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

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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. There is an urgent need to develop novel therapeutic agents to overcome chemoresistance and improve the overall survival of patients. We have designed a novel microtubule destabilizer (2-(4-hydroxy-1*H*-indol-3-yl)-1*H*-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone (QW-296) and combined it with a newly synthesized hedgehog (Hh) signaling pathway inhibitor 2-chloro-*N*¹-[4-chloro-3-(2-pyridinyl)phenyl]-*N*⁴,*N*⁴-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 toward DU145-TXR and PC3-TXR cells and suppressed tumor colony formation when compared with single-drug treatment. Because these drugs are hydrophobic, we synthesized the mPEG-p(TMC-MBC) [methoxy-poly(ethylene glycol)-*block*-poly(trimethylene

carbonate-co-2-methyl-2-benzoxycarbonyl-propylene carbonate)] 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 the respective 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 with their monotherapies or combination therapy formulated in cosolvent. The overall findings suggest that combination therapy with QW-296 and MDB5 has 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*.

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Introduction

Prostate cancer is the most common noncutaneous malignancy and the second leading cause of cancer-related mortality in American men. Prostate cancer at stage I/II can be treated with surgery or radiation therapy, but if the cancer has grown outside the prostate or come back after surgery or radiation, androgen deprivation therapy may be used to reduce androgen levels. Unfortunately, after a certain period the aggressive portion of prostate cancer cells develops resistance

ABBREVIATIONS: ABI-231, 2-(1*H*-indol-3-yl)-1*H*-imidazol-4-yl (3,4,5-trimethoxyphenyl) Methanone; ACN, acetonitrile; CI, combination index; CMC, critical micelle concentration; CSCs, cancer stem cells; DMF, *N,N*-dimethylformamide; DTX, docetaxel; EtOAc, ethyl acetate; GDC-0449, 2-chloro-*N*-(4-chloro-3-pyridin-2-ylphenyl)-4-methylsulfonylbenzamide; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid-hexafluoro-phosphate; Hh, hedgehog; HPLC, high-performance liquid chromatography; MBC, 5-methyl-5-benzoyloxycarbonyl-1,3-dioxane-2-one; MDB5, 2-chloro-*N*¹-[4-chloro-3-(2-pyridinyl)phenyl]-*N*⁴,*N*⁴-bis(2-pyridinylmethyl)-1,4-benzenedicarboxamide; mPEG, poly(ethylene glycol) methyl ether; mPEG-p(TMC-MBC), methoxy-poly(ethylene glycol)-*block*-poly(trimethylene carbonate-co-2-methyl-2-benzoxycarbonyl-propylene carbonate); PI, propidium iodide; QW-296, 2-(4-hydroxy-1*H*-indol-3-yl)-1*H*-imidazol-4-yl(3,4,5-trimethoxyphenyl)methanone; Shh, sonic hedgehog; TEM, transmission electron microscopy; TXR, taxane resistant.

to hormone treatment and becomes 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 a cancer treatment in the long term is often limited by intrinsic or acquired drug resistance due to mutation of β -tubulin, affecting androgen receptor signaling or overexpression of drug efflux pumps (ATP-binding cassette) in cancer cells (Chen et al., 2003; Leonard et al., 2003; Risinger et al., 2008; Darshan et al., 2011). 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 inhibitor can circumvent the limitations associated with clinically available tubulin inhibitors (Buey et al., 2005; Lu et al., 2012). Previously, we discovered a new class of tubulin inhibitors targeting the colchicine-binding site (Li et al., 2011; Lu et al., 2011, 2014; Xiao et al., 2013; Yang et al., 2017a) and demonstrated that these compounds effectively overcome a number of clinically relevant taxane-resistant (TXR) mechanisms using multiple tumor models, including TXR prostate cancer (Chen et al., 2012; Li et al., 2012; Arnst et al., 2018; Banerjee et al., 2018).

We 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 the 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 cross talk with androgen signaling, suggesting that inhibition of Hh pathway has the possibility of inducing antiproliferative and apoptotic effect on prostate cancer cells (Karhadkar et al., 2004; Shaw and Prowse, 2008; Chen et al., 2010; Chung et al., 2010). Furthermore, Hh signaling involves in the initiation and maintenance of cancer stem cells (CSCs), a subset of cancer cells with self-renewal and tumorigenic potential, which have been demonstrated to play a key role in chemoresistance, metastatic progression, and the epithelial-mesenchymal transition (Clement et al., 2007; Peacock et al., 2007; Dierks et al., 2008).

