Published in final edited form as:

Pharm Res. ; 35(1): 17. doi:10.1007/s11095-017-2303-3.

Coadministration of Polymeric Conjugates of Docetaxel and Cyclopamine Synergistically Inhibits Orthotopic Pancreatic Cancer Growth and Metastasis

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Abstract

Purpose—The aim of this study was to determine whether co-administration of hedgehog (Hh) pathway inhibitor cyclopamine (CYP) and microtubule stabilizer docetaxel (DTX) as polymer-drug conjugates, methoxy poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylenecarbonate-graft-dodecanol-graft-cyclopamine) (P-CYP) and methoxy poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propyl-ene carbonate-graft-dodecanol-graft-docetaxel) (P-DTX) could synergistically inhibit orthotopic pancreatic tumor growth in NSG mice.

Methods—P-DTX and P-CYP were synthesized from mPEG-b-PCC through carbodiimide coupling reaction and characterized by ¹H-NMR. The micelles were prepared by film hydration and particle size was measured by dynamic light scattering (DLS). Cytotoxicity, apoptosis and cell cycle analysis of P-DTX and P-CYP were evaluated in MIA PaCa-2 cells. *In vivo* efficacy of P-DTX and P-CYP were evaluated in NSG mice bearing MIA PaCa-2 cells derived orthotopic pancreatic tumor.

Results—P-CYP and P-DTX self-assembled into micelles of <90 nm and their combination therapy efficiently inhibited the proliferation of MIA PaCa-2 cells, induced apoptosis and cell cycle arrest at M-phase more efficiently than P-CYP and P-DTX monotherapies. Furthermore, the combination therapy of P-CYP and P-DTX significantly reduced Hh component expression compared to P-CYP alone as determined by Western blot analysis. Lastly, the combination therapy induced greater inhibition of orthotopic pancreatic tumor growth in NSG mice compared to their monotherapies.

Conclusion—Combination of polymer conjugated anticancer drug (P-DTX) with polymer conjugated Hh inhibitor (P-CYP) enhanced pancreatic cancer cell killing, apoptosis as well as *in vivo* tumor growth inhibition with no obvious toxicities.

Keywords

cyclopamine; docetaxel; pancreatic ductal adenocarcinoma; polymer-drug conjugate

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is among the most devastating diseases, with a 5-year survival rate of less than 6% [1]. The curative therapeutic option is surgical resection; however, because of the lack of early symptoms, the vast majority of patients present in the clinic at a very advanced stage, and less than 25% of patients are surgically resectable by the time of diagnosis [2,3]. By 2030, pancreatic cancer is expected to be the third leading cause of cancer-related death [4]. In 1996, the Food and Drug Administration (FDA) approved gemcitabine hydrochloride as a first line chemotherapy for locally advanced and metastatic pancreatic adenocarcinoma, but its clinical outcomes are poor due to inefficient delivery and rapid metabolic inactivation [5]. In addition, a new combination therapy FOLFIRINOX (5-fluorouracil [5-FU], leucovorin, irinotecan and oxaliplatin) has shown improvement in overall survival compared to gemcitabine alone. However, there were significantly more grade 3–4 toxicities such as cytopenia and neutropenia fever with this therapy [6].

One of the characteristic features of PDAC is development of a dense desmoplasia (abundant fibrotic stroma around the cancer cells) that affects tumorigenesis, therapy resistance and possibly metastasis of tumor cells [7]. Desmoplasia is composed of extracellular matrix (ECM) including collagen and fibronectin. These proteins play a vital role in supporting cancer cell growth, invasion and metastasis [8,9]. Hedgehog (Hh) signaling pathway promotes desmoplasia by causing pancreatic stellate cells to differentiate into myofibroblasts, which hinder the delivery of chemotherapeutic agents by forming a barrier from fibrotic cells around the tumor cells. By blocking the Hh pathway to inhibit desmoplasia formation, extravasation of chemotherapeutic agents can be facilitated, and chemotherapeutic activity may be improved.

Cyclopamine (CYP) is a natural steroidal alkaloid, which can inhibit the Hh signaling pathway by blocking the 12-transmembrane protein smoothened (Smo) that inactivates the transcription factor glioma-associated oncogene family zinc finger-1 (GLI-1) [10]. As a result, CYP downregulates the target genes that are responsible for dense desmoplastic formation. CYP has been shown to inhibit cell proliferation of E3LZ10.7 and L3.6pl pancreatic cancer cell lines and growth of tumors in mice [11]. Docetaxel (DTX) acts as a microtubule stabilizer by binding to the β -tubulin and results in mitotic arrest and subsequent apoptosis. DTX is a second-line therapy for advanced pancreatic cancer [12]. However, DTX alone is not effective due to chemoresistance. Accordingly, we hypothesized that a combination of CYP and DTX might have a synergistic therapeutic efficacy in treating pancreatic cancer. However, both CYP and DTX are hydrophobic drugs that need an appropriate carrier system to improve their aqueous solubility. One of the strategies to improve water solubility is to use a co-solvent such as cremophor and Tween 80. The drawbacks of using these chemicals are their severe toxicities such as peripheral neuropathies, acute hypersensitivity reactions and alteration of pharmacokinetic profiles [13]. Therefore, polymeric micelle delivery systems, which either physically encapsulate drugs or conjugate to amphiphilic polymer can overcome these drawbacks. Physical drug-encapsulated micelles often have poor drug loading, premature drug release and unwanted toxicity. In contrast, covalently linked polymer-drug conjugate micelles are more stable in circulation and can release drugs in a controlled manner at the tumor site with a

therapeutically effective concentration [14]. Furthermore, drug conjugation to an amphiphilic polymer covalently accomplished higher drug payload compared to physically encapsulated micelles. In addition, the nanosize range confirms accumulation of these conjugates in tumor site through the enhanced permeability and retention (EPR) effect [15].

