Polymeric Micellar Delivery of Novel Microtubule Destabilizer and Hedgehog Signaling Inhibitor for Treating Chemoresistant Prostate Cancer

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List of non-standard abbreviations:
ABC, ATP-binding cassette; CSCs, cancer stem cells; Combination Index (CI); CMC, Critical micelle concentration; MBC, 5-methyl-5-benzyloxy carbonyl-1,3-dioxane-2-one; MDB5, 2-chloro-N1-[4-chloro-3-(2-pyridinyl) phenyl]-N4,N4- bis(2-pyridinylmethyl)-1,4-benzenedicarboxamide; mPEG-p(TMC-MBC), methoxy-poly(ethyleneglycol)-block-poly(trimethylene carbonate-co-2-methyl-2-benzoxy carbonyl-propylene carbonate); PI, propidium iodide; ROP, ring-opening polymerization; Shh, Sonic hedgehog; TXR, taxane-resistant; QW-296, 2-(4-hydroxy-1H-indol-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone.
Abstract

Castration-resistant prostate cancer that has become resistant to docetaxel (DTX) represents one of the greatest clinical challenges in the management of this malignancy. Therefore, there is an urgent need to develop novel therapeutic agents, which can overcome chemoresistance and improve overall survival of patients. Herein, we designed a novel microtubule destabilizer [(2-(4-hydroxy-1H-indol-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone (QW-296)] and combined with a newly synthesized hedgehog (Hh) signaling pathway inhibitor 2-chloro-N1-[4-chloro-3-(2-pyridinyl)phenyl]-N4,N4-bis(2-pyridinylmethyl)-1,4-benzenedicarboxamide (MDB5) to treat taxane resistant (TXR) prostate cancer. The combination of QW-296 and MDB5 exhibited stronger anticancer activity towards DU145-TXR and PC3-TXR cells and suppressed tumor colony formation, compared to single drug treatment. Since these drugs are hydrophobic, we synthesized mPEG-p(TMC-MBC) copolymer, which could self-assemble into micelles with loading capacities of 8.13 ± 0.75% and 9.12± 0.69% for QW-296 and MDB5, respectively. Further, these micelles provided controlled drug release of 58% and 42% release of QW-296 and MDB5 within 24 hours when dialyzed against PBS (pH 7.4). We established an orthotopic prostate tumor in nude mice using stably luciferase expressing PC3-TXR cells. There was maximum tumor growth inhibition in the group treated with the combination therapy of QW-296 and MDB5 in micelles compared to their monotherapies or combination therapy formulated in co-solvent. The overall findings suggest that combination therapy of QW-296 and MDB5 have great clinical potential to treat TXR prostate cancer and copolymer mPEG-p(TMC-MBC) could serve as an effective delivery vehicle to boost therapeutic efficacy in vivo.

Keywords: Microtubule destabilizer, Hedgehog signaling inhibitor, Synergistic therapy, Chemoresistant prostate cancer, Polymeric micelles.
Introduction

Prostate cancer is the most common non-cutaneous malignancy and the second leading cause of cancer related mortality in American men. Prostate cancer at stage I/II can be treated by surgery or radiation therapy but if 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 (DTX, 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, the clinical efficacy of DTX or paclitaxel which interacts with the taxane binding site in tubulin as cancer treatment in the long term is often limited 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 (Z. S. Chen et al., 2003; Darshan et al., 2011; Leonard et al., 2003; Risinger et al., 2008). Therefore, there is an urgent need to identify novel therapeutic agents for treating DTX-resistant patients. A number of tubulin inhibitors targeting the colchicine binding site 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 (Buey et al., 2005; Lu et al., 2012). Previously, we have discovered a new class of tubulin inhibitors targeting the colchicine binding site (C. M. Li et al., 2011; Lu et al., 2011; Lu et al., 2014; Xiao et al., 2013; Yang et al., 2017), and demonstrated that these compounds effectively overcome a number of clinically relevant taxane-resistant (TXR) mechanisms using multiple tumor models, including TXR prostate cancer (Arnst et al., 2018; Banerjee et al., 2018; J. Chen et al., 2012; C. M. Li et al., 2012). We have recently obtained high-resolution X-ray crystal structures for some of these compounds in complex with the tubulin protein (Arnst et al., 2018; Banerjee et al., 2018), which will enable us to further optimize the molecule structure for this class of tubulin inhibitors. 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 (M. Chen et al., 2010; Chung et al., 2010; Karhadkar et al., 2004; Shaw and Prowse, 2008). 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 (EMT) (Clement et al., 2007; Dierks et al., 2008; Peacock et al., 2007). In our previous studies (Singh et al., 2012; Yang et al., 2016), we also demonstrated the significant benefit of combination therapy of Hh signaling inhibitor cyclopamine and paclitaxel to combat TXR prostate cancer cells in vitro and vivo. Recently, our group developed a series of GDC-0449 analogs and one of the analogs, 2-chloro-N1-[4-chloro-3-(2-pyridinyl) phenyl]-N4,N4- bis(2-pyridinylmethyl)-1,4-benzenedicarboxamide (MDB5), exhibited stronger inhibition to Hh pathway and anticancer effect in vitro and vivo than GDC-0449 (Kumar et al., 2017). Therefore, in the current study we investigated whether the combination therapy of a novel microtubule destabilizer and a novel Hh inhibitor can work synergistically through different mechanisms to treat TXR prostate, 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.

