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molecular pharmaceutics

Biodistribution of HPMA Copolymer-Aminohexylgeldanamycin-RGDfK Conjugates for Prostate Cancer Drug Delivery

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Received May 14, 2009; Revised Manuscript Received August 4, 2009; Accepted September 10, 2009

Abstract: *N*-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer-RGD (Arg-Gly-Asp) conjugates targeting the $\alpha_v\beta_3$ integrin present on angiogenic blood vessels and some tumor types have shown increased accumulation in solid tumors and possess properties that suggest their use for site-specific drug delivery. Geldanamycin (GDM) is a benzoquinoid ansamycin that binds to heat-shock protein 90 (HSP90), effective for the treatment of multiple cancer types including prostate, but has dose-limiting cytotoxicity. We recently reported the synthesis of HPMA copolymer-aminohexyl-geldanamycin (AH-GDM) conjugates containing RGDfK that demonstrated favorable properties of drug release, in vitro binding to the $\alpha_v\beta_3$ integrin, cytotoxicity in human prostate cancer cells, and tolerability in nude mice greater than 2-fold equivalent free drug doses. In this study the biodistribution of ^{125}I-radiolabeled HPMA copolymer-AH-GDM conjugates with and without RGDfK in both non-tumor-bearing and DU145 prostate tumor xenograft-bearing nude mice was evaluated. At 60 mg/kg drug equivalent polymer doses in non-tumor-bearing mice both conjugates showed fast elimination from blood and decreasing accumulation in all other organs. Kidney accumulation predominated and was higher for the conjugate containing RGDfK. In tumor-bearing mice, trace quantities of the conjugate containing RGDfK showed increased tumor accumulation as compared to the conjugate without RGDfK. Also evaluated were free drug concentrations in prostate tumor xenografts following treatments of 30 and 60 mg/kg drug-equivalent copolymer conjugates (with and without RGDfK) compared with 30 mg/kg free AH-GDM. Overall, 60 mg/kg treatment of RGDfK-containing conjugate showed significantly higher ($p < 0.001$) tumor drug concentrations compared with all other treatments. The targetable conjugates can effectively deliver higher amounts of geldanamycin to the tumor compared to nontargetable systems.

Keywords: Geldanamycin; HPMA copolymer; RGDfK; targeted delivery; prostate cancer

Introduction

Recent advances in polymeric drug delivery systems have improved the chemotherapeutic options available to cancer patients.[1] The use of targeted therapies aids the continued development of highly effective therapies designed to target the tumor.[2] Copolymers based on *N*-(2-hydroxypropyl)-

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10.1021/mp900134c CCC: $40.75
Published on Web 09/11/2009

methacrylamide (HPMA) are such drug carriers undergoing clinical investigations for chemotherapeutic delivery because of their macromolecular and biologically compatible nature.[3] Targeted HPMA copolymer-based drug delivery systems have been previously investigated for their ability to deliver radioisotopes to solid prostate and lung tumor xenografts in mice.[4−7] These conjugates contained cyclic Arg-Gly-Asp (RGD) peptide sequences that target $\alpha_v\beta_3$ integrins expressed on angiogenic blood vessels and tumor cells.[8,9] Increased tumor accumulation of these conjugates compared to non-targeted conjugates and peptide alone was identified and set the stage for the use of these copolymers for the delivery of bioactive agents to solid tumors.

Geldanamycin (GDM) is a benzoquinoid ansamycin that binds to the chaperone protein heat-shock protein 90 (HSP90), inhibiting its ability to fold client proteins into their active conformation.[10,11] Many of these proteins (Lattouf et al.[12]) are required for cancer cell survival and progression.[13,14] Inhibition of HSP90 clients such as the wild-type and mutated androgen receptor, human epidermal growth factor receptor 2 (HER2) and protein kinase B offers particular advantages in GDM therapy for treatment of prostate cancer, among many other cancer types.[12] Geldanamycin analogues 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) were developed following the preclinical failure of the parent compound due to hepatotoxicity[15] and are currently progressing in clinical trials.[16−20] These analogues were designed to decrease the toxicity of the parent compound while maintaining or increasing potency. While these efforts have been successful to a given extent, clinical response continues to be limited.[12,20]

In a previous work,[21] we reported the synthesis of HPMA copolymers containing aminohexyl-geldanamycin (AH-GDM) and the targeting peptide RGDfK. AH-GDM is

(1) Duncan, R. Polymer conjugates as anticancer nanomedicines. *Nat. Rev. Cancer* **2006**, *6*, 688–701.

