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ORIGINAL ARTICLE

The uptake of paclitaxel and docetaxel into ex vivo porcine bladder tissue from polymeric micelle formulations

Antonia Tsallas · John Jackson · Helen Burt

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Abstract Superficial bladder cancer occurs in the urothelial layer of the bladder and is usually treated by transurethral resection and chemotherapy. Although the bladder is well suited for intravesical chemotherapy, effective drug delivery is restricted by urine dilution and poor drug uptake by bladder tissues during a 2 h instillation. In this study, freshly excised pig bladder sections were mounted on Franz diffusion cells and treated with anticancer drugs paclitaxel (PTX) and docetaxel (DTX) formulated in diblock copolymer (methoxy poly(ethylene glycol)-block-poly (D,L-lactic acid) (MePEG-PDLLA) and methoxy poly(ethylene glycol)-block-poly(caprolactone) (MePEG-PCL) nanoparticles for 2 h. The bladder sections were then frozen, cryosectioned (60-μm sections) and the amount of ^{3}H drug taken up into each section was determined using liquid scintillation counting. Tissue concentration versus tissue depth profiles were obtained for all drug formulations and drug exposure obtained from area-under-the-curve (AUC) calculations. PTX or DTX loaded in MePEG-PDLLA micelles produced significantly higher urothelial tissue levels and greater bladder wall exposures compared to their commercial formulations, Cremophor EL/ethanol (PTX) or Tween 80 (DTX). The results of this study support the use of diblock copolymer micellar PTX or DTX formulations as they allow for improved drug penetration of bladder tissues compared to commercial formulations for taxane delivery to superficial bladder tumors.

A. Tsallas · J. Jackson · H. Burt (✉)
Faculty of Pharmaceutical Sciences,
University of British Columbia, 2146 East Mall,
Vancouver, BC V6T 1Z3, Canada
e-mail: Burt@interchange.ubc.ca

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Introduction

The majority of bladder cancer patients present with superficial tumors and are effectively treated by transurethral resection (TUR) methods [28]. However, more than 60% of cases will recur and approximately 15% of patients will develop deeper tumors that require radical surgery and systemic chemotherapy [5, 9]. Patients with superficial cancer are generally given immunotherapy, such as Bacillus Calmette-Guerin (BCG) or chemotherapy, such as mitomycin C or doxorubicin. These agents are introduced into the bladder for 2 h to eliminate any residual cancer cells [3, 22]. Although these chemotherapeutic methods may reduce short-term (2 year) recurrence rates, they have little effect on long-term outcome at 5 years [6, 26, 31, 32]. Systemic administration of anticancer agents is not indicated because the superficial tumors are located close to the surface urothelium, which is poorly vascularized.

Clearly, the location of superficial bladder tumors supports the strategy of intravesical drug administration since concentrated anticancer drug solutions may be readily instilled through a urethral catheter and then held directly next to the target tissues for a period of 2 h, until voiding. However, treatment failure is often ascribed to complete washout of drug upon voiding, poor uptake of drugs like mitomycin C and doxorubicin into the bladder wall and low sensitivity of tumors to these drugs [16]. Furthermore, significant inter-patient variability in the penetration of doxorubicin into bladder tissues has been reported that may be related to urine dilution and urothelium barrier properties [34].

During the last decade, paclitaxel (PTX) and docetaxel (DTX) have emerged as important drugs for intravesical chemotherapy. DTX is currently in phase I clinical trails for intravesical treatment of superficial bladder cancer [4, 18, 23]. PTX has significant activity against bladder tumor cells [13, 25, 27] and is reported to be particularly effective against rapidly proliferating human bladder cancer cells [2]. Furthermore, it was proposed that the hydrophobicity of PTX might enhance drug penetration across the urothelium barrier. Following the intravesical administration of Taxol® (the commercial formulation of PTX), PTX was found to exhibit much greater partitioning across the urothelium than doxorubicin or mitomycin C [30].

It has been suggested that the micellar Cremophor EL formulation of PTX (Taxol®) significantly sequesters the drug within the micellar core, which limits free drug availability at the bladder wall for urothelium uptake. By simply suspending PTX in water, Knemeyer et al. [16] demonstrated much greater drug penetration into the bladder wall following intravesical administration than when the drug was administered solubilized in Cremophor EL/ethanol. Another report showed that dissolving PTX in 50% dimethyl sulphoxide (DMSO) produced similar levels of bladder tissue penetration to those found with water suspensions of the drug [8]. In the same study, the addition of 50% DMSO to the Cremophor EL/ethanol PTX formulation increased the free fraction of PTX and the urothelial tissue concentration, but did not significantly increase the average tissue concentration of PTX in the bladder wall compared to PTX in Cremophor EL/ethanol alone [8].

We have previously described the effective encapsulation of PTX in polymeric micelles composed of methoxy poly(ethylene glycol)-block-poly(D,L-lactic acid) (MePEG-PDLLA) and methoxy poly(ethylene glycol)-block-poly(caprolactone) (MePEG-PCL) for intravenous administration [7, 20, 21, 36]. These biocompatible and biodegradable micellar systems effectively solubilize PTX and DTX allowing for the use of concentrated drug solutions intravesically. PTX-loaded MePEG-PDLLA micelles have shown significant cytotoxicity against bladder cancer cell lines and significant inhibition of tumor growth following intravesical administration in a mouse model of superficial bladder cancer [13]. Furthermore, after a 2 h instillation of PTX-loaded MePEG-PDLLA micelles into healthy mice bladders, significant bladder tissue levels were achieved [13]. However, the mouse bladder is very thin in comparison with humans, dogs and pigs and tissue levels of PTX in mice bladders were suggested to be much greater than in larger species [13]. The objectives of this work were to compare the penetration of PTX and DTX from MePEG-PDLLA, MePEG-PCL and Cremophor EL or Tween 80 formulations into pig bladder tissue. Using freshly excised porcine bladder tissue in Franz diffusion cells, tissue level-depth profiles were obtained and showed that DTX uptake was greater than PTX from MePEG-PDLLA micelles and that these micelles also provided the highest tissue levels compared to the other formulations.

Materials

Chemicals and solvents

HPLC-grade acetonitrile, dichloromethane and ethanol were obtained from Fisher Scientific (Fairlawn, NJ). ACS-grade sodium chloride, magnesium chloride, calcium chloride, sodium dihydrogen orthophosphate, sodium bicarbonate, glacial acetic acid and anhydrous sodium acetate were purchased from Fisher Scientific (Fairlawn, NJ). Triton X-100 and Cremophor EL were obtained from Fluka Biochemika (Buchs, Switzerland) and Tween 80 (polyoxyethylene-sorbitan monoleate) from Sigma–Aldrich (St. Louis, MO). Liquid scintillation fluid, Cyto-Scint™ES, was purchased from MP Biomedicals (Irvine, CA). [^{14}C] D-mannitol, 250 μCi/ml was purchased from Moravek Biochemicals (Brea, CA). Tyrode salts were purchased from Sigma–Aldrich (St.Louis, MO) (Tyrode buffer contains the following in g/l: NaCl:8.0, KCl: 0.3, $NaH_2PO4.5H_2O$: 0.093, KH_2PO_4:0.025, $NaHCO_3$: 1.0, Glucose:2.0).

Paclitaxel was purchased from Polymed Therapeutics Inc. (Houston, TX). Docetaxel was obtained from Natural Pharma (Langley BC. Canada). Commercial Taxol® 6 mg/ml (Biolyse Pharma, St Catharines, ON) and Taxotere® 20 mg/0.5 ml (Sanofi Aventis, Laval, QC) were purchased from the BC Cancer Agency at the Vancouver General Hospital. Tritium-labeled PTX and DTX in ethanol were purchased from Moravek Biochemicals (Brea, CA) with specific activities of 19.7 and 23.2 Ci/mmol.

Diblock copolymer, methoxy poly(ethylene glycol)-block-poly(D,L-lactic acid) (MePEG-PDLLA) was provided by Angiotech Pharmaceuticals (Vancouver, BC). The copolymer was manufactured with a weight ratio of 60:40 (MePEG:PDLLA), using a MePEG molecular weight of 2,000 g/mol [36]. Diblock copolymers of methoxy poly(ethylene glycol)-block-poly(caprolactone), MePEG-PCL_{19} and MePEG-PCL_{104}, were previously synthesized in weight ratios of 70:30 and 30:70 with a MePEG molecular weight of 5,000 g/mol [21].

