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Prostate-Cancer-Targeted *N*-(2-Hydroxypropyl)methacrylamide Copolymer/Docetaxel Conjugates

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Abstract

Biodistribution, pharmacokinetics, and efficacy of prostate-cancer-targeted HPMAC copolymer/DTX conjugates are evaluated in nude mice bearing prostate cancer C4-2 xenografts. PSMA-specific monoclonal antibodies 3F/11 are used as the targeting moiety. Control conjugates contain either non-specific IgG or no IgG. The ratios of tumor accumulation to total background organs (heart, lung, kidney, liver, spleen and blood) accumulation increase substantially with time for the targeted conjugate, and the ratio at 48 h is 7-fold higher than that at 6 h. Preliminary

evaluation of the efficacy of the conjugates in vivo show tumor growth inhibition for all HPMA copolymer/DTX conjugates.

Keywords

antibody 3F/11; biodistribution; prostate cancer treatment; targeted HPMA copolymer/docetaxel conjugates; tumor accumulation

1. Introduction

Polymeric drug delivery systems emerged as promising therapeutic alternatives for cancer treatment due to distinctive advantages over low-molecular-weight drugs, including targetability.^[1–4] Incorporation into a polymeric drug delivery system of a targeting agent (moiety) that recognizes a tumor-surface biomarker, enables the targeted drug conjugate to preferentially accumulate in cancer cells. In addition, targeted drug conjugates will have significantly decreased systematic side effects that are inevitably associated with most low-molecular-weight anticancer chemotherapeutic agents. Indeed, targeted polymer therapeutics have demonstrated improved therapeutic index with minimal side effects in both preclinical and clinical settings.^[5–8] For instance, *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-based doxorubicin conjugates containing *N*-acylated galactosamine as the targeting moiety resulted in biorecognition by hepatocellular carcinoma cells as shown in a clinical study.^[5] The incorporation of OV-TL16 antibody, recognizing the CD47 (OA-3) antigen expressed on most of human ovarian carcinomas, into HPMA copolymer-based therapeutics resulted in their enhanced accumulation in human ovarian OVCAR-3 carcinoma xenografts in mice with concomitant inhibition of tumor growth when compared to the non-targeted HPMA copolymer/drug (doxorubicin) conjugate.^[6]

Prostate cancer is the second leading cause of cancer-related deaths.^[9] Many state-of-the-art clinical and pre-clinical studies have been undertaken aiming to improve treatment efficiency for advanced prostate cancer. Identification of prostate cancer cell surface specific molecules, such as the prostate-specific membrane antigen (PSMA), enables development of targeted anti-prostate cancer polymer therapeutics.^[10–14] PSMA is a dimeric type II integral membrane glycoprotein with an intracellular segment, a transmembrane domain, and an extracellular domain.^[12] It is highly expressed in poorly differentiated, metastatic and

hormone refractory prostate carcinoma with a minimal expression in benign prostatic secretory-acinar epithelium and other tissues.^[13,14] The expression pattern renders PSMA a suitable molecular marker for diagnosis, imaging, and targeted therapy of prostate cancer.

Two generations of monoclonal antibodies (mAbs) specific for PSMA have been developed. The first generation of mAbs, represented by 7E11.C5, recognizes the intracellular segment of PSMA and is unable to bind viable cells. Therefore, its utilization is greatly limited.^[15,16] The second generation of mAbs specifically binds to extracellular epitopes of PSMA.^[17,18] J591 is the most widely used second-generation mAb. Conjugates containing J591 have been widely used for immunotherapy and radioimmunotherapy in prostate cancer, and some are undergoing Phase I clinical trials.^[19–24] Biorecognition of J591-containing drug delivery systems has been evaluated.^[25,26] Elsässer-Beile and coworkers developed several monoclonal and recombinant antibodies against cell-adherent PSMA. In their studies, mAbs and scFv from mice immunized with the native form of PSMA demonstrated binding activity and internalization into prostate cancer LNCaP and C4-2 cells.^[27–29] The 3/A12 Ab labeled with ⁶⁴Cu was successful in positron emission tomography (PET) imaging of prostate cancer in mice.^[30] Another anti-PSMA antibody (3/F11) labeled with ¹⁷⁷Lu was an effective radioimmunotherapeutic agent in a prostate cancer SCID mice model.^[31] Brechbiel and coworkers evaluated the 3C6 Ab and demonstrated biorecognition by the PSMA positive cells.^[32]

In addition to antibodies, several other ligands have been reported to bind PSMA with high binding affinity, such as peptides,^[33] aptamers,^[8] and small molecular homing ligands.^[34] For example, aptamer decorated nanoparticles with encapsulated docetaxel (DTX) demonstrated remarkable prostate cancer growth control in mouse xenografts.^[8]

DTX was approved to represent the first-line treatment for advanced prostate cancer by the European Medicine Agency and the US Food and Drug Administration in 2004, based on two large randomized Phase III clinical trials.^[35–37] DTX demonstrated improvement in chemotherapy with a two- to three-month survival benefit.^[36,37] No other drug has demonstrated enhanced survival in advanced prostate cancer.^[38] However, side effects of DTX are significant, with neutropenia occurring in up to 70% and thrombosis in up to 10% of patients; consequently, DTX therapy is dose-limited.^[39] Hence, DTX in combination with numerous classes of therapeutics with potential for reduction in individual dosing has been actively investigated.^[40–42] As an alternative, delivery of DTX with polymeric drug delivery systems has attracted great attention due to the potential to increase the maximum tolerated dose.^[43–45] For instance, DTX was chemically conjugated with human serum albumin via a succinic spacer. The albumin conjugated formulation of DTX showed enhanced cytotoxicity against tumor cells.^[44] DTX-containing micelles prepared from monomethoxypoly(ethylene glycol)-*block*-poly(L-lactide)/DTX (MPEG-PLLA/DTX) demonstrated a similar antitumor activity as DTX.^[45]