(2) Li, C.; Wallace, S. Polymer-drug conjugates: recent development in clinical oncology. *Adv. Drug Delivery Rev.* **2008**, *60*, 886–98.

(3) Kopecek, J.; Kopeckova, P.; Minko, T.; Lu, Z. HPMA copolymer-anticancer drug conjugates: design, activity, and mechanism of action. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 61–81.

(4) Line, B. R.; Mitra, A.; Nan, A.; Ghandehari, H. Targeting tumor angiogenesis: comparison of peptide and polymer-peptide conjugates. *J. Nucl. Med.* **2005**, *46*, 1552–60.

(5) Mitra, A.; Coleman, T.; Borgman, M.; Nan, A.; Ghandehari, H.; Line, B. R. Polymeric conjugates of mono- and bi-cyclic alphaV-beta3 binding peptides for tumor targeting. *J. Controlled Release* **2006**, *114*, 175–83.

(6) Mitra, A.; Mulholland, J.; Nan, A.; McNeill, E.; Ghandehari, H.; Line, B. R. Targeting tumor angiogenic vasculature using polymer-RGD conjugates. *J. Controlled Release* **2005**, *102*, 191–201.

(7) Mitra, A.; Nan, A.; Papadimitriou, J. C.; Ghandehari, H.; Line, B. R. Polymer-peptide conjugates for angiogenesis targeted tumor radiotherapy. *Nucl. Med. Biol.* **2006**, *33*, 43–52.

(8) Brooks, P. C.; Clark, R. A.; Cheresh, D. A. Requirement of vascular integrin alphaVbeta 3 for angiogenesis. *Science* **1994**, *264*, 569–71.

(9) Hood, J. D.; Cheresh, D. A. Role of integrins in cell invasion and migration. *Nat. Rev. Cancer* **2002**, *2*, 91–100.

(10) Whitesell, L.; Mimnaugh, E. G.; De Costa, B.; Myers, C. E.; Neckers, L. M. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 8324–8.

(11) Schulte, T. W.; Neckers, L. M. The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. *Cancer Chemother. Pharmacol.* **1998**, *42*, 273–9.

(12) Lattouf, J. B.; Srinivasan, R.; Pinto, P. A.; Linehan, W. M.; Neckers, L. Mechanisms of disease: the role of heat-shock protein 90 in genitourinary malignancy. *Nat. Clin. Pract. Urol.* **2006**, *3*, 590–601.

(13) Rocchi, P.; So, A.; Kojima, S.; Signaevsky, M.; Beraldi, E.; Fazli, L.; Hurtado-Coll, A.; Yamanaka, K.; Gleave, M. Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer. *Cancer Res.* **2004**, *64*, 6595–602.

(14) Solit, D. B.; Scher, H. I.; Rosen, N. Hsp90 as a therapeutic target in prostate cancer. *Semin. Oncol.* **2003**, *30*, 709–16.

(15) Supko, J. G.; Hickman, R. L.; Grever, M. R.; Malspeis, L. Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent. *Cancer Chemother. Pharmacol.* **1995**, *36*, 305–15.

(16) Banerji, U.; O'Donnell, A.; Scurr, M.; Pacey, S.; Stapleton, S.; Asad, Y.; Simmons, L.; Maloney, A.; Raynaud, F.; Campbell, M.; Walton, M.; Lakhani, S.; Kaye, S.; Workman, P.; Judson, I. Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. *J. Clin. Oncol.* **2005**, *23*, 4152–61.

(17) Goetz, M. P.; Toft, D.; Reid, J.; Ames, M.; Stensgard, B.; Safgren, S.; Adjei, A. A.; Sloan, J.; Atherton, P.; Vasile, V.; Salazaar, S.; Adjei, A.; Croghan, G.; Erlichman, C. Phase I trial of 17-allylamino-17-demethoxygeldanamycin in patients with advanced cancer. *J. Clin. Oncol.* **2005**, *23*, 1078–87.

(18) Nowakowski, G. S.; McCollum, A. K.; Ames, M. M.; Mandrekar, S. J.; Reid, J. M.; Adjei, A. A.; Toft, D. O.; Safgren, S. L.; Erlichman, C. A phase I trial of twice-weekly 17-allylamino-demethoxy-geldanamycin in patients with advanced cancer. *Clin. Cancer. Res.* **2006**, *12*, 6087–93.