Previously, we synthesized methoxy poly (ethylene glycol)-block-poly (2-methyl-2-carboxyl-propylene carbonate (mPEG-b-PCC) as a polymer delivery system and conjugated CYP and paclitaxel (PTX) to the carboxyl pendant groups of mPEG-b-PCC [16,17]. In the present study, we conjugated CYP and DTX to the carboxyl pendant groups of mPEG-b-PCC. DTX was used instead of PTX because DTX is more potent than PTX. The final polymeric conjugates with CYP or DTX contained biocompatible PEG blocks to contribute stealth property, dodecanol (DC) as lipid chains, and polycarbonate as the biodegradable backbone. The polycarbonate backbone exhibits low toxicity to humans because its byproducts are carbon dioxide (CO₂) and alcohol. Furthermore, DC increases the hydrophobicity of the polymer, which promotes self-assembly into nanosized micelles. The polymer-drug conjugates were tested for their effect on the proliferation of MIA-PaCa-2 pancreatic cell lines, which highly express sonic hedgehog (SHh) and smoothened (Smo) receptor [18]. Finally, this formulation was administered intravenously into mice bearing orthotopic pancreatic tumors to determine whether P-DTX and P-CYP conjugates inhibit tumor growth associated with dense desmoplasia.

MATERIALS AND METHODS

Materials

CYP was purchased from Logan Natural Product (Plano, TX), and DTX was obtained from LC Laboratories (Woburn, MA). Dodecanol (DC), 1-ethyl-3-(3-(dimethylamino)propyl carbodiimide, N,N'-dicyclohexylcarbodiimide (EDC), N,N-dimethylpyridin-4-amine (DMAP), N,N'-Dicyclohexylcarbodiimide (DCC), Boc- β -alanine, dichloromethane (DCM), and hydroxybenzotriazole (HOBt) were obtained from Sigma-Aldrich (St. Louis, MO). Primary antibodies Shh (rabbit polyclonal antibody sc-9024), Bax (rabbit polyclonal antibody sc-6236) and GAPDH (mouse monoclonal antibody sc-365062) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Secondary antibodies goat anti-rabbit IgG-HRP (sc-2054) and goat anti-mouse IgG-HRP (sc-2055) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Matrigel® matrix basement membrane was obtained from Corning (Chicago, IL). All other chemicals were analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

Methods

Synthesis of mPEG-PCC-g-DTX-g-DC (P-DTX)—Previously, ring opening polymerization was utilized to synthesize monomer 2-methyl-2-benzoyloxycarbonyl-propylene carbonate and its copolymer with mPEG (mPEG-b-PBC) followed by reduction to obtain the copolymer containing carboxyl pendant groups (mPEG-b-PCC) [17]. Carbodiimide coupling reaction was used to conjugate DTX and DC to the carboxyl groups of mPEG-b-PCC polymer. mPEG-b-PCC (150 mg, 0.0158 mmol) was dissolved in anhydrous dichloromethane (DCM) followed by the addition of DTX (108 mg, 0.134

mmol), DCC (130 mg, 0.62 mmol) and DMAP (58 mg, 0.48 mmol). This reaction mixture was stirred for four to five days under nitrogen atmosphere at 4°C. Then, DC (47 mg, 0.24 mmol) was added, and the reaction was allowed to proceed for another two days (Fig. 1a). The product was purified by precipitation method in excess cold diethyl ether followed by dialysis against methanol for 4 days using a regenerated cellulose membrane with 7 K MWCO.

