Acetal-Linked Paclitaxel Prodrug Micellar Nanoparticles as a Versatile and Potent Platform for Cancer Therapy

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ABSTRACT: Endosomal pH-activatable paclitaxel (PTX) prodrug micellar nanoparticles were designed and prepared by conjugating PTX onto water-soluble poly(ethylene glycol)-b-poly(acrylic acid) (PEG-PAA) block copolymers via an acetal labile acetal bond to the PAA block and investigated for potent growth inhibition of human cancer cells in vitro. PTX was readily conjugated to PEG-PAA with high drug contents of 21.6, 27.0, and 42.8 wt % (denoted as PTX prodrugs 1, 2, and 3, respectively) using ethyl glycol vinyl ether (EGVE) as a linker. The resulting PTX conjugates had defined molecular weights and self-assembled in phosphate buffer (PB, pH 7.4, 10 mM) into monodisperse micellar nanoparticles with average sizes of 158.3–180.3 nm depending on PTX contents. The in vitro release studies showed that drug release from PTX prodrug nanoparticles was highly pH-dependent, in which ca. 86.9%, 66.4% and 29.0% of PTX was released from PTX prodrug 3 at 37 °C in 48 h at pH 5.0, 6.0, and pH 7.4, respectively. MTT assays showed that these pH-sensitive PTX prodrug nanoparticles exhibited high antitumor effect to KB and HeLa cells (IC_{50} = 0.18 and 0.9 μg PTX equiv/mL, respectively) as well as PTX-resistant A549 cells. Notably, folate-decorated PTX prodrug micellar nanoparticles based on PTX prodrug 3 and 20 wt % folate-poly(ethylene glycol)-b-poly(D,L-lactide) (FA-PEG-PLA) displayed apparent targetability to folate receptor-overexpressing KB cells with IC_{50} over 12 times lower than nontargeting PTX prodrug 3 under otherwise the same conditions. Furthermore, PTX prodrug nanoparticles could also load doxorubicin (DOX) to simultaneously release PTX and DOX under mildly acidic pH. These acetal-linked PTX prodrug micellar nanoparticles have appeared as a highly versatile and potent platform for cancer therapy.

INTRODUCTION

The lack of suitable vehicles is a major challenge for systemic administration of potent and yet poorly water-soluble anticancer drugs including paclitaxel (PTX) and doxorubicin (DOX).1–4 The current clinical PTX formulations based on Cremophor EL and ethanol (Taxol) though improved solubility of PTX are associated with hypersensitivity reactions.5 In the past decades, various biocompatible nanosystems such as polymeric prodrugs,6 liposomes,7 polymeric micelles,8,9 polymersomes,10 and polymeric nanoparticles11,12 have been developed for safer as well as better-controlled delivery of PTX. The in vivo studies have shown that nanosystems exhibit improved drug tolerance, better drug bioavailability, and apparent tumor-targetability via enhanced permeability and retention (EPR) effect (passive targeting).13,14 In particular, polymeric prodrugs and micellar nanoparticles have received the most attention. For example, PTX prodrugs have been prepared by conjugating PTX to water-soluble polymers such as poly(ethylene glycol) (PEG), poly(N-(2-hydroxypropyl)-methacrylamide) (PHPMA), poly(ε-caprolactone) (PCL) and hyaluronic acid via a cleavable peptide, ester or disulfide bond.15–17 It should be noted that PHPMA-PTX (PNU166945)18 and PCL-PTX (CT-2103, Xyotax)19,20 have progressed to phase I and III clinical trials, respectively. However, polymeric prodrugs are often plagued by complex synthesis, low drug conjugation, small size, as well as inferior drug activity due to slow release of drug at the site of action and/or chemical alternation of released drug. In comparison, core–shell micelles with sizes of about 20–200 nm load PTX via physical encapsulation with generally higher drug loading contents.21,22 Notably, micellar PTX formulations, Genexol-PM23 and NK105,24 have been approved for treating breast, lung, and ovarian cancers in South Korea and for phase III clinical trials in patients with metastatic or recurrent breast cancers, respectively. The self-assembled micellar nanoparticles, nevertheless, suffer poor in vivo stability, premature drug release following injection, and significant systemic side effects.3

