Published in final edited form as:

Biomed Pharmacother. 2018 July ; 103: 1348–1354. doi:10.1016/j.biopha.2018.04.139.

α - Tocopherol succinate loaded nano-structured lipid carriers improves antitumor activity of doxorubicin in breast cancer models in vivo

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Abstract

Combination-based chemotherapies have been the standard treatment for multiple solid tumors since the 1960s. Combined therapies where both agents have toxicity results in dose-limiting effects. α -tocopherol succinate (TS) is an analogue of vitamin E that exhibits antitumor properties in the absence of toxicity. Hence, its combination with a frontline chemotherapy, doxorubicin (DOX) is an alternative to increase antitumor efficacy. Therefore, the aim of this work was to evaluate the antitumor activity of nanostructured lipid carriers (NLC) loaded with TS and DOX. The NLC-TS-DOX were prepared, characterized and radiolabeled with technetium-99m. Cytotoxicity studies were performed *in vitro*, using two breast cancer cell lines, MDA-MB-231 and 4T1. Biodistribution and antitumor activity were evaluated in 4T1 tumor-bearing mice. The results showed that NLC-TS-DOX had a small diameter (85 nm) and a long blood clearance ($T_{1/2\beta}$ = 1107.71 min) that consequently resulted in a higher tumor uptake compared to contralateral muscle for up to 48 h. Drug combination studies in MDA-MB-231 and 4T1 cells showed a

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Conflict of interest

The authors declare that they have no conflict of interests.

combination index below 0.8 at ED_{50–90} for both cell lines. Interestingly, a high synergism was found at ED₉₀. Antitumor activity showed a better control of tumor growth for animals treated with NLC-STDOX. The small particle size, along with the EPR effect and the controlled release of DOX from the particle, associated with the synergic combination between TS and DOX led to an increase of the antitumor efficacy. Therefore, NLC-TS-DOX can be considered a plausible alternative to improve antitumor efficacy in DOX therapeutic regimens.

Keywords

Doxorubicin; α -tocopherol succinate; Nanostructured lipid carriers; Drug combination; Breast cancer; Technetium-99m

1. Introduction

The anthracycline glycoside antibiotic doxorubicin (DOX) is one of the most commonly used drugs for several types of tumors, including lung, ovarian, and breast cancer [1]. Its effectiveness is limited by low penetration into solid tumors, drug resistance and cardiotoxic effects [2–4]. Anthracycline-based combination chemotherapy is an alternative to improve doxorubicin therapeutic efficiency. In clinical practice, doxorubicin is used in combination with cyclophosphamide, cisplatin and fluorouracil [5]. Several studies propose new treatment regimens combining DOX with others antitumor agents, such as simvastatin, ribavirin and curcumin [6–8].

Alpha-tocopherol succinate (TS) is a lipophilic ester of vitamin E that exhibits antitumor properties. The molecular mechanism remains unclear, however TX treatment leads to proliferation inhibition and induction of apoptosis in cancer cells [9]. There are some studies reporting that the combination with TS increased the antitumor activity of chemotherapeutics, including paclitaxel [10] and doxorubicin [11].

Although combined chemotherapy often shows an improvement of anticancer activity, the administration of both free drugs will not guarantee that they will simultaneously reach the tumor area in the optimal ratio [12]. Therefore, to overcome this limitation, the combination of drugs co-delivered in a single drug delivery system has emerged as an attractive strategy. Nanostructured lipid carriers (NLC) are promising drug delivery systems, offering many advantages including easy translation to large-scale production, preparation in the absence of organic solvents, and relatively low toxicity [13]. In addition, the blend of solid and liquid lipids provides an efficient payload with less drug expulsion during storage when compared with solid lipid nanoparticles [14].