Synthesis of mPEG-b-PCC-g-CYP-g-DC (P-CYP)—Previously, secondary amine terminal was introduced to CYP structure (16). Briefly, Boc- β -Ala-TT was synthesized by reacting the following chemicals together. Boc- β -Ala-OH, 2-mercaptothiazoline and DMAP were dissolved in DCM under nitrogen protection. The solution was first stirred for 5 min –10°C followed by the addition of EDC 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. Then, the organic layer was deried by adding Na₂SO₄, and the product was purified by column chromatography (silica gel 60 Å, 200–400 mesh, EtOAc: CH₃OH 15: 1) and then recrystallized from DCM to find yellow powder. The structure was confirmed by ¹H-NMR spectroscopy. Second step is the synthesis of Boc- β -Ala-CYP. Briefly, the final product from the first step (Boc- β -Ala-TT 53 mg 0.185 mmol) and CYP (38 mg, 0.09 mmol) were dissolved in pyridine under nitrogen. The reaction mixture was stirred at 50°C for 18 h. The solvent was removed by rotary evaporation and t. The resulted product was cleaned 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. Third step is the synthesis of NH₂- β -Ala-CYP. Briefly, the final product from the previuos step (Boc- β -Ala-CYP) was dissolved in anhydrous DCM (3 mL) and TFA (3 mL) was added at 0 °C. Then, the solvent was removed by N₂ flashing. The crude product was dissolved in chloroform (20 mL) and washed with aqueous saturated NaHCO₃ (3 × 25 mL) and brine (1 × 25 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. Final step was to synthesize P-CYP. Briefly, mPEG-PCC (185 mg, 0.0195 mmol) was dissolved in anhydrous DCM followed by the addition of NH₂- β -Ala-CYP (75 mg, 0.15 mmol), EDCI (75 mg, 0.375 mmol), HOBt (53 mg, 0.375 mmol). The reaction was stirred for two days under nitrogen at the room temperature. Then, DC (72.45 mg, 0.375 mmol) was added and the reaction was allowed to proceed for another two days (Fig. 1b). After completion of the reaction, the final product was purified by precipitation in large excess of diethyl ether and then by dialysis against ethanol using a regenerated cellulose membrane of 3500 MWCO.

Preparation and characterization of P-CYP and P-DTX micelles—Briefly, 15 mg of P-CYP or P-DTX was dissolved in 500 μ L of DCM and the solvent was removed under reduced pressure to obtain a film, which was vacuum dried overnight. The film was hydrated by adding one mL of phosphate-buffered saline (PBS; pH 7.4) followed by probe sonication for 6 min and filtration by using a 0.22 μ m filter. A zetasizer Nano ZS90 (Malvern, Worcestershire, UK) was used to determine the mean particle size at a scattering angle of 90. Particle size distribution is reported as the mean $\pm$ S.D.

Cell Culture—The human pancreatic cancer cell line MIA PaCa-2 was obtained from ATCC (ATCC® CRL-1420™), and cultured in DMEM medium containing 10% FBS and 1% penicillin/ streptomycin. Cells were maintained in an incubator with 5% CO₂ at 37°C.

Isobologram Analysis—To assess the synergistic effect of combination of P-CYP and P-DTX, dose-response curves were plotted for the effect of P-CYP and P-DTX on MIA PaCa-2 and BxPC-3 cell viability separately. Both cells were treated with P-CYP (0, 1.5625, 6.25, 25 and 100 μM) and P-DTX (0, 1, 4, 16 and 64 nM) for 48 h. From these curves, The IC₅₀ values were determined for P-CYP micelles and P-DTX micelles in MIA PaCa-2 and BxPC-3 cells. Then, Isobologram method of analysis of Steel and Peckham were applied to check the synergism. The combination index was calculated based on the following equation

$$CI = \frac{d1}{D_{501}} + \frac{d2}{D_{502}}$$

Where D₅₀₁ is the dose of P-DTX that produce 50% inhibition effect alone, *d1* is the dose of P-DTX that produce same 50% inhibition effect in combination with P-CYP, D₅₀₂ is the dose of P-CYP that produce 50% inhibition effect alone, and *d2* is the dose of P-CYP that produce same 50% effect inhibition in combination with P-DTX. The interpretation of CI was done as follow: < 1.0 synergism, =1.0 additive, >1 antagonism. The parameter *d1* was obtained by using constant dose of P-DTX in combination varied dose of P-CYP (1.5625, 6.25, 25 and 100 μM). The *d2* was determined when the dose of P-CYP combined with *d1* produced the 50% inhibition in cell growth.

Cell Viability Assay—To determine the effect of P-CYP micelles, P-DTX micelles and their combination on cell viability and tumor spheroid formation, MIA PaCa-2 cells were seeded in a 96-well plate (5000 cells/well) and incubated for 24 h. The culture medium was replaced with medium containing either empty polymer micelles as a negative control, 25 μM P-CYP micelles, 16 nM P-DTX micelles, or a mixture of 25 μM P-CYP and 16 nM P-DTX micelles in 200 μL; cells were then grown for 48 h. Cell viability was evaluated by an MTT assay at 560 nm absorbance and 630 nm for the cell debris. The absorbance results from 630 nm were deducted from 560 nm to eliminate cell debris effects. The cell viability for all groups was calculated according to the following formula:

$$\text{Cell viability (\%)} = \frac{\text{absorbance of test sample}}{\text{absorbance of control}} \times 100$$

For 3D spheroid formation, MIA PaCa-2 cells were seeded in Perfecta3D® 96-Well Hanging Drop Plates (3D Biomatrix, Inc., Ann Arbor MI) in triplicate and incubated at 37°C, 5% CO₂ over the course of time. The cell number per well was 3000 cells in 40 μL of DMEM. The tumor spheroids were treated with empty polymer micelles as negative control, 25 μM P-CYP micelles, 16 nM P-DTX micelles, or a combination of 25 μM P-CYP micelles and 16 nM P-DTX micelles on day 4, 6 and 8 after forming tumor spheroid. The image under light microscope were taken in the day before starting the treatment, and the day after

each treatment (day 5th, 7th and 9th) to check the cytotoxic effect of different formulations on 3D tumor model.