The structures of the MePEG-PDLLA and MePEG-PCL104 are shown in Fig. 1a, b, respectively.

Porcine bladder tissue

Porcine bladders were purchased from Britco Inc. (Langley, BC). Freshly excised urinary bladders were removed

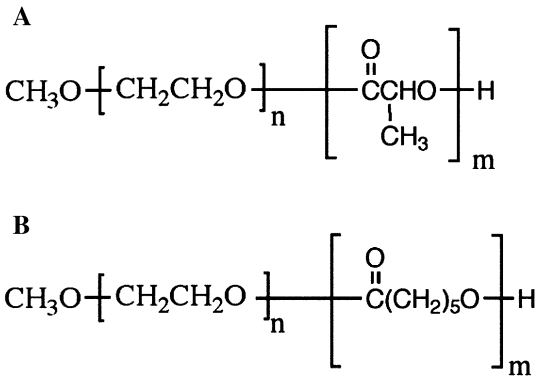

Fig. 1 Structures of diblock copolymers **a** MePEG-PDLLA and **b** MePEG-PCL where n is the number of repeat units of MePEG and m is the number of repeat units of either PDLLA or PCL

on-site from 6- to 10-month-old male pigs weighing between 90 and 113 kg.

Preparation of PTX- and DTX-loaded nanoparticles

Micelles loaded with PTX or DTX were prepared using the solvent evaporation technique. PTX or DTX and MePEG-PDLLA copolymer (10% w/w) were dissolved in acetonitrile (ACN) and dried with nitrogen gas. Prior to drying, the polymer/drug solution was spiked with a small aliquot of ^{3}H PTX or ^{3}H DTX. The resulting polymer/drug matrix was reconstituted with 60°C tyrode buffer (pH 7.4) and vortexed for 2 min. The final concentration of drug was 0.5 and 1 mg/ml and was used at 37°C.

Nanoparticles of MePEG-PCL were prepared by a nanoprecipitation and dialysis technique. Briefly, 0.5 ml solutions of MePEG-PCL (either MePEG-PCL_{19} or -PCL_{104}) were made at concentrations of 20 or 5% w/v in dimethyl formamide (DMF). PTX was also dissolved in the solutions at 2 mg/ml and spiked with a small volume of ^{3}H PTX. The copolymer/drug solutions were added drop-wise to 0.75 ml of rapidly stirring tyrode buffer (pH 7.4). The DMF was removed from the solution by dialysis in tyrode buffer overnight using 3500 MWCO Spectra/Por® dialysis membranes. The resulting dialysates were pooled and diluted with tyrode buffer such that the final copolymer concentration was 5 and 1.2% w/v and the final drug concentration was 0.5 mg/ml.

Preparation of control formulations of PTX and DTX

PTX was prepared in Cremophor EL and ethanol by diluting Taxol® (containing 6 mg of PTX, 527 mg Cremophor EL and 49.7% (v/v) ethanol per ml) with tyrode buffer to yield a final concentration of either 0.5 or 1 mg/ml PTX. Solutions were doped with a small aliquot of ^{3}H PTX prior to dilution. DTX was prepared in Tween 80 by diluting Taxotere® concentrated solution (containing 40 mg of DTX and 1,040 mg of Tween 80 per ml) with tyrode buffer to yield a final concentration of either 0.5 or 1 mg/ml DTX. Solutions were doped with a small amount of ^{3}H DTX prior to dilution. In these studies, aqueous solution of PTX or DTX was not used because the solubility of these drugs cannot be increased above 10 μg/ml without the use of solubilizing excipients.

Tissue preparation

Freshly excised porcine bladders were removed of excess adipose tissue on the exterior wall and opened longitudinally into left and right lateral sides and cut into pieces approximately 2 × 2 cm in a shallow bath of 37°C tyrode buffer bubbled with carbogen (95% O_2/5% CO_2). All studies were performed within 5 h after killing. Bladder pieces were mounted onto a Franz diffusion cell apparatus, such that the luminal side of the bladder wall was exposed to the drug solution. These tissue sections were not stretched and measured approximately 2–3 mm thick. Receptor chambers were filled with 10 ml of 37°C tyrode buffer (pH 7.4). Excess tissue was trimmed around the perimeter of the diffusion cell. The donor chamber of the diffusion cell was filled with 1 ml of either 0.5 or 1 mg/ml drug solution and the tissue exposure area was 0.64 cm^2. Each diffusion cell was set into a shallow water bath and incubated at 37°C for 2 h, with the exception of time-dependent studies, which incubated tissues for 0.5, 1, 2 and 3 h. Upon termination, the donor and receptor solutions were collected and stored at −20°C for further analysis. Tissue samples were washed three times with tyrode buffer to remove all unbound drug. Tissue samples were trimmed and rapidly frozen on metal plates with liquid nitrogen on a bed of dry ice.

Cryotome sectioning of tissue

Frozen bladder tissue was mounted with Shandon Cryomatrix™ (Themo Scientific, Pittsburgh, PA) onto a cryotome object holder. Bladder tissue was sectioned with Shandon MB35 Premier Low Grade Microtome Blades (Themo Scientific, Pittsburgh, PA) at −20°C on a Shandon Cryotome Electronic (Thermo Electron Corporation, Cheshire, England) with a R404A refrigeration system. Tissues were sectioned into 60 μm thicknesses. Tissues between 60 and 240 μm (urothelium) were collected individually for analysis. Two tissue sections between 240 and 1,260 μm (lamina propria) were collected and pooled for analysis. Three tissue sections between 1,260 and 2,160 or 3,060 μm (muscle layer) were collected and pooled for analysis. Tissue sections were placed in pre-weighed 1.5-ml eppendorf tubes and stored frozen at −20°C.

Quantification of drug in tissue

Two hundred microliters of acetonitrile was added to the weighed tissue slices for drug extraction. Samples were vortexed until all tissue slices were freely submerged in ACN and left at room temperature for 24 h to ensure complete extraction of drug. The extracted samples including all tissue slices were transferred to scintillation vials and 5 ml of scintillation fluid was added. Counts of ^{3}H PTX and ^{3}H DTX were measured by liquid scintillation counting and quantitated using calibration graphs from the original stock solution. The extracted samples and small amounts of tissue in the scintillation vials did not cause quenching because counts of calibration standards were unaffected by the inclusion of acetonitrile-treated control tissues.

Analysis of tissue level-depth profiles

The tissue level-depth profiles were analyzed for average PTX and DTX concentrations in the urothelium, lamina propria, muscularis and whole bladder tissue. The average tissue levels were determined as the total amount of drug found in the tissue layer divided by the total tissue weight for that layer. The area under the tissue level-depth profile (AUC) was calculated using the linear trapezoid rule, as follows:

$$\mathrm{AUC}_0^t = \sum_{i=0}^{n-1} \frac{(t_{i+1} - t_i)}{2} \times (C_1 + C_{i+1})$$

where t is tissue depth in μm and C is concentration in μg/g.

An estimation by extrapolation of the drug concentration (μg/g) at 0 μm was required in order to calculate the AUC.

Mass balance

The amount of drug in the donor, receptor and tissue wash solutions was also assayed by liquid scintillation counting. Receptor solutions (10 ml) collected from diffusion cells were treated with 1 ml of dichoromethane (DCM), shaken and centrifuged for 10 min at 1,500 rpm. The supernatant was aspirated and the organic phase containing drug was dried under nitrogen gas and reconstituted in 1 ml 60:40 ACN:H_2O (v/v) and counted using a Beckman Scintillation Counter.

Furthermore, 20-μl aliquots of donor solution and 100–200 μl of tissue wash solutions were also counted. Total drug recovery was accounted for by summing the amounts of drug found in the tissue, donor, receptor and tissue wash fractions.

