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Docetaxel-Carboxymethylcellulose Nanoparticles Display Enhanced Anti-tumor Activity in Murine Models of Castration-Resistant Prostate Cancer

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Abstract

Docetaxel (DTX) remains the only effective drug for prolonging survival and improving quality of life of metastatic castration resistant prostate cancer (mCRPC) patients. Despite some clinical successes with DTX-based therapies, advent of cumulative toxicity and development of drug resistance limit its long-term clinical application. The integration of nanotechnology for drug delivery can be exploited to overcome the major intrinsic limitations of DTX therapy for mCRPC. We evaluated whether reformulation of DTX by facile conjugation to carboxymethylcellulose nanoparticles (Cellax) can improve the efficacy and safety of the drug in *s.c.* and bone metastatic models of CRPC. A single dose of the nanoparticles completely regressed *s.c.* PC3 tumor xenografts in mice. In addition, Cellax elicited fewer side effects compared to native DTX. Importantly, Cellax did not increase the expression of drug resistance molecules in androgen-independent PC3 prostate cancer cells in comparison with DTX. Lastly, in a bone metastatic model of CRPC, Cellax treatment afforded a 2- to 3-fold improvement in survival and enhancements in quality-of-life of the animals over DTX and saline controls. These results demonstrate the potential of Cellax in improving the treatment of mCRPC.

Keywords

castration-resistant prostate cancer; Cellax; docetaxel; nanoparticles

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INTRODUCTION

Prostate Cancer remains the most clinically diagnosed malignancy within the United States for men (Li et al., 2012). In many instances, anti-androgenic therapy remains the standard-of-care for initial treatment of advanced prostate cancer, but the disease invariably progresses after a median of 1-2 years and becomes castration-resistant (Niraula and Tannock, 2011). The development of the castration-resistant phenotype results in poor chemotherapeutic outcomes and significant morbidity and mortality. In patients who exhibit rapid disease progression and/or develop metastases to visceral organs such as bone, chemotherapy is often recommended as the next line of defense.

Taxanes remain a fundamentally important class of antineoplastic agents in the advanced prostate cancer therapy cascade and until recently, the only treatment demonstrated to increase overall survival in CRPC was docetaxel-based chemotherapy (Berthold et al., 2008; Petrylak et al., 2004; Tannock et al., 2004). Docetaxel (DTX, Taxotere®) plus prednisone is the standard of care for metastatic castration-resistant prostate cancer (mCRPC). Although this treatment offers significant palliation, only half mCRPC patients respond to the therapy, and of those that respond, the median survival is <20 months (Niraula and Tannock, 2011; Petrioli et al., 2008; Seruga and Tannock, 2011). Moreover, all patients eventually discontinue treatment due to cumulative toxicity or the emergence of DTX resistance and disease progression. Side effects include neurotoxicity, myelosuppression, acute hypersensitivity reactions, nasolacrimal duct stenosis, asthenia, febrile neutropenia, and myalgia (Baker et al., 2009; Eckhoff et al., 2011; Roglio et al., 2009). Some of these side effects can be directly associated with the solubilizing agent polysorbate 80 (Tween 80) used in the formulation, which often exhibits its own cytotoxic profile (Gelderblom et al., 2001). Mechanisms of innate and acquired resistance to DTX have limited its potential application and overall clinical utility. One putative mechanism of resistance is the up-regulation of P-glycoprotein (P-gp), which possesses high affinity for docetaxel. These efflux proteins expressed on the cell surface effectively decrease the intracellular concentration of drug, thereby promoting the development of resistance (Krishna and Mayer, 2000; Links and Brown, 1999). In addition, β -III tubulin, microtubule-associated proteins (tau) and some anti-stress and anti-apoptotic proteins (eg, Bcl-2, survivin and clusterin) have also been linked to the development of drug resistance (Seruga et al., 2011; Seruga and Tannock, 2011). Impaired drug delivery to tumor cells have also been shown to promote resistance to DTX (Seruga et al., 2011; Seruga and Tannock, 2011).

Treatment options after DTX failure are very limited (Niraula and Tannock, 2011; Seruga and Tannock, 2011). For example, in patients administered mitoxantrone plus prednisone, only 15% of the patients respond with symptom relief with no survival benefit. Cabazitaxel plus prednisone has been approved by the FDA and has shown to increase overall survival by 2.5 months and quality of life compared to mitoxantrone plus prednisone in phase III trials. However, the severe toxicity profile of cabazitaxel combined with the fact that the drug is not cost-effective (\$7,400/60 mg vial), prevents it from becoming a standard of care (Shih and Halpern, 2008). Abiraterone plus prednisone improved survival for mCRPC patients previously treated with DTX by ~4 months compared to placebo, and has recently been approved by the FDA. In a recent phase 3 clinical trial of enzalutamide, a potent

androgen-receptor-signalling inhibitor, significant enhancements in overall survival was demonstrated in men with progressive CRPC after DTX chemotherapy. Among patients receiving enzalutamide, the median overall survival was 18.5 months, in comparison to 13.6 months for patients receiving placebo control, corresponding to a 37% reduction in risk of death (Scher et al., 2012). Currently, both abiraterone and enzalutamide are being evaluated in clinical trials as a first line drug for mCRPC.

Thus far, DTX remains the only effective drug for prolonging survival and improving quality of life of mCRPC patients; however, the cumulative toxicity and development of drug resistance during the therapy prevent it from long-term use. This study is aimed at developing an improved drug delivery system to mitigate the toxicity of DTX and reduce the development of drug resistance, to ultimately prolong the duration of time that patients can remain under DTX chemotherapy. Here, we report the evaluation of an innovative nanoparticle drug delivery system that displayed significant potential in addressing the three major limitations of DTX in the treatment of mCRPC, including poor tolerability, limited efficacy, and high tendency for the development of drug-resistance.

