Cellular cytotoxicity and in-vivo biodistribution of docetaxel poly(lactide-co-glycolide) nanoparticles

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Docetaxel (DTX) is one of the most effective antineoplastic drugs. However, its current clinical administration, formulated in tween80, causes serious side effects. This study is focused on preparation and evaluation of poly(lactide-co-glycolide) nanoparticles (NPs) containing DTX to remove tween80. Drug encapsulation efficiency, in-vitro drug release, cellular cytotoxicity, and in-vivo biodistribution of NPs in mice after intravenous administration were investigated. The average diameter of the NPs was approximately 172–178 nm with encapsulation efficiency of 68%. A burst release of approximately 30% (w/w) of the loaded drug followed by a sustained release profile was observed. Cellular mortality of the NPs was more than or at least as great as DTX free drug; for example, cell viability measured at 100 nmol/l drug concentration was decreased from 50.9% for DTX free drug to 15.9% for the NP formulation after 48 h incubation with T47D cells. The DTX plasma amount remained at a good level (13% of the initial dose) in the NP formulation compared with the DTX conventional formulation, which is approximately 0.5% of the initial dose, was present in plasma up to 2 h. Poly(lactide-co-glycolide) NPs containing DTX prepared in this study may be regarded as a suitable and superior formulation for the current formulation in the market containing tween80 with improved cancerous cell mortality and biodistribution characteristics. *Anti-Cancer Drugs* 21:43–52 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Docetaxel (DTX) is one of the drugs in the taxane family, derived from the needles of the European yew tree, *Taxus baccata* [1]. DTX disrupts the microtubular network that is necessary for mitotic and interphase cellular functions. It inhibits tubulin disassembly, causing inhibition of cell division and finally cell death. DTX has an affinity of 1.9-fold higher than paclitaxel for microtubules [2]. Several problems, some because of DTX itself, others because of the solvent system used in the preparation of the commercial formulation of DTX, limit the clinical use of this drug. The major problem is the wide and nonspecific distribution of DTX through the body, like most other anticancer agents. Such distribution causes some drug-related side effects; for example, fluid retention, neurotoxicity, musculoskeletal toxicity, and neutropenia [3]. As DTX is lipophilic, it is commercially available as an ethanolic solution containing tween80 (i.e. Taxotere) [4]. This solvent system promotes hypersensitivity reactions [5], which makes premedication a necessity, again limiting the maximum tolerable dose of the drug. Clearly, a less toxic formulation of DTX that better targets malignant tissue is desirable.

Recently, polymeric NPs have shown much advantage in drug delivery and have attracted many researchers to find better means for the delivery of chemotherapy agents [6–8]. NPs of biodegradable polymers can provide sustained, controlled, and targeted drug delivery to improve the therapeutic effects and reduce the side effects of the formulated drugs. Polyesters such as polylactide (PLA), poly(lactide-co-glycolide) (PLGA), and polycaprolactone (PCL) are FDA-approved biodegradable polymers, which are often used in the field of drug delivery [9].

The effectiveness of drug delivery systems could be evaluated according to their size, controlled drug release, reduced toxicity, and an improved drug pharmacokinetics and biodistribution. For example, loading of cytotoxic agents in NPs or liposomes can improve the stability of a drug and its pharmacokinetics and reduce the drug toxicity [10,11].

In the field of nanoparticulate drug delivery systems for DTX, in 2003, Jakate *et al.* [12] formulated DTX in olive oil droplets and evaluated the cytotoxicity of the particles. Musumeci *et al.* [13] developed polymeric NP formulations of DTX based on the method of solvent displacement by using tween80 as emulsifier. Although they could decrease the amount of the tween80, they did not succeed in totally removing it.

Farokhzad *et al.* [14] formulated the aptamer bioconjugate formulation of DTX-loaded PLGA NPs. Senthilkumar *et al.* [15] formulated DTX in NPs of PLGA–mPEG block

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copolymer. However, there is no report on the safety of the NPs with regard to cellular mortality, especially on four cancerous cell lines, in-vivo toxicity of the particles, or in-vivo distribution of such a delivery system.

In this study, we developed polymeric nanoparticulate drug delivery systems free of tween80 for intravenous (i.v.) administration of DTX by an emulsification solvent diffusion method [16–18]. Drug encapsulation efficiency (EE) and in-vitro release of DTX from NPs were then investigated. MCF7, T47D, SKOV3, and A549 cells were used for in-vitro cell line experiments. Cytotoxicity of DTX-loaded PLGA NPs was investigated by MTT assay. The in-vivo biodistribution of the NPs containing DTX administered i.v. in mice was also evaluated.

Materials and methods

Materials

RPMI-1640 modified medium and penicillin/streptomycin solution were obtained form Gibco Invitrogen (Carlsbad, California, USA). PLGA (50 : 50; Resomer RG 504 H, MW 48 000) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA) (MW 22 000) and rhodamine B isothiocyanate (RBITC) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Dichloromethane and acetone (analytical grade) were purchased from Merck (Darmstadt, Germany). DTX active pharmaceutical powder was purchased from Cipla Pharmaceutical Co. (Mumbai, India). MTT was obtained from Sigma-Aldrich. Methanol used as mobile phase in high-performance liquid chromatography (HPLC) was purchased from Merck. Deionized water was used throughout the experiment. All other chemicals used were of reagent grade.

