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Backbone Degradable *N*-(2-Hydroxypropyl)methacrylamide Copolymer Conjugates with Gemcitabine and Paclitaxel: Impact of Molecular Weight on Activity toward Human Ovarian Carcinoma Xenografts

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Abstract

Degradable diblock and multiblock (tetrablock and hexablock) *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer–gemcitabine (GEM) and –paclitaxel (PTX) conjugates were synthesized by reversible addition–fragmentation chain-transfer (RAFT) copolymerization followed by click reaction for preclinical investigation. The aim was to validate the hypothesis that long-circulating conjugates are needed to generate a sustained concentration gradient between vasculature and a solid tumor and result in significant anticancer effect. To evaluate the impact of molecular weight of the conjugates on treatment efficacy, diblock, tetrablock, and hexablock GEM and PTX conjugates were administered intravenously to nude mice bearing A2780 human ovarian xenografts. For GEM conjugates, triple doses with dosage 5 mg/kg were given on days 0, 7, and 14 (q7dx3), whereas a single dose regime with 20 mg/kg was applied on day 0 for PTX conjugates treatment. The most effective conjugates for each monotherapy were the diblock ones, 2P–GEM and 2P–PTX (Mw $\approx$ 100 kDa). Increasing the Mw to 200 or 300 kDa resulted in decrease of activity most probably due to changes in the conformation of the macromolecule because of interaction of hydrophobic residues at side chain termini and formation of “unimer micelles”. In addition to monotherapy, a sequential combination treatment of diblock PTX conjugate followed by GEM conjugate (2P–PTX/2P–GEM) was also performed, which showed the best tumor growth inhibition due to synergistic effect: complete remission was achieved after the first treatment cycle. However, because of low dose applied,

Supporting Information

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tumor recurrence was observed 2 weeks after cease of treatment. To assess optimal route of administration, intraperitoneal (i.p.) application of 2P–GEM, 2P–PTX, and their combination was examined. The fact that the highest anticancer efficiency was achieved with diblock conjugates that can be synthesized in one scalable step bodes well for the translation into clinics.

Graphical Abstract

Keywords

N-(2-hydroxypropyl)methacrylamide (HPMA); macromolecular therapeutics; biodegradable copolymers; ovarian cancer; RAFT polymerization

1. INTRODUCTION

To address the lack of specificity of low-molecular weight drugs for malignant cells, the concept of polymer–drug conjugates was developed in the 1970s (early work reviewed in ref 1). When compared to low-molecular weight drugs, the advantages of polymer-bound drugs^{2–7} are (a) active uptake by fluid-phase pinocytosis (nontargeted polymer-bound drugs)^{8,9} or receptor-mediated endocytosis (targeted polymer-bound drugs),¹⁰ (b) increased active accumulation of the drug at the tumor site by targeting,^{11–16} (c) increased passive accumulation of the drug at solid tumor site by the enhanced permeability and retention (EPR) effect,^{17–23} (d) long-lasting circulation in the blood-stream,^{18,19,24} (e) decreased nonspecific toxicity of the conjugated drug,^{25,26} (f) potential to overcome multidrug resistance,²⁷ (g) decreased immunogenicity of the targeting moiety,²⁶ (h) immunoprotecting and immunomobilizing activities,²⁸ and (i) and modulation of the cell signaling and apoptotic pathways.^{9,29–33}

Various water-soluble polymers have been used as drug carriers such as dextran,³⁴ poly(glutamic acid),³⁵ poly(malic acid),³⁶ poly(ethylene glycol),³⁷ polyoxazoline,³⁸ and N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers.^{5,6,39}

HPMA copolymers are biocompatible, nonimmunogenic; their favorable properties have been demonstrated in many preclinical and clinical studies. Researchers studied the relationship between the detailed structure of HPMA copolymer–drug conjugates on one hand and their physicochemical and biological properties on the other hand. The impacts of architecture,^{25,40–42} structure of spacer between drug and carrier,^{3,25,43,44} solution properties (aggregation),^{45,46} combination therapy,^{47–49} targeting stem cells,⁵⁰ multivalency,^{12,51} and hyperthermia⁵² were evaluated.

HPMA copolymer–anticancer drug conjugates were tested in clinical trials.^{53–56} The early (1st generation) conjugates were nondegradable;³ consequently, their whole molecular weight distribution needed to be below renal threshold to ensure biocompatibility. The conjugates demonstrated reduced adverse effects, although only minor improvement was seen in therapeutic efficacy when compared to free drugs. We hypothesized that conjugates efficient in humans need to possess long circulating time in bloodstream to produce a sustained concentration gradient between the vasculature and solid tumor. To this end, we designed second generation backbone degradable multiblock HPMA copolymer carriers that are composed of synthetic polymer segments and degradable oligopeptide sequences in the main polymer backbone as well in side chains terminated in drug.^{57–59} We have synthesized a new bifunctional reversible addition–fragmentation chain transfer (RAFT) agent that contains an enzymatically degradable sequence and permits to synthesize degradable diblock copolymers in one, industrially scalable, step.⁵⁸ Further expansion of molecular weight can be achieved by click reactions, which produce multiblock copolymers.^{57–59} Both degradable diblock and multiblock HPMA copolymer–drug (doxorubicin, paclitaxel, gemcitabine, epirubicin, prostaglandin E1) conjugates have shown excellent efficacy in animal models of cancer and musculoskeletal diseases.^{19,20,22–24}

In this study, we focused on the determination of the optimal molecular weight of backbone degradable HPMA copolymer–paclitaxel (PTX) and HPMA copolymer–gemcitabine (GEM) conjugates. Using A2780 human ovarian carcinoma xenografts in nude mice, we evaluated and compared the pharmacokinetics and efficacy of treatment of diblock, tetrablock, and hexablock HPMA copolymer–PTX and –GEM conjugates following intravenous and intraperitoneal administration.