MATERIALS & METHODS

Materials

Carboxymethylcellulose (CMC) sodium salt 30000-P was purchased from CPKelco (Atlanta, GA). Docetaxel (DTX) was purchased from LC Laboratories (Woburn, MA). Polyethylene glycol methyl ether (mPEG-OH, MW=2000), 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDC.HCl) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich (Oakville, ON).

Preparation and characterization of Cellax nanoparticles and native DTX control

Docetaxel and PEG conjugated carboxymethylcellulose polymer (Cellax) was prepared using a method described in detail elsewhere (Ernsting et al., 2011). Cellax nanoparticles were prepared in a controlled nanoprecipitation process by hydrodynamic flow focusing through a two-channel microfluidic system (NanoAssemblr, Precision Nanosystems, Canada). Hydrodynamic flow focusing was achieved with Cellax polymer (150 mg) in acetonitrile at a concentration of 45 mg/mL in one channel and 0.9% NaCl (aq.) in the adjacent channel. Total flow rate was maintained at 18 mL/min and the flow ratio of the aqueous to organic stream was 3:1 (v/v), respectively. The outlet stream resulting from the rapid mixing of aqueous and organic streams was immediately diluted in an equal volume of saline in a collection conical tube. The resulting nanoparticle solution was subsequently transferred to a dialysis cartridge (Slide-a-lyzer) with a 10000 MWCO and dialyzed against 0.9% NaCl overnight with an exchange of dialysate at 2 h and again the following morning. The particles were filtered through a 0.45 µm and a 0.22 µm Millipore PVDF filter, transferred to a centrifugal filter unit (25 mL, 10000 MWCO, Vivaspinn), and concentrated at 4000 rpm for 1 h to obtain a total volume of approximately 1 mL. Docetaxel (DTX) content was determined by ¹H NMR (Bruker, 500 MHz NMR Spectrometer) using a water-presaturation protocol; 100 µL of Cellax solution was mixed with 900 µL of deuterated

dimethylsulphoxide containing 2-methyl-5-nitrobenzoic acid as an internal standard. Free, unreacted DTX and PEG content was quantified using LC/MS.

Analysis of nanoparticle size and zeta potential

Nanoparticle size and size distribution were determined by dynamic light scattering using a particle analyzer (Zetasizer Nano-ZS, Malvern Instruments Ltd, Malvern, UK). The sample was diluted 10-fold in saline prior to analyses. The results are expressed as Z-Ave diameters (nm). The zeta potentials of nanoparticle preparations were determined by photon correlation spectroscopy in a 10% sucrose solution.

Cell culture and animal protocol

Human PC3 cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Male NOD-SCID mice were purchased from Jackson Laboratories (Bar Harbour, ME). All experimental protocols in this study were approved by the Animal Care Committee of the University Health Network (UHN, Toronto, ON, Canada) in accordance with the policies established in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council of Animal Care. Three days prior to tumor inoculation, mice were supplemented with Baytril in their water supply. Subsequently, they were irradiated (2.27 Gy) in a GammaCell. Two days after irradiation, shaved mice were injected with 2×10^7 PC3 cells *s.c.* into the right lateral flank. Tumor size was determined by caliper measurements, with volume calculated as $(\text{length} \times \text{width} \times \text{width}/2)$. Mice were treated at the maximum tolerated dose (MTD) which was dependent on the treatment formulation, animal species and dosing frequency. At terminal endpoints, the mice were sacrificed under CO₂. Terminal endpoints were determined to be tumor volume greater than 1000 mm³, weight loss exceeding 20%, expression of significant discomfort (i.e. severe piloerection, withdrawn, abnormal behaviour) or presence of other physical abnormalities (i.e. paralysis, morbidity, seizures).

Subacute toxicology study

Non-tumor bearing BALB/c mice were treated with Cellax and native DTX once weekly for three weeks (q1w $\times$ 3 cycle) at 170 mg DTX/kg and 25 mg/kg respectively (n=5). The toxicity was evaluated by analyses of hematology, serology, and changes in body weight. Blood and serum samples were collected at baseline and every 7 days following administration of each dose up to 4 weeks post final dose. Briefly, at 3 and 4 weeks post treatment, mice were sacrificed under anaesthesia; major organs and tissues were harvested, fixed in formalin, and processed for H&E analysis. Tissue histology was evaluated by a certified veterinary pathologist at the Toronto Center for Phenogenomics.

Efficacy study in a *s.c.* PC3 tumor xenograft model

Seven to ten days post tumor inoculation, when tumors became palpable, mice received a single *i.v.* bolus injection of native DTX (20 mg DTX/kg, n=5), Cellax (170 mg DTX/kg, n=5) or saline (n=5). MTD for a single *i.v.* bolus injection of DTX was previously

determined to be 20 mg/kg in the NOD-SCID animal model. Tumor growth was monitored using caliper measurements.