Preparation of the formulations

The DTX-loaded NPs were prepared by a modified emulsification/solvent diffusion method [16–18]. Briefly, 200 mg PLGA/1 mg DTX were added into the mixture of dichloromethane/ethanol/acetone (25/12.5/62.5%), which was suitably stirred to ensure that all the materials were dissolved. This solution of organic phase was poured through a syringe equipped with a 20-G angiocatheter at a rate of 10 ml/min into a 200-ml aqueous solution of PVA (0.5% w/v) using a high-speed homogenizer (Ultra-turrax IKA, Wilmington, Massachusetts, USA) at 24 000 rpm for 5 min. Stirring was continued for 3 h to allow evaporation of the internal phase. The NPs were then isolated by centrifuging (Sigma 3K30, Steinheim, Germany) at 21 000*g* for 15 min, followed by washing three times with deionized water. Mannitol was added to the suspension as the cryoprotectant (2% w/v). The suspension produced was freeze-dried at −40°C for 48 h (Lyotrap Plus; LTE Scientific Ltd., Oldham, UK) to obtain a fine powder of NPs, which was then kept in a desiccator to protect the NPs from degradation due to heat and moisture.

Nanoparticles characterization

The particle size, size distribution, polydispersity, and zeta potential of the NPs were measured by laser light scattering (Malvern Zetasizer ZS, Malvern, UK). The samples were prepared by suspending the freeze-dried nanoparticles in 10 ml deionized water (10 μg/ml).

In addition, for stability investigation of the NPs in serum during in-vivo studies, NPs were suspended in rat serum at a concentration of 2 mg/ml and were placed in a water bath maintained at 37 ± 0.5°C and shaken at 90 cycles/min. At fixed time intervals, the tubes were taken out from the water bath and centrifuged at 21 000*g* for 15 min. The NPs were washed with deionized water, resuspended in it, and their size was analyzed with laser light scattering.

Scanning electron microscopy (SEM; Philips XL 30 Scanning Microscope, Philips, Amsterdam, The Netherlands) was used to determine the shape and surface morphology of the produced NPs. Particles were coated with gold under vacuum before SEM.

Encapsulation efficiency

Lyophilized NPs (20 mg) were dissolved in 1 ml of acetonitrile and shaken gently followed by sonication for 5 min. Then, 2 ml of methanol was added to precipitate the polymer. Although DTX was soluble in both acetonitrile and methanol, the PLGA was precipitated in methanol. The sample was then centrifuged at 21 000*g* for 10 min and the drug amount in supernatant was determined by the HPLC analysis. The DTX loading was determined as the ratio of DTX content of NPs to the total weight of the NPs, and the EE was determined as the mass ratio of entrapped DTX in NPs to the theoretical amount of drug used in their preparation [19].

In-vitro drug release

Five milligrams of freeze-dried DTX-loaded NPs were poured in screw-capped tubes and suspended into 10 ml of isotonic pH 7.4 phosphate buffer saline (PBS) solution. The tubes were placed in a water bath maintained at 37 ± 0.5°C and shaken at 90 cycles/min. At fixed time intervals, the tubes were taken out from the water bath and centrifuged at 21 000*g* for 15 min. The NPs were resuspended into 10 ml of fresh PBS and returned to the water bath to continue release measurement [20]. An aliquot of 9 ml was taken from the supernatant. A volume of 1 ml of methanol was added to precipitate the PLGA. The solution was then centrifuged for 15 min at 21 000*g* and analyzed by HPLC. The experiments were carried out in triplicate.

High-performance liquid chromatography analysis

HPLC analysis was performed at room temperature using a Knauer apparatus model K-1001, WellChrom (Berlin, Germany) equipped with a reversed-phase C_{18} column (25 cm × 0.46 cm internal diameter, pore size 5 μm; Teknokroma, Barcelona, Spain) and eluted isocratically

with methanol/water (80/20 v/v). The flow rate was fixed at 1 ml/min and detection was obtained by UV detection at 230 nm. The linear regression coefficient determined in the range 0.05–10 μg/ml was 0.9994 ($n = 6$). The method sensitivity was 0.05 μg/ml (signal-to-noise ratio 3:1) and the retention time of DTX for both in-vitro and in-vivo experiments was 12.65 ± 0.8 min.

Cell culture

Human breast cancer cells (T47D and MCF7), ovary cancer cells (SKOV3), and lung cancer cells (A549) (American Type Culture Collection) were cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO_2. Cells were maintained in an exponential growth phase by periodic subcultivation.

In-vitro cell viability

SKOV3, T47D, MCF7, and A549 cells were seeded in 96-well plates (Costar, Illinois, USA) at the density of 1×10^4 viable cells/well and incubated for 24 h to allow for the cell attachment. The medium was replenished every other day. The cells were incubated with the DTX-loaded NP suspension or DTX free drug at concentrations of 10–100 nmol/l for 48, 72, 96, and 168 h. For all studies involving cultured tumor cells, we used dimethyl sulfoxide (DMSO) instead of ethanolic tween80 as a solvent for DTX free drug stock solution preparation, as tween80, which has been used at high concentration as surfactant in Taxotere, has shown to be cytotoxic itself [21]. The diluent for preparing the DTX free drug and NPs working solution was RPMI-1640 culture medium.

At designated time intervals, 20 μl of MTT (5 mg/ml in PBS) was added to each well and the culture medium containing MTT solution was removed after 3–4 h. The formazan crystals were dissolved in 100 μl DMSO and read at 570 nm by a microplate reader. Cell viability was calculated by the following equation:

$$\text{Cell viability}(\%) = (\text{Int}_s/\text{Int}_{\text{control}}) \times 100$$

where Int_s is the colorimetric intensity of the cells incubated with the samples, and $\text{Int}_{\text{control}}$ is the colorimetric intensity of the cells incubated with the PBS only (positive control). IC_{50}, the drug concentration at which 50% of the cell growth inhibits, was calculated by the curve fitting of the cell viability data using Prism 4.0 (Graphpad, San Diego, California, USA).