To combine advantageous features of polymeric prodrugs and micellar nanoparticles, prodrug micellar nanoparticles have been conceived based on amphiphilic block copolymer-drug conjugates that contain stimuli-sensitive degradable hydrazone, ester or disulfide bonds.25–30 These prodrug micellar nanoparticles have shown improved systemic stability, longer circulation time, and better tumor targetability. For instance, Jing et al. reported that P(LGG-PTX)-PEG-P(LGG-PTX) prodrug micelles by conjugating PTX via an ester bond did not show initial burst drug release and kept the in vitro antitumor activity of PTX

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Acetal-linked PTX prodrug micellar nanoparticles have been developed for targeted delivery. These nanoparticles are designed to release PTX under acidic conditions, mimicking the endosomal pH. The nanoparticles are formed by conjugating PTX onto water-soluble poly(ethylene glycol)-b-poly(acrylic acid) (PEG-PAA) block copolymers via an acid-labile acetal bond to PAA block core.

**Scheme 1. Illustration of Acetal-Linked PTX Prodrug Micellar Nanoparticles Based on PEG-PAA Block Copolymer**

(i) Self-assembly of acetal-linked PTX prodrug in water to form nano-sized micellar nanoparticles. (ii) Endosomal pH-triggered acetal cleavage and release of pristine PTX.

**EXPERIMENTAL SECTION**

**Materials.** Acrylic acid (AA, 98%, Alfa Aesar) was purified by passing through a basic alumina column, S-1-dodecyl-S-(R,R-dimethyl-R-acetic acid)trithiocarbonate (DMF),17 PEG-DMP (Mw/PD = 5.0 kg/mol) macro-reversible addition/fragmentation chain transfer (RAFT) agent,18 and folate-poly(ethylene glycol)-b-poly(L-lactide) (FA-PEG-PLA)19 were synthesized according to previous reports. FA-PEG-PLA was prepared from PEG-PLA block copolymer with an Mn of 6.0–4.5 kg/mol and an Mw/Mn of 1.20. UV measurements showed that FA functionality was close to 100%. 2,2′-Azobisobutyronitrile (AIBN, 98%, J&K) was recrystallized twice from hexane and methanol, respectively. N,N-Dimethylformamide (DMF) dried by MgSO4 was distilled under reduced pressure, and 1,4-dioxane was dried by refluxing over sodium wire and stored under argon. All other reagents were reagent grade and used as received.

**EXPERIMENTAL SECTION**

**Characterization.** 1H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterium oxide (D2O) or deuterated chloroform (CDCl3) as a solvent. The chemical shifts were calibrated against residual solvent signals. The molecular weights and polydispersity index (PDI) of the polymers were determined by Waters 1515 gel permeation chromatography (GPC) equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using DMF as the eluent at a flow rate of 0.8 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The size of nanofibers was determined using dynamic light scattering (DLS). Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of 0.1 mg/mL nanoparticle solution on the copper grid. The amount of PTX was determined by high-performance liquid chromatography (HPLC; Agilent 1260) with UV detection at 227 nm using a mixture of acetonitrile and water at 1/1 (v/v) as a mobile phase.

**Synthesis of PEG-PAA Diblock Polymer.** PEG-PAA copolymer was prepared by RAFT polymerization of AA using PEG-DMP (Mn = 5.0 kg/mol) as a macro-RAFT agent. Under a nitrogen atmosphere, AA (1630 μL, 2.375 mmol), PEG-DMP (0.5 g, 0.095 mmol), AIBN (3.12 mg, 0.019 mmol), and anhydrous dioxane (6 mL) were added into a 25 mL Schlenk flask. After blowing the mixture with nitrogen for 30 min, the flask was sealed and placed in an oil bath thermostatted at 70 °C. The polymerization proceeded under stirring for 48 h. The resulting polymer was isolated by precipitation in cold diethyl ether, filtration, and
drying in vacuo. Yield: 85%. $^1$H NMR (400 MHz, D$_2$O): PEG (−CH$_2$−CH$_2$−O−): δ 3.63; CH$_3$−O−: δ 3.38. PAA (−CH$_2$−CH−COO−): δ 2.37; −CH$_3$−CH−COO−: δ 1.6−1.9. $M_c$ ($^1$H NMR) = 6.4 kg/mol.

