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Polymer conjugate of a microtubule destabilizer inhibits lung metastatic melanoma

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ABSTRACT

Melanoma is the most aggressive type of skin cancer. It is highly metastatic, migrating through lymph nodes to distant sites of the body, especially to lungs, liver and brain. Systemic chemotherapy remains the mainstay of treatment; however, the development of multidrug resistance (MDR) restricts the efficacy of current chemotherapeutic drugs. We synthesized a series of microtubule destabilizers, substituted methoxybenzoyl-ary-thiazole (SMART) compounds, which inhibited tubulin polymerization and effectively circumvented MDR. Due to poor water solubility of SMART compounds, co-solvent delivery is required for their systemic administration, which is usually associated with hepatotoxicity, nephrotoxicity and hemolysis. To solve this problem and also to increase circulation time, we synthesized a new SMART analogue, SMART-OH, and its polymer-drug conjugate, methoxy-poly (ethylene glycol)-*block*-poly (2-methyl-2-carboxyl-propylene carbonate-*graft*-SMART-*graft*-dodecanol) (abbreviated as P-SMART), with 14.3 ± 2.8% drug payload of SMART-OH. Similar to its parent drug, P-SMART showed significant anticancer activity against melanoma cells in cytotoxicity, colony formation, and cell invasion studies. In addition, P-SMART treatment led to cell cycle arrest at G2/M phase and cell accumulation in sub-G1 phase. We established a model of metastatic melanoma to the lung in C57/BL6 albino mice to determine *in vivo* efficacy of P-SMART and SMART-OH at the dose of 20 mg/kg. P-SMART treatment resulted in significant inhibition of tumor growth and prolonged mouse median survival. In conclusion, P-SMART, a novel polymer-microtubule destabilizer conjugate, has the potential to treat metastatic melanoma.

1. Introduction

Over the past several decades, the development of nanomedicines has been driven by the increasing demands of delivering therapeutic agents to disease sites efficiently. A large amount of pioneering research has highlighted applications of micelles, nanoparticles, liposomes, polymersomes, nanogels and dendrimers as nanocarriers of low molecular-weight drugs, oligonucleotides and genes. Polymer-drug conjugates debuted in 1955 [1], and in the mid-1970s Ringsdorf proposed the idea of conjugating therapeutic agents to water soluble polymers [2]. Since then, the field of polymer-drug conjugates started a new era of drug delivery and has been growing fast. Advantages of conjugates over their corresponding parent drugs include: 1) increased aqueous solubility of hydrophobic drugs; 2) prolonged blood circulation time; 3) enhanced bioavailability; 4) increased protection of drugs from degradation; 5) increased tumor accumulation either due to enhanced permeability and retention (EPR) effect or tunable targeting moieties. Unlike physically drug encapsulation into nanoparticles and micelles, covalent drug conjugation to polymers achieves enhanced drug payload and prevents premature drug release, thereby decreasing undesired toxicities compared to physically drug-encapsulated liposomes, nanoparticles and micelles. The polymer-drug conjugate market is currently becoming well-established with several commercialized products available for a wide range of disease states, such as Adynovate by Baxalta, Movantik™ by AstraZeneca, Oncospar® by Enzon Pharmaceuticals, Plegridy® by Biogen, *etc.*

Malignant melanoma is the most invasive form of skin cancer with high metastatic propensity, typically metastasizing to the lymph nodes, lungs, liver, brain and heart at late stage of melanoma. The median overall survival time of patients suffering from metastatic melanoma is less than one year, and only about 10% of these patients survive more than 5 years after diagnosis [3]. Unfortunately, the survival of patients with advanced metastatic melanoma has not been significantly