2. EXPERIMENTAL SECTION

2.1. Materials.

Common reagents were purchased from Sigma-Aldrich and used as received unless otherwise specified. 2,2'-Azobis(2,4-dimethylvaleronitrile) (V-65) and 2,2'-azobis-[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) were from Wako USA. 4,4-Azobis(4-cyanopentanoic acid) (V-501) was from Fisher Scientific (Pittsburgh, PA). *N*-Ethyl-*N'*-(3-(dimethylamino)propyl)-carbodiimide hydrochloride (EDC) and 4-(dimethylamino)pyridine (DMAP) were obtained from AAPTEC (Louisville, KY). Paclitaxel (PTX, > 99.5%) was purchased from LC Laboratories (Woburn, MA). Gemcitabine hydrochloride (99.0%) was purchased from NetQem LLC (Research Triangle Park, NC). Iodine-125 [¹²⁵I] was obtained from PerkinElmer. 1-Hydroxybenzotriazole (HOBt) and *N*-Boc-ethylenediamine were purchased from AnaSpec (Fremont, CA). RAFT agents, 4-cyanopentanoic acid dithiobenzoate⁶⁰ and peptide2CTA (*N,N'*-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenylalanylleucylglycyl)lysine),⁵⁸ were synthesized according to literature. *N*-(2-Hydroxypropyl)-methacrylamide (HPMA) was synthesized by acylating 1-aminopropan-2-ol with methacryloyl chloride in acetonitrile as previously described.⁶¹ Mp 69–71 °C; *N*-methacryloyltyrosinamide (MA-Tyr-NH₂)⁸ and 3-(*N*-methacryloylglycylphenylalanylleucylglycyl)thiazolidine-2-thione (MA-GFLG-TT)⁶²

were synthesized as previously described. *N*-methacryloylglycylphenylalanylleucylglycine (MA-GFLG-OH) was synthesized, and detailed procedure is in the Supporting Information.

2.2. Synthesis of Polymerizable Drug Derivatives.

2.2.1. N-Methacryloylglycylphenylalanylleucylglycyl Paclitaxel (MA-GFLG-PTX).—MA-GFLG-OH (4.9 g, 10.5 mmol), paclitaxel (6 g, 7 mmol), and a small amount of free-radical inhibitor *t*-octylpyrocatechine (~50 mg) were added into a 500 mL round-bottom flask, and then DMF (80 mL) was added. The solution was stirred at −10 °C for 10 min. EDC·HCl (3.1 g, 16.2 mmol) and DMAP (1.2 g, 9.8 mmol) were dissolved with 100 mL of DCM and then added dropwise into the round-bottom flask via an addition funnel. The reaction mixture was stirred at −5 °C for 2 h, then 4 °C for 24 h, and room temperature for 2 h. After reaction, the mixture was diluted with 600 mL of ethyl acetate. The solution was then washed consecutively with HCl (1 N, 100 mL × 3), DI water (100 mL × 2), NaHCO₃ (sat. 100 mL × 3), DI water (100 mL × 2), and NaCl (sat. 100 mL × 2). The organic phase was then dried over anhydrous Na₂SO₄.

After removal of Na₂SO₄ by filtration, the solution was concentrated by rotary-evaporator at less than 30 °C to about 30 mL. The product was precipitated into ether, washed with ether, and redissolved in ethyl acetate/DCM (1:1). The product was purified by silica gel chromatography (250 g). The column was eluted with ethyl acetate/DCM (1:1; 2 column volumes), ethyl acetate/DCM (3:1; 3 column volumes), then eluted with ethyl acetate. MA-GFLG-PTX was obtained after removal of the solvent by rotary-evaporator at less than 30 °C and further dried under vacuum at room temperature. Yield 6.8 g (74.6%). The product was confirmed by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS), and purity was verified using reversed-phase high-performance liquid chromatography (RP-HPLC).

2.2.2. N-Methacryloylglycylphenylalanylleucylglycyl Gemcitabine (MA-GFLG-GEM).—MA-GFLG-TT (15.0 g, 26.6 mmol), gemcitabine hydrochloride (7.0 g, 23.4 mmol), and small amount of free-radical inhibitor *t*-octylpyrocatechine (~70 mg) were added into a 500 mL round-bottom flask with a magnetic stir bar. After addition of 150 mL of pyridine, the flask was sealed with a rubber septum, then bubbled with nitrogen for 30 min before placing into 50 °C oil bath for reaction. After 20 h, the solvent was removed by rotary-evaporator at 40–50 °C. The residue was purified by silica gel chromatography (~200 g) with gradient elution: the column was first eluted with ethyl acetate (3 column volumes), then ethyl acetate/acetone (3:1; 3 column volumes), and finally eluted with ethyl acetate/acetone 1:3. MA-GFLG-GEM was obtained after removal of the solvent by rotary-evaporation below 30 °C and further dried under vacuum at room temperature. Yield 13.2 g (80.1%).

