A prostate-specific antigen–activated N-(2-hydroxypropyl) methacrylamide copolymer prodrug as dual-targeted therapy for prostate cancer

Sachin S. Chandran,1 Anjan Nan,2 D. Marc Rosen,3 Hamidreza Ghandehari,2 and Samuel R. Denmeade1,3

1Department of Chemical and Biomolecular Engineering, The Johns Hopkins University Whiting School of Engineering; 2Department of Pharmaceutical Sciences and Center for Nanomedicine and Cellular Delivery, University of Maryland-Baltimore; and 3The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

Prostate cancer targeted peptide prodrugs that are activated by the serine protease activity of prostate-specific antigen (PSA) are under development in our laboratory. To enhance delivery and solubility of these prodrugs, macromolecular carriers consisting of N-(2-hydroxypropyl) methacrylamide (HPMA)–based copolymers were covalently coupled to a PSA-activated peptide prodrug. HPMA copolymers are water-soluble, nonimmunogenic synthetic carriers that exhibit promise for drug delivery applications. These macromolecular copolymers enter the interstitium of solid tumors by the enhanced permeability and retention effect. The PSA-activated peptide substrate imparts selectivity because it is specifically hydrolyzed to release a cytotoxin at the site of prostate tumor. Enzymatically active PSA is present in high amounts in the extracellular fluid of a tumor, but PSA is inactivated in blood by binding to serum protease inhibitors. As an initial proof of concept, the HPMA copolymer was synthesized with a peptide substrate sequence of the cleavage sites within the human seminal antigen (PSA; refs. 5, 6). By determining the amino acid sequence His-Ser-Ser-Lys-Leu-Gln-Leu (HSSKLQL) was identified (3, 7, 8). The C-terminal carboxyl of this peptide sequence His-Ser-Ser-Lys-Tyr-Gln-Leu-12-aminododecanoyl thapsigargin (JHPD). L12ADT is a potent analogue of the highly cytotoxic natural product thapsigargin. HPMA-JHPD was hydrolyzed by PSA in vitro and was toxic to prostate cancer cells in the presence of active PSA. The HPMA-JHPD produced no systemic toxicity when given at a 500 μmol/L L12ADT equivalent dose. Analysis of tumor tissue from mice treated with a single or multiple dose of the HPMA-JHPD copolymer showed release and accumulation of the L12ADT toxin within the tumor tissue. [Mol Cancer Ther 2007;6(11):2928–37]

Introduction

Prostate cancer is the most commonly diagnosed nonskin cancer in American men and remains uniformly fatal once it has metastasized outside of the prostate gland (1). Androgen ablation is effective palliative therapy, but all men progress to an androgen deprivation refractory state, at which point treatment with cytotoxic chemotherapy can produce, at best, modest improvement in survival of a few months, at the cost of substantial systemic toxicity to normal host tissues. Previous studies have shown that metastatic androgen-independent prostate cancer cells have a remarkably low rate of proliferation, which may, in part, explain the relative unresponsiveness of prostate cancer cells to conventional antiproliferative therapy (2). To kill these slow-proliferating cells, earlier studies have been focused on developing methods to target a cytotoxic analogue of the naturally occurring plant product thapsigargin to prostate cancers (3, 4). Thapsigargin is a potent inhibitor of the sarcoplasmic/endoplasmic reticulum Ca2+ ATPase, which is a house-keeping enzyme that plays a key role in Ca2+ homeostasis within cells and whose inhibition triggers apoptotic death in all cell lines tested (3, 5). Of particular importance for slowly proliferating prostate cancers, thapsigargin can induce apoptosis in a proliferation-independent manner (2, 4).

