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Polymeric Nanocarriers Delivering Anticancer Agents for the Treatment of Chemoresistant Prostate Cancer and Lung Metastatic Melanoma

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POLYMERIC NANOCARRIERS DELIVERING ANTICANCER AGENTS FOR THE TREATMENT OF CHEMORESISTANT PROSTATE CANCER AND LUNG METASTATIC MELANOMA

by

Ruinan Yang

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
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Under the Supervision of Professor Ram I. Mahato

University of Nebraska Medical Center
Omaha, Nebraska

June, 2018

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ACKNOWLEDGEMENT

This is a delightful moment for me as I have survived all the unforgettable ups and downs and come to the end of the Ph.D. journey that is full of challenges and gains. At this special moment, I would like to express my appreciation to those who have supported me and contributed to this thesis.

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Ruinan Yang

May 2018
ABSTRACT: POLYMERIC NANOCARRIERS DELIVERING ANTICANCER AGENTS FOR THE TREATMENT OF CHEMORESISTANT PROSTATE CANCER AND LUNG METASTATIC MELANOMA

Ruinan Yang, Ph.D.
University of Nebraska, 2018

Supervisor: Ram I. Mahato, Ph.D.

The aims of this thesis is to first develop novel combination chemotherapies of two anticancer agents and their appropriate drug delivery platforms to treat chemoresistant prostate cancer, and second develop a polymeric conjugate of a biodegradable polymer and novel tubulin destabilizer to treat lung metastatic melanoma.

In Chapter 1, a general introduction of polymeric nanocarriers including polymeric micelles and polymer drug conjugates for cancer therapy was given. In Chapter 2, we described a combination therapy of paclitaxel (PTX) polymer conjugate and cyclopamine (CYP) polymer conjugate, which had the potential to treat chemoresistant prostate cancer. We first synthesized mPEG-b-PCC-g-PTX-g-DC (P-PTX) and mPEG-b-PCC-g-CYP-g-DC (P-CYP) polymer-drug conjugates, which they self-assembled into micelles. The combination of P-PTX and P-CYP alleviated PTX resistance and suppressed tumor colony formation. Further, combination therapy inhibited Hedgehog (Hh) signaling and upregulated tumor suppressor miRNAs. We established orthotopic prostate tumor in nude mice and there was significant tumor growth inhibition in the group treated with the combination therapy of P-PTX and P-CYP compared with monotherapy. In
Chapter 3, we designed a novel microtubule destabilizer QW-296 and combined it with a newly synthesized Hh signaling inhibitor MDB5 to treat taxane-resistant prostate cancer. The combination of QW-296 and MDB5 exhibited stronger anticancer activity towards chemoresistant prostate cancer cells than single drug treatment, and the results revealed that they synergistically worked together via distinct but complementary mechanisms. To improve the translation and promote therapeutic efficacy of the two novel anticancer agents, we synthesized an amphiphilic copolymer mPEG-p(TMC-MBC) that could self-assemble into polymeric micelles and encapsulate two hydrophobic drugs with high drug loading. We established a model of orthotopic prostate tumor in nude mice to evaluate in vivo efficacy of QW-296, MDB5 and their combination. The results confirmed that combination of QW-296 and MDB5 in micelles showed maximum inhibition of tumor growth compared with monotherapy or combination therapy in co-solvent.

In Chapter 4, we introduced a new microtubule destabilizer 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). 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 the
end, Chapter 5 summarized the featured results of this thesis and gave some suggestions for future studies.
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LIST OF ABBREVIATIONS

hormone refractory prostate cancer  HRPC

cancer stem cells  CSCs

microRNA  miRNA

Hedgehog signaling  Hh signaling

Cyclopamine  CYP

Paclitaxel  PTX

mPEG-b-PCC-g-PTX-g-DC  P-PTX

mPEG-b-PCC-g-CYP-g-DC  P-CYP

enhanced permeability and retention  EPR

High Performance Liquid Chromatography  HPLC

critical micelle concentrations  CMC

Polydispersity index  PDI

dodecanol  DC

dichloromethane  DCM

poly(ethylene glycol)  PEG

poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate)  mPEG-b-PCC

5-Methyl-5-benzylxocarbonyl-1,3-dioxane-2-one  MBC

Trimethylene carbonate  TMC

poly(ethylene glycol)-block-poly(trimethylene carbonate-co-2-methyl-2-benzoxy carbonyl-propylene carbonate)  mPEG-p(TMC-MBC)

Dacarbazine  DTIC
<table>
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<th>Term</th>
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<tr>
<td>substituted methoxybenzoyl-ary-thiazole</td>
<td>SMART</td>
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<tr>
<td>2-(4-Hydroxyphenyl)-4,5-dihydrothiazol-4-yl-3,4,5-</td>
<td></td>
</tr>
<tr>
<td>trimethoxyphenyl)methanone</td>
<td>SMART-OH</td>
</tr>
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<td>mPEG-b-PCC-g-SMART-g-DC</td>
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<tr>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
<td>DBU</td>
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<td>N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride</td>
<td>EDC</td>
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<td>1-hydroxybenzotriazole</td>
<td>HOBT</td>
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<td>N,N,N’,N’-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate</td>
<td>HBTU</td>
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<td>TBDMSCI</td>
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CHAPTER 1 INTRODUCTION

1.1 POLYMERIC NANOCARRIERS FOR CANCER TREATMENT

Even though the overall cancer death rate has dropped by more than 20% over two decades, cancer accounts for the most common cause of death in the United States. Approximately, there are 439 new cases of cancer and 163 cancer deaths every 100,000 men and women per year. Globally, there were 14.1 million new cases and 8.2 million cancer-related deaths in 2012. These statistics indicate that progresses in preclinical research of cancer biology have slowly been translated into actual improvement in cancer therapy and much work still remains, which encourages more emerging collaborations among oncologists, cancer biologists, biomaterial scientists and biomedical engineers. Among various clinical therapies for cancer treatment, chemotherapy is still the mainstay, however, the inefficiency and side effects of chemotherapy associated with conventional formulations of anticancer small molecules are urgently needed to overcome. Many chemotherapeutic drugs have poor aqueous solubility, which requires appropriate formulation to assist solubilization when intravenous administration needed. Cremophor EL, for example, is the formulation vehicle for paclitaxel and also known to exert a range of side effects even including anaphylactoid hypersensitivity and nephrotoxicity [1]. Off-target to the site of interest and wide biodistribution via bloodstream of anticancer agents also attribute to inefficiency of chemotherapy, suggesting the ideal formulation should have the capability to deliver and release drugs precisely at the target sites in a sustained and controlled manner.
Nanomedicines have been no doubt gaining tremendous recognition as indispensable tools to assist and boost chemotherapies and other traditional cancer therapies. They are designed to much elevate water solubility of given drugs, provide selective delivery of anticancer agents to target tumor site, and has the potential to alter the pharmacokinetics of those drugs leading to extended blood circulation time and accumulation at the site of action. A variety of innovative nano-delivery platforms such as polymeric nanocarriers, liposomes, dendrimers, nano-hydrogels have been developed and applied to deliver specific anticancer agents including small-molecular-weight drugs, proteins, peptides and genes. Synthetic polymer-based nanocarriers have been extensively investigated among a variety of nanocarrier systems due to their amphiphilic characters and chemical versatility [2]. This chapter will mainly focus on the introduction and recent research progress of polymeric micelles and polymer-drug conjugates.

1.2 POLYMERIC MICELLES

Polymeric micelles, which were initially introduced by Ringsdorf in 1984 [3], are assembled by amphiphilic block copolymers in aqueous milieu. They are characterized by the core-shell architecture, where hydrophobic composition are segregated from hydrophilic exterior to form an inner core. Core segregation from aqueous solvent is the major driving force for micelle formation along with a variety of intermolecular forces such as hydrophobic interaction, electrostatic interaction, and hydrogen bonding [4-13]. Several advantages of polymeric micelles make them favorable drug carriers compared with conventional dosage forms. First, polymeric micelles have a size range from several tens to a hundred of nanometers
with a narrow distribution, which can help micelles avoid from rapid renal clearance and retain in the target tissue with an enhanced permeation retention (EPR) effect involved [14]. Second, the amphiphilic character of micelles protects hydrophobic drugs in of the core from degradation of hydrolytic enzymes, and accounts for relatively prolonged retention time in blood circulation. It has been reported that nanoformulations with hydrophobic outer surfaces tend to be easily taken up by reticuloendothelial system (RES) or mononuclear phagocyte system (e.g. monocytes and macrophages) [15,16], whereas those with hydrophilic surfaces could minimize protein adsorption, prevent unwanted aggregation between micelles, and show increased circulation time [17]. Furthermore, shell surface of polymeric micelles can be decorated by a variety of functional groups facilitating receptor-mediated drug delivery. Poly(ethylene glycol) (PEG) has been widely used as hydrophilic backbone of polymeric micelles due to their biocompatible hydrophilic nature and the stealth behavior resulting in reduced interaction with plasma proteins and cell-surface proteins [18-20].

1.3 POLYMER-DRUG CONJUGATES

Polymer and anticancer drug conjugates have also drawn extensive attention to researchers for cancer therapy from bench side to bedside. Generally, the design of polymer conjugates is to chemically combine drug molecules to an amphiphilic block polymer to form a macromolecular prodrug. Although, the conjugated drug may lose some therapeutic activity in the form of the prodrug, there are several advantages of the conjugates over corresponding parent drugs such as less side effects, enhanced accumulation to site of interest and improved
patient compliance, which makes polymer-drug conjugates have become a fast-growing application in clinical studies. Similar with polymeric micelles, PEG is one of the most common hydrophilic polymers to be selected as hydrophilic blocks, because it has chemical flexibility of the chain in favor of subsequent conjugation and it is approved by FDA for human use [21]. However, PEG only has two terminal groups able to conjugate with drugs, thus the selection of hydrophobic segment for PEG copolymer are mainly based on its drug conjugating capacity that enables reaction with reactive groups on the drug molecule. A variety of monomers that have reactive groups such as OH, COOH, NH₂, CH=CH₂, C≡CH can be introduced the polymer chains [22-27]. The linker or the spacer between polymer and small molecule are supposed to be relatively stable during transport in physiological condition and hydrolysed at the site of target. In contrast with PEG, N-(2-hydroxypropyl)-methacrylamide copolymers (HPMA), another widely used hydrophilic block, has multiple reactive groups and can be simultaneously functionalized with therapeutic agents, targeting moieties as well as imaging moieties. Typically, a lysosomally degradable peptidyl Gly-Phe-Leu-Gly is most commonly used linker for HPMA drug conjugation.
2.1 INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths in American men. Although androgen deprivation therapy (or androgen suppression therapy) is effective in treating prostate cancer in early stage [28], shrunken tumors often become androgen-independent at 18-24 months post-treatment [29], leading to aggressive and metastatic forms of prostate cancer, also known as hormone refractory prostate cancer (HRPC). Taxane (docetaxel and paclitaxel (PTX)) is one of the standard therapies for HRPC [30]. While these anticancer agents have shown significant efficacy at the initial stage of chemotherapy, the long-term efficacy is limited and patients will suffer from relapse owing to the development of chemoresistance [31-35]. Therefore, there is an urgent need to improve the long-term efficacy of current chemotherapy.

Hedgehog (Hh) signaling influences the initiation and progression of cancer and is needed for regeneration of prostate epithelium through crosstalk with androgen signaling [36-39]. Therefore, the inhibition of Hh pathway has the potential to induce anti-proliferative and apoptotic effect on prostate cancer cells. Furthermore, Hh signaling involves in the initiation and development of cancer

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1 R. Yang, G. Mondal, D. Wen, R.I. Mahato, Combination therapy of paclitaxel and cyclopamine polymer-drug conjugates to treat advanced prostate cancer, Nanomedicine. (2016)
stem cells (CSCs), which are a distinct subset of cancer cells with self-renewal/differentiation and tumorigenic potentials [40-42]. Thus, depleting CSCs at the early stage is essential to inhibit tumor growth and metastasis.

Cyclopamine (CYP), a naturally occurring antagonist of Hh signaling, binds to cell surface receptor smoothened (SMO) resulting in suppression of SMO activity and inhibition of Hh signaling pathway [43,44]. CYP has shown to inhibit the proliferation of DU145, PC3 and 22RV1 cells and reduce tumor growth in mice [45,46]. In this regard, a combination therapy of PTX and CYP could have synergistic therapeutic efficacy of treating advanced prostate cancer and also lay the foundation for promising chemotherapy. However, these drugs are highly hydrophobic, which require an appropriate drug delivery system, such as liposomes, nanoparticles and micelles, for their formulation and delivery to the tumor after systemic administration. Polymer-drug conjugates acquire wide attention and some of them are undergoing clinical trials or ready to reach the market [47-52]. Unlike physically drug encapsulated liposomes, nanoparticles and micelles, polymer-drug conjugates prevent premature drug release and undesired toxicities as covalently linked polymer-drug conjugates are more stable in the circulation and release drugs in a controlled manner at the tumor site with therapeutically effective concentration. Moreover, covalent drug conjugation to polymers achieves enhanced drug payload compared to physically encapsulated micelles or nanoparticles. Additionally the nano-size ensures preferential accumulation of these conjugates in tumor cells via the enhanced permeability and retention (EPR) effect [53].
In our previous studies [54], we synthesized poly (ethylene glycol)-block-poly (2-methyl-2-carboxyl-propylene carbonate) (mPEG-b-PCC) for drug delivery. In this study, we conjugated PTX and CYP to the carboxyl pendant groups of mPEG-b-PCC (Mw: mPEG 5000 Da, PCC 4480 Da, 28 units). Besides PTX and CYP, the final polymeric conjugates consist of three components, biocompatible PEG blocks, a biodegradable polycarbonate backbone and dodecanol (DC) lipid chains. PEG ensures the stealth properties of polymeric conjugates and the polycarbonate backbone has low toxicity since its degradation products are CO2 and alcohol. In addition, DC can increase requisite hydrophobicity and thus assist in self-assembly into nano-sized micelles. This conjugation strategy has the potential to effectively deliver drugs to tumors after systemic administration and treat orthotopically implanted prostate cancer in mice by killing both bulk tumor cells and CSCs.