In-vitro cellular uptake of nanoparticles

For quantitative study, T47D cells were seeded in 24-well plates (Costar) at 50×10^4 cell/well and after the cells reached 80% confluence, the medium was changed with that containing DTX-loaded NPs or DTX free drug. The particles were dispersed in the medium at concentration of 100 nmol/l, and then the wells were incubated for 2 h. After incubation, the suspension was removed and the wells were washed three times with 1 ml of PBS to eliminate traces of NPs or free drug left in the wells. After that, 1 ml of 0.5% Triton X-100 in 0.2 N NaOH was added to the sample wells to lyse the cells. The amount of DTX present in each well was then measured by HPLC [22].

For qualitative study, cells were reseeded in the chambered cover glass system (Lab-Tek, Nunc International Co., Naperville, Illinois, USA). Cells were washed four times after incubation with RBITC-loaded NPs, wherein their preparation method was similar to that of the DTX-loaded NPs [18] for 2 h, and then fixed by a cold mixture of methanol/acetone (50 : 50 v/v) for 15 min at room temperature. The cells were washed twice with PBS and mounted in mounting medium consisted of Na_2HPO_4 + acetic acid (pH 5.5)/glycerol (50 : 50 v/v) to be observed by fluorescence microscope (λ_{ext}: 540 nm and λ_{em}: 580 nm; BX40, Olympus, Tokyo, Japan).

The fluorescent images were taken by DP70 digital imaging system (Olympus) analyzed by olysia imaging software (Olympus).

Body distribution and pharmacokinetic studies

In-vivo biodistribution studies were conducted using 13-week-old female BALB/c mice and pharmacokinetic studies were conducted using male Wistar rats weighing 200–250 g, provided by the Animal Care Center (Faculty of Pharmacy, Tehran University of Medical Sciences) after a 7-day acclimatization period. All animals were randomly assigned into two groups ($n = 5$–7 animals/group) and fasted overnight but had free access to water. Each group received 2 mg/kg of DTX formulation either as DTX-loaded NPs or DTX injection through tail vein injection. Both formulations were sterile filtered and administered in 13% ethanol in water for DTX injection and in NaCl 0.9% for NPs. As the size of the NPs was less than 220 nm, sterile filtration did not have a significant effect on particle size ($P > 0.05$).

For biodistribution studies, at various times after dosing, mice were killed and major organs were collected for analysis. Organ samples, consisting of lungs, liver, heart, kidneys, intestine, and spleen were removed, washed with NaCl 0.9% and accurately weighed. The organ samples were then homogenized and centrifuged at 21 000*g* for 10 min. Methanol was added to the supernatant (1 : 1) to precipitate the unwanted proteins and centrifuged (21 000*g* 10 min). The aliquots were assayed for DTX by the HPLC to estimate the amount of DTX in each organ. For calculations, standard curves of DTX were prepared by the addition of DTX solutions in methanol to tissues following the same treatment steps [18,23]. Blood samples were obtained by cardiac puncture in preweighed heparinized tubes; plasma was separated by centrifugation of the blood samples at 7000*g* (10 min). DTX was extracted from

plasma by adding $ZnSO_4$ (2% w/v) in a mixture of methanol/water (50 : 50 v/v) at ratio of 1 : 2, and standard curves were prepared by the addition of DTX in plasma following the same process. Extraction efficiency from plasma and various tissues was variable (80–95%).

For pharmacokinetic analysis, animals were anesthetized by an intraperitoneal injection of a mixture of 90 mg/kg ketamine hydrochloride and 10 mg/kg xylazine. One-half of the above original dose was administered every 45–60 min to maintain proper anesthesia/analgesia. The different formulations were injected through the tail vein at the DTX dose of 2.5 mg/kg, 400 µl of blood was collected at time 0, 5, 30, 60, 90, 120, 180, and 210 min postdrug administration. A PE-50 tubing cannula was made in the femoral artery for blood sampling. Blood was collected in a microcentrifuge tube, and the plasma was separated by centrifugation (10 min at 2500*g* and 4°C), and stored at −20°C until analyzed using the extraction procedure and HPLC system described above.

Pharmacokinetic parameters were determined from the plasma concentration–time data. The area of the first moment of the concentration–time curves (AUMC) and the area under the concentration–time curve from time zero to time t (AUC_{0-t}) were determined by the trapezoidal rule. The area from time t to infinity ($AUC_{0-\infty}$) was estimated according to the equation:

$$AUC_{0-\infty} = AUC_{0-t} + C_t/k_{el}$$

where C_t is the plasma concentration observed at time t, and k_{el} is the apparent elimination rate constant of DTX obtained from the slope of the linear portion of the curve by least square regression analysis. The mean residence time (MRT) of the drug of the formulations was calculated using the following equation:

$$MRT = AUMC/AUC$$

Acute toxicity

Groups of 10 mice were injected intraperitoneally with 0.5 ml doses of 100–400 mg/m^2 of DTX entrapped in NPs. Death was observed over 24 h.

Statistical analysis

Results are expressed as mean ± SD. Statistical data analysis was conducted using statistical software program (SPSS 11.5, Microsoft; SPSS Inc., Chicago, Illinois, USA). After confirmation of normal distribution using the Kolmogoroff–Smirnoff test, comparison of the data was performed using Student's *t*-test.

Results and discussion

Physicochemical properties of the nanoparticles and their in-vitro release properties

