Sequential combination therapy of ovarian cancer with degradable N-(2-hydroxypropyl)methacrylamide copolymer paclitaxel and gemcitabine conjugates

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For rapid and effective clinical translation, polymer-based anticancer therapeutics need long circulating conjugates that produce a sustained concentration gradient between the vasculature and solid tumor. To this end, we designed second-generation backbone-degradable diblock N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer carriers and evaluated sequential combination therapy of HPMA copolymer-paclitaxel and HPMA copolymer-gemcitabine conjugates against A2780 human ovarian carcinoma xenografts. First, extensive in vitro assessment of administration sequence impact on cell cycle, viability, apoptosis, migration, and invasion revealed that treatment with paclitaxel conjugate followed by gemcitabine conjugate was the most effective scheduling strategy. Second, in an in vivo comparison with first-generation (nondegradable, molecular weight below the renal threshold) conjugates and free drugs, the second-generation degradable high-molecular weight conjugates showed distinct advantages, such as favorable pharmacokinetics (three- to five-times half-life compared with the first generation), dramatically enhanced inhibition of tumor growth (complete tumor regression) by paclitaxel and gemcitabine conjugate combination, and absence of adverse effects. In addition, multimodality imaging studies of dual-labeled model conjugates confirmed the efficacy of second-generation conjugates by visualizing more than five-times enhanced tumor accumulation, rapid conjugate internalization, and effective intracellular release of payload. Taken together, the results indicate that the second-generation degradable HPMA copolymer carrier can provide an ideal platform for the delivery of a range of antitumor compounds, which makes it one of the most attractive candidates for potential clinical application.

In the past decades, numerous polymers have been developed as drug carriers, but so far only a few progressed to clinical evaluation, such as N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-drug conjugates (1–3). Results from clinical trials with first-generation HPMA conjugates (4–6) indicated a significant decrease of adverse effects compared with small-molecule drugs; however, the therapeutic efficacy did not match the data in preclinical animal studies. The most likely reason is that the molecular weight (Mw) of first-generation HPMA copolymer conjugates used in the trials was only 25 kDa, not large enough to ensure sufficient circulation time in the human body and sufficient extravasation of the conjugates at the tumor by enhanced permeability and retention (EPR) effect (7). Consequently, tumors were not exposed to effective drug concentrations. For prolonged plasma circulation and enhanced tumor accumulation, it is imperative to use polymeric carriers with increased Mw, which makes high-Mw biodegradable polymeric conjugates the most attractive candidates for future clinical applications (8, 9). Thus, we designed second-generation HPMA copolymer carriers that contain enzymatically degradable oligopeptide sequences in the linear main chain by combining reversible addition-fragmentation chain transfer (RAFT) polymerization and click reactions (10–12). In addition, we developed a new RAFT chain transfer agent (Peptide2CTA) composed of an enzymatically degradable oligopeptide sequence flanked by two dithiobenzoate groups. The monomer units incorporate in both positions at the same rate. Consequently, the process allows to synthesize well-defined degradable diblock copolymers with narrow polydispersity in one step (12), which is suitable for scale-up of the synthesis.

To date, ovarian cancer remains the deadliest gynecologic malignancy in the United States, with an estimated 15,500 deaths in 2012. The disease is usually diagnosed in advanced stages and the 5-y survival remains 44% for all stages and 27% for advanced stages (13). Because the majority of patients with this disease have advanced intraabdominal metastatic disease at diagnosis, chemotherapy has been considered as an essential treatment. Previous clinical trials have already demonstrated that combinations of two or more drugs were more effective in the treatment of ovarian cancer than just one drug alone (14, 15). Paclitaxel (PTX) and gemcitabine (GEM) possess distinct mechanisms of anticancer effect; they are among the most common antineoplastic agents and potent combination regimens in clinics. Therefore, we hypothesized that the combination of second-generation degradable diblock HPMA copolymer-PTX and HPMA copolymer-GEM conjugates would present an efficient way to treat solid tumors.
To prove this hypothesis, we synthesized diblock backbone-degradable HPMA copolymer-PTX and HPMA copolymer-GEM conjugates (2P-PTX, 2P-GEM), respectively, and evaluated their combination as therapeutics against A2780 human ovarian carcinoma xenografts. Combination of first-generation low-Mw HPMA copolymer conjugates (P-PTX and P-GEM) and combination of free drugs (PTX and GEM) served as controls. In addition, studies of dual-labeled (125I and 111In: FITC and Cy5) model conjugates were performed to evaluate the fate of second-generation conjugates at whole-body, tissue, and cellular levels. The new generation degradable carriers with high Mw have a great potential to improve therapeutic performance and narrow the gap between preclinical studies and clinical trials (1, 2).