In our previous studies (Singh et al., 2012; Yang et al., 2017b), we demonstrated the significant benefits of combination therapy with the Hh-signaling inhibitor cyclopamine and paclitaxel to combat TXR prostate cancer cells in vitro and vivo. Recently, our group developed a series of

2-chloro-*N*-(4-chloro-3-pyridin-2-ylphenyl)-4-methylsulfonylbenzamide (GDC-0449) analogs. One of these analogs, 2-chloro-*N*¹-(4-chloro-3-(2-pyridinyl) phenyl)-*N*⁴,*N*⁴-bis(2-pyridinylmethyl)-1,4-benzenedicarboxamide (MDB5), exhibited stronger inhibition of the Hh pathway and an anticancer effect in vitro and vivo than GDC-0449 (Kumar et al., 2017). Therefore, in the current study we investigated whether combination therapy with a novel microtubule destabilizer and a novel Hh inhibitor can work synergistically through different mechanisms to treat TXR prostate cells, 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.

Because 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, prolonging their circulation, and reducing the dose-related side effects. The anticancer effect and corresponding mechanisms of the combination chemotherapy were determined by using different prostate cancer cells and an orthotopic mouse model.

Materials and Methods

Materials. Poly(ethylene glycol) methyl ether (mPEG, Mn = 5000, polydispersity index = 1.03) was dried by azeotropic distillation from anhydrous toluene just before use. Trimethylene carbonate (TMC) was obtained from Polysciences, Warrington, PA. 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; *N*-bromosuccinimide; 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid-hexafluoro-phosphate (HATU); 4-chloro-3-(pyridin-2-yl) benzeneamine; triethanolamine; dimethylacetamide; magnesium sulfate (MgSO₄); potassium hydroxide (KOH); *N,N*-dimethylformamide (DMF); tetrahydrofuran; acetonitrile (ACN); ethyl acetate (EtOAc); anhydrous chloroform; dichloromethane; toluene; stannous 2-ethylhexanoate (Sn(Oct)₂); and DMSO were commercially available from Sigma-Aldrich and were used as received. Deuterated DMSO (DMSO-*d*₆), acetonitrile-*d*₃ (CD₃CN), deuterium oxide (D₂O), and chloroform (CDCl₃) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Other chemicals were all of analytic grade and were used without further purification.

Synthesis and Characterization of MDB5. MDB5 was synthesized as reported elsewhere (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 triethanolamine (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 dichloromethane (50 ml) and washed with brine (3 × 50 ml), then the organic layer was dried with 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 hours. 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) benzeneamine (197 mg, 0.96 mmol), and HATU (547 mg, 1.4 mmol) in dimethylacetamide (5 ml) was stirred at room temperature overnight and then quenched with 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 Fig. 1): ¹H NMR (500 MHz, DMSO-*d*₆) 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); ^{13}C NMR (125.7 MHz, $\text{DMSO}-d_6$) 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, 137.25, 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 The compound (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, which then reacts with POCl_3 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. Subsequently compound 4 reacts with *N*-bromosuccinimide in tetrahydrofuran to provide the dibromo imidazole derivative compound 5. Protection of the imidazole in the presence of 2-(trimethylsilyl)ethoxymethyl chloride and NaH afforded intermediate compound 6, which could be smoothly reacted with 3,4,5-trimethoxybenzoyl chloride and isopropylmagnesium chloride-lithium chloride complex to give compound 7. Then removal of benzenesulfonyl and the bromo group provided the intermediate compound 8.

Subsequent deprotection of the SEM group with 1 M HCl provided compound 9, which after removal of the 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 the final compound

QW-296 were outlined in Fig. 1A: ^1H NMR (400 MHz, $\text{CD}_3\text{CN} + \text{D}_2\text{O}$) δ 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, $\text{CD}_3\text{CN} + \text{D}_2\text{O}$) 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; mass spectrometry (electrospray ionization) calculated for $\text{C}_{21}\text{H}_{19}\text{N}_3\text{O}_5$, 393.4; found, 394.3 $[\text{M} + 1]^+$, liquid chromatography with mass spectrometry: purity >99%.

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

Tubulin Polymerization Assay. The tubulin polymerization assay was performed according to the manufacturer's instructions (Cytoskeleton, Denver, CO) (Chen et al., 2012). Briefly, bovine brain tubulins (97%) were resuspended in G-PEM buffer to reach a final

Fig. 1. Synthesis and characterization of QW-296 and its inhibition of tubulin. (A) Synthetic scheme and ^1H 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 groups.

concentration of 3 mg/ml at 4°C. The tubulin protein solution was then exposed to vehicle control (5% DMSO) or 10 μ M 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 (Fig. 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 was maintained by adding 200 nM paclitaxel to the growth medium 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 4×10^3 per well in 96-well plates. After attachment to the bottom of plate, the cells were incubated with different concentrations of QW-296, MDB5, or combination for another 72 hours. 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 are reported as mean $\pm$ S.D.