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improved by current Food and Drug Administration (FDA)-approved systemic chemotherapies [4]. Dacarbazine (DTIC), a widely used chemotherapeutic agent for treatment of metastatic melanoma, shows transient efficacy in most patients, however, only 1–2% of patients achieve a durable long-term response to this therapy [5]. The combination of paclitaxel and carboplatin is used as second-line therapy for patients who suffer from disease progression while receiving DTIC treatment. Clinical benefit of this combination therapy was noted in more than 40% of all patients in the original study [6,7]. Nevertheless, a potential problem when using paclitaxel or other microtubule inhibitors for cancer treatment in the long term is that their anticancer effects could be undermined by intrinsic or acquired drug resistance due to overexpression of drug efflux pumps (P-glycoprotein, MRP and BCRP) in cancer cells [8–10]. To address this problem, we have synthesized a series of novel microtubule destabilizers, substituted methoxybenzoyl-ary-thiazole (SMART) compounds, with nanomolar anticancer activity against melanoma, breast cancer, ovarian cancer, colon cancer and prostate cancer [11]. In addition, their ability to circumvent P-gp mediated drug resistance was confirmed by using prostate cancer cells with P-gp overexpression [12]. However, the clinical translation of SMART compounds is limited due to their poor aqueous solubility as many other anticancer agents. Moreover, these small molecular weight drugs are rapidly eliminated from the circulation, requiring frequent dosing, leading to increased risk of side effects. To address this issue, we formulated SMART-100 in micelles using poly (ethylene)-*b*-poly(D,L-lactide) (PEG-PLA) in a previous study, but the utility of physical encapsulation is limited by low drug payload. Therefore, in the current study we synthesized a new SMART analogue, SMART-OH and conjugated this compound to the carboxyl pendant groups of poly (ethylene glycol)-*block*-poly (2-methyl-2-carboxyl-propylene carbonate) (mPEG-b-PCC). The polymeric conjugate consists of three components including biocompatible PEG blocks, a biodegradable polycarbonate backbone and lipid chains of dodecanol (DC). The anticancer effect of SMART-OH and its polymer-drug conjugate mPEG-b-PCC-g-SMART-g-DC (abbreviated as P-SMART) on melanoma cells was determined. Furthermore, a mouse model of metastatic melanoma to the lungs was established to study *in vivo* efficacy of P-SMART as well as SMART-OH.

2. Materials

4-Cyanophenol, L-cysteine, *N*,*N*,*N*′,*N*′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), tert-butyldimethylsilyl chloride (TBDMSCl), *n*-butyllithium, tetra-*n*-butylammonium fluoride, 2, 2-bis(hydroxymethyl) propionic acid, methoxy poly(ethylene glycol) (mPEG, Mn = 5000), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) 98%, 1-hydroxybenzotriazole (HOBT), Benzyl bromide, Dodecanol (DC), diisopropylethylamine (DIPEA) and cremophor® EL were purchased from Sigma Aldrich (St. Louis, MO). FxCycle™ PI/RNase staining solution was purchased from ThermoFisher Scientific (Waltham, MA). Bovine brain tubulin was purchased from Cytoskeleton (Denver, CO).

3. Methods

3.1. Synthesis and characterization of SMART-OH

2-(4-Hydroxyphenyl)-4,5-dihydrothiazol-4-yl-3,4,5-trimethoxyphenyl)methanone (abbreviated as SMART-OH) was synthesized as shown in Fig. 1A (compound **5**). Briefly, 4-cyanophenol (1 equiv.) was mixed with L-cysteine (1 equiv.) in a 1:1 solution of MeOH/pH 6.4 PBS. The reaction mixture was heated to 40 °C and stirred for 3 days. The mixture was then filtered to remove the precipitate and MeOH was removed using a rotary evaporator. The remaining solution was then acidified to pH 4 using 1 M HCl and CH_2Cl_2 was added to the solution. The resulting precipitate was filtered to yield a white solid, compound **1**. This solid was dried overnight in a vacuum desiccator and then used directly for the next step.