**Synthesis of Vinyl Ether-Functionalized PEG-PAA.** Under a nitrogen flow, to a 50 mL round-bottom flask equipped with a magnetic stirrer were added PEG-PAA (300 mg, equiv. 1.4 mmol COOH), DMAP (8.4 mg, 0.07 mmol), DCC (863.4 mg, 4.2 mmol), and anhydrous dioxane (10 mL). The flask was sealed and the reaction was allowed to proceed under stirring for 10 h at 25 °C. After dialysis (MWCO 3500) against PB (pH 7.4, 10 mM), order with 20 min ultrasonication (200 W, 50 Hz) after each addition. A prodrug nanoparticle dispersions (0.5 mg/mL) was dialyzed against 25 °C acetate buffer (10 mM, pH 5.0) and neutralized with 20 min ultrasonication (200 W, 50 Hz) after each addition. The final drug concentration was determined by the calibration curve of PTX standard solution.

**Preparation of PTX Prodrug Micellar Nanoparticles.** PTX prodrug micellar nanoparticles with or without FA-PEG-PLA (20 wt %) were prepared via the direct hydration method as described in our previous report. Typically, PTX prodrug (1.0 mg) and PEG 550 (15 µL) were charged in 4 mL of buffer. The mixture was heated to 95 °C, stirred at 95 °C for 20 min, and cooled to room temperature. Then, 10, 20, 70, and 1900 µL of phosphatase buffer (PB; pH 7.4, 10 mM) was added in that order with 20 min ultrasonication (200 W, 50 Hz) after each addition. After dialysis (MWCO 3500) against PB (pH 7.4, 10 mM), homogenous prodrug nanoparticle dispersions were obtained. The size and size distribution were determined by DLS measurements.

**In Vitro PTX Release from PTX Prodrug Nanoparticles.** The release profiles of PTX from prodrug nanoparticles were studied using a dialysis tube (MWCO 12,000) under shaking (200 rpm) at 37 °C in three different media, i.e., pH 5.0 (acetate buffer, 10 mM), pH 6.0 (acetate buffer, 10 mM), and pH 7.4 (PB, 10 mM). Typically, 0.5 mL of prodrug nanoparticle dispersions (0.5 mg/mL) was dialyzed against 25 mL of release media. At desired time intervals, 8 mL of release media was taken out and replenished with an equal volume of fresh media. The amount of PTX released was determined by HPLC measurements. The cumulative PTX release ratio was calculated according to the following formula:

$$E_i = \frac{V_i C_i}{m_{drug}} \times 100\% \quad (i = 1)$$

$$E_f = \frac{V_f \sum_{i=1}^{n-1} C_i + V_f C_n}{m_{drug}} \times 100\% \quad (i = 2)$$

where $E_i$ is the cumulative released ratio of PTX (%), $V_i$ and $V_f$ are the volumes of the exchanged medium and the total medium, respectively (in mL), $C_i$ is the PTX concentration of the release medium taken at the ith time (in mg/mL), and $m_{drug}$ is the total mass of PTX in the nanoparticles (in mg).

The release experiments were conducted in triplicate, and the results presented are the average data with standard deviation. The change of prodrug nanoparticle sizes following release of PTX in time was monitored by DLS.

**MTT Assays of PTX Prodrug Nanoparticles.** The antitumor activity of PTX prodrug nanoparticles was evaluated in human nasopharyngeal epidermal carcinoma cells (KB), human cervical carcinoma cells (HeLa), as well as paclitaxel resistant human lung carcinoma A549 cells (A549/PTX$^{\text{R}}$) by MTT assays. The cells were plated in a 96-well plate (5 × 10$^4$ cells/well) using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, and the antibiotics penicillin (100 IU mL$^{-1}$) and streptomycin (100 µg mL$^{-1}$) for 24 h. The medium was aspirated and replaced by 90 µL of fresh medium supplemented with 10% FBS. Ten microliters of prodrug nanoparticles in PB (10 mM, pH 7.4) was added to yield final drug concentrations of 5 × 10$^{-5}$ to 20 µg PTX equiv/mL. The cells were cultured at 37 °C in an atmosphere containing 5% CO$_2$ for 48 h. Then 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) solution (5 mg/mL) was added. The cells were incubated for 4 h. The medium was aspirated, washed with PBS, and 150 µL of dimethyl sulfoxide (DMSO) was added. The absorbance at a wavelength of 492 nm of each well was measured using a microplate reader. PTX formulations prepared by dissolving PTX in Cremophor EL/ethanol (1:1 v/v) with the concentrations ranging from 5 × 10$^{-5}$ to 20 µg PTX equiv/mL were used as controls. The relative cell viability (%) was determined by comparing the absorbance at 492 nm with control wells containing only cell culture medium. Data are presented as average ± SD ($n = 4$).