Recently, our research group demonstrated that TS improves encapsulation of DOX into lipid nanoparticles [15], with increased cytotoxicity in monolayer and 3-D spheroid models, against MCF-7/Adr and NCI/Adr line cells [16]. Therefore, the aim of this work was to evaluate the *in vivo* properties of this NLC formulation in an experimental model. To achieve this purpose, we evaluated the blood clearance and bio-distribution in tumor-bearing mice. Furthermore, the antitumor activity of TS/DOX-loaded NLC was evaluated in 4T1 breast tumor-bearing model.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (DOX) was obtained from ACIC Chemicals (Ontario, Canada). α -Tocopherol succinate (TS) was purchased from Sigma-Aldrich (St. Louis, USA). Triethanolamine (TEA) was obtained from Merck (Darmstadt, Germany). Glyceryl behenate (Compritol 888 ATO) and monooleate of sorbitanethoxylated (Super refined Polysorbate 80TM; Tween 80TM) were kindly provided by Gattefossé (Saint Priest, France) and Croda Inc. (Edison, USA), respectively. Dulbecco's Modified Eagle's Medium (DMEM), RPMI1640 medium, fetal bovine serum, and trypsin EDTA (0.25%) were purchased from Gibco-Invitrogen (Grand Island, USA). ^{99m}Tc was obtained from an alumina-based ⁹⁹Mo/^{99m}Tc generator from IPEN (São Paulo, Brazil). All other chemicals were of analytical grade.

2.2. Preparation of NLC

NLC was prepared, by the hot melting homogenization method using emulsification-ultrasound, as published elsewhere [15]. Briefly, the oily phase (Compritol 120 mg; medium-chain triglycerides (MCT) 30 mg; Tween 80 100 mg; DOX 10 mg; TEA 0.6 mg; TS 40 mg) was heated to 80 °C. Then, the aqueous phase (water 8 mL at 80 °C) was gently dropped onto the oily phase under constant agitation, at 8000 rpm, with an Ultra Turrax T-25 homogenizer (IkaLabortechnik, Germany), followed by high intensity probe sonication at 20% amplitude for 10 min using a high intensity ultrasonic processor (CPX 500 model, Cole-Palmer Instruments, USA). The suspension was cooled to room temperature; the volume was completed to 10 mL and stored protected from light, at 4 °C.

2.3. Characterization of the NLC

The NLC formulation was characterized as mean hydrodynamic diameter, polydispersity index (PDI), zeta potential, drug encapsulation efficiency (EE) and DOX *in vitro* release. Nanoparticle tracking analysis (NTA) and images of atomic force microscopy (AFM) were also obtained.

2.3.1. Mean diameter and zeta potential—The mean particle diameter and PDI were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments, UK), at a fixed angle of 90° and 25 °C. Samples of NLC were diluted 100 times in distilled water. Zeta potential measurements were carried out by DLS associated with electrophoretic mobility, at 25 °C. All measurements were performed in triplicate.

2.3.2. Drug encapsulation efficiency (%EE)—The EE of DOX in NLC was determined by an ultrafiltration method using centrifugal devices (Amicon[®] Ultra – 4 100 kDa, Millipore, USA). By using this strategy, DOX is freely filtered through the membrane while NLC remains in the filter [15]. Briefly, for the determination of total concentration of DOX in the NLC, aliquots of the formulation were dispersed in a mixture of THF and methanol (4:6), that was centrifuged for 10 min at 2400g and the supernatant was analyzed. To evaluate the non-encapsulated doxorubicin, an aliquot of the NLC was submitted to ultrafiltration (10 min at 2400g) to separate the aqueous phase from particles. The drug concentration was determined by HPLC (Waters, 515 isocratic pump, 717 plus automatic

injector, and 474 fluorescent detector, Milford, EUA) using a methanol: phosphate buffer pH 3.0 (65:35) as the mobile phase, and a reversed-phase C₈ column (5 µm; 250 × 4.6 mm) with fluorescence detection in emission at 470 nm and excitation at 555 nm. %EE was calculated by the formula:

$$\%EE = (C_T - C_{AP} / C_T) \times 100$$

Where: C_T = total doxorubicin concentration in SLN, C_{AP} = doxorubicin concentration in aqueous phase (non-encapsulated).

2.3.3. Nanoparticle Tracking Analysis (NTA)—NTA experiments were performed using NanoSight LM 10 & NTA 2.2 Analytical Software (Nanosight Ltd., Amesbury, UK). NLC were diluted in ultrapure water and introduced into the Nanosight sample chamber with a disposable syringe. The samples were measured at room temperature for 60 s with automatic detection. The suspension was irradiated by a laser source and light scattering. Images were captured by a charge-coupled device camera.