Combination Effect and Their Interaction Analysis. The 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 3×10^5 and were treated with QW-296, MDB5, or their combination for 48 and 72 hours. The cells were harvested, fixed in 70% ice-cold ethanol for 1 hour, and washed with PBS. A cell pellet containing 1×10^6 cells was then resuspended in 0.5 ml of FxCycle PI/RNase staining solution and incubated for 15 minutes at room temperature. The cell cycle was measured by a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA). 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 2 days. Treatment with QW-296, MDB5, or combination was given to different wells. After 7 days of incubation, the colonies in each well were fixed with 10% formalin, stained with a 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 hours of treatment with QW-296, MDB5, or combination, and the protein concentration was determined with the Micro BCA Protein assay kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein (50 μ g) were separated in 4%–15% Mini PROTEAN TGX Precast Gel followed by transfer to polyvinylidene fluoride membranes with the iBlot Gel Transfer system (Thermo Fisher Scientific). After the membrane had been blocked with Odyssey Blocking Buffer (TBS), the following primary antibodies were used: sonic hedgehog (Shh) (sc-9024) and β -actin (sc-47778) (Santa Cruz Biotechnology, Dallas, TX). Subsequently, the membranes were incubated with their corresponding IRDye 800CW secondary antibodies, and they were detected with the Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, NE).

Synthesis and Characterization of mPEG-poly(TMC-MBC). Monomer 5-methyl-5-benzoyloxycarbonyl-1,3-dioxane-2-one (MBC) and the copolymer mPEG-poly(TMC-MBC) were synthesized as reported elsewhere without any modifications (Li et al., 2010). In brief, mPEG (1 g, 0.0002 mol), TMC (307 mg, 0.003 mol), and MBC (750 mg, 0.003 mol) were mixed in a dried round-bottom flask under vacuum, and then Sn(Oct)₂ (10 mol% relative to mPEG) as a catalyst was added to the mixture to initiate polymerization. The reaction

mixture was heated to 100°C for 24 hours under stirring. Afterward, the product was dissolved in chloroform (10 ml) and precipitated in a large amount of diethyl ether (400 ml) 3 times and dried under vacuum. The purified copolymer was characterized by ¹H NMR, and the spectrum was recorded on a Bruker (500 MHz, T = 25°C) using deuterated chloroform (CDCl₃) as the solvent.

Preparation and Characterization of Polymeric Micelles. Polymeric micelles were prepared by a thin-film hydration method. Briefly, a given amount 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 formed after removing the solvent under reduced pressure. The thin film was hydrated by PBS (2 ml), and the micelles were formed by applying a probe sonicator (100 W, 5 minutes) at 37°C. The formulation was then centrifuged at 3000 rpm for 5 minutes followed by membrane filtration to remove any precipitated or unformulated drug.

The hydrodynamic diameters and ζ potential were measured by a Zetasizer Nano ZS90 (Malvern, Worcestershire, United Kingdom) at a scattering angle of 90°. The particle size distribution was reported as the mean $\pm$ S.D. of three independent samples. The critical micelle concentration (CMC) was determined using pyrene as a hydrophobic fluorescent probe as described elsewhere (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 the drug content was determined by high-performance liquid chromatography (HPLC)/UV-Vis analysis (Shimadzu, Kyoto, Japan) on a Phenomenex Aqua C18 column (5 μ m, 250 mm $\times$ 4.6 mm) using acetonitrile and water (55:45, v/v) as the mobile phase for QW-296 (R_t = 7.8 minutes) at a detection wavelength of 222 nm. For MDB5, the same column was used for quantification at a detection wavelength of 261 nm using acetonitrile and water (70:30, v/v) as the mobile phase (R_t = 4.0 minutes). The payload was calculated using the following equation:

$$\text{Drug loading} \left(\frac{w}{w} \% \right) = \frac{\text{Amount of extracted drug}}{\text{Total weight of formulation}} \times 100$$

Drug Release from Polymeric Micelles. Drug-loaded micelles with a final drug concentration of 1 mg/ml were placed into a dialysis membrane with the molecular mass cutoff of 3500 Da and dialyzed against 50 ml of 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 of sample was withdrawn at specified times for a period of 4 days and was subsequently replaced with 1 ml of the respective buffer solution. The drug concentration was analyzed by HPLC as described previously. All experiments were performed in triplicate on the same day, and the data are reported as the mean $\pm$ 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 at the University of Nebraska Medical Center. Orthotopic prostate tumors were established in 8- to 9-week-old male athymic nude mice by injecting 1.5×10^6 PC3TXR-luc cells suspended in 50 μ l of PBS into the dorsum of the prostate gland.