## Results

### Synthesis and Characterization of HPMA Copolymer-Drug Conjugates

The general approaches used for synthesis of HPMA copolymer-drug conjugates are depicted in Fig. 1 and detailed in SI Materials and Methods, Fig. S1, and Table S1. The use of the Peptide2CTA RAFT chain transfer agent (12) permitted one-step synthesis of diblock degradable HPMA copolymer-drug (PTX and GEM) conjugates with narrow Mw distribution. As shown in Fig. 1B, the diblock conjugates can be eliminated via renal filtration following enzymatic degradation. To monitor the fate of conjugates, we synthesized two sets of dual-labeled model conjugates: (i) dual-isotope-labeled conjugates and (ii) dual-fluorophore-labeled conjugates. For dual-isotope-labeled conjugates, we incorporated into HPMA copolymer backbone a small amount of N-methacryloyltyrosinamide (MA-Tyr-NH2) comonomer (16) and radioiodinated it with 125I. To obtain information on in vivo drug delivery, we chose 111In-DTPA complex to mimic drug (Fig. 1C). We attached a bifunctional chelating agent p-SCN-Bn-DTPA to cleavable tetrapeptide GFLG side chains via ethylenediamine linker and labeled it with 111In (Fig. 1C and SI Materials and Methods). For dual-fluorophore-labeled conjugates to label the backbone, we used a small amount of comonomer-containing FITC (termed as MA-FITC) (17). To mimic the drug, we incorporated the thiostainoldisine-2-thione (TT) containing comonomer (MA-GFLG-TT) and conjugated imaging probe Cy5 by polymer analogous reaction of TT groups with Cy5 amine (Fig. 1D and SI Materials and Methods).

### In Vitro Cytotoxicity

The cytotoxicity of free drugs (PTX, GEM) and their HPMA copolymer conjugates (2P-PTX, 2P-GEM) against A2780 human ovarian cancer cells was determined. Representative cell-growth inhibition curves are shown in Fig. S2. Overall, free drugs (PTX, GEM) and their conjugates (2P-PTX, 2P-GEM) showed a dose-dependent cytotoxicity against A2780 cells. The IC50 values (Fig. 24) revealed that HPMA copolymer-drug conjugates exhibited less cytotoxicity than their corresponding free drugs, which is because of different mechanisms of cell entry: diffusion (free drugs) vs. endocytosis (conjugates). If IC50 is calculated based on intracellular concentrations of drugs, the cytotoxicity of HPMA copolymer-drug conjugates may be equivalent to or higher than that of free drugs (18).

### Cell Cycle Perturbation Following Different Exposure Schedules

In combination treatment, different administration sequences of drugs might lead to distinct interactions, ranging from antagonism to synergism (19–22). Because both PTX and GEM are cell cycle-specific drugs, we analyzed A2780 cell cycle changes after exposure to drug alone, conjugate alone, or different sequential combinations to investigate the schedule-dependence. Fig. 2B and Figs. S1, S2, and Table S1 show the percentage of cells in the different phases of cell cycle. Histograms of DNA content (Fig. S3) revealed that PTX or 2P-PTX alone could induce accumulation of cells in the G2/M phase with a concomitant decrease in G0/G1 compartment, which was in agreement with the known response of other tumor cells to PTX (20–22). Similarly, GEM alone caused a reduction in the G0/G1 phase, with cells arresting mainly in S phase and G2/M phases (20–22). 2P-GEM did not dramatically alter cell cycle distribution like GEM, probably because of the relatively low dose (20 nM GEM equivalent) of conjugate applied within this experimental setting. Interestingly, PTX and its conjugate induced formation of hyperploidy (4N) (PTX: 14.2%, 2P-PTX: 7.3%), which involves abnormal chromatid segregation, incomplete cell division, and aberrant exit from mitosis to a G1-like stage of 4N cells (termed “mitotic slippage”) (Fig. S3). Hyperploidy can be produced by prolonged exposure to microtubule inhibitor, like PTX. However, when GEM was given before or simultaneously with PTX, GEM arrested cells at G0/G1 phase and reduced PTX-induced hyperploidy (Fig. 2B) (21). Variant patterns of cell cycle distribution under different combination sequences revealed that the interaction between GEM and PTX is likely schedule-dependent.