In our previous study, we have proved that anti-PSMA 3F/11 monoclonal antibodies retained their binding affinity after conjugation to HPMa copolymer; they guided HPMa copolymer conjugates to PSMA+ prostate cancer cells.^[46] In the present study, we synthesized and characterized HPMa copolymer conjugates containing specific 3F/11

mAbor non-specific IgG and DTX as the drug. Their binding affinity to C4-2 prostate cancer cells, and biodistribution, pharmacokinetics in mice bearing C4-2 prostate tumor xenografts were investigated. Preliminary experiments on the growth inhibition of prostate tumor xenografts were also undertaken.

2. Experimental Section

2.1. Materials

DTX was purchased from AK Scientific. ^{125}I Iodine ($10^{-4} \text{ Ci} \cdot \mu\text{L}^{-1}$) was obtained as Na^{125}I from Perkin-Elmer. Iodogen-precoated tubes were purchased from Pierce (Rochford, IL). Cathepsin B (from bovine spleen) was purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade and were used as obtained from Sigma. Human male and female plasmas were obtained from the University of Utah Hospital Blood Bank (protein content $5.7 \pm 0.5 \text{ mg} \cdot \text{mL}^{-1}$). LNCaP-derived androgen-independent C4-2 cells were kindly provided by Dr. Elsässer-Beile. The prostate cancer cell lines 22Rv1 and PC-3 were purchased from American Type Culture Collection (ATCC, Manassas, VA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). C4-2, 22Rv1 and PC-3 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS at 37°C in an incubator with 5% CO_2 . The monoclonal antibodies against PSMA (3F/11) were generated as previously described.^[27]

2.2. Methods

UV-vis spectra were measured on a Varian Cary 400 Bio UV-visible spectrophotometer. Mass spectra of all synthesized compounds were obtained using a mass spectrometer Voyager-DE (STR Biospectrometry Workstation, PerSeptive Biosystems, Framingham, MA). The molecular weight and molecular weight distribution of polymers were measured on the ÅKTA fast protein liquid chromatography (FPLC) system (GE Healthcare, formerly Amersham) equipped with UV, refractive-index (RI), and light-scattering MiniDawn TREOS (Wyatt Inc., Santa Barbara) detectors using a Superose 6 HR10/30 column with phosphate-buffered saline (PBS)/acetonitrile, 70/30 (pH = 6.5) as the mobile phase. The average molecular weights were calculated using the ASTRA software from Wyatt Inc.

2.3. Synthesis of HPMA Copolymer/Drug Conjugates Containing Antibodies (P-DTX-3F/11)

The conjugates were synthesized in three steps (Figure 1). First, three monomers were synthesized: the DTX-containing monomer *N*-methacryloylglycylphenylalanylleucylglycine-DTX (MA-GFLG-DTX), a reactive thiazolidine-2-thione (TT group)^[47] containing monomer *N*-methacryloylglycylglycinethiazolidine-2-thione (MA-GG-TT), and the key monomer unit HPMA.^[48] Next, the reactive polymeric precursors containing DTX and TT groups for binding antibodies were prepared by solution free-radical polymerization. Finally, the antibodies were coupled to the polymer precursors by aminolysis of TT groups in PBS.

2.4. Synthesis of Monomer MA-GFLG-DTX

MA-GFLG-OH (*N*-methacryloylglycylphenylalanylleucylglycine, 248 mg, $0.54 \times 10^{-3} \text{ mol}$), DTX (396 mg, $0.49 \times 10^{-3} \text{ mol}$) and 4-dimethylaminopyridine (DMAP, 60 mg, $0.49 \times$

10^{-3} mol) were dissolved in *N,N*-dimethylformamide (DMF) and cooled to 0 °C. Then, *N,N'*-dicyclohexylcarbodiimide (DCC, 131 mg, 0.64×10^{-3} mol) dissolved in DMF was added dropwise. The reaction system was subsequently stirred for 2 h at 0 °C, then for 24 h at room temperature. Dicyclohexylurea was removed by filtration and crude MA-GFLG-DTX was dissolved in ethyl acetate (EtOAc) followed by washing with KHSO_4 , NaHCO_3 and NaCl solutions, respectively. Next, the organic layer containing MA-GFLG-DTX was dried over MgSO_4 , filtered and evaporated to remove EtOAc. Finally, trituration was performed with ether to yield a solid product (430 mg, 70%). The crude product was purified on semi-preparative high-performance liquid chromatography (HPLC) column Zorbax 300SB-C18, (9.4×250 mm) [buffer A: $\text{H}_2\text{O} + 0.1\%$ trifluoroacetic acid (TFA); buffer B: 90% MeOH + 0.1% TFA; gradient elution: 75–85% B for 15 min, 85–90% B for 20 min, 100% B for 10 min; $5 \text{ mL} \cdot \text{min}^{-1}$; product elution time 32–38 min (free DTX 20 min, disubstituted product 43 min)]. Purified monomer (36% yield): $m/z = 1272.6$ [$\text{M} + \text{Na}$] $^{+}$.