2.2 METHODS

2.2.1 Synthesis of mPEG-b-PCC-g-PTX-g-DC (P-PTX)

Synthesis scheme of polymer-drug conjugates is illustrated in Figure 1. Monomer 2-methyl-2-benzyloxycarbonyl-propylene carbonate and its copolymer with mPEG (mPEG-b-PBC) were synthesized by ring opening polymerization followed by hydrogenation to obtain the copolymer containing carboxyl pendant groups (mPEG-b-PCC) as previously described. PTX and DC were conjugated to the carboxyl groups of mPEG-b-PCC polymer using carbodiimide coupling. mPEG-b-PCC (75 mg, 0.0079 mmol) was dissolved in anhydrous dichloromethane (DCM) followed by addition of PTX (54 mg, 0.063 mmol), DCC (65 mg, 0.31 mmol),
DMAP (29 mg, 0.24 mmol). The reaction was stirred for three days under nitrogen atmosphere at 4°C and then DC (23.5 mg, 0.12 mmol) was added and the reaction was allowed to proceed for another two days (Figure 1A). Crude product was purified by precipitation in large excess of diethyl ether and then by dialysis against ethanol using a regenerated cellulose membrane with 7K MWCO.

2.2.2 Synthesis of mPEG-b-PCC-g-CYP-g-DC (P-CYP)

**Synthesis of Boc-β-Ala-TT.** Boc-β-Ala-OH (94.6 mg, 0.5 mmol), 2-mercaptothiazoline (59.6 mg, 0.5 mmol) and DMAP (61.1 mg, 0.5 mmol) were dissolved in DCM under nitrogen protection. The solution was first stirred for 5 min at -10 °C followed by adding EDCI (95.9 mg, 0.5 mmol) and the solution was stirred at -10 °C for 3 h and another 14 h at room temperature. After reaction, 40 mL DCM was added and the solution was washed with NaHCO3 aq. (0.1 M), HCl (0.1 M) and then NaCl aq. The organic layer was dried by Na2SO4 and the crude product was purified by column chromatography (silica gel 60 Å, 200-400 mesh, EtOAc: CH3OH 15: 1) and then recrystallized from DCM to give yellow powder. The structure was confirmed by 1H NMR spectroscopy.

**Synthesis of Boc-β-Ala-CYP.** Boc-β-Ala-TT (107 mg, 0.37 mmol) and CYP (76 mg, 0.18 mmol) were dissolved in pyridine under nitrogen atmosphere. The solution was stirred at 50 °C for 28 h and then the solvent was removed by rotary evaporation. The crude product was purified by column chromatography (silica gel 60 Å, 70-230 mesh, EtOAc: Hexane 7:3) to give solid of Boc-β-Ala-CYP in 75 % yield. The structure was confirmed by 1H NMR spectroscopy and LC-MS.
Synthesis of NH2-β-Ala-CYP. Boc-β-Ala-CYP was dissolved in anhydrous DCM (3 mL) and TFA (3 mL) was added at 0 °C. The resulting solution was left stirring overnight to ensure complete Boc removal. Excess TFA was removed by nitrogen flushing. The resulting compound was dissolved in chloroform (15 mL) and washed with aqueous saturated NaHCO3 (3 x 20 mL) and brine (1 x 20 mL). The organic layer was dried over anhydrous sodium sulfate and filtered. NH2-β-Ala-CYP was obtained after the solvent was removed by rotary evaporation. The structure was confirmed by 1H NMR spectroscopy and LC-MS.

Synthesis of P-CYP. mPEG-b-PCC (123 mg, 0.013 mmol) was dissolved in anhydrous DCM followed by the addition of NH2-β-Ala-CYP (50 mg, 0.10 mmol), EDCI (50 mg, 0.25 mmol), HOBt (35 mg, 0.25 mmol). The reaction was stirred for two days under nitrogen atmosphere at the room temperature. Then, DC (48.3 mg, 0.25 mmol) was added and the reaction was allowed to proceed for another two days (Figure 1B). After completion of the reaction, crude product was purified by precipitation in large excess of diethyl ether. The precipitate was then purified by dialysis against ethanol using a regenerated cellulose membrane with 3,500 MWCO.
2.2.3 Characterization of polymer-drug conjugates

$^1$H NMR spectra were recorded on a Bruker (500 MHz, $T = 25 \degree C$) using DMSO-d6 as a solvent for P-PTX and Chloroform-d as a solvent for P-CYP in a chemical shift range of 0-12 ppm.

2.2.4 Preparation and characterization of polymer-drug conjugate micelles

The film hydration method was used for preparing P-PTX and P-CYP conjugate micelles. Briefly, 10 mg of P-PTX or P-CYP was dissolved in
dichloromethane (DCM) and the solvent was evaporated under reduced pressure. The resulting film was hydrated with 1 ml of phosphate buffered saline (PBS; pH 7.4) followed by sonication and filtration through 0.22 μm filter. Mean particle size was measured by using a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK) at a scattering angle of 173°. A total of 12 measurements were taken per sample with a time span of 10 s. Particle size distribution was reported as the mean ± SEM. of three independent samples. Critical micelle concentration (CMC) of P-PTX and P-CYP was determined using pyrene as a hydrophobic fluorescent probe as described previously [54].

PTX or CYP encapsulated micelles were also prepared by film hydration method. Briefly, 1 mg of PTX or CYP and 9 mg of poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft dodecanol) (mPEG-b-PCC-g-DC) (units of DC is 22) were dissolved in chloroform. Solvent was evaporated under vacuum and resulting film was hydrated in 1 mL of PBS followed by sonication. Free drug was removed by centrifugation at 3,000 rpm for 5 min and filtration using a 0.22 μm filter. To determine the drug loading, PTX or CYP loaded micelles were dissolved in methanol and the concentration was measured by HPLC. Drug loading was calculated using the following equation:

\[
\text{Drug loading (w/w %) = } \frac{\text{Weight of encapsulated drug}}{\text{Total weight of formulation}} \times 100
\]

**2.2.5 In vitro stability of p-px and p-cyp micelles**

To determine the stability of P-PTX and P-CYP conjugate micelles under the physiological conditions, micelles with final concentration of 1 mg/ml were
incubated with 45 mg/mL bovine serum albumin (BSA) at 37 °C with gentle agitation. The particle size distribution of these formulations was measured by DLS.

2.2.6 In vitro drug release

The release of PTX from P-PTX and CYP from P-CYP was determined at pH 5.3 and 7.4 as previously described [55]. Briefly, 1 mg of P-PTX or P-CYP was re-suspended in 1 ml buffer solution (0.1 M acetic acetate, pH 5.3 or 0.1 M phosphate buffer, pH 7.4) and diluted with methanol in a volume ratio of methanol: aqueous solution (1:3, v/v). All samples were incubated at 37 °C shaken at 100 rpm for 0, 6, 12, 24, 48, 72, 96, 120 and 144 h and neutralized to pH 7.4 prior to HPLC analysis.

In vitro release of PTX or CYP from mPEG-b-PCC-g-DC was determined by dialysis (7K MWCO) against 50 mL PBS containing 2% Tween 80 at pH 5.3 and 7.4 with gentle agitation. 1 mL samples were taken at regular time intervals and replaced with fresh media. Drug concentration was measured by HPLC as described for drug loading. (Waters HPLC system with 996 photodiode array detector (Milford, MA, USA)). All experiments were performed in triplicate and the data reported as the mean of three individual experiments. Micelles (final concentration of 1 mg/ml) were incubated with 45 mg/mL bovine serum albumin (BSA) at 37 °C with gentle agitation. HPLC conditions for PTX detection: column: Inertsil®ODS-3 (4.6 × 250 mm; 5 μm), mobile phase: 60:40 acetonitrile: water, wavelength: 227 nm. HPLC conditions for CYP detection: column: SymmetryShield™ RP18 (4.6 x 250 mm; 5 μm), mobile phase: 30:70 acetonitrile: water (0.1% trifluoroacetic acid); wavelength: 210 nm.
2.2.7 Cell culture

The human metastatic prostate cancer cell lines DU145 and PC3 and their PTX resistant versions DU145-TXR and PC3-TXR were a kind gift of Prof. Evan T. Keller of the University of Michigan. DU145-TXR cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. PC3-TXR and PC3-luc-GFP cells were cultured in F-12K medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were grown and maintained in a humidified incubator containing 5% CO₂ at 37°C.

2.2.8 Cellular uptake of P-PTX

To determine the cellular uptake of polymer-drug conjugates, we conjugated a fluorescent taxol derivative flutax-1 and dodecanol to copolymer mPEG-b-PCC, treated PC3-TXR cells with 0.5 µM of this conjugate (P-flutax-1) for 12 h and the cellular internalization was observed under a fluorescent microscope (Axio Vert.A1, Zeiss, USA) after washing the cells with PBS.

2.2.9 Cytotoxicity assay

DU145-TXR and PC3-TXR cells were used to determine the cell growth inhibition ability of polymer-drug conjugates. Cells (5X10³/well) were seeded in 96-well plates and 0.5 µM P-PTX, 10 µM P-CYP and the combination of 0.5 µM P-PTX and 10 µM P-CYP were added to different groups of wells after 24 h. Cell viability was assessed by MTT assay after another one to three-day incubation. The absorbance was measured at 560 nm and corrected for the cell debris by subtracting absorbance at 630 nm. Cell viability was calculated using the following formula:
Cell viability (%) = Absorbance of test sample/Absorbance of control X 100

2.2.10 Colony formation assay

PC3-TXR cells were seeded into 6-well plates. Each well had 300 cells and treatments of 0.5 µM P-PTX, 10 µM P-CYP and the combination of 0.5 µM P-PTX and 10 µM P-CYP were added to the wells after 24 h. At 10 days post incubation, colonies in each well were fixed by 10% formalin, stained with 0.5% crystal violet solution and visualized under microscopy.

2.2.11 Real time RT-PCR of microRNA quantification

DU145-TXR and PC3-TXR cells were treated with 0.5 µM P-PTX, 10 µM P-CYP and the mixture of 0.5 µM P-PTX, 10 µM P-CYP. After 72 h treatment, total RNA was isolated from untreated and drug-treated cancer cells using miRNEasy RNA isolation kit (Qiagen, Germany) following manufacturer’s instructions. Then, 200 ng of total RNA was converted to cDNA using miScript II RT Kit (Qiagen, Germany). Diluted cDNA was mixed with universal primer and SYBR Green dye and added to the wells of 96-well plates containing miScript primer. The plates were run on a Roche Light Cycler 480® instrument and the expression of individual miRNAs was analyzed using the obtained C_t values. For each of the selected miRNA, a miScript PCR primer was purchased from Qiagen. This assay targets only mature miRNAs, not their precursors. Untreated DU145-TXR and PC3-TXR were used as the control to calculate the fold change in drug-treated cells, respectively. As a normalizer, RNU6-1 was used as a housekeeping miRNA. The following miRNA primers were used:

hsa-miR-29b-3p: UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-34a-5p: UGGCAGUGUCUUAGCUGGUUGU
hsa-miR-148a-3p: UCAGUGCACUACAGAACUUUGU
hsa-miR-200c-3p: UAAAUACUGCCGGUAAUGAUGGA

RNU6-1:
GUGCUCGCUUCCGCAGCACAUAUAUCUAAAAAUUGGAACGAUACAGAGA
UUAGCAUGGCCGGAUGACACGCAAUUUUCUGUGAAAGCGGUUCC
AUAUUUU

2.2.12 Expression of miRNA target genes

We also determined the levels of several miRNA target genes, such as Gli1, PTCH1, ZEB1, ZEB2, E-CAD, CD133 and NOTCH2 at 72 h post treatment of DU145-TXR and PC3-TXR cells with the 0.5 µM P-PTX and 10 µM P-CYP. Then total RNA were extracted using RNeasy RNA isolation kit (Qiagen, Germany), reverse transcribed into cDNA template and amplified by real time RT-PCR using SYBR Green dye universal master mix and the primers for different genes. GAPDH was used as a housekeeping gene. The following primers were used:

CD133 forward primer: 5’AAGCATTGGCAGCTTCTATGG-3’
CD133 reverse primer: 5’-AAGCACAGAGGGTCATTGAGA-3’

NOTCH2 forward primer: 5’-GGCATTAATCGCTACAGTTGTGCT-3’
NOTCH2 reverse primer: 5’-GGAGGCACACTCATCAATGTCA-3’

Gli1 forward primer: 5’-TCCTACGGGTCTCATTCTCTATGG-3’
Gli1 reverse primer: 5’-GCCAGGGAGCTTACATACATAC-3’

PTCH1 forward primer: 5’-TGCTTGGAGGTATTACATACATAC-3’
PTCH1 reverse primer: 5’-CCCACAATCAACTCCTCTG-3’
E-CAD forward primer: 5'-AAGAAGCTGGCTGACATGTACGGA-3'
E-CAD reverse primer: 5'-CCACCAGCAACGTGATTTCGTCACTG-3'
ZEB1 forward primer: 5'-ATGCACAACCAAGTGAGGAGCAGGAC-3'
ZEB1 reverse primer: 5'-TTGCCTGGTTCAGGAGATGCTG-3'
ZEB2 forward primer: 5'-CTAACCCAAGGACGAGGATATTTATT-3'
ZEB2 reverse primer: 5'-GGGAAGAACCCGTCTTGATATT-3'
GAPDH forward primer: 5'-GATTCCACCCATGGCAATTC-3'
GAPDH reverse primer: 5'-GTCATGAGTCCTTCCACGATAC-3'

2.2.13 Evaluation in orthotopic prostate cancer bearing athymic nude mice

All animal experiments were performed in accordance with the NIH animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center (UNMC), Omaha, NE. Orthotopic prostate tumors were established in 8 week-old male athymic nude mice by injecting 1 × 10⁶ PC3-luc-GFP cells suspended in 50 μl PBS into dorsum of the prostate gland. Animals were randomly divided into four groups of five animals per group when the radiance of tumor volume had reached 10⁷. Formulations were administered to these mice via the tail vein thrice a week for two weeks. Group 1 was kept as the control and received normal saline, group 2 received 10 mg/kg P-PTX PBS solution (equivalent to free PTX), group 3 received 10 mg/kg P-CYP PBS solution (equivalent to free CYP) and group 4 received the mixture of 10 mg/kg P-PTX and 10 mg/kg P-CYP PBS solution. Bioluminescent radiance of tumor was measured every alternate day using IVIS® Spectrum imaging system (PerkinElmer Inc., MA). Body weight of these mice was recorded
every alternate day. At the end of the animal study (i.e., day 24), mice were sacrificed and tumors as well as vital organs (liver, spleen, kidney and heart) were excised.