Since these drugs are highly hydrophobic, we synthesized a diblock copolymer methoxy-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 micelles to encapsulate two small molecules to enhance their therapeutic potential by improving their water solubility, prolonged circulation and reduced dose-related side effects. The anticancer effect and corresponding mechanisms of the combination chemotherapy was determined by using different prostate cancer cells and orthotopic mouse model.

Materials and methods

Materials

Poly(ethylene glycol) methyl ether (mPEG, Mn=5000, PDI=1.03) was dried by azeotropic distillation from anhydrous toluene just before use. Trimethylene carbonate (TMC) was obtained from Polysciences, Inc Warrington, PA, USA. Bis(2-pyridylmethyl)amine, 3-chloro-4-(methoxycarbonyl) benzoic acid, 4-benzyloxyindole, benzenesulfonyl chloride, 3,4,5-trimethoxybenzoyl chloride, phosphorus(V) oxychloride (POCl₃), 2-(trimethylsilyl)ethoxymethyl chloride (SEMCI), N-bromosuccinimide (NBS), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid-hexafluorophosphate) (HATU), 4-chloro-3-(pyridin-2-yl) benzenamine, triethanolamine (TEA), dimethylacetamide (DMA), magnesium sulfate (MgSO₄), potassium hydroxide (KOH), N,N-dimethylformamide (DMF), tetrahydrofuran (THF), acetonitrile (ACN), ethyl acetate (EtOAc), anhydrous chloroform, dichloromethane (DCM), toluene, stannous 2-ethylhexanoate (Sn(Oct)₂) and dimethyl sulfoxide (DMSO) were commercially available from Sigma-Aldrich and used as received. Deuterated dimethyl sulfoxide (DMSO-d₆), acetonitrile-d₃ (CD₃CN), deuterium oxide (D₂O) and chloroform (CDCl₃) were purchased from Cambridge Isotope Laboratories. Other chemicals were all of analytic grade and were used without further purification.

Synthesis and Characterization of MDB5

MDB5 was synthesized as previously reported (Kumar et al., 2017). Briefly, HATU (1.43 g, 3.75 mmol) was added to a mixture of 3-chloro-4-(methoxycarbonyl) benzoic acid (540 mg, 2.5 mmol), bis(2-pyridylmethyl)amine (740 mg, 3.7 mmol), and TEA (505 mg, 5 mmol) in ACN (25 mL), followed by overnight stirring at room temperature. After
completion of the reaction, the residue was dissolved in DCM (50 mL) and washed by brine (3 × 50 mL), then the organic layer was dried by MgSO₄. Next, a mixture of the above crude product and KOH (291 mg, 5.2 mmol) in CH₃OH (25 mL) was heated at 50 °C for 17 h. After removal of the solvent, the residue was recrystallized from ACN to give the desired white solid as potassium salt. Then, the mixture of the potassium salt, 4-chloro-3-(pyridin-2-yl) benzenamine (197 mg, 0.96 mmol) and HATU (547 mg, 1.4 mmol) in DMA (5 mL) was stirring at room temperature for overnight and then quenched by brine (50 mL). The resulting precipitate was dried and purified by silica gel column chromatography (EtOAc/ACN = 50:50) to yield the target compound MDB5 (149 mg, 10%) as a white solid (Supplementary Figure 1). ¹H NMR (500 MHz, DMSO-d₆) 4.63 (s, 2H), 4.70 (s, 2H), 7.27–7.39 (m, 3H), 7.42–7.46 (m, 2H), 7.56 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 7.0 Hz, 1H), 7.64–7.69 (m, 2H), 7.72–7.82 (m, 4H), 7.90–7.94 (m, 1H), 8.00 (d, J = 2.0 Hz, 1H), 8.53 (d, J = 3.5 Hz, 1H), 8.61 (d, J = 3.0 Hz, 1H), 8.70 (d, J = 3.5 Hz, 1H), 10.77 (s, 1H); ¹³C NMR (125.7 MHz, DMSO-d₆) 50.2, 54.02, 121.03, 121.91, 122.38, 122.48, 122.56, 123.04, 123.15, 124.67, 125.72, 127.96, 129.25, 130.18, 130.48, 136.58, 137.10, 137.12, 137.36, 138.00, 139.25, 139.35, 149.21, 149.65, 149.72, 155.86, 156.02, 156.71, 164.69, 169.52.

