**In vivo evaluation of mucoadhesive nanoparticulate docetaxel for intravesical treatment of non-muscle-invasive bladder cancer**

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**Running title:** Mucoadhesive nanoparticulate docetaxel for intravesical therapy

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Statement of Translational Relevance

Development of novel therapeutics for patients with high-risk non-muscle-invasive bladder cancer is an important topic considering the rather limited options currently available. Microtubules are one of the most successful targets in cancer therapy to date and the recent Phase I trial of intravesical docetaxel for the treatment of non-muscle-invasive bladder cancer, refractory to Bacillus Calmette-Guérin (BCG) therapy, has shown this to be a promising intravesical agent. Treatment failure is thought to be in part due to the short dwell-time of intravesical drugs and low drug uptake in the bladder wall. Conventional formulations are typically maintained in the bladder for 2 h and urothelial drug exposure rarely lasts beyond the first voiding of urine after instillation. In this study, we developed a novel mucoadhesive controlled-release formulation of docetaxel. Mucoadhesive polymers have several advantages when compared to conventional drug carriers, including localization at the specific target site, prolonged residence time and increase drug uptake. Overall, our data show promising antitumor efficacy and safety in a recently validated orthotopic model of bladder cancer and provide a clear rationale for the intravesical use of this nanoparticulate formulation over the commercial formulation of docetaxel in the treatment of non-muscle-invasive bladder cancer.
Abstract

Purpose: The present work describes the development, in vitro and in vivo evaluation of a mucoadhesive nanoparticulate docetaxel (DTX) formulation for intravesical bladder cancer therapy.

Experimental Design: Mucoadhesive formulations based on hyperbranched polyglycerols (HPGs), hydrophobically derivatized with C8/C10 alkyl chains in the core and modified with MePEG and amine groups in the shell (HPG-C8/10-MePEG-NH2) were synthesized and DTX was loaded into these by a solvent evaporation method. Both low-grade (RT4, MGHU3) and high-grade (UMUC3) human urothelial carcinoma cell lines were treated with various concentrations of DTX formulations in vitro. KU7 cells that stably express firefly luciferase (KU7-luc) were inoculated in female nude mice by intravesical instillation and quantified using bioluminescence imaging. Mice with established KU7-luc tumors were given a single intravesical instillation with PBS, Taxotere® (DTX from Sanofi-Aventis), DTX loaded HPG-C8/10-MePEG and/or HPG-C8/10-MePEG-NH2. Drug uptake was conducted using LC/MS/MS and tumor microenvironment and uptake of rhodamine labeled HPGs was assessed.

Results: In vitro, all DTX formulations potently inhibited bladder cancer proliferation. However, in vivo, DTX loaded HPG-C8/10-MePEG-NH2 (mucoadhesive DTX) was the most effective formulation to inhibit tumor growth in an orthotopic model of bladder cancer. Furthermore, mucoadhesive DTX significantly increased drug uptake in mouse bladder tissues. In addition, rhodamine labeled HPG-C8/10-MePEG-NH2 demonstrated enhanced uptake of these nanoparticles in bladder tumor tissues.
Conclusions: Our data show promising in vivo antitumor efficacy and provide preclinical proof-of-principle for the intravesical application of mucoadhesive nanoparticulate DTX formulation in the treatment of bladder cancer.

Introduction

Bladder cancer is a significant public health problem responsible for more than 130,000 deaths annually worldwide (1). In the US, bladder cancer is the fourth most common cancer diagnosed in men, with an estimated 70,530 new cases and 14,680 deaths from the disease in 2010 (2). Approximately 70-80% of patients with bladder cancer initially present with superficial disease that does not invade the muscularis propria (3). Transurethral resection (TUR) is the primary treatment method for non-muscle-invasive or “superficial” carcinoma, which consists of the surgical removal of tumor nodules from the bladder wall (4, 5). However, there is a high rate of tumor recurrence and high chance of disease progression after TUR alone (6). This is probably due to incomplete removal of the tumors and/or implantation of cancer cells in normal tissues of the bladder. Intravesical therapy, which involves instillation of one or more chemo- and/or immunotherapeutic agents into the bladder following resection, has become the standard of care for the treatment of non-muscle-invasive bladder cancers (7). Systemic administration of anticancer drugs offers little therapeutic benefit in these patients, as the urothelial layer of the bladder is not vascularized (8). By contrast, intravesical chemotherapy has the potential to selectively deliver high drug concentrations to tumor-bearing bladder tissues while minimizing the systemic exposure.

Immunotherapy with bacillus Calmette-Guérin (BCG) is the most effective form of intravesical therapy for prophylaxis of recurrence and progression in non-muscle-invasive
bladder cancer patients (9). However, it is associated with frequent local and/or systemic adverse effects including BCG sepsis (10, 11). The incidence of local and systemic adverse effects is significantly lower with chemotherapeutic agents. However, the response to intravesical chemotherapy is incomplete and tumor recurrence is still high (up to 80%) in patients with non-muscle-invasive bladder cancer (12).

Treatment failure is thought to be in part due to the short dwell-time of intravesical drugs and the low drug permeability of urothelium. Conventional formulations are typically maintained in the bladder for 2 h and urothelial drug exposure rarely lasts beyond the first voiding of urine after instillation (13). Our approach to increase the dwell-time and uptake of intravesical drugs is to target the mucin glycoproteins on the surface of urothelium by exploiting the concept of mucoadhesion, which is an adhesive phenomenon occurring between certain type of polymers known as “mucoadhesive” polymers and the mucin-gel layer covering mucosal membranes.