2.3.4. Atomic force microscopy (AFM)—AFM observation of the NLC was performed in air, at room temperature, using both a Dimension 3000 apparatus, as well as an AFM Multimode Equipment scope, both monitored by a Nanoscope IIIa controller from Digital Instruments (Santa Barbara, CA). A droplet (10 µL) of the sample was deposited on a freshly cleaved mica surface, spread and dried at room temperature. The images were obtained in tapping mode, using commercial silicon probes from Nanosensors™, with cantilevers having a length of 228 nm, resonance frequencies of 75–98 kHz, spring constants of 29 – 61 N/m and a nominal tip curvature radius of 5–10 nm. The scan rate used was 1 Hz. Dimensional analyses were performed using the ‘section of analyses’ of the system. A minimum of ten images from each sample was analyzed to assure reproducible results.

2.3.5. In vitro release of DOX—The release of DOX was quantified by dialysis method. Dialysis tubes with a cutoff size of 14 kDa and diameter of 21 mm (cellulose ester membrane; Sigma–Aldrich, St Louis, USA) were filled with 2 mL of formulation, sealed and incubated with 50 ml of PBS (pH 7.4) for 24 h at 37 °C, under magnetic stirring at 250 rpm. An aqueous solution of DOX (1.0 mg/ml) was used as a control (free DOX). At 1 h, 2 h, 4 h, 8 h and 24 h, aliquots were withdrawn and DOX concentration was analyzed by the above HPLC method. Values were plotted as cumulative percentage of drug release.

2.4. Cell culture

A human breast tumor cell line MDA-MB-231 was grown in DMEM, and a murine breast tumor cell line 4T1 was grown in RPMI. The cells lines were supplemented with 10% fetal bovine serum (FBS, Gibco) and maintained at 37 °C and 5% CO₂ in a humidified atmosphere.

2.5. Cytotoxicity studies and data analysis

MDA-MB-231 and 4T1 cells were seeded in 96-well plates (1 × 10⁴ cells/well and 5 × 10³ cells/well, respectively) at 24 h before treatment. The cells were then exposed to a series of

concentrations of DOX (single drug), TS (single drug), NLC-TS-DOX (at 1:4 DOX:TS ratio) and NLC-blank, for 48 h. Cell viability was assessed using sulforhodamine B (SRB) assay. Briefly, after incubation, 10% trichloroacetic acid (TCA) was added to each well to fix cells for one hour. Plates were then washed with water to remove TCA and stained with SRB for 30 min. Afterwards, the plate was washed with 1% acetic acid to remove the unbound SRB. Then, the protein-bound dye was solubilized in 10 mM of Tris-Base [tris(hydroxymethyl) aminomethane] solution and optical densities (OD) were read at 510 nm on a microplate spectrophotometer Spectra Max Plus 384 (Molecular Devices, Sunnyvale, CA, USA).

Drug combination studies were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells versus the drug concentration after 48 h of treatment. Synergism, additivity, and antagonism were quantified by determining the combination index (CI) calculated by the Chou-Talalay equation with the software CalcuSyn (Biosoft, Cambridge, UK). Assuming 1.0 as the cutoff, $CI < 1.0$, $CI = 1.0$, or $CI > 1.0$ indicates synergistic, additive, or antagonistic effects, respectively [17].

2.6. In vivo studies

2.6.1. Animals—Female BALB/c mice with 6–8 weeks (18–22 g) were obtained from the central vivarium of Federal University of Minas Gerais - Brazil. All animal studies were approved by the local Ethics Committee for Animal Experiments (CEUA/UFMG).

2.6.2. Radiolabeling of NLC-TS-DOX—The radiolabeling of NLC-TS-DOX with technetium-99m and the evaluation of radiochemical purity were performed as previously reported [18]. ^{99m}Tc -NLC-TS-DOX was used for blood clearance, and biodistribution and scintigraphic images.

2.6.3. Blood clearance—The ^{99m}Tc -NLC-TS-DOX was administrated into healthy BALB/c mice ($n = 6$) through the tail vein. At pre-determined time-points, blood samples (~20 μl each) were collected from the tail vein. Each sample was weighed, and the radioactivity was measured with an automatic scintillation counter (Wallace Wizard 1470 – 020 Gamma Counter; PerkinElmer Inc., Waltham, Massachusetts, USA). The percentage of injected activity per gram (% ID/g) in each sample and its mean $\pm$ SD were determined, and the data were plotted as a function of time.

