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A highly tumor-targeted nanoparticle of podophyllotoxin penetrated tumor core and regressed multidrug resistant tumors

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ABSTRACT

Podophyllotoxin (PPT) exhibited significant activity against P-glycoprotein mediated multidrug resistant (MDR) tumor cell lines; however, due to its poor solubility and high toxicity, PPT cannot be dosed systemically, preventing its clinical use for MDR cancer. We developed a nanoparticle dosage form of PPT by covalently conjugating PPT and polyethylene glycol (PEG) with acetylated carboxymethyl cellulose (CMC-Ac) using one-pot esterification chemistry. The polymer conjugates self-assembled into nanoparticles (NPs) of variable sizes (20–120 nm) depending on the PPT-to-PEG molar ratio (2–20). The conjugate with a low PPT/PEG molar ratio of 2 yielded NPs with a mean diameter of 20 nm and released PPT at ~5%/day in serum, while conjugates with increased PPT/PEG ratios (5 and 20) produced bigger particles (30 nm and 120 nm respectively) that displayed slower drug release (~2.5%/day and ~1%/day respectively). The 20 nm particles exhibited 2- to 5-fold enhanced cell killing potency and 5- to 20-fold increased tumor delivery compared to the larger NPs. The biodistribution of the 20 nm PPT-NPs was highly selective to the tumor with 8-fold higher accumulation than all other examined tissues, while the larger PPT-NPs (30 and 120 nm) exhibited increased liver uptake. Within the tumor, >90% of the 20 nm PPT-NPs penetrated to the hypovascular core, while the larger particles were largely restricted in the hypervascular periphery. The 20 nm PPT-NPs displayed significantly improved efficacy against MDR tumors in mice compared to the larger PPT-NPs, native PPT and the standard taxane chemotherapies, with minimal toxicity.

1. Introduction

Tubulin, a major component of the cellular cytoskeleton, plays an important role in the survival and growth of cells. Its functions extend from cellular transport to cell motility and mitosis. The importance of tubulin in mitosis and cell division makes them an attractive target for anticancer therapy. Chemotherapeutic agents that disrupt the normal function of tubulin are amongst the most potent and broadest spectrum anticancer agents in the clinic, and have been used to treat major cancers including lung, breast, prostate, and ovarian cancer [1]. A structurally diverse class of compounds has been found to antagonize tubulin functions with various tubulin binding sites and different mechanisms of action [1].

Anti-tubulin agents can be divided into two major categories, microtubule-destabilizing agents and microtubule-stabilizing agents, based on their effect on microtubule dynamics. Microtubule-destabilizing agents, such as colchicine and the vinca alkaloids, inhibit polymerization and decrease the mass of microtubules. Microtubule-stabilizing agents like taxanes stabilize microtubules, increase microtubule polymer mass, and induce the formation of microtubule bundles in cells. Both classes of anti-microtubule agents function by disrupting the dynamic equilibrium of the microtubules, resulting in arrest of cells in mitosis through blocking cell cycle at the metaphase–anaphase transition and leading to cellular apoptosis [2,3].

However, clinical success of these anti-microtubule agents has been compromised by the emergence of drug resistance [4]. Often, the resistance renders ineffectiveness to a variety of anticancer

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agents and is termed multidrug resistance (MDR). The therapeutic options after development of MDR are limited. MDR can be induced by various mechanisms, including decreased drug uptake, increased drug efflux, activation of detoxifying systems, activation of DNA repair mechanisms and evasion of drug-induced apoptosis [5]. Among these, overexpression of P-glycoprotein (Pgp) is the most commonly found mechanism for MDR in clinical samples [6,7]. Another shortcoming of these anti-tubulin drugs are their significant side effects, including drug induced neutropenia and neurotoxicity [8], as well as hypersensitivity reactions provoked by the surfactants used in the formulation to increase their solubility [9,10].