The systems we are currently developing are based on hydrophobically derivatized hyperbranched polyglycerols (HPGs) which are dendritic-like macromolecules, with a hydrophobic core and a hydrophilic shell connected by covalent bonds in a single molecule (14). The hydrophobic core is based on a mixture of alkyl (C₈/C₁₀) chains which is important for the binding of hydrophobic molecules, including drugs, while the hydrophilic shell based on methoxy-poly(ethylene glycol) (MePEG, MW 350) keeps the system soluble in water (15). In addition, HPGs possess many hydroxyl groups on the outer surface that can be further derivatized to increase their mucoadhesiveness.

Mucoadhesive systems possess numerous advantages when compared to conventional dosage forms. Localization at a specific region of the body helps improve drug
uptake at the site of interest (16). Mucoadhesion also increases the intimacy of contact between a drug-containing polymer and mucous surface, which can enhance the permeability of the drug (17). Mucoadhesive polymers can also prolong residence time of the incorporated drugs, leading to less frequent dosing and improved patient compliance (18).

Recently, we have reported the synthesis and characterization of HPG-C$_8$/10-MePEG nanoparticles with surface derivatized amine groups (19). The surface amine groups on HPG-C$_8$/10-MePEG resulted in positively charged nanoparticles (HPG-C$_8$/10-MePEG-NH$_2$) with improved mucoadhesive properties. We conducted a dose-range finding and tolerability study of intravesical docetaxel (DTX) in HPG formulations in an orthotopic model of bladder cancer and showed that intravesical DTX in either commercial or in HPG formulations (up to 1.0 mg/ml) was well tolerated with no apparent signs of toxicity in mice (19).

The objective of the present work was to evaluate the effectiveness of intravesical mucoadhesive DTX loaded in HPG nanoparticles in an orthotopic model of bladder cancer and to investigate bladder tissue and tumor uptake of these nanoparticulate formulations.

**Materials and Methods**

**Chemicals**

All chemicals were purchased from Sigma-Aldrich (Oakville, ON) and all solvents were HPLC grade from Fisher Scientific (Ottawa, ON). -epoxy, ω-methoxy polyethylene glycol 350 (MePEG epoxide), was synthesized from a reaction of MePEG 350, sodium hydroxide, and epichlorohydrin. Potassium methylate, trimethyloyl propane (TMP) and octyl/decyl
glycidyl ether were obtained from Sigma-Aldrich and used without further purification. N-(2,3-epoxypropyl)-phthalimide) (EPP) was obtained from Sigma-Aldrich. Tetramethylrhodamine-carbonyl-azide (TMRCA) was purchased from Invitrogen Canada Inc. (Burlington, ON). DTX powder was obtained from Natural Pharmaceuticals Inc. (Beverly, MA) and Taxotere® (DTX in Tween 80) was purchased from Sanofi-Aventis Canada Inc. (Laval, Quebec).

**Cell lines**

The human bladder cancer cell lines RT4 and UMUC3 were purchased from the American Type Culture Collection. Cells were maintained in McCoy’s medium (Invitrogen, Burlington, ON) containing 10% heat-inactivated fetal bovine serum and kept at 37°C in a humidified 5% CO2 atmosphere. MGHU3 cells were obtained as a generous gift from Dr. Y. Fradet (L’Hotel-Dieu de Quebec, Quebec, Canada) and maintained in MEM supplemented with 10% fetal bovine serum and 2mM L-glutamine (Invitrogen). KU7 was kindly provided by Dr. C. Dinney (MD Anderson Cancer Center, Houston, TX, USA) and maintained in DMEM containing 5% fetal bovine serum. Although controversial, KU7 cells were initially thought to be of bladder origin, but recent studies in our lab suggest they may be of HeLa origin (data not shown). However, this cell line provides for an excellent *in vivo* representation of an aggressive epithelial carcinoma that can be modelled intravesically. For visualization purposes, KU7 cells were infected with a lentivirus containing the firefly luciferase gene by Dr. Graig Logsdon (M.D. Anderson Cancer Center, Houston, TX, USA), and these subclones were named KU7-luc as described previously (20).
**Synthesis and characterization of HPG-C_{8/10}-MePEG and HPG-C_{8/10}-MePEG-NH_{2}**

The synthesis and characterization of HPG-C_{8/10}-MePEG has been previously reported by our group (15). Recently, we have described the surface derivatization of HPG-C_{8/10}-MePEG with amine groups using N-(2,3-epoxypropyl)-phthalimide (EPP) followed by cleavage of the phthalimide functional groups by hydrazinolysis (19). The details on the synthesis and characterization of HPG-C_{8/10}-MePEG and HPG-C_{8/10}-MePEG-NH_{2} have been provided in the Data Supplements section.

**Rhodamine labeling of HPGs**

HPG-C_{8/10}-MePEG and HPG-C_{8/10}-MePEG-NH_{2} polymers were covalently labeled with tetramethyl-rhodamine-carbonyl-azide (TMRCA) as previously reported (21, 22).