The cytotoxicity of vinyl ether functionalized PEG-PAA prepolymer was determined in a similar way at polymer concentrations ranging from 0.05 to 1.0 mg/mL. Here, acetaldehyde, which is a hydrolysis byproduct of PTX prodrug nanoparticles, was added at a concentration of 32.5 µM (corresponding to a maximal amount of acetaldehyde generated from 1.0 mg/mL of PTX prodrug 3 nanoparticles).

**Folate-Decorated PTX Prodrug Nanoparticles Targeting to KB Cells.** Folate-decorated PTX prodrug micellar nanoparticles were prepared from PTX prodrug 3 and 20 wt % FA-PEG-PLA. The antitumor activity of folate-decorated PTX prodrug nanoparticles to folate receptor-overexpressing KB cells was evaluated by MTT assays. The folate concentration of polymer was varied from 1.0 × 10$^{-3}$ to 0.1 mg/mL and the concentration of pyrene was fixed at 0.6 µL. The fluorescence spectra were recorded using a FLS920 fluorescence spectrometer with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm was monitored. The CAC was estimated as the cross-point when extrapolating the intensity ratio $I_{372}/I_{383}$ at low and high concentration regions.
PEG-PAA diblock copolymers were prepared by controlled acetal bond to PAA block using EGVE as a linker (Scheme 2). Water-soluble PEG-PAA block copolymers via an acid-labile PEG-PAA block copolymers. PTX was conjugated onto triplicate and the results presented are the average data with standard measurements, respectively. The release experiments were conducted in fresh replenished with an equal volume of fresh media. The amounts of PTX in DMSO solutions with known DOX concentrations. DLC was calculated according to the following formula: DLC (wt %) = (weight of loaded prodrug/total weight of prodrug and loaded DOX) × 100%.

The co-release profiles of PTX and DOX from DOX-loaded prodrug nanoparticles were studied using a dialysis method (MWCO 12000) under shaking (200 rpm) at 37 °C in three different media, i.e., pH 5.0 (acetate buffer, 10 mM), pH 6.0 (acetate buffer, 10 mM), and pH 7.4 (PB, 10 mM). Typically, 0.5 mL of DOX-loaded prodrug nanoparticle dispersions (0.5 mg/mL) was dialyzed against 25 mL of release media. The whole procedure was performed in the dark. For determination of drug loading content (DLC), DOX-loaded nanoparticles were lyophilized, dissolved in DMSO, and analyzed with fluorescence spectroscopy (FLS 920, excitation at 480 nm). The calibration curve was obtained with DOX/DMSO, and analyzed with fluorescence spectroscopy (FLS 920, excitation at 480 nm, emission at 492 nm with control wells containing only cell culture medium. Data are presented as average ± SD (n = 4).

**Loading of DOX and pH-Triggered Co-release of PTX and DOX.** DOX could be physically loaded into PTX prodrug nanoparticles. In a typical example, PTX prodrug (1.0 mg) and PEG 550 (15 μL) were charged into a 4 mL centrifuge tube. The mixture was heated to 95 °C, stirred at 95 °C for 20 min, and cooled to room temperature. Ten microliters of PB (pH 7.4, 10 mM) and 22 μL of DOX solution in DMSO (5.0 mg/mL) were added, and the mixture was stirred at 60 °C for 20 min. Then, 20, 70, and 1900 μL of PB (pH 7.4, 10 mM) was added in that order with 20 min ultrasonication (200 W, 50 Hz) after each addition. After dialysis (MWCO 3500) against PB, homogeneous DOX-loaded prodrug nanoparticle dispersions were obtained. The whole procedure was performed in the dark. For determination of drug loading content (DLC), DOX-loaded nanoparticles were lyophilized, dissolved in DMSO, and analyzed with fluorescence spectroscopy (FLS 920, excitation at 480 nm). The calibration curve was obtained with DOX/DMSO solutions with known DOX concentrations. DLC was calculated according to the following formula: DLC (wt %) = (weight of loaded DOX/total weight of prodrug and loaded DOX) × 100%.