2.3. Synthesis of Diblock Degradable HPMA Copolymer Drug Conjugates.

Diblock degradable HPMA copolymer–drug conjugates were synthesized using RAFT polymerization strategy using peptide2CTA as RAFT chain transfer agent (Scheme 2). In this case, a degradable conjugate with molecular weight around 100 kDa can be obtained

in one step. Different polymerization conditions were explored (Tables 1 and 2). Typical examples for synthesis of 2P–GEM and 2P–PTX are described further.

2.3.1. Synthesis of Diblock Degradable HPMA Copolymer–Gemcitabine Conjugates (2P–GEM/2P–GEM–Tyr).—Polymerizable gemcitabine derivative MA–GFLG–GEM (66 mg, 0.093 mmol) and HPMA (134 mg, 0.94 mmol) were added into a 2 mL ampule with a stirring bar, followed by addition of 0.75 mL of degassed DMSO containing 0.2% (v/v) acetic acid. The ampule was bubbled with N₂ for 30 min, then 100 μ L of stock solution of peptide2CTA and 50 μ L of stock solution of initiator V-65 were added via syringe. An additional 100 μ L of acidified DMSO was added to reach final monomer concentration of 16.6% (wt). The ampule was sealed after another 5 min of N₂ bubbling and kept stirring at 50 °C oil bath for 24 h. The polymer was isolated by precipitation into acetone and purified by redissolving in methanol and precipitating into acetone two more times. The conjugate (2P–GEM) was obtained as a light pink powder with yield of 130 mg (65%).

The molecular weight and the polydispersity index (PDI) of 2P–GEM were determined using size-exclusion chromatography (SEC) on an ÄKTA FPLC system (GE Healthcare) equipped with Superose 6 HR10/300 column, UV (280 nm, GE Healthcare), miniDAWN TREOS, and OptilabEX detectors (Wyatt Technology, Santa Barbara, CA). Sodium acetate buffer containing 30% acetonitrile (pH 6.5) served as mobile phase and flow rate 0.4 mL/min. HPMA homopolymer fractions were used as molecular weight standards.

Gemcitabine content in the conjugate was estimated by UV spectrophotometry in methanol ($\epsilon_{300} = 5710 \text{ L mol}^{-1} \text{ cm}^{-1}$).⁵⁷

For in vivo evaluation, the conjugate was postpolymerization end-modified with excess of V-65 to remove dithiobenzoate end-groups as previously reported.¹⁹

To synthesize a radioactively labeled diblock conjugate 2P–GEM–Tyr, comonomer MA–Tyr–NH₂ (1% molar ratio in feed) was added into ampule, and the procedure shown above was used.

2.3.2. Synthesis of Diblock Degradable HPMA Copolymer–Paclitaxel Conjugates (2P–PTX/2P–PTX–Tyr).—The RAFT copolymerization of MA–GFLG–PTX with HPMA was accomplished in DMSO/deionized H₂O (3:1 v/v) using VA-044 as initiator. HPMA (156 mg, 1.09 mmol) and MA–GFLG–PTX (44 mg, 0.034 mmol) were added into an ampule containing a stirring bar. Degassed solvent was added into the ampule followed by addition of 100 μ L of stock solution of peptide2CTA and 50 μ L of stock solution of VA-044. The ampule was flushed with additional 100 μ L of solvent to reach final monomer concentration of 16.6% (wt). After bubbling with N₂ for 30 min, the ampule was sealed and the mixture kept stirring at 40 °C oil bath for 48 h. The copolymer was precipitated in acetone, isolated by centrifugation, and purified by dissolution–precipitation in methanol–acetone twice, then dried under vacuum at room temperature. The copolymer was obtained as a slightly pink powder with yield 162 mg (81%).

The molecular weight and the PDI of 2P-PTX were determined as described above. The drug content was estimated through enzymatic cleavage by incubation of 2P-PTX with papain in McIlvaine's buffer (50 mM citrate/0.1 M phosphate; 2 mM EDTA, pH 6.0) at 37 °C, and analyzed using RP-HPLC.

To synthesize a radioactively labeled diblock conjugate 2P-PTX-Tyr, comonomer MA-Tyr-NH₂ (1% molar ratio in feed) was added into ampule, and the procedure shown above was used.

2.3.3. Synthesis of First Generation (Nondegradable) HPMA Copolymer–Drug Conjugates.—Traditional HPMA copolymer–drug conjugates (P-GEM and P-PTX with Mw < 50 kDa) were synthesized by copolymerization of HPMA with MA-GFLG-GEM or MA-GFLG-PTX using 4-cyanopentanoic acid dithiobenzoate as RAFT agent and following above procedures.

2.4. Synthesis of Multiblock Degradable HPMA Copolymer–Drug Conjugates (mP-GEM/mP-PTX).

Multiblock degradable copolymers were prepared from diblock degradable copolymers in two steps (Scheme 3). The preparation of mP-PTX is described as an example. 2P-PTX (Mw 118.7 kDa Mn 98.9 kDa, PTX content 6.3%) was used.

2.4.1. Chain-End Modification.—The mixture of 2P-PTX (709 mg, 7.2 μmol) and dialkyne-V-501 (203 mg, 573 μmol) was added into a 25 mL round-bottom flask with a magnetic stirring bar inside. The flask was connected to Schlenk line and purged with N₂. Degassed DMSO (7 mL) was added into the flask via a syringe. After the dissolution of the reactants, the flask was put in a preheated 70 °C oil-bath for 2 h. The product was purified by precipitation into acetone twice, which resulted in α,ω -dialkyne telechelic HPMA copolymer–PTX conjugate.