Because thapsigargin does not specifically target prostate cancer and is toxic to normal cells, a method must be developed to target thapsigargin selectively to prostate cancer cells. To accomplish this targeting, we have previously taken advantage of the ability of both normal and malignant prostate epithelial cells to synthesize copious amounts of the serine protease prostate-specific antigen (PSA; refs. 5, 6). By determining the amino acid sequence of the cleavage sites within the human seminal proteins semenogelins I and II, the physiologic substrates for PSA, a PSA-selective peptide substrate with the sequence His-Ser-Ser-Lys-Gln-Leu (HSSKLQ) was identified (3, 7, 8). The C-terminal carboxyl of this peptide was coupled to a thapsigargin analogue HPMA–morpholinocarbonyl-Ser-Ser-Lys-Tyr-Gln-Leu-12-aminododecanoyl thapsigargin (JHPD)]. L12ADT was toxic to prostate cancer cells in the presence of active PSA.
was then coupled to a primary amine, containing analogue of thapsigargin to produce a novel prodrug that is selectively activated by PSA within PSA-producing tumors. The prodrug HSSKLQ-L12ADT, on hydrolysis by PSA, releases the potent cytotoxic thapsigargin analogue (L12ADT) into the tumor microenvironment (9, 10). Because PSA is a secreted protein, this approach has the added benefit that nearby cells that do not produce PSA can also be targeted, resulting in a local bystander effect that could kill prostate cancer cells, as well as supporting endothelial cells and stromal cells within the tumor microenvironment.

The PSA-activated peptide prodrug showed antitumor efficacy in vivo against PSA-producing human prostate cancer xenografts. The prodrug had a serum half-life of ~1.5 h. In addition, although conjugation to the PSA-peptide greatly improves the solubility of the highly lipophilic thapsigargin analogue, solubility has been dose-limiting at the higher concentrations required for administration in vivo. Therefore, to increase serum half-life of this protease-activated prodrug and improve solubility in aqueous media, we developed a strategy to attach the PSA-activated peptide prodrug to a water soluble macromolecule. An added potential benefit of this strategy is the improved tumor targeting that may be achieved based on the enhanced permeability and retention (EPR) effect (11, 12). This EPR effect is due to the “leaky” nature of tumor blood vessels compared with a normal blood vessel (Fig. 1A). Macromolecules can extravasate into the tumor due to the EPR effect and accumulate as they are cleared very poorly due to the lack of a well-organized lymphatic system within solid tumors (13).

To evaluate this strategy, we therefore copolymerized the PSA-activated prodrug with N-(2-hydroxypropyl)methacrylamide (HPMA; Fig. 1B). The polymeric form of HPMA, pHPMA, is a biocompatible, nonbiodegradable, watersoluble macromolecule that has been increasingly used as a drug carrier for antioxidant agents in clinical development as an alternative to polyethylene glycol (PEG). Copolymers of HPMA with other chemotherapeutic molecules, such as doxorubicin, have been targeted to human colon cancer cells or human B-cell leukemia (14, 15). Satchi-Fainaro et al. have also developed a copolymer of HPMA and TNP-470 as an antiangio genesis strategy (16). Currently, there are at least six different HPMA-based copolymers in either phase I or phase II trials (17). The goal of this work, therefore, is to present the characterization of a PSA-activated peptide, macromolecular prodrug copolymerized with HPMA in terms of PSA hydrolysis, stability, and in vivo biodistribution.

Materials and Methods

Monomers and Chemicals

HPMA (M<sub>w</sub> 143) was synthesized using previously described methods (18). His-Ser-Lys(Fmoc)-Leu-Gln-7-amino-4-methylcoumarin (JHAMC; M<sub>w</sub> 1,078), N-methacryloyl-Ser-Lys-Tyr-Gln-Leu-12-aminododecanoyl thapsigargin (M<sub>w</sub> 1,552), and morpholinocarbonyl-Ser-Ser-Lys-Tyr-Gln-Leu-12-aminododecanoyl thapsigargin (JHPD; M<sub>w</sub> 1,597) were purchased from California Peptide Company. The JHAMC content of the copolymer was determined by UV spectrophotometry (331 nm). The product was further reprecipitated in ether, and dried. The product was further dissolved in water and dialyzed for 72 h using a Spectrapor cellulose membrane (molecular weight cut-off at 5,000 Da) to remove all traces of the starting material and solvents followed by lyophilization. The lyophilized material was then dissolved in 500 μL of 20% piperidine in dry dimethylformamide for depertiation of the Fmoc group on lysine, precipitated in cold ether, filtered through a 0.2-μm filter, and then relyophilized to obtain an off-white powder. The JHAMC content of the copolymer was determined by UV spectrophotometry (331 nm). The weight and number average molecular weights of the...
polymer (M<sub>n</sub> and M<sub>w</sub>) were estimated by size exclusion chromatography using a Superose 12 column (10 mm × 30 cm; Amersham Biosciences) on a fast protein liquid chromatography (FPLC) system (Amersham Biosciences). 1× PBS buffer was used as the mobile phase at a flow rate of 0.4 mL/min.