Five representative tumor tissues were collected per experimental group and fixed with 10% buffered formalin for 24h. The fixed samples were embedded in paraffin and thin sections of 4 μm were obtained and immunostained for cell proliferation marker (Ki-67) and hematoxylin and eosin (H&E). To identify apoptotic cells by fluorescein-12−dUTP labeling of fragmented DNA, DeadEnd fluorometric TUNEL assay kit was used according to the manufacturer’s instructions. Tumor sections were also counterstained with propidium iodide (PI) and imaged under a fluorescence microscope.

2.2.14 Statistical analysis

Data are represented as means ± SEM. The statistical comparisons of the experiments were performed by two-tailed Student’s test. P <0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 Synthesis and characterization of P-PTX and P-CYP

P-PTX was synthesized by direct carbodiimide coupling of PTX onto the pendant carboxylic acid groups on the hydrophobic block of mPEG-b-PCC copolymer and esterification took place preferentially at the 2’-hydroxyl group due to its less steric hindrance. In 1H NMR spectra of mPEG-b-PCC, protons corresponding to −CH2−CH2−O− of PEG were observed at δ 3.4−3.6, and −CH2− units of PCC δ 4.2−4.4 and −COOH were observed at δ 12−14 as reported earlier
by our group. After conjugation of PTX to mPEG-b-PCC, protons corresponding to PTX were all observed in $^1$H NMR spectra and PTX content in the conjugates was determined to be $25.4 \pm 3.7\%$ (w/w), as calculated from the peak intensities of all phenyl proton signals from 7.3 to 8.4 ppm.

$^1$H NMR (500 MHz, DMSO-$d_6$) spectra of mPEG-b-PCC-g-PTX-g-DC showed peaks corresponding to PEG ($-\text{CH}_2-\text{CH}_2-O$) at δ3.5, PCC ($-\text{CO}-\text{CH}_2- & \text{CH}_3-\text{C(CO)}-\text{CH}_2$) at δ4.2 (m, 4H), CH$_3$-(CH$_2$)$_9$ at δ0.9 (t, 3H), CH$_3$-(CH$_2$)$_9$- at δ1.2-1.45 (bs, 18H), CH$_3$-(CH$_2$)$_9$-CH$_2$ at δ1.5-1.8 (m, 4H), CH$_3$-(CH$_2$)$_9$-CH$_2$-CH$_2$- at δ4.02 (m, 2H), PTX-(CH$_3$)$_2$-C$_6$H$_3$ at δ 1.01 (t, 6H), PTX-C$_6$H$_5$-CH$_3$ at δ 1.2 (t, 3H), PTX-CH$_3$-C$_6$H$_3$- at δ 1.8 (t, 3H), PTX-CH$_3$-CO-O- at δ 2.3 (t, 3H), PTX-C$_6$H$_5$-CH-NH- at δ4.78 (m, 1H), PTX-C$_6$H$_5$-CO-O-CH- at δ 4.79 (m, 1H), PTX-CHC$_2$H$_2$O- at δ 5.34 (m, 2H), PTX-CH$_3$-CO-O-CH- at δ 6.31 (m, 1H), PTX-C$_6$H$_5$- at δ7.3-8.4 (m, 15H), PTX-C$_6$H$_5$-CO-NH- at δ 9.0 (m, 1H) (Figure 2A).

We have tried to conjugate CYP directly onto mPEG-b-PCC but we failed to form amide bond between secondary amine of CYP and carboxyl group of mPEG-b-PCC (data not shown). Therefore, CYP was conjugated onto mPEG-b-PCC through a Boc-b-Ala linker because the secondary amine on CYP was not easy to conjugate with carboxylic acid group on mPEG-b-PCC, thus after Boc-removal the primary amine on the linker made the conjugation of CYP to the copolymer much easier. After conjugation of CYP to mPEG-b-PCC, protons corresponding to CYP were observed in $^1$H NMR spectra and CYP content in the conjugates were $14.5 \pm 1.6\%$ (w/w) calculated from proton signal 1.7-2.4 ppm.
$^1$H NMR (500 MHz, DMSO-$d_6$) spectra of mPEG-b-PCC-g-CYP-g-DC showed peaks corresponding to CYP-$\text{CH}_3$-$\text{C}_4\text{H}_3$O$^-$, CYP-$\text{CH}_3$-$\text{C}_5\text{H}_7$N$^-$, CYP-$\text{CH}_3$-$\text{C}_6\text{H}_5$- at δ1.01-1.3 (m, 9H), CYP-$\text{CH}_3$-$\text{C-CH-CH-CH-CH-}$ at δ1.48-1.88 (m, 3H), CYP-$\text{CH}_2$-$\text{CH=C-CH}_2$- at δ1.94-2.21 (m, 4H), CYP-$\text{O-C-CH-CH}_3$ at δ 2.50 (m, 1H), CYP-$\text{CH}_3$-$\text{CH-CH}_2$-$\text{N-CO-CH}_2$- at δ 2.46 (m, 2H), CYP-$\text{CH}_3$-$\text{CH-CH}_2$-$\text{N-CO}$ at δ 3.68, CYP-$\text{CH}_3$-$\text{CH-CH}_2$-$\text{N-CH}$- at δ 3.75 (m, 1H), CYP-$\text{CH}_3$-$\text{C-CO-NH-CH}_2$- at δ 8.01 (m, 1H) (Figure 2B).
Figure 2. $^1$H NMR spectra of two polymer-drug conjugates. (A) P-PTX, (B) P-CYP.
2.3.2 Formulation characterization

High drug payload of these polymer-drug conjugates was observed by using $^1$H NMR characterization (Figure 2): 25.40±3.70% (w/w) and 14.50±1.60% (w/w) for PTX and CYP, respectively. Unlike polymer-drug conjugates, PTX and CYP loadings in physically encapsulated micelles were 8.21±0.01% (w/w) and 5.36±0.07% (w/w), respectively.

P-PTX and P-CYP conjugate micelles were prepared by dissolving 10 mg of P-PTX or P-CYP in dichloromethane followed by solvent evaporation under reduced pressure to form a thin film, followed by hydration with 1 mL of PBS (pH 7.4). The mean particle sizes of P-PTX and P-CYP were 70.02 ± 0.22 nm (PDI 0.223) and 76.37 ± 0.15 nm (PDI 0.273), respectively. CMC values were $3 \times 10^{-4}$ g/L for P-PTX and $4 \times 10^{-4}$ g/L for P-CYP.

2.3.3 In vitro stability of P-PTX and P-CYP micelles

Within 48 hours, there was no significant change in the mean particle size of P-PTX and P-CYP micelles before and after their incubation with BSA, suggesting that plasma proteins were unlikely to affect the integrity of these polymer-drug conjugate micelles (Figure 3).
2.3.4 PH dependent drug release

*In vitro* drug release studies were carried out in PBS buffer with pH 7.4 and acetate buffer with pH 5.3 to simulate drug release in blood and tumor cells. P-PTX or P-CYP showed a slow but sustained release of PTX and CYP, respectively. Due to the stability of conjugated PTX and CYP, there was no noticeable initial burst release of these drugs at different pH 7.4 and 5.3. At neutral environment, both ester and amide bonds were stable and no significant release of PTX and CYP was observed. Since the liberation of free drugs required pH-dependent cleavage of ester and amide linkages, the percentage of PTX and CYP released from the conjugated micelles significantly increased as pH was decreased from 7.4 to 5.3. After six days, more than 25% of PTX and CYP was observed at pH 5.3, while about 10% of PTX and CYP was released at pH 7.4 (*Figure 4A*).
To better confirm the advantages of drug-conjugated micelles, we also determined the release of PTX and CYP from physical-encapsulated micelles. As expected, both PTX and CYP were released very fast. In the first 12 hours, burst release of two drugs was observed and almost 60% of drugs were released after 24 hours (Figure 5). In addition, with incubation of BSA both PTX and CYP were released from the conjugates very slowly as determined by HPLC (Figure 4B). The mean particle sizes of P-PTX and P-CYP micelles before and after their incubation with BSA were also monitored by DLS but there was no significant change during 48 h (Figure 3). These results suggested that the conjugated micelles were stable under physiologically simulating conditions.

Figure 4. In vitro release of PTX from P-PTX and CYP from P-CYP at different pH and with incubation of BSA. Drug concentrations from each time point were measured by HPLC and drug release profiles were represented as the mean±SEM.
2.3.5 Cellular internalization

P-flutax-1 conjugate was efficiently taken up by the cells as evidenced by green fluorescent positive cells (Figure 6).

Figure 5. *In vitro* release of PTX and CYP from physically drug encapsulated micelles at pH 7.4 and 5.3. (A) PTX, (B) CYP. Drug concentrations from each time point were measured by HPLC and drug release profiles were represented as the mean± SEM.

Figure 6. Intracellular accumulation of P-flutax-1. PC3-TXR cells were incubated with 0.5 µM P-flutax-1 for 12 h, and the intracellular accumulation of P-flutax-1 was observed by Axio Vert.A1 Microscope.
2.3.6 Cytotoxicity and colony formation assays

Cytotoxicity of 0.5 μM P-PTX, 10 μM P-CYP and the combination of 0.5 μM P-PTX and 10 μM P-CYP was determined by incubating DU145-TXR and PC3-TXR cells with these polymer-drug conjugate micelles for 24, 48 and 72 h. Due to the slow release of these drugs from the polymer conjugates, combination therapy of P-PTX and P-CYP had the highest cell-killing effect at 72 h compared to 24 or 48 h post incubation. Treatment with the combination formulation after 72 h killed 45.6% of DU145-TXR cells and 47.1% of PC3-TXR (Figure 7A-B). Both P-PTX and P-CYP monotherapy failed to kill many cells at those doses, suggesting that the combination formulation was quite effective in killing chemoresistant cancer cells.

Tumorigenic potential of PC3-TXR cells was determined by colony formation assay after treatment with P-PTX, P-CYP and their combination. Due to the long-term treatment of polymer-drug conjugates, a large % of PTX and CYP was released from the conjugates, resulting in significant anti-proliferative effect. The number of colonies formed from untreated group was largest compared with 0.5 μM P-PTX, 10 μM P-CYP or the combination treated group, and the number of colonies formed from combination treated group was significantly less compared with other three groups (Figure 7C-D).
2.3.7 Effect of combination therapy on miRNAs and their target genes

The effect of 10 µM P-CYP and its combination with 0.5 µM P-PTX on the expression of miR-29b, miR-34a, miR-148a and miR-200c was determined in DU145-TXR and PC3-TXR cells using real time RT-PCR. All these tumor suppressor miRNAs were upregulated when treated with either 10 µM P-CYP or its combination with 0.5 µM P-PTX (Figure 8). However, this effect was better when the cells were treated with the combination therapy.

We also determined the levels of several known downstream targets of these miRNAs and Hh signaling. There was downregulation of miR-200c target
genes like ZEB-1 by 1.30 fold in PC3-TXR cells and ZEB-2 by 2.32 fold in DU145-TXR cells, while the expression of another miR-200c target E-CAD was upregulated in DU145-TXR cells by 4.75 fold and PC3-TXR cells by 3.62 fold (Figure 8). miR-34a target gene CD133 was significantly downregulated and NOTCH2 which is the target gene of miR-29b was also downregulated in DU145-TXR cells by 2.0 fold and PC3-TXR cells by 7.69 fold. In addition, gene targets of Hh signaling such as Gli1 and PTCH1 were regulated in a reverse pattern. While Gli1 was highly downregulated in PC3-TXR after combination therapy, PTCH1 was upregulated in DU145-TXR cells by 3.22 fold and PC3-TXR cells by 1.89 fold indicating Hh signaling was inhibited when these cells were treated with the mixture of P-PTX and P-CYP.
2.3.8 Efficacy in orthotopic prostate tumor bearing mice

We established orthotopic prostate tumor in 8 weeks old male athymic nude mice by injecting $1 \times 10^6$ PC3-luc-GFP cells. At day 7, all mice were imaged for...
luciferase bioluminescence to determine the tumor growth rate (Figure 9) and mice whose bioluminescent radiance achieved $10^7$ were randomized into four groups: i) control, ii) P-PTX, iii) P-CYP and iv) combination of P-PTX and P-CYP.

These polymer-drug conjugates were administered intravenously thrice a week for two weeks at equivalent doses of 10 mg/kg PTX and 10 mg/kg CYP. All the treatment groups had significantly lower tumor growth compared with the control group. However, a significantly higher tumor growth inhibition was observed in the group treated with the combination therapy compared with the formulations containing either P-PTX or P-CYP (Figure 10A-B).
The body weights of the mice did not drop after systemic administration of saline or polymer-drug conjugates, with steady increase at similar rates in all groups (**Figure 10C**). This indicates that P–PTX, P–CYP and their combination are well tolerated. In addition, the chronic toxicities of these formulations were also evaluated by histological analysis of the major organs (**Figure 11**). No obvious histological changes were observed in the livers, spleens, kidneys and hearts from...
all the treatment groups.

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**Figure 11. Hematoxylin and Eosin (H&E) staining of major organs (liver, spleen, kidney and heart).** Organ samples from control (saline), P-PTX, P-CYP and P-PTX + P-CYP treated groups were excised, fixed and stained for H&E.

Immunohistochemical analysis showed tumor sections from P-PTX, P-CYP and combination of P-PTX with P-CYP treated group had significant necrotic area with less number of live tumor cells, whereas control tumor remained viable. Notably, the combination therapy showed more necrotic areas compared with monotherapy. Furthermore, mice treated with the combination therapy showed least Ki-67 staining compared with P-PTX, P-CYP and control treated groups (**Figure 12A**). In TUNEL assay, control group did not have much TUNEL-positive
green fluorescent cells while P-PTX or P-CYP treated group showed modest increase in the number of apoptotic cells. The combination of P-PTX and P-CYP showed significantly enhanced apoptosis supporting the tumor growth inhibition potential of this combination therapy (Figure 12B).

Figure 12. Analysis of tumor samples for hematoxylin and eosin (H&E) and Ki-67 (cell proliferation marker) staining and TUNEL assay. Control (Saline), P-PTX, P-CYP and P-PTX + P-CYP treated tumor samples were excised, fixed and immunostained for H&E and Ki-67 (A), and stained for TUNEL-positive nuclei (green) and propidium iodide (red) positive nuclei (B).
2.4 DISCUSSION

HRPC poses a medical challenge due to its insensitivity to the majority of chemotherapeutic agents after long-term treatment. Emerging therapies directed against different pathways include mTOR, MAPK/ERK, NFκB/IL-6, endothelin A receptor, and somatostatin receptor [56]. While these therapies have partially restored the sensitivity of prostate tumors to taxanes like PTX and DTX, the benefit was only moderate or little compared to monotherapy.