2.4.2. Chain Extension.—Clickable copolymer conjugate 2P-PTX (621 mg, 6.4 μmol) and 11.8 mg (9.6 μmol, 1.5×) of chain-extender Peptide2N₃ (a cleavable peptide sequence flanked at both termini with azido group) were added into a 25 mL round-bottom flask with a magnetic stirring bar inside. The flask was capped with a rubber septum and purged with N₂ for 30 min.

Degassed DMF containing L-ascorbic acid was added into the flask via a syringe. After clear solution formed, the click reaction was initiated by adding 250 μL (4.6 mg, 5 equiv) of CuBr stock solution (suspension) in DMF. The reaction was carried out at room temperature overnight. The mixture was then centrifuged to remove any precipitated copper compound. The supernatant was precipitated into large excess (>20×) of cold acetone/ether (1:1 v/v). The resulting product was purified once more by dissolving in methanol and precipitating into acetone.

Following chain extension, the product was fractionated on XK50 column (GE). Sodium acetate buffer containing 30% acetonitrile (pH 6.5) served as mobile phase at a flow rate 2.5 mL/min. Different fractions were collected and confirmed on Superose 6 HR10/300

column. The fractions consisting of hexablock- (mP-GEM₃₀₀; mP-PTX₃₀₀) and tetrablock- (mP-GEM₂₀₀; mP-PTX₂₀₀) conjugates were concentrated and washed with DI water using ultrafiltration under nitrogen (Amicon). The desalted fractions were lyophilized. The molecular weight, PDI, and drug content of each fraction were redetermined. The results are summarized in Tables 4 and 5.

2.5. Radiolabeling.

¹²⁵I labeling of polymer pendant tyrosinamide moieties were conducted immediately before use. HPMa copolymer–drug conjugates (2P-PTX, 2P-GEM, mP-PTX₂₀₀, mP-GEM₂₀₀, mP-PTX₃₀₀, mP-GEM₃₀₀), containing tyrosinamide in the side chains, were reacted with Na¹²⁵I (PerkinElmer) at room temperature in iodination tube and purified with Sephadex PD-10 columns (GE Healthcare). The specific activity of the hot samples was in the range of 40–80 μ Ci/mg.

2.6. Tumor Model.

All animal studies were carried out in accordance with the University of Utah IACUC guidelines under approved protocols. A2780 human ovarian cancer cells (5×10^6) in 100 μ L of phosphate buffered saline were subcutaneously inoculated in right flank of 6–8-week-old syngeneic female nude mice (22–25 g, Charles River Laboratories).

2.7. Pharmacokinetics.

The 6–8-week-old healthy female nude mice (22–25 g; Charles River Laboratories) ($n = 5$) were intravenously injected with ¹²⁵I-labeled conjugates mP-PTX₂₀₀, 2P-GEM, mP-GEM₂₀₀, or mP-GEM₃₀₀ (1 mg, 20 μ Ci/mouse), respectively. At predetermined intervals, blood samples (10 μ L) were taken from the tail vein, and the radioactivity of each sample was measured with Gamma Counter (Packard). The blood pharmacokinetic parameters for the radiotracer were analyzed using a two-component model with WinNonlin 5.0.1 software (Pharsight).

To investigate the impact of administration route on in vivo fates, the mice were also intraperitoneally injected with ¹²⁵I-labeled diblock degradable conjugate 2P-PTX (1 mg, 20 μ Ci/mouse). Blood samples (10 μ L) were taken from the tail vein, and the radioactivity of each sample was measured as above-described.

2.8. In Vivo Antitumor Activity.

The antitumor efficacy of monochemotherapy, and sequential combination chemotherapies, was evaluated in female nude mice bearing subcutaneous A2780 ovarian tumors. Three weeks after inoculation, when tumors reached approximately 100–200 mm³, mice were randomly assigned to 10 intravenously administered groups and three intraperitoneally administered groups. The intravenous administration treatment groups included saline (control), PTX (formulated with Cremophor EL/ethanol 1:1 v/v), P-PTX, 2P-PTX, mP-PTX₂₀₀, free GEM, P-GEM, 2P-GEM, mP-GEM₂₀₀, mP-GEM₃₀₀, and combination treatment (2P-PTX/2P-GEM). In PTX monotherapy, the mice received one dose of PTX or HPMa copolymer–PTX conjugates (20 mg/kg PTX equivalent) through intravenous injection on day 0 ($n = 5$). In GEM monotherapy, the mice received three doses of GEM

or HPMA copolymer–GEM conjugates (5 mg/kg GEM equivalent) through intravenous injection on days 0, 7, and 14 ($n = 5$). In combination therapy, the mice received one dose of diblock degradable conjugate 2P–PTX (20 mg/kg PTX equivalent) on day 0, and three doses of 2P–GEM (5 mg/kg GEM equivalent) on days 1, 8, and 15 through intravenous injection ($n = 5$). In parallel, the mice were also intraperitoneally injected with 2P–PTX, 2P–GEM, and combination 2P–PTX/2P–GEM, respectively, at the aforementioned doses. The day that mice received the first treatment was set as day 0. The tumor size was measured to monitor the tumor growth. The tumor volume at day 0 was normalized to 100%. All subsequent tumor volumes and body weight were then expressed as the percentage relative to those at day 0.

