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Original Article

Cabazitaxel-conjugated nanoparticles for docetaxel-resistant and bone metastatic prostate cancer

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ABSTRACT

Effective treatment of metastatic castration resistant prostate cancer (mCRPC) remains an unmet challenge. Cabazitaxel (CBZ) is approved for mCRPC after docetaxel (DTX) failure, but the improvement in survival is only moderate (~2 months) and patients suffer from significant side effects. Here, we report the development of a polymer based delivery system for CBZ to improve its safety and efficacy against DTX-resistant mCRPC. CBZ was conjugated to a carboxymethylcellulose-based polymer (Cellax-CBZ), which self-assembled into ~100 nm particles in saline and exhibited sustained drug release in serum at 10%/day. Cellax-CBZ delivered 157-fold higher CBZ to PC3-RES prostate tumor in mice and could be safely administered at a 25-fold higher dose compared to free CBZ, resulting in superior tumor inhibition in multiple mice models of DTX-resistant CRPC. In a metastatic bone model of CRPC, Cellax-CBZ significantly improves overall survival with a 70% long-term survival rate to day 120, while mice treated with free CBZ had a median survival of 40 days. Cellax-CBZ induced mild and reversible neutropenia in mice but no other tissue damage. Cellax-CBZ showed significant potential for improving therapy of mCRPC over clinically approved CBZ.

Introduction

Prostate cancer (PC) is the third leading cause of cancer-related deaths in men in developed countries [1,2]. Advanced PC can progress to castration-resistant PC (CRPC) of which 50–70% will metastasize to the bone [3]. Metastasis to the bone is often associated with disease progression and morbidity [4,5]. The current standard therapy for metastatic CRPC (mCRPC) is docetaxel (DTX) based chemotherapy [6,7]; however, patients eventually discontinue DTX because of cumulative toxicity and the development of drug resistance [8]. There are a number of treatment options for mCRPC, including the CYP17 lyase inhibitor abiraterone acetate involved in androgen biosynthesis inhibition [9–11], the androgen signaling cascade inhibitor enzalutamide [12–19], the immunotherapeutic sipuleucel-T [20–23], the bone targeting radiopharmaceutic radium-223 [24] and the chemotherapeutic cabazitaxel (CBZ). CBZ based chemotherapy was found to significantly improve overall survival by 2.4 months and was approved for DTX-resistant CRPC in 2010 [25–27]. Despite these advances, there has been no clinical evidence of any curative therapies available once the cancer has spread to the bone [4].

CBZ is a semisynthetic tubulin-binding taxane derivative. Unlike DTX, CBZ exhibits poor substrate affinity for P-glycoprotein (P-gp), an ATP-dependent efflux pump which has been found to be overexpressed in DTX-resistant tumors, including PC [3,25,26,28]. However, the clinical efficacy of CBZ against DTX-resistant CRPC is moderate and only increases the overall survival by 2.4 months [25–27]. This is because that CBZ is a highly toxic drug, limiting its clinical dose that can be safely administered to human patients (25 mg/m^2 every 3 weeks). The significant toxicity of CBZ is due to the poor selectivity of this cytotoxic [27,29], and we hypothesized that the effectiveness of CBZ could be significantly enhanced by improving its delivery. By enhancing CBZ targeting to tumors and sparing the rest of the body, the maximum tolerated dose of CBZ may be increased to exert improved therapy. This study focused on

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developing a new delivery technology to enhance targeting of CBZ to PC, thereby increasing its efficacy. Here we report the synthesis of a polymer-CBZ conjugate that self-assembled into a 100-nm particle. We characterized the nanoparticles and compared the biodistribution, in vivo antitumor effect and safety with free CBZ in multiple CRPC models in mice.