**DTX loading into HPGs**

DTX (0.2 mg) and HPGs (100 mg) (HPG-C_{8/10}-MePEG and HPG-C_{8/10}-MePEG-NH_{2}) were dissolved in 1 ml acetonitrile solution in 4 ml vials and dried in an oven at 60°C for 1 h and flashed with nitrogen stream to eliminate traces of the organic solvent. The resulting HPG/DTX matrix was hydrated with 1 ml of 10 mM phosphate buffered saline (PBS, pH 6) and vortexed for 2 min. The amounts of DTX incorporated in HPGs were determined by reversed phase HPLC. Drug content analysis was performed using a symmetry C18 column (Waters Nova-Pak, Milford, MA) with a mobile phase containing a mixture of acetonitrile, water, and methanol (58:37:5, v/v/v) at a flow rate of 1 ml/min. Sample injection volumes were 20 µl and detection was performed using UV detection at a wavelength of 232 nm.
Total run time was set to 5 min and DTX retention time was 2.9 min. Our HPLC method for quantification of DTX has been previously validated. This method was evaluated over a linear range of 0.5 -100 µg/ml. Over this range, the accuracy (percent deviation from theoretical value) was found to be within 3% and the precision (%RSD) was less than 2% using clean DTX standards.

**In vitro cytotoxicity studies**

Cells were plated at 5,000 cells/well in 96-well plates in a 100 µl volume of McCoy’s Medium supplemented with 10% FBS and allowed to equilibrate for 24 h before freshly prepared solutions of Taxotere®, or DTX in HPG-C₈/₁₀-MePEG and/or HPG-C₈/₁₀-MePEG-NH₂ (dissolved in PBS, pH 7.4) were added. Cells were exposed to the drugs for 2 h and cell viability was determined after 72 h using the CellTiter96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) as described previously (23). Each experiment was repeated three times and MTS values fell within a linear absorbance range for all cell lines.

**In vivo studies**

**Efficacy of intravesical DTX in orthotopic murine model of bladder cancer**

The orthotopic mouse model used in the present study has been described in earlier publications (20, 23-26). Animal studies were carried out in accordance with the Canadian Council on Animal Care. Briefly, eleven-week-old female nude mice (Harlan, Indianapolis, IN) were anaesthetized with isoflurane. A superficial 6-0 polypropylene purse-string suture
was placed around the urethral meatus before a lubricated 24 G Jelco angiocatheter (Medex Medical Ltd., Lancashire, UK) was passed through the urethra into the bladder. After a single irrigation of the bladder with PBS, two million KU7-luc cells were instilled as a single cell suspension in 50 µl and the purse-string suture was tied down for 2.5 h. To quantify in vivo tumor burden, animals were imaged in supine position 15 min after intraperitoneal injection of 150 mg/kg luciferin on days 4, 11, 18, and 25 with an IVIS200 Imaging System (Xenogen/Caliper Life Sciences, Hopkinton, MA). Data were acquired and analyzed using Living Image software (Xenogen).

On day five post-tumor inoculation, mice were randomized to receive a single 50 µl intravesical treatment with PBS (control); Taxotere® (0.2 mg/ml); DTX (0.2 mg/ml) loaded HPG-C₈/₁₀-MePEG; and DTX (0.2 mg/ml) loaded HPG-C₈/₁₀-MePEG-NH₂. Levels of bioluminescence were equivalent among the groups; however, as tumors varied between individual mice, for statistical analyses, tumor bioluminescence after treatment was normalized against the initial flux on day four in each mouse.

Necropsy was performed on day 25 after tumor inoculation. The whole bladders were removed, fixed in 10% buffered formalin and embedded in paraffin. 5 µm sections were prepared and stained with H&E using standard techniques. All slides were reviewed by a pathologist (Dr. L. Fazli, The Vancouver Prostate Centre, Vancouver, BC, Canada) and scanned on a BLISS microscope imaging workstation (Bacus Laboratories Inc., Lombard, IL).

Drug uptake studies
Drug uptake studies were conducted in fifteen-week-old female nude mice with established KU7-luc tumors (33 days post-tumor inoculation). Tail blood samples were taken at 0, 30, and 60 min post intravesical instillation. During this period mice were still anaesthetized with isoflurane. After 2 h, all mice were euthanized using CO₂ asphyxiation and additional blood was removed by cardiac puncture. Blood samples were centrifuged in micro-haematocrit tubes (Fisher Scientific, Pittsburg, PA) or serum-separator tubes (Becton Dicknson) and the serum was snap-frozen in liquid nitrogen. Urine and bladder of each mouse were also harvested and before freezing, the bladders were cut, opened to expose the lumen and were vigorously washed in five sequential 10 ml PBS washes. All samples were stored at -80°C.

The UPLC-MS/MS system used for analysis consisted of an integrated Waters Acquity UPLC separation system (Acquity BEH C18, 1.7 µm, 2.1 X 50 mm column) coupled to a mass spectometry analysis using Waters TQD mass spectrometer. The system was operated at an electrospray ion source block temperature of 150°C, a desolvation temperature of 350°C, a cone voltage of 14 V, a capillary voltage of 0.70 kV, extractor voltage of 3 kV, RF voltage of 0.1 kV, a cone gas flow at 25 l/h, a desolvation gas flow at 600 l/h and a collision gas flow at 0.2 ml/min. The molecules undergo electron spray ionization in the positive ion mode. DTX was quantified in multiple reaction monitoring with the transition of m/z 808.5 → 527.2, as previously established (22).