Synthesis and Characterization of QW-296 (2-(4-Hydroxy-1H-indol-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl) methanone (QW-296) was designed and synthesized from compound 1 that reacts with POCl₃ and DMF to give compound 2 followed by reaction with benzene sulfonyl chloride to afford compound 3 as a major product. Compound 3 will react with ammonium hydroxide and glyoxal to form compound 4. Subsequent compound 4 reacts with NBS in THF provided the dibromo imidazole derivative 5. Protection of the imidazole in the presence of SEMCl and NaH afforded intermediate 6 which could be smoothly reacted with 3,4,5-Trimethoxybenzoyl chloride and isopropylmagnesium chloride-lithium chloride complex to give 7, and then removal of benzenesulfonyl and bromo group provided the intermediate 8. Subsequent deprotection of the SEM group with 1 M HCl provided the compound 9, which after removal of benzyl group provided the target compound QW-296 as a yellow solid. The chemical structures of the synthesized compounds were characterized and confirmed by NMR and high-resolution mass spectrometry to ensure the authenticity and purity for subsequent in vitro and in vivo
studies. The general synthetic scheme and the proton NMR for final compound QW-296 were outlined in Figure 1A. $^1$H NMR (400 MHz, CD$_3$CN+D$_2$O) $\delta$ 7.86 (s, 1 H), 7.71 (s, 1 H), 7.18 (s, 2 H), 6.99 (t, $J = 8.0$ Hz, 1 H), 6.86 (d, $J = 8.0$ Hz, 1 H), 6.41 (d, $J = 7.6$ Hz, 1 H), 3.81 (s, 6 H), 3.74 (s, 3 H); $^{13}$C NMR (100 MHz, CD$_3$CN+D$_2$O) 184.9, 152.7, 151.1, 147.2, 141.2, 138.4, 133.4, 124.4, 113.5, 106.2, 105.3, 104.8, 102.5, 59.8, 55.5. MS (ESI) calcd for C$_{21}$H$_{19}$N$_3$O$_5$, 393.4; found, 394.3 [M + 1]$^+$, LCMS: purity >99%.

**Docking**

The molecular modeling studies were performed in the tubulin crystal structure (PDB code: 5H7O) as compared to the original ligand (DJ-101, Figure 1B) using Schrödinger Molecular Modeling Suite 2018 (Schrödinger LLC, New York, NY) with similar procedures described before (Arnst et al., 2018; Hwang et al., 2015; Lu et al., 2014). Briefly, both QW-296 and original ligand DJ101 were built and prepared using LigPrep tool, and the protein structure was prepared using the Protein Preparation Wizard workflow. The tubulin receptor grid was generated with binding site enclosing box centered on the active ligand binding site using the Receptor Grid Generation. Then, all ligands we prepared were docked into this tubulin receptor grid at the same colchicine binding pocket using Glide Docking module. The docking poses with lowest binding energy defined by lower glide docking score were kept and analyzed. Hydrogen bonds and data analysis were performed using Maestro interface of Schrödinger software.

**Tubulin Polymerization Assay**

The tubulin polymerization assay was carried out according to the manufacturer’s instructions (Cytoskeleton Inc., Denver, CO) (J. Chen et al., 2012). Briefly, bovine brain tubulins (97%) were resuspended in G-PEM buffer to reach a final concentration of 3 mg/ml at 4°C. The tubulin protein solution was then exposed to vehicle control (5% DMSO), 10 μM of colchicine, paclitaxel or QW-296, respectively. The polymerization of tubulin was incubated at 37 °C in SYNERGY 4 Microplate Reader (Bio-Tek Instruments, Winooski, VT) immediately after adding the tubulin protein solution. Absorbance at 340 nm was measured every minute for a 60-minute period. (Figure 1C)

**Cell culture**
Human prostate cancer cell lines with taxane resistance DU145-TXR and PC3-TXR were kindly provided by Dr. Evan T. Keller from the University of Michigan. The cells were cultured in RPMI-1640 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.

