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Selective targeting and therapy of metastatic and multidrug resistant tumors using a long circulating podophyllotoxin nanoparticle

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ABSTRACT

Treatment options for metastatic and multidrug resistant (MDR) tumors are limited, and most of the chemotherapeutic drugs exhibit low efficacy against MDR cancers. An anti-tubulin agent podophyllotoxin (PPT) displays high potency against MDR tumor cells. However, due to its poor solubility and non-specificity, PPT cannot be used systemically. We have developed a self-assembling nanoparticle dosage form for PPT (named Celludo) by covalently conjugating PPT and polyethylene glycol (PEG) to acetylated carboxymethyl cellulose (CMC-Ac) via ester linkages. Celludo displayed extended blood circulation with an 18-fold prolonged half-life ($t_{1/2}$), 9000-fold higher area under the curve (AUC), and 1000-fold reduced clearance compared to free PPT. Tumor delivery was 500-fold higher in the Cellduo group compared to free PPT. Against the lung metastatic model of EMT6-AR1, Celludo showed selective localization in the metastatic nodules and increased the median survival to 20 d compared to 6–8 d with docetaxel and PPT treatment. In the intraperitoneal metastatic model of human ovarian NCI-ADR/RES tumor, Celludo prolonged the median survival from 50 d to 70 d, whereas the standard therapy PEGylated liposomal doxorubicin showed no effect. No major toxicity was detected with the Celludo treatment. These results demonstrate that Celludo is effective against metastatic and MDR tumors.

1. Introduction

Although early detection and novel therapeutic modalities has improved treatment of some cancers, no significant reduction in the mortality rate has been reported for majority of the advanced cancers that have developed metastases, including lung, prostate, colorectal and ovarian cancer [1]. In many of these cases, the tumors initially respond to chemotherapy and exhibit reduction in the mass, but they eventually relapse and become resistant to chemotherapy. During this period, many diseases develop into the metastatic stage. Treatment options are limited for these resistant and metastatic tumors, posing a formidable challenge. There are many mechanisms for the development of resistance in tumor cells, and the most studied and prevalent one is the over-expression of the membrane efflux pump, P-glycoprotein (Pgp) [2]. The majority of the commonly used chemotherapeutic drugs are substrates for Pgp, such as taxanes, vinca alkaloids, anthracyclines and epi-podophyllotoxins, hence are ineffective against Pgp over-expressing tumors [3,4]. Apart from drug resistance, effective delivery of chemotherapeutics to metastatic tumors remains a major challenge. Increasing dose of the drug has limited effect and in many instances drug toxicity becomes the limiting factor for the treatment of these patients [5]. The 5-year survival rate for localized breast cancer is 99%, but that declines to 24% for metastatic diseases [1]. For colorectal cancer, the 5-year survival rate is 90% when detected at a localized stage, and it reduces to 13% after tumors metastasize [1]. At the metastatic stages, surgery is no longer an effective treatment, and as most of the advanced metastatic tumors have developed multidrug resistance (MDR) [6,7], an effective therapy must be safe and can overcome the major MDR mechanisms such as Pgp overexpression. We have previously demonstrated that podophyllotoxin (PPT) is a potent drug against a panel of MDR tumor cells with an IC50 ~10 nM [8]. PPT is a natural product extracted from roots and rhizomes of Podophyllum species and is an anti-tubulin agent acting on the cholchicine-binding site in the

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tubulin, preventing the polymerization, which leads to mitotic arrest and cellular apoptosis [9]. PPT thus remains active against tumors that overexpress β-III tubulin [10]. However, PPT cannot be used systemically due to its poor solubility and selectivity, inducing significant side effects with a low maximum tolerated dose (MTD, 20 mg/kg in mice) [8]. It has been demonstrated that tumor vasculature is highly permeable, and nanoparticles (NP) can selectively accumulate in tumor tissues [11]. Furthermore, lymphatic drainage in tumors is usually compromised, and as a result, NPs can readily enter but cannot exit the tumor compartment [12]. This enhanced permeability and retention (EPR) effect of NPs provides a significant advantage over small molecule drugs. Additionally, NPs provide a detergent and solvent free formulation for drugs that are water insoluble. We have thus developed a NP drug delivery system for PPT, and this system (named Celludo) increased the PPT dose that could be safely administered to mice, resulting in enhanced efficacy in mice bearing different s.c. MDR tumors [8]. The previous work also reported the tissue distribution of the fluorescently labeled Celludo NPs [8], however the results were based on the dye that was passively loaded into Celludo NPs, but not PPT delivery. This manuscript focuses on comparing the pharmacokinetics and biodistribution of free PPT and Celludo. The efficacy of Celludo against metastatic and MDR tumor models was also examined in comparison with the standard therapies. Toxicology study was also performed to examine the safety of Celludo.