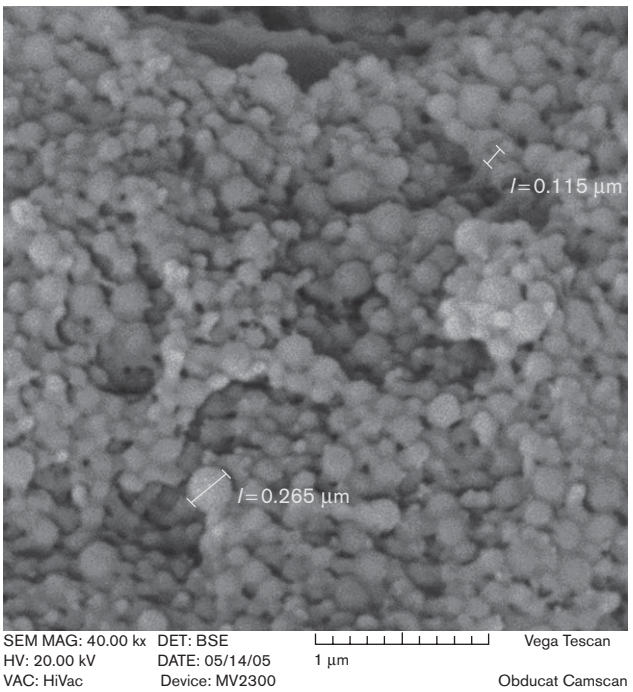
PLGA NPs were fabricated by a modified emulsification/solvent diffusion method [16–18]. The particle size of NPs was 175 ± 13 nm. They were monodispersed with a polydispersity index of less than 0.15, suitable for effective intracellular uptake of NPs.

Under SEM observation (Fig. 1), the NPs all had a fine spherical shape with a relatively monodispersed size distribution.

Zeta potential is one of the important indices in the evaluation of NP suspension stability. The value of the zeta potential was below −12.2 ± 0.6 mV. Zeta potential of NPs was negative because of the presence of terminal carboxylic groups in the polymer [24]. The presence of DTX in the NPs always reduced the negative zeta potential value, probably because of the masking effect of the superficial carboxylic groups by the drug adsorbed on NP surface. The higher absolute value of zeta potential indicates the higher electric charge on the surface of the drug-loaded NPs, which can prevent aggregation of the NPs in buffer solution by causing strong repellent forces among particles [24].

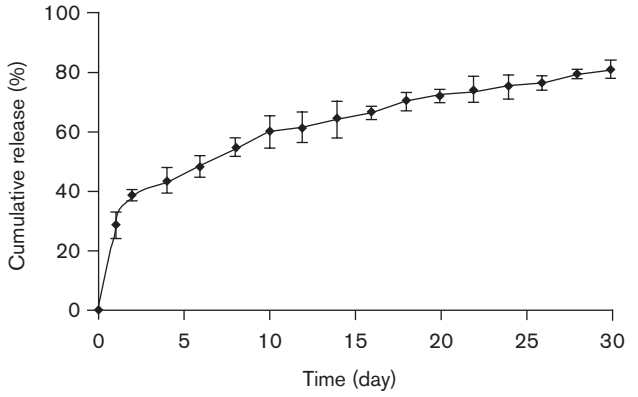
Drug loading was 0.34% with EE of 68 ± 2%. The in-vitro release behavior of the DTX-loaded NPs within 30 days is presented as cumulative percentage release in Fig. 2. In fact, NP formulations produced an initial burst effect in which DTX release was more than 28%, within 24 h.

Fig. 1



Scanning electron microscopy micrograph of docetaxel (DTX)-loaded poly(lactide-co-glycolide) (PLGA) nanoparticles produced by modified solvent/diffusion method (DTX/PLGA: 0.5% w/w) and polyvinyl alcohol 0.5%.

Fig. 2



In-vitro drug release profile of docetaxel (DTX)-loaded poly(lactide-co-glycolide) (PLGA) nanoparticles produced by modified solvent/diffusion method (DTX/PLGA: 0.5% w/w) and polyvinyl alcohol 0.5%, in phosphate buffer saline solution (pH 7.4). Data represents mean ± SD.

This burst release could be related to DTX adsorbed on the NP surface [25] and/or to the drug encapsulated near to NP surface. After the initial burst release, a constant slow release up to 80% of the loaded DTX was observed within 30 days, showing a typical sustained and prolonged drug release that depends on the mechanism of matrix erosion and drug diffusion [26].

In-vitro cell viability of the docetaxel-loaded nanoparticles

Figure 3 shows the in-vitro cytotoxic effect of DTX free drug and DTX-loaded PLGA NPs for T47D (a), SKOV3 (b), MCF7 (c), and A549 (d) cells ($n = 6$), respectively. The result indicates that the drug formulated in the PLGA NPs has shown advantages in achieving lower cell viability, or equivalently higher cytotoxicity, versus the DTX free drug. The cell viability measured at the 100 nmol/l drug concentration was decreased from 50.9, 47.1, 48.9, and 48.0% for DTX free drug to 15.91, 40.15, 29.50, and 21.75% for the NP formulation after 48 h incubation with T47D, SKOV3, MCF7, and A549 cells, respectively. PLGA NPs have shown inhibition of proliferation in all cell lines even at low drug concentration (10 nmol/l) after 72, 96, and 168 h incubation as shown in Fig. 3.

The IC_{50} (the dose that produces 50% inhibition of growth) of DTX free drug and the PLGA NP formulation of DTX have been calculated and listed in Table 1. Table 1 shows that the NP formulation has decreased the IC_{50} value for all cell lines at various incubation times ($P < 0.05$). This effect is more distinct in T47D and MCF7 cells. The diminished IC_{50} has been observed in DTX-loaded NPs for SKOV3 with lower intensity. In contrast, in the A549 cell line, only at 48 h incubation, the IC_{50} has been decreased from 97.90 nmol/l in DTX free drug to 57.28 nmol/l in NPs, but there was no significant difference in the IC_{50} values between DTX free drug and DTX-loaded NPs formulation at 72, 96 h after incubation, whereas the IC_{50} of the DTX free drug is less than NPs after 168 h.

As observed, in-vitro release studies show (Fig. 2), approximately 40 and 50% of the drug is released from the NPs after 48 and 168 h, respectively. Thus, the increased cytotoxicity of the NP formulation is not only through the DTX released from the particles but probably also because of cell uptake of the NPs and/or better penetration of NPs into the cells which in either case DTX will be delivered to its site of action [27].