2.6.4. Tumor cell inoculation—Female BALB/c mice (18–22 g) received, subcutaneously, into the right thigh, aliquots (100 μl) of 2.5×10^6 4T1 cells. Tumor cells incubated *in vivo* for 7 days, leading to tumors with a volume of no more than 100 mm^3 . Breast tumor-bearing BALB/c mice were used for bio-distribution studies and *in vivo* antitumor activity.

2.6.5. Biodistribution studies—Aliquots (100 μl) of 3.7 MBq of ^{99m}Tc -NLC-TS-DOX were injected intravenously into tumor-bearing BALB/c mice. At 4, 8 and 24 h after injection, mice were anesthetized with a mixture of xylazine (15 mg/kg) and ketamine (80 mg/kg). Organs such liver, spleen, kidneys, heart, lungs, muscle, thyroid, intestines, and

tumor were removed, and placed in pre-weighed plastic test tubes. The radioactivity was measured using an automatic scintillation counter.

2.6.6. Antitumor activity—On the 7th day after 4T1 cell inoculation, once the tumor volume reached $\sim 100 \text{ mm}^3$, the mice were randomly assigned into four groups ($n = 6$ for each group): group 1: blank-NLC (negative control group); group 2: aqueous solution of free DOX (positive control); group 3: NLC-DOX; and group 4: NLC-TS-DOX. For all treatments, the dose of DOX was 4 mg/kg/day, in a total of 5 administrations, every 2 days, injected by the tail vein. Hence, the total dose of DOX in all treatment groups was 20 mg/kg. Throughout the study, tumors were measured with caliper every 2 days. Tumor volumes were calculated from the formula:

$$V = (d_1)^2 \times d_2 \times 0.5$$

where d_1 and d_2 , represent the smaller and larger diameter, respectively.

2.6.7. Scintigraphic images—Two days after the end of the treatment, aliquots of 100 μL of $^{99\text{m}}\text{Tc}$ -NLC-TS-DOX were administered intravenously into BALB/c mice ($n = 4$). They were anesthetized and placed horizontally under a gamma camera (Mediso, Budapest, Hungary) coupled with a low-energy high-resolution collimator. Images were acquired at 8 h after injection using a $256 \times 256 \times 16$ matrix size, with a 20% energy window set at 140 keV for a period of 300 s each. To quantify the uptake of the $^{99\text{m}}\text{Tc}$ -NLC-TS-DOX by tumor focus, the images were analyzed by determining the target to non-target ratios. Regions of interest (ROIs) were drawn in scintigraphic images to outline the tumor. The ROIs were automatically copied to the contralateral muscle. Tumor-to-muscle ratios (T/M) were calculated using the formula:

$$\text{T/M ratio} = \text{total ROI counts for tumor} / \text{total ROI counts for muscle}$$

2.7. Statistical analysis

Statistical analyses were performed using GraphPad PRISM, version 5.00 software (GraphPad Software Inc., La Jolla, California, USA). The difference among experimental groups was tested using the one-way analysis of variance (ANOVA), followed by the Tukey's test. Differences were considered statistically significant when P-values were < 0.05 .

3. Results

3.1. Characterization of NLC-TS-DOX

NLC-TS-DOX was characterized as mean diameter, PDI, zeta potential and %EE. The results are shown in Table 1. The average size was around 85 nm and the PDI was less than 0.3, indicating a homogeneous system. The zeta potential was highly negative, likely due to the excess of ionized TS on the surface of NLC. The high value of EE (98.97 ± 0.33) can be attributed to an ion-pare formation between DOX and TS [15].

Nanoparticle size distribution was also evaluated by NTA and the results are summarized in Table 2. It was observed that the average particle size values found for NLC-TS-DOX (113.6 ± 38.2 nm) were consistent with DLS analysis. The SD was a little larger than DLS data probably due to differences in techniques. However, the profile distribution ($90\% < 151$ nm, and $50\% < 98$ nm) was coherent with the DLS profile.

The images produced by the AFM, shown in Fig. 1A, were three-dimensional, with high resolution in the nanometrical scale. The particles have a mean diameter of ~ 75 nm, which was also consistent with DLS data. Moreover, they presented in a rounded form indicated by the height of the droplets (~ 38 nm) in the AFM images (Fig. 1B). These data are consistent with prior publications using NLC formulations.