(19) Ramanathan, R. K.; Egorin, M. J.; Eiseman, J. L.; Ramalingam, S.; Friedland, D.; Agarwala, S. S.; Ivy, S. P.; Potter, D. M.; Chatta, G.; Zuhowski, E. G.; Stoller, R. G.; Naret, C.; Guo, J.; Belani, C. P. Phase I and pharmacodynamic study of 17-(allylamino)-17-demethoxygeldanamycin in adult patients with refractory advanced cancers. *Clin. Cancer Res.* **2007**, *13*, 1769–74.

(20) Ronnen, E. A.; Kondagunta, G. V.; Ishill, N.; Sweeney, S. M.; Deluca, J. K.; Schwartz, L.; Bacik, J.; Motzer, R. J. A phase II trial of 17-(Allylamino)-17-demethoxygeldanamycin in patients with papillary and clear cell renal cell carcinoma. *Invest. New Drugs* **2006**, *24*, 543–6.

(21) Borgman, M. P.; Ray, A.; Kolhatkar, R. B.; Sausville, E. A.; Burger, A. M.; Ghandehari, H. Targetable HPMA copolymer-aminohexylgeldanamycin conjugates for prostate cancer therapy. *Pharm. Res.* **2009**, *26*, 1407–18.

(22) Strohalm, J.; Kopecek, J. Poly N-(2-hydroxypropyl) methacrylamide: 4. Heterogenous polymerization. *Angew. Makromol. Chem.* **1978**, *70*, 109–18.

(23) Lee, J. H.; Kopeckova, P.; Kopecek, J.; Andrade, J. D. Surface properties of copolymers of alkyl methacrylates with methoxy (polyethylene oxide) methacrylates and their application as protein-resistant coatings. *Biomaterials* **1990**, *11*, 455–64.

attached to copolymer side chains via a Gly-Phe-Leu-Gly (GFLG) peptide spacer that is degraded intracellularly by lysosomal enzymes resulting in drug release.[3] These systems demonstrated the ability for in vitro drug release, actively bound to the $\alpha_v\beta_3$ integrin in vitro, and displayed in vitro cytotoxicity comparable to that of free AH-GDM. Upon single administration of drug-equivalent doses of AH-GDM and copolymer conjugates in vivo the conjugates demonstrated a marked increase in tolerability over 2-fold when compared with free drug.

The purpose of the current study was to further the advancement of these targeted drug delivery systems by testing their in vivo properties for delivering the active agent AH-GDM to prostate tumors. We hypothesized that the copolymer−drug conjugates targeted to tumor vasculature can deliver significantly higher amounts of drug to the tumor environment than can be achieved through delivery of free drug, thereby increasing the therapeutic index with higher local doses and decreased toxicity to background organs. Studies demonstrating the in vivo biodistribution of radiolabeled conjugates and in vivo tumor drug accumulation are reported herein.

Experimental Section

Chemicals. Geldanamycin (NSC 122750) was kindly supplied by the National Cancer Institute Developmental Therapeutics Program and was protected from light during all procedures. RGDfK (MW 604.5) was obtained from AnaSpec, Inc. (San Jose, CA). ^{125}I-Echistatin (2000 Ci/mmol) was purchased from Perkin-Elmer (Waltham, MA). Iodine-125 (100mCi/mL) was obtained as $Na^{125}I$ from Perkin-Elmer in 10^{-5} M NaOH. All amino acids used were of L-configuration. All other chemicals were of reagent grade as obtained from Sigma Chemical Co (St. Louis, MO).

Synthesis and Characterization of AH-GDM Derivative and Comonomers. *N*-(2-Hydroxypropyl)methacrylamide (HPMA);[22] *N*-methacryloyl-tyrosinamide (MA-Tyr);[23] *N*-methacryloylglycylglycyl-*p*-nitrophenyl ester (MA-GG-ONp);[24] *N*-methacryloyl-glycylphenylalanylleucylglycine-*p*-nitrophenyl ester (MA-GFLG-ONp);[25] 17-(6-aminohexylamino)-17-demethoxygeldanamycin (AH-GDM);[21,26−28] and *N*-methacryloylglycylphenylalanylleucylglycl-17-(6-aminohexylamino)-17-demethoxygeldanamycin (MA-GFLG-AH-GDM)[21,26−28] were synthesized and characterized according to previously described methods. Water-soluble GDM derivative AH-GDM hydrochloride was synthesized according to a previously described method[21] and used for animal studies.