Synthesis and Characterization of poly(HPMA-co-MA-SSKYQL-12ADT; HPMA-JHPD)

The copolymer poly(HPMA-co-MA-SSKYQL-12ADT; HPMA-JHPD) was prepared by the free radical copolymerization of HPMA and N-methacryloyl-Ser-Ser-Lys-Tyr-Gln-Leu-12-aminododecanoyl thapsigargin in 8% (v/v) DMSO in acetone using N,N′-azobisisobutyronitrile as the free radical initiator. The feed composition was maintained at 0.875 mol%. The polymerization was carried out as described above for 24 h at 50°C to obtain a white precipitate. The purification and characterization methods were identical as above. Amino acid analysis (Commonwealth Biotechnologies) was carried out to determine the peptide content of the copolymer, and UV spectroscopy measurements were carried out at 276 nm.

PSA Hydrolysis of HPMA-JHAMC

Fluorophore release from HPMA-JHAMC was analyzed by measuring increase in fluorescence upon addition of PSA. PSA concentration was 5 μg/mL, and HPMA-JHMC concentration was 1.25 mg/mL. The same concentration of HPMA-JHMC without PSA was used as a control. Both conditions were done in triplicate. The excitation was carried out at 355 nm, and emission was measured at 460 nm, which are characteristic values for 7-amino-4-methylcoumarin (AMC). Release of AMC was monitored for 4 h.

Liquid Chromatography–Mass Spectrometry/Mass Spectrometry Method

All procedures were carried out on wet ice in 1.5-mL microfuge test tubes. Calibration standards were prepared by spiking control plasma or tissue homogenate samples with 10× solutions of L12ADT in 50% acetonitrile/water. Tissue homogenates consisted of one part tissue and three volumes of Complete Protease Inhibitor Buffer (Boehringer Mannheim) in water. A 0.1-mL sample size was deproteinated by its addition to 0.2 mL of 100% acetonitrile containing the internal standard of 500 nmol/L S-12ADT. The samples were vortexed briefly and centrifuged at 21,000×g for 3 min, and the resulting supernatant was transferred to an autosampler vial for subsequent analysis.

Plasma and tissue samples were analyzed by liquid chromatography coupled to a quadrupole mass spectrometer.
[liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS); Perkin-Elmer Applied Biosystems APIO 3000]. The mobile phase consisted of 0.1% formic acid in 5% acetonitrile/water and 0.1% formic acid in acetonitrile. Separation was carried out by a Luna 5 µm C18 column, 150 × 2.0 mm (Phenomenex) subjected to a flow rate of 0.375 mL/min with a linear gradient starting from 25% acetonitrile at 2 min progressing to 100% acetonitrile at 8 min. Compounds of interest eluted around 6 min. The column eluate entered the MS through a 450°C heated turbo ion spray with a +5,500 V differential. Analysis of the subsequent ion stream was accomplished by selecting for the Q1/Q3 transition pairs 891/216 (amu) for L12ADT and 856/216 for S12ADT.

**Stability of HPMA-JHPD**

(a) Stability in 1× PBS: A solution of HPMA-JHPD corresponding to a 12ADT amount of 50 µmol/L was dissolved in 1× PBS. PSA was added such that the final PSA level was 5 µg/mL. At 24 h, the reaction was stopped by adding acetonitrile with 0.1% formic acid and 500 mmol/L serine-12ADT, followed by LC-MS/MS analysis as described. The concentration of L12ADT released from HPMA-JHPD was determined based on the standard curves of L12ADT on the LC-MS/MS.

(b) Stability in plasma: The experiment was carried out in identical fashion as stated above with the following exceptions. The HPMA-JHPD was dissolved in 50% freshly extracted mouse plasma in 1× PBS. The plasma proteins were separated by precipitation using acetonitrile, followed by spinning for 5 min at 21,000 × g.