The animals were randomly divided into five groups after 1 week, and different treatments were administered intravenously every 3rd day for 5 times. Group 1 was kept as the control and received normal saline; group 2 received 10 mg/kg of QW-296 in micelle solution; group 3 received 10 mg/kg of MDB5 in micelle solution; group 4 received 5 mg/kg of QW-296 and 5 mg/kg of MDB5 in cosolvent (12% Cremophor EL, 12% ethanol, and 76% PBS); and group 5 received 5 mg/kg of QW-296 and 5 mg/kg of MDB5 in micelle solution.

At the end of the animal study (i.e., day 24), the mice were sacrificed, and the 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 hours. The fixed samples were embedded in paraffin, and thin sections of 4 μ m were obtained and

immunostained with H&E and cleaved caspase 3. The toxic effects of each treatment were evaluated by H&E staining of the major organs.

Statistical Analysis. Data are represented as the mean $\pm$ S.D. The statistical comparisons of the experiments were performed via 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 2-(1*H*-Indol-3-yl)-1*H*-imidazol-4-yl (3,4,5-trimethoxyphenyl) Methanone (ABI-231) (Chen et al., 2012), which is a highly potent tubulin inhibitor that can also effectively overcome taxane resistance in PC3-TXR tumors (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 are shown in Fig. 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. The molecular modeling studies (Fig. 1B) showed that the binding site of QW-296 was located at the interface between the 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 DJ-101 (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 DJ-101 (−12.9), indicating it may have similar tubulin binding effects of DJ-101.

To elucidate whether the 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 μ M of QW-296, colchicine, paclitaxel, or vehicle control (5% DMSO), respectively, and was 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 (Fig. 1C). These results clearly indicated that QW-296 is a strong tubulin-depolymerizing agent.

Better Anticancer Activity of Two Novel Compounds Compared with 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 (Fig. 2A). By contrast, QW-296 exhibited strong cell killing activity against DU145-TXR and PC3-TXR cell lines with IC₅₀ at 80 and 100 nM, respectively (Fig. 2B).

We also compared the cytotoxic effects of MDB5 and GDC-0449 using these two cell lines (Fig. 2, C and 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 with its

Fig. 2. Cytotoxicity assay of tubulin inhibitors and Hh signaling inhibitors on PC3-TXR and DU145-TXR cells. (A and D) 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 $\pm$ S.D. (*n* = 3).

Fig. 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 hours of incubation. (B–D) Combination effect of QW-296 and MDB5 at fixed ratios was determined, respectively. Results are represented as mean $\pm$ S.D. ($n = 3$). M, MDB5; Q, QW-296.

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 (Fig. 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 μ M) 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 μ M MDB5, or 40 nM QW-296 + 10 μ M MDB5) killed 40% of the cells, but when the concentration of single drug was doubled (40 nM QW-296 or 20 μ M 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, a strong synergistic effect (the lower the CI, the stronger the synergism) was observed in three combinations when the Fa level ranged from 75% to 95%. These results indicate that when the concentration of each drug was around half its corresponding IC_{50} dose, their combination not only resulted in a high level of growth inhibition (75% or above) but also worked synergistically Supplemental Fig. 2.

Inhibition of Colony Formation. The inhibitory activity of QW-296, MDB5, and their combination on the tumorigenic potential in PC3-TXR cells was determined by colony formation assay. As shown in Fig. 4A, the number of colonies in the untreated group was maximum compared with the groups treated with QW-296 or MDB5 alone or their combination. MDB5 at a concentration of 15 μ M exhibited slight inhibition against colony formation, while QW-296 at a concentration of 100 nM markedly suppressed colony formation. However, the combination of 50 nM QW-296 and 15 μ M MDB5 resulted in no colony formation throughout 7 days. These data further confirm 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 hours and 72 hours of treating PC3-TXR cells with the polymeric micelles formulation containing QW-296, MDB5, or their combination (Fig. 4, B and C). The data showed that 100 nM QW-296 caused 35.84% cell arrest in the G2/M phase at 48 hours, but decreased to 25.76% with an increase in treatment time from 48 to 72 hours. Furthermore, QW-296 treatment could induce sub-G1 phase arrest to 27.85% at 48 hours and 40.02% at 72 hours.