Supplementation of Hh signaling inhibitor to chemotherapy has the potential to eliminate the chemoresistance in advanced prostate cancer and to improve the therapeutic efficacy of PTX by targeting both bulk tumor cells and CSCs, and restoring the expression of dysregulated miRNA. Emerging evidence suggests that numerous dysregulated miRNAs are implicated in the pathogenesis of prostate cancer. Since expression profiles of miRNAs in tumors are tissue-specific [57], miRNA could be not only an ideal class of biomarkers for cancer detection, but also promising targets for cancer therapy. Therefore, CYP as an Hh signaling inhibitor will augment PTX therapy by restoring the expression of tumor suppressor miRNAs and thereby would improve the overall efficacy of chemotherapy.

For improved clinical translation of the combination therapy with reduced toxicity and better safety, we conjugated PTX and CYP to a biodegradable amphiphilic diblock copolymer mPEG-b-PCC with pendant carboxyl acid groups. We have previously conjugated a water soluble drug gemcitabine as well as dodecanol to mPEG-b-PCC. This polymer-drug conjugate self-assembled into micelles and showed enhanced stability and antitumor effect of gemcitabine [58].
In this study, PTX was conjugated to mPEG-b-PCC through an ester bond at its 2'-OH position which is more active than other two hydroxyl groups. For binding CYP to mPEG-b-PCC we used a linker containing thiazolidine-2-thione to react with secondary amine in CYP (Figure 1). Additionally, the attachment of DC enhanced requisite hydrophobicity and assisted in the self-assembling of polymer-drug-conjugate micelles. These polymer-drug conjugates were characterized by $^1$H NMR (Figure 2). Therefore, this conjugation strategy imparted high payload of PTX (25.4±3.7%) and CYP (14.5±1.6%) and small micellar sizes of 70-76 nm. Since the preparation of physical encapsulation of drugs into micelles is easier compared to polymer-drug conjugation, we also physically encapsulated PTX and CYP into mPEG-b-PCC-g-DC by film hydration. As expected, drug loading in these physically drug encapsulated micelles was 8.21±0.01% for PTX and 5.36±0.07% for CYP.

Physically encapsulated drugs into polymeric micelles are usually released fast, with burst release due to the dynamic instability of micelles. This means higher initial drug loading is needed because drugs should ideally be released at the disease site to reduce their adverse effects. In contrast, chemical conjugation of drugs to amphiphilic polymers via ester, amide, and disulfide bonds prevents immediate drug release during the transport of polymer-drug conjugates and provides long-term sustained release of drugs. Therefore, polymer-drug conjugation prolongs drug circulation and therapeutic efficacy. To confirm the different drug-release profiles between chemical conjugation and physical encapsulation we also determined the release of PTX and CYP from physically
drug encapsulated micelles. As expected, both PTX and CYP were released very fast, with the burst release of these two drugs in the first 12 h and almost 60% release at 24 hours (Figure 5), which is in good agreement with the literature [59,60]. In contrast, PTX and CYP were released from conjugated micelles at a slow but appreciable rate, which suggested that these polymer-drug conjugate micelles could prolong the circulation of PTX and CYP. The percentage of PTX and CYP released from significantly increased as pH was decreased from 7.4 to 5.3. However, even after six days, only about 25% of PTX and CYP were released at pH 5.3, but only 10% of PTX and CYP were released at pH 7.4 (Figure 4A).

So far polymer-drug conjugates with linear backbone have undergone clinical evaluation, such as polyethylene glycol (PEG), poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) copolymers, dextran and poly(glutamic acid) (PGA) and they display numerous features and advantages among all nano-sized carriers for cancer therapy [61-63]. Our polymer-drug conjugate micelles offer distinct advantages in terms of a) PEG corona on the polymer imparts stealth property; b) conjugation ensures in vivo stability and no premature drug release in the circulation; c) small size of these micelles can take advantage of the EPR effect to maximize drug delivery to prostate tumor.

We previously demonstrated that CYP could target CSCs derived from PTX-resistant prostate cancer cell lines DU145-TXR and PC3-TXR and combination therapy of PTX and CYP could reverse PTX chemoresistance and eliminate CSC fraction in chemoresistant prostate cancer cells [64]. Therefore, in this study we evaluated the inhibitory effect of P-PTX and P-CYP on DU145-TXR
and PC3-TXR cells. We first determined cellular uptake of the conjugates. PTX was replaced by a fluorescent taxol derivative flutax-1 which was conjugated to mPEG-b-PCC along with dodecanol to obtain P-flutax-1. Green-fluorescent positive PC3-TXR cells confirmed the successful cellular internalization of the conjugates (Figure 6). In followed cytotoxicity assay P-PTX with 0.5 μM equivalent PTX was not able to kill many cells due to the development of resistance to PTX. However, the treatment with the combination of 0.5 μM PTX and 10 μM P-CYP for 72 hours killed almost half of the cells (Figure 7), which is in good agreement with our previous observation [64]. This further suggests that this combination therapy had potentials to treat chemoresistant prostate cancer.

Treatment of DU145-TXR and PC3-TXR cells with 10 μM P-CYP alone or in combination with 0.5 μM P-PTX resulted in upregulation of tumor suppressor miRNAs like miR-29b, miR-34a, miR-148a and miR-200c (Figure 8), leading to increase in chemosensitivity to PTX. Emerging evidence demonstrates that miR-200c, a member of miR-200 family, is one of the essential regulators of chemoresistance as well as epithelial-mesenchymal transition (EMT). Expression level of miR-200c is significantly downregulated in DTX-resistant prostate cancer cells compared with non-resistant cells and restoration of miR-200c results in apoptosis of DTX-resistant cells and reversal of EMT [65]. As tumor suppressor miRNA, miR-34a not only has strong inhibitory effects of prostate cancer but also can negatively affect prostate CSCs on the tumor-initiating ability and inhibit their CSC properties such as sphere formation and clonogenic capacity [66]. miR-29b can repress expression of Hh pathway and the inhibition of this signaling leads to
the restoration of miR-29b expression [45,67,68]. miR-148a is reported to inhibit the proliferation and metastasis of PTX-resistant prostate cancer and also attenuate the resistance to PTX [69]. Based on these evidences and combining our results, we confirmed that chemoresistance to PTX could be due to the altered miRNA expression. Therefore, the combination of P-PTX and P-CYP could alleviate chemoresistance by targeting miRNAs participated in chemoresistance. 

*In vivo* efficacy of P-PTX and P-CYP conjugate micelles was determined in orthotopic tumor model developed by PC3-luc-GFP cells in athymic nude mice. Promising results were obtained in three treated groups wherein the combination therapy of P-PTX and P-CYP resulted in a significant reduction in tumor growth rate and tumor size compared with the control group (*Figure 10*). In addition, the significantly low level of cellular proliferation and high level of apoptotic cells found in combination group demonstrated combined P-PTX and P-CYP reduces tumor growth synergistically (*Figure 12*). Furthermore, our formulation carrying P-PTX and P-CYP conjugate micelles were well tolerated as the vital organs such as livers, spleens, kidneys and hearts did not show any histological changes after treatments (*Figure 11*). These results are in good agreement with the work of Kopecek and associates who demonstrated that the combination of HPMA-CYP and HPMA-DTX conjugates significantly inhibited prostate tumor growth [70]. Unlike our current studies these authors used subcutaneous tumor model, while we have evaluated our formulations in orthotopic prostate tumor model.
CHAPTER 3 POLYMERIC MICELLAR DELIVERY OF NOVEL MICROTUBULE DESTABILIZER AND HEDGEHOG SIGNALING INHIBITOR FOR SYNERGISTICALLY TREATING CHEMORESISTANT PROSTATE CANCER

3.1 INTRODUCTION

Prostate cancer (PCa) is the most common non-cutaneous malignancy and the second leading cause of cancer related mortality in American men. PCa at stage I/II can be treated by surgery or radiation therapy but if the cancer has grown outside the prostate or come back after surgery or radiation, androgen deprivation therapy may be used to reduce androgen levels. Unfortunately, after a certain period the aggressive portion of prostate cancer cells develops resistance to hormone treatment and become androgen independent. Alternatively, chemotherapy is given along with hormone therapy to enhance therapeutic efficacy. Docetaxel (Taxotere®) is a clinically approved drug to treat castration-resistant prostate cancer including metastatic prostate cancers and it has been proven to provide a modest survival benefit for patients with advanced prostate cancers. However, a potential challenge of using docetaxel or paclitaxel as cancer treatment in the long term is that their anticancer activities could be hindered by intrinsic or acquired drug resistance due to mutation of β-tubulin, affected androgen receptor signaling or overexpression of drug efflux pumps (ATP-binding cassette) in cancer cells [71-74]. Therefore, there is an urgent need to identify novel therapeutic agents for treating docetaxel-resistant patients.
A number of agents targeting colchicine binding cite of tubulin have been reported to effectively inhibit tumors that are resistant to taxanes and vinca alkaloids, suggesting that this type of tubulin inhibitors can circumvent the limitations associated with clinically available tubulin inhibitors [75,76]. Previously, we have synthesized and evaluated a series of novel microtubule destabilizers [77-81], which can target colchicine domain of tubulin and interfere with tubulin polymerization. Although these new compounds have shown potent anticancer activity, tumor heterogeneity and the complexity of cell signaling pathways in tumor microenvironment make curing cancer through monotherapy a formidable challenge. Combination chemotherapy using two or more anticancer agents that work together synergistically by different mechanisms of action can increase the chance of long-term remission and prevent potential drug resistance. Hedgehog (Hh) signaling participates in the initiation and progression of various cancers, thus its aberrant activation is considered as a hallmark of cancers. Importantly, Hh signaling is needed for regeneration of prostate epithelium through crosstalk with androgen signaling, suggesting that inhibition of Hh pathway has the possibility to induce anti-proliferative and apoptotic effect on prostate cancer cells [38,39,82,83]. Furthermore, Hh signaling involves in the initiation and maintenance of cancer stem cells (CSCs), a subset of cancer cells with self-renewal and tumorigenic potentials, which have been demonstrated to play key roles in chemoresistance, metastatic progression and epithelial-mesenchymal transition [40-42]. In our previous studies [64,84], we also have proved the significant benefit using combination of Hh signaling inhibitor cyclopamine and paclitaxel to combat taxane-
resistant prostate cancer cells in vitro and vivo. Recently, our group developed a series of analogs of GDC-0449, a FDA-approved Hh pathway inhibitor, and one of the analogs, MDB5, exhibited stronger inhibition to Hh pathway and anticancer effect in vitro and vivo than GDC-0449 [85]. Therefore, in the current study we propose that the new combination therapy of a novel microtubule destabilizer and a novel Hh inhibitor can work synergistically through different mechanisms to treat taxane-resistant prostate (Figure 13), as the microtubule destabilizer can kill bulk tumor cells and the Hh inhibitor can suppress Hh signaling and the proliferation of CSCs resulting in alleviation of chemoresistance.

![Figure 13. Structures of QW-296 and MDB5.](image)

In further, considering the hydrophobicity of the two novel agents requiring an appropriate drug delivery carrier, we synthesized a diblock copolymer poly(ethylene glycol)-block-poly(trimethylene carbonate-co-2-methyl-2-
benzoxycarbonyl-propylene carbonate) (abbreviated as mPEG-p(TMC-MBC)) via ring-opening polymerization and prepared polymeric nanoparticles to physically encapsulate two small molecules leading to enhanced water solubility, prolonged circulation and reduced side effects.

To sum up, the anticancer effect and corresponding mechanisms of the combination chemotherapy was thoroughly determined by using different prostate cancer cells and orthotopic mouse model. It is the first time we introduced a new therapeutic strategy to treat taxane-resistant prostate cancer, which would bring potential promise to improve current regimens.

3.2 METHODS

3.2.1 Materials

Poly(ethylene glycol) methyl ether (mPEG, Mn=5000, PDI=1.03) was dried by anzeotropical distillation from anhydrous toluene just before use. Trimethylene carbonate (TMC) was obtained from Polysciences. Anhydrous chloroform, dichloromethane (DCM), toluene, stannous 2-ethylhexanoate (Sn(Oct)2), and diethyl ether were available commercially from Aldrich and used as received. Deuterated chloroform (CDCl3) was purchased from Cambridge Isotope Laboratories. Other chemicals were all of analytic grade and were used without further purification.

3.2.2 Cell culture

Human prostate cancer cell lines with taxane resistance DU145-TXR and PC3-TXR were kindly provided by Dr. Evan T. Keller from University of Michigan. The cells were cultured in RPMI1640 medium supplemented with 10% FBS and
1% penicillin/streptomycin in a humidified incubator containing 5% CO₂ at 37 °C and their resistance to taxane were maintained by adding 200 nM paclitaxel to growth media biweekly.

3.2.3 Cytotoxicity of QW-296 and MDB5

PC3-TXR and DU145-TXR cells were used to determine the cytotoxicity of QW-296, MDB5 or their combination. After attached to the bottom of plate, cells were incubated with different concentrations of QW-296, MDB5 or combination for another 72 h. Cell viability was then determined by MTT assay and the absorbance was measured at 560 nm with subtraction of absorbance at 630 nm. Each group was performed in triplicate and the data reported as the mean ± SEM.

3.2.4 Combination effect and their Interaction analysis

Chou-Talalay method and CompuSyn software were used to determine whether the combination had synergism, additivity, or antagonism [86]. PC3-TXR cells were treated with different combinations at a constant molar ratio, and the combination index (CI) was then determined by the software. CI values below 0.9, between 0.9 and 1.1, or above 1.1 indicate synergism, additivity or antagonism, respectively.

3.2.5 Cell cycle analysis by propidium iodide staining

PC3-TXR cells were cultured in a 24-well plate and treated with QW-296, MDB5 or their combination for 48 h and 72 h. Cells were harvested, fixed in 70% ice-cold ethanol for 1 h and washed by PBS. A cell pellet containing 1 × 10⁶ cells was then re-suspended in 0.5 mL of FxCycle™ PI/RNase staining solution and incubated for 15 min at room temperature. Cell cycle was measured by a flow
cytometer (BD FACSCalibur NJ). Results from 20,000 fluorescent events were obtained for analysis.

### 3.2.6 Colony formation assay

PC3-TXR cells were seeded at 300 cells/well into 6-well plates and allowed to grow for two days. Treatment of QW-296, MDB5 or combination was given to different wells. After a 7 day-incubation, colonies in each well were fixed by 10% formalin, stained with 0.5% crystal violet solution and visualized under a microscope. Each group was performed in triplicate.