_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. PC3-TXR and DU145-TXR cells were seeded at a cell density of 4 × 10³ per well in 96 well plate and after attachment 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 ± SD.

_Comination Effect and Their Interaction Analysis_

Chou-Talalay method and CompuSyn software were used to determine whether the combination had synergism, additivity, or antagonism effect (Zhang et al., 2014). PC3-TXR cells were treated with different combinations at a constant concentration 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.

_Cell Cycle Analysis by Propidium Iodide Staining_

PC3-TXR cells were cultured in a 24-well plate at a cell density of 3 × 10⁵ 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.
Colony Formation Assay

PC3-TXR cells were seeded at a density of 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 7 days of 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.

Western Blotting

Protein was isolated from PC3-TXR cells after 72 h of 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 polyvinylidene fluoride (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 they were detected by Odyssey® CLx Imaging System (LI-COR Biosciences, Lincoln, NE).

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 without any modification (F. Li et al., 2010). In brief, mPEG (1 g, 0.0002 mol), TMC (307 mg, 0.003 mol) and MBC (750 mg, 0.003mol) 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 100 °C for 24 h under stirring. Afterward, the product was dissolved in chloroform (10 mL) and precipitated in a large amount of diethyl ether (400 mL) three times 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.

Preparation and Characterization of Polymeric Micelles
Polymeric micelles were prepared by thin-film hydration method. Briefly, a given amounts of mPEG-poly(TMC-MBC) and the anticancer compound (10% w/w relative to the copolymer) were dissolved in chloroform in a glass vial and a thin polymeric film was formed after removing solvent under reduced pressure. The thin film was hydrated by PBS (2 mL) and the micelles were formed by applying probe sonicator (100W, 5 mins) at 37 °C. The formulation was then centrifuged at 3000 rpm for 5 min followed by membrane filtration to remove any precipitated or unformulated drug. The hydrodynamic diameters and ζ potential was measured by a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK) at a scattering angle of 90°. Particle size distribution was reported as the mean ± S.D. of three independent samples. Critical micelle concentration (CMC) was determined using pyrene as a hydrophobic fluorescent probe as described previously (F. Li et al., 2010).

**Drug Loading of QW-296 and MDB5**

Briefly, QW-296 or MDB5 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 for QW-296 (Rt =7.8 min) at a detection wavelength of 222 nm. For MDB5, same column was used for quantification at detection wavelength of 261 nm using acetonitrile and water (70:30, v/v) as mobile phase (Rt = 4.0 min). Payload was calculated using the following equations:

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

**Drug Release from Polymeric Micelles**

Drug-loaded micelles with a final drug concentration of 1 mg/mL were placed into dialysis membrane with molecular weight cut-off of 3500 Da and dialyzed against 50 mL buffer solution (0.1 M acetic acetate pH 6.5 or PBS pH 7.4) with 1% Tween-80 in a thermo-controlled shaker with a stirring speed of 100 rpm. Then, 1 mL sample was withdrawn at specified times for a period of four days and subsequently replaced with the 1 ml respective buffer solution. The drug concentration was analyzed by HPLC as described.
above. All experiments were performed in triplicate at the same day and the data reported as the mean ± S.D. of the three individual experiments.

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 the University of Nebraska Medical Center. Orthotopic prostate tumors were established in 8-9 week old male athymic nude mice by injecting $1.5 \times 10^6$ 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 third day 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. 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 24 h. 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 toxic effects of each treatment was evaluated by H&E staining of the major organs.

Statistical analysis

Data were represented as the mean ± S.D. The statistical comparisons of the experiments were performed by two-tailed Student's t-test. $P < 0.01$ was considered statistically significant.

Results

Molecular Docking of QW-296 in Tubulin and Its Inhibition of Tubulin Polymerization

We previously reported the discovery of ABI-231 (J. Chen et al., 2012), which is a highly potent tubulin inhibitor that can also effectively overcome taxane resistance in PC3-TXR
tumors (C. M. Li et al., 2012). Based on the structure of ABI-231, we designed and synthesized QW-296 by adding a hydroxyl moiety to the indole ring. The synthesis and structure characterization of QW-296 along with the structure of ABI-231 is shown in Figure 1A. To demonstrate that QW-296 maintains its mechanisms of action as a tubulin inhibitor, we performed molecular docking analysis and evaluated its ability to inhibit tubulin polymerization. Molecular modeling studies (Figure 1B) showed that the binding site of QW-296 was located at the interface between α- and β-subunits of tubulin dimer and extended slightly into β-subunit. QW-296 (orange tube model) demonstrated a similar binding pose with native ligand DJ101 (light blue model) and overlapped very well. The interaction was strongly stabilized by three hydrogen bonds (blue dashed lines): one found between the imidazole NH moiety and Thr179 in α-subunit, one formed between the carbonyl of QW-296 and β-Asp249, and one contributed by one of methoxy from the 3,4,5-trimethoxybenzoyl group of QW-296 and β-Cys239. The glide docking score of QW-296 (-13.1) was comparable with that of the native ligand DJ101 (-12.9), indicating it may have similar tubulin binding effects of DJ101.