Mannitol paracellular permeability assay

To evaluate whether there were vehicle-dependent changes in the integrity of the bladder wall, the penetration of ^{14}C mannitol was determined in the presence of Cremophor EL, Tween 80 and MePEG-PDLLA micelles. MePEG-PDLLA micelles were prepared by direct dissolution of the copolymer in tyrode buffer to a final concentration of 10 mg/ml and a small aliquot of ^{14}C mannitol was added. Cremophor EL and Tween 80 micelles were prepared by direct dissolution at concentrations of 8% w/v Cremophor EL and ethanol and 2.5% w/v Tween 80 in tyrode buffer and spiking the solution with a small aliquot at ^{14}C mannitol. A small aliquot of ^{14}C mannitol was added to a 3 ml sample of tyrode buffer (negative control). As a positive control, 0.2% Triton X-100 solution was prepared and spiked with a trace amount of ^{14}C mannitol. At this concentration, Triton X-100 increases cell permeability and weakens tight junction integrity without causing total lysis of surface cells. The luminal surfaces of freshly excised bladder pieces were exposed to 1,000 μl of vehicle solution and 0.2% Triton X-100 solution at 37°C for 2 h on a Franz diffusion cell apparatus. Tissue samples were trimmed and rapidly frozen on metal plates with liquid nitrogen. Tissues were sectioned by cryostat into 60 μm thicknesses. ^{14}C mannitol was extracted from tissue slices with 100 μl ethanol in eppendorf tubes for 24 h and measured by liquid scintillation counting.

Statistical analysis

Data collected from in vitro drug release, formulation and time-dependent bladder tissue uptake studies and mannitol permeability studies are presented as mean ± standard deviation. All other data are expressed as the mean ± standard error. Statistical analysis was performed using GraphPad Prism version 4.0b (GraphPad Software, La Jolla, CA) with a significance level of $P < 0.05$. The two-tailed Student's t test was used for comparisons between two groups with a significance level of $\alpha < 0.05$ considered to be statistically significant and marked with an asterisk (*). For comparisons between three or more groups, the results were analyzed for statistical significance using a one-way ANOVA. Differences were considered significant at $P < 0.05$. A Bonferroni post hoc test was performed when a difference was detected.

Results

Bladder tissue distribution of paclitaxel and docetaxel

Both drugs were used at concentrations of 0.5 and 1 mg/ml because previously completed in vivo efficacy studies using PTX and DTX in a mouse model of bladder cancer were performed with these concentrations [24]. Figure 2a, b shows the tissue levels of PTX in bladder tissue as a function

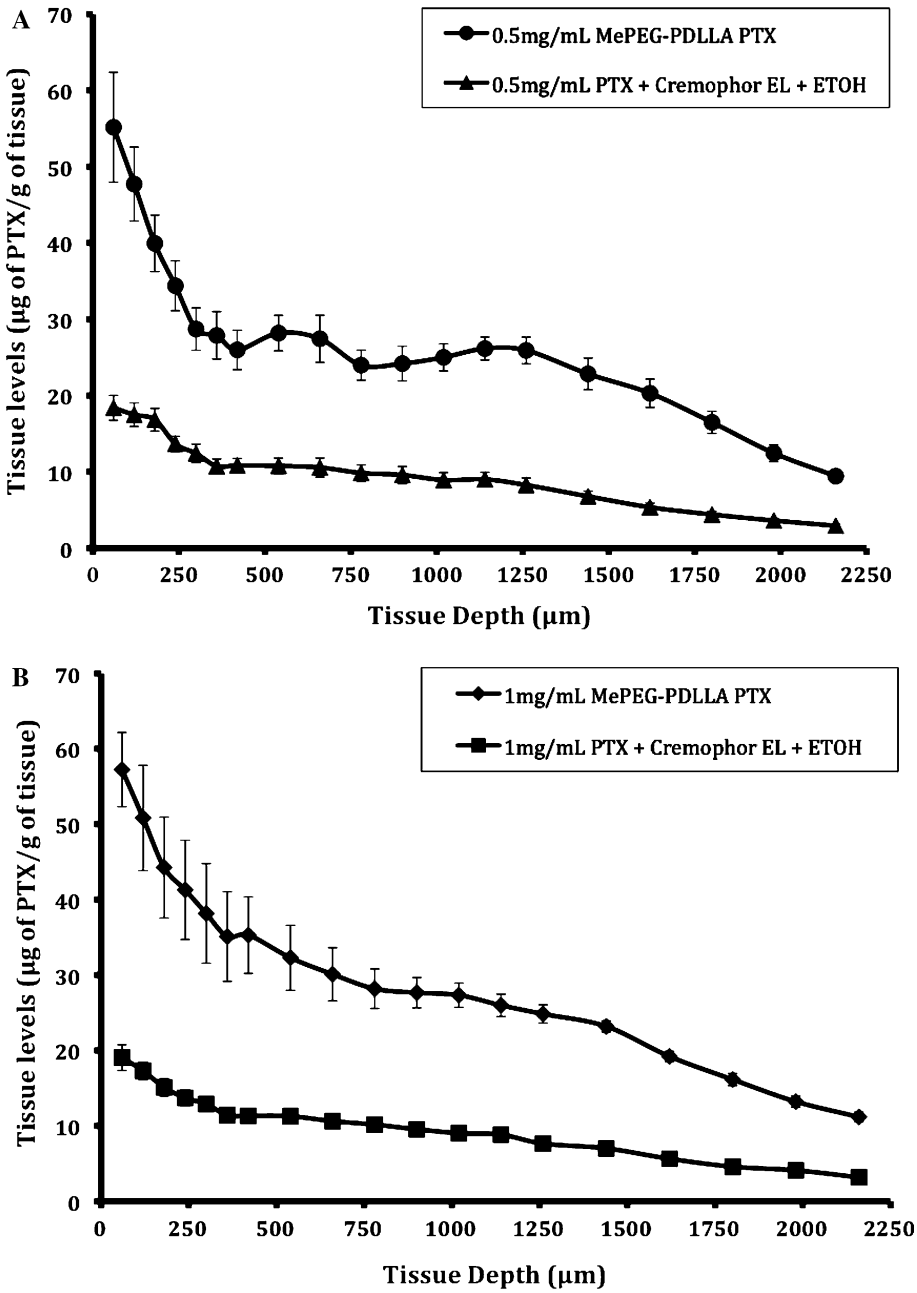

Fig. 2 Tissue level-depth profiles of PTX in bladder tissue following exposure to **a** 0.5 mg/ml PTX in MePEG-PDLLA micelles (*filled circle*) and 0.5 mg/ml Cremophor EL and EtOH (*filled triangle*) and **b** 1 mg/ml PTX in MePEG-PDLLA micelles (*filled diamond*) and 1 mg/ml PTX in Cremophor EL and EtOH (*filled square*). Tissues were incubated for 2 h and sectioned at 60 µm thickness. Values are means ± SEM ($n = 18$)

of tissue depth following exposure to 0.5 or 1 mg/ml solutions. The penetration of PTX decreased with increasing tissue depth. PTX loaded into MePEG-PDLLA micelles showed greater bladder tissue uptake compared to PTX in Cremophor EL and ethanol. However, there was no difference in tissue uptake profiles observed between the 0.5 and 1 mg/ml PTX formulations.

Figure 3a shows the average tissue levels of PTX within each layer of the bladder wall including the entire tissue (60–2,160 µm). The average concentration of PTX taken up into the bladder wall using the MePEG-PDLLA micelle formulation significantly exceeded that observed for the Cremophor EL and ethanol formulation. In the different tissue sections, the values for tissue levels following exposure to 0.5 and 1 mg/ml diblock micellar PTX, respectively, were as follows: 44.1 and 48.3 µg/g (urothelium), 26.4 and 29.7 µg/g (lamina propria) and 17.2 and 17.4 µg/g (superficial muscle). Much lower values were obtained for the same layers after exposure to 0.5 and 1 mg/ml Cremophor EL and EtOH solutions: 16.3 and 16.2 µg/g (urothelium), 10.1 and 10.0 µg/g (lamina propria) and 5.0 and 5.3 µg/g (muscle). Figure 3b shows the AUCs for PTX calculated using the linear trapezoid rule for the urothelium (0–240 µm), the lamina propria (240–1,260 µm) and the muscle tissue (1,260–2,160 µm). The AUC for the whole tissue is designated 0–2,160 µm.