In contrast to QW-296, 15 μ M MDB5 resulted in 57.99% of PC3-TXR cell arrest in the G0/G1 phase at 48 hours, along with 72.85% of cells in G0/G1 at 72 hours, suggesting that MDB5 affected the cell cycle through a different mechanism.

The combination of 50 nM QW-296 and 15 μ M MDB5 slightly induced overall G2/M arrest at 48 and 72 hours. Unlike the single-drug treatments changing the cell arrest dramatically from 48 to 72 hours, the combination therapy caused cell arrest in the G2 phase, and these cells ended up in static status throughout 72 hours.

Fig. 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 six-well culture plates. At 24 hours, the drug formulations were added; at 7 days, the cell colonies were fixed, stained, and counted. (B and C) Cells were treated for 48 and 72 hours, stained with propidium iodide (PI), and analyzed by a flow cytometer. Results are expressed as mean $\pm$ S.D. ($n = 3$). (D) Cells were treated for 72 hours, protein from each group was extracted, and Western blot analysis was carried out for Shh.

Noticeably, after combination treatment the percentage of PC3-TXR cells in the sub-G1 phase was significantly enhanced as compared with the percentage after monotherapy. Because MDB5 was designed as an Hh signaling inhibitor and

GDC-0449 analog, we also measured the Shh protein expression by Western blot analysis. We found a clear reduction in Shh protein in the MDB5-treated group and the combination-treated group (Fig. 4D).

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

Characterization of Copolymer mPEG-p(TMC-MBC).

The monomer 2-methyl-2-benzoyloxycarbonyl propylene carbonate (MBC) was synthesized first as described previously. Then, MBC and trimethylene carbonate (TMC) were copolymerized with mPEG using $\text{Sn}(\text{Oct})_2$ as a catalyst to yield 1.82 g of mPEG-p(TMC-MBC) (88% yield) (Fig. 5A). In the ^1H NMR spectrum of mPEG-p(TMC-MBC), the following peaks were observed at δ 2.03 (CH_2 , br, 2H) corresponding to TMC, δ 1.2 (CH_3 , s, 3H) and δ 7.3 (phenyl, m, 5H) corresponding to MBC, δ 4.2–4.3 (CH_2 , t, 4H) corresponding to both TMC and MBC, and δ 3.63 (CH_2 , s, 2H) corresponding to PEG. The molecular mass of mPEG₁₁₄-b-p(TMC₁₅-MBC₁₅) determined by ^1H NMR was 10,280 Da with 15 U of each block, respectively (Fig. 5B).

Characterization, Quantification, and Release Profile of Drug-Loaded mPEG-p(TMC-MBC) Micelles. The amphiphilic nature of mPEG-p(TMC-MBC) drives its self-assembly into micelles in aqueous buffer. Surface morphology and the mean particle size of the micelles were determined by transmission electron microscopy (TEM; Tecnai G² Spirit; FEI Company, Hillsboro, OR) and dynamic light scattering (Zetasizer Nano ZS90; Malvern Panalytical, Worcestershire, United Kingdom). Empty and drug (QW-296 or MDB5)-loaded polymeric micelles had similar particle-size distributions, with the mean particle size of 81 nm (Fig. 6A). The TEM image also showed that the micelles are spherical in shape with uniform particle size below 60 nm in PBS (Fig. 6B). The critical micelle concentration (CMC) of mPEG-p(TMC-MBC) was 6.65×10^{-4} g/l (Fig. 6C), further indicating that the micelles were quite stable in PBS. Moreover, a high drug payload of these polymeric micelles was determined by HPLC: $8.13\% \pm 0.75\%$ (w/w) for QW-296 and $9.12\% \pm 0.69\%$ (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, determining the release profile of QW-296 and MDB5 from mPEG-p(TMC-MBC) micelles at different pH values was performed by dialysis in PBS buffer of pH 7.4 and acetate buffer of pH 6.5. As shown in Fig. 6D, 27% of QW-296 was rapidly released from the micelles within the initial 6 hours under neutral conditions; the release then was increased up to 58% at 24 hours followed by slow, sustained release until the end of 96 hours. However, the overall release of MDB5 was slightly slower compared with QW-296. The release of QW-296 or MDB5 was slightly faster under acidic conditions. These data suggest that the drug-loaded micelles are quite stable under physiologic pH conditions.