2.5. Synthesis of Reactive Polymer Precursors

P-DTX-TT (P is the HPMA copolymer backbone), polymer precursor containing TT and DTX was prepared by radical polymerization of HPMA (156 mg, 1.1×10^{-3} mol), MA-GG-TT (25 mg, 85×10^{-6} mol), MA-GFLG-DTX (37.5 mg, 30×10^{-6} mol (molar ratio of monomers 90.5/7/2.5), 2,2'-azoisobutyronitrile (AIBN) (22 mg, 0.13 mmol) and 1.1 mg (9×10^{-6} mol) 3-mercaptopropionic acid in 1.6 mL of dimethyl sulfoxide (DMSO) containing 5 μL of CH_3COOH . The concentration of monomers in the polymerization mixture was 15 wt %. The conjugate was isolated and purified by precipitation into acetone/ether (3:1). The polymer contained 0.38×10^{-3} mol of TT $\cdot \text{g}^{-1}$ (6.2 mol% TT; determined by UV spectrophotometry using molar extinction coefficient of monomer $10\,800 \text{ L} \cdot \text{mol}^{-1} \text{ cm}^{-1}$ in MeOH, 305 nm) and 72×10^{-6} mol $\cdot \text{g}^{-1}$ of DTX monomer DTX (1.5 mol%, 6 wt%, determined after complete enzymatic hydrolysis by papain); yield 174 mg. The weight-average molecular weight, $M_w = 40$ kDa (determined after aminolysis of TT groups) and $M_w/M_n = 1.6$, were measured on AKTA/FPLC system as described above.

The P-TT polymer precursor without drug was prepared similarly as described above, molar ratio of HPMA:MA-GG-TT was 93:7. The content of TT group was 0.37×10^{-3} mol $\cdot \text{g}^{-1}$ (6.1 mol%), M_w of the polymer was 37 kDa, $M_w/M_n = 1.6$.

P-DTX-Tyr was prepared similarly as described above with molar ratio of monomers in the polymerization mixture HPMA:MA-GFLG-DTX:MA-TyrNH₂ (*N*-methacryloyltyrosinamide) 97:2:1. The content of the DTX was 82×10^{-6} mol $\cdot \text{g}^{-1}$, 6.7 wt %; the content of TyrNH₂ was 49×10^{-6} mol $\cdot \text{g}^{-1}$; $M_w = 39$ kDa, $M_w/M_n = 1.5$.

2.6. Coupling of Antibodies to Polymer Precursors

A typical procedure was as follows: The polymer precursor (15 mg), dissolved in 0.150 mL DMSO, and a cold solution of 10 mg of 3F/11 Ab or IgG in 1.5 mL of PBS, pH = 7.4 were mixed and stirred at 4 °C for 30 min. Then the pH of the mixture was gradually raised up to 8.0 within 5 h at room temperature. The reaction mixture was kept at 4 °C overnight and pH was adjusted up to 8.3 on the next day. The released TT groups were removed using a Sephadex G-25 column equilibrated in PBS (pH = 6.5). Finally, the conjugate was subjected

to fractionation on a Superose 6 (16/60) column (GE Healthcare). The small amount (<10%) of high-molecular-weight fraction and non-bound polymer were separated from the conjugate fraction and the product was concentrated using an Amicon ultrafiltration system (membrane cut-off 30 kDa) to approximately 2.5 mL. The final molecular-weight profile was measured using Superose 6 (HR 10/30) column in PBS (pH =6.5, Figure 2A) and the content of Ab was determined by UV spectroscopy measurement [using $A(280\text{nm}, 1\text{ cm})$ for $1\text{ mg mL}^{-1}=1.36$] and confirmed by the Lowry method (after subtraction of the background corresponding to polymer). The content of polymer was calculated by the determination of DTX in the conjugate fraction. The molecular weight of the mAb containing conjugate was approx. 700 kDa and IgG conjugate 710 kDa and the polydispersity index (PDI) varied between 1.9 and 2.0 as determined by online light-scattering detector (MiniDawn). The weight ratio of Ab:polymer was approximately 1:1; molecular ratio approximately 1:5.

2.7. Determination of DTX Content in Polymer Conjugates

The content of DTX in polymer precursors and in antibody conjugates was determined after complete enzymatic hydrolysis with an excess of papain (Figure 2B). 10 μL of DMSO polymer stock solution ($25\text{ mg}\cdot\text{mL}^{-1}$, corresponding approximately to $2\times 10^{-3}\text{ M}$ DTX) were mixed with 100 μL 0.1 M citrate/phosphate buffer pH =6.0 [containing $2\times 10^{-3}\text{ M}$ ethylenediaminetetraacetic acid (EDTA) and $5\times 10^{-3}\text{ M}$ glutathione (GSH)] and with 20 μL papain ($\approx 0.5\times 10^{-3}\text{ M}$ buffer pH =6.0). The final concentrations in the incubation mixture were $0.08\times 10^{-3}\text{ M}$ enzyme (determined using $A=25$, for 1 wt%, 280 nm, 1 cm) and $2\text{ mg}\cdot\text{mL}^{-1}$ polymer. In the case of antibody conjugates 10 μL of the conjugate solution was used for the assay under the same conditions. The samples were incubated at 37°C in microcentrifuge tubes, one for each time interval. At time intervals (15, 30 min, 1, 2, 4, 6, 8 h) the hydrolysis was stopped by the addition of 10 μL of $3\times 10^{-3}\text{ M}$ sodium iodoacetate (enzyme inhibitor) solution and samples were stored in the freezer. For HPLC analysis the samples were diluted with MeOH containing 0.02% CH_3COOH (final concentration of MeOH was 70%). 20 μL of the methanolic solution was injected to analytical C18 column (Zorbax 300SB, $4.6\times 150\text{ mm}$; $5\text{ }\mu\text{m}$; $1\text{ mL}\cdot\text{min}^{-1}$) buffer A: $\text{H}_2\text{O} + 0.1\%$ TFA; buffer B: 90% acetonitrile +0.1% TFA, gradient elution 30% B to 90% B in 30 min, 220 nm detection. The amount of DTX was calculated from AUC of the peak at 12.4 min, the value that was achieved at the plateau (Figure 2B). The calibration curve (range $0\text{--}1\times 10^{-9}\text{ mol}$ DTX per 20 μL injection) was generated under the same conditions as the assay.