**PSA Hydrolysis of HPMA-JHPD**

Solutions of JHPD and HPMA-JHPD containing equimolar amounts of 12ADT were prepared in PSA buffer (50 mmol/L Tris, 100 mmol/L NaCl, 0.2% bovine serum albumin). PSA was added to both solutions such that the final PSA level was 5 µg/mL. For each time point, the reaction was stopped by adding acetonitrile. Further analysis was carried out in similar fashion as described above for the stability studies.

**Micelle Formation of HPMA-JHPD**

The critical micelle formation was determined by fluorometry using the ratio of the fluorescence of pyrene at 373 and 384 nm (I1/I3) when excited by a wavelength of 335 nm. Briefly, HPMA copolymers were dissolved in 1 µmol/L pyrene in 1× PBS in a quartz cuvette. Subsequent dilutions gave samples of lower concentrations. Intensity ratios were plotted against sample concentration, and micelle formation was evaluated graphically. To validate the assay, SDS was used as a positive control and ethanol as a negative control. Fluorescence was measured using a cuvette-based fluorometer (PTI).

**In vitro Hydrolysis of HPMA-JHAMC**

Approximately 20,000 LNCaP cells and 5,000 TSU cells growing in serum-containing media were transferred to serum-free media containing the synthetic androgen R1881 at 10 nmol/L. R1881 is added to stimulate maximum PSA production by LNCaP cells. Subsequently, varying amounts of HPMA-JHAMC were added to the cells. In additional studies, 5 µg/mL of exogenous enzymatically active PSA was also added to the LNCaP cell media. The cells were incubated at 37°C, and the fluorescence from the wells was periodically measured at the AMC excitation wavelength of 355 nm and emission wavelength of 460 nm.

**In vitro Viability of HPMA-JHPD – Treated CWR22Rv1 Cells**

Approximately 5,000 CWR22Rv1 cells were seeded in 96-well plates and permitted to attach overnight. On the following day, the media was replaced with 100 µL of serum-free media containing PSA at a final concentration of 5 µg/mL. The cells were then exposed to HPMA-JHPD or JHPD at concentrations of 10, 1, and 0.1 µmol/L. At the end of 4 days, effects on cell growth were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega) according to manufacturer’s instructions.

**Determination of L12ADT Levels in Xenografts**

HPMA-JHPD at a concentration of 12.1 mg/mL in 1× PBS and corresponding to 500 nmol/L of drug equivalent of L12ADT was injected via tail vein into cas-tated CWR22H-bearing mice. The average starting tumor size was ~0.1 cm³. For acute toxicity studies, mice (n = 3 per group) received either a single or four consecutive daily doses of HPMA-JHPD and were sacrificed after 24 h. The plasma, tumor, and skeletal muscle were harvested from sacrificed animals. The tissue was homogenized in a 3-fold excess of 2× COMPLETE protease inhibitor cocktail in water (Roche). The homogenate was then added to a 2-fold excess of 500 nmol/L S12ADT internal standard in acetonitrile containing 0.1% formic acid to precipitate protein. Separation was carried out by spinning at 21,000 × g for 5 min. The L12ADT content of the supernatant was carried out by LC-MS/MS as described above.

**Results**

**Characterization of HPMA-JHAMC and HPMA-JHPD**

The estimated number average molecular weight of HPMA-JHAMC was determined to be 87 kDa based on size exclusion chromatography. The polydispersity was 1.85. Absorbance studies suggested the mole fraction of JHAMC to be 0.7%. Similar analysis for HPMA-JHPD resulted in a number average molecular weight of 196 kDa with a polydispersity of 1.21. Spectrophotometry by absorption at 276 nm confirmed the presence of tyrosine on the copolymer. These studies suggested the mole fraction of JHAMC to be 0.7%. Similarly, amino acid analysis for the HPMA-JHPD suggested the JHPD content of HPMA-JHPD to be 0.63 mol% which translates to 6.4% (w/w). The efficiency of the integration of JHAMC into the HPMA-JHPD backbone was 71.4%. These data have been summarized in Table 1.