Colony Formation Assay—To evaluate the ability of P-CYP micelles, P-DTX micelles or their combination to inhibit cells undergoing cell division, MIA PaCa-2 cells were plated in 6-well plate (500 cells/well), and cultured for 24 h to adhere in the bottom of the plate. Thereafter, the medium was replaced with the medium containing empty micelles as control, 25 μ M P-CYP micelles, 16 nM P-DTX micelles or a mixture of both conjugates in 2 mL then incubated for 14 days. Visible colonies which are consisted of at least 50 cells, were fixed with 10% formalin and stained with 0.5% crystal violet. All colonies were counted following the staining, and the percentage of colonies was calculated with respect to the control colonies as described earlier [19].

Apoptosis Assay—To assess apoptosis, MIA PaCa-2 cells were seeded into 6-well plates (3.5×10^5 cells/well) and incubated for 24 h. The culture medium was replaced with medium containing either plain polymer micelles, 25 μ M P-CYP micelles, 16 nM P-DTX micelles, or a mixture of both in 2 mL and incubated for 48 h. Then, the cells were stained with an Annexin-V and propidium iodide (PI) by using a Vybrant® Apoptosis Assay Kit according to the manufacture's protocol (Molecular Probe, Waltham, MA). Apoptotic cells were quantified by flow cytometry (Becton, Dickison, NJ, USA).

Cell Cycle Analysis—To determine the effect of treatment on cell cycle distribution, 3.5×10^5 MIA PaCa-2 cells were plated into 6-well plates with no FBS for 24 h. Cells were then incubated with empty polymer micelles as a negative control, 25 μ M P-CYP micelles, 16 nM P-DTX, or their mixture in 2 mL for 48 h. Then, cells were detached and fixed in 90% ice-cold ethanol overnight. Cells were washed with cold PBS twice and stained with propidium Iodide (PI) (Molecular Probes, Waltham, MA). Cell cycle analysis was assessed by flow cytometry, with 10,000 fluorescent events per analysis.

Western Blot Analysis—MIA PaCa-2 cells were seeded in 6-well plates (3.5×10^5 cell/well) for 24 h. Cells were then treated with various micelles for 48 h. Next, RIPA buffer (Sigma-Aldrich, St. Louis, MO) was used to lyse the cells, and a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) was used to measure protein concentrations. The lysate was denatured by heating at 90°C for 5 min, then subjected to 15% SDS-PAGE. Thereafter, the lysate was transported to a PVDF membrane using the iBlot™ system (Invitrogen, Carlsbad, CA). Membranes were blocked with Odyssey® blocking buffer for 1 h at room temperature. Then, membranes were incubated with primary antibodies for Shh (sc-9024) BAX (sc-6236), and GAPDH (sc-365,062) overnight at 4°C followed by washing with PBS with 0.05% Tween 20 (PBST). The membranes were then incubated with their corresponding horseradish peroxidase-conjugated secondary antibodyies (sc-2055, and sc-2054). Target proteins were detected by ImmunoCruz Western blotting luminol reagent kit (Santa Cruz Biotechnology, Dallas, TX).

Evaluation in Orthotopic Pancreatic Cancer-Bearing Nsg Mice—All animal experiments were performed according to the NIH animal use guidelines, and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the

University of Nebraska Medical Center, Omaha, NE. The orthotopic pancreatic tumor was created in NSG mice 6–8 weeks old by implanting 2×10^6 MIA Paca-2-GFP-luciferase cells into the dorsum of the pancreas using PBS: Matrigel (1:1 v/v, 50 μ L). The tumor size was monitored by noninvasive bioluminescence. In brief, 150 mg/kg of D-luciferin was administered intraperitoneally, and an IVIS imaging system (Caliper Life Sciences, Waltham, MA) was used to measure the intensity of bioluminescence. All animals were then divided randomly into four groups with six animals in each group once the radiance of the tumor reached 2×10^7 p/s/cm²/sr: saline as control, P-CYP micelles, P-DTX micelles, or a mixture of P-CYP and P-DTX micelles. Those formulations were given intravenously through the tail vein, 3 times per week for 2 weeks and the maximum volume injected was 200 μ L based on the mouse body weight. Group 1 was injected with saline as the vehicle control. Group 2 received six doses of formulated P-CYP micelles in 2 weeks, and the equivalent dose of P-CYP was 20 mg/kg of free CYP. Group 3 received 6 doses of P-DTX micelles in 2 weeks equivalent to 5 mg/kg of free DTX. Group 4 received a combined-chemotherapy of P-CYP and P-DTX with respect to their actual therapeutic doses of 5 mg/Kg and 20 mg/Kg P-DTX and P-CYP, respectively. Bioluminescent radiance of the tumor and body weight were measured every other day following cells implementation for 45 days. After finishing the study, all animals were sacrificed, and the tumor and all vital organs (liver, spleen, kidney, heart) were collected and stored in -80°C .

Immunohistochemical and Immunofluorescence Assay—Tumor tissue was fixed with 10% buffered formalin for 24 h. The samples were embedded in the paraffin, and thin sections of 4 μ m were prepared and immunostained for cell proliferation marker (Ki-67), apoptosis (cleaved caspase 3), tissue morphology hematoxylin and eosin (H&E), total collagen (Masson's trichomes), epithelial–mesenchymal transition (EMT) modulator (ZEB-1) and metastatic marker (E-cadherin).