To elucidate whether QW-296 compound maintains its mode of action by the inhibition of tubulin polymerization, we conducted in vitro tubulin polymerization assays on QW-296. Tubulin (3 mg/ml) was exposed to 10 μM of QW-296, colchicine, paclitaxel or vehicle control (5% DMSO), respectively, and incubated in general tubulin buffer. UV absorption at 340 nm was measured at 37 °C every minute for an hour. Both QW-296 and colchicine effectively inhibited polymerization, while robust polymerization was observed in the control and paclitaxel group (Figure 1C). These results clearly indicated that QW-296 is a strong tubulin-depolymerizing agent.

Better Anticancer Activity of Two Novel Compounds Compared to Their Monotherapies

The PC3-TXR and DU145-TXR cells exhibited resistance to DTX as evidenced by the high IC50 value (>1000 nM) for cell killing (Figure 2A). In contrast, QW-296 exhibited strong cell killing activity against DU145-TXR and PC3-TXR cell lines with IC50 at 80 nM and 100 nM, respectively (Figure 2B).

We also compared the cytotoxic effects of MDB5 and GDC-0449 using these two cell lines (Figure 2C-D). The results confirmed that both MDB5 and GDC-0449 suppressed the growth of PC3-TXR and DU145-TXR cells in a dose-dependent manner. MDB5
showed enhanced activity compared to its parent drug GDC-0449, indicating the expected benefit of developing novel analog of GDC-0449.

**Synergism of QW-296 and MDB5 against Taxane-Resistant Prostate Cancer Cells**

After determining the cytotoxicity of individual drugs, we confirmed the advantages of combination therapy with different concentrations (Figure 3). The concentrations of QW-296 and MDB5 applied in combination were lower than their IC$_{50}$ in monotherapy, hence the combination worked efficiently than individual treatment. Combination of QW-296 (25 nM) and MDB5 (7.5 μM) only killed 20% of PC3-TXR cells, but when their concentrations were doubled the combination therapy killed 72% of the cells. Two different combinations (20 nM QW-296 + 20 μM MDB5, or 40 nM QW-296 + 10 μM MDB5) killed 40% of the cells, but when the concentration of single drug was doubled (40 nM QW-296 or 20 μM MDB5) the combination killed 70% of the cells. The interactions between these 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 are summarized in Supplemental 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 synergistically Supplemental Figure 2.

**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 4A, the number of colonies in untreated group was maximum compared to the groups treated with QW-296 or MDB5 alone or their combination. 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, the combination of 50 nM QW-296 and 15 μM MDB5 resulted in no colony formation throughout 7 days. These data further confirmed the synergistic effect of QW-296 and MDB5.

Effect of Combination Therapy on Cell Cycle and Shh Protein Expression

Cell cycle analysis was determined by propidium iodide (PI) staining after 48 h and 72 h of treating PC3-TXR cells with the polymeric micelles formulation containing QW-296, MDB5 or their combination (Figure 4B-C). The data showed that 100 nM QW-296 caused 35.84% cell arrest in G2/M phase at 48 h, but decreased to 25.76% with increase in treatment time from 48 h to 72 h. Furthermore, QW-296 treatment could induce sub-G1 phase arrest to 27.85% at 48 h and 40.02% at 72 h. In contrast to QW-296, 15 μM MDB5 resulted in 57.99% of PC3-TXR cell arrest in G0/G1 phase at 48 h, along with 72.85% of cells in G0/G1 at 72 h, suggesting MDB5 affected cell cycle through different mechanism. The combination of 50 nM QW-296 and 15μM MDB5 slightly induced overall G2/M arrest at 48 h and 72 h. Unlike single drug treatment changing cell arrest dramatically from 48 h to 72 h, combination therapy caused cell arrest in G2 phase and these cells ended up in static status throughout 72 h. Noticeably, after combination treatment the % of PC3-TXR cells in sub-G1 phase was significantly enhanced compared to that of after monotherapy. Since MDB5 was designed as Hh signaling inhibitor and GDC-0449 analog, we also measured Shh protein expression by Western blot analysis. We found clear reduction of Shh protein in MDB5 treated group and combination treated group (Figure 4D).