**Enzymatic Hydrolysis of HPMA-JHAMC by PSA**

The addition of PSA to the HPMA-JHAMC produced an increase in relative fluorescence units over time (Fig. 2, inset).
consistent with release of AMC from HPMA-JHAMC by PSA. In contrast, no change in fluorescence value over time was observed in the absence of PSA, demonstrating the stability of the copolymer in aqueous media. These results show that PSA is capable of specifically hydrolyzing the peptide when coupled to the high molecular weight of HPMA.

**Hydrolysis of HPMA-JHAMC by Cell Lines In vitro**

Previously, we have shown that the human prostate cancer cell line LNCaP produces low levels of PSA (i.e., ng/mL) in vitro compared with levels produced by human prostate cancer tissue isolated from patients with prostate cancer (i.e., µg/mL; ref. 7). In addition, it has also been shown that only ~10% to 15% of the PSA produced by these LNCaP cells in vitro is enzymatically active. Therefore, minimal hydrolysis of the HPMA-JHAMC was observed when added to conditioned media of LNCaP cells containing 5 ng/mL of PSA or to the media of non-PSA-producing TSU human bladder cancer cells (Fig. 2). These results document the need for higher levels of active PSA to hydrolyze the HPMA-JHAMC. In addition, they show that HPMA-JHAMC is stable to nonspecific hydrolysis by other proteases present in the conditioned media of these cancer cell lines.

Previous studies have shown that HPMA-based copolymers can be endocytosed by cancer cells (19). To document that significant concentrations of HPMA-JHAMC remain in the extracellular fluid accessible to secreted PSA, exogenous enzymatically active PSA was added to the serum-free tissue culture media of LNCaP cells at PSA concentrations that more closely model those observed in the extracellular fluid of human prostate cancer (i.e., 5 µg/mL). In this experiment, HPMA-JHAMC was readily hydrolyzed by PSA consistent with the presence of significant quantities of HPMA-JHAMC in the conditioned media (Fig. 2). These results also confirm the requirement for significant levels of active PSA in the extracellular peritumoral fluid for hydrolysis of HPMA-JHAMC (Fig. 2).

**PSA Hydrolysis of HPMA-JHPD**

Because PSA was able to hydrolyze AMC from the HPMA-JHAMC, we proceeded to test the hydrolysis of HPMA-JHPD by PSA. Previously, we had observed similar kinetics for PSA hydrolysis of the HSSKLQ peptide coupled to either AMC or L12ADT (3). However, in the case of HPMA-JHPD, the presence of the polymer meant that the rate of enzymatic hydrolysis was expected to be slower compared with the rate of hydrolysis of the uncoupled prodrug. Whereas formal kinetic studies were not done, the rate of hydrolysis of HPMA-JHPD (Fig. 3A) was of the same order of magnitude as the rate of hydrolysis of HPMA-JHAMC (Fig. 2, inset). In contrast, at equimolar concentrations of 12ADT (i.e., 50 µmol/L), HPMA-JHPD was hydrolyzed ~13-fold slower than the uncoupled prodrug due to the presence of the

**Figure 2.** JHAMC copolymer is hydrolyzed by active PSA. Measurement of the release of fluorescent molecule AMC from the HPMA-copolymer (final concentration, 1.25 mg/mL) when incubated in serum-free media of low PSA-producing LNCaP prostate cancer cells; PSA-negative TSU human bladder cancer cells and LNCaP cells growing in serum-free media containing exogenously added active PSA at a final concentration of 5 µg/mL. Points, average fluorescence units at indicated time points; bars, SE. All experiments run in triplicate. Inset, release of AMC from HPMA-JHAMC in the presence of purified active PSA (5 µg/mL) in buffer.
polymer. However, the rate of hydrolysis was sufficient to generate 5 μmol/L of L12ADT in the buffer after 2 days (Fig. 3A).