Efficacy study in a xenograft model of bone metastases

Male NOD-SCID mice were pre-treated with buprenorphine (0.1 mg/kg) prior to surgery. The mice were anesthetised with isoflurane and the lateral hind legs scrubbed with a 70% ethanol solution. The mice were inoculated with 1×10^6 PC3 cells directly into the bone marrow via intra-femoral injection. Briefly, a 28-gauge needle was inserted approximately 3-5 mm into the proximal end of the femur. The cell suspension was subsequently injected directly into the marrow via the femoral bone opening. The mice were maintained with buprenorphine (0.1 mg/kg) as an analgesic for 3 days post surgery. The following day, mice were treated with native DTX (10 mg DTX/kg, n=10), Cellax (170 mg DTX/kg, n=10) or saline (n=10). The MTD of native DTX in male NOD-SCID mice in a multi-dose study was previously determined to be 10 mg/kg. Mice were retreated at the same dose one week later. Changes in body weight, incidence and severity of limping and overall survival were monitored over time. Terminal endpoints were defined as described previously. The incidence and severity of limping was graded using a qualitative three tiered grading criteria as described in Table 1 by a senior animal laboratory technician. Incidence and degree of limping was used as an index for tumor-induced pain burden and quality of life. Two and four weeks post treatment, evidence of tumor induced bone change was determined via *in vivo* microCT imaging using a GE Locus Ultra MicroCT imaging system. Mice were anesthetized under isoflurane gas in a lucite module and placed into a 3-chamber bed assembly. The image acquisition protocol consisted of 360 individual projections acquired at 1° increments to complete one rotation around the mice. X-ray settings were 80 kVp voltage and 50 mA current. Projection data was reconstructed using a filtered-back projection reconstruction algorithm resulting in an isotropic voxel dimension of 0.54 μ m.

Evaluation of gene expression by quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Expression of P-glycoprotein (p-gp) [human ATP-binding cassette, sub-family B (MDR/ TAP), β III-tubulin [human tubulin, beta 3 class III (TUBB3), transcript variant 1, NM_006086], tau [human microtubule-associated protein tau (MAPT), transcript variant 2, NM_005910], Bcl-2 [human Bcl2 binding component 3 (BBC3), transcript variant 1, NM_001127240], clusterin [human clusterin (CLU), transcript variant 1, NM_001831], and survivin [human baculoviral IAP repeat containing 5 (BIRC5), were evaluated by quantitative RT-PCR after incubation with either native DTX (dissolved in DMSO) or Cellax (10 nM DTX) for 36 h. Briefly, PC3 cells were washed three-times with PBS and harvested for RNA extraction. Total RNA was isolated from subconfluent cells using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. Complementary DNA (cDNA) was reverse-transcribed with a QuantiTect reverse transcription kit (Qiagen) and amplified with specific primers using the LightCycler SYBR Green Core Reagent Kit (Qiagen). The PCR primer sequences used for amplification are listed in Table 2. Gene expression was calculated using the formula, 2^{-CT} , where CT is the difference in CT (cycle number at which the amount of amplified target reaches a fixed threshold) between

the target and reference. Expression levels were normalized to endogenous actin. A relative change in expression level of 2 or more is considered significant.

Statistical analysis

Il results were reported as mean $\pm$ SD. Statistical analysis was conducted using SPSS (Statistical Package for the Social Sciences, v. 14.0). One-way parametric analysis of variance (ANOVA) with the Bonferroni correction for multiple comparisons was used to compare three or more groups if the ANOVA F-test indicated a statistical significance. All other statistical comparisons were made using the Student's *t*-test with a significance value of $p < 0.05$.

RESULTS

Preparation and physicochemical characterization of Cellax nanoparticles

Cellax nanoparticles were approximately $122 \text{ nm} \pm 3 \text{ nm}$ in diameter with a polydispersity index (PDI) of 0.14. Chemical analysis using ^1H NMR revealed 39.3 wt% DTX and 5.7 wt % PEG conjugated to the carboxymethylcellulose backbone. The nanoparticles exhibited a zeta-potential of $-0.8 \pm 3.1 \text{ mV}$. Free unconjugated DTX and PEG concentrations were found to be less than 2 wt% in the polymer. A schematic representation of a Cellax nanoparticle detailing the composition of Cellax polymer is illustrated in Figure 1.

Toxicology of native DTX and Cellax

Mice treated with a weekly dose of Cellax at 170 mg/kg for three consecutive weeks did not exhibit any considerable loss in body weight (Figure 2). In contrast, mice treated with DTX (25 mg/kg) exhibited greater cumulative toxicity. At day 16, a $13 \pm 2 \%$ decrease in body weight was observed for DTX treated mice. Haematological and serological examination revealed no abnormal changes after administration of Cellax for all haematological parameters measured. Importantly, neutropenia, a common side-effect of taxane therapy was not observed in Cellax treated mice. In comparison, the neutrophil count for DTX treated mice decreased from $1.2 \times 10^9/\text{L}$ to $0.4 \times 10^9/\text{L}$, after administration of the first dose (Table 3) and remained abnormally low during the treatment. Cellax is primarily cleared via the mononuclear phagocyte system resulting in significant accumulation in liver tissues. However, serological analyses of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) enzyme levels revealed no appreciable change from baseline implying that liver function was conserved (Table 4). Histopathological analyses of normal tissues revealed mild inflammation in the lungs and liver as indicated by lesions in hypertrophic Kupffer cells and alveolar macrophage activation, 1 week post final treatment. However, 3-4 weeks post final treatment, the formation of lesions within these tissues were no longer detected indicative of tissue recovery.

Efficacy study in a s.c. PC3 tumor xenograft model

Cellax treated mice exhibited complete tumor regression up to 120 days (Figure 3). In contrast, DTX treated mice demonstrated an initial inhibition of tumor growth but the tumors all rebounded past day 20. By day 44, all DTX treated mice reached terminal

endpoints and were sacrificed. Saline treated mice exhibited aggressive tumor growth resulting in severe ulcerative dermatitis by day 20, necessitating sacrifice.