Characterization of Copolymer mPEG-p(TMC-MBC)

The monomer, 2-methyl-2-benzyloxy carbonyl propylene carbonate (MBC) was synthesized first as described previously. Then, MBC and trimethylene carbonate (TMC) were copolymerized with mPEG using Sn(Oct)2 as a catalyst to yield 1.82 g of mPEG-p(TMC-MBC) (88% yield) (Figure 5A). In 1H NMR spectrum of mPEG-p(TMC-MBC), the following peaks were observed at δ2.03 (CH2, br, 2H) corresponding to TMC, δ 1.2 (CH3, s, 3H) and δ 7.3 (phenyl, m, 5H) corresponding to MBC, δ 4.2-4.3 (CH2, t, 4H) corresponding to both TMC and MBC, and δ 3.63 (CH2, s, 2H) corresponding to PEG.
The molecular weight of mPEG114-b-p(TMC15-MBC15) determined by $^1$H NMR was 10280 Da with 15 units of each block, respectively (Figure 5B).

**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 the mean particle size of micelles were determined by transmission electron microscopy (TEM, Tecnai G² Spirit) and dynamic light scattering (DLS, Zetasizer Nano ZS90). Empty and drug (QW-296 or MDB5)-loaded polymeric micelles had similar particle size distributions, with the mean particle size of 81 nm (Figure 6A). The TEM image also showed that the micelles are spherical in shape with uniform particle size below 60 nm in PBS (Figure 6B). The critical micelle concentration (CMC) of mPEG-p(TMC-MBC) was 6.65×10⁻⁴ g/L (Figure 6C), further indicating that the micelles were quite stable in PBS. Moreover, 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.

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 dialysis in PBS buffer of pH 7.4 and acetate buffer of pH 6.5. As shown in Figure 6D, 27% of QW-296 was rapidly released from the micelles within the initial 6 h under neutral condition, and then the release increased up to 58% at 24 h followed by slow, sustained release until the end of 96 h. However, the overall release of MDB5 was slightly slower compared to QW-296. The release of QW-296 or MDB5 was slightly faster under acidic condition. These data suggest that the drug-loaded micelles are quite stable under physiological pH conditions.

**In vivo antitumor efficacy in orthotopic prostate cancer mouse model**

We successfully established orthotopic prostate tumor in 8-9 week old male athymic nude mice by injecting 1.5×10⁶ PC3-TXR-luc cells into dorsal prostate lobe. The mice whose bioluminescent radiance reached 1×10⁸ p/sec/cm²/sr were randomized into five groups: 1) control, 2) QW-296 loaded micelles (10 mg/kg), 3) MDB5 loaded micelles (10 mg/kg),
4) combination of QW-296 and MDB5 in cosolvent (5 mg/kg + 5 mg/kg), and 5) combination of QW-296 and MDB5 loaded micelles (5 mg/kg + 5 mg/kg). All the treated groups showed inhibition of tumor growth compared to the control group (Figure 7). However, the maximum tumor inhibition was observed in the group treated with the micelles encapsulating both QW-296 and MDB5. Next, we performed immunohistochemical analysis to further elucidate the superior anticancer efficacy of combined micelles. Hematoxylin and eosin (H&E) staining of tumor sections indicated that tumor samples from four treated groups had more necrotic area compared to the tumor samples from the control group (Figure 8). Tumors from combination micelles group showed maximum necrosis. Furthermore, cleaved caspase 3 staining of tumor tissue indicated the induction of significant apoptosis by combination micelles of QW-296 and MDB5 compared to other treatments (Figure 8). 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 9).

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.) (Schinkel and Jonker, 2003), alteration in drug targets, as well as intrinsic chemoresistance due to their poor effect on CSCs (Abdullah and Chow, 2013). With the advances in cancer research, there are numerous approaches to overcome drug resistance. Combination therapy of multiple drugs to target different signaling pathways is considered as one of the preferred choices in both preclinical research and clinical practice. In addition, it is worth mentioning that if the combinatory drug effect is greater than the sum of their individual effects, known as drug synergism, there will be a higher chance of success of combination strategy. In this regard, our study proposed a synergistic combination chemotherapy using two novel anticancer agents to overcome TXR and treat prostate cancer. To replace taxane treatment, we first designed and synthesized a new microtubule destabilizer, QW-296, whose function on microtubule is to inhibit tubulin polymerization. QW-296 is a next-
generation of ABI-231. We have modified ABI-231 by adding a hydroxyl group on one of the indoles. In a separate study, we found that QW-296 showed a significantly low resistance index compared to ABI-231 (1.3 vs. 7.4) in PTX resistant PC3-TXR cells. The potency of QW-296 was 7.4 times higher than that of ABI-321 in PTX resistant cells. This observation guided us to continue this study with QW-296. Molecular modeling suggested that QW-296 targets tubulin at the colchicine-binding site with a similar binding pose as the native ligand DJ101, which is a potent tubulin-depolymerizing agent (Hwang et al., 2015). Further, in vitro tubulin polymerization assay confirmed that QW-296 is a highly potent tubulin polymerization inhibitor exhibiting significant tubulin assembly inhibition by destabilizing microtubule structures (Figure 1C).