DTX was extracted from the mouse serum by solvent/solvent extraction method. Briefly, 50 µl aliquots of the mouse plasma and standards were mixed with 150 µl of 0.1% formic acid in acetonitrile in a 96-well plate and vortexed for 1 min at room temperature. The samples were centrifuged at 5,500 rpm (Allegra™ 25 R centrifuge, Beckman-Coulter) for 10 min at...
4°C. Then 100 µl of the supernatant was mixed with 50 µl of distilled water, mixed and vortexed for 30 s.

Bladder tissues were weighed and homogenized in 0.1% formic acid/methanol using zirconia beads (Biospec Products) and mini-bead beater equipped with microvial holder (Biospec Products) for 60 s. The samples were centrifuged at 14,000 rpm (Allegra™ 25 R centrifuge, Beckman-Coulter) for 2 min at 4°C. 150 µl of 0.1% trifluoroacetic acid in methanol was added to the samples, mixed and vortexed at 14,000 rpm (Allegra™ 25 R centrifuge, Beckman-Coulter) for 15 min at 4°C. All sample analysis was performed using UPLC-MS/MS. The limit of quantification for DTX was 10 ng/ml with a recovery of 97% from spiked control samples. Within run precision (%RSD) was less than 15% in all cases.

Assessing tumor microenvironment and uptake of rhodamine labeled HPGs

Fifteen-week-old female nude mice with orthotopic bladder tumors (33 days post-tumor inoculation) were anaesthetized with isoflurane. A superficial 6/0 polypropylene purse-string suture was placed around the urethral meatus and the bladder was emptied by manual compression. A lubricated 24-gauge Jelco angiocatheter was passed through the urethra into the bladder and then 50 µl of either PBS, free rhodamine (TMRCA), HPG-C₈/₁₀-MePEG-TMRCA, and/or HPG-C₈/₁₀-MePEG-NH₂-TMRCA was instilled and the purse-string suture was tied down for a 2-h period, during which the mice were kept anaesthetized. After the 2-h period the purse-string suture was removed, the bladder was emptied by manual compression and washed twice with 150 µl of PBS (pH 6.0). The mice were euthanized and the bladders were excised and frozen on an aluminum block, then embedded in OCT for cryosectioning. 10 µm cryosections were cut at distances of 1, 2, and 3 mm from the bladder edge. Sections
were dried at room temperature and imaged for rhodamine fluorescence using 10 x objective (0.75 µm/pixel resolution). Slides were fixed in 1:1 acetone : methanol solution for 10 min and stained using a custom capillary-action staining apparatus for CD31 (1:50 hamster anti-CD31 with an anti-hamster Alexa 647 secondary) and Hoechst 33342 (nuclear dye). Following fluorescent imaging of CD31 and Hoechst 33342, sections were counterstained lightly with hematoxylin, mounted & imaged in bright field.

Image analyses: images were reduced to 1.5 µm/pixel resolution to improve manageability in Image J software. With user-supplied algorithms, image stacks were then created, aligned and cropped to tumor tissue boundaries with artifacts removed; necrosis was further cropped based on the hematoxylin image. The bladder lumen was artificially traced along the tumor tissue boundary on Hoechst 33342 images. User-supplied analysis macros were run to generate the following types of data: a) threshold: was manually determined to include positive stain but that does not pick up background outside of necrosis areas; the macro determines the number of positive pixels meeting or exceeding this threshold and was reported as an average for the whole tumor section. b) intensity: was reported as the average intensity of staining for a whole tumor section, or the average intensity of pixels sorted based on their distance from a secondary stain (ie: CD31) or artificially traced boundary (bladder lumen). Calculations to determine averages ± standard error were performed and graphic displays created using Microsoft Excel; non-parametric analysis of variance (Kruskal-wallis tests) statistical analyses were performed using Prism v5 for Macs software.

Results
In vitro cytotoxicity

We evaluated the cytotoxic effects of the commercial formulation of Taxotere® and DTX loaded HPG formulations against the KU7-luc cell line, and both low-grade (RT4, MGHU3) and high-grade (UMUC3) human urothelial carcinoma cell lines. Cells were exposed to the drug formulations for 2 h, to simulate the current clinical standard for instillation therapy, and cell viability was determined after 72 h by MTS assay. All formulations resulted in concentration-dependent inhibition of the proliferation of cells. DTX loaded HPG-C$_{8/10}$-MePEG or HPG-C$_{8/10}$-MePEG-NH$_2$ were found to be as cytotoxic as the commercial formulation of Taxotere® (Fig. 1). The IC$_{50}$ of DTX formulations were in the low nanomolar range (4-12 nM) for all cell lines tested. Control HPGs nanoparticles (no drug) showed no cytotoxicity across the tested concentration range (15-1,500 nM, data not shown).

