Combination therapy of paclitaxel and cyclopamine polymer-drug conjugates to treat advanced prostate cancer

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Abstract

Repeated treatments with chemotherapeutic agent(s) fail due to cancer stem cells (CSCs) and chemoresistance regulated by microRNAs (miRNA) whose expression alters owing to dysfunctional signaling pathways including Hedgehog (Hh) signaling. We previously demonstrated the combination of Hh inhibitor cyclopamine (CYP) and paclitaxel (PTX) effectively inhibit PTX-resistant cells and side population, a cell fraction rich in CSCs. In this study, we 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 Hh signaling and up-regulated 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 conclusion, this combination therapy of P-PTX and P-CYP has the potential to treat chemoresistant prostate cancer.

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Key words: Prostate cancer; Polymer-drug conjugate; Cyclopamine; Paclitaxel; Chemoresistance

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, shrunken tumors often become androgen-independent at 18–24 months post-treatment, 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. 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. 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. 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 stem cells (CSCs), which are a distinct subset of cancer cells with self-renewal/differentiation and tumorigenic potentials. 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. CYP has shown to inhibit the
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.30–25 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.26

In our previous studies,27 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.

**Methods**

**Preparation and characterization of polymer-drug conjugate micelles**

Synthetic schemes of P-PTX and P-CYP are shown in Figure S1. 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.27

PTX or CYP encapsulated micelles were also prepared by film hydration. 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 3000 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:

Drug loading (w/w %) = Weight of encapsulated drug/Total weight of formulation X 100.

**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.28 Briefly, 1 mg of P-PTX or P-CYP was re-suspended in 1 ml buffer solution (0.1 M acetic acid, 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 (Waters HPLC system with 996 photodiode array detector (Milford, MA, USA). The release of PTX and CYP from two conjugated micelles under physiologically simulating condition was also studied. Micelles (final concentration of 1 mg/ml) were incubated with 45 mg/mL bovine serum albumin (BSA) at 37 °C with gentle agitation and HPLC was used to determine released drug. 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 × 250 mm; 5 µm), mobile phase: 30:70 acetonitrile: water (0.1% trifluoro-acetic acid); wavelength: 210 nm.

**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-12 K 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.

**Cytotoxicity assay**

DU145-TXR and PC3-TXR cells were used to determine the cell growth inhibition ability of polymer-drug conjugates. Cells (5X10^3/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:

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\text{Cell viability (%)} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100.
\]

**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.

**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 Ct 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 primers were used:

- **RNU6–1**: GUGCUCGCUCUUGGCAGCACAUAUA
- **hsa-miR-29b-3p**: UAGCACCAUUUGAAUAUCAGUGUU
- **hsa-miR-34a-5p**: UGGCAGUGUCUUGAGAGUUGU
- **hsa-miR-148a-3p**: UCAGUGCACUACAGACUUUGU
- **hsa-miR-200c-3p**: UAAUACUCGCGCGUAUAGUGGA

**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’-AAGCATTTGACATTTCTTCTTCTTGG-3’
- **CD133 reverse primer**: 5’-AAGCCAGAGGCTATGAGAAGATGAG-3’
- **NOTCH2 forward primer**: 5’-GGCTTACCGTAGTCACTCAC-3’
- **NOTCH2 reverse primer**: 5’-GGAGCGCACTCATATGCTATG-3’
- **Gli1 forward primer**: 5’-TTCTACCGTTAATCTCTCCTTATT-3’
- **Gli1 reverse primer**: 5’-GGCCAGAGGTACCTGACATGTAC-3’
- **PTCH1 forward primer**: 5’-ATGCAGTACCTTTTCTTCTTGG-3’
- **PTCH1 reverse primer**: 5’-GGTCTTCTGAGATGTGCTACA-3’
- **E-CAD forward primer**: 5’-AAGAAAGCTGGCTGACATGTAC-3’
- **E-CAD reverse primer**: 5’-CCACACAAACCTGAGATGCTTTCTGAC-3’
- **ZEB1 forward primer**: 5’-ATGCACAACCAAGGCTGAGAAG-3’
- **ZEB1 reverse primer**: 5’-TGCTTGTCTGAGATGTGCTACA-3’
- **ZEB2 forward primer**: 5’-CTAACCAACGAGGCTGAGAAG-3’

**Figure 1. 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.
ZEB2 reverse primer: 5′-GGGAAGAACCCGTCTTGATA TT-3′.

GAPDH forward primer: 5′-GATTCCACCCATGGCAAAT TC-3′.

GAPDH reverse primer: 5′-GTCATGAGTCCTTCCACGA TAC-3′.

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 $1 \times 10^6$ 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 $10^7$. 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 24 h. 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.

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.
Results

Formulation characterization

High drug payload of these polymer-drug conjugates was observed by using $^{1}$H NMR characterization (Figure S2): $25.40 \pm 3.70\%$ (w/w) and $14.50 \pm 1.60\%$ (w/w) for PTX and CYP, respectively. Unlike polymer-drug conjugates, PTX and CYP loadings in physically encapsulated micelles were $8.21 \pm 0.01\%$ (w/w) and $5.36 \pm 0.07\%$ (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 $70.02 \pm 0.22$ nm (PDI 0.223) and $76.37 \pm 0.15$ 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.

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 1, A). In contrast, both PTX and CYP were released from physically encapsulated micelles very fast, with almost 60% of their release at 24 h post incubation at pH 7.4 and 5.3 (Figure S4). In addition, with incubation of BSA both PTX and CYP were released from the conjugates very slowly as determined by HPLC (Figure 1, B). 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 S3). These results suggested that the conjugated micelles were stable under physiologically simulating conditions.
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 2, A–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 2, C–D).

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 3). 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 3). 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.

Efficacy in orthotopic prostate tumor bearing mice

We established orthotopic prostate tumor in 8 weeks old male athymic nude mice by injecting 1x10⁶ PC3-luc-GFP cells.

Figure 4. In vivo representative bioluminescent images at day 7 and day 24. Mice (n = 5) from control (saline), P-PTX, P-CYP and P-PTX + P-CYP group were taken bioluminescent images every alternative day from day 7 to day 24.
day 7, all mice were imaged for luciferase bioluminescence to determine the tumor growth rate (Figure 4) 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 5, A–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 5, C). 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 6). No obvious histological changes were observed in the livers, spleens, kidneys and hearts from all the treatment groups.

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 7, A). 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 7, B).

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. 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, 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. 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 S1). 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 1H NMR (Figure S2). 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 S4), which is in good agreement with the literature. 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 1, A).

So far polymer-drug conjugates with linear backbone have undergone clinical evaluation, such as polyethyleneglycol (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.34–36 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.37 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 S5). 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 2), which is in good agreement with our previous observation.37 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 3), leading to

Figure 7. 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).
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. 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. miR-29b can repress expression of Hh pathway and the inhibition of this signaling leads to the restoration of miR-29b expression. miR-148a is reported to inhibit the proliferation and metastasis of PTX-resistant prostate cancer and also attenuate the resistance to PTX. 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 5). 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 7). 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 6). 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. Unlike our current studies these authors used subcutaneous tumor model, while we have evaluated our formulations in orthotopic prostate tumor model.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2016.07.017.

References