In vitro release data are presented in Fig. 2. The DOX released from NLC-TS-DOX was around 5% after 2 h, reaching 10% in 24 h. In contrast, the drug release from aqueous solution (free DOX), was around 90% after 4 h. The high release ratio of DOX solution was already expected since the drug can pass freely through the dialysis membrane (cutoff size – 14 kDa). In contrast, the encapsulated drug remains into the dialysis bag.

3.2. Cytotoxicity studies

Cytotoxic studies were performed in human MDA-MB-231 and murine 4T1 breast cancer lines. Combination index (CI) values are presented in Fig. 3. DOX and TS encapsulated into NLC at a ratio DOX:TS of 1:4. This is synergic at all concentrations, showing moderated synergism ($CI = 0.78$ for both cell lines) at 50% effective dose (ED_{50}). At ED_{75} a strong synergism ($CI = 0.44$) is observed against MDA-MB-231 cells. At ED_{90} the DOX:TS (1:4) combination is synergic ($CI = 0.58$) against 4T1 cells and strongly synergic ($CI = 0.27$) against MDA-MB-231 cells.

The human and murine breast carcinoma cell lines were incubated for 48 h at 37°C with NLC-blank, free DOX, free TS and NLC-TS-DOX, and analyzed for their viability using sulforhodamine B assay. The data of cell viability are shown in Fig. 4. The *in vitro* behavior of NLC-DOX and NLC-TS was similar to those of the free drugs, with no statistical differences, thus, these data were suppressed in Fig. 4. Interestingly, TS alone did not have a major effect on cell viability for either cell line. In contrast, DOX-TS-loaded NLC demonstrated cytotoxic effects higher than those observed for the free DOX, indicating the synergic effect of DOX and TS. NLC-blank has no effect in cellular viability in both evaluated cell lines.

3.3. In vivo studies

3.3.1. Radiolabeling of NLC-TS-DOX—Radiochemical purity of $^{99\text{m}}\text{Tc}$ -NLC-TS-DOX was 94.7 ± 1.2 ($n = 3$) and it was reproducible during all experiments. Moreover, the radiolabeled system showed high *in vitro* stability up to 24 h (data not shown). This finding was in agreement with our previous studies with radiolabeled NLC [18].

3.3.2. Blood clearance—Following administration into BALB/c female mice, $^{99\text{m}}\text{Tc}$ -NLC-TS DOX clearance was prolonged, with blood levels decaying in a biphasic manner. $T_{1/2\alpha}$ was 72.17 min, $T_{1/2\beta}$ of 1107.71 min and the area under the curve (AUC) was

2750.32%ID min⁻¹. The $T_{1/2\beta}$ of NLC-TS-DOX was ~4 times longer than for free ^{99m}Tc-DOX data obtained in our previous study [18]. This finding indicated that NLC prolonged the circulation time of DOX. This may be important for improving anti-tumor outcomes.

3.3.3. Biodistribution studies—The biodistribution profile of ^{99m}Tc-NLC-TS-DOX is shown in Fig. 5A. There was high uptake by liver and spleen as from mono-nuclear phagocyte system (MPS) organs. Renal uptake was also noted. This finding might be explained by DLS data, which demonstrated some particle formation that was smaller than kidney filtration cut-off size [19]. No significant uptake was observed in other organs, including thyroid, confirming the *in vitro* stability data, since it is well known that free technetium is preferentially taken up by this organ. Tumor uptake was higher than contralateral muscle, at all investigated time points.

Target-to-non-target ratio obtained from biodistribution studies were presented in Fig. 5B. A significant increase was observed from 4 to 8 h after injection. After this time-point the ratio remained high and constant (over 4 in all time-points) up to 48 h. These data indicate consistent high accumulation of the NLC in the tumor area that might be a consequence of the long circulation of the nanoparticle.

3.3.4. Antitumor activity—The antitumor activity of NLC-TS-DOX was evaluated in 4T1-tumor-bearing BALB/c mice. As shown in Fig. 6, the control treated animals (blank-NLC) had the highest tumor volume reaching 1324 ± 156 mm³ at the end of the experiment. NLC-DOX and DOX groups showed similar efficacy with tumor volumes of 721 ± 82 and 642 ± 27 mm³, respectively. In striking contrast, the NLC-TS-DOX group had a reduced tumor volume at D10 relative to other treatment groups (293 ± 91 mm³). These findings reinforced the synergistic effect of TS and DOX against 4T1 cells, corroborating *in vitro* data.