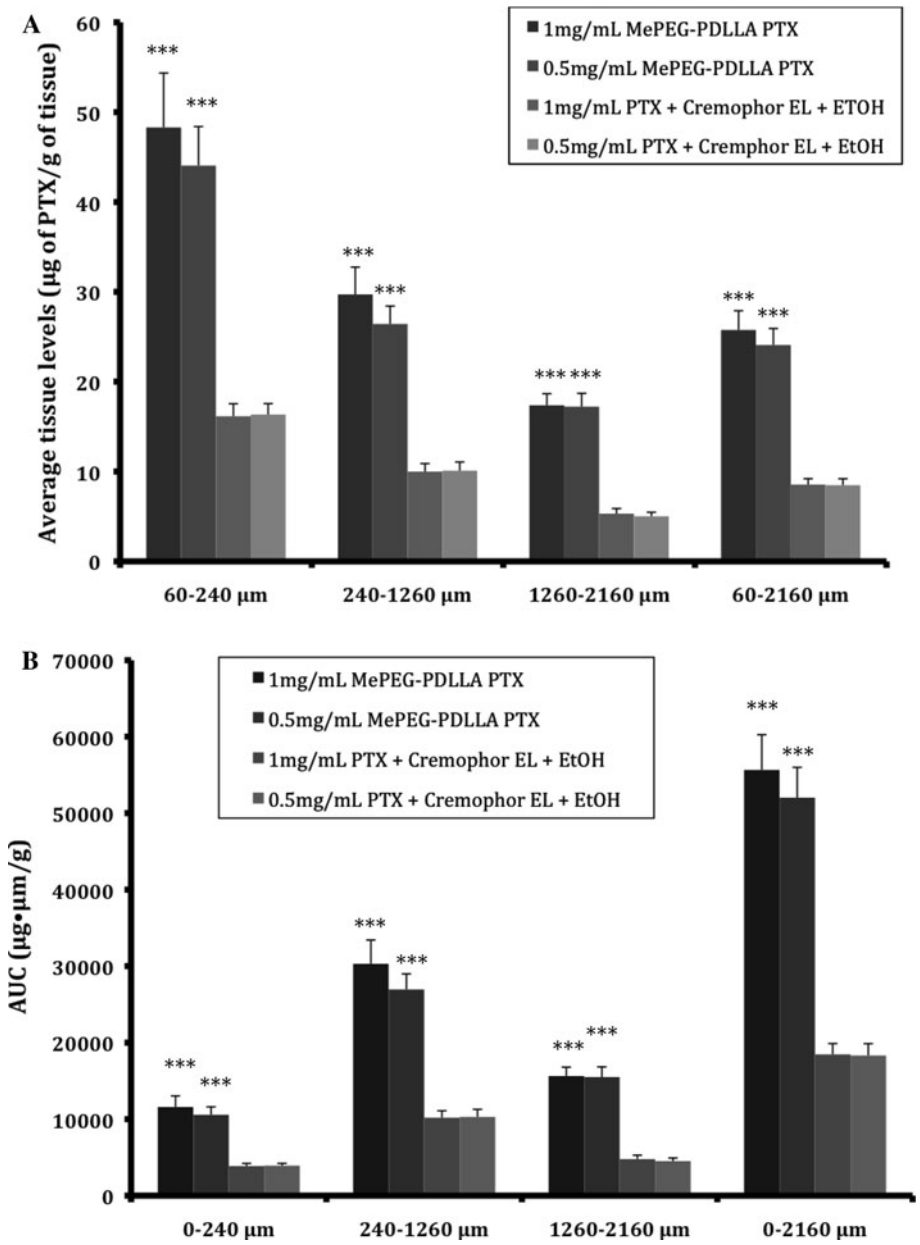

Fig. 3 **a** Average tissue levels of PTX in various layers of the bladder wall (60–240 µm: urothelium, 240–1,260 µm: the lamina propria, 1,260–2,160 µm: the superficial muscle layer and 60–2,160 µm: the whole tissue) following incubation with PTX at either 0.5 or 1 mg/ml from either MePEG-PDLLA or Cremophor EL and EtOH formulations for 2 h. The average tissue levels were determined as the total amount of drug found in the tissue layer divided by the total tissue weight for that layer. **b** AUCs of PTX in various tissue layers of the bladder wall, as described above. The AUCs were calculated using the linear trapezoid rule. The AUC for the whole tissue is designated 0–2,160 µm. Data are means ± SEM ($n = 18$). ***$P < 0.001$, 0.5 mg/ml MePEG-PDLLA PTX versus 0.5 mg/ml PTX + Cremophor EL + EtOH and 1 mg/ml MePEG-PDLLA PTX versus 1 mg/ml PTX + Cremophor EL + EtOH

AUCs give a measure of drug "exposure" and it is clear that much greater PTX exposure is achieved using MePEG-PDLLA micelles compared to Cremophor EL and EtOH solutions of the drug (Fig. 3b).

Tissue levels of DTX within each layer of the bladder wall following incubation with DTX in MePEG-PDLLA micelles or Tween 80 (at either 0.5 or 1 mg/ml) are shown in Fig. 4a, b. For both formulations, the penetration of DTX decreased with increasing tissue depth. Although DTX formulated in MePEG-PDLLA micelles penetrated tissue in significantly higher concentrations than for the drug dissolved in Tween 80 solutions, there was no significant difference observed between DTX concentrations of 0.5 and 1 mg/ml. In Fig. 5a, the average tissue levels in the different tissue layers following exposure to 0.5 and 1 mg/ml diblock, respectively, were as follows: 81.9 and 68.5 µg/g (urothelium), 48.9 and 42.6 µg/g (lamina propria) and 29.1 and 28.6 µg/g (superficial muscle). Much lower values were obtained for the same layers after exposure to 0.5 and 1 mg/ml DTX in Tween 80 solutions: 44.1 and 36.3 µg/g (urothelium), 26.4 and 23.5 µg/g (lamina propria) and 17.2 and 15.8 µg/g (muscle). In Fig. 5b, AUCs are shown for DTX calculated for each tissue layer as described above.

Figures 3a and 5a show the average tissue levels of the two drugs in the urothelium, lamina propria and superficial muscle layers. At both 0.5 and 1 mg/ml incubation concentrations, DTX was taken up into the bladder wall in