In vivo efficacy of Taxotere® and DTX loaded HPGs

In vivo studies were done in a total of 42 nude mice to evaluate the efficacy of a single intravesical treatment with Taxotere® (0.2 mg/ml) and DTX (0.2 mg/ml) loaded HPG-C$_{8/10}$-MePEG and/or HPG-C$_{8/10}$-MePEG-NH$_2$. The KU7-luc tumor cell line, initially thought to be of human bladder origin (i.e. high-grade urothelial carcinoma), was recently found to be of HeLa origin using array CGH (comparative genomic hybridization) in our lab (data not shown). Nonetheless, KU7-luc is the only cell line to date that reproducibly provide reliable tumor ‘take’ rates as orthotopic xenografts without the use of secondary agents such as trypsin or electrocautery that traumatize the urothelium to promote tumor cell adhesion. KU7-luc xenografts represent an epithelial ‘intravesical carcinoma model’ that clinically
resembles human bladder cancer and the key characteristic of this model is the lack of deeper invasion at early time points, which makes these tumors amenable to intravesical therapy. Thus, this bladder cancer ‘model’, we believe, is still appropriate to test pharmacokinetic and pharmacodynamic properties of intravesical agents. After intravesical inoculation of KU7-luc cancer cells, all mice developed bladder tumors. However, one mouse in DTX loaded HPG-C8/10-MePEG-NH2 group died unexpectedly on day four post-treatment. Overall, intravesical DTX administered as either the commercial Taxotere® or the HPGs formulations were well tolerated by mice and no major toxicities were observed. Compared with control mice, DTX loaded HPGs inhibited tumor growth. However, DTX loaded HPG-C8/10-MePEG-NH2 was the most effective formulation to inhibit tumor growth in KU7-luc orthotopic bladder cancer xenografts and reached statistical significance compared to either the PBS control or Taxotere® groups (Fig. 2, P < 0.01, post-hoc Bonferoni analysis after 2-Way ANOVA). At the end of the study, a single intravesical instillation of DTX loaded HPG-C8/10-MePEG-NH2 nanoparticles inhibited tumor growth by 88% compared to the PBS control groups. DTX loaded HPG-C8/10-MePEG nanoparticles exhibited a 54% tumor inhibition in this treatment arm. On the other hand the commercial formulation of Taxotere® failed to inhibit tumor growth in this orthotopic xenograft model. Representative bioluminescence images of mice over time in each treatment group are shown in Fig. 2. Histological examination of bladder tissues show that KU7-luc tumors exhibited an aggressive growth pattern and frequent multifocality, but after 25 days post-tumor inoculation, they were generally confined to the lamina propria and correlated with high-grade T1 stage disease (Fig. 3). Although DTX (0.2 mg/ml) did not cause any remarkable histological change in KU7-luc xenograft compared with the PBS treatment, DTX (0.2 mg/ml) loaded HPG-C8/10-MePEG and/or HPG-C8/10-
MePEG-NH₂ inhibited tumor growth. Tumors treated by DTX loaded in HPG-C₈/₁₀-MePEG-NH₂ decreased significantly in size, with heterogeneous cellular size, nuclear shape and infiltrating inflammatory cells.

**Drug uptake studies**

To evaluate the bladder tissue and serum uptake following intravesical DTX formulations; mice with orthotopic bladder tumors were instilled with either Taxotere® (0.2 mg/ml, n=3) or DTX (0.2 mg/ml) loaded HPG-C₈/₁₀-MePEG (n=4) and/or HPG-C₈/₁₀-MePEG-NH₂ (n=4). The amount of DTX in urine, bladder tissue, and serum were measured two hours post-instillation. Several serum samples had non-quantifiable or no detectable signals. Mice instilled with Taxotere® had no detectable DTX in serum at all time points. DTX loaded HPG-C₈/₁₀-MePEG-NH₂ exhibited the highest serum levels at the 2 h time point (150.87 ± 34.98 vs 23.97 ± 16.71 ng/ml, P < 0.01, 2-way ANOVA, Bonferroni post-test). However, serum concentrations of DTX were several orders of magnitude lower than the concentrations in urine and bladder tissue (Table 1). DTX loaded HPG-C₈/₁₀-MePEG-NH₂ resulted in significantly higher amounts in bladder tissue accumulation compared to Taxotere® or DTX loaded HPG-C₈/₁₀-MePEG (P < 0.001, 1-way ANOVA, Bonferroni’s multiple comparison test). There was no significant difference (P > 0.05, 1-way ANOVA) in bladder tissue accumulation between Taxotere® and DTX loaded HPG-C₈/₁₀-MePEG treatment groups. The final urine concentrations were about 5-7-fold lower than the initial dosing solution. This was due to the urine dilution during the 2 h period of intravesical instillation. However, there was no significant difference (P > 0.05, 1-way ANOVA) in the final urine concentrations of DTX between different treatment groups.
Tumor microenvironment and uptake of rhodamine labeled HPGs