3.3.5. Scintigraphic images—As previously demonstrated, NLC-TS-DOX was capable of accumulating in tumor areas for up to 48 h. This has the potential to be a powerful tool for monitoring tumor growth. As such, 4T1-tumor-bearing mice from all groups were imaged 8 h after treatment of ^{99m}Tc-NLC-TS-DOX. As shown in Fig. 7, the tumor area was clearly seen in the control group, indicating the higher tumor volume of this group. It was observed that NLC-TS-DOX group presented the lowest tumor uptake, when compared with other treatment groups. Scintigraphic images also showed uptake mainly in liver and spleen, which is expected for nanoparticles due to the recognition by MPS.

By calculating the tumor-to-muscle ratio, quantitative data could be extracted from the images as shown in Fig. 8. The control treatment group presented the highest ratio (4.76 ± 0.42), while NLC-TS-DOX showed the lowest ratio (2.16 ± 0.14). There was no statistical difference between NLC-DOX and DOX. These findings suggest that NLC-TS-DOX had the most favorable antitumor infiltration (drug delivery), corroborating tumor volume measurements.

4. Discussion

Combination-based chemotherapies became the standard treatment for hematological cancers and solid tumors since the 1960s as a key strategy to circumvent drug resistance [20]. The combination of TS – an analogue of vitamin E – and DOX loaded into a lipid nanoparticle demonstrated promising *in vitro* results [16] with modest potential for off target toxicity, therefore forming the foundation of these studies. We developed a novel drug delivery system with *in vivo* properties that show favorable pharmacodynamics and antitumor activity. NLC-TS-DOX developed in this present study had a small diameter (85 nm), which was confirmed by complementary techniques (Tables 1 and 2, Fig. 1), and a PDI lower than 0.3 (0.204), which indicates a homogeneous system. Moreover, through the AFM technique it was possible to observe that the particles exhibit smooth surface and rounded shape, data in agreement with other studies [21]. These properties are required characteristics for further *in vivo* administration [22,23]. %EE and *in vitro* release data (Fig. 2) demonstrated that, the ion-pair formed between DOX and TS successfully encapsulated and entrapped DOX into NLC matrix [24].

For the NLC preparation, the optimal molar ratio identified to elicit synergistic effects between DOX and TS was 1:4 [15]. Then, to further confirm prior results, synergism was tested in murine 4T1 and human MDA-MB-231 breast cancer cell lines using DOX and TS into NLC at a ratio of 1:4, respectively. CI below 0.8 (Fig. 3) at ED₅₀₋₉₀ were identified for both cell lines. Nevertheless, at effective dose 90 (ED₉₀) there was more synergy (lower CI) compared with ED₅₀ and ED₇₅. This fact is especially interesting for antitumor drugs since, for cancer therapies, synergism at high effect levels (ED₉₀) is much more therapeutically relevant than at low effect levels (ED₅₀), once is necessary to kill the greatest number of cells [17]. Fig. 4 corroborates with this fact, when cell viability is lower when higher concentrations of the drugs are applied, especially when the cells were treated with the combination DOX:TS (1:4).

The pharmacokinetic properties of ^{99m}Tc-NLC-TS-DOX were more favorable than free DOX. Previous study by our group found T_{1/2α} = 4.5 min; T_{1/2β} = 277.2 min and AUC = 2633%ID min⁻¹ values for ^{99m}Tc-DOX [18]. Specifically, ^{99m}Tc-NLC-TS-DOX exhibited a prolonged clearance, with T_{1/2β} about 4 times higher than T_{1/2β} found for free DOX. This is probably due to the small particle size, which contains Tween 80 and polyethyleneglycol chains in its structure [18]. The longer circulation time, associated with enhanced permeability and retention effect (EPR effect), contributed to the higher tumor accumulation, compared with contralateral muscle, observed in biodistribution studies (Fig. 5A) and target-to-non-target ratio (Fig. 5B). The longer circulation allows more passages through the tumor area, and the EPR effect led to a more permeable blood vessel wall in tumor tissue. Consequently, this allows the accumulation of particles ranging from 10 to 500 nm [25–27]. This property impacts the antitumor activity of the compound as well. Specifically, the small particle size of NLC-TS-DOX, along with the EPR effect and the controlled release of DOX from the particle, play an important role to increase the antitumor effectiveness. Additionally, it is possible to observe the contribution of TS in the antitumor activity which was previously suggested by synergism study. The mechanism of action of TS is not fully

understood, however, there are a few studies demonstrating that, when combined with DOX, TS increases apoptosis induction [28].