Efficacy study in a xenograft model of bone metastases

Intra-femoral injection of PC3 cells to the bone marrow of male NOD-SCID mice was used as a surrogate model for bone metastases. Importantly, in this model of mCRPC, Cellax prolonged survival by greater than 2-fold over native DTX treated mice and 3-fold over control mice (Figure 4). The mean survival time for Cellax treated mice was 68 days, in comparison to 30.5 and 21 days for mice treated with native DTX and saline control, respectively.

A generalized qualitative assessment of the incidence and impact of tumor burden and tumor-associated pain (defined as limping) was developed. This method categorized the incidence and severity of the tumor-associated-pain using a three-tiered classification scale from mild, moderate, and severe (Table 1). Figure 5 illustrates the incidence and severity of limping over time. Results are shown up to day 38, as this was the terminal endpoint for all mice in the native DTX group. Terminal endpoint was reached at day 24 for all saline treated control mice. This range afforded comparisons across all treatment groups. As illustrated in Figure 5, rapid progression of the disease was observed in the saline and DTX treated groups; evidenced by an increase in limping scores. At 24 days post treatment, the average limp grade score for control, native DTX, and Cellax treated mice was 2.4, 1.6, and 0.2 respectively. At 38 days post treatment, the average limp grade score for native DTX and Cellax treated mice was 2.3 and 0.4, respectively.

MicroCT imaging enabled real-time non-invasive visualization and quantification of bone mineral degradation and metastases. As illustrated in Figure 6A, saline and native DTX treated mice displayed severe bone loss at the site of injection in comparison to Cellax treated mice, which displayed no sign of bone loss at 4 weeks post treatment. For saline treated mice, bone mineral density loss could be observed as early as 2 weeks post treatment. Using region-of-interest analysis between the head of the femur to the calcaneum, it was possible to quantify absolute bone mineral density loss associated with mCRPC progression. At two weeks post treatment, there was no significant difference in bone mineral density between Cellax and native DTX treated mice (271 ± 7.6 vs 275 ± 9.2 mg/cm²; $P > 0.05$, respectively). Both Cellax and native DTX treated mice significantly outperformed saline treated mice at two weeks post treatment. However, at 4 weeks post treatment, Cellax treated mice significantly outperformed DTX treated mice, as evidenced by the conservation of bone mineral density (262 ± 5.3 vs 230 ± 11 mg/cm²; $P < 0.05$, respectively). The bones of Cellax treated mice remained normal compared to the healthy mice 2 weeks post therapy.

Expression of drug resistance markers to DTX after exposure to native DTX and Cellax

Quantitative RT-PCR analyses enabled quantitative assessment of P-gp, β III-tubulin, tau, Bcl-2, clusterin and survivin expression in PC3 cells incubated with native DTX (dissolved in DMSO) or Cellax at equal concentration of DTX (10nM) for 36 h. Unlike native DTX, Cellax treatment did not up-regulate P-gp and β -III tubulin expression. In fact, P-gp and β -III

tubulin mRNA expression was found to be 9-fold and 2.5-fold higher for DTX-treated cells over Cellax, respectively. No significant differences were observed in mRNA expression of tau, Bcl-2, clusterin, or survivin between DTX and Cellax treated cells.

DISCUSSION

Prostate cancer remains a major concern amongst men in relation to overall incidence and cancer-related deaths. Hormone-based therapeutic interventions have shown positive clinical outcomes for prostate cancer, providing significant improvements to survival in patients with highly advanced prostate cancer (Niraula and Tannock, 2011). However, mCRPC remains difficult to treat, and is typically characterized with poor disease prognosis (Niraula and Tannock, 2011; Seruga et al., 2011; Seruga and Tannock, 2011). Up to now, DTX is the only FDA approved chemotherapeutic that has been shown to improve the overall survival of men with mCRPC (Berthold et al., 2008; Seruga and Tannock, 2011). However, only half of mCRPC patients respond to DTX, and all will eventually discontinue treatment due to cumulative toxicity or development of resistance and disease progression. It was hypothesized that the limitations of DTX can be addressed by reformulating the drug in a detergent free excipient with tumor-selective delivery. A number of formulations relying on solvent-free nanotechnologies for DTX delivery are currently at various stages of preclinical and clinical evaluation (Conte et al., 2013; Deeken et al., 2013; Elsabahy et al., 2007; Mikhail and Allen, 2010; Sanna and Sechi, 2012; Tan et al., 2012; Wang et al., 2012). However, few are designed to treat mCRPC, with no efficacy data in bone metastatic models to date. Nanotechnology is a promising tool to significantly enhance antitumor efficacy of cytotoxic drugs. Passive targeting of anticancer agents via the enhanced permeability and retention (EPR) effect to tumor tissues has demonstrated increased tumor localization, resulting in significant enhancements in therapeutic efficacy. Indeed, in this study, a single *i.v.* treatment of Cellax completely regressed tumors in a *s.c.* PC3 tumor xenograft model for up to 120 days post treatment. In contrast, a maximum tolerated dose of DTX limited tumor progression for only 20 days before becoming ineffective. Previously published data suggested that Cellax exerts its cytotoxicity via two possible mechanisms: (a) Cellax released DTX extracellularly in a sustained manner through enzymatic hydrolysis of the ester linkages; (b) Cellax is efficiently internalized by tumor cells wherein the drug is released in the acidic endosomal/lysosomal environment (Ernsting et al., 2011).