Bladder tumor microenvironment and distribution of rhodamine labeled HPGs into tumor tissue was assessed. Bladder tumor tissues were highly vascularised with an average distance of 40-60 µm to the nearest blood vessel (Fig. 4A). No significant difference was seen between different groups (P = 0.8). The amount of fluorescence inside whole bladder tumors was measured. Rhodamine labeled HPG-C₈/₁₀-MePEG-NH₂ (HPG-C₈/₁₀-MePEG-NH₂-TMRCA) exhibited the highest tumor uptake compared to the other groups (P = 0.037). There was no significant difference (P > 0.05) in tumor uptake of the bladders instilled with free rhodamine (TMRCA) and rhodamine labeled HPG-C₈/₁₀-MePEG (Fig. 4B). The depth profile of rhodamine uptake into the tumor tissues was assessed as a function of distance from the bladder lumen. HPG-C₈/₁₀-MePEG-NH₂-TMRCA nanoparticles demonstrated enhanced tumor uptake at all distances from lumen, showing a 5-6-fold increase over HPG-C₈/₁₀-MePEG-TMRCA nanoparticles (Fig. 4C). Fig. 5 shows sections of an orthotopic bladder tumor that had been instilled with either PBS; TMRCA; HPG-C₈/₁₀-MePEG-TMRCA; and/or HPG-C₈/₁₀-MePEG-NH₂-TMRCA, where the red fluorescence shows the location of the rhodamine labeled HPG nanoparticles.

Discussion

The objective of the present work was to evaluate the effectiveness of intravesical mucoadhesive DTX formulations in an orthotopic model of bladder cancer and to investigate bladder tissue and tumor uptake of these nanoparticulate formulations.
HPGs were synthesized via anionic ring opening multibranching polymerization of glycidol using trimethylolpropane (TMP) as an initiator. The HPG core was derivatized with C₈/C₁₀ alkyl chains to create a hydrophobic core, to allow the loading of DTX while the MePEG chains linked to hydroxyl groups on HPGs create a hydrophilic shell intended to increase the aqueous solubility of these molecules. To increase their mucoadhesiveness, amine groups were conjugated to some of the hydroxyl groups on HPG-C₈/₁₀-MePEG polymer in a two-step reaction procedure (Fig. S1, Data Supplements). HPGs are small nanoparticles with hydrodynamic radii less than 10 nm. The surface modification of HPG-C₈/₁₀-MePEG with amine groups resulted in highly positive charged nanoparticles with improved mucoadhesive properties (Table S1, Data Supplements). DTX was loaded into these HPG nanoparticles due to hydrophobic interactions between the hydrophobic drug and the hydrophobic core of the HPGs. The maximum drug loading of DTX in HPGs was ~5%, which corresponded to about 5-6 DTX molecules per HPG molecule. Previously, we have shown that paclitaxel (PTX) can be loaded in HPGs, however the loading capacity was lower (2 vs 5% w/w) and this may be due to the fact that PTX is slightly larger (MW, 854 vs 808 Da) and more hydrophobic than DTX (22). The release profiles of DTX from HPGs were characterized by a continuous controlled release with little or no burst phase of release. Approximately 55% of initially encapsulated drug was released within the first 24 h of incubation in artificial urine. However, the presence of amine groups on the surface of HPGs had no effect on drug release (19).

We evaluated the cytotoxic effects of the standard Taxotere® and DTX loaded HPGs against the KU7-luc cell line, and both low-grade and high-grade human urothelial carcinoma cell lines. All DTX formulations resulted in concentration-dependent inhibition of
proliferation in all cell lines tested. In agreement with our previous results with paclitaxel, the more aggressive and fast growing KU7-luc cell line was the most sensitive to DTX formulations (23, 24). Loading of DTX in HPGs had no effect on its cytotoxicity. Indeed, DTX loaded HPG-C8/10-MePEG or HPG-C8/10-MePEG-NH2 were found to be as cytotoxic as the commercial formulation of Taxotere® (Fig. 1).

Next, we evaluated the effectiveness of a single intravesical therapy with DTX formulations in a mouse xenograft model of bladder cancer. Orthotopic implantation of human cancer cells into immunodeficient mice is one of the best models currently available to assess the effects of intravesical anticancer therapy in vivo. Using both histologic evaluation and magnetic resonance imaging, our group has previously validated an orthotopic mouse model of bladder cancer in which luciferase expressing KU7-luc cells are transurethrally inoculated in nude mice and tumor burden is longitudinally quantified using bioluminescence imaging (20). However, the fact that KU7 may not be of a human bladder origin has to be acknowledged, and our group is actively seeking new human urothelial carcinoma cell models for evaluation of intravesical therapy. The major issue with most human urothelial carcinoma cell lines is the low and non-reproducible tumor take in nude mice which makes intravesical evaluation challenging. Our objectives in this study, however, were not to develop nor to establish a ‘true’ non-muscle-invasive bladder cancer model but rather to evaluate the drug uptake and the efficacy of our newly developed mucoadhesive DTX formulations. Compared with control mice, DTX loaded HPGs inhibited tumor growth. However, DTX loaded HPG-C8/10-MePEG-NH2 was the most effective formulation to inhibit tumor growth in KU7-luc orthotopic bladder cancer xenografts and reached statistical significance compared to either the PBS control or Taxotere® groups (Fig. 2, P < 0.01, post-
hoc Bonferroni analysis after 2-Way ANOVA). This increase in efficacy likely resulted from enhanced drug uptake in bladder and tumor tissues of mice treated with DTX loaded HPG-C₈/₁₀-MePEG-NH₂ nanoparticles. Our uptake studies have demonstrated significantly increased drug uptake in bladder tissues and enhanced tumor uptake of rhodamine labeled HPG-C₈/₁₀-MePEG-NH₂ nanoparticles (Table 1 & Fig. 4). These results are in agreement with our recent studies in which DTX loaded HPG-C₈/₁₀-MePEG-NH₂ nanoparticles significantly increased the urinary bladder wall permeability and uptake of DTX in both isolated pig bladder as well as in a live mouse bladder tissues without tumors. Our preliminary studies on the effects of HPGs on the urothelium has demonstrated that HPG-C₈/₁₀-MePEG-NH₂ nanoparticles increase the permeability of the urinary bladder wall by causing changes to the urothelial barrier function and morphology through opening of tight junctions and exfoliation of the urothelium. These effects are similar to the effects on bladder mucosa caused by chitosan dispersions, a well-established, cationic, mucoadhesive polymer, which increased the permeability of isolated pig bladder to a model drug by causing desquamation of the urothelium (27-30).