The goal of this study was to develop a new treatment for Pgp-mediated MDR tumors that are resistant to the standard taxane chemotherapies. Nanoparticle (NP) drug delivery system has been postulated to enhance activity of standard drugs such as taxanes and anthracyclines against MDR tumors by modulating the cellular uptake pathway [11]. It is hypothesized that NPs can carry drugs into an MDR cell via cellular endocytosis, bypassing the Pgp mediated drug efflux, and thus enhancing the drug activity. However, this class of products has not produced significant clinical success [12]. We aimed to explore an alternative approach: first identifying a potent anti-tubulin compound that is not a Pgp substrate, and then developing a NP formulation to selectively deliver the drug to the tumor. Additionally, NP drug delivery has been compromised by significant uptake by the reticuloendothelial system (RES) and poor tumor penetration, leading to impaired drug delivery and efficacy [13]. In this study, we also focused on optimizing the NP formulation to improve its targeting to tumors and its penetration into tumor core for enhanced safety and therapy.

We first screened a wide range of tubulin inhibitors against different MDR cell lines, and demonstrated that podophyllotoxin (PPT) remained active against those highly resistant lines. However, PPT exhibits poor solubility and a has a very narrow therapeutic window, preventing its systemic use for treating MDR cancer [14]. We hypothesized that PPT could be targeted to MDR tumors by NPs in a detergent and solvent free formulation to exert significant therapeutic activity with reduced side effects. We covalently conjugated PPT and polyethylene glycol (PEG) to an acetylated carboxymethyl cellulose (CMC-Ac) backbone via ester linkages in a one-pot reaction. The resultant polymer conjugates self-assembled into NPs of various sizes (20–120 nm) depending on the PPT-to-PEG ratio. Their drug release kinetics, cytotoxic potency and in vivo biodistribution were analyzed and the optimal formulation was tested for its efficacy and safety against MDR tumor models in mice with comparison to other standard taxane chemotherapies.

2. Materials and methods

2.1. Reagents and reference drugs

Podophyllotoxin (PPT) was obtained from Carbosynth (Berkshire, UK). Docetaxel (DTX), Paclitaxel (PTX) and Cabazitaxel (CBZ) were obtained from LC Laboratories (Woburn, MA). Cholchicin (Cho) and Vinblastin (Vin) were purchased from Sigma Aldrich (Oakville, ON). Carboxymethyl cellulose (CMC) sodium salt (CEKOL 30000-P) was purchased from CPKelco (Atlanta, GA). Poly(ethylene glycol) methyl ether (mPEG–OH, MW = 2000), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDC.HCl), and 4-dimethylaminopyridine (DMAP) were purchased from Sigma Aldrich (Oakville, ON). Hydrophobic fluorescent dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, D-307) was purchased from Invitrogen (Burlington, ON). For all the in vitro studies, free drugs were first dissolved in DMSO and then diluted with DMEM medium.

2.2. Synthesis of PPT-CMC-Ac-PEG polymer conjugate

The polymer conjugate was synthesized in a two-step reaction protocol. In the first step, sodium salt of CMC was acetylated as described by Namikoshi et al. [15]. Briefly, sodium CMC was first de-salted using 20% sulfuric acid solution, and the free acid was then acetylated with acetic anhydride to yield acetylated CMC (CMC-Ac). The purified CMC-Ac was subsequently conjugated to PEG and PPT via EDC/DMAP coupling chemistry. CMC-Ac (300 mg, 1.2 mmol acid) was weighed into a 25 mL round bottom flask, and dissolved in a mixture of anhydrous MeCN (9 mL) and DMSO (6 mL). EDC HCl (448 mg, 2.4 mmol) and DMAP (580 mg, 4.8 mmol) were added into that solution followed by addition of variable amount of PPT and m-PEG-OH. The solution was stirred overnight at room temperature with protection from light. The reaction mixture was then precipitated through 135 mL diethyl ether. The precipitate was dried, re-dissolved in MeCN, and the precipitation process was repeated twice. The final precipitate was dried under vacuum, and the fine powder was suspended in water (25 mL) and dialyzed (MW cut-off = 10 kDa) against MilliQ water for 24 h with 3 changes. The product was analyzed by LC-MS for uncoupled PEG and DTX, and washing was repeated if residual reagent was detected. The chemical composition of the polymer conjugate was determined by ^{1}H NMR using 2-methyl 5-nitro benzoic acid as an internal standard. The NPs were prepared by the nano-precipitation method using nanoAssemblr (Precision Nanosystems, Vancouver, Canada). Thirty mg of the polymer was dissolved in 1 mL MeCN and precipitated into 3 mL of normal saline in the nanoAssemblr at the flow rate of 18 mL/min. The formed particles were dialyzed in a Slide-A-Lyzer 10,000 MWCO cartridge against 0.9% saline overnight to extract solvent. The particles were filtered through a 0.22 μm Millipore PVDF filter, and were concentrated using a Vivaspin unit (10,000 MWCO). Particle size and zeta potential were measured with a Zetasizer (Nano-ZS, Malvern Instruments, Malvern, UK). PPT content of the NPs was determined by ^{1}H NMR using 2-methyl 5-nitro benzoic acid as an internal standard. DiI loaded NPs were prepared by dissolving 30 mg of the polymer in MeCN (1 mL) containing 0.1 mg/mL DiI and was precipitated into 3 mL of normal saline in the nanoAssemblr at the flow rate of 18 mL/min. DiI content of the NPs was determined by dissolving the NPs in DMSO and assaying for fluorescence (Excitation filter: 535 nm; Emission Filter 590 nm) and comparing to a calibration curve of fluorescence versus DiI concentration, subtracting the background signal of un-loaded particle fluorescence.