The EPR effect describes the distinctive increase in vascular permeability and impaired lymphatic drainage from the interstitial space in tumor and inflammatory tissues. During tumor angiogenesis, newly formed blood vessels do not conform to standard morphology and are typically characterized by dilated, disorganized, misaligned and heterogeneous endothelial formation resulting in wide fenestrations that permit transvascular transport of macromolecules to tumor interstitium (Hashizume et al., 2000; Skinner et al., 1990; Yuan et al., 1994). Upregulation of angiogenesis mediators and promoters of vascular permeability effectively enhance systemic blood flow and localized diffusion and retention of nanoparticles at tumor sites (Taurin et al., 2012). Other morphological characteristics of tumor vasculature include the absence of a basement membrane, lack of a smooth muscle layer, a wide lumen, tortuous microvessel formation, and high transvascular pressure gradient (Greish, 2010; Hashizume et al., 2000; Skinner et al., 1990; Taurin et al., 2012).

These physiological and morphological considerations can alter the transport kinetics, retention and elimination of any drug delivery vehicle from the tumor site (Taurin et al., 2012). In contrast, low molecular weight drugs often have rapid distribution and elimination kinetics, can freely diffuse across the vascular endothelium, are broadly distributed across most tissues, and lack tumor specificity (Taurin et al., 2012). As a result, the broad distribution of the drug to healthy tissues often causes systemic toxicity.

The integration of chemotherapeutics with nanotechnology using drug delivery systems promises tissue selective delivery of chemotherapeutic agents while minimizing systemic toxicity (Alexis et al., 2010). These nanosized drug delivery vehicles have several desirable advantages in comparison to systemic chemotherapy. First, low-molecular-weight chemotherapeutic agents are rapidly distributed and subsequently eliminated via glomerular filtration through the kidneys. Incorporation of these low molecular weight drugs in nanoparticles substantially prolongs their pharmacokinetics and substantially improves their bioavailability. Second, nanoparticles are known to exploit the EPR effect for passive targeting to tumors thereby increasing tumor drug concentrations while minimizing systemic drugs toxicity. Lastly, formulation of hydrophobic often requires the use of solvents or excipients such as ethanol, Tween-80 or Cremophor EL as drug solubilizers, that often exhibit their own inherent toxicity (Gelderblom et al., 2001; Strickley, 2004). In contrast, hydrophobic drugs can be easily incorporated into nanoparticles without the use of toxic additives. Indeed, as illustrated in Figure 2 and Table 3, greater than 8-fold higher dose of DTX could be administered to mice when DTX was delivered using Cellax nanoparticles resulting in reduced side-effects as determined by weight loss, haematology and serology in mice.

Up to 84% of new prostate cancer cases will develop advanced metastatic disease to bone as a progression of the primary tumor. Prostate cancer metastases to bone have been shown to be debilitating in patients, leading to fractures and severe bone pain in men with advanced disease progression (Raheem et al., 2011). As such, a direct bone marrow injection via the femur was used here to simulate the occurrence of bone metastases. Prostate cancer cells injected directly into the femur of NOD-SCID male mice are capable of proliferating and stimulating remodeling of mouse bone. The bone lesions induced by the PC3 cell line could be monitored and quantified by microCT image analysis. In this study, MicroCT imaging was used to non-invasively monitor the progression of bone metastatic disease and evaluate bone mineral loss. One of the strengths of the microCT imaging modality is the ability to image bone structures. MicroCT has been used to monitor bone lesion development and progression in intratibial or intra-femoral injected mouse models (Bi et al., 2013; Raheem et al., 2011; Virk et al., 2009; Zilber et al., 2008). In this study, microCT imaging was able to non-invasively follow the onset and progression of bone metastatic lesions and bone degradation over time. Interestingly, Cellax treatment significantly inhibited bone degradation at the site of injection in comparison to native DTX and control. Such skeletal damage significantly impacts quality of life and survival. Cellax treated mice had approximately 2-fold greater survival than native DTX treated mice. Thus, the integration of DTX in this unique drug delivery system significantly improved its performance *in vivo* while minimizing dose-limiting toxicity, such as neutropenia. This is further demonstrated

with the evaluation of the incidence and severity of limping. Taken together, this indicates that Cellax inhibited or limited the progression of tumor and metastases.