Systemic absorption of chemotherapeutic drugs through the bladder wall is thought to be unlikely for agents with a molecular weight over 300 Da (31). Thus, DTX (808 Da) theoretically has little risk of systemic uptake. However, similar to our previous studies with paclitaxel (854 Da) in healthy mice (24), and in this model (23), we detected some systemic uptake especially in DTX loaded HPG-C₈/₁₀-MePEG-NH₂ treatment group. Systemic levels of DTX in the Taxotere® group were below our limit of quantification which was 10 ng/ml. This may be due to DTX sequestration in micelles as has been reported for Taxol® (32). The presence of Cremophol-EL in the commercial formulation of PTX (Taxol®) has been shown
to reduce its penetration into the bladder tissues in dogs. This was due to PTX sequestration into micelles which reduced the free fraction of PTX and consequently, lowered the drug penetration into the bladder tissues (32). DTX is commercially formulated in Tween 80 and mixed with 13% v/v ethanol and further diluted prior to administration and is also entrapped in micelles. The highest serum levels were obtained in the DTX loaded HPG-C_{8/10}-MePEG-NH_{2} arm at the 2 h time point (150.87 ± 34.98 vs 23.97 ± 16.71 ng/ml (DTX loaded HPG-C_{8/10}-MePEG), P < 0.01, 2-way ANOVA, Bonferroni post-test). This was not surprising as the levels in bladder tissues were more than 10-fold higher than those of DTX loaded HPG-C_{8/10}-MePEG (Table 1). Nevertheless, we did not observe any local or systemic toxicity in either group. Compared with humans, the mouse bladder wall is very thin and with an assumed logarithmic decrease in drug penetration across the tissue (33), lower serum levels would be expected in humans. In the recent phase I trial of intravesical DTX for the treatment of non-muscle-invasive bladder cancer refractory to BCG therapy, DTX was administered as in six weekly instillations with a dose-escalation up to 75 mg (34). The authors reported minimal toxicity with dysuria being the most common; however, no systemic absorption was reported for intravesical DTX. BCG therapy is the most effective intravesical therapy to date; unfortunately, head-to-head comparison with our DTX formulations was not possible because our orthotopic murine model lacks an immune response, which is important for the effectiveness of BCG therapy.

In conclusion, the surface modification of HPG-C_{8/10}-MePEG with amine groups resulted in highly positive charged nanoparticles (HPG-C_{8/10}-MePEG-NH_{2}) with improved mucoadhesive properties. DTX loaded HPGs were found to have equivalent cytotoxicities as the commercial Taxotere® formulation against four bladder cancer cell lines in vitro.
However, *in vivo*, DTX loaded HPG-C$_{8/10}$-MePEG-NH$_2$ was the most effective formulation to inhibit tumor growth in an orthotopic model of bladder cancer. Furthermore, this formulation significantly increased drug uptake in mouse bladder tissues. In addition, rhodamine labeled HPG-C$_{8/10}$-MePEG-NH$_2$ demonstrated enhanced tumor uptake of these nanoparticles. Our data show promising *in vivo* antitumor efficacy and provide preclinical proof-of-principle for the intravesical application of mucoadhesive DTX in the treatment of bladder cancer. We are in the process of understanding the effects of these mucoadhesive nanoparticles on the bladder urothelium and its integrity, which will give us valuable information about their biocompatibility, toxicity and mode of action. Once these studies have been completed, we hope to move forward with early phase clinical trials to evaluate the safety and efficacy of our mucoadhesive nanoparticulate DTX formulation in patients refractory to BCG.

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Conflict of interest

None declared

References

Table 1. Drug uptake of intravesical DTX formulations in orthotopic xenografts

<table>
<thead>
<tr>
<th>DTX formulations (No. of mice)</th>
<th>$^{1}C_{\text{urine}}$ (µg/ml)</th>
<th>$^{2}C_{\text{bladder}}$ (µg/g)</th>
<th>$^{3}C_{\text{serum}}$ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 h</td>
<td>1 h</td>
<td>2 h</td>
</tr>
<tr>
<td>Taxotere® (3)</td>
<td>31.4 ± 15.5</td>
<td>1.24 ± 0.54</td>
<td>BLOQ</td>
</tr>
<tr>
<td>DTX/HPG-C8/10-MePEG (4)</td>
<td>53.8 ± 8.1</td>
<td>1.09 ± 0.70</td>
<td>55.87</td>
</tr>
<tr>
<td>DTX/HPG-C8/10-MePEG-NH2 (4)</td>
<td>27.6 ± 4.0</td>
<td>13.07 ± 4.32</td>
<td>81.47 ± 23.76</td>
</tr>
</tbody>
</table>