Among other advantages, encapsulation of DOX and TS into the same nanosystem guarantees that a fixed ratio will reach the tumor area at the same time, leading to a synergic effect. This is particularly important since changes in the drugs ratio may result in an undesired antagonistic response [12]. Our study describes a novel drug delivery system that has favorable pharmacokinetic and dynamic properties and demonstrated a substantial increase in the antitumor activity.

5. Conclusion

In the present study, we developed an NLC loaded with DOX and TS at a 1:4 ratio that showed favorable pharmacokinetic and antitumor activity *in vivo*. *In vitro* assays demonstrated a synergic combination between DOX and TS, at the fixed ratio proposed by the formulation development. From labeling NLC-TS-DOX surface with technetium-99m, it was possible to evaluate the biodistribution profile and to confirm the preferential accumulation in the tumor. The antitumor activity of TS has potential antitumor activity as a single agent. Therefore, NLC-TS-DOX can be considered an interesting alternative to improve antitumor efficacy in DOX therapeutic regimens.

Acknowledgments

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG-Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil).

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Fig. 1. AFM images of (A) 5 $\mu\text{m} \times 5 \mu\text{m}$ scan size of NLC-TS-DOX and (B) topographical profile given by the 'section analysis' software showing measurements of NLC-TS-DOX diameter (green and red arrows) and height (black arrow). Scan size: 900 $\times$ 900 nm.

Fig. 2.
In vitro release of free and encapsulated DOX, in isotonic PBS (pH 7.4), at 37 °C, by dialysis. (n = 3).

Fig. 3. Combination index values (CI) of NLC-TS-DOX (DOX:TS 1:4 ratio) treatment against 4T1 and MDA-MB-231 cell lines, at effect levels ED₅₀, ED₇₅ and ED₉₀. Data are shown as the average of at least 3 independent experiments and error bars represent SD.

Fig. 4. Cytotoxicity of NLC-blank, TS, DOX and NLC-TS-DOX against (A) 4T1 and (B) MDA-MB-231 cells. Data are shown as the average of at least 3 independent experiments and error bars represent SD. Different letters represent statistical differences among the cell viability in the evaluated groups ($P < 0.05$).

Fig. 5.

(A) Biodistribution profile of ^{99m}Tc -NLC-TS-DOX following intravenous tail-vein administration in tumor-bearing BALB/c mice ($n = 6$). All data are the mean percentage of the injected dose per gram of organ, $\pm$ SD of the mean. (B) Tumor-to-muscle ratio for ^{99m}Tc -NLC-TS-DOX at 4, 8, 24 and 48 h after administration.

Fig. 6. Antitumor effect of blank-NLC (control), free DOX, NLC-DOX and NLCTS-DOX on the growth of 4T1 tumor-bearing BALB/c mice (female, n = 6). Each treatment was intravenously administered five times, every 2 days, at a dose of 4 mg/kg.

Fig. 7.
(A) Scintigraphic images obtained eight hours after intravenous administration of ^{99m}Tc -NLC, after five doses of the treatments (Blank NLC – control, NLC-DOX, NLC-TS-DOX and free DOX). The white circle indicated by an arrow indicates the target area (tumor).

Fig. 8.

Target to non-target ratios obtained after treatments for each group. Data were expressed as mean $\pm$ SD (n = 4 animals/group). Different letters indicate statistical significant differences between groups ($P < 0.05$).

Table 1

Mean diameter, PDI, zeta potential and EE for the NLC-TS-DOX.

Mean diameter (nm)	PDI	Zeta potential	%EE
85.0 ± 4.1	0.204 ± 0.02	-41.0 ± 2.6	98.97 ± 0.33

Data represented as mean ± SD, n=3.

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Table 2

NTA measurements (mean size, D10, D50, D90) of NLC-TS-DOX.

Parameters (nm)			
Mean size	D10	D50	D90
113.6 ± 38.2	60.8 ± 1.0	98.1 ± 1.5	151.3 ± 0.9

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