PLGA NPs are taken up by cells by endocytosis, which results in a higher cellular uptake of the therapeutic agent, which is loaded into the particles [28,29], and therefore helps them to escape from P-glycoprotein pumps and render a high cytotoxic effect in comparison with DTX free drug, in agreement with other related results [30].

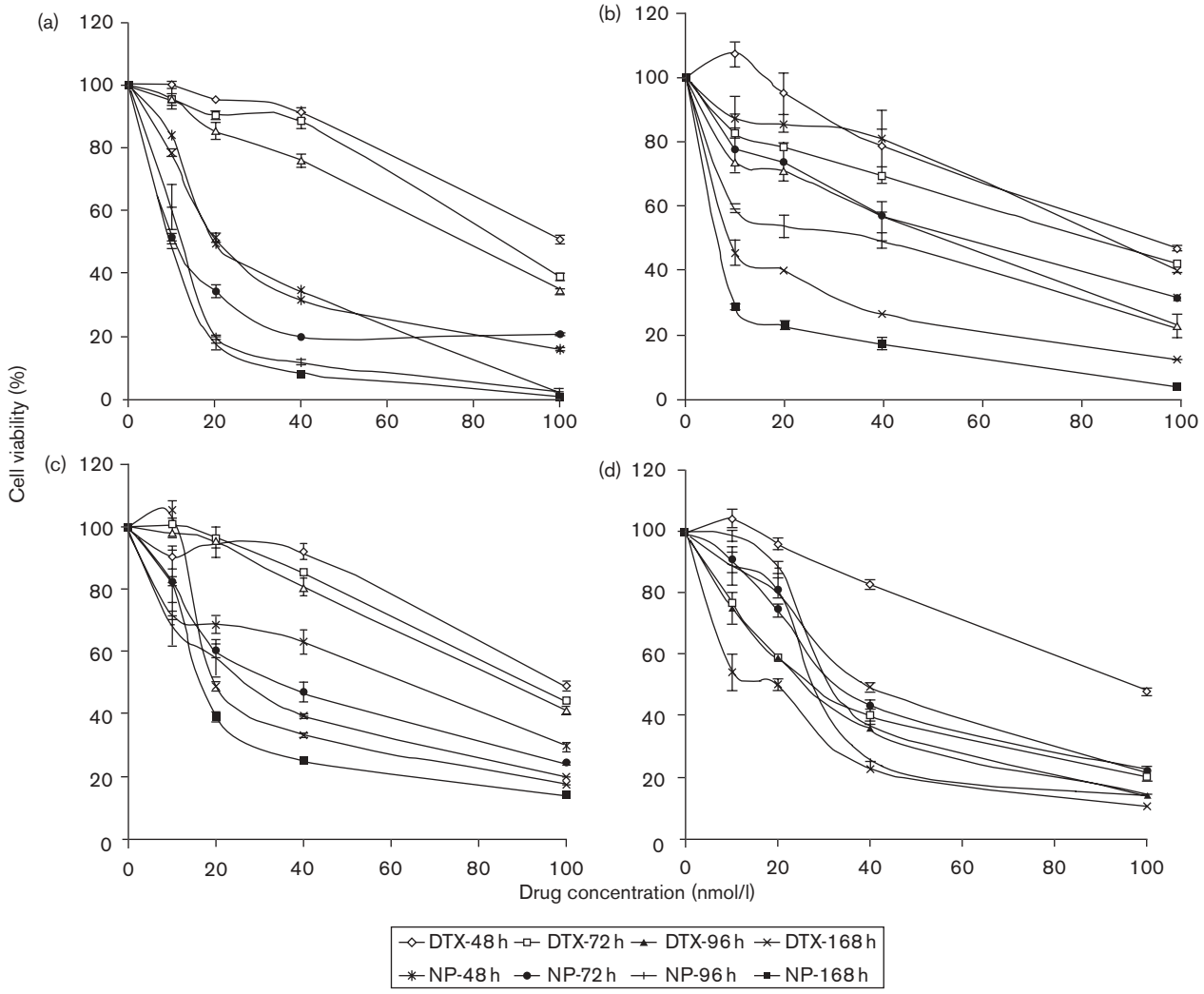
Thus, NPs act as intracellular drug depots that release the loaded therapeutic agent in the cellular cytoplasm slowly. This results in an increase of therapeutic efficacy for some drugs, such as dexamethasone [31] and paclitaxel in drug-sensitive cells, because cytoplasm is the site of action for these drugs [32]. As all the cell lines used in this study were sensitive to DTX and also cytoplasm is the site of action for DTX, better cytotoxic effects of DTX-loaded PLGA NPs are in agreement with other scientific reports on paclitaxel or dexamethasone [32].

DTX-unloaded NPs did not show any cytotoxic effect (data not shown). PVA – used as an emulsifier in NP preparation – remained on the NPs but was not cytotoxic; Westedt *et al.* have already shown the good biocompatibility of PVA-g-PLGA polymer, which contains both PLGA and PVA [33]. As no or little degradation of the PLGA NPs in the neutral (buffered) cell incubation medium is expected to occur [34] within the incubation times, no significant cytotoxicity could have resulted from polymer degradation products. The cytotoxicity of the NPs is, then, probably related to the uptake of the NPs by cells and not to the toxicity of the PLGA after NPs uptake [35].

Optimally, localization of the dosed drug to its site of action is a complicated process because of several factors, especially the presence of efflux transporters such as multidrug resistance proteins or membrane bound P-glycoprotein in tumor cells [36].

Another probability for the better effect of the NPs is that when the cell medium was changed, the cytotoxic effect of the drug in solution might be decreased, whereas in the case of drug-loaded NPs, this effect might be retained even after medium change [37].

Fig. 3



Cytotoxic effect of docetaxel (DTX)-free drug and DTX-loaded poly(lactide-co-glycolide) nanoparticles (NPs) incubated with (a) T47D, (b) SKOV3, (c) MCF7 and (d) A549 cells ($n=6$).