To detect tumor cell apoptosis induced by different micelle formulation, a TUNEL assay kit (Abcam, Cambridge, MA) was used according to the manufacturer's protocol. PI was used to counterstain the tumor sections and then imaged under a fluorescence microscope (Zeiss, Germany).

Statistical Analysis—Data are represented as the mean $\pm$ standard deviation (SD). The statistical comparisons of the data were performed by student's *t* test. *P*-value <0.05 was considered statistically significant.

RESULTS

Synthesis and Characterization of Polymer-Drug Conjugates

P-DTX was synthesized by direct carbodiimide coupling of DTX to one of carboxylic acid groups on the hydrophobic block of mPEG-b-PCC copolymer. The esterification took place favorably at 2'-hydroxyl group due to its less steric hindrance. In ¹H NMR spectra of mPEG-b-PCC, protons corresponding to $-\text{CH}_2 - \text{CH}_2 - \text{O}-$ of PEG were observed at δ 3.4–3.6, and $-\text{CH}_2-$ units of PCC δ 4.2–4.4 and $-\text{COOH}$ were observed at δ 12–14 as reported earlier by our group. After conjugation of DTX to mPEG-b-PCC, protons related to DTX were all seen in ¹H NMR spectra and DTX content in the conjugates was determined to be

30.4 ± 3.7% (w/w), as calculated from the peak intensities of all phenyl proton signals from 7.3 to 8.4 ppm (Fig. 1a,c). Conjugation of CYP was done 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. Therefore, 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 seen in ¹H NMR spectra and CYP content in the conjugates were 25 ± 1.6% (w/w) calculated from proton signal 1.7–2.4 ppm (Fig. 1b,d). P-DTX and P-CYP micelles were prepared by film hydration method, and the mean particle sizes of P-DTX and P-CYP were 66.18 ± 0.4 nm (PDI 0.190) and 73.11 ± 0.7 nm (PDI 0.209), respectively (Fig. S2).

Combined Treatment with P-CYP and P-DTX Synergistically Inhibits the Growth of Pancreatic Cancers Cells

To determine whether the combination of P-CYP and P-DTX could have a synergistic effect, combination index (CI) was determined after treating 5000 MIA PaCa-2 or BxPC-3 cells with P-CYP, P-DTX and combination of P-CYP and P-DTX. MIA PaCa-2 and BxPC-3 were treated with P-DTX (0–64 nM) to produce dose-response curves using MTT assay, with 50% inhibitory concentration (IC₅₀) 39 nM and 34 nM, respectively (Fig. S1 A). In addition, MIA PaCa-2 and BxPC-3 were treated with P-CYP (0–100 μM) to produce the dose-response curve. From these curves, the (IC₅₀) were 55 μM and 70 μM, respectively (Fig. S1 B). CI was calculated by using this formula: $CI = (d1/D_{501}) + (d2/D_{502})$. As shown in Table I, CI value was calculated to be 0.86 and 0.89 when MIA PaCa-2 and BxPC-3 were treated with 25 μM P-CYP and 16 nM P-DTX combination, respectively.

Combined-Treatment with P-CYP and P-DTX Decreases the Proliferation of Pancreatic Cancer Cells

Cell viability after incubation of MIA PaCa-2 and BxPC-3 cells with empty micelles, 25 μM P-CYP, 16 nM P-DTX micelles or their combination for 48 h was measured by an MTT assay. Combination treatment for 48 h reduced MIA PaCa-2 cells viability by 64% and BxPC-3 cells viability by 60% (Fig. 2a). However, both P-CYP and P-DTX micelles as monotherapy did not achieve the same level of cell viability reduction when 100 μM P-CYP and 64 nM P-DTX were used (Fig. S1 A,B). Thus, combination of P-CYP and P-DTX micelles had better effect on decreasing the cell viability of pancreatic cancer cells.

Comined Treatment with P-CYP and P-DTX Reduces Colony Formation Ability of MIA PaCa-2 Cells

The efficacy of combination treatment with 25 μM P-CYP and 16 nM P-DTX to inhibit 50 of MIA PaCa-2 cells to come close together and form one colony for 14 days was evaluated by unaided eye after staining with 0.5% crystal violet. The combination treatment had more effect on MIA PaC-2 cells in terms of decreasing the percentage of colonies to 12% compared to 34% and 67% in case of P-DTX micelles and P-CYP, respectively (Fig. 2b).

Combination of P-CYP and P-DTX Reduced the Size of MIA PaCa-2 Spheroid Structure

To simulate the real pancreatic tumor structure, 3D model was performed by using hanging drop method. Combination treatment with 25 μ M P-CYP and 16 nM P-DTX had the smallest spheroid size compared to either P-DTX or P-CYP only (Fig. 2c).