### 3.2.7 Western blotting

Protein was isolated from PC3-TXR cells after 72 h treatment of QW-296, MDB5 or combination and protein concentration was determined by Micro BCA™ Protein assay kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein (50 µg) were separated in 4–15% Mini PROTEAN® TGX™ Precast Gel followed by transferring to polyvinylidenefluoride (PVDF) membranes by iBlot® Gel Transfer system (Thermo Fisher Scientific, Waltham, MA). After the membrane was blocked by Odyssey® Blocking Buffer (TBS), the following primary antibodies were used: Shh (sc-9024), β-actin (sc-47778) (Santa Cruz Biotechnology, Dallas, TX.) Subsequently, the membrane was incubated with their corresponding IRDye® 800CW secondary antibodies and target proteins were detected by Odyssey® CLx Imaging System (LI-COR Biosciences, Lincoln, NE).

### 3.2.8 Synthesis and characterization of mPEG-poly(TMC-MBC)

Monomer 5-Methyl-5-benzylloxycarbonyl-1,3-dioxane-2-one (MBC) and the copolymer mPEG-poly(TMC-MBC) were synthesized as reported previously with
minor modification [87]. In brief, mPEG (1 g, 0.0002 mol), TMC (307 mg, 0.003 mol) and MBC (750 mg, 0.003 mol) were mixed in a dried round bottom flask under vacuum, and then Sn(Oct)$_2$ (10 mol% relative to mPEG) as a catalyst was added to the mixture to initiate polymerization. The reaction mixture was heated to 120 °C for 24 h under stirring. Afterward, the product was dissolved in chloroform and precipitated in a large amount of diethyl ether and dried under vacuum. The purified copolymer was characterized by $^1$H NMR and spectrum was recorded on a Bruker (500MHz, T=25 °C) using deuterated chloroform (CDCl$_3$) as solvent.

### 3.2.9 Preparation and characterization of polymeric micelles

Polymeric micelles were prepared using thin-film hydration method. Briefly, a given amounts of mPEG-p(TMC-MBC) and the anticancer compound (10% w/w relative to the copolymer) were dissolved in chloroform in a glass vial and a thin film was formed after removing solvent under reduced pressure. The lipid film was hydrated by PBS buffer and the micelle solution was formed under ultrasonic bath at 37 °C. The formulation was then centrifuged at 3000 rpm for 5 min followed by membrane filtration to remove any unformulated drug. The hydrodynamic diameters and zeta potentials were measured by a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK) at a scattering angle of 90°. Particle size distribution was reported as the mean ± SEM. of three independent samples. Critical micelle concentration (CMC) was determined using pyrene as a hydrophobic fluorescent probe as described previously [87].
3.2.10 Drug loading and encapsulation efficiency

Briefly, MDB5 and QW-296 loaded micelles were dissolved in acetonitrile for drug extraction and drug content was determined by HPLC/UV-Vis analysis (Shimadzu, Kyoto Japan) on Phenomenex Aqua C18 column (5µm, 250 mm×4.6 mm) using acetonitrile and water (55:45, v/v) as mobile phase. Detection wavelength of QW-296 and MDB5 were 222 nm and 261 nm, respectively. Payload and encapsulation efficiency were calculated using the following equations:

\[
\text{Drug Loading (} \frac{w}{w} %) = \frac{\text{amount of extracted drug}}{\text{total weight of formulation}} \times 100\%
\]

\[
\text{Encapsulation Efficiency (%) = } \frac{\text{amount of extracted drug}}{\text{initial weight of drug}} \times 100\%
\]

3.2.11 Drug release from polymeric micelles

Drug-loaded micelles with a final concentration of 1 mg/mL were placed into a dialysis membrane with a molecular weight cut-off of 3500 Da and dialyzed against 50 mL buffer solution (0.1 M acetic acetate, pH 6.5 or 0.1 M PBS, pH 7.2) with 0.5% Tween-80 in a thermo-controlled shaker with a stirring speed of 100 rpm. 1 mL samples were withdrawn at specified times for a period of five days and drug concentration was analyzed by HPLC. All experiments were performed in triplicate and the data reported as the mean of the three individual experiments.

3.2.12 In vivo study

All animal experiments were performed in accordance with the NIH animal use guideline and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at University of Nebraska Medical Center. Orthotopic prostate tumors were established in 8-week-old male athymic nude mice by injecting 1.5 ×
10^6 PC3TXR-luc cells suspended in 50 μl PBS into dorsum of the prostate gland. Animals were randomly divided into five groups after one week and different treatments were administered intravenously every three days for five times. Group 1 was kept as the control and received normal saline, group 2 received 10 mg/kg QW-296 in micelle solution, group 3 received 10 mg/kg MDB5 in micelle solution, group 4 received 5 mg/kg QW-296 and 5 mg/kg MDB5 in cosolvent (12% Cremophor® EL, 12% ethanol and 76% PBS), and group 5 received 5 mg/kg QW-296 and 5 mg/kg MDB5 in micelle solution. Body weight of mice was recorded twice a week. At the end of the animal study (i.e., day 24), mice were sacrificed and tumors as well as vital organs (liver, spleen, kidney and heart) were excised. Three representative tumors were collected per group and fixed with 10% buffered formalin for 24h. The fixed samples were embedded in paraffin and thin sections of 4 μm were obtained and immunostained for hematoxylin and eosin (H&E) and cleaved Caspase 3. The side effects of each treatment were evaluated by H&E staining of the major organs.

3.2.13 Statistical analysis

Data were represented as the mean± SEM. The statistical comparisons of the experiments were performed by two-tailed Student's t-test. P < 0.05 was considered statistically significant.
3.3 RESULTS

3.3.1 Better anticancer activity of two novel compounds compared with their counterparts

We firstly confirmed that PC3-TXR and DU145-TXR cells exhibited high resistance to docetaxel (Figure 14). In the contrast, QW-296 exhibited strong cell killing activity against two taxane-resistant cell lines with IC$_{50}$ at 80 nM and 100 nM, respectively, as shown in Figure 1B. In addition, PC3-TXR cells showed more sensitivity to QW-296 than DU145-TXR cells.

![Figure 14. Cytotoxicity assay of Docetaxel and QW-296 on PC3-TXR and DU145-TXR for 72h.](image)

We also compared the anticancer effects of MDB5 and GDC-0449 using the two cell lines (Figure 15). The results confirmed that both MDB5 and GDC-0449 suppressed the growth of PC3-TXR and DU145-TXR cells in a dose-dependent manner, while MDB5 showed significantly enhanced activity with IC$_{50}$
48 µM than its parent drug, indicating the expected benefit of developing novel analog of GDC-0449.

![Figure 15. Cytotoxicity assay of MDB5 and GDC-0449. A) PC3-TXR and B) DU145-TXR for 72h.](image)

3.3.2 Synergism of QW-296 and MDB5 against taxane-resistant PCa cells

After testing cytotoxicity of individual drug, we confirmed the advantages of combination therapy with different concentrations (Figure 16). The concentrations of QW-296 and MDB5 applied in combination were lower than their IC50 in monotherapy, but the results proved combination at low concentrations worked efficiently than individual treatment. Combination of QW-296 (25 nM) and MDB5 (7.5 µM) only killed 20% of PC3-TXR, but when their concentrations were both doubled the combination killed 72% of cells. Two different combinations (Q 20 nM/M 20 µM, or Q 40 nM/M 10 µM) killed 40% of cells, but when the concentration of single drug was doubled (Q 40 nM/M 20 µM) the combination killed 70% of cells. All the above results suggested the two small molecules worked together not in an additive manner and the anticancer effect of their combination could enhance significantly at a certain combination ratio.
The interactions between the two drugs were determined by Chou-Talalay method and CompuSyn software, and we analyzed their combinations with different concentration ratios. The Combination Index (CI) values below 0.9, between 0.9 and 1.1, or above 1.1 indicate synergism, additivity or antagonism, respectively. The Fa (i.e. fraction affected, degree of growth inhibition) and CI values of different combinations were summarized in Figure 16 B-D and Table 1. When the combinations inhibited 50% of cells (i.e. Fa=50%), their CI values were in the range of additive effect, however, strong synergistic effect (the lower CI, the stronger synergism) was observed in three combinations when Fa level ranging from 75% to 95%. These results indicated that when concentration of each drug was around half of its corresponding IC$_{50}$ dose, their combination not only resulted in high level of growth inhibition (75% or above) but also worked together in the trend of synergism. Overall, this preliminary study explained the mechanism behind the drug interaction between QW-296 and MDB5 and the benefit of combination therapy was approved.
Table 1. Combination index of QW-296 and MDB5 with various ratios against PC3-TXR

<table>
<thead>
<tr>
<th>Ratios of Q/M</th>
<th>Combination index (CI)</th>
<th>Fa_{25}</th>
<th>Fa_{50}</th>
<th>Fa_{75}</th>
<th>Fa_{90}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:200</td>
<td></td>
<td>1.81</td>
<td>0.92</td>
<td>0.54</td>
<td>0.34</td>
</tr>
<tr>
<td>1:300</td>
<td></td>
<td>2.39</td>
<td>0.94</td>
<td>0.43</td>
<td>0.32</td>
</tr>
<tr>
<td>1:400</td>
<td></td>
<td>2.49</td>
<td>0.87</td>
<td>0.35</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Note: Q: QW-296; M: MDB5; Fa: fraction affected (%); CI > 1.1: antagonism; 0.9 < CI < 1.1: additivity; CI < 0.9: synergism
3.3.3 Inhibition of colony formation

The inhibitory activity of QW-296, MDB5 and their combination on tumorigenic potential in PC3-TXR cells was determined by colony formation assay. As shown in Figure 17, it was not surprising that the number of colonies in untreated group was the maximum compared with that of QW-296 alone, MDB5 alone, or the combination treated group. MDB5 at concentration of 15 μM exhibited slight inhibition against colony formation, while QW-296 at concentration of 100 nM markedly suppressed the colony formation. However, combination of QW-296 at 50 nM and MDB5 at 15 μM almost resulted in no colony formation throughout 7 days. These data further confirmed the synergistic effect of QW-296 and MDB5 for treating advanced prostate cancer.

Figure 17. Effect of QW-296 and MDB5 on colony formation of PC3-TXR cells. 300 PC3-TXR cells per well were seeded to 6-well culture plates. At 24h, drug formulations were added and at 7 days, cell colonies were fixed, stained and counted.
3.3.4 Effect of combination therapy on cell cycle, apoptosis and protein expression

Cell cycle analysis and apoptosis was determined by PI-staining after 48 h or 72 h of treatments to PC3-TXR cells (Figure 18). The data showed that QW-296 (100 nM) caused immediate 35.84% G2/M phase arrested at 48 h, but the percentage of arrested cells in G2 phase decreased to 25.76% with increase of treatment time at 72 h. Meanwhile, QW-296 treatment could induce sub-G1 phase arrest to 9.79% at 48 h and 11.43% at 72 h. In contrast to QW-296, MDB5 (15 µM) resulted in 57.99% of cells arrested in G0/G1 phase at 48 h, along with 72.85% of arrested cells in G0/G1 at 72 h, suggesting MDB5 affected on cell cycle through different mechanism and it worked at a slow manner. The combination of QW-296 at 50 nM and MDB5 at 15 µM induced overall G2/M arrest at 48 h and 72 h. Unlike single treatment changing cell arrest dramatically from 48 h to 72 h, combination therapy caused cell arrested in G2 phase and these cells ended up in static status throughout 72 h. Noticeably, after combination treatment the cell percentage of PC3-TXR in sub-G1 phase was significantly enhanced to 19% compared with cell percentage after monotherapy.
Meanwhile, as mentioned at the beginning of this paper, MDB5 was designed as Hh signaling inhibitor and GDC-0449 analog, thus we determined the effect of monotherapy or combination therapy on Hh signaling-related protein expression. We found clear reduction of Shh proteins in MDB5 treated group and combination treated group (Figure 19).

Figure 18. Effect of QW-296 and MDB5 on cell cycle and apoptosis of PC3-TXR cells. Cells were treated for 48 h or 72 h, stained with propidium iodide (PI), and analyzed on a flow cytometer. (A) 48 h, (B) 72 h. Results were expressed as the mean ± SEM (n=3).
3.3.5 Characterization of copolymer mPEG-p(TMC-MBC)

Monomer 2-Methyl-2-benzyloxy carbonyl-propylene carbonate (MBC) was synthesized first as described previously. Then MBC and trimethylene carbonate (TMC) were copolymerized with methoxy PEG\textsubscript{5000} using Sn(Oct\textsubscript{2}) as a catalyst to yield 1.82 g of copolymer mPEG-p(TMC-MBC) (88% yield). In \textsuperscript{1}H NMR spectrum of mPEG-p(TMC-MBC), the following peaks were observed at δ 2.03 (CH\textsubscript{2}, br, 2H) corresponding to TMC, δ 1.2 (CH\textsubscript{3}, s, 3H) and δ 7.3 (phenyl, m, 5H) corresponding to MBC, δ 4.2-4.3 (CH\textsubscript{2}, t, 4H) corresponding to both TMC and MBC, and δ 3.63 (CH\textsubscript{2}, s, 2H) corresponding to PEG. The M\textsubscript{n} of mPEG-p(TMC\textsubscript{15}-MBC\textsubscript{15}) determined by \textsuperscript{1}H NMR was 10280 g/mol with 15 units of each block, respectively. (Figure 20).
Figure 20. Synthetic route of copolymer mPEG-p(TMC-BC) (A) and its 1H NMR spectra (B).
3.3.6 Characterization, quantification and release profile of drug-loaded mPEG-p(TMC-MBC) micelles

The amphiphilic nature of mPEG-p(TMC-MBC) drives self-assembly into micelles in aqueous buffer. Surface morphology and mean particle size of micelles were checked by transmission electron microscopy (TEM, Tecnai G² Spirit) and dynamic light scattering (DLS, Zetasizer Nano ZS90). Unloaded, QW-296-loaded and MDB5-loaded polymeric micelles had similar size distributions, which were 81 nm. The TEM image also showed that the micelles had a narrow size distribution below 60 nm (Figure 21C) and confirmed that mPEG-p(TMC-MBC) formed spherical micelles in PBS with distinct boundaries as anticipated. CMC value of mPEG-p(TMC-MBC) was $6.65 \times 10^{-4}$ g/L (Figure 21D), further indicating that the micelles were quite stable in PBS. Meanwhile, high drug payload of these polymeric micelles was determined by HPLC: 8.13±0.75% (w/w) for QW-296 and 9.12±0.69% (w/w) for MDB5, respectively (Table 2).