Table 1 **IC_{50} of T47D, SKOV3, MCF7, and A549 cells incubated with (DTX) and DTX-NP ($n=6$)**

	IC_{50} (nmol/l)							
	T47D cells		SKOV3 cells		MCF7 cells		A549 cells	
Incubation time (h)	DTX	DTX-NP	DTX	DTX-NP	DTX	DTX-NP	DTX	DTX-NP
48	107.25 ± 4.07	20.61 ± 1.03	94.47 ± 3.52	85.35 ± 2.62	105.49 ± 8.6	61.93 ± 7.2	97.90 ± 2.62	57.28 ± 2.81
72	90.46 ± ± 1.82	10.47 ± 0.52	81.65 ± 3.97	63.59 ± 4.95	93.56 ± 2.27	52.48 ± 9.12	47.05 ± 11.07	54.99 ± 4.91
96	82.06 ± 1.60	12.54 ± 0.75	56.12 ± 3.55	38.84 ± 1.94	88.09 ± 1.6	44.45 ± 23.8	43.04 ± 7.89	53.81 ± 6.80
168	24.45 ± 1.22	9.85 ± 0.49	8.75 ± 0.53	6.15 ± 0.37	19.75 ± 0.97	17.25 ± 0.86	21.82 ± 0.87	32.23 ± 1.29

Data expressed as mean ± SD.
DTX, docetaxel-free drug; DTX-NP, docetaxel-loaded poly(lactide-co-glycolide) nanoparticles; IC_{50}, the drug concentration at which 50% of the cell growth inhibits.

In the case of the A549 cells, according to the results, as after 168 h the cell mortality is higher in DTX free drug compared with NPs, there is a probability that the cells have a limited capacity to uptake the NPs. Further studies are needed to confirm these preliminary results.

In 2003, Jakate *et al.* [12] showed that the in-vitro cytotoxicity of DTX delivered in olive oil droplets – regardless of coating the oil droplets with or without fibrinogen – is at least as great as, if not greater than, that of DTX delivered in DMSO.

Cellular uptake of nanoparticles

Therapeutic effects of the DTX-loaded PLGA NPs depend on the internalization and sustained retention of the NPs by the cells. Therefore, the in-vitro investigation can show some evidence for advantages of the NP drug formulation versus the free drug. In this study, T47D cells were used to investigate the cellular uptake of the DTX-loaded NPs. It has been reported that PLGA NPs could have a higher cell uptake efficiency compared with free drug [38]. For T47D cells, the cell uptake efficiency was increased from 16% for free drug to 53% for DTX-loaded NPs after 2 h incubation at 100 nmol/l drug concentration. As viability of T47D cells incubated with NPs was approximately three-fold to four-fold lower than cells incubated with free drug at a concentration of 100 nmol/l, the higher cellular uptake of the NPs correlated well with the increased cytotoxicity and perhaps can be assigned for this increase in cell cytotoxicity of the NPs. Figure 4 shows fluorescence microscopy images of T47D cells after 2 h incubation with RBITC-loaded PLGA NPs at 37°C. We can observe from this figure that the fluorescence of the RBITC NPs (red) is distributed in cytoplasm more closely located around the nuclei (black), which indicates that the NPs have been taken up by the cells and are located inside the cells [22].

Body distribution and pharmacokinetics

To investigate the differences in organ distribution of DTX entrapped in NPs when compared with DTX injection (Taxotere), a biodistribution study was carried out. The original dose after 2 h postinjection, which was found in blood, lung, spleen, liver, kidney, intestine, and heart, was 96.9 and 57.3% in NP and Taxotere formulation, respectively. Figure 5 shows the result of the biodistribution studies at 2 h after i.v. administration of the formulations. There were significant differences in drug distribution for lung, spleen, kidney, liver, and plasma ($P < 0.05$). The amount of DTX and/or its metabolite was five-fold to six-fold higher in plasma for DTX-loaded NPs compared with Taxotere ($P < 0.05$). The DTX concentration in lungs was also higher in the NP formulation than Taxotere ($P < 0.05$), probably because of the filtration effect of the lung capillary bed that removes the large particles or their aggregates [38]. Similarly, the high DTX concentration in spleen for NPs ($P < 0.05$) could also be described by the splenic filtration [38].

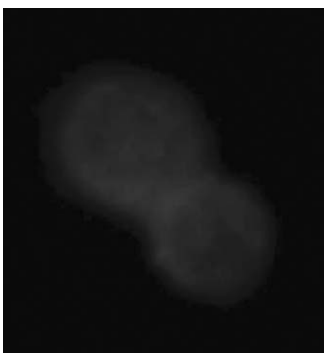
The significant differences in the DTX biodistribution pattern of DTX-loaded NPs and DTX injection in these organs can be explained by the apparent entrapment efficiency of DTX in the NPs and the release rate of DTX from NPs. As reported earlier, during the in-vitro studies approximately 6% of the drug was released from NPs after 2 h; in addition, the DTX entrapment efficiency was determined as approximately 68%, and for the biodistribution studies, DTX-loaded NPs were administered without purification. This indicates that after 2 h, only 37% of the DTX could potentially be either adsorbed to the NP surface or free, then it could be concluded that the amount of the DTX measured in body distribution study is the DTX associated with the particles in the case of NPs [20].

The plasma level after i.v. administration of DTX NPs maintained at a good level (13%) for up to 2 h compared with DTX formulated in tween80, which is approximately 0.5% of the initial dose, was present in plasma up to 2 h.

Entrapping of DTX in NPs could not change the distribution amount of DTX to kidney, liver, heart, and intestine ($P > 0.05$) in comparison with Taxotere at 2 h.