Combined Treatment with P-CYP and P-DTX Enhances Cell Cycle Arrest at G2/M Phase

The ability of the combination treatment with 25 μ M P-CYP and 16 nM P-DTX on cell cycle arrest was measured by flow cytometry using PI staining. Treatment for 48 h with the combination of P-CYP and P-DTX increased the fraction of cells in the G2/M phase compared to that of their monotherapies. The combination treatment resulted in increased number of cells in G2/M phase from $22.0 \pm 0.2\%$ in control to $63.3 \pm 2.5\%$. However, the increase in the number of cells in the G2/M phase with P-CYP or P-DTX alone was $30.2 \pm 0.2\%$ and $50.9 \pm 1.3\%$, compared to $22.0 \pm 0.2\%$ in control respectively (Fig. 3a).

Combination of P-CYP and P-DTX Enhances Apoptosis in Pancreatic Cell Lines

The ability of combined-treatment with 25 μ M P-CYP and 16 nM P-DTX to enhance apoptosis over 48 h was measured by an Annexin V binding assay. Combined-treatment with P-CYP and P-DTX resulted in more apoptotic cells compared to the single treatments (Fig. 3b,c). The percentage of cell death in the combination treatment group was $64 \pm 2\%$, whereas in the case of P-CYP or P-DTX, the percent of cell death was $33 \pm 1.5\%$ and $51 \pm 2\%$, respectively.

Combination Treatment with P-CYP and P-DTX Downregulates Their Respective Target Proteins—The effect of combination treatment of MIA PaCa-2 cells with 25 μ M P-CYP plus 16 nM P-DTX, vs. 25 μ M P-CYP or 16 nM P-DTX CYP and DTX alone on their target proteins, such as Shh and BAX were determined by Western blot analysis at 48 h post-treatment. Combined treatment of P-CYP and P-DTX significantly decreased Shh expression more than their monotherapies. On the other hand, combined-treatment of P-CYP and P-DTX significantly increased BAX expression compared to monotherapies (Fig. 3d).

In vivo Evaluation

To investigate the efficacy of combination therapy with P-CYP and P-DTX, orthotopical pancreatic cancer mouse model was established by implementing stably luciferase expressing MIA PaCa-2 cells in the pancreas. Once the tumor radiance reached around 2×10^7 p/s/cm²/sr, mice were injected intravenously with the following formulations via the tail vein thrice a week for 2 weeks: saline, 20 mg/Kg of P-CYP micelles, 5 mg/Kg of P-DTX micelles, or a mixture of 20 mg/Kg P-CYP and 5 mg/Kg P-DTX micelles. After 12 days of cell implementation in the pancreas, all mice were imaged for luciferase bioluminescence to determine the tumor growth and divided the mice randomly into different groups. At day 33, the final bioluminescence images were recorded and showed significant tumor growth inhibition in mice receiving the combination therapy to monitor the tumor burden over time (Fig. 4a). Luciferase bioluminescence intensity was measured throughout the study from day 12 to day 33. After plotting the photon intensity values of different mouse groups *versus*

time, mice administered combination therapy had the lowest photon intensity value compared to other groups receiving saline, P-CYP micelles, or P-DTX micelles (Fig. 4b). At the end of the study, all mice were sacrificed and the tumors and vital organs were collected. Then, the tumor weight and volume were measured, and we found that mice receiving combination therapy had the lowest tumor weight (0.23 ± 0.03 g) compared to the mice which were injected with P-DTX micelles (0.58 ± 0.16 g), P-CYP micelles (0.78 ± 0.10 g), or PBS (1.13 ± 0.04 g) (Fig. S3 A). Furthermore, the combination therapy had the lowest-tumor volume (202 ± 52 mm³) compared to P-DTX micelles (661 ± 112 mm³), P-CYP micelles (732 ± 104 mm³), or PBS (1596 ± 394 mm³) (Fig. 4c & Fig. S3 B). To evaluate the general toxicity profile of the formulation, mouse body weights were monitored throughout the treatment. No significant differences were observed in body weights in the treatment groups compared to the control (Fig. S3 C). In addition, the acute toxicity was evaluated in vital organs (liver, spleen, heart, or kidney). There were no huge histological changes post treatment for all groups (Fig. S4).

To expound the remarkable effect of combination therapy, immunofluorescence and immunohistochemistry experiments were carried out. Tumors from mice treated with combination therapy had loose groups of epithelial cells; however, mice treated with P-CYP micelles or P-DTX micelles had dense epithelial cells. Treatment with combination of P-CYP and P-DTX had the lowest staining of Ki-67 that reflects the cancer cell proliferation (Fig. 5). Moreover, mice receiving the mixture of P-CYP and P-DTX had the highest staining of cleaved caspase-3 that reflects the apoptosis of cancer cells (Fig. 5).

To investigate whether treatment with the combination therapy of P-CYP and P-DTX micelles can enhance the ECM degradation, tumor section slides were stained with Masson's trichrome. Combination treatment with P-CYP and P-DTX micelles degraded the components of ECM much more efficiently than either P-CYP micelles or P-DTX micelles alone (Fig. 6). An increase in E-cadherin signal was detected in the mice treated with the combination therapy of P-CYP and P-DTX micelles compared with the control or monotherapies. Furthermore, ZEB-1, which regulates the EMT was seen in low expression in mice treated with combination therapy of P-CYP and P-DTX micelles (Fig. 6). TUNEL assay was performed to evaluate the tumor growth inhibition. The apoptotic cells were stained with green fluorescence. P-CYP micelles or P-DTX micelles treated group had small number of apoptotic cells, but the combination of P-CYP and P-DTX micelles treated group had significant number of apoptotic cells (Fig. 7).