3. RESULTS

3.1. Synthesis and Characterization of HPMA Copolymer–Drug Conjugates.

The design of backbone degradable HPMA copolymer drug carrier is based on the state-of-the-art approaches, a combination of controlled/living radical polymerization with click reactions. Use of the RAFT agent Peptide2CTA, which contains an enzymatically degradable oligopeptide flanked by two dithiobenzoate groups, permits the synthesis of degradable diblock copolymers in one step.^{19,24,58} In this work, we focus on optimization of polymerization conditions and selection of lead conjugates for further clinical translation development.

In general, there are two approaches to incorporate chemotherapeutic agents onto polymeric carrier backbone: one-pot copolymerization or two-steps preparation of polymer precursor followed by polymer-analogous reaction. In our project, we selected the copolymerization strategy to minimize reaction steps and to avoid tiresome purification of the conjugate from free drug after polymer-analogous reaction. To this end, two polymerizable drug derivatives, MA–GFLG–PTX and MA–GFLG–GEM, were synthesized (Scheme 1).

It is advantageous to use RAFT polymerization to design and predict (co)polymer molecular weight. The theoretical molecular weight of a copolymer synthesized by RAFT polymerization can be expressed as

$$M_p = M_m \frac{[\text{Monomer}]_0}{[\text{CTA}]_0} p + M_{\text{CTA}}$$

Here, M_p is target molecular weight of a copolymer, M_m is the average molecular weight of all monomers, $[\text{Monomer}]_0$ is the initial concentration of total monomers, $[\text{CTA}]_0$ is the initial concentration of a RAFT agent, p is polymerization conversion, and M_{CTA} is the molecular weight of the RAFT agent.

Because of different solubility and stability of MA–GFLG–PTX and MA–GFLG–GEM, a series of polymerizations was conducted (Scheme 2). Tables 1 and 2 summarize the exploration of HPMA copolymerization with MA–GFLG–PTX and MA–GFLG–GEM, respectively.

Further synthesis of 2P-PTX was conducted under modified conditions: VA-044 as initiator, DMSO/H₂O 3:1 (v/v) as solvent, 40 °C for 48 h with feed molar ratio of PTX 3%, and monomer concentration 16.6% (wt); whereas for synthesis of 2P-GEM, the polymerization conditions were V-65 as initiator, acidified DMSO as solvent, 50 °C for 24 h with feed molar ratio of GEM 9–10%, and overall monomer concentration 16.6% (wt). Accordingly, polymer–drug conjugates at different scales were synthesized. As an example, Table 3 shows results from copolymerization of HPMA with MA-GFLG-PTX in the scale range from 200 mg to 5 g. Figure 1 shows the SEC profiles of PTX conjugates and GEM conjugates with different scales.

Multiblock backbone degradable HPMA copolymer–drug conjugates with higher Mw (mP-PTX/mP-GEM) were synthesized from diblock degradable polymer conjugates (**I** in Scheme 2) in two steps: first, the conjugate **I** was postpolymerization end-modified with dialkyne-V-501 to produce a telechelic dialkyne conjugate (**II**); in the second step, the conjugate was chain extended by click reaction with Peptide2N₃ (a cleavable peptide sequence flanked at both termini with azido group) in DMF in the presence of Cu (I) (Scheme 3). Tables 4 and 5 list conjugates prepared for pharmacokinetics study and for in vivo evaluation of tumor growth inhibition, respectively.

3.2. Antitumor Activity.

The treatment efficacy of HPMA copolymer–drug conjugates was evaluated in female nude mice bearing subcutaneous A2780 ovarian tumors. Both monotherapy of GEM/GEM conjugates or PTX/PTX conjugates and their combination treatment were performed. The applied dose and administration schedule followed our previous studies^{19,20} in which paclitaxel was given a single dose of 20 mg/kg on day 0, the day when mice received the first treatment, whereas gemcitabine was given with triple doses of 5 mg/kg on days 0, 7, and 14. For combination therapy, paclitaxel was given on day 0, and gemcitabine was given on days 1, 8, and 15. Tumor growth was frequently monitored (Figure 2).

Treatment with HPMA copolymer–GEM conjugates revealed a clear advantage of macromolecular therapeutics, as all conjugates were considerably more active when compared with free GEM (Figure 2A). Because of the low dose and fast metabolism, free GEM showed similar activity to saline. The first-generation conjugate P-GEM possessed the lowest activity among all polymer conjugates indicating the importance of the molecular weight of the carrier on activity. 2P-GEM inhibited tumor growth more than both multiblock conjugates mP-GEM₂₀₀ and mP-GEM₃₀₀. The highest tumor inhibitory activity was demonstrated in the combination treatment 2P-PTX/2P-GEM.

The blood radioactivity–time profiles of HPMA copolymer–GEM conjugates evidently demonstrate long retention of high Mw conjugates in the bloodstream compared with P-GEM, the first generation conjugate with low Mw below the renal threshold (Figure 3A). Pharmacokinetic parameters indicate prolonged blood circulation time of second-generation conjugates (Table 6). For example, the terminal half-life of 2P-GEM (34.53 ± 4.23 h) was considerably better than that of P-GEM (6.36 ± 0.66 h). On the basis of AUC (area under the blood vs time curve) data, the exposure of the organism to all backbone degradable conjugates was about 10-times longer when compared to P-GEM.