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 EMT, and has cross-talk with androgen pathway. In our previous study, we also demonstrated that the combination of Hh inhibitor cyclopamine and paclitaxel synergistically suppressed the growth of TXR prostate cancer in vitro and vivo by playing different roles on cancer cells, suggesting a promising combination strategy. Thus, in the current study, we have used a newly designed Hh signaling inhibitor MDB5, having stronger inhibition activity against Hh signaling compared with its parent drug GDC-0449 (Kumar et al., 2017).

First, the inhibitory effect of QW-296 and MDB5 on prostate cancer cells was confirmed by cell viability and colony formation assays, clearly indicating the benefit of this combination (Figures 3 and 4). The IC₅₀ of QW-296 in DU-145-TXR and PC3-TXR was in the range of 80-100 nM, whereas that of MDB5 was in the range of 30-50 µM. This means the IC₅₀ values of these two drugs have approximate 300-600 fold difference. Therefore, we used 1:200, 1:300 and 1:400 ratios for vitro combination therapy of QW-296 and MDB5. We determined the synergism between these two drugs using Chou–Talalay method (Supplemental Figure 2), which strengthened our hypothesis. In the cell cycle analysis, QW-296 and MDB5 made distinct impacts on cell cycle as MDB5 treatment caused G0/G1 phase arrest while QW-296 treatment lead to G2/M phase arrest (Figure 4B-C), which demonstrated two
anticancer agents worked at complementary mechanism of action against PC3-TXR cells, and this result is in agreement with previous reports. Further, we analyzed the change in protein expression to highlight the combination advantage at the molecular level. We observed downregulation of Shh (Figure 4D), a key component of Hh signaling pathway after MDB5 monotherapy or combination of QW-296 and MDB5.

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, nanocarrier-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, which are suitable for systemic drug delivery. mPEG 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 is expected to prolong the circulation time of micelles and consequently increase drug’s accumulation in the tumor tissue. On the other hand, TMC and MBC are both cyclic carbonate blocks that provide desired hydrophobicity to encapsulate lipophilic molecules in the core and balance hydrophilic composition. Furthermore, polycarbonates have been proven to be non-toxic, biocompatible and biodegradable, which provides the potentials of future clinical translation. They degrade into carbon dioxide and benzyl alcohol, which has less effect on microenvironment pH and as such will not result in local inflammation and are easily cleared from the body. Our results also confirmed that mPEG-p(TMC-MBC) polymeric micelles enabled loading of 8~9% for both drugs and sustained drug release in acidic or neutral condition (Figure 6).

To better investigate the combination efficacy and confirm the benefits of using micelles as delivery vehicle, we used half dose of QW-296 and MDB5 in combination treatment in co-solvent or micelles to compare with monotherapies, while using saline injected mice as control. All the four treated groups exhibited excellent tumor inhibitory results (Figure 7). 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 support our hypothesis that QW-296 and MDB5 could synergistically treat chemoresistant prostate cancer in vivo and mPEG-p(TMC-MBC) could serve as an effective delivery vehicle to boost anticancer activity of two drugs than co-solvent.

Conclusion
In the present study, we successfully synthesized and screened a novel microtubule destabilizer QW-296 and an Hh pathway inhibitor MDB5 and demonstrated their anticancer activities in combination or individually. mPEG-p(TMC-MBC) was successfully synthesized and formed polymeric micelles to encapsulate QW-296 and MDB5 with payload of 8.13 ± 0.75 and 9.12 ± 0.69% (w/w), respectively. 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.