DISCUSSION

PDAC is a highly aggressive cancer usually diagnosed at an advanced state and poses a medical challenge due to its insensitivity to the majority of chemotherapeutic agents after long-term treatment. Hh signaling promotes pancreatic cancer cell proliferation by promoting EMT and decreasing apoptosis by regulation of Bcl-2 and Bcl-X. Hh pathway also promotes cell invasion, migration, and chemoresistance. Therefore, inhibition of Hh signaling using CYP has the potential to synergistically inhibit tumor growth and metastasis.

Unlike physically encapsulated drugs into polymeric micelles which are released faster due to the dynamic instability of micelles, bioconjugation to amphiphilic polymers prevents drug release in the circulation and provides sustained drug release. Therefore, in this study, we conjugated DTX and CYP to a biodegradable amphiphilic diblock copolymer mPEG-b-PCC with pendant carboxyl acid groups. We previously conjugated a water soluble drug gemcitabine as well as dodecanol to mPEG-b-PCC, which self-assembled into micelles and showed enhanced drug delivery to pancreatic tumor, which resulted in significant reduction in tumor growth [20–22]. In this study, DTX was conjugated to mPEG-b-PCC through an ester bond at its 2'-OH position. For CYP conjugation to mPEG-b-PCC, we used a linker containing thiazolidine-2-thione to react with secondary amine in CYP [16]. The attachment of DC enhanced requisite hydrophobicity and assisted in the self-assembly of polymer-drug-conjugate micelles. Combination of P-CYP and P-DTX micelles significantly inhibited MIA PaCa-2 and BxPC-3 cell viability (Fig. 2a). Isobologram method of analysis of Steel and Peckham suggests that the combination of P-CYP and P-DTX have a synergistic effect on the growth inhibition of pancreatic cell lines (MIA PaCa-2 and BxPC-3) since the CI values were less than one (Table I). This combination therapy inhibited colony formation, enhanced apoptosis and cell cycle arrest in G2/M phase, upregulated BAX proteins, and downregulated the Shh ligand much more than micelles with either CYP or DTX micelles alone (Fig. 2b,c & Fig. 3).

In vivo efficacy of P-DTX and P-CYP conjugate micelles was determined in orthotopic pancreatic tumor model developed by MIA PaCa-2-luc-GFP cells in NSG mice. Promising results were obtained in three treated groups wherein the combination therapy of P-DTX and P-CYP resulted in a significant reduction in tumor growth rate and tumor size compared with the control group (Fig. 4). In addition, the significantly low level of cellular proliferation and high level of apoptotic cells found in combination group demonstrated combined P-DTX and P-CYP reduces tumor growth synergistically (Figs. 5, 6 and 7). Furthermore, our formulation carrying P-DTX 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 (Fig. S4). The tumor tissues of mice treated with combination therapy were loose epithelial cells aggregate with a high amount of interspersed mesenchymal cells, whereas compact epithelial cells were seen in the control group. Ki-67 is a specific nuclear protein for cancer cell proliferation. Since the overexpression of this protein is linked to high cancer cell proliferation with low survival of the patients in many kinds of malignancies such as brain cancer [23]. A low expression of Ki-67 was seen in mice treated with a mixture of P-CYP and P-DTX mice, indicating low tumor cell proliferation. Cleaved caspase 3 is a hallmark of programmed cell death. The expression of this protein after immunostaining was higher in group treated with combination of P-CYP and P-DTX micelles compared to other groups. Masson's trichrome staining method was used to test where this combination has better effect on reducing ECM deposition. Mice treated with the mixture of P-CYP and P-DTX micelles had lowest Masson's trichrome staining compared to either control or monotherapies groups. This finding supports that using combination of P-CYP and P-DTX micelles decreases the stress in the cancer microenvironment because of reducing the ECM deposition. Moreover, measurement of E-cadherin expression of the mice treated with the combination therapy

showed significant enhancement in the expression that explain metastasis to the liver is low compared to other treated groups. High expression of ZEB-1 was observed in mice treated with combination of P-CYP and P-DTX micelles compared to either control group or their monotherapies. The number of apoptotic cells were seen very high in mice treated with the combination of P-CYP and P-DTX micelles, whereas these cells were found to be in small number in the control group or monotherapies group. Thus, the possibility to reduce the proliferation and increase the apoptosis of cancer cells *in vitro* and *in vivo* by using the combination therapy of P-CYP and P-DTX micelles may be a potential advantage to improve chemotherapy outcomes.