$^{1}$ Final concentration of DTX in mouse urine after 2 h of intravesical instillation measured by HPLC

$^{2}$ Concentration of DTX in mouse bladder tissue following a 2 h intravesical instillation measured by LC/MS/MS

$^{3}$ Concentration of DTX in mouse serum taken at 0.5, 1, and 2 h post-intravesical instillation measured by LC/MS/MS

BLOQ, below the limit of quantification (lowest limit of quantification was 10 ng/ml)

Data shown are the mean ± SD
Legend to Figures

Fig. 1. *In vitro* cytotoxicity of DTX formulations against the KU7-luc cell line, and both low-grade (RT4, MGHU3) and high-grade (UMUC3) human urothelial carcinoma cell lines. Cells were exposed to either Taxotere® or DTX loaded HPG-C_{8/10}-MePEG and/or HPG-C_{8/10}-MePEG-NH₂ for 2 h and cell viability was determined after 72 h using the CellTiter96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay. Data shown are representative of three independent experiments.

Fig. 2. Treatment effects of single intravesical DTX formulations on orthotopic bladder cancer xenografts. Female nude mice (n=9-13/group) were intravesically inoculated with 2 x 10^6 KU7-luc tumor cells on day 0 and randomized on day 5 to receive 50 µl instillation volumes of formulations. Taxotere® (0.2 mg/ml, Sanofi-Aventis), or DTX (0.2 mg/ml) loaded HPG-C_{8/10}-MePEG and/or HPG-C_{8/10}-MePEG-NH₂ (mucoadhesive DTX). Tumor growth was determined in an IVIS200 Imaging System (Xenogen, Caliper Life Science). Bioluminescence imaging of mice is shown on the left panel.

Fig. 3. Representative histological sections of bladders harvested at the end of study, from mice receiving various formulations of 0.2 mg/ml DTX: A) HPG-C_{8/10}-MePEG-NH₂ B) HPG-C_{8/10}-MePEG C) Taxotere®. After H & E staining of whole bladder section, the low magnification (2x on a BLISS microscope imaging workstation) show multifocal and aggressive pT1 disease. KU7-luc tumors grow partially below the urothelial lining (magnification 6x) after 25 days post-tumor inoculation, there was no evidence of muscularis propria invasion despite some extremely large tumors observed in Taxotere® and PBS control groups. High-power view (magnification 20x) demonstrating evidence of hyperchromasia.
and mitotic figures within KU7-luc tumors. Within treatment groups, A, B, and C, numbers 1-3 designate different magnifications.

**Fig. 4.** Orthotopic bladder carcinoma instilled with PBS; free rhodamine (TMRCA); rhodamine labeled HPG-C_{8/10}-MePEG (HPG-C_{8/10}-MePEG-TMRCA); rhodamine labeled HPG-C_{8/10}-MePEG-NH₂ (HPG-C_{8/10}-MePEG-NH₂-TMRCA). Bladders were rinsed twice with PBS, excised whole and frozen immediately. 10 µm cryosections were subsequently imaged for fluorescent signal of rhodamine, then stained and imaged for vasculature CD 31 (A). B, Amount of fluorescence inside the bladder tumors. C, Observed rhodamine fluorescence in tumor tissues as a function of distance from bladder lumen.

**Fig. 5.** Orthotopic bladder carcinoma instilled with PBS (top left panel); free rhodamine (TMRCA, top right panel); rhodamine labeled HPG-C_{8/10}-MePEG (bottom left panel); rhodamine labeled HPG-C_{8/10}-MePEG-NH₂ (bottom right panel). Bladders were rinsed twice with PBS, excised whole and frozen immediately. 10 µm cryosections were subsequently imaged for fluorescent signal of rhodamine (red), then stained and imaged for cell nuclei (Hoechst 33342, grey) and vasculature (CD31, blue). *Scale bar, 250 µm*
Fig. 1

- **KU7-luc**
- **RT4**
- **MGHU3**
- **UMUC3**

Graphs showing the effect of different drug concentrations on cell lines. Each graph includes lines representing Taxotere, HPG-C<sub>6</sub>-MePEG/DTX, and HPG-C<sub>6</sub>-MePEG-NH<sub>2</sub>/DTX.
Fig. 2

Days post-tumor inoculation:
- PBS Control
- Taxotere®
- HPG-C₈/₁₀-MePEG/DTX
- HPG-C₈/₁₀-MePEG-NH₂/DTX

Tumor bioluminescence (fold change of initial flux):
- PBS (n=13)
- Taxotere (0.2 mg/ml, n=10)
- HPG-C₈/₁₀-MePEG/DTX (0.2 mg/ml, n=9)
- HPG-C₈/₁₀-MePEG-NH₂/DTX (0.2 mg/ml, n=9)

Time (days post-tumor inoculation):
- Treatment

Days: 4, 11, 18, 25
Fig. 3A
Fig. 3B
Fig. 3C
Fig. 5

PBS

HPG-C<sub>8/10</sub>-MePEG-TMRCA

TMRCAN

HPG-C<sub>8/10</sub>-MePEG-NH<sub>2</sub>-TMRCA
In vivo evaluation of mucoadhesive nanoparticulate docetaxel for intravesical treatment of non-muscle-invasive bladder cancer

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