Synthesis and Characterization of HPMA Copolymer Conjugates. HPMA copolymer precursor was synthesized via free radical precipitation copolymerization of comonomers in 10% v/v anhydrous dimethyl sulfoxide (DMSO) in acetone using *N*,*N*′-azobisisobutyronitrile (AIBN) as the initiator.[22] The feed composition of comonomers for copolymer precursor was 20 mol % for MA-GG-ONp, 5 mol % for MA-GFLG-AH-GDM, 2 mol % for MA-Tyr and 73 mol % for HPMA. The comonomer mixture was sealed in an ampule under nitrogen and stirred at 50 °C for 24 h. Solvent was removed by rotary evaporation, copolymer precursor dissolved in methanol and precipitated and washed in diethyl ether. MA-GG-ONp content in the polymeric precursor was assessed by release of *p*-nitrophenol (ONp) from the copolymer in 1.0 N sodium hydroxide by UV spectrophotometry (400 nm). Weight average molecular weight (M_w) and polydispersity (M_w/M_n) were estimated by size exclusion chromatography (SEC) on a Superose 12 column (10 mm × 30 cm) (GE Healthcare, Piscataway, NJ) with fractions of known molecular weight HPMA copolymers using a fast protein liquid chromatography (FPLC) system (GE Healthcare).

HPMA copolymer-AH-GDM-RGDfK conjugate (P1) (Figure 1) was synthesized via *p*-nitrophenyl ester aminolysis of polymeric precursors in dry DMF in the presence of pyridine for 48 h.[6] The reaction was terminated with 0.1 N sodium hydroxide and DMF removed under vacuum. Copolymer precipitates were dissolved in deionized water and purified using Amicon Ultra-15 (MWCO 3000, Millipore, Billerica, MA) ultracentrifugal tubes to remove small molecular weight impurities. HPMA copolymer-AH-GDM conjugate (P2) was formed by hydrolyzing the precursor ONp groups with 0.1 N sodium hydroxide followed by purification using the same method as for P1. AH-GDM content was determined by UV spectrophotometric analysis of copolymer product P2 (without RGDfK) using $\varepsilon^{340nm} = 2.16 \times 10^4\ M^{-1}\ cm^{-1}$ in DMSO. The peptide and MA-Tyr content of copolymer conjugate P1 was determined by amino acid analysis (Commonwealth Biotechnologies, Richmond, VA).

^{125}I Radiolabeling of Conjugates. The copolymers were labeled via conjugation of ^{125}I to tyrosinamide groups incorporated using the Iodogen method as previously described.[29] Briefly, 3 mg of copolymer conjugates were

(24) Rejmanova, P.; Labsky, J.; Kopecek, J. Aminolyses of monomeric and polymeric p-nitrophenyl esters of methacryloylated amino acids. *Makromol. Chem.* **1977**, *178*, 2159–68.

(25) Ulbrich, K.; Subr, V.; Strohalm, J.; Plocova, D.; Jelinkova, M.; Rihova, B. Polymeric drugs based on conjugates of synthetic and natural macromolecules. I. Synthesis and physico-chemical characterisation. *J. Controlled Release* **2000**, *64*, 63–79.

(26) Kasuya, Y.; Lu, Z. R.; Kopeckova, P.; Minko, T.; Tabibi, S. E.; Kopecek, J. Synthesis and characterization of HPMA copolymer-aminopropylgeldanamycin conjugates. *J. Controlled Release* **2001**, *74*, 203–11.

(27) Kasuya, Y.; Lu, Z. R.; Kopecková, P.; Tabibi, S. E.; Kopecek, J. Influence of the structure of drug moieties on the in vitro efficacy of HPMA copolymer-geldanamycin derivative conjugates. *Pharm. Res.* **2002**, *19*, 115–23.

(28) Kasuya, Y.; Lu, Z.; Kopeckova, P.; Kopecek, J. Improved synthesis and evaluation of 17-substituted aminoalkylgeldanamycin derivatives applicable to drug delivery systems. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2089–91.

(29) Salacinski, P. R.; McLean, C.; Sykes, J. E.; Clement-Jones, V. V.; Lowry, P. J. Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenyl glycoluril (Iodogen). *Anal. Biochem.* **1981**, *117*, 136–46.