<table>
<thead>
<tr>
<th>Parameters of mPEG-p(TMC-BC) micelles</th>
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<tr>
<td><strong>Mean size (nm)</strong></td>
</tr>
<tr>
<td>81.51</td>
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To improve the bioavailability of QW-296 and MDB5 for cancer treatment, controlled and sustained drug release is very important. Therefore, the release profile of QW-296 and MDB5 from mPEG-p(TMC-MBC) micelles at different pH was carried out by a dialysis method in PBS buffer (pH 7.4) and acetate buffer (pH 6.5). As shown in Figure 22, 40% of QW-296 or 25% of MDB5 was rapidly released from the micelles within the initial 12 h. Then the release of QW-296 or MDB5 increased up to 75% or 65% at 48 h followed by slower sustained profile until the end of the 96 h. Furthermore, at pH 6.5, the liberation of QW-296 or MDB5 from polymeric micelles was accelerated as expected due to the instability of micelles occurred in an acidic or basic environment. These data suggested that

Figure 21. Characterization of polymeric micelles. (A) Hydrodynamic diameter, (B) zeta potential, (C) TEM morphology, (D) critical micelle concentration.
the drug-loaded micelles could release these drugs at expected speed under physiologically simulating conditions.

3.3.7 *In vivo* antitumor efficacy in Orthotopic Prostate Cancer Mouse Model

We successfully established orthotopic prostate tumor in 8-week-old male athymic nude mice by injecting $1.5 \times 10^6$ PC3TXR-luc cells into dorsal prostate lobe (Figure 23). The mice whose bioluminescent radiance reached $10^8$ were randomized into five groups: 1) control, 2) QW-296 micelles (10 mg/kg), 3) MDB5 micelles (10 mg/kg), 4) combination of QW-296 and MDB5 in cosolvent (5 mg/kg + 5 mg/kg), 5) combination of QW-296 and MDB5 in micelles (5 mg/kg + 5 mg/kg).
All the treated groups showed inhibition of tumor growth compared with control group, however, the maximum tumor inhibition was observed in the group treated with combination in micelles. Notably, combination of QW-296 and MDB5 in micelles exhibited stronger antitumor activity in comparison with combination with same doses in cosolvent (Figure 24).

**Figure 23. In vivo imaging of athymic nude mice bearing PC3-TXR orthotopic prostate cancer.** Representative bioluminescence images were took at day 7 and day 24 (n = 6).
Next, we performed immunohistochemical analysis to further elucidate the superior anticancer efficacy of combination micelles. Hematoxylin and eosin (H&E) stain of tumor sections indicated that tumor samples from four treated groups had more necrotic area compared with tumor samples from control group, and tumors from combination micelles group showed maximum necrosis. Furthermore,

Figure 24. *In vivo* efficacy of QW-296 and MDB5 after systemic administration in PC3-TXR orthotopic prostate cancer bearing athymic nude mice. Representative tumors of each group were excised after sacrificing the mice at the end of the experiment.
cleaved caspase 3 stain indicated the induction of significant apoptosis by combination micelles of QW-296 and MDB5 compared to other treatments (Figure 25). Meanwhile, the chronic toxicities of these treatments were also examined by histological analysis of major organs. No distinct histological changes were observed in the liver, spleen, kidney and heart from all treated groups, suggesting that mice tolerated all treatments well (Figure 26).

Figure 25. Analysis of tumor samples by hematoxylin and eosin (H&E) and Caspase 3 stain. Tumor samples from control and treated groups were excised, fixed and immunostained for (A) H&E and (B) Caspase 3.
3.4 DISCUSSION

Drug resistance is still one of the major impediments for the success of chemotherapy and several factors account for the occurrence of resistance, including ATP-binding cassette transporter family (P-gp, ABCC1, ABCG2, etc.)
 alteration in drug targets, as well as intrinsic chemoresistance (cancer stem cells, CSCs) [89]. With the advances in cancer research, there are numerous approaches to overcome drug resistance. Combination chemotherapy is considered as one of the preferred choice in both preclinical research and clinical practice. Due to heterogeneity and molecular complexity of cancers, combination therapy referring to administration of two or more anticancer agents with different mechanisms of action can modulate various signaling pathways and maximize therapeutic effects. In addition, it is worth mentioning that if the combinatory drug effect is greater than the sum of their individual effects, as known as drug synergism, there will be a higher chance in success of combination strategy. In this regard, our study proposed a synergistic combination chemotherapy using two novel anticancer agents to overcome taxane-resistance and treat advanced prostate cancer.

To replace taxane treatment, we first designed and synthesized a new microtubule destabilizer, QW-296, whose function on microtubule mass, different from the stabilizers such as taxanes, was to suppress tubulin polymerization. On the other hand, a novel Hh pathway inhibitor MDB5 was selected to ally with QW-296 to treat advanced prostate cancer due to the emerging clinical reports that over-expressed Hh pathway promotes prostate tumor formation from epithelial cells, renders the epithelial-to-mesenchymal transition, and has cross-talk with androgen pathway. In our previous study, we also demonstrated that the combination of Hh inhibitor cyclopamine and paclitaxel effectively worked together to suppress the growth of taxane-resistant prostate cancer in vitro and vivo by
playing different roles on cancer cells, suggesting a promising combination strategy. Thus, in the current study, we upgraded Cyclopamine to a newly designed Hh signaling inhibitor MDB5, having stronger inhibition activity against Hh signaling but less unfavorable toxicity compared with its parent drug GDC-0449. First, the inhibitory effect of QW-296 and MDB5 on prostate cancer cells was confirmed by cell viability and colony formation assay, clearly indicating the benefit of this combination, and then their synergism was proved by Chou–Talalay method, which strengthened our hypothesis preliminarily. In the following cell cycle analysis, QW-296 and MDB5 made distinct impacts on cell cycle as QW-296 treatment caused G0/G1 phase arrest while MDB5 treatment lead to G2/M phase arrest, which demonstrated two anticancer agents worked at complementary mechanism of action against PC3-TXR cells, and this result was in the agreement with previous reports. In further we analyzed the expression change of relevant protein to highlight the combination advantage at molecular level. We observed Shh, a key downstream component of Hh signaling pathway, were highly downregulated after MDB5 monotherapy or combination of QW-296 and MDB5, indicating the treatment did have effect on Hh pathway. All these results reinforced our preliminary findings and provided us with a reasonable explanation on benefits of the combination therapy.

Although QW-296 and MDB5 demonstrated excellent synergy of anticancer activity against chemoresistant prostate cancer, their clinical translation will be limited due to their intrinsic poor aqueous solubility as many other anticancer agents. Therefore, to solve this problem, nanoparticle-based therapeutic systems
have emerged as a promising platform for delivering hydrophobic drugs over several decades. In this study, we developed an amphiphilic copolymer mPEG-p(TMC-MBC) to encapsulate two hydrophobic small molecules, thereby forming polymeric micelles in aqueous solution with nano-ranged particle size that are suitable for systemic therapy in animal study. PEG$_{5000}$ was used as hydrophilic backbone and this long length enabled us to synthesize the copolymer with molecular weight in the range of 10,000-11,000 Da. Its stealth-like property helped resulting micelles prolong circulation time and accumulate the amount of drug at target tumor tissue. On the other hand, two carbonate blocks TMC and MBC provided desired hydrophobicity to wrap lipophilic molecules in the core and balance hydrophilic composition as well. Our results also confirmed that polymeric micelles mPEG-p(TMC-MBC) enabled appropriate drug loading for both drugs and sustained drug release in acidic or neutral condition, which laid a foundation for the success of combination therapy applied in animals or future clinical translation.

To better investigate the combination efficacy, we used half dose of QW-296 and MDB5 in combination treatment as compared with monotherapy. All the four treated groups exhibited excellent tumor inhibitory results. However, combination therapy in micelles showed significantly enhanced reduction in tumor size compared with the combination in co-solvent as well as QW-296 or MDB5 monotherapy. Apart from tumor growth suppression, H&E stain of vital organs demonstrated the micelles carrying QW-296 and MDB5 were well tolerated, as other healthy organs did not show obvious histological changes after treatments. These results strongly supported our hypothesis that QW-296 and MDB5 could
synergistically treat chemoresistant prostate cancer *in vivo* and copolymer mPEG-p(TMC-MBC) could serve as a more effective delivery vehicle to boost anticancer activity of two drugs than co-solvent.

### 3.5 CONCLUSION

In the present study, we successfully synthesized and screened a novel microtubule destabilizer QW-296 and a Hh pathway inhibitor MDB5 and demonstrated their anticancer activities in combination or individually. The copolymer mPEG-p(TMC-MBC) was successfully synthesized and formed polymeric micelles to encapsulate QW-296 and MDB5 with desirable drug payload and small particle sizes. The overall findings indicated that the combination of QW-296 and MDB5 exhibited the synergistic therapeutic effect against chemoresistant prostate cancer via different mechanism, and with the help of mPEG-p(TMC-MBC) the combination could effectively inhibit the growth of chemoresistant prostate cancer *in vivo*. Given these encouraging results, our micelles of QW-296 and MDB5 provide a promising therapeutic strategy for chemoresistant prostate cancer therapy.
CHAPTER 4 POLYMER CONJUGATE OF A MICROTUBULE DESTABILIZER INHIBITS LUNG METASTATIC MELANOMA²

4.1 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 [90], and in the mid-1970s Ringsdorf proposed the idea of conjugating therapeutic agents to water soluble polymers [91]. 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 [92]. Unfortunately, the survival of patients with advanced metastatic melanoma has not been significantly improved by current Food and Drug Administration (FDA)-approved systemic chemotherapies [93]. 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 [94]. 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 [95,96]. 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 [71-73]. 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 [97]. In addition, their ability to circumvent P-gp mediated drug resistance was confirmed by using prostate cancer cells with P-gp overexpression [98]. 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.
4.2 METHODS

4.2.1 Materials

4-Cyanophenol, L-cysteine, N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uranium 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).

4.2.2 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 Figure 27 (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 1M HCl and CH2Cl2 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$_2$Cl$_2$. HBTU (1.1 equiv) was then added and stirred for 15 minutes. This was followed by addition of DIPEA (2.2 equiv) which was stirred for 2-3 minutes. Finally, HNCH$_3$OCH$_3$ HCl salt (1.1 equiv.) was added and the reaction was stirred at room temperature for 12-18 hours. The reaction mixture was washed once with ddH$_2$O 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 hours. The solvent was removed by a rotary evaporator and the resulting solid was dissolved in CH$_2$Cl$_2$ and washed once with ddH$_2$O and once with saturated NH$_4$Cl solution. The organic layer was dried over Na$_2$SO$_4$ 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 minutes. The solution containing compound 3 was then added to the mixture and stirred for 2 hours while returning to room temperature. The reaction was quenched with saturated NH$_4$Cl solution, extracted three times with ethyl acetate, dried over
NaSO₄, 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 minutes. The reaction was quenched with saturated NH₄Cl solution, extracted three times with ethyl acetate, dried over NaSO₄, and purified using flash chromatography to yield the final pure product (compound 5, Figure 27).

### 4.2.3 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 [99-101]. 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.

### 4.2.4 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 MgCl₂, 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).
4.2.5 Synthesis and characterization of mPEG-b-PCC-g-SMART-g-DC (P-SMART)

2-Methyl-2-benzylxycarbonyl-propylene carbonate (MBC), poly(ethylene glycol)-block-poly(2-methyl-2-benzoxycarbonyl-propylene carbonate) (mPEG114-b-PBC28) and poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate) (mPEG114-b-PCC28) were synthesized as described previously [54]. SMART-OH and DC were conjugated to the carboxyl groups of mPEG114-b-PCC28 copolymer using carbodiimide coupling. MPEG-b-PCC (180 mg, 0.019 mmol) was dissolved in anhydrous CH2Cl2 followed by addition of EDC (215 mg, 1.12 mmol), HOBT (101 mg, 0.75 mmol) and the solution was stirred at room temperature. After two hours, N,N-diisopropylethylamine (DIPEA, 98 μl, 0.56 mmol) and SMART-OH (84 mg, 0.23 mmol) were added and the reaction continued for two days. Then, DC (70 mg, 0.37 mmol) was added and the reaction was allowed to proceed for one additional day (Figure 29A). Crude product was purified by precipitation in large excess of diethyl ether and then by dialysis against MeOH using a regenerated cellulose membrane with 3.5 K MWCO.

1H NMR spectrum was recorded on a Bruker (500 MHz, T = 25 °C) using DMSO-d6 as solvent for P-SMART in a chemical shift range of 0-12 ppm.

Dynamic light scattering (DLS) was used for measuring the particle size distribution of P-SMART. Briefly, 10 mg of P-SMART was dissolved in CH2Cl2 and the solvent was evaporated under reduced pressure. The resulting film was hydrated with 1 ml PBS (pH 7.4) followed by sonication and filtration through 0.22 μm filter. Mean particle size was measured by using a Zetasizer Nano ZS90.
at a scattering angle of 173°. A total of 12 measurements were taken per sample with a time span of 10 s. Particle size distribution was reported as the mean ± SEM of three independent samples.

4.2.6 Quantification of SMART payload in P-SMART

To quantify the conjugated drug, alkaline hydrolysis method was used. 1 mg/ml P-SMART was mixed with 1 ml NaOH (1 N) at 37 °C overnight. Samples were then neutralized to pH 7.4 followed by HPLC-PDA analysis. Chromatography was performed on Phenomenex® column (250×4.6 mm; 5 μm) using acetonitrile and water (60:40, v/v) as mobile phase and wavelength of 300 nm. The stability of SMART-OH was also tested under the same alkaline hydrolysis conditions at 37 °C overnight to determine if there was any degradation of SMART-OH. The data was reported as the mean ± SEM of three individual experiments. Payload was calculated using equation 1.

\[
\text{Payload} \left(\frac{w}{w} \%\right) = \frac{\text{amount of hydrolysed drug}}{\text{total weight of polymer drug conjugate}} \times 100\%
\]

4.2.7 In vitro drug release

Drug release from the P-SMART conjugate was determined at pH 6.5 and 7.4. Briefly, 1 mg of P-SMART was suspended in 1 ml buffer solution (0.1 M acetic acetate, pH 6.5 or 0.1 M PBS, pH 7.4) and diluted with MeOH in a volume ratio of MeOH: aqueous solution (1:4, v/v). All samples were incubated at 37 °C, shaken at 100 rpm for 0, 3, 6, 9, 12, 24, 48, 72, 96, 120 h, and neutralized to pH 7.4 prior to HPLC analysis as described in previous section. All experiments were performed in triplicate and the data reported as the mean ± SEM of three individual experiments.
4.2.8 Cell culture

Human melanoma cell line A375, mouse melanoma cell lines B16-F10 and B16-F10-luc were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were grown and maintained in a humidified incubator containing 5% CO₂ at 37 °C.