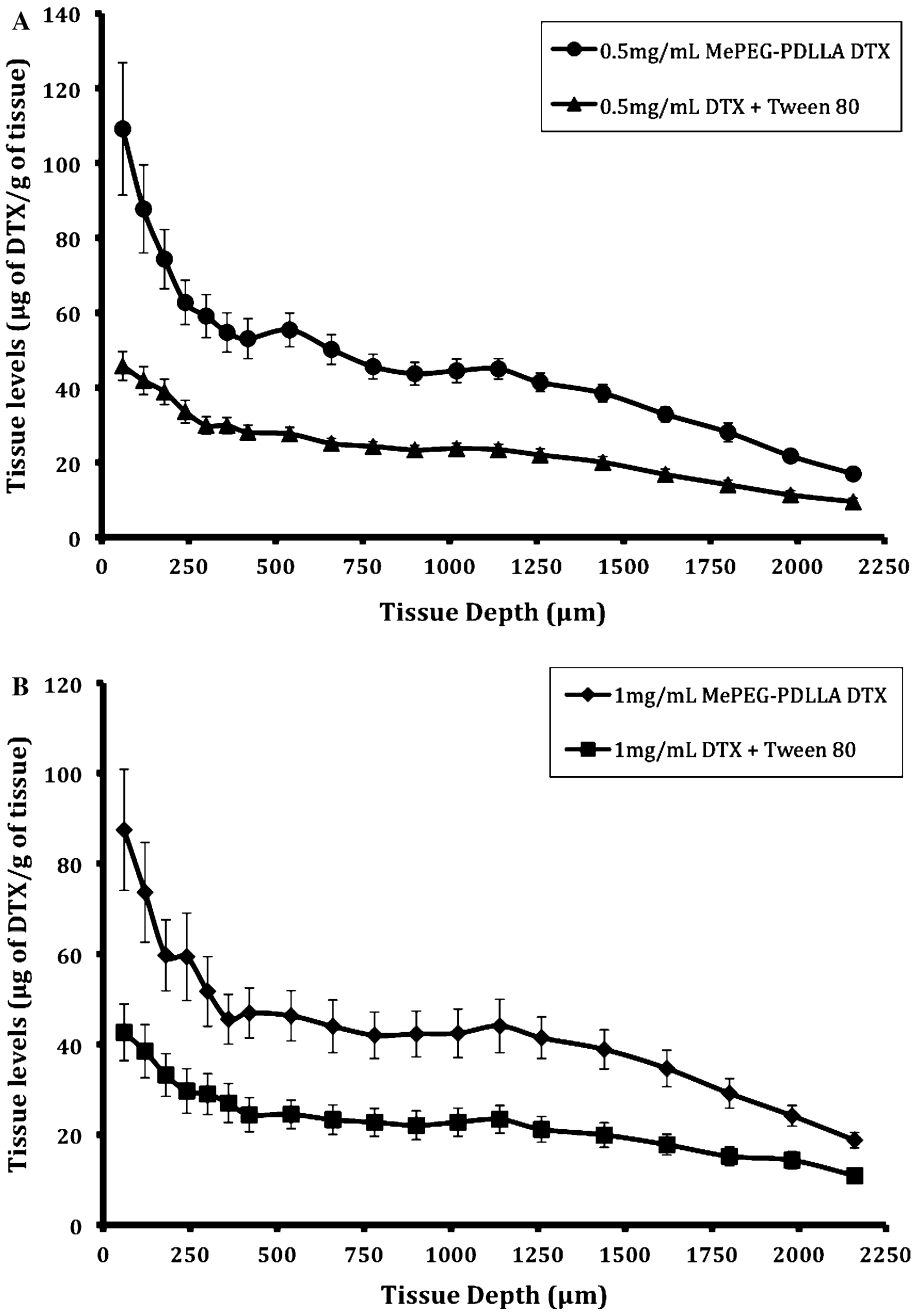

Fig. 4 Tissue level-depth profiles of DTX in bladder tissue following exposure to **a** 0.5 mg/ml DTX in MePEG-PDLLA micelles (*filled circle*) and 0.5 mg/ml DTX in Tween 80 (*filled triangle*) and **b** 1 mg/ml DTX in MePEG-PDLLA micelles (*filled diamond*) and 1 mg/ml DTX in Tween 80 (*filled square*). Tissues were incubated for 2 h and sectioned at 60 µm thickness. Values are means ± SEM ($n = 18$)

greater amounts than PTX as shown by comparing levels in Figs. 3a and 5a.

All components of the diffusion cell apparatus including donor, receptor, washes and tissue were analyzed for drug content. Mass balance analysis showed that the drugs in both the 0.5 and 1 mg/ml PTX and DTX diffusion experiments could be recovered (Fig. 6a, b). The majority of drug was recovered in the donor chamber, less than 3% in the washes and less than 0.04% in the receptor solutions. In some cases, more than 100% of drug was accounted for in the donor chamber but this result probably arose from a minor amount of evaporation of water that occurred during incubation and pipetting. Approximately, 0.5 and 0.3% of the initial dose was recovered in bladder tissue treated with 0.5 and 1 mg/ml PTX loaded MePEG-PDLLA micelles, respectively, whereas 0.2 and 0.1% of the initial dose was recovered in bladder tissue treated with 0.5 and 1 mg/ml PTX in Cremophor EL and ethanol. Approximately, 1 and 0.5% of the initial dose was recovered in bladder tissue treated with 0.5 and 1 mg/ml DTX-loaded MePEG-PDLLA micelles, respectively, whereas 0.4 and 0.3% of the initial dose was recovered in bladder tissue treated with 0.5 and 1 mg/ml DTX in Tween 80.

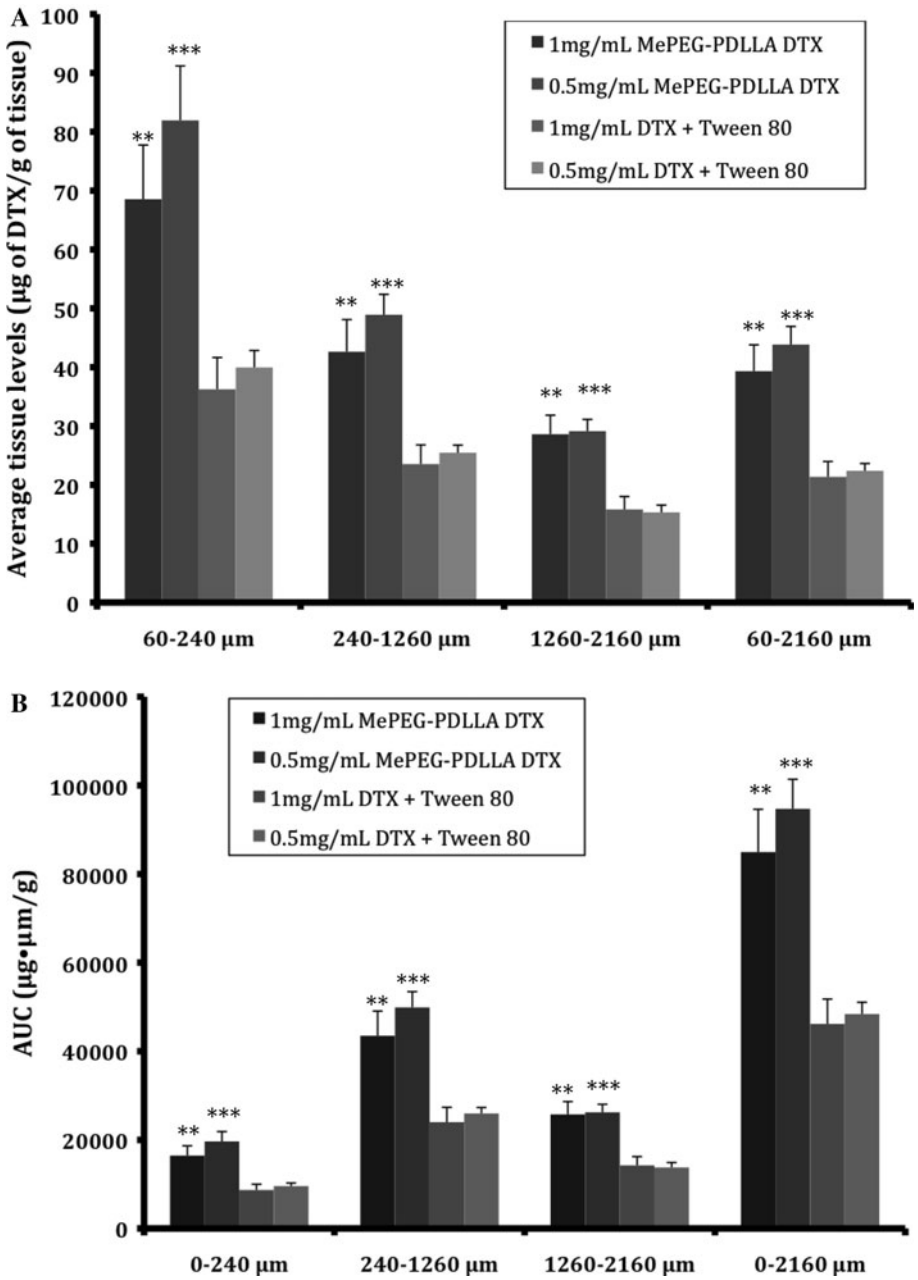

Fig. 5 **a** Average tissue levels of DTX in various layers of the bladder wall (60–240 µm: urothelium, 240–1,260 µm: the lamina propria, 1,260–2,160 µm: the superficial muscle layer and 60–2,160 µm: the whole tissue) following incubation with DTX at either 0.5 or 1 mg/ml from either MePEG-PDLLA or Tween 80 formulations for 2 h. The average tissue levels were determined as the total amount of drug found in the tissue layer divided by the total tissue weight for that layer. **b** AUCs of DTX in various tissue layers of the bladder wall, as described above. The AUCs were calculated using the linear trapezoid rule. The AUC for the whole tissue is designated 0–2,160 µm. Data are means ± SEM ($n = 18$). $^{**}P < 0.01$, 1 mg/ml MePEG-PDLLA DTX versus 1 mg/ml DTX + Tween 80 and $^{***}P < 0.001$, 0.5 mg/ml MePEG-PDLLA DTX versus 0.5 mg/ml DTX + Tween 80

The time course of PTX and DTX uptake into bladder tissues using MePEG-PDLLA solutions at 1 mg/ml are shown in Fig. 7a, b respectively. All uptake profiles were similar, tissue levels decreased with increasing tissue depth and DTX uptake was greater than PTX. For both PTX and DTX, high levels of drug were found in the urothelium layers at 30 min with small increases after that time. The average concentrations of PTX or DTX that penetrated the bladder wall are shown in the insets of Fig. 7a, b. PTX and DTX demonstrated increased uptake at longer incubation times. Tissue concentrations of PTX differed significantly between 180- and 30-min incubation times. Tissue concentrations of DTX were significantly higher following a 120-min incubation than a 30- and 60-min incubation.