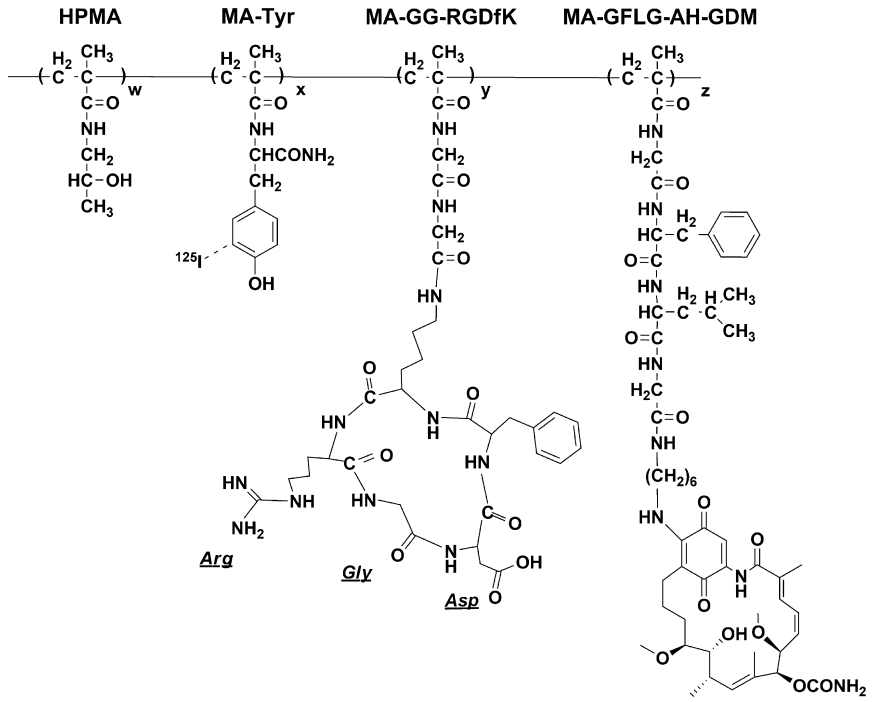

Figure 1. Structure of HPMA copolymer-RGDfK-AH-GDM conjugates. Copolymers P1 and P2 contained the geldanamycin derivative AH-GDM as well as MA-Tyr to label the conjugate with ^{125}I. P1 contained the monocyclized RGDfK peptide targeting moiety to target the $\alpha_v\beta_3$ integrin.

dissolved in 0.5 mL of PBS and added to IODO-GEN tubes (Pierce, Rockford, IL) along with 0.5 mCi of ^{125}I in 0.2 mL saline. The solutions were mixed and incubated for 30 min at room temperature with intermittent gentle agitation. Following incubation, the labeled conjugates were isolated in normal saline over a Sephadex G-25 (PD-10) column (GE Healthcare, Piscataway, NJ).

Cell Lines. DU145 prostate cancer cells (ATCC, Manassas, VA) were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 4 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% 100× antibiotic-antimycotic (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 (v/v). Human umbilical vein endothelial cells[30] (HUVECs) were cultured in endothelial cell growth media-2 (EGM-2: Lonza, Walkersville, MD) at 37 °C in a humidified atmosphere of 5% CO_2 (v/v). For all experimental procedures, subconfluent cells in 24 h culture were harvested with 0.05% trypsin/0.02% EDTA in PBS.

Mouse Xenograft Model of Human Prostate Cancer. DU145 prostate cancer cells were collected, washed, counted and resuspended in RPMI 1640 media. 5×10^6 cells (50 μL) mixed with 50 μL of Matrigel (Becton Dickinson Biosciences, Bedford, MA) were injected subcutaneously in the flank of each female athymic NCr-*nu/nu* mice (Animal Production Facility at NCI Frederick, MD) (6−8 weeks old, 20−25 g). After 28 days postinjection, tumor volume reached approximately 400 mm^3 and experiments were initiated. All studies were conducted under an approved protocol of the University of Maryland Baltimore Institutional Animal Care and Use Committee (IACUC).

Cell Receptor Binding Assay. The binding affinities of free RGDfK peptide and HPMA copolymer-AH-GDM-RGDfK conjugate (P1) were assessed using a competitive binding assay to $\alpha_v\beta_3$ integrin expressed on HUVECs with ^{125}I-echistatin.[31−33] HUVECs were harvested, washed with PBS, resuspended in binding buffer (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L $MnCl_2$, 0.1% BSA) and seeded in 96-well Multiscreen HV filter plates (0.45 μm; Millipore) at 50,000 cells per well. Cells were coincubated at 4 °C for 2 h with ^{125}I-echistatin (0.05 nM) and increasing peptide equivalent concentrations of copolymers or free RGDfK (0−500 μM), and final volume was adjusted to 200 μL all in binding buffer. Following incubation, the plates were filtered using a Multiscreen vacuum manifold (Millipore) and washed twice with cold binding buffer. Filters were harvested and radioactivity was determined by γ-counting (Perkin-Elmer Wizard,

(30) Rhim, J. S.; Tsai, W. P.; Chen, Z. Q.; Chen, Z.; Van Waes, C.; Burger, A. M.; Lautenberger, J. A. A human vascular endothelial cell model to study angiogenesis and tumorigenesis. *Carcinogenesis* **1998**, *19*, 673–81.