Despite the clinical effectiveness of taxanes such as DTX in the treatment of specific indications, mechanisms of innate and acquired resistance often limit their overall clinical utility. Chemoresistance is multifactorial in etiology and can include overexpression of transmembrane transporters and other resistance markers. Evaluation of these chemoresistance pathways have led to the identification of several proteins which often associate with the development of taxane resistance including amplification and overexpression of membrane transporters such as P-gp, apoptotic regulatory proteins such as bcl-2, clusterin and survivin, and tubulin and microtubulin associated proteins including β -III tubulin and tau (Yusuf et al., 2003). The significant enhancement in P-gp and β -III tubulin expression after DTX treatment suggests that upregulation of P-gp is a major factor in the development of DTX resistance in prostate cancer and could be exploited as a biomarker for quantifying the resistant phenotype. In prostate cancer, P-gp is over-expressed in prostate tumor epithelium compared to normal tissue and has been shown to drive tumor progression. The overexpression of P-gp is often a mitigating factor for chemotherapy failure in patients (Baekelandt et al., 2000; Kamazawa et al., 2002). In clinical evaluation, tumor samples harvested from taxane-resistant patients exhibited a 28-fold higher level of P-gp in comparison to patients who did not exhibit taxane resistance (Kamazawa et al., 2002). Similarly, the role of β -III tubulin in generating selective drug resistance in lung and ovarian cancer patients is well established (Burkhart et al., 2001; Mozzetti et al., 2005). Taxanes preferentially bind to the β -subunit of tubulin. Kavallaris et al. demonstrated this phenomenon at the mRNA level (Kavallaris et al., 1997). They found taxol-resistant epithelial ovarian tumors were directly associated with the altered expression of β -tubulin (Kavallaris et al., 1997). In particular, β -III tubulin is overexpressed in drug-resistant tumors. The exact mechanism by which β -III tubulin upregulation confers taxane resistance is yet to be fully elucidated. However, it is believed that β -III tubulin mediates enhanced microtubule dynamic instability, thereby overcoming the suppressive microtubule dynamicity induced by taxanes (Panda et al., 1994). In addition, studies have demonstrated upregulation of β -III tubulin mitigates the polymerization rate of microtubules, thereby facilitating drug-resistance (Hari et al., 2003). The drug-resistant nature of prostate cancer remains a challenge to the effectiveness of such therapies. Interestingly, Cellax was found to limit P-gp and β -III tubulin overexpression in comparison to native DTX. This has profound implications as this technology may have the potential to overcome one of the primary putative mechanisms for the development of taxane-resistance, thereby prolonging the effectiveness of taxanes such as DTX for an extended period of time. It is postulated that Cellax is able to circumvent the intrinsic ability of DTX to upregulate P-gp through a slow drug release mechanism. Indeed, previous studies evaluating the drug release kinetics of Cellax in serum revealed near zero-order kinetics over 3 weeks (~5 %/day) (Ernsting et al., 2011). Studies have demonstrated slow and continuous exposure to DTX curbs induction of P-gp upregulation in cancer cells when compared to a single bolus dose. For example, Ho et al. demonstrated in a human ovarian cancer xenograft model that intraperitoneal implantation of a slow releasing formulation of paclitaxel did not induce overexpression of P-gp. (Ho et al., 2007)

We have demonstrated that Cellax exhibited enhanced activity against a model of CRPC in mice by completely regressing *s.c.* tumor xenografts with one dose. In addition, Cellax was also found to elicit fewer side effects compared to native DTX. Importantly, it was demonstrated that Cellax did not increase the expression of drug resistance molecules in androgen-independent PC3 prostate cancer cells in comparison with the native drug. Finally, in a mouse model of prostate cancer metastases to the bone, 2- to 3-fold improvements in survival and significant enhancements in the quality-of-life of the animals were observed for Cellax treated mice over native DTX and control. These results provide an encouraging outlook on the potential translation of Cellax in improving the care of mCRPC.

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Figure 1. Schematic illustrating structure of Cellax polymer and preparation of Cellax nanoparticles.

Figure 2.

Evaluation of gross toxicity of DTX and Cellax. Mice were treated with *i.v.* Cellax (170 mg DTX/kg), native DTX (25 mg/kg), or saline once per week for three consecutive weeks. Body weight was measured at various timepoints post treatment. Data is expressed as change in body weight (%) vs. time (n=5).

Figure 3.

Evaluation of efficacy of DTX and Cellax in a *s.c* PC3 tumor xenograft model. Mice were treated with either a single dose of Cellax (170 mg DTX/kg), native DTX (20 mg/kg), or saline. Data is expressed as percent tumor growth vs. time (n=5).

Figure 4.

Evaluation of efficacy of DTX and Cellax in a model of bone metastases (PC3). Mice were treated with a weekly dose of Cellax (170 mg DTX/kg), native DTX (10 mg/kg) or saline for two weeks. Survival was monitored over time and a Kaplan-Meier survival curve was generated using GraphPad Prism v. 5.0 (GraphPad Software, Inc) and plotted as % survival vs. time, n=10.

Figure 5.
Evaluation of limping in a bone metastases model of PC3 prostate cancer after treatment with Cellax, native DTX or saline, n=10.

Figure 6. MicroCT analysis of bone in mice with PC3 bone tumors. A) MicroCT axial projections of a representative mouse in the bone metastases model after treatment with saline at 2 weeks post treatment, DTX at 4 weeks post treatment and Cellax at 4 weeks post treatment. B) Region of interest analysis of bone mineral density in a bone metastases model of prostate cancer after treatment with saline control, native DTX (20 mg/kg), or Cellax (170 mg DTX/kg). * indicates $p<0.05$.

Figure 7. Quantitative RT-PCR analysis of major taxane resistance markers in PC3 cells after incubation with 10 nM of DTX and Cellax (10 nM DTX equivalent) for 36 h. * indicates >2-fold difference with p<0.05.

Table 1

Qualitative assessment of tumor pain burden.

Grade	Classification	Characteristics
0	Normal	No observable change from normal gait.
1	Slight	Barely perceptible lameness in hind-limb gait.
2	Moderate	Apparent lameness in hind-limb gait.
3	Severe	Inability to apply pressure on hind-limb. Non-weight bearing lameness.

Table 2

Real-time RT-PCR Primer Sequences.