In Vivo Antitumor Efficacy in Orthotopic Prostate Cancer Mouse Model. We successfully established orthotopic prostate tumors in 8- to 9-week-old male athymic nude mice by injecting 1.5×10^6 PC3-TXR-luc cells into the dorsal prostate lobe. The mice whose bioluminescent radiance reached 1×10^8 p/s square centimeter per steradian 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 + 5 mg/kg), and 5) combination of QW-296- and MDB5-loaded micelles (5 + 5 mg/kg).

All the treated groups showed inhibition of tumor growth compared with the control group (Fig. 7). However, the maximum tumor inhibition was observed in the group treated with the micelles encapsulating both QW-296 and MDB5.

Next, we performed an immunohistochemical analysis to further elucidate the superior anticancer efficacy of combined micelles. H&E staining of tumor sections indicated that tumor samples from four treated groups had more necrotic area compared with the tumor samples from the control group (Fig. 8). Tumors from combination micelles group showed maximum necrosis. Furthermore, cleaved caspase 3 staining

Fig. 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 mean $\pm$ S.D. ($n = 3$).

Fig. 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.

of tumor tissue indicated the induction of significant apoptosis by combination micelles of QW-296 and MDB5 compared with other treatments (Fig. 8).

Meanwhile, the chronic toxicities of these treatments were also examined by histologic analysis of major organs. No distinct histologic changes were observed in the liver, spleen, kidney, or heart from any of the treated groups,

suggesting that the mice tolerated all treatments well (Fig. 9).

Discussion

Drug resistance is still one of the major impediments for the success of chemotherapy, and several factors account for the

Fig. 8. Analysis of tumor samples with (A) H&E and (B) caspase 3 stains.

Fig. 9. Analysis of major organs with H&E stain. Organ samples from five groups were excised, fixed, and stained with H&E. No obvious histologic changes were observed in the livers, spleens, kidneys, and hearts from any of the treated groups.

occurrence of resistance, including ATP-binding cassette transporter family (P-glycoprotein, ABCC1, ABCG2, etc.) (Schinkel and Jonker, 2003), alteration in drug targets, and 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 with 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 with a 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 microtubules 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 with ABI-231 (1.3 vs. 7.4) in paclitaxel-resistant PC3-TXR cells. The potency of QW-296 was 7.4 times higher than that of ABI-231 in paclitaxel-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 DJ-101, 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 (Fig. 1C).

We selected the novel Hh pathway inhibitor MDB5 to ally with QW-296 to treat advanced prostate cancer due to the emerging clinical reports that the overexpressed Hh pathway promotes prostate tumor formation from epithelial cells, renders the epithelial-mesenchymal transition, and has cross talk with the androgen pathway. In our previous study, we also demonstrated that the combination of the Hh inhibitor cyclopamine and paclitaxel synergistically suppressed the growth of TXR prostate cancer *in vitro* and *in 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, which has 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 (Figs. 3 and 4). The IC_{50} of QW-296 in DU-145-TXR and PC3-TXR cells was in the range of 80–100 nM, whereas that of

MDB5 was in the range of 30–50 μM . This means the IC_{50} values of these two drugs have an approximately 300- to 600-fold difference. Therefore, we used 1:200, 1:300, and 1:400 ratios for *in vitro* combination therapy with QW-296 and MDB5. We determined the synergism between these two drugs using the Chou–Talalay method (Supplemental Fig. 2), which strengthened our hypothesis. In the cell cycle analysis, QW-296 and MDB5 made distinct impacts on the cell cycle; MDB5 treatment caused G0/G1 phase arrest while QW-296 treatment led to G2/M phase arrest (Fig. 4, B and C), which demonstrated the two anticancer agents worked at a complementary mechanism of action against PC3-TXR cells. This result was in agreement with the previous reports.

Further, we analyzed the change in protein expression to highlight the combination advantage at the molecular level. We observed down-regulation of Shh (Fig. 4D), a key component of the Hh signaling pathway, after MDB5 monotherapy or the combination of QW-296 and MDB5.

Although QW-296 and MDB5 have demonstrated excellent synergy in anticancer activity against chemoresistant prostate cancer, their clinical translation will be limited due to their intrinsic poor aqueous solubility, like many other anticancer agents. To solve this problem, nanocarrier-based therapeutic systems have emerged over several decades as a promising platform for delivering hydrophobic drugs. 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 that are suitable for systemic drug delivery.

We used mPEG as the hydrophilic backbone, and the long length enabled us to synthesize the copolymer with a molecular mass 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 the drug's accumulation in 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 nontoxic, biocompatible, and biodegradable, which provides potential for future clinical translation. They degrade into carbon dioxide and benzyl alcohol, which have 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 the drug release in acidic or neutral conditions (Fig. 6).