### Combination Effect of PTX and GEM

To determine synergistic, additive, or antagonistic interactions between PTX and GEM, we analyzed their combinations with different exposure sequences using the Chou–Talalay method (Table S2) (23). This procedure allows characterization of drug interactions with a single number, the Combination Index (CI). CI values of 0.9–1.1 indicate additivity, and CI values >1.1 or <0.9 were interpreted as antagonism.

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**Fig. 1.** Synthesis of HPMA copolymer-drug conjugates and dual-labeled model conjugates. (A) Synthesis of first-generation conjugate (P-GEM and P-PTX) using 4-4-vinylpyridinium chloride to act as the RAFT chain transfer agent, modified with V65, and synthesis of backbone degradable diblock conjugates (2P-GEM and 2P-PTX) using peptide2CTA as RAFT chain transfer agent (II), MA-Tyr-NH2 was optionally used for 125I labeling for pharmacokinetics and biodistribution studies. (B) Following incubation of 2P-GEM with papain, the Mw decreased to half of the original one. (C) Dual-isotope-labeled model conjugates were synthesized in multiple steps. Chelator DTPA was covalently attached to polymer backbone via GFLG cleavable linker to mimic the drug. The polymer precursor was then labeled with 125I and 111In. (D) Dual-fluorophore-labeled model conjugates were synthesized by RAFT copolymerization of HPMA with MA-GFLG-TT and MA-FITC followed by polymer analogous reaction with Cy5 amine.
PTX followed by GEM appears to be necessary for maximal augmentation of anti-tumor activity against A2780 cancer cells.