(31) Wu, Y.; Zhang, X.; Xiong, Z.; Cheng, Z.; Fisher, D. R.; Liu, S.; Gambhir, S. S.; Chen, X. microPET imaging of glioma integrin alphaVbeta3 expression using (64)Cu-labeled tetrameric RGD peptide. *J. Nucl. Med.* **2005**, *46*, 1707–18.

(32) Kumar, C. C.; Nie, H.; Rogers, C. P.; Malkowski, M.; Maxwell, E.; Catino, J. J.; Armstrong, L. Biochemical characterization of the binding of echistatin to integrin alphaVbeta3 receptor. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 843–53.

(33) Borgman, M. P.; Coleman, T.; Kolhatkar, R. B.; Geyser-Stoops, S.; Line, B. R.; Ghandehari, H. Tumor-targeted HPMA copolymer-(RGDfK)-(CHX-A″-DTPA) conjugates show increased kidney accumulation. *J. Controlled Release* **2008**, *132*, 193–99.

1470 automatic gamma counter) to determine percent bound ^{125}I-echistatin. Nonspecific binding was determined by incubating cells with a 200-fold excess of cold echistatin. Nonlinear regression analysis and determination of IC_{50} values was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Biodistribution Studies. Two biodistribution studies with radiolabeled copolymers were conducted. In the first study, non-tumor-bearing athymic nude mice were injected via the lateral tail with 200 μL of normal saline containing HPMA copolymer-AH-GDM-RGDfK (P1) or HPMA copolymer-AH-GDM (P2) at 60 mg/kg drug equivalent with 1 μCi ^{125}I-labeled copolymer conjugates as a tracer. Time dependent biodistribution studies were carried out by sacrificing mice at 15 min, 1, 6, 24, 48, and 72 h postinjection (p.i.). At the time of euthanasia, blood samples were collected by cardiac puncture. During necropsy, whole organ tissue samples were obtained from the heart, lung, liver, kidneys, spleen, small intestine, large intestine, stomach and skeletal muscle. Blood concentrations of ^{125}I-radiolabeled conjugates were calculated as μCi/mL from radioactivity counts and 2-compartmental pharmacokinetic analyses performed using WinNonlin (Pharsight Corp., Mountain View, CA).

In the second study, tumor-bearing mice were injected via the lateral tail vein with 200 μL of normal saline containing 4 nmol each of ^{125}I labeled HPMA copolymer-AH-GDM-RGDfK (P1) conjugate (4.5−4.7 μCi) or HPMA copolymer-AH-GDM (P2) conjugate (4.2−4.4 μCi) per mouse. Time dependent biodistribution studies were carried out by sacrificing mice at 1, 24, 48, and 72 h p.i. At the time of euthanasia, blood was collected as described above. During necropsy, whole organ tissue samples were obtained from the heart, lung, liver, kidneys, spleen, small intestine, large intestine, stomach and tumor.

In both studies tissue samples were washed with water, counted (Perkin-Elmer Wizard, 1470 automatic gamma counter), and weighed and the percentage-injected dose per gram tissue (% ID/g) was calculated. Tumor-to-background (T/B) ratios were calculated by dividing % ID/g results for the tumor by indicated background organ for each time point. All biodistribution studies were performed with three mice per group.

Evaluation of Tumor Drug Concentrations. Relative concentrations of drug in tumors following treatment with free drug or copolymer−drug conjugates were evaluated in DU145 prostate tumor-bearing mice. Mice were injected intravenously (iv) via the tail vein with sterile solutions of 30 and 60 mg/kg drug equivalent P1 and P2, and 30 mg/kg AH-GDM hydrochloride. Cohorts of 3 mice per treatment group were sacrificed at 4, 12, and 24 h p.i. Tumors were harvested and stored at −80 °C until analysis.