To better investigate the combination efficacy and confirm the benefits of using micelles as a delivery vehicle, we used a half-dose of QW-296 and MDB5 in combination treatment in cosolvent or micelles to compare with the monotherapies, while using saline-injected mice as the control. All four treatment groups exhibited excellent tumor inhibitory results (Fig. 7). However, combination therapy in micelles showed significantly enhanced reduction in tumor size compared with the combination in cosolvent 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 the other healthy organs showed no obvious histologic changes after the 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 the two drugs rather than cosolvent.

Conclusion

In the present study, we successfully synthesized and screened the novel microtubule destabilizer QW-296 and an Hh pathway inhibitor MDB5 and demonstrated their anticancer activities in combination or individually. We successfully synthesized mPEG-p(TMC-MBC) and formed polymeric micelles to encapsulate QW-296 and MDB5 with payloads of $8.13\% \pm 0.75\%$ and $9.12\% \pm 0.69\%$ (w/w), respectively. The overall findings indicated that the combination of QW-296 and MDB5 exhibited a synergistic therapeutic effect against chemoresistant prostate cancer via different mechanisms, and with the help of mPEG-p(TMC-MBC) the combination could effectively inhibit the growth of chemoresistant prostate cancer.

Authorship Contributions

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

References

- Abdullah LN and Chow EK (2013) Mechanisms of chemoresistance in cancer stem cells. *Clin Transl Med* 2:3.
- Arnst KE, Wang Y, Hwang DJ, Xue Y, Costello T, Hamilton D, Chen Q, Yang J, Park F, Dalton JT, et al. (2018) A potent, metabolically stable tubulin inhibitor targets the colchicine binding site and overcomes taxane resistance. *Cancer Res* 78: 265–277.
- Banerjee S, Arnst KE, Wang Y, Kumar G, Deng S, Yang L, Li GB, Yang J, White SW, Li W, et al. (2018) Heterocyclic-fused pyrimidines as novel tubulin polymerization inhibitors targeting the colchicine binding site: structural basis and antitumor efficacy. *J Med Chem* 61:1704–1718.
- Buey RM, Barasoain I, Jackson E, Meyer A, Giannakakou P, Paterson I, Mooberry S, Andreu JM, and Diaz JF (2005) Microtubule interactions with chemically diverse stabilizing agents: thermodynamics of binding to the paclitaxel site predicts cytotoxicity. *Chem Biol* 12:1269–1279.
- Chen J, Ahn S, Wang J, Lu Y, Dalton JT, Miller DD, and Li W (2012) Discovery of novel 2-aryl-4-benzoyl-imidazole (ABI-III) analogues targeting tubulin polymerization as antiproliferative agents. *J Med Chem* 55:7285–7289.
- Chen M, Feuerstein MA, Levina E, Baghel PS, Carkner RD, Tanner MJ, Shtutman M, Vacherot F, Terry S, de la Taille A, et al. (2010) Hedgehog/gli supports androgen signaling in androgen deprived and androgen independent prostate cancer cells. *Mol Cancer* 9:89.
- Chen ZS, Hopper-Borge E, Belinsky MG, Shchavaleva I, Kotova E, and Kruh GD (2003) Characterization of the transport properties of human multidrug resistance protein 7 (MRP7, ABCC10). *Mol Pharmacol* 63:351–358.
- Chung MK, Kim HJ, Lee YS, Han ME, Yoon S, Baek SY, Kim BS, Kim JB, and Oh SO (2010) Hedgehog signaling regulates proliferation of prostate cancer cells via stathmin1. *Clin Exp Med* 10:51–57.
- Clement V, Sanchez P, de Tribolet N, Radovanovic I, and Ruiz i Altaba A (2007) HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. *Curr Biol* 17:165–172.
- Darshan MS, Loftus MS, Thadani-Mulero M, Levy BP, Escuin D, Zhou XK, Gjyzezi A, Chanev-Vos C, Shen R, Tagawa ST, et al. (2011) Taxane-induced blockade to nuclear accumulation of the androgen receptor predicts clinical responses in metastatic prostate cancer. *Cancer Res* 71:6019–6029.
- Dierks C, Beigi R, Guo GR, Zirikli K, Stegert MR, Manley P, Trussell C, Schmitt-Graeff A, Landwerlin K, Veelken H, et al. (2008) Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer Cell* 14: 238–249.
- Hwang DJ, Wang J, Li W, and Miller DD (2015) Structural optimization of indole derivatives acting at colchicine binding site as potential anticancer agents. *ACS Med Chem Lett* 6:993–997.
- Karhadkar SS, Bova GS, Abdallah N, Dhara S, Gardner D, Maitra A, Isaacs JT, Berman DM, and Beachy PA (2004) Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* 431:707–712.
- Kumar V, Chaudhary AK, Dong Y, Zhong HA, Mondal G, Lin F, Kumar V, and Mahato RI (2017) Design, synthesis and biological evaluation of novel hedgehog inhibitors for treating pancreatic cancer. *Sci Rep* 7:1665.