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**RESULTS AND DISCUSSION**

**Synthesis of Acetal-Linked PTX Prodrugs Based on PEG-PAA Block Copolymers.** PTX was conjugated onto water-soluble PEG-PAA block copolymers via an acid-labile acetal bond to PAA block using EGVE as a linker (Scheme 2). PEG-PAA diblock copolymers were prepared by controlled RAFT polymerization of AA using PEG-DMP (Mn = 5.0 kg/mol) as a macro-RAFT agent and AIBN as the radical source in dioxane at 70 °C for 48 h. 1H NMR displayed clearly peaks characteristic of PEG (δ 3.63) and PAA blocks (δ 1.6–1.9 and 2.37). The degree of polymerization (DP) of PAA block was determined, by comparing integrals of peaks at δ 2.37 and 3.63, to be 20, which was close to our designed DP of 25. Using PEG-DMP under similar conditions, we have previously synthesized thermo-sensitive PEG-PAA-PNIPAM triblock copolymers (PNIPAM = poly(N-isopropylacrylamide)), based on which reduction-sensitive reversibly cross-linked polymersomes were developed for efficient intracellular protein release. EGVE was coupled to PEG-PAA using DCC and DMAP in dioxane for 48 h to yield vinyl ether-functionalized PEG-PAA. 1H NMR spectrum displayed new signals at δ 6.47/4.18/4.05 and δ 4.25/3.87 besides peaks of PEG-PAA, attributable to the vinyl and methylene protons of EGVE moieties, respectively (Figure 1A). The number of vinyl ether substitution per polymer chain was estimated to be 12 by comparing the intensities of signals at δ 6.47 (vinyl proton of EGVE moieties) with δ 3.63.

PTX was readily conjugated to vinyl ether-functionalized PEG-PAA copolymers via an acetal bond through a “click”-type conjugate reaction between 2′-hydroxyl groups of PTX and pendant vinyl ethers. Frey and Wurm et al. reported that vinyl ether side chains of PEG reacted efficiently with alcohols. 1H NMR showed besides peaks of vinyl ether-functionalized PEG-PAA also signals due to PTX at δ 1.1–1.2, 2.2–2.4, 3.5–3.8, and 7.3–8.4 (Figure 1B). Furthermore, a peak assignable to acetal methine proton was detected at δ 4.37, supporting conjugation of PTX to PEG-PAA via an acetal bond. PTX content was determined by HPLC measurements. The results showed that PTX prodrugs with 21.6, 27.0, and 42.8 wt % PTX (denoted as prodrugs 1, 2, and 3) were obtained at varying PTX-to-polymer molar ratios of 4/1, 6/1, and 12/1, respectively (Table 1). 1H NMR analysis by comparing integrals of signals at δ 7.3–8.4 with 3.63 indicated similar PTX contents to those determined with HPLC (Table 1). Notably, these PTX prodrugs have significantly...
higher drug contents than most prodrugs including PHPMA-peptide-PTX prodrugs (5 wt % PTX, PNU166945) used in phase I clinical trials and PHPMA-hydrazide-LEV-PTX (7.6−16.3 wt % PTX). GPC revealed that all PTX prodrugs had a unimodal distribution with moderate $M_w/M_n$ of 1.25−1.42 (Table 1). Moreover, $M_n$ of PTX prodrugs increased in parallel (from 17.3 to 22.1 kg/mol) with increasing PTX contents.

**Formation of PTX Prodrug Micelles and pH-Triggered Drug Release.** PTX prodrugs formed nanosized micellar nanoparticles in PB (pH 7.4, 10 mM) (Figure 2A). The average hydrodynamic sizes increased from 158.3 to 180.3 nm with increasing PTX contents from 21.6 to 42.8 wt % (Table 2). TEM micrograph revealed that these prodrug nanoparticles had a spherical morphology and particle sizes close to those determined by DLS (Figure 2B). Notably, these PTX prodrugs had low CACs of 3.16−3.47 mg/L (Table 2), and were stable during storage for several months at 4 °C.