We recently reported higher drug loading and prolonged drug release of P-CYP and P-PTX conjugate micelles than physically drug encapsulated micelles [16]. In the present study, we replaced PTX with DTX, since DTX is more potent than PTX [24]. Using CYP as an Hh signaling pathway inhibitor in combination with DTX as a microtubule stabilizer has the potential to chemosensitize resistant cells and improve the efficacy of DTX by targeting both desmoplastic cells and bulk tumor cells. In our recent study, CYP and paclitaxel were conjugated as well as dodecanol to mPEG-b-PCC, and drug release was in sustained manner. Furthermore, drug loading of PTX and CYP was high and their release from the micelles was significantly prolonged, compared to physically drug encapsulated micelles [16]. Olive *et al.* showed that the inhibition of Hh signaling pathway using IPI-926 improved gemcitabine delivery to pancreatic cancer KPC mouse model [25]. Similarly, the Jiang group demonstrated that CYP disrupts the ECM and enhances the delivery and efficacy of nanoparticles in pancreatic cancer [10].

CONCLUSION

In conclusion, these studies show that P-CYP and P-DTX combination therapy has the potential to treat advanced pancreatic cancer associated with dense desmoplasia. Although tumor regression phenomenon could be achieved by the synergistic effect of CYP and DTX, the mechanistic regression pathway needs further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Saud Almawash is supported by Shaqra University through Saudi Arabian government for his graduate studies. This work was partly supported by the National Institutes of Health (1R01GM113166) and the University of Nebraska Medical Center.

ABBREVIATIONS

CYP	Cyclopamine
DC	Dodecanol
DCM	Dichloromethane

DTX	Docetaxel
ECM	Extracellular matrix
EPR	Enhanced permeability and retention
Hh	Hedgehog
mPEG-b-PCC	Poly (ethylene glycol)-block-poly (2-methyl-2-carboxyl-propylene carbonate
PBST	Phosphate Buffered Saline with Tween
P-CYP	Methoxy poly(ethylene glycol)- block-poly(2-methyl- 2-carboxylpropylenecarbonate- graftdodecanol- graft-cyclamine
PDAC	Pancreatic ductal adenocarcinoma
P-DTX	Methoxy poly(ethylene glycol)-blockpoly(2-methyl- 2-carboxylpropylenecarbonate- graftdodecanol- graft-docetaxel
PI	Propidium Iodide
PTX	Paclitaxel
SHh	Sonic hedgehog

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Fig. 1. Synthesis schemes and ¹H NMR analysis of polymer-drug conjugates. (a) synthesis scheme of P-DTX, (b) synthesis scheme of P-CYP, (c) ¹H NMR spectrum of P-DTX, and (d) ¹H NMR spectrum P-CYP.

Fig. 2.

Cell viability assay at 48 h post incubation of MIA PaCa-2 and BxPC-3 cells with combination of 16 nM P-DTX and 25 μM P-CYP (**a**). Colony formation potential of MIA PaCa-2 cells. 500 cells were seeded to 6-well plate. After 24 h, formulations were added and at day 14 the colonies were fixed, stained and counted (**b**). Treatment of tumor spheroids of MIA PaCa-2 with the same formulation at days 4, 6 and 8 and images were taken at days 4, 5, 7 and 9 under inverted microscope at 40 x magnification (**c**). Results are presented as the mean $\pm$ S.D. ($n = 5$).

Fig. 3.

Cell cycle, apoptosis and Western blot analysis of MIA PaCa-2 cells after 48 h exposure to empty micelles, 25 μ M P-CYP micelles, 16 nM P-DTX micelles, and their combination. **(a)** Cells were grown in 6-well plate (3.5×10^5 cells/well), and cell cycle analysis were evaluated by flow cytometry after staining with propidium iodide (PI). **(b-c)** Apoptotic cells were measured by flow cytometry after staining with Annexin-V and PI. **(d)** BAX and SHH protein levels were assessed by Western blot analysis. All data were presented as the mean $\pm$ S.D. ($n = 3$) (*) $P < 0.05$, (**) $P < 0.001$.

Fig. 4.

(a) *In vivo* representative bioluminescence image at last day from mice treated with free saline (control), P-CYP, P-DTX, and P-CYP + P-DTX. (b) Photon intensity of orthotopically implanted pancreatic tumor was measured by IVIS machine at various times post treatment. (c) a representative tumor image after sacrificing all mice at the end of experiment.

Fig. 5. Immunohistochemical staining of tumor section for hematoxylin and eosin (H&E), Ki-67 (proliferation related marker), and cleaved caspase-3 (apoptotic marker). The apoptotic cells are stained brown. Images were taken at 40× magnification.

Fig. 6. Immunohistochemical staining of tumor section with Masson's Trichome (related to collagen deposition and desmoplasia), E-cadherin (related to metastasis maker) and ZEB-1 (EMT modulator).

Fig. 7. TUNEL assay of tumor specimens of orthotopic MIA PaCa-2 cells derived tumor bearing mice treated with P-CYP, P-DTX and their combination. Tumor tissues were fixed, paraffin cryosectioned and immunostained for TUNEL to detect apoptotic tumor cells. TUNEL positive nuclei (green), DAPI positive nuclei (blue), scale bar 100 μ M.

Table I
Synergistic Anticancer Activity of P-CYP and P-DTX in Human Pancreatic Cancer

Drug combination	Cell line	D501 (nM)	d 1 (nM)	D502 (μ M)	d 2 (μ M)	CI
P-DTX + P-CYP	MIA PaCa-2	39	16	55	13.7	0.66
					25	0.86
	BxPC-3	34		70	16.34	0.7
					25	0.83