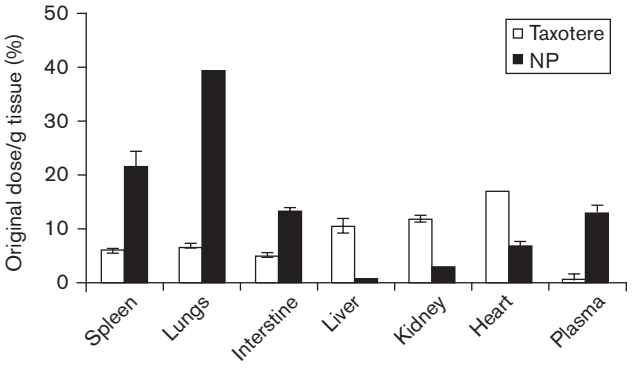
As NPs are usually distributed to reticuloendothelial system (RES) organs, such as lung, spleen, and especially liver, after i.v. administration, but in this case after 2 h,

Fig. 4



Fluorescence microscopy images of T47D cells after 2 h incubation with rhodamine B isothiocyanate (RBITC)-loaded nanoparticles (NPs) at 37°C. As shown in the image, the 'red' fluorescence of the RBITC NPs is distributed in cytoplasm, indicating the uptake of NPs by the cells.

Fig. 5



The level of docetaxel (DTX) detected in the organs of mice after intravenous administration of DTX-loaded nanoparticles (NPs) and Taxoterte (2.5 mg/kg). The data obtained from lung, liver, spleen, intestine, heart, kidney, and plasma in 2 h after injection calculated as a percentage of the initial dose applied/g tissue and presented as mean ± SD ($n = 6$).

DTX amount was lower in NPs formulation compared with Taxotere; therefore, to evaluate the behavior of the NP biodistribution at various time postinjection, percentage of the dose/g tissue at 1, 2, and 3 h after administration was calculated for plasma and the major RES organs, including liver, lung, and spleen. Figure 6 shows the biodistribution of the NPs (a) and Taxotere (b) to these organs at various times. Plasma level of DTX was higher for NPs ($P < 0.05$) at all time intervals with a decrease in amount during the time, and this decrease was because of the elimination process.

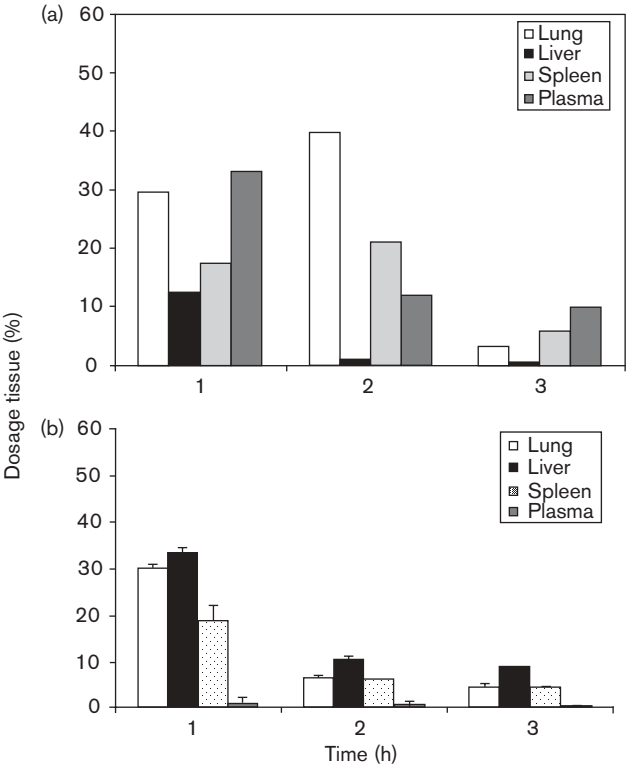
At 1 h, a large amount of the NPs were removed by the RES organs, especially by liver. As the DTX-loaded NPs distributed to the major RES organs were more than or at least as much as Taxotere, RES-evading ability of such NPs is low. As the size of the colloidal carriers is a key for the biological fate of the NPs, and NPs of smaller than 100 nm or surface-modified NPs usually bypass the RES macrophages uptake, the DTX-loaded particles of 172–178 nm with no surface treatment were not small or hydrophilic enough to bypass the RES macrophages [39].

The NPs that were removed by liver could leave the liver with a high speed; therefore, after 2 h the amount of the DTX in liver was lower for NPs compared with Taxotere, whereas removed NPs by spleen and lung leave these organs slowly in a way that there was not a significant difference between distribution behavior of the NPs at 1 and 2 h for lung and at 2 and 3 h for spleen. Study of the NPs stability in serum showed that there was an increase in particle size during the first 2 h of NPs circulation in serum, which reached to a plateau by 3 h (Fig. 7). This increase could be because of the attachment of a blood component, such as proteins to the NPs or production of particles aggregation. Then, formation of the largest particles after 2 h was probably the reason for much more distribution of the NPs to the lung at this time compared with 1 h postinjection (when the particle size in the serum was smaller than their size at 2 h).

The most important difference between the in-vivo distribution behavior of the NPs and Taxotere is the plasma level of the DTX. As it is shown in Fig. 6, the DTX level in plasma was at a good amount after 3 h for NPs (a), whereas DTX level in plasma for Taxotere was dropped significantly (b).

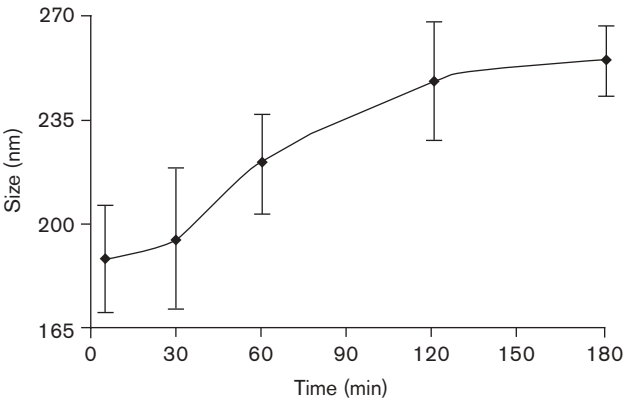
Then, as it is obvious, prepared DTX-loaded NPs were able to present a good plasma level *in vivo* in comparison with DTX conventional formulation. In addition, as the level of DTX formulated in NPs was higher for RES organs, such as liver, lung, and spleen, in comparison with Taxotere, and DTX is a drug of choice in the treatment of lung carcinoma, it is an added value for incorporation of DTX in PLGA NPs for RES organs carcinomas therapy, especially lung cancer.