The in vitro drug release from PTX prodrug nanoparticles was studied at 37 °C under three different conditions, i.e., pH 5.0, 6.0, and 7.4. The results showed that PTX release was significantly accelerated under mildly acidic environments (Figure 3). For example, ca. 86.9%, 66.4% and 29.0% of PTX was released from prodrug nanoparticles in 48 h at pH 5.0, 6.0, and pH 7.4, respectively. Moreover, all three prodrug nanoparticles exhibited

**Figure 1.** $^1$H NMR spectra (400 MHz, CDCl$_3$) of vinyl ether-functionalized PEG-PAA block copolymer (A) and PTX prodrug (B).

**Table 1. Synthesis of PTX Prodrugs Based on PEG-PAA Block Copolymers**

<table>
<thead>
<tr>
<th>PTX prodrug</th>
<th>PTX/polymer molar feed ratio</th>
<th>$M_n$ (kg/mol)</th>
<th>$M_n$/M$^b_n$ (PDI)</th>
<th>PTX (wt %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>9.3</td>
<td>17.3</td>
<td>1.42</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>10.0</td>
<td>19.5</td>
<td>1.25</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>13.2</td>
<td>22.1</td>
<td>1.30</td>
</tr>
</tbody>
</table>

$^a$Determined by $^1$H NMR. $^b$Determined by GPC measurements (eluent: DMF, flow rate: 0.8 mL/min, polystyrene standards). $^c$Determined by HPLC measurements. $^d$Calculated by $^1$H NMR via comparing integrals of signals at δ 7.3−8.4 with 3.63.

**Figure 2.** Size distribution profiles of PTX prodrug nanoparticles determined by DLS (A) and of PTX prodrug 1 nanoparticles determined by TEM (B).

**Table 2. Formation of PTX Prodrug Nanoparticles**

<table>
<thead>
<tr>
<th>PTX prodrug nanoparticle</th>
<th>size (nm)$^a$</th>
<th>PDI$^c$</th>
<th>CAC$^d$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>prodrug 1</td>
<td>158.3 ± 11.5</td>
<td>0.17 ± 0.01</td>
<td>3.16</td>
</tr>
<tr>
<td>prodrug 2</td>
<td>167.1 ± 16.7</td>
<td>0.18 ± 0.03</td>
<td>3.27</td>
</tr>
<tr>
<td>prodrug 3</td>
<td>180.3 ± 11.4</td>
<td>0.14 ± 0.05</td>
<td>3.47</td>
</tr>
</tbody>
</table>

$^a$Determined by DLS at a concentration of 0.5 mg/mL in PB at 25 °C. $^b$CACs determined by fluorescence microscopy using pyrene as a probe.

**Figure 3.** pH-dependent drug release from acetal-linked PTX prodrug nanoparticles at 37 °C. The initial prodrug nanoparticle concentration was 0.5 mg/mL.
similar PTX release profiles under otherwise the same conditions, in which slightly faster drug release was observed for PTX prodrug nanoparticles with higher PTX contents. It should be noted that the drug release rates from these acetal-linked PTX prodrug nanoparticles were much faster than those of ester or peptide-linked PTX prodrug micelles. As expected, no burst release was observed in all cases owing to covalent linking of PTX to PEG-PAA. The release of PTX from PTX prodrug nanoparticles at pH 5.0 resulted in marked increase of particle sizes from 160 to 1600 nm and decline of scattering intensities in 48 h (Figure 4). ¹H NMR studies showed that signals assignable to PTX moieties as well as acetal methine proton (δ 4.37) were diminishing following acidic treatment of PTX prodrug nanoparticles. In contrast, little size change was observed for PTX prodrug nanoparticles in PB (10 mM) containing 10% serum or in PBS (10 mM, 150 mM NaCl) at pH 7.4 and 37 °C over a period of 4 days, in accordance with slow release of PTX at physiological pH. These results demonstrate that acetal-linked PTX prodrug nanoparticles, while stable at physiological pH, effectively release PTX under a mildly acidic environment.