Concentrations of AH-GDM (via enzymatic drug release from copolymers or accumulation of free drug) in tumors was determined with HPLC using modification of previously described procedures.[21,34] Tumor samples were thawed and homogenized using a Powergen 125 homogenizer (Fisher Scientific, Pittsburgh, PA) in 1 part (weight to volume) PBS. 5 μL of 200 μg/mL 17-DMAG (internal standard, IS) in acetonitrile was added to a 250 μL sample of tumor homogenate and mixed. Samples were extracted with 1 mL of ethyl acetate (EtOAc) by vortexing and centrifuged at 14000*g* for 5 min, and the resulting organic layer was transferred to 12 × 75 mm glass culture tubes. The samples were extracted with an additional 1 mL of EtOAc, vortexed, centrifuged, organic layers combined with the first, and dried under nitrogen. The resulting residue was dissolved in 250 μL of mobile phase and filtered with 0.2 μm polypropylene syringe filter units (Whatman, Florham Park, NJ). The resulting filtrate was placed in Waters Total Recovery glass vials (Waters Corporation, Milford, MA), and 150 μL was injected into the HPLC system. The mobile phase consisted of 0.05 M ammonium acetate buffer pH = 4.7 and acetonitrile (70:30, v/v) at a flow rate of 1 mL/min.[15] HPLC analyses were performed with a Waters 717 autosampler with Waters 2487 dual wavelength detector set at 350 nm using a Waters XBridge column (C18, 4.6 × 150 mm, 5 μm). The analytical column was protected with a Waters Xbridge guard column (C18, 4.6 × 20 mm, 5 μm).

A calibration curve was generated for AH-GDM at concentrations of 0.15, 0.25, 0.5, 1.5, 5, 15, 25, and 50 μg/mL in control tissue homogenate and processed as described above. The curve was constructed by plotting the peak area ratio of AH-GDM to the IS and was linear from 0.15 to 50 μg/mL. Recovery of 17-DMAG internal standard was 91 ± 2.5%. AH-GDM in tissue samples was quantitated using the ratio of found AH-GDM peak area to IS peak area. Drug release was not detected from copolymer standard solutions under these extraction conditions (data not shown).

Statistical Analysis. Differences in organ accumulation of the copolymer conjugates and tumor drug concentration were analyzed using one-way ANOVA. Where differences were detected, Tukey's test was used to test for pairwise differences between the groups.

Results

Characteristics of HPMA Copolymer Conjugates. Geldanamycin derivative AH-GDM and corresponding drug comonomer MA-GFLG-AH-GDM were synthesized and characterized as previously reported.[21,27] The characteristics of HPMA copolymers P1 and P2 synthesized from the same polymeric precursor are summarized in Table 1. Copolymer precursor had an estimated molecular weight of 28.4 kDa and polydispersity of 1.7 as determined from size exclusion chromatography (SEC). Drug content in the conjugates was determined to be 0.239 mmol/g corresponding to 15.4% (w/w), approximately 6.7 units per backbone, as determined spectrophotometrically using P2 (without RGDfK) and SEC.

(34) Egorin, M. J.; Lagattuta, T. F.; Hamburger, D. R.; Covey, J. M.; White, K. D.; Musser, S. M.; Eiseman, J. L. Pharmacokinetics, tissue distribution, and metabolism of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (NSC 707545) in CD2F1 mice and Fischer 344 rats. *Cancer Chemother. Pharmacol.* **2002**, *49*, 7–19.

Table 1. Physicochemical Characteristics of HPMA Copolymer Conjugates[a]

	P1	P2
structure	P-(GFLG-AH-GDM)-(GG-RGDfK)	P-(GFLG-AH-GDM)-(GG-OH)
estimated M_w (kDa)[b]	28.4[d]	28.4
M_w/M_n[b,c]	1.7	1.7
AH-GDM content[e]		
mmol/g	0.239	0.239
% wt/wt	15.4	15.4
RGDfK content[f]		
mmol/g	0.361	
peptides/backbone[g]	10.1	

[a] For structure see Figure 1. [b] As determined by size exclusion chromatography. [c] Polydispersity. [d] Reported as precursor M_w. [e] Results of UV spectrophotometric analysis. [f] Results of amino acid analysis. [g] Average as estimated from molecular weight and amino acid analysis data.