2.8. Enzymatic Cleavage of Conjugates by Cathepsin B

A similar procedure was used as described for the enzymatic hydrolysis by papain, with the exception of the lower concentration of the enzyme cathepsin B in the incubation mixture. The final concentrations in the incubation mixture were as follows: $8\times 10^{-6}\text{ M}$ cathepsin B; $2\text{ mg}\cdot\text{mL}^{-1}$ DTX polymer; 0.1 M citrate/ phosphate buffer pH =6.0, $2\times 10^{-3}\text{ M}$ EDTA, $5\times 10^{-3}\text{ M}$ GSH (Figure 2C).

2.9. Stability of Conjugates at Different pH and in Human Plasma

For the determination of the stability, the conjugates were incubated under similar procedure as described above in 0.1 M phosphate buffer pH =6.5, 7.3 and 8.5. The stability in human

plasma was measured by incubation of a conjugate in non-diluted plasma instead of buffer (Figure 2D).

2.10. Radio-iodination of Antibodies and Conjugates

Free 3F/11 and all drug conjugates were labeled with ^{125}I by the iodogen method.^[49,50] Antibody or conjugate were dissolved in 0.3 mL of PBS, pH =7.4 and added into an iodogen-precoated tube followed by addition of 10 μL (0.5 mCi) of Na^{125}I . The mixtures were incubated for 10 min. After incubation, the labeled antibody or conjugates were purified using a PD-10 column (Amersham GE Healthcare) pre-equilibrated with PBS (pH =6.5) containing 1% bovine serum albumin (BSA). The specific radioactivity was $1.5 \mu\text{Ci} \cdot \mu\text{g}^{-1}$.

2.11. Radioimmunoassay

The antigen binding affinity of 3F/11 (free mAb), P-3F/11 (conjugate without drug) and P-DTX-3F/11 (conjugate containing drug) was determined using a saturation radioimmunoassay. Briefly, cells grown in 24-wells at 80–90% confluence were pre-incubated with Hanks balanced salt solution (HBSS) containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; $20 \times 10^{-3} \text{ M}$), NaN_3 ($15 \times 10^{-3} \text{ M}$) and BSA (1%) for 30 min on ice and subsequently incubated with serial concentrations of labeled P-DTX-3F/11 dissolved in the same buffer on ice for 4–6 h. After incubation, cells were extensively washed with PBS to remove unbound conjugates. Cells were solubilized with 1 M NaOH for 1 h and counted for radioactivity (γ counter Packard, Minaxi γ , Autogamma 5000 series, calibrated with ^{137}Cs , nominally 0.25 μCi). The results were analyzed using the GraphPad Prism (GraphPad Software, La Jolla, CA). The nonlinear regression analysis was performed and K_d value determined.

2.12. Mouse Xenograft Model of Human Prostate Cancer

C4-2 prostate cancer cells grown in log phase were harvested and resuspended in RPMI 1640 medium containing 10% fetal bovine serum. Cells (1.5×10^6 in 50 μL of medium) were mixed with 50 μL of Matrigel (Becton Dickinson Biosciences, Bedford, MA). Each male nu/nu mouse (Charles River Frederick, MD, 7 weeks old, 20–25 g) was injected with the mixture on their flank subcutaneously. After 21 d post-injection, tumor volume reached $\approx 300 \text{ mm}^3$ and biodistribution experiments were carried out in these mice. The mice were taken care and experimental studies were conducted according to the University of Utah Institutional Animal Care and Use Committee (IACUC) approved protocols.

2.13. Biodistribution Studies

The study was performed with radiolabeled HPMA copolymer/ drug conjugates in tumor-bearing nu/nu mice. Mice were injected via tail vein with 20 μg conjugates dissolved in normal saline in the volume of 100 μL containing 0.1 μCi ^{125}I labeled conjugates as a tracer. Time-dependent experiments were carried out by euthanizing mice at 6, 24, 48, and 96 h post injection. During necropsy, blood samples were collected via the caudal vena cava. Major organs, such as heart, lungs, kidneys, liver, spleen, and tumor as a whole were isolated, washed with PBS, weighted, and counted. The volume of blood sample was also

measured. Subsequently, the percentage-injected dose per gram tissue ($\% \text{ ID} \cdot \text{g}^{-1}$) was calculated. Each animal group was composed of three mice for all biodistribution studies.

Tumor-to-background (T/B) ratios were calculated by dividing $\% \text{ ID} \cdot \text{g}^{-1}$ results for the tumor by other organs in the study (heart, lung, kidney, liver, spleen and blood) for each time point.