**In Vitro Anti-Tumor Activity of PTX Prodrug Micellar Nanoparticles to Drug-Sensitive and Resistant Cancer Cells.** The antitumor activity of PTX prodrug nanoparticles was investigated using MTT assays. Figure 5 showed that PTX prodrug nanoparticles induced pronounced antitumor effects to both KB and HeLa cancer cells. Notably, PTX prodrug nanoparticles displayed similar drug efficacy to free PTX in HeLa cells at a concentration of 0.1 μg PTX equiv/mL and lower (Figure 5A). At drug concentrations higher than 0.1 μg PTX equiv/mL, antitumor activity of PTX prodrug nanoparticles became somewhat lower than free PTX. The maximal half inhibitory concentrations (IC₅₀) were determined to be 0.9, 1.6, and 0.3 μg of PTX equiv/mL for prodrug 1 nanoparticles, prodrug 3 nanoparticles, and free PTX, respectively. KB cells were much more sensitive to PTX prodrug nanoparticles and free PTX than HeLa cells, in which low IC₅₀ values of 0.47, 0.18, and 0.03 μg PTX equiv/mL were obtained for prodrug 1 nanoparticles, prodrug 3 nanoparticles, and free PTX, respectively (Figure 5B). The higher antitumor activity of prodrug 3 nanoparticles as compared to prodrug 1 nanoparticles might be due to their slightly faster drug release at endosomal pH condition (Figure 3). It is remarkable that these acetal-linked PTX prodrug nanoparticles retain high antitumor efficacy as prodrugs, and prodrug nanoparticles usually exhibit one to two magnitude lower antitumor efficacy as compared to the parent prodrugs.
free drugs.\textsuperscript{5,25,46} For example, Yang et al. reported that poly(L-γ-glutamyl-glutamine)-docetaxel prodrug micelles exhibited approximately 22-fold higher IC\textsubscript{50} than free docetaxel against NCI-H460 cells.\textsuperscript{47}

In the following, we studied the antitumor activity of acetal-linked PTX prodrug nanoparticles to PTX-resistant A549 cells (A549/PTX\textsuperscript{R}). As expected, little cell death was observed for A549/PTX\textsuperscript{R} cells following 48 h incubation with free PTX, even at a high PTX dosage of 20 μg/mL (Figure 5C). The IC\textsubscript{50} of free PTX toward A549/PTX\textsuperscript{R} cells was determined to be 175.8 μg/mL, which was over 800 times higher than that observed for drug-sensitive A549 cells (IC\textsubscript{50} = 0.2 μg/mL), further confirming that A549/PTX\textsuperscript{R} cells are PTX-resistant (Figure 5C). By contrast, pH-sensitive PTX prodrug 3 nanoparticles caused significant death of A549/PTX\textsuperscript{R} cells under otherwise identical conditions, in which cell viability was reduced to ca. 50.3% at a dosage of 10 μg PTX equiv/mL (Figure 5C). The IC\textsubscript{50} was determined to be 10.9 μg PTX equiv/mL. It is remarkable that these acetal-linked PTX prodrug nanoparticles can effectively overcome multidrug resistance, which is probably due to effective cellular uptake via the mildly acidic endo/lysosomal compartments. In comparison, vinyl ether-functionalized PEG-PAA prepolymer was practically nontoxic to KB, HeLa, and MCF-7 cells (85–100% cell viabilities) up to a tested concentration of 1.0 mg/mL (Figure 5D). Notably, 32.5 μM acetaldehyde, which is the maximal possible concentration of acetal degradation product resulting from 1.0 mg/mL PTX prodrug 3 nanoparticles, was added during cell culture. These results indicate that vinyl ether-functionalized PEG-PAA has good biocompatibility and is suitable for drug delivery.