The influence of four different nanoparticulate/micellar formulations of PTX on the overall permeation of PTX into the bladder wall is shown in Fig. 8. In order to encapsulate PTX in the MePEG-PCL copolymers, it was necessary to use a high polymer to drug ratio so the uptake levels for the MePEG-PDLLA cannot be compared to those in Fig. 3. The average tissue levels of drug in bladders incubated with PTX at a concentration of 0.5 mg/ml was significantly higher for MePEG-PDLLA micelle formulations compared to MePEG-PCL_{19} micelles and MePEG-PCL_{104} nanosphere

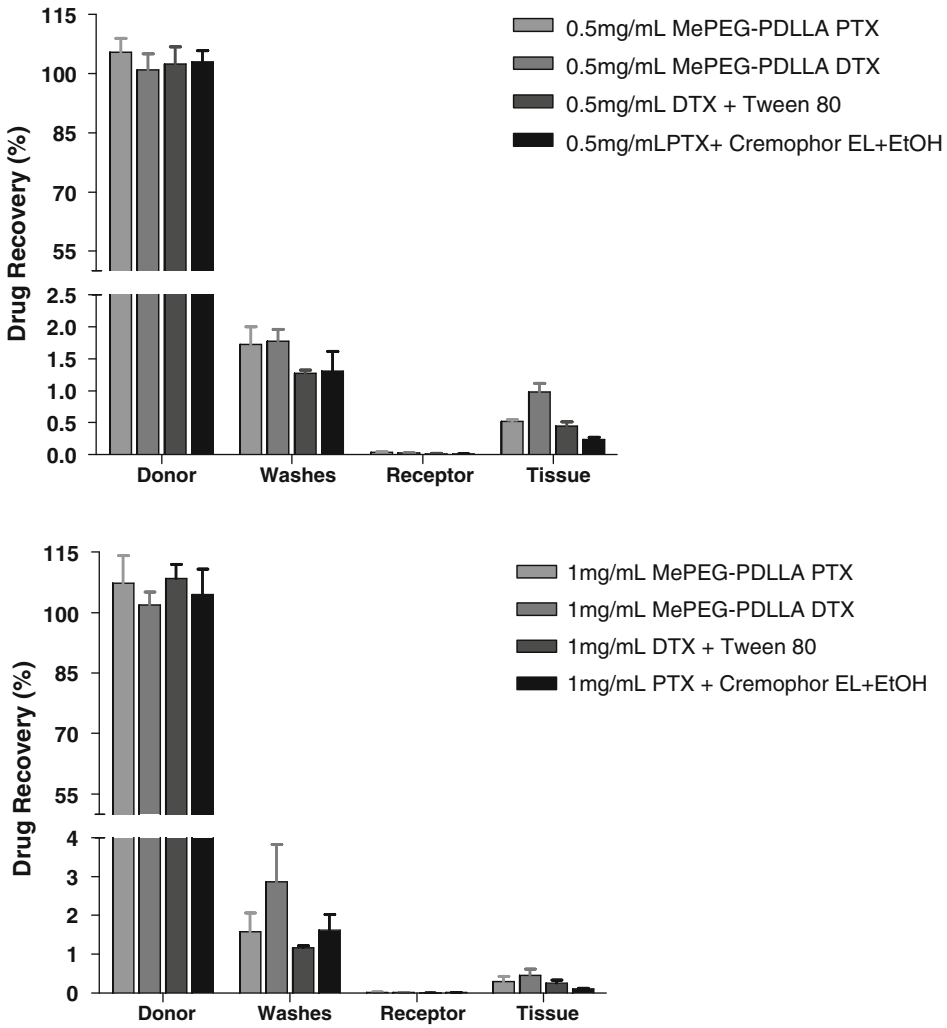

Fig. 6 Mass balance analysis of drug recovered from donor, washes, receptor and tissue fractions following a 2-h incubation with PTX or DTX at **a** 0.5 mg/ml and **b** 1 mg/ml PTX and DTX following a 2-h incubation. All portions of the diffusion cell including washes and tissue were analyzed for drug content. Data are means ± SEM ($n = 18$)

formulations. No differences in average tissue levels were observed between tissues incubated with MePEG-PDLLA micelles and Cremophor EL and ethanol, Cremophor EL and ethanol and MePEG-PCL_{19} formulations or between MePEG-PCL_{19} micelles and MePEG-PCL_{104} nanosphere formulations.

Mannitol paracellular permeability assay

The various formulations of PTX and DTX used in these studies contain amphipathic excipients that might permeabilize tissues. Therefore, the uptake of ^{14}C mannitol (poorly taken up by cells) was used to investigate paracellular transport in the presence of the excipients following a 2-h incubation. In the presence of 0.2% Triton X-100 (sublytic, tissue permeabilizing concentration [11]), mannitol was taken up into tissue at levels almost 4 times higher than those observed for mannitol in tyrode buffer alone (Fig. 9). Mannitol dissolved in solutions of either Cremophor EL or Tween 80 penetrated tissues at levels slightly higher than those observed for mannitol in buffer. However, in the presence of MePEG-PDLLA, the uptake of mannitol was slightly lower than that observed from mannitol dissolved in buffer alone.

Discussion

By nature, the bladder is physiologically adapted to retain large volumes of urine without transfer of the waste solutes through the bladder wall back into the blood stream. The surface urothelium together with the bound mucous layer maintains tight junction function and acts as a natural barrier to the transfer of molecules through the bladder wall. However, despite this barrier effect, superficial bladder cancer is amenable to localized chemotherapy because the tumors are located within the surface urothelial layer, and concentrated drug solutions may be readily instilled into the bladder and held for up to 2–2.5 h. Unfortunately, many hydrophilic anticancer drugs such as doxorubicin and mitomycin C do not penetrate the urothelial tissue well [14, 30] following intravesical