**Pharmacokinetics and Imaging Studies of 125I-labeled Conjugates.** Before in vivo therapeutic evaluation, we compared pharmacokinetic profiles of first-generation low-Mw conjugates (P-PTX, P-GEM) and second-generation high-Mw diblock conjugates (2P-PTX, 2P-GEM). The blood radioactivity-time profiles of four conjugates in mice are illustrated in Fig. 3 and the pharmacokinetic parameters are summarized in Table S4. The previously reported half-lives of Cremophor EL-based PTX vehicle and GEM are cited here for comparison (24, 25). Both second-generation conjugates, 2P-PTX and 2P-GEM, showed improvement in pharmacokinetics. 2P-PTX showed a longer terminal half-life (37.90 h) than P-PTX (13.30 h), PTX (2 h), and Cremophor EL (17 h) (24). 2P-GEM (32.07 h) also had prolonged half-life compared with P-GEM (6.36 h) and GEM (1.2 h) (25). The total area under the blood concentration versus time curve (AUC) of 2P-PTX [1206% injected dose per milliliter (ID/mL)] was significantly higher than that of P-PTX (420% ID/mL) (P < 0.001). 2P-GEM (AUC = 1481% ID/mL) even had a 13-fold higher systemic exposure than P-GEM (108% ID/mL) (P < 0.001). The increased exposure resulted mainly from a significantly slower mean systemic clearance (CL) of second-generation conjugates (2P-PTX: 0.08 mL/h vs. P-PTX: 0.24 mL/h, 2P-GEM: 0.07 mL/h vs. P-GEM: 0.92 mL/h) (P < 0.001). In addition, the mean residence time of second-generation conjugates was also significantly longer than that of first-generation conjugates (2P-PTX: 52.86 h vs. P-PTX: 18.25 h; 2P-GEM: 45.39 h and synergism, respectively. The CI values of different sequential combinations are summarized in Table S3 and isobolograms were constructed for fraction affected (Fa, representing degree of growth inhibition) (Fig. 2C). Strong synergism was observed for the sequence PTX→GEM, with CI values ranging from 0.51 to 0.28 (Table S3). We also investigated these three schedules in conjugate combinations (i.e., 2P-PTX→2P-GEM, 2P-GEM→2P-PTX, and 2P-PTX+2P-GEM). The combinations showed synergistic effect (up to 70% Fa level) when 2P-PTX was given before or simultaneously with 2P-GEM, whereas posttreatment with 2P-PTX resulted in antagonism (Fig. 2C). Within the Fa levels from 75% to 90%, 2P-PTX→2P-GEM produced additive outcome, whereas antagonistic interaction occurred in the sequence 2P-PTX+2P-GEM. Both annexin V/7-AAD (7-aminoactinomycin D) apoptosis assay and cell migration/invasion assay supported the findings from CI experiments: (i) PTX→GEM (65.5%) induced more apoptosis than PTX+GEM (54.3%) (Fig. S4A) (P < 0.05), whereas there was no statistical difference between two synergistic sequences (PTX→GEM: 65.5% vs. GEM→PTX: 63.2%). In conjugate combination, both 2P-PTX→2P-GEM (65.6%) and 2P-PTX+2P-GEM (65.5%) showed significantly stronger cytotoxicity than antagonistic schedule 2P-GEM→2P-PTX (44.0%) (P < 0.05). (ii) Among all three administration sequences, PTX→GEM (30.9%) also displayed the strongest inhibition effect on A2780 cell migration (Fig. S4B) (GEM→PTX: 50.9%; PTX+GEM: 48.9%, P < 0.01). In addition, PTX→GEM (48.4%) was able to effectively inhibit cell invasion as well. Nevertheless, there was no statistically significant difference between PTX→GEM and the other two sequences (GEM→PTX, PTX+GEM). Overall, administration of and synergism

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<th>PTX</th>
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<td>10.4±1.6</td>
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![Graph](https://placek.it/100/50)
A2780 human ovarian carcinoma xenografts. The combination index data shown above indicate that exposure of cells to PTX first followed by GEM results in a synergistic effect. Consequently, the mice were intravenously injected with one dose of PTX or HPMCA copolymer-PTX conjugates (20 mg/kg PTX equivalent) on day 0, and three doses of GEM or HPMCA copolymer-GE conjugates (5 mg/kg GEM equivalent) on days 1, 7, and 14 (Fig. 3C). Tumor growth and ascites development were closely monitored during treatment (Fig. 3D). At day 21, complete tumor regression was achieved in two mice treated with the combination of second-generation conjugates (2P-PTX→2P-GEM), and relative tumor volume of the other three mice in this group decreased to 3% of initial size (Fig. 3D). Free-drug control (2P-GEM→2P-GEM) and first-generation conjugate (2P-PTX→2P-GEM) treatment resulted in tumor formation and proliferation in tumors after 2P-PTX→2P-GEM and other combination group (Fig. 3D and E) (P < 0.01). We also found that the mice treated with saline developed bloody ascites, whereas the mice treated with 2P-PTX and 2P-GEM had no detectable ascites. In addition, histological analysis showed that combination treatment of second-generation conjugates inhibited angiogenesis and proliferation of tumor cells and tumors, and tumor cells underwent apoptosis compared with saline treatment (Fig. S5). (i) Angiogenesis marker CD31, which indicates the presence of similar blood half-lives in mice (27.96 h vs. 30.68 h) (Table S5). We also compared biodistribution of payload 111In-DTPA and carrier 125I-Tyr-P (Fig. 4B). At 48 h and 96 h after intravenous injection of conjugate 125I-Tyr-P-DTPA-111In, 111In-DTPA showed similar uptake in major organs as 125I-Tyr-P. It revealed that the GFLG bond between payload and backbone remained stable in the bloodstream during transport, which is in agreement with results obtained with isolated plasma (26). Dual-isotope SPECT/CT images in Fig. 4D showed that both 111In-DTPA and 125I-Tyr-P accumulated at the tumors. At 48 h after injection, the tumor uptake of 111In-DTPA and 125I-Tyr-P reached 4.08% injected dose per gram tissue (ID/g) and 4.57% ID/g (Fig. 4C), respectively. Notably, the tumor uptake of the second generation is significantly higher than that of first-generation low-Mw model conjugate (111In: 0.77% ID/g, 125I: 0.53% ID/g) (Fig. 4C) (P < 0.001). At the cellular level, we took 3D stochastic optical reconstruction microscopy (3D-STORM) images of model conjugate FITC-P-Cy5 to elucidate conjugate internalization and drug release. The high-resolution 3D-STORM imaging allowed us to track single molecules with ~10-nm precision (below the diffraction limit) (27). As shown in Fig. 5, model conjugate FITC-P-Cy5 was internalized via endocytosis, and most FITC-P signal colocalized with lysosomes and late endosomes. At 4 h, the majority of FITC-P and Cy5 molecules were located at the outer space of the cytoplasm and FITC-P molecules were surrounded by clusters of Cy5 molecules. Over time, more Cy5 molecules were found inside the cell and located at a distance from FITC-P, indicating the release of Cy5 from polymer side chains. By 12 h, we found that Cy5 molecules diffused throughout the entirety of the cell. These images reveal that the GFLG bond can be cleaved in the lysosomes and subsequently the functional payload can translocate to the cytoplasm.