4.2.9 Cytotoxicity assay

A375 and B16-F10 cells were used to determine the cytotoxicity of P-SMART and parent drug. After attached to the bottom of plate, cells were incubated with different concentrations of SMART-OH or P-SMART for another 72 h. Cell viability was determined by MTT assay and the absorbance was measured at 560 nm with subtraction of absorbance at 630 nm. Each group was performed in triplicate and the data reported as the mean ± SEM.

4.2.10 Colony formation assay

A375 or B16-F10 cells were seeded at 250 cells/well into 6-well plates and allowed to attach for 24 h. Cells were then treated with SMART-OH or P-SMART at different concentrations. After a 7 day-incubation, colonies in each well were fixed by 10% formalin, stained with 0.5% crystal violet solution and visualized under a microscope. Each group was performed in triplicate.

4.2.11 Transwell invasion assay

Cell invasion experiments were carried out using 24-well plates and cell culture inserts with 8 μm pore size (Corning®). The upper sides of the inserts were coated with 40 μL Matrigel® diluted 1:4 (v/v) with serum-free DMEM, were placed in a 24-well plate, and were incubated for 2 h at 37 °C. B16-F10 cells were
suspended in serum-free DMEM and placed in the upper chamber of the Transwell insert (1×10^5 cells/ml) with treatment of SMART-OH or P-SMART at a dose of 0.5 μM. Cell suspension without treatment was used as a control. DMEM containing 10% FBS was added to the corresponding lower chamber. After 24 h, the non-invaded cells in the upper chamber were removed by a cotton swab and the invaded cells were fixed with 10% formaldehyde in PBS and stained with 0.5% crystal violet solution. Each group was conducted in triplicate wells and three 40X imaging areas were randomly selected for each well.

### 4.2.12 Cell cycle analysis by propidium iodide staining

A375 and B16-F10 cells were used for cell cycle analysis. Cells were cultured in a 24-well plate to 80% confluence and treated with P-SMART for 48 h. Cells were harvested, fixed in 70% ice-cold ethanol for 1 h and washed by PBS. A cell pellet containing 1x10^6 cells was then re-suspended in 0.5 mL of FxCycle™ PI/RNase staining solution and incubated for 15 min at room temperature. Cell cycle was measured by a flow cytometer (BD FACSCalibur NJ). Results from 20,000 fluorescent events were obtained for analysis.

### 4.2.13 In vivo study

All animal experiments were performed in accordance with the NIH animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center (UNMC), Omaha, NE. A mouse model of metastatic melanoma to the lung was established in 8 week-old female C57BL/6 albino mice by injecting 2 × 10^5 B16-F10-luc cells suspended in 100 μL PBS into their tail vein. Mice were randomly divided into three
groups of five animals per group when the radiance of tumor had reached $10^5$. SMART-OH and P-SMART were administered intravenously to mice once every three days for a total of five times. Group 1 was kept as the control and received normal saline, group 2 received 20 mg/kg SMART-OH in 35% of cosolvent (50% propylene glycol, 30% Cremophor® EL, and 20% ethanol) and 65% of dextrose solution, and group 3 received 20 mg/kg P-SMART (equivalent to free SMART-OH). Bioluminescent radiance of tumor was measured every other day using IVIS® Spectrum imaging system (PerkinElmer Inc., MA). At the end of the animal study (i.e., day 24), mice were sacrificed and tumors as well as vital organs (liver, spleen, kidney and heart) were excised.

In a separate survival study, mice were randomly divided into three groups of seven mice for different treatments as described above. Survival observation of mice ceased when death occurred due to uncontrolled tumor growth or the toxicity of treatments. Three representative tumor tissues were collected per group and fixed with 10% buffered formalin for 24h. The fixed samples were embedded in paraffin and thin sections of 4 μm were obtained and immunostained for hematoxylin and eosin (H&E) and cleaved Caspase 3.

4.2.14 Statistical analysis

Data were represented as the mean ± SEM. The statistical comparisons of the experiments were performed by two-tailed Student’s t test. P < 0.05 was considered statistically significant.
4.3 RESULTS

4.3.1 Characterization and molecular docking of SMART-OH in tubulin

The structures for all synthesized compounds were characterized and confirmed by NMR and high resolution mass spectrometry. The proton NMR for final compound 5 (SMART-OH) was shown in Figure 27.

![Figure 27. Synthesis and characterization of SMART-OH.](image)

Docking studies (Figure 28A) indicated excellent binding and interactions between SMART-OH and the tubulin dimer. SMART-OH and the native ligand in the crystal structure of 1SA0 showed good overlap when they bind to the colchicine
binding site in tubulin (Figure 28A, enlarged portion). Three hydrogen bonds were formed between SMART-OH and the tubulin dimer, namely oxygen in the 4-methoxy moiety to Cys241; the carbonyl to Asp-251; and the phenol to Val315. These three hydrogen bonds anchor SMART-OH tightly in this colchicine binding pocket, predicting effective disruption of tubulin polymerization.

**Figure 28. Inhibition of SMART-OH on tubulin.** A) Molecular docking. Docking image showed that SMART-OH bind to the colchicine binding site in tubulin. B) Tubulin polymerization assay. Tubulin (3.33 mg/ml) was exposed to 10 µM of SMART-OH, colchicine or vehicle control (5% DMSO), respectively, and incubated in general tubulin buffer. Absorbance at 340 nm was monitored at 37°C every minute for 15 min. Both SMART-OH and colchicine effectively inhibited polymerization, while robust polymerization was observed in the control group.

### 4.3.2 Inhibition of tubulin polymerization

To evaluate the ability of SMART-OH to directly interact with tubulin and confirm its mode of action, we performed a microtubule polymerization assay *in vitro*. A vehicle and colchicine (10µM), a well-known microtubule destabilizing agent, were used as controls and assayed under the same conditions. Robust polymerization is observed in the control group, while both SMART-OH and colchicine effectively inhibit polymerization (Figure 28B). This result is consistent
with the proposed mechanism of action of SMART-OH compound as a potent tubulin polymerization inhibitor.

### 4.3.3 Characterization and quantification of P-SMART

P-SMART was synthesized by direct carbodiimide coupling of SMART-OH onto the pendant carboxylic acid groups of the hydrophobic block of mPEG-b-PCC copolymer (Figure 29A). In 
\(^{1}\)H NMR spectrum of mPEG-b-PCC, protons corresponding to \(-\text{CH}_2\text{-CH}_2\text{-O-}\) of PEG at \(\delta 3.4\text{–}3.6\), \(-\text{CH}_2\text{-}\) units of PCC at \(\delta 4.2\text{–}4.4\) and \(-\text{COOH}\) at \(\delta 12\text{–}14\) were observed and reported earlier by our group. After conjugation of SMART-OH to mPEG-b-PCC, protons corresponding to SMART were all observed in \(^{1}\)H NMR spectrum. \(^{1}\)H NMR (500 MHz, DMSO-\(d_6\)) spectrum of mPEG-b-PCC-g-SMART-g-DC showed peaks corresponding to PEG: \((-\text{CH}_2\text{-CH}_2\text{-O-})\) at \(\delta 3.5\), PCC: \((-\text{CO-O-CH}_2\text{-C-})\) at \(\delta 4.2\) (m, 4H), \((-\text{CO-O-CH}_2\text{-C-CH}_3\)) at \(\delta 1.1\text{–}1.3\) (t, 3H), Dodecanol: \(\text{CH}_3\text{-}(\text{CH}_2)_9\text{-}\) at \(\delta 0.9\) (t, 3H), \(\text{CH}_3\text{-}(\text{CH}_2)_9\text{-}\) at \(\delta 1.0\text{–}1.3\) (bs, 18H), \(\text{CH}_3\text{-}(\text{CH}_2)_9\text{-CH}_2\) at \(\delta 1.6\) (m, 2H), \(\text{CH}_3\text{-}(\text{CH}_2)_9\text{-CH}_2\text{-CH}_2\) at \(\delta 4.3\) (m, 2H), SMART-OH: benzene \(-\text{CH-}\) at \(\delta 8.1\) (dt, 2H), \(\delta 7.6\) (dt, 2H), \(\delta 7.2\) (t, 2H), thiazole \(-\text{CH-}\) at \(\delta 8.7\) (s, 1H), \(-\text{OCH}_3\) at \(\delta 3.83\) (s, 6 H) and \(\delta 3.71\) (s, 3 H) (Figure 29B).

DLS showed the mean particle size of P-SMART was 71.51±0.47 nm (PDI: 0.055 ± 0.011) (Figure 29C). Naked SMART-OH was stable under alkaline hydrolysis condition and drug payload of conjugated SMART-OH was determined by HPLC analysis as 14.3±2.8 % (w/w).
4.3.4 PH dependent drug release

*In vitro* drug release studies were carried out in PBS at pH 7.4 and acetate buffer at pH 6.5 to simulate drug release in blood and tumor environment. P-SMART showed a slow but sustained release of SMART-OH. There was no noticeable initial burst release at the different pH and no significant drug release at neutral conditions afterwards. At pH 6.5, the liberation of SMART-OH from P-SMART was accelerated as expected due to the increased cleavage of ester linkages known to occur in an acidic or basic environment. After five days, more than 25% of SMART-OH was released at pH 6.5, but only 15% of SMART-OH at pH 7.4 (*Figure 29D*).
4.3.5 Anticancer activity

We determined the anticancer activity of P-SMART as well as parent drug SMART-OH on A375 and B16-F10 cells for 72 h. Due to the slow release of SMART-OH from the polymer conjugates, the IC$_{50}$ of P-SMART increased to 0.75 μM in two cell lines while IC$_{50}$ of SMART-OH was 75 nM in A375 cells and 150 nM in B16-F10 cells. P-SMART effectively killed 80% of melanoma cells at 2 μM parent drug equivalent dose (Figure 30A).
4.3.6 Inhibition of colony formation

The inhibitory effect of SMART-OH and P-SMART on tumorigenic potential in melanoma cells was determined by colony formation assay. The parent drug SMART-OH greatly reduced colony formation compared with the control group in both cell lines. Meanwhile, long-term treatment of P-SMART allowed much amount of conjugated drug to be released from the conjugate and then expose to melanoma cells, which resulted in significant anti-proliferative effect. Treatment of A375 with P-SMART at a dose of 75 nM reduced colony formation by 94.5% compared to the control and treatment of B16-F10 cells with 200 nM P-SMART reduced colony formation by 79%. The doses of P-SMART in this assay were far below the IC\textsubscript{50} of P-SMART in cytotoxicity assay (Figure 30B).
We also determined the inhibitory effect of SMART-OH and P-SMART on cell invasion using B16-F10 cells. At a dose of 0.5 μM, both parent drug and prodrug showed effective inhibition of cell invasion. Treatment of SMART-OH suppressed 87% of cell invasion while the ability of P-SMART to prevent cell invasion was slightly less with 73% of cell invasion blocked at 24 h (Figure 31).

Figure 30. Cytotoxicity and colony formation assay. A) Cytotoxicity of SMART-OH and P-SMART was determined in A375 and B16-F10 cells for 72 h. The IC50 of P-SMART was 0.75 μM in two cell lines. B) To determine colony formation of melanoma cells, 250 cells per well were seeded to 6-well culture plates. At 24 h, drug formulations were added and at 7 days, cell colonies were fixed, stained and counted. The long-term treatment of P-SMART resulted in significant anti-proliferative effect. Data represented as the mean ± SEM (n=3). *p<0.05, **p<0.01 compared to Control.
4.3.7 Effect of P-SMART on cell cycle and apoptosis

The effect of P-SMART on cell cycle and apoptosis was determined by PI-staining using A375 and B16-F10 cells. There was observable G2/M phase arrest after treatment of these cells with P-SMART for 48 h and the % of cells in G2/M phase was augmented in a dose dependent manner. Specifically, the number of A375 cells increased from 14.9% in the control group to 19.1% with 1 μM of P-SMART and to 39.0% with 1.5 μM of P-SMART (Figure 32A). Similarly, the % of B16-F10 cells increased from 11.3% in the control group to 22.9% with 1.5 μM of
P-SMART and to 53.0% with 2 μM of P-SMART (Figure 32B). In addition, the number of A375 cells in sub-G1 phase elevated from 0.04% in the control group to 24.0% with 1.5 μM of P-SMART (Figure 32A). The number of B16-F10 cells in sub-G1 phase increased from 5.2% in the control group to 28.3% with 2 μM of P-SMART (Figure 32B). The accumulation of cell population in sub-G1 phase indicated that apoptotic cells significantly increased after P-SMART treatment.

Figure 32. Cell cycle analysis and apoptosis of P-SMART. Cells were treated with P-SMART for 48 h, stained with propidium iodide (PI), and analyzed on a flow cytometer. A) A375. B) B16F10. Results were expressed as the mean ± SEM (n=3). The percent of cells in G2/M phase and sub-G1 phase was augmented in a dose dependent manner after treatment with P-SMART.
4.3.8 *In vivo* efficacy in B16-F10 lung metastatic mouse model

We successfully established a metastatic melanoma model in 8-week-old female C57BL/6 albino mice by injecting B16-F10-luc cells via tail vein. At day 10, all mice were imaged for luciferase bioluminescence to determine the tumor growth rate. The mice whose bioluminescent radiance reached $10^5$ were randomized into three groups: 1) control, 2) SMART-OH and 3) P-SMART. All treatments were administered intravenously at the equivalent dose of 20 mg/kg SMART-OH. Both parent drug and prodrug groups showed inhibition of tumor growth compared with the control group. Significantly higher tumor growth inhibition was observed in the group treated with P-SMART compared with the group treated with SMART-OH (Figure 33 and 34A).

![First day of treatment](image1)

![Last day of treatment](image2)

*Figure 33. In vivo representative bioluminescent images at first day and last day of treatments.* Mice (n=5) from Control (saline), SMART-OH and P-SMART groups were taken bioluminescent images every alternate day during the treatment. Images of four mice from each group were shown.
In addition, treatment with P-SMART significantly reduced the number of lung tumor nodules compared to the control and SMART-OH groups (Figure 34B-C). The survival study showed that the median survival was 28 days in the control group and 31 days in the SMART-OH group. The median survival was significantly prolonged (38 days) when mice were treated with P-SMART (Figure 34D).