- Leonard GD, Fojo T, and Bates SE (2003) The role of ABC transporters in clinical practice. *Oncologist* **8**:411–424.
- Li CM, Chen J, Lu Y, Narayanan R, Parke DN, Li W, Ahn S, Miller DD, and Dalton JT (2011) Pharmacokinetic optimization of 4-substituted methoxybenzoyl-aryl-thiazole and 2-aryl-4-benzoyl-imidazole for improving oral bioavailability. *Drug Metab Dispos* **39**:1833–1839.
- Li CM, Lu Y, Chen J, Costello TA, Narayanan R, Dalton MN, Snyder LM, Ahn S, Li W, Miller DD, et al. (2012) Orally bioavailable tubulin antagonists for paclitaxel-refractory cancer. *Pharm Res* **29**:3053–3063.
- Li F, Danquah M, and Mahato RI (2010) Synthesis and characterization of amphiphilic lipopolymers for micellar drug delivery. *Biomacromolecules* **11**:2610–2620.
- Lu Y, Chen J, Wang J, Li CM, Ahn S, Barrett CM, Dalton JT, Li W, and Miller DD (2014) Design, synthesis, and biological evaluation of stable colchicine binding site tubulin inhibitors as potential anticancer agents. *J Med Chem* **57**:7355–7366.
- Lu Y, Chen J, Xiao M, Li W, and Miller DD (2012) An overview of tubulin inhibitors that interact with the colchicine binding site. *Pharm Res* **29**:2943–2971.
- Lu Y, Li CM, Wang Z, Chen J, Mohler ML, Li W, Dalton JT, and Miller DD (2011) Design, synthesis, and SAR studies of 4-substituted methoxybenzoyl-aryl-thiazoles analogues as potent and orally bioavailable anticancer agents. *J Med Chem* **54**:4678–4693.
- Peacock CD, Wang Q, Gesell GS, Corcoran-Schwartz IM, Jones E, Kim J, Devereux WL, Rhodes JT, Huff CA, Beachy PA, et al. (2007) Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma. *Proc Natl Acad Sci USA* **104**:4048–4053.
- Risinger AL, Jackson EM, Polin LA, Helms GL, LeBoeuf DA, Joe PA, Hopper-Borge E, Ludueña RF, Kruh GD, and Mooberry SL (2008) The taccalonolides: microtubule stabilizers that circumvent clinically relevant taxane resistance mechanisms. *Cancer Res* **68**:8881–8888.
- Schinkel AH and Jonker JW (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* **55**:3–29.
- Shaw G and Prowse DM (2008) Inhibition of androgen-independent prostate cancer cell growth is enhanced by combination therapy targeting hedgehog and ErbB signalling. *Cancer Cell Int* **8**:3.
- Singh S, Chitkara D, Mehrazin R, Behrman SW, Wake RW, and Mahato RI (2012) Chemoresistance in prostate cancer cells is regulated by miRNAs and Hedgehog pathway. *PLoS One* **7**:e40021.
- Xiao M, Ahn S, Wang J, Chen J, Miller DD, Dalton JT, and Li W (2013) Discovery of 4-Aryl-2-benzoyl-imidazoles as tubulin polymerization inhibitor with potent antiproliferative properties. *J Med Chem* **56**:3318–3329.
- Yang R, Mondal G, Ness RA, Arnst K, Mundra V, Miller DD, Li W, and Mahato RI (2017a) Polymer conjugate of a microtubule destabilizer inhibits lung metastatic melanoma. *J Control Release* **249**:32–41.
- Yang R, Mondal G, Wen D, and Mahato RI (2017b) Combination therapy of paclitaxel and cyclopamine polymer-drug conjugates to treat advanced prostate cancer. *Nanomedicine (Lond)* **13**:391–401.
- Zhang R, Yang J, Sima M, Zhou Y, and Kopeček J (2014) Sequential combination therapy of ovarian cancer with degradable *N*-(2-hydroxypropyl)methacrylamide copolymer paclitaxel and gemcitabine conjugates. *Proc Natl Acad Sci USA* **111**:12181–12186.

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