Discussion
As noted above, the second-generation conjugates completely surmounted the first generation in therapeutic efficacy (Fig. 3D). Such effective inhibition of tumor growth was attributed to several features: improved pharmacokinetic profile, enhanced bioavailability of polymer-bound drugs, and synergistic action of PTX and GEM in combination. (i) For improved pharmacokinetic profile, increased Mw of the conjugates resulted in enhanced drug exposure to tumor cells (Table S4, 2P-PTX: 2.9±SD < 0.01). We also compared biodistribution of payload 111In-DTPA and carrier 125I-Tyr-P in vivo after intravenous administration of second-generation conjugate 125I-Tyr-P-DTPA-111In. (A) Blood activity-time profiles of 111In-DTPA and 125I-Tyr-P in mice. The open circles represent the mean radioactivity expressed as a percentage of the injected dose per gram of blood from mice (n = 5). (B) Biodistribution of 111In-DTPA and 125I-Tyr-P in mice at 48 h and 96 h after injection of 125I-Tyr-P-DTPA-111In. (C) Tumor uptake of 111In-DTPA and 125I-Tyr-P in mice bearing subcutaneous A2780 tumor 48 h after injection of second-generation 125I-Tyr-P-DTPA-111In or first-generation model conjugates. Data obtained using the radioactivity count method plotted as percentage of injected dose per gram of tumor (n = 5). All of the data are expressed as mean ± SD (2-4×). (D) Dual-isotope SPECT/CT images of 111In-DTPA and 125I-Tyr-P in mice bearing subcutaneous A2780 tumor in the right flank 24 h and 48 h after injection of 125I-Tyr-P-DTPA-111In. K, kidney; L, liver; T, tumor.

vs. P-GEM: 8.49 h) (P < 0.001). Because of prolonged blood circulation time, the accumulation of 125I-labeled conjugates (2P-PTX, 2P-GEM) at tumors was readily visualized in single-photon emission computed tomography (SPECT)/CT images 24 h after intravenous administration and further increased at 48 h because of the EPR effect (Fig. 3B) (7). At day 7, there was still a relatively high signal of 125I-labeled conjugates remaining at the tumor compared with other tissues.