**Targetability and Antitumor Activity of Folate-Decorated PTX Prodrug Micelles.** The present pH-sensitive PTX prodrug micelles can be used as a versatile platform for efficient chemotherapy. For instance, tumor-targeting PTX prodrug nanoparticles could be developed to further enhance drug specificity and efficacy. To verify our concept, folate (FA)-decorated PTX prodrug nanoparticles were prepared by combining 80 wt % PTX prodrug 3 with 20 wt % FA-PEG-PLA. The PEG in FA-PEG-PLA was designed longer than that in PEG-PAA (6.0 versus 5.0 kg/mol), to facilitate exposure of FA ligands on nanoparticle surfaces for optimal targeting.\textsuperscript{39} The resulting nanoparticles had average sizes of ca. 180 nm with a PDI of 0.18. Here, folate receptor-overexpressing KB cells and MCF-7 cells (negative control) were incubated for 3 h with prodrug nanoparticles or free PTX at a dosage of 5 μg PTX/mL, media was removed and replenished with fresh culture media, and cells were further cultured for another 45 h. Notably, MTT assays demonstrated that antitumor activity of FA-decorated prodrug 3 nanoparticles was significantly more potent against KB cells than nontargeting PTX prodrug 3 nanoparticles (34.6% versus 61.8% cell viability) in folate free medium (Figure 6A). In fact, FA-decorated PTX prodrug 3 nanoparticles had antitumor efficacy close to free PTX in KB cells. The inhibition experiments showed that the presence of folic acids in culture media (+FA) resulted in largely reduced antitumor efficacy of FA-decorated PTX prodrug 3 nanoparticles to a comparable level to nontargeting prodrug 3 nanoparticles, indicating that FA-decorated PTX prodrug 3 nanoparticles delivered PTX into KB cells via a receptor-mediated mechanism. Moreover, both FA-decorated and nontargeting PTX prodrug 3 nanoparticles exhibited similarly low inhibition of MCF-7 cells (ca. 71.1% cell viability). The plot of cell viabilities against drug concentrations showed that FA-decorated PTX prodrug 3 nanoparticles had an IC\textsubscript{50} of 1.42 μg PTX equiv/mL (1.69 μM), which was about 12 times lower than that of nontargeting prodrug 3 nanoparticles (17.16 μg PTX equiv/mL, 20.5 μM) (Figure 6B). These results confirm that FA-decorated PTX prodrug 3 nanoparticles had apparent targetability and high antitumor activity to folate-overexpressing cancer cells.

**pH-Triggered Co-release of PTX and Physically Loaded DOX.** These acetal-linked PTX prodrug nanoparticles can also be used as a pH-sensitive nanocarrier for controlled release of other anticancer drugs, providing an elegant approach for combination cancer therapy. In this study, DOX was physically loaded into PTX prodrug 3 nanoparticles with loading contents of 2.3, 3.8, and 7.2 wt % at theoretical drug loading contents of 5, 10, and 20 wt %, respectively. The in vitro release results showed that both PTX and DOX were released in a pH dependent manner (Figure 7). For instance, 85.3, 63.4, and 24.7% of PTX were released from pH-sensitive polymersomes at pH 5.0, 6.0 and 7.4, respectively. We previously reported pH-triggered release of both PTX and DOX-HCl from pH-sensitive degradable polymersomes.\textsuperscript{36} Discher et al. reported that co-delivery of PTX and DOX by polymersomes resulted in synergistic treatment effect.\textsuperscript{85} These acetal-linked PTX prodrug nanoparticles present a versatile platform for pH-triggered co-release of PTX and various potent drugs.
CONCLUSIONS

We have demonstrated that acetal-linked PTX prodrug micellar nanoparticles based on PEG-PAA block copolymer possess excellent in vitro antitumor activity to drug-sensitive as well as drug-resistant cancer cells. Notably, these PTX prodrug nanoparticles have several unique features: (i) They contain remarkable drug contents (up to 42.8 wt % PTX), which are significantly higher than macromolecular prodrugs as well as micelles. (ii) They, though robust under physiological pH environments, show accelerated drug release under endosomal pH conditions. Importantly, because PTX is linked to PEG-PAA environments, show accelerated drug release under endosomal conditions, significantly higher than macromolecular prodrugs as well as micelles. (iii) They exhibit potent antitumor activity to multidrug-resistant (MDR) cells likely due to effective cellular uptake via the endocytosis pathway as well as fast intracellular release of PTX. (iv) Tumor-targeting PTX prodrug micellar nanoparticles can be formulated to achieve specific and efficient cancer chemotherapy. (v) They can also be used as a pH-sensitive nanocarrier for controlled release of other anticancer drugs to achieve synergistic treatment effect. (vi) They can readily be prepared with controlled structures and molecular weights from PEG-PAA block copolymer. These acetal-linked PTX prodrug micellar nanoparticles have appeared as a highly versatile and potent platform for targeted cancer chemotherapy.

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Notes
The authors declare no competing financial interest.

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Figure 7. pH-dependent release of PTX (A) and DOX (B) from DOX-loaded PTX prodrug 3 nanoparticles at 37 °C. DOX-loaded PTX prodrug 3 nanoparticle concentration was 0.5 mg/mL and DOX loading content was 3.8 wt %.