Gene	Forward Primer	Reverse Primer
P-gP	5'-GTGGGGCAAGTCAGTTCATT-3'	5'-TCTTCACCTCCAGGCTCAGT-3'
-III tubulin	5'-ACCTCAACCACCTGGTATCG-3'	5'-TTCTTGGCATCGAACATCTG-3'
tau	5'-GCTGAGTCCCAGCAATTCTC-3'	5'-GAAGAGCAGGGCACAAGAAC-3'
Bcl-2	5'-GCCCAGACTGTGAATCCTGT-3'	5'-CTCCTCCCTCTTCCGAGATT-3'
Clusterin	5'-ACATTTGGTGCCCAGAAAGTC-3'	5'-CTGTGGTCCAGGGAAAGGTA-3'
Survivin	5'-ACCTGAAAGCTTCCTCGACA-3'	5'-TAACCTGCCATTGGAACCTC-3'

Table 3

Hematology measurements for DTX and Cellax treated mice. Baseline values were analyzed pre-dosing. Dose 1 was administered after baseline measurement (t=0), and dose 2 and 3 were administered in the subsequent 2 weeks. Data = mean $\pm$ SD (n=5).

		Baseline (pre-dose) Dose 1 iv	+ 1 week Dose 2 iv	+ 2 weeks Dose 3 iv	+ 3 weeks	+ 4 weeks	+ 5 weeks	+ 6 weeks
RBC ($10^{12}/L$)	Cellax	10.6 $\pm$ 0.2	9.8 $\pm$ 0.7	10.0 $\pm$ 0.5	10.5 $\pm$ 0.3	10.4 $\pm$ 0.3	10.4 $\pm$ 0.9	10.8 $\pm$ 0.4
	DTX	10.7 $\pm$ 0.2	9.4 $\pm$ 0.2	10.2 $\pm$ 0.1	8.0 $\pm$ 0.5	-	-	-
Hgb (g/L)	Cellax	169.8 $\pm$ 5.0	166.8 $\pm$ 7.7	162.8 $\pm$ 6.1	174.2 $\pm$ 7.6	164.0 $\pm$ 6.3	162.8 $\pm$ 12.0	159.6 $\pm$ 5.6
	DTX	160.4 $\pm$ 2.9	144.7 $\pm$ 1.5	154.0 $\pm$ 4.0	116.0 $\pm$ 9.9	-	-	-
HCT (L/L)	Cellax	0.65 $\pm$ 0.04	0.62 $\pm$ 0.02	0.62 $\pm$ 0.03	0.64 $\pm$ 0.02	0.64 $\pm$ 0.02	0.63 $\pm$ 0.05	0.62 $\pm$ 0.03
	DTX	0.64 $\pm$ 0.01	0.57 $\pm$ 0.01	0.64 $\pm$ 0.01	0.49 $\pm$ 0.03	-	-	-
MCV (fL)	Cellax	61.2 $\pm$ 0.7	62.6 $\pm$ 0.9	62.2 $\pm$ 0.8	60.8 $\pm$ 0.3	61.3 $\pm$ 0.6	60.5 $\pm$ 0.8	57.7 $\pm$ 0.4
	DTX	59.6 $\pm$ 1.7	60.4 $\pm$ 0.25	62.8 $\pm$ 1.2	60.6 $\pm$ 1.1	-	-	-
MCH (pg/cell)	Cellax	16.1 $\pm$ 0.3	17.0 $\pm$ 0.4	16.2 $\pm$ 0.2	16.5 $\pm$ 0.2	15.7 $\pm$ 0.3	15.6 $\pm$ 0.2	14.8 $\pm$ 0.3
	DTX	15.0 $\pm$ 0.4	15.4 $\pm$ 0.4	15.2 $\pm$ 0.4	14.4 $\pm$ 1.1	-	-	-
MCHC (g/L)	Cellax	262.6 $\pm$ 3.4	271.2 $\pm$ 7.9	261.0 $\pm$ 2.0	272.0 $\pm$ 3.3	256.4 $\pm$ 5.6	258.4 $\pm$ 4.7	257.2 $\pm$ 4.0
	DTX	251.6 $\pm$ 7.8	254.7 $\pm$ 5.9	241.7 $\pm$ 4.2	237.0 $\pm$	-	-	-
PLT ($10^9/L$)	Cellax	878.8 $\pm$ 70.8	782.8 $\pm$ 74.3	850.0 $\pm$ 66.1	825.0 $\pm$ 48.7	939.6 $\pm$ 51.8	967.0 $\pm$ 85.3	935.4 $\pm$ 66.3
	DTX	830.8 $\pm$ 78.7	1181 $\pm$ 59.2	1149 $\pm$ 122	237 $\pm$ 130.0	-	-	-
WBC ($10^9/L$)	Cellax	7.5 $\pm$ 1.5	5.9 $\pm$ 0.9	10.0 $\pm$ 1.5	8.8 $\pm$ 1.7	10.8 $\pm$ 2.6	8.4 $\pm$ 0.6	7.1 $\pm$ 1.6
	DTX	8.34 $\pm$ 1.5	5.9 $\pm$ 0.9	10.0 $\pm$ 1.5	8.8 $\pm$ 1.7	-	-	-
MPV (fL)	Cellax	4.9 $\pm$ 0.1	5.1 $\pm$ 0.9	4.8 $\pm$ 0.2	5.0 $\pm$ 0.2	5.0 $\pm$ 0.1	5.0 $\pm$ 0.2	5.1 $\pm$ 0.2
	DTX	5.0 $\pm$ 0.1	4.9 $\pm$ 0.1	5.2 $\pm$ 0.1	4.5 $\pm$ 0.2	-	-	-
RDW (%)	Cellax	16.8 $\pm$ 0.9	17.2 $\pm$ 0.9	16.8 $\pm$ 0.6	16.5 $\pm$ 0.5	16.7 $\pm$ 0.5	16.9 $\pm$ 0.3	16.4 $\pm$ 0.3
	DTX	17.3 $\pm$ 0.2	16.1 $\pm$ 0.6	18.5 $\pm$ 0.4	15.5 $\pm$ 1.7	-	-	-
NE ($10^9/L$)	Cellax	1.4 $\pm$ 0.1	1.2 $\pm$ 0.2	1.9 $\pm$ 0.4	1.6 $\pm$ 0.3	2.0 $\pm$ 0.4	1.8 $\pm$ 0.7	1.3 $\pm$ 0.4
	DTX	1.2 $\pm$ 0.1	0.4 $\pm$ 0.3	0.4 $\pm$ 0.3	0.4 $\pm$ 0.3	-	-	-
LY ($10^9/L$)	Cellax	7.6 $\pm$ 1.4	4.3 $\pm$ 0.7	7.6 $\pm$ 1.1	6.7 $\pm$ 1.6	8.2 $\pm$ 2.1	6.1 $\pm$ 0.8	5.3 $\pm$ 1.3
	DTX	6.4 $\pm$ 0.6	5.7 $\pm$ 0.3	8.1 $\pm$ 1.1	2.9 $\pm$ 0.6	-	-	-
MO ($10^9/L$)	Cellax	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1	0.5 $\pm$ 0.1	0.6 $\pm$ 0.1	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1
	DTX	0.4 $\pm$ 0.0	0.3 $\pm$ 0.0	0.6 $\pm$ 0.3	0.2 $\pm$ 0.1	-	-	-
BA ($10^9/L$)	Cellax	0.01 $\pm$ 0.01	0.01 $\pm$ 0.02	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.02 $\pm$ 0.04
	DTX	0.02 $\pm$ 0.01	0.03 $\pm$ 0.05	0.01 $\pm$ 0.02	0.04 $\pm$ 0.06	-	-	-