2.3. Cell culture and animals

Human MDA-MB-231 and PC3 and mouse EMT6 cancer cell lines were obtained from the American Type Culture Collection (ATCC). Resistant EMT6/AR1 cells were a gift from Ian Tannock, Princess Margaret Hospital, Toronto. DTX resistant PC3 and MDA-MB-231 cells were generated from the native phenotype by treating them with gradually increasing concentrations of DTX until the cells become fully resistant to 100 nM and 10 nM of DTX respectively. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. NOD-SCID and BALB/c mice were purchased from Jackson Laboratories (Bar Harbour, ME). All protocols were approved by the Animal Care Committee of the University Health Network.

2.4. In vitro release of PPT from the NPs

PPT-NPs were 1:1 (v:v) mixed with fetal bovine serum (FBS) at the final concentration of 100 μg PPT/mL. Samples were incubated at 37 °C, and at selected time points triplicate samples were removed and serum protein was precipitated using 600 μL MeCN containing 1% acetic acid. The sample was centrifuged for 5 min at 10,000 rpm and the supernatant was analyzed for released PPT by a Waters Acquity UPLC/MS system equipped with a PDA and SQ MS detector. The samples were injected into an Agilent XDB-C18 column (1.8 μm, 4.6 × 50 mm) at a flow rate of 0.4 mL/min, with a gradient program of 95/5 to 10/90 water/MeCN over 5 min. A calibration curve for PPT was prepared by spiking known amounts of PPT in a saline/FBS mixture, followed by the same extraction protocol.

2.5. Transmission electron microscopy (TEM)

The size and morphology of the NPs were determined by TEM using a Hitachi 7000 microscope (Schaumburg, IL) operating at an acceleration voltage of 75 kV. PPT-NPs were prepared as described previously with slight modification of using double distilled water as the precipitating media instead of normal saline. The NPs were negatively stained with a 1% uranyl acetate (UA) solution immediately prior to analysis. The samples were first deposited on copper grids (Ted Pella Inc., Redding, CA) that had been pre-coated with carbon and negatively charged and then stained with UA. The copper grids were briefly left to stand to allow the solvent to evaporate. The imaging was done at 100,000 magnification.

2.6. In vitro analysis of viability

Cell growth inhibition activity of different drugs was analyzed by measuring cell viability with the XTT assay. Briefly, cells were dislodged and re-suspended at a concentration of 1×10^4 cell/mL, and 100 μL of cell suspension per well was added to a 96 well plate. A slight modification (5×10^4 cell/mL) was made for the MDA-MB-231 analysis, as these cells were slow-growing compared to the other lines. The cells were maintained for 24 h in culture (37 °C, 5% CO_2, humidified) before treatment. Cells were treated with different concentration of the free drugs (primarily dissolved in DMSO and further diluted in DMEM) or NPs (suspended in normal saline and diluted in DMEM). After 72 h of treatment, viability was assayed by the XTT assay. Briefly, a 1 mg/mL solution of XTT reagent and 1.53 mg/mL solution of phenazine methylsulfate in water were prepared, and 5 μL of phenazine methylsulfate was