2. Materials and methods

2.1. Materials and reagents

Podophyllotoxin (PPT) was purchased from Carbosynth Limited (Compton, Berkshire, UK). Docetaxel (DTX) and Paclitaxel (PTX) were obtained from LC Laboratories (Woburn, MA). Doxorubicin (DOX) was purchased from Tocris Bioscience (Ellisville, MO). Poly(ethylene glycol) methyl ether (mPEG−OH, MW = 2000; no polydispersity index (PDI) data available), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDC.HCl), and 4-dimethylaminopyridine (DMAP) were purchased from Sigma Aldrich (Oakville, ON, Canada). Hydrophobic fluorescent dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, D-307) was purchased from Invitrogen (Burlington, ON, Canada). Ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO). Methyl-tert-butyl ether, acetonitrile, and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NY), hydrochloric acid (1.0 M) from VWR (West Chester, PA). Ultra pure water was prepared using Milli-Q Synthesis system (Millipore, Billerrica, MA). Sodium carboxymethylcellulose (CMC) (CEKOL 30000, degree of substitution = 0.82 was received from CPKelco (Atlanta, GA). Slide-a-Lyzer dialysis cartridges were purchased from pierce Biotechnology (Rockford, IL). Vivaspin 10 kDa MWCO ultracentrifugation filters were purchased from Fisher Scientific (Ottawa, ON, Canada). Formic acid (99.99%) and morpholine (99.99%) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Blank BALB/c mouse plasma and liver homogenate were purchased from Bioreclamation IVT (Chestertown, NY). Homogenate Navy RINO Lysis kit 50 for sample homogenizing was purchased from FroggaBio Inc (Toronto, ON, Canada). All other general laboratory chemicals were purchased from Fisher Scientific Fisher Scientific (Ottawa, ON, Canada) and VWR scientific (Mississauga, ON, Canada). Resistant EMT6/AR1 cells overexpressing P-glycoprotein (Pgp) were a gift from Dr. Ian Tannock (Princess Margaret Hospital, Toronto, ON, Canada). NCI-ADR/RES cells were obtained from National Cancer Institute (Frederick, MD).

2.2. Synthesis and preparation of celludo nanoparticles (NPs)

Celludo NPs were prepared as described previously [8]. Briefly, m-PEG-OH and PPT were conjugated to acetylated carboxymethyl cellulose (CMC-Ac) 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 PPT (340 mg, 0.8 mmol) and m-PEG-OH (1200 mg, 0.6 mmol). After an overnight reaction, the mixture was 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 dialyzed (MW cut-off = 10 kDa) against MilliQ water for 24 h with 3 changes. It was then lyophilized into a dry powder form. The NPs were prepared by the nano-precipitation method using a microfluidic mixing device NanoAssemblr (Precision Nanosystems International, Vancouver, BC, Canada). Thirty mg of the polymer conjugate was dissolved in 1 mL MeCN and precipitated into 3 mL of normal saline in the NanoAssemblr at a flow rate of 18 mL/min. The formed particles were dialyzed in a Slide-A-Lyzer 10,000 MWCO cartridge against 0.9% saline for 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 in 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. Determination of critical aggregation concentration (CAC)

CAC of Celludo was determined by using the fluorescence depolarization method [13]. Briefly, 1,6-Diphenyl-1,3,5-hextriene (DPH, 1.175 mg) was dissolved in MeCN (10 mL) to form a stock solution. Celludo polymer (10 mg) was serially diluted with the DPH solution to form a series of 10 concentrations of Celludo (1, 0.5, 0.25, 0.05, 0.025, 0.005, 0.0025, 0.0005, 0.00025, 0.00005 mg/mL) in a constant concentration of DPH, 100 μL of each sample were precipitated dropwise in 900 μL normal saline on a vortexer at room temperature for 1 min. One hundred μL of each particle solution was transferred to a 96-well microplate, and fluorescence was measured (Ex 360 nm, Em 460 nm) on a plate reader.

2.4. Instrumentation and experimental conditions

Plasma and tissue concentrations of Celludo and free PPT were determined by an ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC/MS/MS) method using cabazitaxel (CBZ) as an internal standard (IS). The UHPLC/MS/MS system consisted of an Agilent 1290 Infinity Binary Pump, a 1290 Infinity Sampler, a 1290 Infinity Thermostat, and a 1290 Infinity Thermostatted Column Compartment (Agilent, Mississauga, ON, Canada) connected to an AB Sciex QTrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer equipped with a Turbo Spray source (AB Sciex, Concord, ON, Canada). The mass spectrometer was operated in positive ionization mode and data were