meattini I, Cardillo Cde L, Mangoni M, Greto D, Petrucci A, *et al.* Non-pegylated liposomal doxorubicin in combination with cyclophosphamide or docetaxel as first-line therapy inmetastatic breast cancer: a retrospective analysis. Tumori. 2009;95(4):422–6.
2. Shi Y, Moon M, Dawood S, McManus B, Liu PP. Mechanisms and management of doxorubicin cardiotoxicity. Herz. 2011;36(4):296–305.
3. Primeau AJ, Rendon A, Hedley D, Lilge L, Tannock IF. The distribution of the anticancer drug doxorubicin in relation to blood vessels in solid tumors. Clin Cancer Res. 2005;11(24 Pt 1):8782–8.
4. Trédan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst. 2007;99(19):1441–54.
5. Jassem J, Pieńkowski T, Płuzańska A, Jelic S, Gorbunova V, Mrsic-Krmpotic Z, *et al.* Doxorubicin and paclitaxel *versus* fluorouracil, doxorubicin, and cyclophosphamide as first-line therapy for women with metastatic breast cancer: final results of a randomized phase III multicenter trial. J Clin Oncol. 2001;19(6):1707–15.
6. Germain E, Chajès V, Cognault S, Lhuillery C, Bougnoux P. Enhancement of doxorubicin cytotoxicity by polyunsaturated fatty acids in the human breast tumor cell line MDA-MB-231: relationship to lipid peroxidation. Int J Cancer. 1998;75(4):578–83.
7. Colas S, Mahéo K, Denis F, Goupille C, Hoinard C, Champeroux P, *et al.* Sensitization by dietary docosahexaenoic acid of rat mammary carcinoma to anthracycline: a role for tumor vascularization. Clin Cancer Res. 2006;12(19):5879–86.
8. Siddiqui RA, Harvey KA, Xu Z, Bammerlin EM, Walker C, Altenburg JD. Docosahexaenoic acid: a natural powerful adjuvant that improves efficacy for anticancer treatment with no adverse effects. Biofactors. 2011;37(6):399–412.
9. Mahéo K, Vibet S, Steghens JP, Dartigeas C, Lehman M, Bougnoux P, *et al.* Differential sensitization of cancer cells to doxorubicin by DHA: a role for lipoperoxidation. Free Radic Biol Med. 2005;39(6):742–51.
10. Hardman WE, Avula CP, Fernandes G, Cameron IL. Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 breast cancer xenografts. Clin Cancer Res. 2001;7(7):2041–9.
11. Hajjaji N, Besson P, Bougnoux P. Tumor and non-tumor tissues differential oxidative stress response to supplemental DHA and chemotherapy in rats. Cancer Chemother Pharmacol. 2012;70(1):17–23.

12. Bougnoux P, Hajjaji N, Ferrasson MN, Giraudeau B, Couet C, Le Floch O. Improving outcome of chemotherapy of metastatic breast cancer by docosahexaenoic acid: a phase II trial. Br J Cancer. 2009;101(12):78–85.
13. Parhi P, Mohanty C, Sahoo SK. Nanotechnology-based combinational drug delivery: an emerging approach for cancer therapy. Drug Discov Today. 2012;17(17–18):1044–52.
14. Okuda T, Kidoaki S. Multidrug delivery systems with single formulation—current status and future perspective. J Biomater Nanobiotechnol. 2012;3(1):50–60.
15. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release. 2000;65(1–2):271–84.
16. Torchilin V. Tumor delivery of macromolecular drugs based on the EPR effect. Adv Drug Deliv Rev. 2011;63(3):131–5.
17. Li Y, Wang J, Wientjes MG, Au JL. Delivery of nanomedicines to extracellular and intracellular compartments of a solid tumor. Adv Drug Deliv Rev. 2012;64(1):29–39.
18. Mehnert W, Mäder K. Solid lipid nanoparticles: production, characterization and applications. Adv Drug Deliv Rev. 2001;47(2–3): 165–96.
19. Müller RH, Radtke M, Wissing SA. Nanostructured lipid matrices for improved microencapsulation of drugs. Int J Pharm. 2002;242(1–2):121–8.
20. Wissing SA, Kayser O, Müller RH. Solid lipid nanoparticles for parenteral drug delivery. Adv Drug Deliv Rev. 2004;56(9):1257–72.
21. Radtke M, Müller RH. NLC-nanostructured lipid carriers: the new generation of lipid drug carriers. New Drugs. 2001;2:48–52.