However, there is no significant Mw dependency for blood clearance of second-generation GEM conjugates when Mw changed in the range from 120 to 330 kDa (Table 6).

In PTX monotherapy, as expected, the diblock degradable conjugate 2P-PTX demonstrated superiority of tumor growth inhibition over free drug PTX and the first-generation conjugate P-PTX, but it was unable to provide complete tumor regression probably due to the single low dose treatment (Figure 2B). Because of poor water solubility of free paclitaxel (<0.03 mg/mL), it was formulated with Cremophor EL (CrEL)/ethanol (1:1 v/v) (30 mg PTX/5 mL vehicle) for administration. It has been reported that CrEL, a heterogeneous nonionic surfactant, provided therapeutic advantage by reducing paclitaxel elimination despite severe toxicity. This alteration of pharmacokinetics may contribute to the tumor inhibitory effect that is comparable with P-PTX, the first generation conjugate. It is worth noting that, unlike the GEM conjugate (mP-GEM₂₀₀), the activity of mP-PTX₂₀₀ possessed antitumor activity as low as free PTX and P-PTX. Further pharmacokinetics study demonstrated the fast clearance of mP-PTX₂₀₀ from blood circulation (Figure 3B). This might be the result of association of hydrophobic PTX moieties at side-chain termini of HEMA copolymer conjugate resulting in formation of “unimer micelles”. Conjugates with higher molecular weight result in a higher amount of PTX per macromolecule and enhance intramolecular association.

To assess the optimal route of administration for ovarian cancer treatment, we compared intravenous and intraperitoneal administrations of degradable diblock conjugates 2P-GEM and 2P-PTX, respectively, and their sequential combination using the dosing scheme as above, that is, single 20 mg/kg PTX equivalent on day 0 and triple doses of 5 mg/kg GEM equivalent on days 1, 8, and 15. The tumor inhibition effect is shown in Figure 4. The blood radioactivity–time profile of 2P-PTX administered via i.p. was determined. It was found that the area under the blood radioactivity curve is only 54% compared with that from i.v. (Figure 3C), which indicated low bioavailability of 2P-PTX via i.p. administration. T_{max} is ~9 h, which suggests the conjugate that injected into the peritoneum very slowly entered blood circulation. In clinics, patients with ovarian cancer have high trend of peritoneal metastases and ascites formation; therefore, i.p. injection has advantages due to high local concentration. However, in this study, the animal model was a s.c. tumor; thus, the i.p. administered drug (or conjugate) was less active before it reached tumor tissue followed by uptake.

Body weights of nude mice following treatment with degradable diblock conjugates (Figure 4B) indicate biocompatibility of the conjugates. Following administration of the conjugates or of the combination therapy, a slight decrease of weight in the first week was observed. However, all mice reversed the trend and started to gain weight. Histopathology evaluation of major organs in mice following combination therapy 2P-PTX/2P-GEM did not reveal any abnormal features, which indicated a promising toxicity profile.¹⁹

3.3. Statistical Evaluation.

The statistical significance of data in Figures 2 and 4 was evaluated by comparing results at the time when tumor reached the 10-times the baseline size. The logrank test was used to compare the time to reach 10-times the initial size as implemented in the “survdiff” function

in the “survival” R package (<http://www.R-project.org>). Tumors that did not reach the 10-times baseline size were censored at the time of last observation. For i.v. administration, comparison of 2P–GEM with any other groups (except the combination 2P–GEM + 2P–PTX) resulted in statistical significance well below 0.05. For 2P–PTX versus P–PTX, $p = 0.011$; for 2P–PTX versus mP–PTX₂₀₀, $p = 0.125$. The combination treatment was the most effective one; the statistical significance of 2P–PTX/2P–GEM versus 2P–PTX, mP–PTX₂₀₀, P–PTX, and PTX was 0.051, 0.016, 0.002, and 0.003, respectively. Comparison of 2P–PTX/2P–GEM versus 2P–GEM, mP–GEM₂₀₀, mP–GEM₃₀₀, P–GEM, and GEM was 0.170, 0.003, 0.007, 0.016, and 0.003, respectively.

Comparison of i.v. versus i.p. administration revealed that only 2P–PTX (i.v.) versus 2P–PTX (i.p.) showed $p < 0.05$ (0.018), whereas comparison of 2P–GEM (i.v.) versus 2P–GEM (i.p.) and 2P–PTX/2P–GEM (i.v.) versus 2P–PTX/2P–GEM (i.p.) did not produce statistically significant differences.

4. DISCUSSION

The architecture of water-soluble polymeric drug carriers is an important factor that defines their biological properties.⁶³ Linear, branched, and star-like macromolecules have been used as drug attachment/release entities. The fate of these constructs in the organism depends on the presence of degradable bonds in their structure. The first generation HPMA copolymer–drug conjugates have a nondegradable backbone. To insert degradability, new synthetic approaches have been used. Linear backbone degradable HPMA copolymers have been synthesized by RAFT polymerization combined with chain extension via click reactions.^{57–59,64} Such high molecular weight polymer–drug conjugates have a long intravascular half-life thus providing a sufficient time period for the extravasation of the conjugates into solid tumors due to the EPR effect.^{17–24,65} It has been shown that the higher the molecular weight of the polymer–drug conjugate, the higher the tumor accumulation and efficacy in an animal model of ovarian carcinoma.¹⁸ However, other factors, such as the conformation of the macromolecule,^{45–47,65} association of side-chains by hydrophobic interactions (formation of unimolecular micelles), or random association of flexible chains by “point–point” contacts^{66–68} influence the rate of enzymatic drug release, penetration of the solid tumor,^{18,65} and, ultimately, the efficacy.