Compound **1** (1 equiv.) was dissolved in anhydrous CH_2Cl_2. HBTU (1.1 equiv.) was then added and stirred for 15 min. This was followed by addition of DIPEA (2.2 equiv.) which was stirred for 2–3 min. Finally, $HNCH_3OCH_3$ HCl salt (1.1 equiv.) was added and the reaction was stirred at room temperature for 12–18 h. The reaction mixture was washed once with ddH_2O and twice with saturated NaCl solution. The organic layer was then dried over $MgSO_4$. The solvent was removed by a rotary evaporator to yield crude yellow oil. This was then purified by flash chromatography to obtain compound **2**.

A solution of compound **2** (1 equiv.) in anhydrous tetrahydrofuran (THF) was kept under argon and cooled to 0 °C. Imidazole (2.5 equiv.) and TBDMSCl (2 equiv.) were then added to the solution and the mixture was stirred for 12–18 h. The solvent was removed by a rotary evaporator and the resulting solid was dissolved in CH_2Cl_2 and washed once with ddH_2O and once with saturated NH_4Cl solution. The organic layer was dried over $NaSO_4$ followed by purification using flash chromatography to yield compound **3**.

Compound **3** (1 equiv.) was dissolved in freshly distilled THF at room temperature while 3,4,5-methoxylphenyl (1.2 equiv.) was dissolved in freshly distilled THF in a separate flasks and cooled to −78 °C under argon. *n*-Butyllithium (1.5 equiv.) was then added to the cooled mixture and stirred at −78 °C for 30 min. The solution containing compound **3** was then added to the mixture and stirred for 2 h while returning to room temperature. The reaction was quenched with saturated NH_4Cl solution, extracted three times with ethyl acetate, dried over $NaSO_4$, and purified by flash chromatography to yield a bright yellow solid, compound **4**.

Compound **4** (1 equiv.) was then dissolved in THF and cooled to 0 °C. Tetra-*n*-butyl ammonium fluoride (2 equiv.) was then added and the mixture was stirred for 10 min. The reaction was quenched with saturated NH_4Cl solution, extracted three times with ethyl acetate, dried over $NaSO_4$, and purified using flash chromatography to yield the final pure product (compound **5**, Fig. 1A).

3.2. Docking

Docking studies were carried out using the crystal structures of the α,β-tubulin dimer in complex with DAMA-colchicine (Protein Data Bank code 1SA0). Schrodinger Molecular Modeling Suite 2016 (Schrodinger Inc., Portland, OR) running on Microsoft Windows 7 platform was used to perform these studies, similar to what we described in previous reports [13–15]. Briefly, the protein-ligand complex was prepared using the Protein Preparation module, and SMART-OH was docked into the colchicine binding site in the structure of 1SA0 using Glide module. Data analyses were performed using the Maestro interface of the software.

3.3. Tubulin polymerization assay

Bovine brain tubulin (3.33 mg/m) was exposed to 10 μM of SMART-OH, colchicine or vehicle control (5% DMSO), respectively, and incubated in 100 μl of general tubulin buffer (80 mM PIPES, 2.0 mM $MgCl_2$, 0.5 mM EGTA and 1 mM GTP; pH 6.9). Absorbance at 340 nm was monitored at 37 °C every minute for 15 min by the SYNERGY 4 Microplate Reader (Bio-Tek Instruments, Winooski, VT).

3.4. Synthesis and characterization of mPEG-b-PCC-g-SMART-g-DC (P-SMART)

2-Methyl-2-benzyloxycarbonyl-propylene carbonate (MBC), poly (ethylene glycol)-*block*-poly(2-methyl-2-benzoxycarbonyl-propylene carbonate) ($mPEG_{114}$-b-PBC_{28}) and poly(ethylene glycol)-*block*-poly(2-methyl-2-carboxyl-propylene carbonate) ($mPEG_{114}$-b-PCC_{28}) were synthesized as described previously [16]. SMART-OH and DC