**Stability of HPMA-JHPD in 1× PBS and Plasma**

The lack of significant hydrolysis of HPMA-JHAMC in the conditioned media of cancer cell lines suggested that the copolymer was relatively stable to nonspecific hydrolysis. Because the PSA-activated prodrug copolymer will be injected systemically into the blood, the goal of the next set of experiments was to determine if this prodrug construct was also stable in human plasma. Therefore, the HPMA-JHPD was incubated in human plasma for 24 h, then extracted and subjected to LC-MS/MS analysis. In this study, no appreciable L12ADT (i.e., <1 nmol) was detected, suggesting that HPMA-JHPD was stable to nonspecific hydrolysis in both buffer and plasma (Fig. 3A). In addition, to determine if additional degradation products of the HPMA-JHPD were produced after 24-h incubation in plasma, the total ion chromatogram for the extracted eluent was also obtained. In this evaluation, no new mass peaks corresponding to unknown degradation products were observed (data not shown).

**Micelle Formation of HPMA-JHPD**

Thapsigargin is a highly lipophilic compound, and the addition of the 12-carbon aliphatic side chain to produce the 12ADT analogue would significantly increase its hydrophobicity. Previously, we showed that coupling this hydrophobic 12ADT to a charged peptide produces an amphipathic species that is capable of forming micelles at high micromolar concentrations (20). To evaluate HPMA-JHPD micellar formation, we did assays using pyrene based on prior observations that the ratio I$_1$/I$_3$ of pyrene decreases with a decrease in the polarity of the solvent. Upon addition of increasing concentrations of HPMA-JHPD at room temperature to a solution of pyrene, a decrease in the ratio was observed consistent with the formation of micelles in solution (Fig. 3B). This result suggests that amphipathic prodrug part of HPMA-JHPD, although incorporated into a high molecular weight polymer network, could orient itself such that the hydrophobic 12ADT side chains of the copolymer may be “hidden” from the bulk aqueous phase containing active PSA. These results suggest that further studies are needed to identify an optimal formulation for in vivo administration of HPMA-JHPD that will minimize micellar formation in the dosing solution.

**Antitumor Effects of HPMA-JHPD Against PSA-Producing Tumor Cells In Vitro**

The observed formation of micelles at high micromolar concentrations set an upper limit for cell-based cytotoxicity assays. These assays were done using the cell-based prostate cancer cell line CWR22R. This cell line produces low levels of total PSA (i.e., low ng/mL). Immunofluorescence analysis of CWR22R conditioned media revealed that this line produces even less percentage of enzymatically active PSA (i.e., <5% active) compared with LNCaP. CWR22R cells, however, are androgen receptor positive and grow well in tissue culture compared with LNCaP and were therefore selected for this experiment as a representative human prostate cancer cell line.

In the absence of exogenous PSA, the HPMA-JHPD exhibited no cytotoxicity (i.e., growth rate equal to controls at 10 μmol/L) to CWR22R cells. These results show that no significant hydrolysis of the HPMA-JHPD to release active L12ADT occurred in the absence of active PSA. However, addition of exogenous enzymatically active PSA in the CWR22R media to a final concentration of 5 μg/mL resulted in a concentration-dependent inhibition of cell growth (Fig. 4). These results show that HPMA-JHPD prodrug activation only occurs to a significant degree in the presence of enzymatically active PSA. The GI$_{50}$ value for HPMA-JHPD is <100 nmol/L as determined by the ratio of growth of treated cells to that of the untreated cells (Fig. 4).

**Accumulation of L12ADT in Tumor Tissue In Vivo**

Previous studies have shown that polymers can accumulate in solid tumors via the EPR effect (11, 13). Based on the observed hydrolysis of HPMA-JHPD by PSA in vitro, we evaluated the uptake and release of L12ADT within PSA-producing tumors after i.v. administration of HPMA-JHPD. Due to its high molecular weight and lack of spectrophotometric qualities, tumor and plasma levels of noncleaved HPMA-JHPD versus cleaved HPMA-JHPD cannot be easily measured or discriminated by LC-MS/MS analysis. Therefore, in these in vivo studies, we focused on measurement of free L12ADT in plasma and PSA-producing tumors after single and multiple i.v. injections. As a tissue comparison, we also determined levels of L12ADT in PSA-negative skeletal muscle from treated animals.