2.14. Pharmacokinetic Analysis

Blood concentrations of labeled conjugates were calculated as $\mu\text{Ci} \cdot \text{mL}^{-1}$ based on radioactivity and volume measurements. The blood concentrations vs. time profiles for each animal group were assessed by two-compartment analysis using WinNonlin software (version 5.0, Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameters were calculated from standard equations using WinNonlin.

2.15. Preliminary Evaluation of Treatment Efficacy

Male nu/nu mice (6 weeks old; 20–25 g) were subcutaneously injected in their flank with a mixture of 1.5×10^6 C4-2 cells in 50 μL media and 50 μL Matrigel. When the tumor size reached about 100 mm^3 (approximately on day 19) mice received five intravenous injections (tail vein) of polymer conjugates ($5 \times 2 \text{ mg} \cdot \text{kg}^{-1}$ DTX equivalent) on treatment days 0, 3, 6, 9, and 13. The tumor volume was measured with calipers; the body weight was monitored.

2.16. Statistical Analysis

The one-way ANOVA test was utilized to determine the statistical significance of the differences in the organ accumulation among the different conjugates. Student's unpaired t test was used to determine the statistical significance of the differences in the organ deposition between targeted and non-targeted HPMA/copolymer conjugates. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Synthesis of Conjugates

The antibody conjugates (Figure 1) were synthesized by nucleophilic substitution of reactive TT groups at the termini of glycylglycine side chains of the HPMA copolymer precursor by accessible amino groups on the surface of Ab. The degree of modification (the molecular weight and shape of conjugates) generally depends on the concentration of reactants and to a lower extent on the reactivity of the amino groups of the used Ab. In this study we aimed to achieve a high molecular weight branched conjugate, consequently we chose a relatively high concentration of reactants during the synthesis (concentration of Ab plus polymer was 1.5 wt%). The crude conjugates were fractionated by size exclusion chromatography (SEC; Figure 2A) to achieve a reasonable molecular weight profile and to remove a small amount of very high molecular weight fraction ($<10\%$) and also non-reacted polymer precursor. From the composition of the conjugates, the molecular structure of the final sample (ca. 700 kDa) is estimated to be formed by two antibody molecules bridged by HPMA copolymer and containing on average five polymer chains attached to each molecule of antibody.

The final antibody conjugates were stored in PBS solution pH =6.5 at 4 °C to minimize the hydrolysis of ester bond between the polymer and drug. Under such conditions the hydrolysis was negligible. The DTX containing polymers (without Ab) were stored as powder.

A considerable attention was paid to the stability of ester linkage between the drug and polymer. As expected for this type of structure, the ester linkage originating from an amino acid, is activated and releases the drug with a considerable rate even at the physiological pH (Figure 2D; changes of DTX chemical structure were detected by HPLC after incubation at longer time intervals in plasma and pH above neutral). Before the in vivo study we have checked the cytotoxic activity of conjugates toward C4-2 cells (12 and 24 h incubation). All conjugates were efficiently cytotoxic with the IC₅₀ values very close to each other ($5\text{--}10 \times 10^{-9}$ M; data not shown). Cathepsin B, the model of lysosomal enzymatic activity, cleaved the GlyPheLeuGly oligopeptide spacer and released the DTX as expected (Figure 2C). The characteristics of HPMA copolymer conjugates are listed in Table 1.

3.2. Biorecognition of PSMA-Positive Prostate Cancer Cells by Antibody Conjugates

The antigen binding affinity of P-DTX-3F/11 (conjugate containing drug) and P-3F/11 (conjugate without the drug) were determined using saturated binding experiments with PSMA positive prostate cancer cells. It was to verify that anti-PSMA antibody is able to guide conjugates to actively bind PSMA positive prostate cancer cells. As seen in Figure 3, free 3/F11 and both its conjugates showed typical saturation binding curves for active binding to PSMA positive prostate cancer C4-2 cells. K_d values (10^{-9} M antibody) were 2.8, 14.9 and 20.7 for free 3/F11, P-3F/11 and P-DTX-3F/11, respectively. Despite free antibody demonstrating slightly greater binding affinity, its polymeric counterparts with or without DTX possessed efficient binding affinity to PSMA in prostate cancer cells. The binding capability of conjugates was validated also in PSMA-positive prostate cancer 22Rv1 cells. However, the binding affinity was slightly lower in 22Rv1 cells than C4-2 cells due to slightly lower expression of PSMA in 22Rv1 cells than LNCaP cells (C4-2 cells were derived from LNCaP).^[32]

3.3. Biodistribution of Targeted and Non-Targeted HPMA Copolymer/DTX Conjugates

Time-dependent biodistribution of radiolabeled targeted and control polymer drug conjugates was evaluated in nude mice bearing C4-2 xenografts. The results are shown in Figure 4. Significantly slower blood clearance was observed for P-DTX-3F/11 and P-DTX-IgG, compared to P-DTX. For instance, more than 5% ID $\cdot$ g⁻¹ (injected dose per g) remained in blood for P-DTX-3F/11 and P-DTX-IgG 96 h after injection, whereas less than 2.5% ID $\cdot$ g⁻¹ for P-DTX at the time point of 48 h. Due to the fact that very little activity remained in blood and other organs 48 h after injection, we omitted the time point of 96 h for P-DTX. Similar to the prolonged circulation time, increased organ retention occurred for P-DTX-3F/11 and P-DTX-IgG, compared to P-DTX for each time point ($p < 0.05$). At 48 h, minimal amount of conjugates resided in organs for P-DTX, suggesting that majority of conjugates were eliminated from organs after 48 h. On the contrary, higher accumulation of conjugates was shown in organs for P-DTX-3F/11 and P-DTX-IgG after 48 h.