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Authorship Contributions
Participated in research design: Yang, Chen, Guo, Li and Mahato.
Conducted experiments: Yang, Chen, Guo, and Dong.
Contributed new reagents or analytic tools: Yang, Dong, Miller, and Li.
Performed data analysis: Yang, Chen, Miller, Li and Mahato.
Wrote or contributed to the writing of the manuscript: Yang, Li, and Mahato.
References


Legends for Figures

Figure 1. Synthesis and characterization of QW-296 and its inhibition of tubulin. (A) Synthetic scheme and $^1$H NMR spectrum of QW-296. (B) Proposed binding poses of QW-296 in the tubulin crystal structure (PDB code: 5H7O). Superposition of native ligand DJ-101 (light blue thin tube; glide docking score -12.9) with QW-296 (orange ball-and-stick; glide docking score -13.1). Hydrogen bonds are shown by blue dashed line. (C) Effect of QW-296 compound on tubulin polymerization in vitro. Tubulin (3 mg/ml) was exposed to 10 μM of QW-296, colchicine, paclitaxel or vehicle control (5% DMSO), respectively, and incubated in general tubulin buffer. Turbidity change at 340 nm was measured at 37 °C every minute for an hour. Both QW-296 and colchicine effectively inhibited polymerization, while robust polymerization was observed in the control and paclitaxel group.

Figure 2. Cytotoxicity assay of Tubulin Inhibitors and Hh Signaling Inhibitors on PC3-TXR and DU145-TXR cells. (A-B) Cytotoxicity of docetaxel, QW-296, GDC-0449 and MDB5 was determined in PC3-TXR and DU145-TXR cells after 72 h of incubation. Results are presented as the mean ± S.D. (n = 3).

Figure 3. Combination effect of QW-296 and MDB5 at various ratios against PC3-TXR cells. (A) Effect of QW-296 and MDB5 concentrations at different concentration ratios was determined after 72 h of incubation. (B-D) Combination effect of QW-296 and MDB5 at fixed ratios was determined respectively. Results are represented as the mean ± S.D. (n = 3). Q: QW-296, M: MDB5.

Figure 4. Effect of QW-296 and MDB5 on colony formation, cell cycle and Shh protein expression of PC3-TXR cells. A) 300 PC3-TXR cells per well were seeded in 6-well culture plates. At 24h, drug formulations were added and at 7 days, cell colonies were fixed, stained and counted. B-C) Cells were treated for 48 h and 72 h, stained with propidium iodide (PI), and analyzed by a flow cytometer. Results are expressed as the mean ± S.D. (n = 3). D) Cells were treated for 72h and then protein from each group was extracted and Western blot analysis was carried out for Shh.

Figure 5. Synthetic route of mPEG-p(TMC-BC) copolymer (A) and its $^1$H NMR spectra (B).

Figure 6. Characterization of polymeric micelles. A) Particle size distribution as determined by dynamic light scattering, B) TEM morphology, C) Critical micelle concentration (CMC), (D) In vitro release of QW-296 and MDB5 from micelles at pH 7.4 and 6.5. Drug concentrations from each time point were measured by HPLC. Results are expressed as the mean ± SD (n=3).

Figure 7. In vivo efficacy of polymeric micelles carrying QW-296 and MDB5 after systemic administration in PC3-TXR orthotopic prostate cancer bearing athymic nude mice. (A) Representative bioluminescence images were taken at day 24 (n = 6),
(B) Tumors of each group were excised after sacrificing the mice at the end of the experiment.

**Figure 8.** Analysis of tumor samples by (A) hematoxylin and eosin (H&E) and (B) Caspase 3 stain.

**Figure 9.** Analysis of major organs by H&E stain. Organ samples from five 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.
Figure 1

A: Chemical structures and reactions involved in the synthesis of QW-296.

B: Molecular docking of QW-296 with the Go subunit of G-GoT complex.

C: Graph showing the time (mins) vs. A340 values for different treatments: Control, Colchicine, Paclitaxel, and QW-296.
Figure 2
Figure 3

A

Viability (%) vs. Concentration

PC3-TXR

B

Viability (%) vs. Concentration

QW-296/MDB5 1:200

C

Viability (%) vs. Concentration

QW-296/MDB5 1:300

D

Viability (%) vs. Concentration

QW-296/MDB5 1:400
Figure 4

A

Control 100 nM QW-296 15 μM MDB5 50nM QW-296 + 15μM MDB5

B

Population %

48 h

G0/G1  G2/M  S

C

Population %

72 h

G0/G1  G2/M  S

D

Control 100 nM QW-296 15 μM MDB5 50 nM QW-296 + 15μM MDB5

Shh β-actin
Figure 5

A. Reaction of mPEG, MBC, and TMC to form mPEG-p(TMC-BC) with Sn(Oct)$_2$ at 120°C, 16 h.

B. NMR spectrum of mPEG-p(TMC-BC) showing peaks at 7.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.0, and 0.0 ppm.
Figure 9

Control

QW-296

MDB5

Formulation Combination of QW-296 and MDB5

Free Drug Combination of QW-296 and MDB5