22. Serpe L, Satalano MG, Cavalli R, Ugazio E, Bosco O, Canaparo R, *et al.* Cytotoxicity of anticancer drugs incorporated in solid lipid nanoparticles on HT-29 colorectal cancer cell line. Eur J Pharm Biopharm. 2004;58(3):673–80.
23. Wong HL, Rauth AM, Bendayan R, Manias JL, Ramaswamy M, Liu Z, *et al.* A new polymer-lipid hybrid nanoparticle system increases cytotoxicity of doxorubicin against multidrug-resistant human breast cancer cells. Pharm Res. 2006;23(7):1574–85.
24. Mussi SV, Silva RC, Oliveira MC, Lucci CM, Azevedo RB, Ferreira LA. New approach to improve encapsulation and antitumor activity of doxorubicin loaded in solid lipid nanoparticles. Eur J Pharm Sci. 2013;48(1–2):282–90.
25. Elbayoumi T, Torchilin VP. Tumor-targeted immuno-liposomes for cancer therapy and imaging. J Pharm Innov. 2008;3:51–8.
26. Perche F, Patel NR, Torchilin VP. Accumulation and toxicity of antibody-targeted doxorubicin-loaded PEG-PE micelles in ovarian cancer cell spheroid model. J Control Release. 2012;164(1):95–102.
27. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, *et al.* Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. N Engl J Med. 2012;367(18):1694–703.
28. Joshi MD, Müller RH. Lipid nanoparticles for parenteral delivery of actives. Eur J Pharm Biopharm. 2009;71(2):161–72.
29. Zhang X-g, Miao J, Dai Y-Q, Du Y-Z, Yuan H, Hu F-Q. Reversal activity of nanostructured lipid carriers loading cytotoxic drug in multi-drug resistant cancer cells. Int J Pharm. 2008;361(1–2):239–44.
30. Yang XY, Li YX, Li M, Zhang L, Feng LX, Zhang N. Hyaluronic acid-coated nanostructured lipid carriers for targeting paclitaxel to cancer. Cancer Lett. 2013;334(2):338–45.
31. Bunjes H. Lipid nanoparticles for the delivery of poorly water-soluble drugs. J Pharm Pharmacol. 2010;62(11):1637–45.
32. Rosenblatt KM, Douroumis D, Bunjes H. Drug release from differently structured monoolein/poloxamer nanodispersions studied with differential pulse polarography and ultrafiltration at low pressure. J Pharm Sci. 2007;96(6):1564–75.
33. Souto EB, Müller RH. Lipid nanoparticles: effect on bioavailability and pharmacokinetic changes. Handb Exp Pharmacol. 2010;197: 115–41.
34. Subedi RK, Kang KW, Choi H. Preparation and characterization of solid lipid nanoparticles loaded with doxorubicin. Eur J Pharm Sci. 2009;37(3–4):508–13.
35. Needham D, Ponce A. Nanoscale drug delivery vehicles for solid tumors: a new paradigm for localized drug delivery using temperature sensitive liposomes. In: Amiji MM, editor. Nanotechnology for cancer therapy. Boca Raton: CRC Press, LLC (a subsidiary of Taylor and Francis); 2007. p. 677–719.
36. Miao J, Du YZ, Yuan H, Zhang XG, Hu FQ. Drug resistance reversal activity of anticancer drug loaded solid lipid nanoparticles in multi-drug resistant cancer cells. Colloids Surf B: Biointerfaces. 2013;110:74–80.
37. Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA. Multicellular tumor spheroids: an underestimated tool is catching up again. J Biotechnol. 2010;148(1): 3–15.
38. Perche F, Torchilin VP. Cancer cell spheroids as a model to evaluate chemotherapy protocols. Cancer Biol Ther. 2012;13(12):1205–13.
39. Dufau I, Frongia C, Sicard F, Dedieu L, Cordelier P, Ausseil F, *et al.* Multicellular tumor spheroid model to evaluate spatio-temporal dynamics effect of chemotherapeutics: application to the gemcitabine/CHK1 inhibitor combination in pancreatic cancer. BMC Cancer. 2012;12(15):1–11.
40. Kim TH, Mount CW, Gombotz WR, Pun SH. The delivery of doxorubicin to 3-D multicellular spheroids and tumors in a murine xenograft model using tumor-penetrating triblock polymeric micelles. Biomaterials. 2010;31(28):7386–97.