At 6 h, higher accumulation was observed in lung and kidney than in other organs except blood for all three conjugates ($p < 0.05$), indicating rapid accumulation in these two organs. Liver and spleen accumulation rose slowly beyond 6 h for the three conjugates. Three conjugates did not amass in liver and spleen at prolonged time points (24, 48 and 96 h). Accumulation of conjugates appears to decline with time in all organs for all three conjugates.

3.4. Antibody-Containing HPMA Copolymer/DTX Conjugates

Tumor accumulation was analyzed to evaluate tumor-targeting capability (Figure 4). As expected, the accumulation of P-DTX-3F/11 demonstrated a significantly increasing trend, reached a peak ($11 \times 0.12\% \text{ ID} \cdot \text{g}^{-1}$) at 48 h and decreased afterwards (see Figure 4 inset). At 48 and 96 h, tumor accumulation of P-DTX-3F/11 was only slightly lower than concentration in the blood but significantly higher than in all other organs ($p < 0.05$). Additionally, tumor accumulation for P-DTX-3F/11 was considerably higher than that for P-DTX-IgG and P-DTX at each time point of the study ($p < 0.01$).

The ratio of tumor accumulation to total background organs (heart, lung, kidney, liver, spleen and blood) accumulation, designated as tumor-to-background ratio, was analyzed to confirm the tumor accumulation of targeted HPMA copolymer/DTX conjugates. The ratio increased dramatically with time for P-DTX-3F/11; the ratio at 48 h was 7-fold higher than that at 6 h, indicating extended tumor residence. In contrast, the tumor-to-background ratio for P-DTX-IgG and P-DTX appeared slightly increased (1-fold) with time due to enhanced permeability and retention (EPR) effect, suggesting lower tumor accumulation than the targeted conjugate.

3.5. Pharmacokinetic Study of Targeted and Non-Targeted HPMA Copolymer/DTX Conjugates

P-DTX-3F/11 and P-DTX-IgG demonstrated nearly the same pharmacokinetic profiles (Figure 5). Prolonged $t_{1/2\alpha}$ indicated sufficient organ and tissue exposure α of both conjugates while prolonged $t_{1/2\beta}$ indicated slow organ and tissue metabolism. The large volume of distributions and slow clearance were observed for both conjugates, suggesting adequate tissue distribution. The pharmacokinetic parameters of both conjugates are summarized in Table 2.

3.6. Preliminary Evaluation of in vivo Efficacy

Small groups of nu/nu mice ($n=3$) were used to evaluate the dosing schedule and obtain information for the planning of a decisive experiment on the relationship between the structure of conjugates and their efficacy in the treatment of C4-2 prostate cancer xenografts (Figure 6). All conjugates inhibited tumor growth when compared to untreated controls.

4. Discussion

The ultimate goal of this study is to improve the therapeutic effectiveness of macromolecular therapeutics by enhancement of tumor localization of anticancer drugs. We attempted to verify the hypothesis that the biorecognition of this conjugate by PSMA

positive cells will result in favorable tumor accumulation and pharmacokinetics of the targeted conjugate in prostate cancer xenografts. To this end, we designed and synthesized an HPMa copolymer/DTX conjugate targeted by 3F/11 mAb (P-DTX-3F/11) and evaluated its properties in vitro and in nude mice bearing C4-2 human prostate cancer xenografts.

We have previously demonstrated that HPMa copolymers containing anti-PSMA monoclonal antibodies (3A/12, 3F/11, and 3E/7) can specifically recognize and be internalized by PSMA expressing C4-2 prostate cancer cells ($K_d \approx 10^{-8}$).^[46] The targeted P-DTX-3F/11 conjugate demonstrated efficient binding affinity to PSMA expressing cells, similar to HPMa copolymer conjugates containing only antibody. This indicated that incorporation of DTX does not compromise the binding activity of HPMa copolymer/antibody conjugates.

Distribution designates the transport of macromolecular therapeutics between blood and major organs. The kinetics of distribution to a tissue is regulated by blood perfusion, extravasation, and tissue permeability. Furthermore, the biodistribution of conjugates in organs is mainly determined by the physicochemical characteristics of conjugates, such as molecular weight and charge.^[51,52] Indeed, P-DTX-3F/11 and P-DTX-IgG with molecular weight of 700 kDa possessed a more prolonged circulation time and, consequently, increased organ retention than P-DTX (molecular weight 40 kDa) for each time point (Figure 5). Rapid accumulation in lung and kidney and slow accumulation in liver and spleen shown in our data are in accordance with varied perfusion rate and physiology of organs. Lammers et al.^[51] studied the effect of physicochemical modification of HPMa copolymers on their biodistribution in rats bearing Dunning R3327-AT1 prostate carcinoma. In agreement with our present and previous^[52] data, they observed increased tumor accumulation with increasing molecular weight of the carrier. Both in rat and mice prostate cancer models, the increased molecular weight of HPMa copolymer/drug conjugates also led to increased kidney, spleen, and lung accumulation. However, the high biorecognition of P-DTX-3F/11 by PSMA, as determined by the K_d constant, resulted in favorable tumor accumulation with a peak at 48 h ($11 \times 0.12\% \text{ ID} \cdot \text{g}^{-1}$; inset of Figure 4) and decrease afterwards. It is likely due to the fact that antibody is degraded in lysosomes after endocytic uptake and ^{125}I attached to small degradation products diffuses out of tumor tissue. The accumulation of P-DTX-IgG at 48 h was lower than expected based on the molecular weight of the conjugate. A partial explanation may be the low number of animals per group, but the low standard deviation of the data suggests that this is not the only reason.