In Vivo Antitumor Activity. Therapeutic potential of second-generation conjugates was evaluated in female nude mice bearing A2780 human ovarian carcinoma xenografts. The combination index data shown above indicate that exposure of cells to PTX first followed by GEM results in a synergistic effect. Consequently, the mice were intravenously injected with one dose of PTX or HPMCA copolymer-PTX conjugates (20 mg/kg PTX equivalent) on day 0, and three doses of GEM or HPMCA copolymer-GE conjugates (5 mg/kg GEM equivalent) on days 1, 7, and 14 (Fig. 3C). Tumor growth and ascites development were closely monitored during treatment (Fig. 3D). At day 21, complete tumor regression was achieved in two mice treated with the combination of second-generation conjugates (2P-PTX→2P-GEM), and relative tumor volume of the other three mice in this group decreased to 3% of initial size (Fig. 3D). Free-drug control (2P-GEM→2P-GEM) and first-generation conjugate (2P-PTX→2P-GEM) treatment resulted in tumor formation and proliferation in tumors after 2P-PTX→2P-GEM and other combination group (Fig. 3D and E) (P < 0.01). We also found that the mice treated with saline developed bloody ascites, whereas the mice treated with 2P-PTX and 2P-GEM had no detectable ascites. In addition, histological analysis showed that combination treatment of second-generation conjugates inhibited angiogenesis and proliferation of tumor cells and tumors, and tumor cells underwent apoptosis compared with saline treatment (Fig. S5). (i) Angiogenesis marker CD31, which indicates the presence of endothelial cells in blood vessels, showed that in mice treated with second-generation conjugates there was a smaller number of blood vessels and shorter length of blood vessels lumen. (ii) K,67 proliferation marker indicated that there was much less cell proliferation in tumors after 2P-PTX→2P-GEM treatment. (iii) TUNEL staining showed that more tumor cells underwent apoptosis in second-generation conjugate-treated tumors than in saline-treated tumors. For safety evaluation, body weight of the mice was also recorded during treatment (Fig. S6). The body weights recovered quickly after treatment withdrawal. By day 28, the mice treated with second-generation conjugates gained an average of 4.0% body weight, whereas the saline-treated mice gained 19.4%. The body-weight difference between saline-treated and conjugates-treated groups may be attributed to the difference of tumor weight and ascites. Fig. S5 shows that histopathologic features of major organs in mice treated with 2P-PTX→2P-GEM were similar to those observed in the saline-treated mice. No abnormal features were identified, indicating a favorable toxicity profile of combination treatment with second-generation conjugates.

Imaging Studies of Dual-Labeled Model Conjugates. To gain a deeper insight into fate of second-generation conjugates in the body and cancer cells, we used dual-labeled model conjugates, including 125I-Tyr-P-DTPA-111In and FITC-P-Cy5, and investigated their behavior at cell and animal levels. The dual-labeling strategy allowed us to separately track the payload 111In-DTPA and polymeric carriers (125I-Tyr-P, FITC-P) at the same time. The blood (radio)activity-time profile of model conjugate 125I-Tyr-P-DTPA-111In is illustrated in Fig. 4A and pharmacokinetic parameters are summarized in Table S5. We found that payload 111In-DTPA and carrier 125I-Tyr-P possess similar blood half-lives in mice (27.96 h vs. 30.68 h) (Table S5).
sequential therapy with PTX followed by GEM was highly effective (32–34). Overall, all of the aforementioned features make this conjugate-mediated combination system superior to previously reported combination therapeutics against the same ovarian carcinoma (see SI Discussion for details).

This study used dual-labeled model conjugates to separately investigate behavior of carrier and payload. (i) To follow the in vivo fate of the conjugates, we tracked the dual-isotope–labeled model conjugate [125I-Tyr-P-DTPA]-111In. HPMA copolymer backbone was labeled with 125I, whereas 111In-DTPA complex was bound at the oligopeptide GFLG side-chain termini and served as the drug model. The results demonstrated that payload (111In) had similar blood half-life and tissue uptake as polymeric carrier (125I), indicating the integrity of GFLG bond in blood circulation during transport (Fig. 4). The tumor accumulation of second-generation conjugate was five–(111In) to eight–(125I) times more efficient than accumulation of first-generation conjugates (Fig. 4C). (ii) To follow the conjugates at the cellular level, we used a fluorescently dual-labeled conjugate (FITC-P-Cy5). FITC served as the backbone tag and Cy5 was bound to the GFLG spacer as the model drug. Three-dimensional STORM images of FITC-P-Cy5 visualized endocytosis of intact conjugates and diffusion of drug model from lysosomes into the cytoplasm (Fig. 5), suggesting the specific cleavage of cathepsin B-sensitive GFLG linker and intracellular release of model drug. The GFLG segment between two HPMA copolymer blocks can be cleaved as well, leading to the degradation of polymer backbone. The expression of cathepsin B is tightly regulated in normal physiological conditions (35), and its expression increases in malignant ovarian and other tumors, which contributes to the degradation of extracellular matrix in the process of tumor cell invasion (36, 37). High expression of cathepsin B in tumors can lead to a fast release of drugs from conjugates (SI Discussion). As shown in Fig. 5, the drugs diffused over the entire cell at 12 h. Drug molecules that are released intracellularly may avoid the membrane efflux pumps, such as P-glycoprotein, and overcome efflux-pump–mediated drug resistance, as we previously observed (38). The results obtained with the dual-labeled model conjugates further demonstrated that second-generation conjugates possess favorable pharmacokinetics, preferential tumor accumulation, and controlled drug release, which are the most important factors to therapeutic index.