The evaluation of backbone degradable HPMA copolymer–gemcitabine/paclitaxel conjugates validated the above arguments. The fact that biodegradable diblock conjugates (2P–GEM and 2P–PTX) were more efficient than biodegradable multiblock conjugates reflects most probably the complex solution properties of hydrophilic polymers that contain hydrophobic substituents (drugs) at side chain termini.^{45,46} Hydrophobic interactions result in conformation changes of the macromolecule into compact coils as determined previously by light scattering,⁴⁵ fluorescence resonance energy transfer (FRET),⁴⁶ and quantum yield of singlet oxygen formation⁶⁹ techniques. These effects result in decreased solubility in aqueous environment, decrease of rate of enzymatic drug release, or decrease in quantum yield of singlet oxygen formation (when photosensitizers are used). Another possible factor is the size of the multiblock conjugate. As shown by Noguchi et al., polymers with mol. weight >50 kDa showed significantly higher tumor accumulation than those below the renal

threshold (<40 kDa).⁷⁰ However, a too large size might prevent diffusion into the tumor mass and restrict the conjugate to the tumor periphery. Similar results on decreased efficacy of very high molecular weight conjugates were observed in our previous study²² where the HPMA copolymer–doxorubicin conjugate with molecular wt. of 349 kDa was less efficient in the treatment of human ovarian A2780/AD resistant tumors than conjugates of 94 and 185 kDa molecular weight. Thus, it appears that an optimal molecular weight of a polymer–drug conjugate needs to be determined. It bodes well for the translation of this research into clinical use that the optimal molecular weight is that of biodegradable diblock copolymer conjugates that can be prepared by a scalable one step polymerization.⁷¹

The combination of 2P–PTX/2P–GEM provided the highest tumor growth inhibition efficacy as a result of synergism of this combination.^{19,49} Other combination systems using HPMA copolymer carriers have also shown enhanced activity when compared to monotherapy.^{47,48,50,72}

5. CONCLUSIONS

Gemcitabine and paclitaxel are both active as single chemotherapeutics for treatment of recurrent or metastatic breast cancer, lung cancer, and other tumors. Their combination showed great potential for synergistic antitumor activity. However, the barriers for clinical application are the instability of GEM and poor solubility of PTX. For example, following intravenous administration, GEM is quickly converted to an inactive uracil metabolite (2'-deoxy-2',2'-difluorouridine).⁷³ Conjugation of these drugs to newly developed long-circulating degradable HPMA copolymers can prevent GEM metabolism, enhance solubility of PTX, and dramatically improve their pharmacokinetics and bioavailability, and ultimately provide a novel avenue for development of highly effective and safe anticancer entities. The robust and versatile RAFT polymerization technology allows for more precise control of molecular weight and molecular weight distribution, which is critical for later clinical translation. This study investigated extensively polymerization conditions and the relationship of polymer structure with the antitumor activity. All these efforts will contribute to promising future of these polymer–drug conjugates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SEC profiles of synthetic diblock degradable HPMA copolymer–drug conjugates using RAFT polymerization strategy. (A) PTX conjugates with scales from 200 mg to 5 g (Table 1); (B) GEM conjugates at variable scales: G2–200 mg; G7–600 mg; G25–1.2 g.

Figure 2.

In vivo efficacy of HPMA copolymer–drug conjugates against A2780 human ovarian tumor xenografts. Tumor cells (5×10^6 /mouse) were inoculated subcutaneously on the back, and administration started when the tumor size reached $\sim 100\text{--}200 \text{ mm}^3$ ($n = 5$). As a comparison, sequential combination therapy of 2P–PTX followed by 2P–GEM was also plotted in both cases. (A) Tumor growth was inhibited by gemcitabine monotherapy. Intravenous injection three times with a 7-day interval. (B) Tumor growth was inhibited by paclitaxel monotherapy with a single intravenous administration on day. The dose of conjugate for each injection is expressed as a dose equivalent to free drug gemcitabine (5 mg/kg) or paclitaxel (20 mg/kg).

Figure 3.

Blood activity–time profiles of ^{125}I -labeled HPMA copolymer–drug conjugates in mice.

The experimental points represent the mean radioactivity as a percentage of the injected dose per gram of blood (%ID/g) from mice ($n = 5$). (A) Intravenously administered HPMA copolymer–GEM conjugates with different Mw. (B) Intravenously administered HPMA copolymer–PTX conjugates with different Mw. (C) Impact of different administration routes (i.v. vs i.p.) on blood activity–time profiles of ^{125}I -labeled 2P–PTX.

Figure 4.

(A) In vivo efficacy of degradable diblock copolymer-GEM conjugates and PTX conjugates against A2780 human ovarian tumor xenografts. Tumor cells (5×10^6 /mouse) were inoculated subcutaneously on the back of nude mice, and administration started when the tumor size reached ~ 100 – 200 mm^3 ($n = 5$). Tumor growth was inhibited by either monotherapy or combination therapy. Intravenous and intraperitoneal injections were conducted, respectively. The doses of conjugates (gemcitabine $5 \text{ mg/kg} \times 3$ on days 0, 7, 14 and paclitaxel 20 mg/kg on day 0) are in drug equivalent. (B) Body-weight change.