At 96 h its accumulation in all organs, except tumor and blood was low. The enhanced accumulation in organs with leaky vasculature through increasing molecular weight has been described by the EPR effect.^[53] The accumulation is dependent on the concentration gradient between blood and tissue and time. Thus, long-circulating (high-molecular-weight) conjugates are more effective.

The accumulation of targeted conjugates in tumor tissue is mediated by specific binding of targeting moieties to tumor cell surface prostate specific membrane antigens. Consequently, active transport is a key factor in their biodistribution. In addition to antigen density, turnover and antigen expression profile that are of importance but often predetermined by

pathological situation of diseases, many other factors need to be considered, including the binding affinity of the targeting moiety, number of targeting moieties per macromolecule, molecular weight, and conformation of the conjugate in solution, in order to develop a conjugate possessing better tumor targeting capability. The antibody chosen in the present study was verified to have nanomolar affinity to its antigen. The targeted conjugate (P-DTX-3F/11) affinity was only slightly lower than that of free Ab (Figure 3). Definitely, our results showed significant tumor accumulation of P-DTX-3F/11 and noteworthy increased tumor-to-background ratio for P-DTX-3F/11.

The favorable biodistribution of anti-PSMA antibody drug conjugates has been proved by several studies aiming to develop tumor targeted diagnostics or targeted therapies. For instance, an antibody specific for PSMA, 3/A12, similar to 3F/11, was conjugated with the chelating agent 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) and radiolabeled with ^{64}Cu . The biodistribution of labeled antibody conjugates were studied in severe combined immunodeficient (SCID) mice bearing PSMA-positive C4-2 or PSMA-negative DU 145 prostate carcinoma xenografts. At 48 h after injection of labeled conjugates, a significantly higher uptake of $35.1 \pm 8.0\% \text{ ID} \cdot \text{g}^{-1}$ was demonstrated in C4-2 tumors, compared with $12.8 \pm 2.7\% \text{ ID} \cdot \text{g}^{-1}$ in DU145 tumors ($P < 0.001$). The activity remaining in the blood after 48 h was $16.2 \pm 9.5\% \text{ ID} \cdot \text{g}^{-1}$ in mice with C4-2 tumors and $17.9 \pm 1.8\% \text{ ID} \cdot \text{g}^{-1}$ in mice with DU 145 tumors, similar to our results.^[30]

Monoclonal antibody 3C6 is another PSMA-specific Ab. 3C6 was conjugated with the acyclic CHX-A'' DTPA chelate, radiolabeled with ^{111}In , and the in vivo biodistribution, blood pharmacokinetics, and imaging characteristics of ^{111}In -labeled 3C6 were evaluated in athymic mice bearing LNCaP, 22Rv1, or DU145 xenografts. The tumor values were 22.93 ± 6.23 , 10.44 ± 2.32 and $4.27 \pm 0.37\% \text{ ID} \cdot \text{g}^{-1}$ (mean $\pm$ SD) for LNCaP, 22Rv1 and DU145 tumors, respectively, at 72 h, indicating successful PSMA targeting of ^{111}In -CHX-A''-3C6. As expected, tumor-to-blood ratios for ^{111}In -CHX-A''-3C6 in both the LNCaP and 22Rv1 tumors were higher than that in DU145 tumor. These data correlated well with the level of expression of PSMA in the cell lines. LNCaP and 22Rv1 express PSMA, whereas DU 145 expresses very little PSMA.^[32]

Many biodistribution and pharmacokinetic studies have been done on HPMA copolymer drug conjugates.^[52,54–57] The pharmacokinetics study of P-DTX-3F/11 and control conjugate, P-DTX-IgG, revealed that the $t_{1/2}$ for both conjugates is long (Table 2), in agreement with similar antibody conjugates. The $t_{1/2\alpha}$ and $t_{1/2\beta}$ of ^{111}In -CHX-A''-3C6 were ≈ 5.3 and ≈ 92.3 h, respectively.^[32] Smith-Jones et al. reported that the $t_{1/2\beta}$ of ^{111}In -J591 is 2.3 d, comparable to our results.^[58] The prolonged $t_{1/2\alpha}$ and $t_{1/2\beta}$, large volume distribution and slow clearance suggested that retention of both conjugates in tissues and organs in accordance with biodistribution data. Both conjugates have the same pharmacokinetics profile as expected. It is due to the fact that pharmacokinetics parameters are determined by physicochemical properties of drug or drug conjugates. As the critical control, P-DTX-IgG was designed to possess the same physicochemical properties as P-DTX-3F/11.

The treatment efficacy was evaluated on a small number of animals. All three HPMA copolymer/DTX conjugates inhibited tumor growth when compared to untreated controls

(Figure 6). However, the dosing schedule was not optimal; the results are probably based mainly on the contribution from the EPR effect. The biorecognition impact was minor. On the other hand, the results of biodistribution of the conjugates strongly suggest the potential of the delivery system to enhance DTX tumor accumulation. A study with different dosing schedules and with larger number of animals per group is needed to evaluate the correlation between biorecognition and treatment efficacy. Optimization of the treatment protocol should lead to higher treatment efficiency.