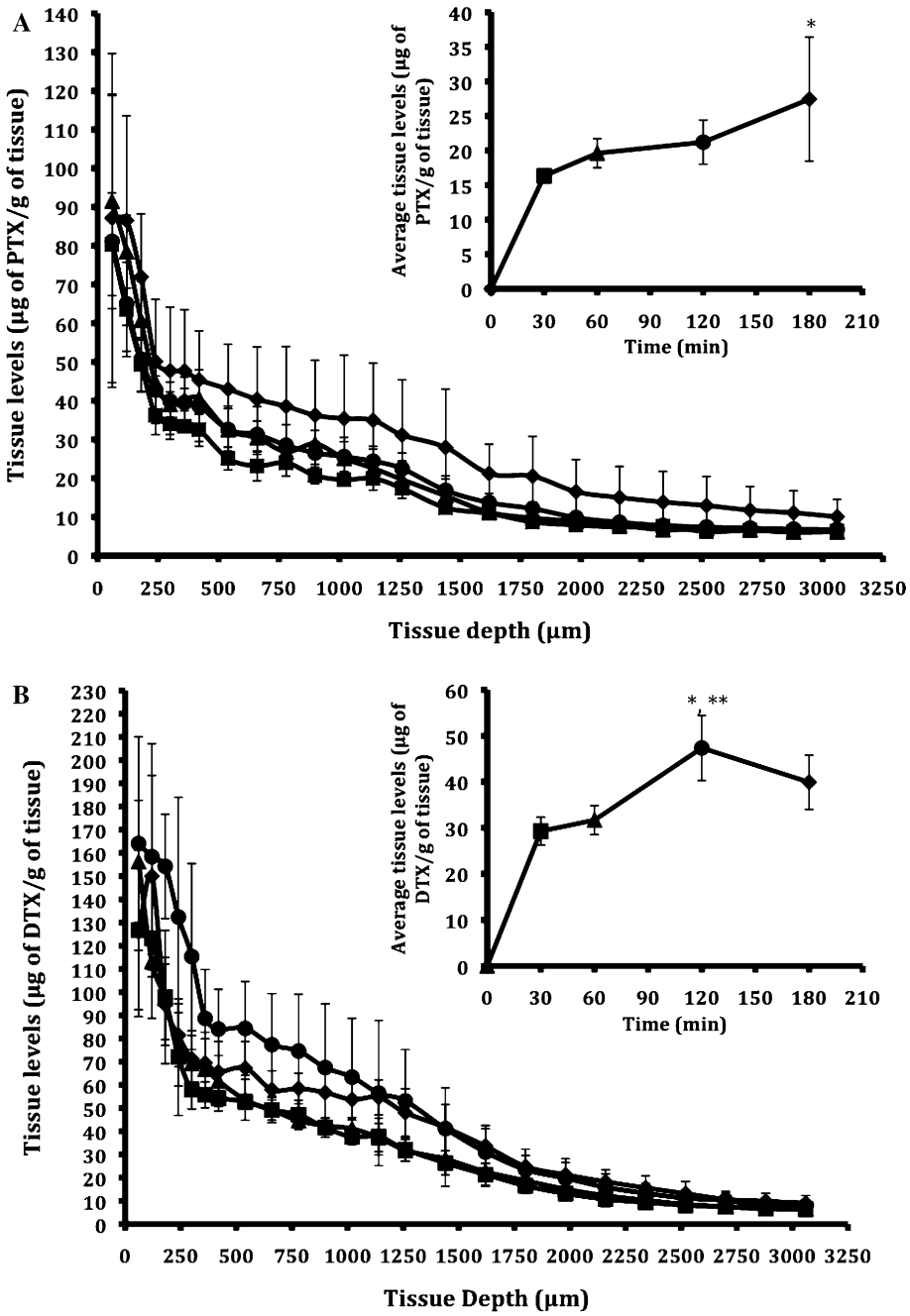

Fig. 7 Tissue level-depth profile of **a** PTX and **b** DTX in bladder tissue from MePEG-PDLLA micelle formulations (1 mg/ml) following incubation for 30 min (*filled square*), 60 min (*filled triangle*), 120 min (*filled circle*) and 180 min (*filled diamond*) at 37°C. *Inset* The time course of drug uptake into bladder tissue for **a** PTX and **b** DTX at 1 mg/ml from MePEG-PDLLA micelles. Points show average tissue levels of drug recovered from all bladder tissue. Data are expressed as µg of drug per g of tissue and the means ± SD ($n = 4$). *$P < 0.05$, PTX 180 min versus 30 min and DTX 120 min versus 60 min. **$P < 0.01$, DTX 120 min versus 30 min

administration and hydrophobic anticancer drugs (such as the taxanes) possess the disadvantage that they cannot be dissolved in water without the use of solubilizing agents. Furthermore, continuous urine dilution reduces drug concentrations significantly prior to first void [1, 34] and voiding results in complete washout of drugs from the bladder [35].

In this study, we have solubilized PTX and DTX in biodegradable diblock copolymer (MePEG-PDLLA) micelles for drug delivery to the bladder wall. Taxanes may be readily solubilized in these systems [7, 36] and the copolymers offer highly biocompatible vehicles due to the presence of polyethylene glycol on the micelle outer surface. In vitro, both drugs exhibited controlled and complete release from these micelles over 7 days (Data not shown).

In these drug uptake studies, we used the ex vivo pig bladder tissue supported on a diffusion cell apparatus to incubate mucosal tissues with drug formulations. Fresh bladder sections were stored in tyrode buffer on ice and drug-incubation commenced within 2 h of animal slaughter and were complete after a 3-h incubation. In viability studies, the pig bladder tissue was determined to be viable for up to 8 h in the Franz diffusion cell at 37°C as measured by the release of lactate dehydrogenase (LDH) (data

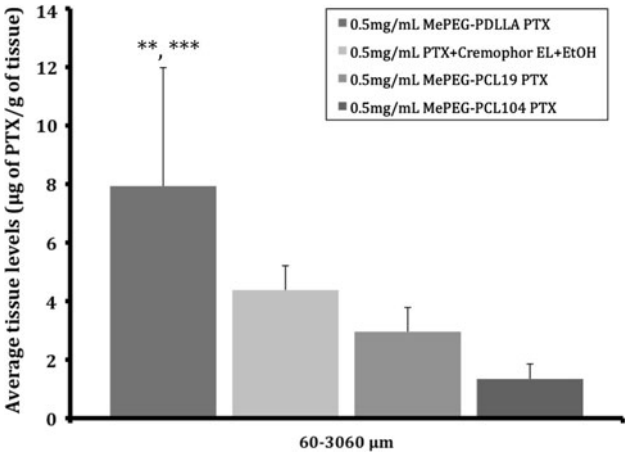

Fig. 8 Average tissue levels of PTX in whole bladder tissue (60–3,060 µm) following a 2-h incubation at 0.5 mg/ml. The average tissue levels were determined as the total amount of PTX found in the tissue divided by the total tissue weight. Data are means ± SD ($n = 6$). $^{**}P < 0.01$, MePEG-PDLLA versus MePEG-PCL_{19} and $^{***}P < 0.001$, MePEG-PDLLA versus MePEG-PCL_{104}

not shown). Furthermore, scanning electron micrographs of the surface of bladder tissues incubated for 3 h in tyrode buffer at 37°C showed normal surface urothelial tissue morphology as evidenced by hexagonal-shaped umbrella cells with intact tight junctions (data not shown). This type of ex vivo bladder tissue incubation is frequently used to characterize drug uptake properties into bladder tissue and allow for comparisons between different drug formulations in the absence of variable urine dilution effects.

The MePEG-PDLLA PTX and DTX formulations showed penetration of the drugs into the bladder wall (Figs. 2 and 4). There was no apparent drug concentration dependence in penetration levels as both 0.5 and 1 mg/ml concentrations gave similar drug depth profiles (Figs. 2 and 4) for both PTX and DTX. These tissue level-depth profiles demonstrated taxane levels at about 60 and 100 µg/g tissue levels for PTX and DTX, respectively, at the urothelium surface decreasing to approximately 30 and 50 µg/g levels at 1 mm depths.

To our knowledge, there are no previous reports of tissue level-depth profiles for DTX. We found much higher PTX tissue levels for either MePEG-PDLLA or Cremophor EL/ethanol formulations compared to PTX tissue levels from water, Cremophor EL or DMSO vehicles used by other groups [8, 16, 30]. However, there were major differences between these studies. The PTX concentrations placed in the donor chambers in contact with pig bladder tissue were about 20-fold higher than PTX concentrations instilled into dog bladders and there was no urine dilution effect in our studies.

The bladder tissue level-depth profiles for PTX and DTX showed an approximately linear decrease in drug concentration over the initial 200–240 µm depth of tissue, corresponding roughly to the urothelial thickness. This was

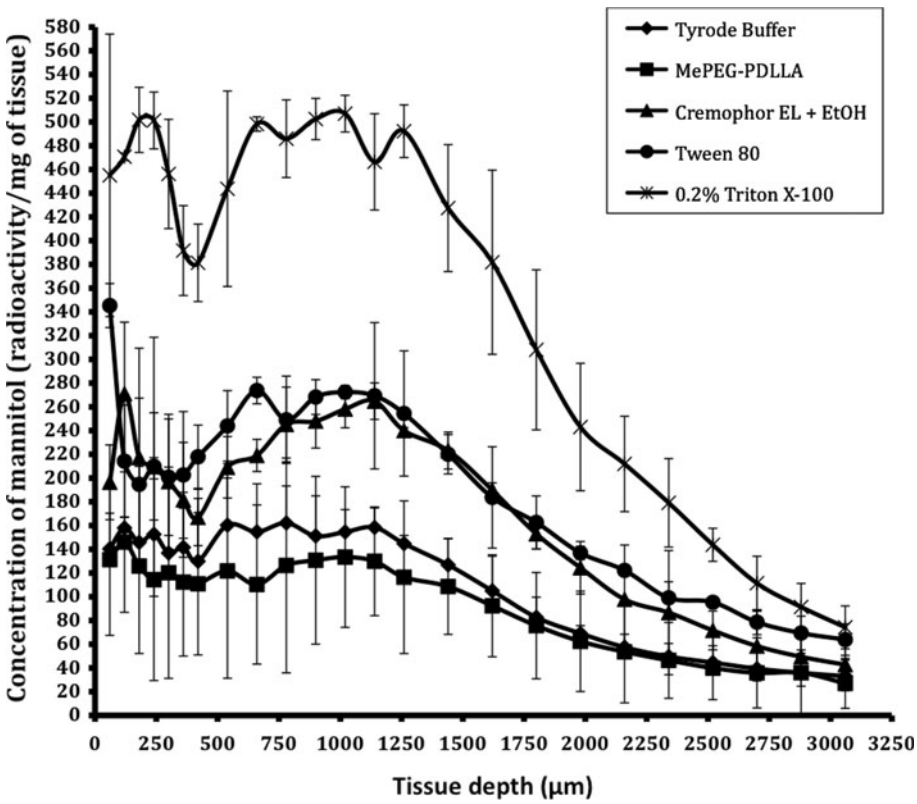

Fig. 9 Tissue level-depth profile of ^{14}C mannitol in bladder tissue. Mannitol delivered to bladder in tyrode buffer containing various excipients. Data are mean ± SD ($n = 3$)