Figure 34. In vivo efficacy of SMART-OH and P-SMART in B16-F10 lung metastatic animal model. Mice received saline, 20 mg/kg SMART-OH or 20 mg/kg P-SMART intravenously once every three days for a total of five times when the radiance of tumor had reached 10^5 (day 10 after tumor implantation). A) Radiance intensity plot of all groups was measured from day 10 to day 24. Data represented as the mean ± SEM (n=5). B) Representative tumors of each group were excised after sacrificing the mice at the end of the efficacy study. Significantly higher tumor growth inhibition and less number of lung tumor nodules was observed in the group treated with P-SMART compared to SMART-OH treated group. C) The weight of mouse lungs from each group was measured at the end of the study. D) Survival analysis of control group, SMART-OH group and P-SMART group. The median survival was 31 days in SMART-OH group and 38 days in P-SMART group while in control group median survival was 28 days.
Hematoxylin and eosin (H&E) stain of lung tissues confirmed the extensive metastasis throughout the lung lobe in the control group and the inhibition of metastasis and proliferation of tumor cells in the treated groups. As compared with the control and parent drug groups, the lung samples from P-SMART treated group exhibited alveolar lumen with limited mass of metastatic cells (Figure 35A). Furthermore, cleaved caspase-3 stain indicated the induction of significant apoptosis by treatment of P-SMART compared to the treatment of SMART-OH (Figure 35B). Additionally, the chronic toxicities of these treatments were also evaluated by histological analysis of the major organs (Figure 35C). No obvious histological changes were observed in the livers, spleens, kidneys and hearts from all the treated groups, which suggested that mice tolerated all treatments well.
Figure 35. Analysis of lung samples by hematoxylin and eosin (H&E), Caspase 3 stain and analysis of major organs by H&E stain. Lung samples from control, SMART-OH and P-SMART treated groups were excised, fixed and immunostained for A) H&E and B) Caspase 3. In control group the metastasis was throughout the lung lobe but in the treated groups the metastasis and proliferation of tumor cells was inhibited. Cleaved caspase-3 stain indicated significant the induction of apoptosis by P-SMART. C) Organ samples from three groups were excised, fixed and stained for H&E. No obvious histological changes were observed in the livers, spleens, kidneys and hearts from all the treated groups.
4.4 DISCUSSION

Conventional therapies of melanoma such as dacarbazine (DTIC) and combination of paclitaxel and carboplatin are small molecular weight drugs, which are rapidly eliminated from the circulation, requiring frequent dosing, leading to increased risk of side effects. From a clinical standpoint, many anticancer agents that are hydrophobic require appropriate drug delivery system to help them reach tumors after systemic administration. To solve this problem, hydrophobic drugs are encapsulated into liposomes, nanoparticles and micelles. However, physical encapsulation into these nanoparticulate systems usually results in fast drug release with burst effect. This means higher initial drug loading is needed because most drugs should ideally be released at the disease site to reduce their adverse effects. In contrast, chemical conjugation of drugs to polymers or lipids prevents immediate drug release during the transportation of polymer-drug conjugates and provides long-term sustained drug release ([Figure 29D]). Therefore, conjugation of a drug to the polymer prolongs drug circulation and enhances therapeutic efficacy. So far polymer-drug conjugates with linear backbone have undergone clinical evaluation, such as poly(ethylene glycol) (PEG), poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) copolymers, dextran and poly(glutamic acid) (PGA) [61-63].

We previously physically encapsulated SMART-100 into poly(ethylene glycol)-b-poly(D,L-lactide) (PEG-PLA) micelles by film dispersion.[98] As expected, drug loading in these physically drug encapsulated micelles was 1.5%. To increase drug loading, in previous studies we conjugated gemcitabine or paclitaxel to
mPEG-b-PCC, and in the current study we conjugated a novel microtubule inhibitor, SMART-OH, to the copolymer.

PEG was used as a hydrophilic block for synthesizing methoxy-poly (ethylene glycol)-block-poly (2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) copolymer. To form micelles, we need to maintain a delicate balance between hydrophilic and lipophilic components of the copolymer. Since the molecular weight of our polymer is in the range of 10,000-11,000 Da, we chose PEG of 5000 Da for synthesizing this polymer before conjugating our drug SMART-OH. If we use PEG of 2,000 Da, we could not have conjugated SMART-OH as much as what we have done in this work, otherwise no micelles could be formed. This PEG length helps us maintain higher HLB easily and allows us conjugating large amount of SMART-OH. The attachment of dodecanol (DC) also enhanced requisite hydrophobicity to form micelles of polymer-drug conjugate. Our polymer-drug conjugation system offers the following distinct advantages: a) PEG corona on the polymer imparts stealth property; b) conjugation ensures in vivo stability and no premature drug release in the circulation; c) small size of this conjugate facilitates the EPR effect to maximize drug delivery to the tumor. Therefore, these polymer-drug conjugate showed increased stability and antitumor effect compared to parent drugs.

Microtubule targeting agents that alter microtubule dynamics have been developed as anticancer drugs for more than several decades, and they have achieved exceptional clinical success acting as essential roles in combination therapy and adjuvant therapy [102-104]. These compounds are currently classified
as microtubule stabilizers and destabilizers based on their function on microtubule mass at high concentrations. Stabilizing agents such as taxanes, epothilones, discodermolide, laulimalide, peloruside A, etc., enhance tubulin polymerization and block microtubule dynamics [105]. On the other hand, microtubule-destabilizing agents suppress tubulin polymerization and can be further characterized into two groups: vinca-domain binders and colchicine-domain binders. Although the wide application of microtubule inhibitors is observed, there is urgent need to overcome several emerging challenges including drug-resistance and neurotoxicity [106,107].

In this study, we synthesized a novel microtubule destabilizer, SMART-OH, with a hydroxyl group for conjugation with the copolymer (Figure 27). Molecular modeling suggested strong interactions between SMART-OH and the tubulin dimer, with the phenolic moiety forming a strong hydrogen bond interaction to Val315 in tubulin, in addition to the other two hydrogen bonds and hydrophobic interactions between SMART-OH and tubulin dimer (Figure 28A). Further, in vitro tubulin polymerization assay confirmed experimentally that SMART-OH effectively disrupted tubulin polymerization, serving as a potent microtubule-targeting agent (Figure 28B). It is also known that microtubule-targeting agents suppress microtubule dynamics leading to cell cycle arrest at the mitotic phase. In cell cycle analysis, cells were arrested in G2/M phase after treatment with P-SMART (Figure 32), which confirmed that the mechanism of action of P-SMART was through destabilization of microtubules. Treatment of P-SMART also resulted in cell
accumulation in sub-G1 phase indicating cell apoptosis and DNA damage was induced by P-SMART treatment.

In addition to its effects on microtubules, P-SMART demonstrated other anticancer activities in cytotoxicity, colony formation and cell invasion assays. As expected, P-SMART resulted in lower toxicity in A375 and B16-F10 cells when compared with the parent drug SMART-OH in a dose dependent manner. The IC\textsubscript{50} of P-SMART was 0.75 μM in both cell lines which was about 10-fold higher than SMART-OH in A375 cells and 4-fold higher in B16-F10 cells (Figure 30A). This finding is in good agreement with previous reports suggesting that conjugated anticancer drugs have higher IC\textsubscript{50} than their corresponding parent drugs due to slow drug release kinetics [108,109]. Unlike cytotoxicity assay, SMART-OH and P-SMART showed nearly equivalent activity in the colony formation assays (Figure 30B) likely due to the 7-day incubation period, which provided P-SMART with more time for cellular uptake and drug release. Therefore, the difference in anticancer effect between free drug and conjugated drug was reduced and this result demonstrated that the potency of SMART-OH was maintained after conjugation.

As discussed above, melanoma has high metastatic propensity and easily metastasizes to other organs. Thus, we did a transwell invasion assay to determine whether SMART-OH and P-SMART can impede the migratory potential and the invasive property of melanoma cells. Both SMART-OH and P-SMART showed significant cell invasion inhibition (Figure 31), which confirmed this drug had good anti-metastatic properties \textit{in vitro}. 
To investigate *in vivo* efficacy of SMART-OH and P-SMART, B16-F10-luc cells were selected to establish a metastatic model in C57BL/6 albino mice. We chose 20 mg/kg as the dose of SMART-OH and P-SMART based on our previous studies. We plan to do dose escalation studies and will report in our future publication. Both treated groups exhibited promising tumor inhibitory results. However, P-SMART treatment showed further enhanced reduction in tumor growth rate and tumor size compared with SMART-OH treatment (*Figures 33-34*). Apart from tumor growth suppression, P-SMART also extended mouse survival compared to other groups. In addition, a significantly lower burden of metastatic cells and increased level of apoptotic cells were found in P-SMART group. H&E stain of vital organs demonstrated our formulation carrying P-SMART was well tolerated, as other healthy organs did not show obvious histological changes after treatments (*Figure 35*). This is in agreement with our recent report indicating that our biodegradable copolymer mPEG-b-PCC as the backbone of delivery system has less toxicity and good safety [84].

**4.5 CONCLUSION**

We have synthesized a novel microtubule destabilizer SMART-OH and its corresponding polymer-drug conjugate P-SMART. Our results demonstrate that SMART-OH binds to microtubules and suppresses tubulin polymerization. Both SMART-OH and P-SMART inhibit *in vitro* proliferation and invasion of melanoma cells. When tested *in vivo*, P-SMART treatment shows increased anticancer efficacy in a melanoma model with lung metastases compared to the control and SMART-OH treatment. Future work to fight with metastatic melanoma will focus
on improving the potency of novel microtubule inhibitors and optimization of our delivery system by including targeting moieties.
CHAPTER 5 SUMMARY AND FUTURE DIRECTIONS

5.1 SUMMARY

Prostate cancer is one of the most common cancer types and the second leading cause of cancer-related death in American men. Although several hormone therapies (or androgen deprivation therapy) as first line treatments are effective for prostate cancer in early stage, the shrunken tumors might become androgen-independent after 18 to 24 month treatment leading to aggressive and metastatic forms of prostate cancer, also known as hormone refractory prostate cancer (HRPC). Taxane (docetaxel and paclitaxel), used as chemotherapy, is one of the standard therapies for HRPC. These anticancer agents have shown significant efficacy at initial period of chemotherapy, however, the long-term efficacy is limited and patients will suffer from relapse owing to the development of chemoresistance.

One notable cellular mechanism behind chemoresistance is related to the presence of cancer stem cells (CSCs). CSCs, a rare and distinct subset of cancer cells, have stem-cell-like properties such as self-renewal/differentiation, and tumorigenic potentials, which are responsible for cancer initiation, maintenance and relapse. The birth of cancer-stem-cell theory can be traced back to 1994. John Dick, a Canadian scientist, strikingly identified leukemia stem cell in human leukemia and inaugurated a new era of cancer research. With decades of further development, it has been established that CSCs are found in many other types of cancer including breast, ovary, prostate, pancreas, colon cancer and melanoma. A growing body of evidence suggests that several molecular signaling pathways such as
Sonic Hedgehog (Hh) signaling, Notch signaling and Wnt/β-Catenin signaling mainly involve in the initiation and development of CSCs. Therefore, in Chapter 2 and 3 we studied the combination therapy of microtubule inhibitor (paclitaxel or QW-296) and Hh signaling inhibitor (Cyclopa mine or MDB5) to overcome drug resistance and improve the therapeutic effect of chemoresistant prostate cancer. Since both tubulin inhibitors and Hh signaling inhibitors are highly hydrophobic, we also developed PEG-based polymeric drug conjugates or polymeric micelles to deliver these anticancer drugs and enhance therapeutic efficacy. Our results indicated that the combination formulations could synergistically work together with different mechanisms of action and suppress chemoresistant prostate tumor growth in vitro as well as in orthotopic mouse model.

Melanoma originated from melanocytes is the most aggressive type of skin cancer. It has high potential to metastasize through lymph nodes to the distant sites of the body, especially the lungs, liver and brain. Systemic chemotherapy remains the mainstay of its treatment; however, multidrug resistance (MDR) and dose limiting toxicity restrict the efficacy of current chemotherapeutic drugs. We recently synthesize a novel microtubule destabilizer, substituted methoxybenzoyl-ary-thiazole (SMART-100) and it can effectively circumvent MDR that hinders the clinical efficacy of existing tubulin inhibitors. Nevertheless, poor water solubility of SMART-100 requires co-solvent delivery for its systemic administration, which associates with toxicity to liver and kidney, hemolysis and peripheral neuropathies. Therefore, in Chapter 4, to solve this problem and prolong circulation of this small molecule, we developed methoxy-poly (ethylene glycol)-block-poly (2-methyl-2-carboxyl-propylene carbonate-graft-SMART-graft-dodecanol) (P-SMART) with
high drug payload of SMART. This polymer-drug conjugate self-assembled into micelles with small particle size and the release of SMART was slow but at appreciable rate. Similar with its parent drug, P-SMART killed 60-70% of A375 cells and B16-F10 cells at 1 µM of equivalent concentration of SMART and arrested cell cycle in G2/M phase. In addition, P-SMART significantly suppressed colony formation and cell invasion of melanoma cells. We established lung metastatic melanoma in C57/BL6 albino mice by injecting B16-F10-luc through tail vein. During the treatments, there was maximum inhibition of tumor growth in P-SMART group compared with control group and parent drug group. In conclusion, this novel polymer-microtubule inhibitor conjugate P-SMART has the potential to treat lung metastasis melanoma.

5.2 FUTURE DIRECTIONS

The preliminary findings reported in the prostate cancer projects and melanoma project confirmed that the combination strategy or the novel tubulin destabilizer had the potential to treat chemoresistant prostate cancer or metastatic melanoma. The PEG-based polymeric formulations could promote the delivery efficiency of these anticancer agents to the tumor sites leading to enhanced therapeutic efficacy compared with their corresponding parent drugs. However, from a perspective of clinical translation, our current polymeric delivery platform could be improved from the following aspects.

First, it is known that nano-sized agents could preferentially leak into tumor sites through permeable tumor vasculature; nevertheless, the EPR effects as known as passive targeting are relatively moderate and at the same time a small
portion of drugs can also extravasate into normal tissues. Therefore, in the future we will attach targeting moieties such as peptides or antibodies to the polymeric platform to develop a specific active-targeting drug delivery system, which can reduce off-target delivery and release more anticancer drugs at the site of action. Furthermore, besides targeting efficiency, the drug release profile is another limiting factor for therapeutic efficacy, which suggests the composition of formulation can be designed to release the drug according to the tumor microenvironment or the therapeutic needs. Since the pH of tumor site is usually acidic, in future studies we will modify polymer components to be sensitive to acidic environment allowing pH-triggered drug release.
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