Fig. 6



Biodistribution in Balb/c mice of docetaxel-loaded nanoparticles (a) and Taxotere (b) in plasma, liver, lung, and spleen at 1, 2, and 3 h after intravenous administration. Data are given as mean ± SD ($n=6$).

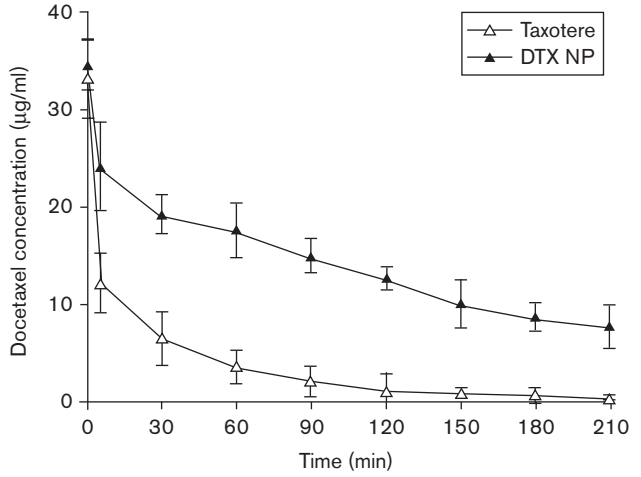
Fig. 7



Study of the size variation of docetaxel-loaded nanoparticles in rat serum. Data are given as mean ± SD ($n=3$).

The plasma concentration–time curves for Taxotere and NPs after i.v. injection are shown in Fig. 8. Free drug from DTX injection was quickly removed from the circulating system, whereas in contrast, NPs exhibited a markedly delayed blood clearance. It could be observed that the

Fig. 8



Plasma concentration–time curves of docetaxel (DTX) in DTX-loaded nanoparticles (NPs) and Taxotere after intravenous administration of 2.5 mg/kg of each dosage form. Data are given as mean ± SD ($n=6$).

DTX level from NPs remained higher at 210 min compared with those of free drug from DTX injection. The DTX–time curves for NPs and injection in rats were fitted with the two-compartment model according to the equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_p is the plasma concentration of DTX at time t; A and B are empirical constants, and α and β are hybrid rate constants. The rate constants α and β were derived from the slopes of the decline in plasma concentration during the initial distribution and the terminal elimination phase. From the estimated slopes, the half-lives of α and β phase were calculated as $t_{1/2\alpha} = 0.693/\alpha$ and $t_{1/2\beta} = 0.693/\beta$. DTX-loaded in NPs was eliminated rather slowly with $t_{1/2\ \beta}$ for NPs (153.4 min) and DTX injection (75.32 min). The $t_{1/2\alpha}$ of NPs showed longer than that of DTX injection ($P < 0.05$). The short half-life of DTX injection explains the rapid decline of plasma concentrations after i.v. administration.

The MRT of NP formulation (77.4 min) was significantly longer compared with Taxotere (46.1 min) (Table 2). However, it should be noted that for the NP formulation, the value obtained for MRT is underestimated because this formulation is based on PLGA, wherein degradation time is much longer than the duration of the in-vivo study.

Acute toxicity

As the loading amount of DTX in NPs was low (0.34%), for administration of the DTX at doses of 100–400 mg/kg in mice, a large amount of the NPs had to be administered intraperitoneally. At doses of less than 400 mg/kg, all mice were alive for 24 h postadministration but after 48 h all developed abdominal ascites. This was probably because of the water absorption by the colloidal solution of PLGA. At higher doses, abdominal ascites occurred soon after injection and the mice were dead.

Table 2 Mean pharmacokinetic parameters after single intravenous administration of DTX-loaded NPs and DTX injection (Taxotere) in rats (2.5 mg/kg)

Pharmacokinetic parameter	DTX-loaded NPs	DTX injection
β (1/min)	0.006 ± 0.002	0.009 ± 0.003
α (1/min)	0.078 ± 0.021	0.009 ± 0.002
$t_{1/2\beta}$ (min)	153.9 ± 19.5	75.3 ± 9.5
$t_{1/2\alpha}$ (min)	8.9 ± 1.5	75.3 ± 12.1
Cl (ml/min)	1.1 ± 0.2	0.8 ± 0.1
AUC (μg min/ml)	3286.5 ± 235.3	729.4 ± 98.9
V_c (ml/kg)	168.9 ± 21.5	84.4 ± 12.4
MRT (min)	77.4 ± 21.0	46.1 ± 16.5

Data expressed as mean ± SD.
AUC, area under thecurve; Cl, clearance; DTX, docetaxel; MRT, mean residence time; NP, nanoparticles; V_c, volume of distribution.

These results indicate that the association of DTX within NPs was able to reduce general in-vivo toxicity compared with DTX injection ($LD_{10} = 345$ mg/m^2), and this effect is likely because of tween80 removal from the DTX formulation.

Conclusion

It was shown that PLGA NPs containing DTX, with the desired size and drug loading characteristics suitable for i.v. administrations, could be prepared without using tween80 and cellular mortality of the NPs was more than or at least as great as free drug. Prepared DTX-loaded NPs presented a good plasma level *in vivo* in comparison with DTX conventional formulation.

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