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5. Nomenclature

DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DTX	docetaxel
EDTA	ethylenediaminetetraacetic acid
EPR	enhanced permeability and retention
GSH	glutathione
HPMA	<i>N</i> -(2-hydroxypropyl)methacrylamide
ID	injected dose
MA	methacryloyl
MA-GFLG-OH	<i>N</i> -methacryloylglycylphenylalanylleucylglycine
MA-GG-TT	<i>N</i> -methacryloylglycylglycinethiazolidine-2-thione
MA-TyrNH₂	<i>N</i> -methacryloyltyrosinamide
PBS	phosphate-buffered saline P-DTX-3F/11 HPMA copolymer/ docetaxel/3F/11 antibody conjugate
P-DTX-IgG	HPMA copolymer-docetaxel-nonspecific immunoglobulin conjugate
P-DTX-TT	HPMA copolymer/docetaxel conjugate containing reactive TT groups
P-DTX-Tyr	HPMA copolymer/docetaxel conjugate containing a small amount of tyrosinamide groups to permit radioiodination
P-TT	copolymer of HPMA and MA-GG-TT
PSMA	prostate-specific membrane antigen
SEC	size-exclusion chromatography
TT	thiazolidine-2-thione

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Figure 1.
Synthesis scheme.

Figure 2.

Characteristics of the conjugates. **A:** SEC profiles measured on the AKTA system, Superose 6 HR10/30 column, buffer PBS (pH = 6.5) + 30% acetonitrile. **B:** An example of DTX release by enzymatic hydrolysis with an excess of papain: enzyme 80×10^{-6} M; polymer (P-DTX) $2.01 \text{ mg} \cdot \text{mL}^{-1}$; pH = 6.0; GSH 2×10^{-3} M; EDTA 2×10^{-3} M. **C:** Cleavage by cathepsin B: enzyme 8×10^{-6} M, polymer $2 \text{ mg} \cdot \text{mL}^{-1}$, buffer as for B. **D:** DTX hydrolysis by human plasma and in buffer different pH.

Figure 3.

Binding of targeted and non-targeted HPMA copolymer/ DTX conjugates. Saturated binding was performed using C4-2 cells. **A:** Free antibody; **B:** P-3F/11; and **C:** P-DTX-3F/11.

Results are expressed as means of triplicate $\pm$ SD. Some of SDs are too small to be visible.

Figure 4.

Biodistribution of targeted and non-targeted HPMA copolymer/DTX conjugates after intravenous administration to nude mice bearing C4-2 xenografts. P-DTX-3F/11 and P-DTX-IgG were administered at 20 μg protein (3F/11 or IgG) and 21 μg HPMA copolymer for each mouse with 0.1 μCi ^{125}I -labeled conjugates as a tracer. P-DTX was administered with 0.1 μCi ^{125}I -labeled conjugates and 21 μg HPMA copolymer per mouse. Activity per organ is expressed as % injected dose per gram of tissue (% ID $\cdot \text{g}^{-1}$). Necropsy was performed at indicated time intervals post injection. Results are displayed as mean $\pm$ SD ($n=3$).

Figure 5. Blood clearance of ^{125}I -labeled HPMA copolymer/DTX-immunoglobulin conjugates. Squares: P-DTX-IgG; diamonds: P-DTX-3F/11; triangles: P-DTX. Results are expressed as mean $\pm$ SD ($n=3$).

Figure 6.

Growth inhibition of subcutaneous prostate cancer C4-2 xenografts in nude mice. Treatment was started when the tumor reached a size of $\approx 100 \text{ mm}^2$ (day 0). The mice ($n=3$) received intravenous injections of $2 \text{ mg} \cdot \text{kg}^{-1}$ DTX equivalent on days 0, 3, 6, 9, and 13. Values are the means $\pm$ SD. Squares: untreated control; diamonds: P-DTX; Triangles: P-DTX-IgG; circles: P-DTX-3F/11.

Table 1

Physicochemical characteristics of HPMA/copolymer conjugates (NA =not applicable).

Property	P-DTX-3F/11	P-DTX-IgG	P-DTX
structure	P-(GFLG-DTX)-(GG-3F/11) ^{a)}	P-(GFLG-DTX)-(GG-IgG) ^{a)}	P-(GFLG-DTX)-TyrNH ₂ ^{b)}
estimated M_w [kDa]	700	710	39
M_w/M_n	1.9	2.0	1.5
DTX content	0.182 mg · mL ⁻¹	0.179 mg · mL ⁻¹	83×10^{-6} mol · g ⁻¹ (6.7 wt%)
Ab content [mg · mL ⁻¹]	1.74	1.85	NA
weight ratio Ab:polymer	1:1.7	1:1.6	NA
molecular ratio Ab:polymer	≈1:5	≈1:5	NA

^{a)}For structure see Figure 1;

^{b)}TyrNH₂ incorporated into the polymer by copolymerization with 1 mol% *N*-(methacryloyl)tyrosinamide.

Table 2

Pharmacokinetic parameters of HPMA copolymer/drug conjugates in blood.

Sample	AUC [$\mu\text{Ci}\cdot\text{mL}^{-1}\cdot\text{h}^{-1}$]	$t_{1/2\alpha}$ [h]	$t_{1/2\beta}$ [h]	CL [$\text{mL}\cdot\text{h}^{-1}$]	V_d [mL]
P-DTX-3F/11	5.28 ± 2.81	3.23 ± 0.71	66.63 ± 3.71	0.22 ± 0.10	9.81 ± 0.61
P-DTX-IgG	4.41 ± 0.24	2.75 ± 0.14	57.79 ± 3.84	0.29 ± 0.02	9.96 ± 0.79