		Baseline (pre-dose) Dose 1 iv	+ 1 week Dose 2 iv	+ 2 weeks Dose 3 iv	+ 3 weeks	+ 4 weeks	+ 5 weeks	+ 6 weeks
EO (10 ⁹ /L)	Cellax	0.02 ± 0.01	0.02 ± 0.04	0.02 ± 0.02	0.01 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.06 ± 0.09
	DTX	0.05 ± 0.02	0.09 ± 0.12	0.08 ± 0.04	0.10 ± 0.13	-	-	-

Table 4

Serology measurements for DTX and Cellax treated mice. Baseline values were analyzed pre-dosing. Dose 1 was administered after baseline measurement ($t=0$), and dose 2 and 3 were administered in the subsequent 2 weeks. Data = mean $\pm$ SD (n=5).

		Baseline (pre-dose) Dose 1 iv	+ 1 week Dose 2 iv	+ 2 weeks Dose 3 iv	+ 3 weeks	+ 4 weeks	+ 5 weeks	+ 6 weeks
ALP (IU/L)	Cellax	260 $\pm$ 24	307 $\pm$ 24	332 $\pm$ 23	258 $\pm$ 42	178 $\pm$ 22	171 $\pm$ 38	138 $\pm$ 7
	DTX	290 $\pm$ 20	250 $\pm$ 10	250 $\pm$ 78	145 $\pm$ 4	-	-	-
ALT (IU/L)	Cellax	39 $\pm$ 16	24 $\pm$ 5	38 $\pm$ 8	44 $\pm$ 15	31 $\pm$ 4	43 $\pm$ 11	46 $\pm$ 4
	DTX	37 $\pm$ 11	30 $\pm$ 14	43 $\pm$ 6	51 $\pm$ 20	-	-	-
AST (IU/L)	Cellax	104 $\pm$ 13	72 $\pm$ 9	88 $\pm$ 13	110 $\pm$ 20	88 $\pm$ 8	131 $\pm$ 23	132 $\pm$ 21
	DTX	83 $\pm$ 11	110 $\pm$ 35	87 $\pm$ 29	128 $\pm$ 43	-	-	-
Urea (mmol/L)	Cellax	10 $\pm$ 1	7.8 $\pm$	-	-	-	9 $\pm$ 1	12 $\pm$ 2
	DTX		6.3 $\pm$ 1.0	-	-	-	-	-
Creatinine (umol/L)	Cellax	15 $\pm$ 3	8.6 $\pm$ 2.7	-	-	-	16 $\pm$ 2	13 $\pm$ 4
	Cellax DTX		9.0 $\pm$ 3.7	-	-	-	-	-
Total Protein (g/L)	Cellax	54 $\pm$ 3	46.0 $\pm$ 1.6	-	-	-	48 $\pm$ 2	46 $\pm$ 2
	DTX		47.2 $\pm$ 1.9	-	-	-	-	-
Albumin (g/L)	Cellax	38 $\pm$ 1	33.8 $\pm$ 1.5	-	-	-	36 $\pm$ 2	34 $\pm$ 1
	DTX		36 $\pm$ 1.0	-	-	-	-	-
Globulin (g/L)	Cellax	16 $\pm$ 1	12.2 $\pm$ 0.4	-	-	-	12 $\pm$ 1	12 $\pm$ 1
	DTX		11.2 $\pm$ 1.3	-	-	-	-	-
A/G ratio	Cellax	2.4 $\pm$ 0.1	2.8 $\pm$ 0.1 3.2	-	-	-	3.0 $\pm$ 0.1	2.9 $\pm$ 0.2
	DTX		3.2 $\pm$ 0.4	-	-	-	-	-
Bilirubin (Total) umol/L	Cellax	1.5 $\pm$ 1.0	1.9 $\pm$ 0.2	-	-	-	1.5 $\pm$ 0.4	1.7 $\pm$ 0.5
	DTX		1.3 $\pm$ 0.8	-	-	-	-	-