Scheme 1.
Synthesis of Polymerizable Drug Derivatives MA-GFLG-PTX and MA-GFLG-GEM

Scheme 2.
One-Step Synthesis of Degradable Diblock HPMA Copolymer–Drug Conjugates

Scheme 3.

Synthesis of Multiblock Degradable HPMA Copolymer-Drug Conjugates

Table 1.Copolymerization of HPMA with MA-GFLG-PTX^{a,b,c}

	[CTA]/[I]	initiator	solvent	temp (°C)	time (h)	M _w (kDa)	PDI	yield (%)	drug% (wt)
P-1	2.5	V65	DMSO/H ⁺	50	24	51.8	1.10	35	7.2
P-2	1.25	V65	DMSO/H ⁺	50	24	66.3	1.17	39	6.6
P-3	1.25	VA044	DMSO/H ₂ O 3:1 (v/v)	40	48	105	1.12	81	6.6

^a 200 mg scale: 156 mg HPMA/44 mg MA-GFLG-PTX; PTX 3% molar ratio in feed.^b Monomer concentration 16.6% (wt).^c [M]/[CTA] = 1106.

Table 2.Copolymerization of HPMA with MA-GFLG-GEM^{a,b,c}

	[CTA]/[I]	initiator	solvent	temp (°C)	time (h)	Mw (kDa)	PDI	yield (%)	drug% (wt)
G-1	2.5	V65	DMSO/H ⁺	50	24	73.6	1.22	43	10.9
G-2	1.25	V65	DMSO/H ⁺	50	24	97.6	1.20	65	11.4
G-3	2.5	VA044	DMSO/H ₂ O 1:1 (v/v)	70	2	81.2	1.25	54	12.2
G-4	1.25	VA044	DMSO/H ₂ O 3:1 (v/v)	40	48	148.3	1.27	76	12.6
G-5	1.25	VA044	DMSO/H ₂ O 3:1 (v/v)	40	24	61.5	1.15	40	12.3

^a 200 mg scale, 134 mg HPMA/66 mg MA-GFLG-GEM; GEM 9.1% molar ratio in feed.^b Monomer concentration 16.6% (wt).^c [M]/[CTA] = 1030.

Table 3.Copolymerization of HPMA with MA-GFLG-PTX at Different Scales^{a,b,c}

no.	scale (mg)	feed ratio (mg)				[CTA]/[I]	M _w (kDa)	PDI	yield (%)	drug% (wt)
		HPMA	MA-GFLG-PTX	MA-Tyr						
P-3	200	156	44			1.25	105	1.12	81	6.6
P-8	1200	857	327	16		1.5	90.9	1.09	63	6.1
P-16	1180	937.2	244.5			1.25	118.7	1.20	68.5	6.3
P-37	5000	4220	780			1.25	109.8	1.27	78	7.9

^a DMSO/H₂O 3:1 (v/v) as solvent, monomer concentration 16.6% (wt).^b VA-044 as initiator, 40 °C for 48 h.^c [M]/[CTA] = 1040.

Table 4.

Multiblock Degradable Conjugates for PK Studies

	conjugate	Mw (kDa)	PDI	drug (%)
GEM	mP-GEM ₃₀₀	330	1.18	9.2
	mP-GEM ₂₀₀	227	1.25	7.6
	2P-GEM	127	1.21	10.1
	P-GEM ^a	32	1.05	8.2
PTX	mP-PTX ₂₀₀	240	1.18	6.5
	2P-PTX	119	1.20	6.3
	P-PTX ^a	48	1.05	7.3

^aFrom ref 19.

Table 5.

HPMA Copolymer–GEM/PTX Conjugates for Evaluation of Treatment Efficacy

	conjugate	Mw (kDa)	PDI	drug (%)
GEM	mP–GEM ₃₀₀	310	1.16	7.0
	mP–GEM ₂₀₀	218	1.12	7.4
	2P–GEM	121	1.24	8.8
	P–GEM	48	1.20	8.0
PTX	mP–PTX ₂₀₀	213	1.32	7.0
	2P–PTX	92	1.15	7.5
	P–PTX	29	1.06	7.6

Table 6.Pharmacokinetic Parameters for ^{125}I -Labeled Conjugates in Mice

	P-GEM ^a	2P-GEM	mP-GEM ₂₀₀	mP-GEM ₃₀₀
$T_{1/2,\alpha}$ (h)	0.26 ± 0.02	1.09 ± 0.28	1.38 ± 0.40	1.74 ± 0.58
$T_{1/2,\beta}$ (h)	6.36 ± 0.66	34.53 ± 4.23	39.69 ± 4.00	33.94 ± 3.39
AUC (%ID h/mL blood)	108.7 ± 6.7	1189.0 ± 113.3	1290.2 ± 113.3	1251.3 ± 86.8
CL (mL/h)	0.92 ± 0.06	0.08 ± 0.008	0.07 ± 0.005	0.07 ± 0.005
MRT (h)	8.49 ± 0.88	48.74 ± 5.93	56.25 ± 5.58	47.93 ± 4.60
V_{ss} (mL)	7.82 ± 0.38	4.09 ± 0.18	4.36 ± 0.18	3.83 ± 0.17

^aFrom ref 19.