Materials & methods

Synthesis of Cellax-CBZ polymer

The acetylated carboxymethylcellulose (CMC-Ac) precursor was synthesised using a method described in detail elsewhere [30]. Subsequently, the CMC-Ac backbone was functionalized with $mPEG_{2000}$ and CBZ (Taizhou Bolon Pharmachem, Zhejiang, China) through the formation of ester bonds. CMC-Ac (300 mg, 1.17 mmol) was dissolved in acetonitrile (MeCN). EDC HCl (672 mg, 3.51 mmol), NHS (404 mg, 3.51 mmol), DMAP (143 mg, 1.17 mmol), mPEG2000-OH (702 mg, 0.35 mmol) and CBZ (391 mg, 0.47 mmol) were added to the reactor with trace amounts of dimethylformamide and water for complete solubilisation of the reactants. The reactor was maintained under constant stirring overnight and precipitated in diethyl ether to remove unreacted CBZ. The polymer was dried vacuum and triturated with water to extract unreacted PEG. Removal of free CBZ and PEG was repeated until negligible amounts could be detected by LC/MS. Chemical composition and coupling efficiency were determined by ^{1}H NMR with 2-methyl-5-nitrobenzoic acid as an internal standard (IS).^{1}H NMR was used to confirm the chemical composition of the drug-polymer conjugate (Cellax-CBZ polymer) (See supplementary information for ^{1}H NMR spectrum).

Preparation of Cellax-CBZ nanoparticles

Cellax-CBZ polymer was dissolved in MeCN at a concentration of 11 mg polymer/mL, and precipitated into 0.9% saline under constant vortexing. The resulting solution was centrifuged (4000 rpm, 5 min) to remove macro-aggregates, then loaded onto Slide-A-Lyer cartridges (10,000 MWCO, Pierce Biotechnology) and dialyzed overnight against 0.9% saline with two changes of external medium. The particle solution was then filtered through 0.22 mm Millipore PVDF filters, and concentrated in 10,000 MWCO Vivaspin filters (Fisher). Particle size and zeta potential were measured with a Zetasizer (Nano-ZS, Malvern Instruments, Malvern, UK). The concentration of CBZ within the nanoparticles was determined by ^{1}H NMR (Bruker, 500 MHz NMR spectrometer). Briefly, 100 μL of Cellax-CBZ suspension was diluted with 900 μL of deuterated DMSO and analyzed by ^{1}H NMR using a custom water-presaturation method with 2-methyl-5-nitrobenzoic acid as an internal standard.

Cell culture and animal protocols

PC3 purchased from American Type Culture Collection and C4-2B cells obtained from MD Anderson Cancer Center (Houston, TX) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The DTX-resistant variants of the cell lines were established by incubating the cells with gradually increasing concentrations of DTX until the cells became completely resistant to 6 nM DTX. The drug resistant lines were maintained with 6 nM of DTX, and exhibited ~100-fold increased IC50 for DTX compared to the parent lines [31]. 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.

Drug release

Cellax-CBZ nanoparticles were added to 1 mL of plasma or saline at a final concentration of 0.1 mg CBZ/ml containing 10 μg/ml d6-CBZ as an internal standard, and incubated at 37 °C for 0, 1, 24, 48, and 72 h. At each timepoint, a 20 μL aliquot was removed and added to 400 μL of MeCN/0.5% fomic acid. The samples were centrifuged at 14,000 rpm for 6 min. The supernatant was collected and analyzed by LC-MS using the method as described below to determine the released drug.

Biodistribution of Cellax-CBZ nanoparticles

Groups of NOD-SCID mice bearing PC3-RES tumor xenografts with a size of 60–100 mm^3 were treated with an i.v. dose of 20 mg CBZ/kg of Cellax-CBZ nanoparticles (n = 5/groups) or free CBZ, and sacrificed at various timepoints post injection. Organs and tissues were collected and analyzed for CBZ concentration by LC-MS-MS (supplementary information).