In these experiments, animals bearing PSA-producing CWR22R-H xenografts received i.v. dose of 100 μL of a 500 μmol/L L12ADT equivalent solution (50 nmol of total L12ADT) of HPMA-JHPD as either a single injection or 4× daily. The CWR22R-H model was derived from the CWR22 xenograft model, which is an androgen-sensitive tumor that produces relatively high levels of PSA (~10% of that produced by human prostate cancer) compared with other prostate cancer xenografts. CWR22 xenografts regress after castration with well-defined kinetics. A CWR22R-H was produced by serial passage of CWR22 xenograft tissue in castrated mice. Evaluation of PSA isoforms in the blood of CWR22R-H–bearing animals showed that the majority (~85%) of the PSA produced by these CWR22R-H xenografts is enzymatically active as it is able to form covalent complexes with the serum protease inhibitor α-1-antichymotrypsin.

At 24 h after the last dose (i.e., nadir), treated tumor-bearing animals were sacrificed and plasma and tissue were obtained. Acetonitrile extracted plasma and tissue...
was analyzed using LC-MS/MS to determine the levels of L12ADT. After a single dose, ~3-fold to 4-fold higher concentrations of L12ADT was detected in the tumor compared with plasma or skeletal muscle (Fig. 5). After four consecutive doses, nadir plasma levels were not significantly different than those measured after a single dose (Fig. 5). In contrast, L12ADT nadir levels in the tumor (f350 nmol/L) after four doses were 15-fold higher than plasma levels and 3.5-fold higher than levels in skeletal muscle (Fig. 5). These results are consistent with release of L12DT within the tumor tissue by enzymatically active PSA present in the peritumoral fluid. In this experiment, no significant effect on tumor size in the treated versus control group was observed on the day of tumor harvest (i.e., day 5). In addition, no animal deaths or significant weight loss (<5% weight loss compared with starting weight) was observed in the HPMA-JHPD–treated group.

Discussion
HPMA-based copolymers have been used in a variety of different strategies for purposes of targeted drug delivery. Copolymers of HPMA have been synthesized

![Figure 3. A, hydrolysis of HPMA-JHPD at a concentration equivalent to 50 μmol/L L12DAT. HPMA-JHPD copolymer was incubated with 5 μg/mL active PSA in aqueous buffer, and aliquots were analyzed by LC-MS/MS for L12ADT release at indicated time points. B, determination of micelle formation using a pyrene-based fluorometric assay. SDS (millimolar concentrations) was used as a positive control to validate the assay. G115 and HPMA-JHPD (micromolar concentrations) showed decrease in the ratio of fluorescence (373 nm/384 nm) with increasing concentrations. No micellar formation was observed with ethanol over a range of concentrations (0.2–50% v/v; data not shown).](image)
with small molecule drugs linked via nonspecifically hydrolyzed ester linkages to Gly-Phe-Leu-Gly–based linkages, which are then specifically hydrolyzed by lysosomal cathepsin B (19) after endocytosis. This use of HPMA compares with approaches in which higher molecular weight PEG is attached to cytotoxins via labile ester linkages. However, the distinct advantage that HPMA enjoys over PEG is very high multivalency, wherein multiple drug or targeting molecules can be conjugated to the same polymer molecule. This leads to drug loading levels as high as 10% by weight of the polymer, which is in contrast to linear PEG, which at maximum contains only two functional groups at the ends of the PEG polymer suitable for polymerization (e.g., Prothecan, which is a PEG-camptothecin conjugate, is only 1.7% by weight camptothecin; refs. 21, 22).

In this work, we further develop the strategy of HPMA conjugation by synthesizing a copolymer of HPMA with a peptidic prodrug that can be cleaved in the extracellular matrix by a specific secreted protease, PSA. This strategy has several advantages. First, it incorporates a dual targeting approach based on both the EPR effect and protease activation. The EPR effect has been suitably shown to target tumors passively as depicted in Fig. 1. We further enhance this passive EPR-based targeting by adding a prodrug component that offers potential for more tumor-selective delivery of the cytotoxic molecule. In such a therapeutic copolymer, the passive targeting to

![Figure 4](image-url) Growth inhibition of CWR22R cells in culture after 4-d incubation with indicated concentrations of HPMA-JHPD compared with JHPD in serum-free media containing 5 μg/mL active PSA. Data show percentage inhibition of growth assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 4 d exposure to compounds in the presence of active PSA compared with growth of untreated control cells. Columns, average of eight replicates for indicated concentration; bars, SE.