In summary, we designed and synthesized a new generation of backbone degradable diblock HPMA copolymer-PTX and HPMA copolymer-GEM conjugates. The in vitro studies demonstrated that the drug combination was sequence-dependent and PTX followed by GEM had synergism. The in vivo studies showed that second-generation backbone-degradable conjugates possessed prolonged blood-circulation time, enhanced tumor accumulation, and improved antitumor efficacy compared with first-generation low-Mw conjugates and free drugs. The new second-generation conjugates were degradable in vivo and possessed no obvious systemic toxicity. Thus, we anticipate that second-generation long-circulating degradable conjugates can open up new opportunities to improve current cancer chemotherapy.

Materials and Methods
A full description of materials and methods is provided in SI Materials and Methods.

IC50 Study and CI Analysis. In single treatment, A2780 cells were incubated with free drugs (PTX, GEM) or their conjugates (2P-PTX, 2P-GEM) at a series of drug concentrations for 24 h to assess IC50 of individual drug or conjugate. In combination treatment, A2780 cells were exposed to different sequential combinations (Table S2). The cell viability was measured by CCK-8 assay. Synergism, additivity, or antagonism of the combination was determined by the Chou–Talalay method.

Cell Cycle Analysis. A2780 cells were treated as shown in Table S2. After treatment, cells were fixed and stained with propidium iodide. Cell cycle analysis was performed using flow cytometer and FlowJo software.
High-Resolution 3D-STEM Imaging. A2780 cells were incubated with FITC-P-Cys for 4, 8, and 12 h at 37 °C, and stained with Lysotracker Red DND-99. Then, the cells were fixed with 4% (w/v) paraformaldehyde and visualized under a Vutara SR-200 fluorescence microscope.

Pharmacokinetics and Biodegradation Study. In pharmacokinetic study, female nude mice were intravenously injected with 125I-Tyr-DTPA.111 In and 125I-labeled conjugates, respectively. At predetermined time intervals, blood samples were taken from the tail vein and the radioactivity of each sample was measured with a Gamma Counter. As described previously (39), 125I activity was counted in a channel of windows set for 15-85 keV and 111In activity was counted in a channel of 237–257 keV. The data were analyzed using a noncompartimental model with WinNonlin software. In a bio- distribution study, female nude mice bearing A2780 ovarian tumors. At 24 h and 48 h after ad- ministration, SPECT/CT images of mice were acquired by using an Inveon tri- modality PET/SPECT/CT scanner. The data of 125I image were histogrammed with a window setting of 15-85 keV, and the data of 111In image used a window setting of 149–194 keV.

In Vivo Antitumor Activity. The mice in the drug-treated groups received se- quential combination treatment as shown in Fig. 3C (n = 5), and the control mice received saline (n = 5). The tumor size was measured to monitor the tumor growth. The day that mice received PTX or its conjugates treatment was set as day 0 and the tumor volume at day 0 was normalized to 100%. All subsequent tumor volumes were expressed as the percentage relative to those at day 0. At the end of the experiment, the tumors were photographed and harvested for histological analysis.

Statistical Analysis. Data were presented as mean ± SD. Statistical analyses were done using a two-tailed unpaired Student t test, with P values of <0.01 indicating statistically significant differences.

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