Maximum tolerated dose

Maximum tolerated dose (MTD) of drugs in NOD-SCID mice was determined in a dose escalation study, and is identified as the maximum dose of a drug that does not induce humane endpoints during the treatment. Health of mice was monitored for their appearance, body weight and natural behavior, and mice were scored using a clinical monitoring sheet (Supplementary Table 1). Humane endpoints include body weight loss >20% or an overall score ≥8.

Subcutaneous PC3-RES model

NOD-SCID mice were injected with 2.5×10^6 PC3-RES cells *s.c.* into the right lateral flank. Seven to ten days post tumor inoculation when tumors became palpable, mice were treated with Cellax-CBZ on a q159d cycle at MTD (55 mg CBZ/kg, n = 5), 75% MTD (41 mg CBZ/kg), and 60% MTD (33 mg CBZ/kg). Similarly, mice were treated with free CBZ on a q159d cycle at MTD (2 mg/kg, n = 5), 75% MTD (1.5 mg/kg), and 60% MTD (1.2 mg/kg). Control mice were treated with a single dose of saline (n = 5). Tumor size was determined by caliper measurements, with volume calculated as (length × width × width/2). Mice body weight and general health were monitored.

Bone metastatic C4-2B-RES prostate tumor model

Male NOD-SCID mice were anesthetized by isoflurane. The region from the inguen to the knee was cleared of fur, the knee was flexed to 90°, and the proximal side of the tibia was drawn to the anterior. A 25 5/8 gauge needle was inserted into the knee joint surface of the thigh bone through the patellar tendon, and inserted into the bone marrow cavity. A 28-gauge needle was subsequently inserted into the proximal terminus of the femoral opening and 30 μL 5×10^6 C4-2B-RES cells were directly injected into the bone marrow. The wound was cleaned with iodine scrub and the mice were maintained under analgesic (buprenorphine, 0.1 mg/kg) for 3 days after surgery. One day post intra-femoral tumor cell inoculation, groups of mice were treated with Cellax-CBZ (55 mg CBZ/kg, n = 10) or CBZ (2 mg/kg, n = 10) on a q159d cycle. Control mice were treated with a single dose of saline (n = 10). The mice were monitored for weight loss, limping, other behavioural changes and overall survival. Terminal endpoints were determined to be weight loss exceeding 20%, expression of significant discomfort (i.e. severe piloerection, withdrawn, abnormal behavior) or presence of other physical abnormalities (i.e. hind-limb paralysis, morbidity, seizures). Four weeks after 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.

Toxicology, hematology and serology

Mice were *i.v.* treated with free CBZ (2 mg/kg) and Cellax-CBZ (55 mg CBZ/Kg) at their MTDs, or saline control respectively (n = 3/group) on a q159d schedule. Blood and serum samples were collected at baseline, one day post administration of every dose, and at 1, 2, and 3 weeks after administration of the final dose. In a separate study, one day after a third dose of CBZ or Cellax-CBZ, mice were sacrificed under anesthesia and 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 (TCP).

Statistical analysis

Statistical comparisons were performed using SPSS (Statistical Package for the Social Sciences, v 14.0). All graphed data are expressed as the mean ± standard error. Statistical comparisons between two groups were made using the student's *t*-test, where $p < 0.05$ was considered to be statistically significant. Comparisons between three or more groups were made with one-way parametric analysis of variance (ANOVA). Post hoc analysis was conducted using Bonferroni's multiple comparisons correction.

Results

Preparation of the Cellax-CBZ

By ^{1}H NMR analysis, the Cellax-CBZ polymer (Fig. 1) was determined to contain 36 wt% CBZ and 11 wt% PEG. Analysis by LC/MS revealed less than 1% residual CBZ and PEG within the final polymer product. Cellax-CBZ particles were prepared from the polymer by the nanoprecipitation technique, and were dispersed in normal saline. The particles were 96 ± 5.3 nm in diameter with a polydispersity index (PDI) of 0.115 ± 0.036. Analysis of the Cellax-CBZ particle dose solutions indicated less than 0.1% residual free CBZ relative to coupled CBZ.