![Figure 5](image-url) Levels of L12ADT at 24 h after i.v. injection (nadir) of HPMA-JHPD into nude mice bearing s.c. growing PSA-positive CWR22R-H xenografts. Columns, average concentration (n = 3 per group) of L12ADT in the plasma, tumor, and skeletal muscle 24 h after single or four daily injections; bars, SE. For tumor versus skeletal muscle or plasma levels of L12ADT, P < 0.05 after both one and four treatments.
the peritumoral space is achieved by the polymer through the EPR effect with specificity achieved by the requirement for activation by PSA, which is only present within sites of prostate cancer and the normal prostate.

There are several additional advantages to this approach. First, prodrug activation occurs in the PSA-rich extracellular fluid surrounding tumor cells. Therefore, every cell in the tumor does not need to produce PSA to be killed by the prodrug, thus overcoming potential resistance related to heterogeneity in level of PSA production by prostate cancer cells. The “bystander” effect achieved by this extracellular activation results in death of both PSA-positive prostate cancer cells and PSA-negative endothelial and stromal cancer cells. In addition, the released drug L12ADT is highly lipophilic and, upon hydrolysis, rapidly partitions into plasma membranes. Little, if any, of the L12ADT leaks back into circulation, further limiting nonspecific toxicity to normal tissue.

The in vitro and in vivo characterization of this HPMA copolymer-peptide prodrug is complicated by the high molecular weight of the HPMA and the lack of spectral characteristics of the L12ADT molecule. Therefore, quantification of the intact, nonhydrolyzed HPMA-prodrug copolymer was not possible using high performance liquid chromatography/LC-MS analysis. For this reason, our initial characterization work with the copolymer was carried out using the fluorophore AMC. Based on previous reports and studies in our laboratory with PEG-peptide conjugates, it was not clear at the outset if PSA would be able to hydrolyze the peptide-AMC substrate when incorporated into the HPMA copolymer structure. However, fluorescence data from the initial experiments established that the copolymer was cleaved by PSA while in solution, liberating the free AMC. The HPMA-JHAMC copolymer was also stable to nonspecific hydrolysis in both plasma and conditioned media from cancer cell lines. These in vitro studies with the HPMA-JHAMC copolymer suggested that a therapeutic index could be achievable in vivo with the HPMA-JHPD copolymer.

Toxicity studies against LNCaP cells in an in vitro assay clearly showed comparable growth inhibition between the HPMA copolymer and the free prodrug in the presence of sufficient concentration of active PSA. This suggests that a requisite amount of L12ADT is released from both forms to effect inhibition of growth. Furthermore, the copolymer has an IC₅₀ below 100 nmol/L (Fig. 4), which is also comparable with the previously reported IC₅₀ of L12ADT (3). Finally, in vivo work in mice establishes that multiple doses with the copolymer can achieve drug levels in the tumor that are considerably higher than the IC₅₀ of the HPMA copolymer. There is also a build-up of levels in the tumor that are considerably higher than the multiple doses with the copolymer can achieve drug L12ADT (3). Finally, the in vivo L12ADT leaks back into circulation, further limiting nonspecific toxicity to normal tissue.

The results from the multidosing component of the in vivo study show that this highly lipophilic L12ADT is not rapidly cleared by the tumor and accumulates with time.

Further studies are under way in our laboratory to assess the optimal drug formulation and dosing regimen for this HPMA copolymer based on toxicologic and pharmacokinetic studies that can then be used to assess antitumor efficacy against PSA-producing and PSA-nonproducing human cancer xenografts. In addition, we are developing methods to determine the levels of total HPMA-prodrug present within the tumor and normal tissue to understand the extent of targeting via the EPR effect. Determination of total HPMA-prodrug levels will also allow us to gauge the extent of PSA hydrolysis within the PSA-producing tumor. Such insights will be useful for future studies that attempt to optimize targeted delivery using this HPMA-copolymer–based approach.

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References


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A prostate-specific antigen–activated \(N\)-(2-hydroxypropyl) methacrylamide copolymer prodrug as dual-targeted therapy for prostate cancer

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