followed by an exponential decline in drug concentrations over the remaining tissue thickness, corresponding to the lamina propria and muscle layers of the bladder that contain blood vessels and lymphatics [28]. These profiles possessed similar characteristics to those obtained for moxifloxacin in isolated porcine bladder [15] and PTX given intravesically into the bladders of dogs and PTX measured as a function of bladder tissue depth at the end of a 2-h PTX instillation [8, 16, 30]. Au, Wientjes and coworkers have examined the bladder tissue pharmacokinetics of PTX in dogs and have described drug transport across the urothelium layer as being diffusion controlled with a linear decline in concentration as a function of urothelial tissue depth, with a first-order decrease of concentration through the lower layers of submucosa and superficial muscle tissue [28]. In these studies, there is no functioning blood supply to the tissue so there is no drug clearance mechanism that might drive a first-order drug distribution profile in this ex vivo model. Therefore, it seems likely that drug distribution is diffusion driven and that the nonlinear nature of the profile probably arises from differences in drug diffusion coefficients in the different layers of the tissues.

Tissue level-depth profiles shown in Figs. 2 and 4 were also analyzed by calculating both the average tissue levels and the AUCs under the tissue concentration-depth curves (Figs. 3 and 5). Determination of the AUC provides a measure of exposure of the tissue to the drug. There were no differences in AUC observed between the two PTX (Fig. 3) or DTX (Fig. 5) initial donor chamber concentrations (0.5 and 1 mg/ml), but MePEG-PDLLA micellar formulations of both drugs produced significantly greater AUCs in urothelial tissue layers than commercial formulations in Cremophor EL (PTX) or Tween 80 (DTX). Assuming diffusion-controlled drug transport across the urothelium, the lack of initial drug concentration dependence for any of the formulations suggests that the concentration gradients across the tissue in the diffusion cells were similar for both 0.5 and 1 mg/ml donor chamber experiments. We suggest that since the free drug concentrations released from all formulations within the short incubation period are likely to be similar then this does not satisfactorily explain the difference in tissue drug levels for different formulations. It is likely that other drug transport processes may have become rate limiting: for example, partitioning into the urothelium due to close association of micellar formulations with the bladder tissue. Using equilibrium dialysis methods, Chen et al. [8] and Knemeyer et al. [16] reported that Cremophor EL micelles bind PTX very strongly, such that free fractions of PTX decreased to 23 and 11%, respectively, in 0.25 and 1% Cremophor. Given that we employed Cremophor concentration as high as 8% this may explain the significantly lower tissue levels for PTX in our work.

The recovered range of concentrations in bladder tissue from formulations of 0.5 and 1 mg/ml PTX and DTX represented 0.1–1% of the total delivered dose. In the receptor chamber less than 0.04% of drug recovered suggested negligible drug penetration through the full thickness of the tissue. Other groups have shown drug uptake into bladder tissue following intravesical administration within the same range (less than 1% of the dose). Song et al. [30] achieved a 1.2% uptake of PTX in the bladder tissue of dogs using a nonmicellar solution at 25 μg/ml and in a later study by the same group a 0.4% uptake with a Cremophor EL formulation at the same concentration [16]. Using PTX conjugated to hyaluronic acid to increase its water solubility, Tringali et al. [33] showed that using a PTX concentration of either 1 or 3 mg/ml in rabbit bladders, only 0.68% of the dose was recovered in bladder tissue. There was a rapid rate of drug penetration observed for both drugs whereby near maximal penetration was achieved in the urothelium layer after just 1 h (Fig. 7) for PTX, with maximal tissue uptake for DTX in 2 h.

Drug formulation dependence in penetration levels for 0.5 mg/ml donor cell concentration of PTX was highest for MePEG-PDLLA micelles compared to Cremophor EL and ethanol, MePEG-PCL_{19} micelles and MePEG-PCL_{109} nanospheres, all of which showed the lowest concentrations below 6 μg/g. A number of factors may be responsible for the reduced PTX uptake into the bladder tissue for MePEG-PCL nanoparticles and MePEG-PDLLA micelles, including differences in the core-forming polymer, the length of the hydrophobic and hydrophilic block, the compatibility between the drug and the core-forming block and the size of the nanoparticulate system. The length of the hydrophobic block, which as previously investigated by Letchford et al. [21], has been shown to retard the release of PTX due to the increased interaction between the hydrophobic core of the nanosphere and the drug. PCL as a semicrystalline polymer compared to amorphous PDLLA can affect the permeability and degradation of the polymer and thus influence the release and penetration of the drug. The length of the hydrophilic block may prevent a close and immediate contact between the mucin of the bladder wall and the polymer and provide difficulty for the drug to penetrate the tissue. Furthermore, the increased size of the MePEG-PCL nanoparticles (data not shown) may reduce the penetration ability of the system keeping the particles at a further distance from the urothelium.

Since amphipathic molecules like diblock copolymers may have surfactant-like effects on tissues, it was considered possible that this drug carrier might permeabilize the bladder tissue and allow for the enhanced penetration of drugs into the tissue through a paracellular process. The sugar mannitol is not readily taken up into cells and may be used to study paracellular transport effects [17, 29]

(i.e. tissue permeabilization). Using radioactive mannitol solutions, we demonstrated that the surfactant Triton X-100 (used at a nonlytic concentration of 0.2% [11]) allowed for fourfold increases of mannitol penetration into bladder tissues when compared to control (Fig. 9). However, mannitol penetration in the presence of the diblock copolymers was slightly lower than those observed in control samples establishing that taxane penetration from micellar formulations probably did not arise from any permeabilization effect of the polymeric excipient. Interestingly, both Cremophor EL and Tween 80 carriers caused increases in mannitol penetration into bladder tissue suggesting a small tissue permeabilization effect of these agents. Therefore, it is likely that the low levels of taxane penetration observed with these carriers may arise from sequestration of the drug in the micelles of these agents and reduced free drug to penetrate tissues.

In this study, the use of micellar formulations of taxanes allowed for PTX and DTX solubilization at high concentrations in stable, biocompatible formulations of the drugs. The excellent biocompatibility of this formulation may arise, in part, from the outer layer of PEG which is known to shield unwanted surface effects with cells [12]. The MePEG-PDLLA micellar formulation of PTX had no adverse toxicity following intravenous [7] or intravesical administration in mice at higher concentrations (5 mg/ml) [13] than concentrations used in this study. While the outer MePEG surface of micelles may allow for improved biocompatibility [19], it is also possible that the MePEG chains associated or interpenetrated the mucin chains of the urothelium to effectively "entrap" the formulation at that surface. This mucoadhesive effect of MePEG has been previously reported by others [10].

Overall, it is likely that the rapid and high levels of PTX and DTX penetration observed in bladder tissue using the MePEG-PDLLA micellar formulation of these drugs arose from three factors: the ability to use high concentrations of drugs with these polymeric micelles, greater free drug released from the MePEG-PDLLA micelles and the ability to increase the contact of the formulation at the bladder wall surface allowing for improved partitioning of the drug into the tissues. We have previously shown efficacy following intravesical administration of MePEG-PDLLA PTX at 5 mg/ml in mice bearing xenograft human bladder tumors without carrier-induced local toxicity [13]. Taken together, our studies support the use of the micellar formulation for taxane delivery to superficial bladder tumors.

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Conflict of interest None.

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