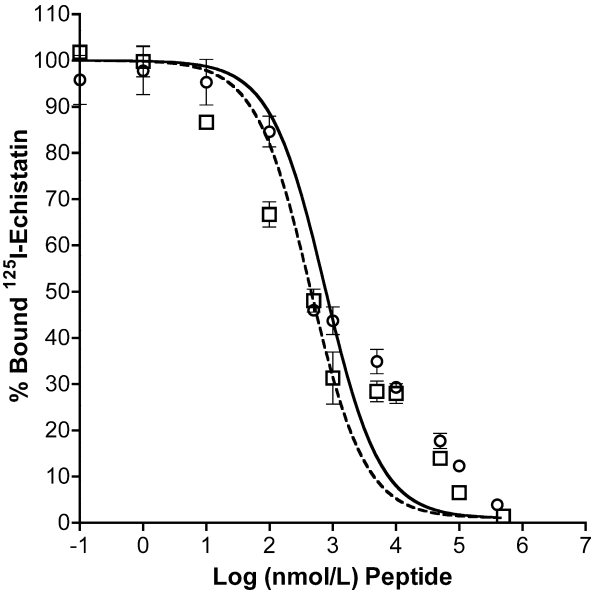

Figure 2. Competitive binding of HPMA copolymer conjugates and free RGDfK peptide. Binding of HPMA copolymer-AH-GDM-RGDfK conjugate P1 was compared to that of free peptide using HUVEC cells. Open squares and dashed line: RGDfK. Open circles and solid line: P1. Results are expressed as means of triplicate ± SD.

RGDfK content in P1 as determined by amino acid analysis was 0.361 mmol/g, corresponding to approximately 10 peptide units per polymeric backbone. Tyrosine content in the conjugates was 0.027 mmol/g corresponding to one Tyr unit per backbone. The size exclusion profiles indicated the absence of small molecular weight impurities in the polymer conjugates (not shown). Radiolabeling the conjugates P1 and P2 with ^{125}I was achieved using the Iodogen method resulting in 1.16 μCi/nmol (41 μCi/mg) for P1 and 1.07 μCi/nmol (38 μCi/mg) for P2.

Competitive binding studies with HUVECs showed active binding of RGDfK peptide and copolymer−peptide conjugate (P1) to the $\alpha_v\beta_3$ integrin (Figure 2). IC_{50} values (nM peptide) as determined by nonlinear regression for binding to HUVECs were 323.8 ± 3.4 and 438.5 ± 3.5 for RGDfK and P1, respectively. At equivalent peptide concentration, free RGDfK showed greater displacement and binding affinity as compared to polymeric conjugates. However it is clear as has been in previous studies[21,33] that targeted conjugates containing RGDfK actively bind to the $\alpha_v\beta_3$ integrin.

Biodistribution of HPMA Copolymer-AH-GDM Conjugates. The results of a 3-day biodistribution study of ^{125}I-labeled conjugates containing RGDfK (P1) and nontargeted conjugate (P2) at 60 mg/kg doses in non-tumor-bearing mice is shown in Figure 3. Rapid blood clearance was observed for both conjugates with very little activity remaining in blood after 24 h. This is also evident in the pharmacokinetic profiles (Figure 4) and pharmacokinetic parameters (Table 2) showing short half-lives of 1.75 and 3.23 h for P1 and P2, respectively. Accumulation in all other organs appears to decrease with time. Significant differences in accumulation for the targeted conjugate P1 compared with the nontargeted conjugate P2 were analyzed at each time point for a given organ beyond one hour. For both treatments, high accumulation is observed in the kidneys despite a decreasing trend. Significantly higher accumulation is observed in the kidneys for the targeted conjugate containing RGDfK (P1) after one hour and for the remainder of the study. Polymer accumulation in the liver for P1 was significantly higher at later time points, but the overall level remained low. Activity in stomach was high out to 6 h but rapidly decreased at 24 h and beyond with no significant differences between the treatments.

Individually for P1 or P2, significant differences in organ accumulation exist at each time point. Most notably kidney accumulation is significantly higher for both copolymer treatments at each time point compared with all other organs ($p < 0.001$). Liver accumulation of P1 at 24 h and beyond was significantly higher than all other organs with the exception of kidney ($p < 0.01$).

The biodistribution of ^{125}I-radiolabeled copolymers P1 and P2 at a lower dose of total polymer (4 nmol) was evaluated in prostate tumor-bearing mice to demonstrate the targeting capability of the targeted conjugate P1 containing RGDfK to the $\alpha_v\beta_3$ integrin in vivo (Figure 5). As is seen in the biodistribution study with high polymer doses (Figure 3), both conjugates undergo rapid blood clearance. In this study, kidney accumulation is lower for both and the conjugates displayed similar renal elimination profiles in contrast to what was observed for higher doses. Additionally, polymer accumulation in all background organs decreased with time with no significant differences existing in organ accumulation between the two conjugates with the exception of tumor. Tumor accumulation was significantly higher for the targeted conjugate P1 after 1 h ($p < 0.05$) and demonstrated prolonged accumulation for the duration of the study. At 24 h, tumor accumulation of P1 was significantly higher than blood, heart, lung, spleen and stomach ($p < 0.05$). Additionally, at 72 h tumor accumulation was significantly higher than all organs except kidneys ($p < 0.01$). Very little tumor accumulation for the nontargeted conjugate P2 was observed beyond 1 h.

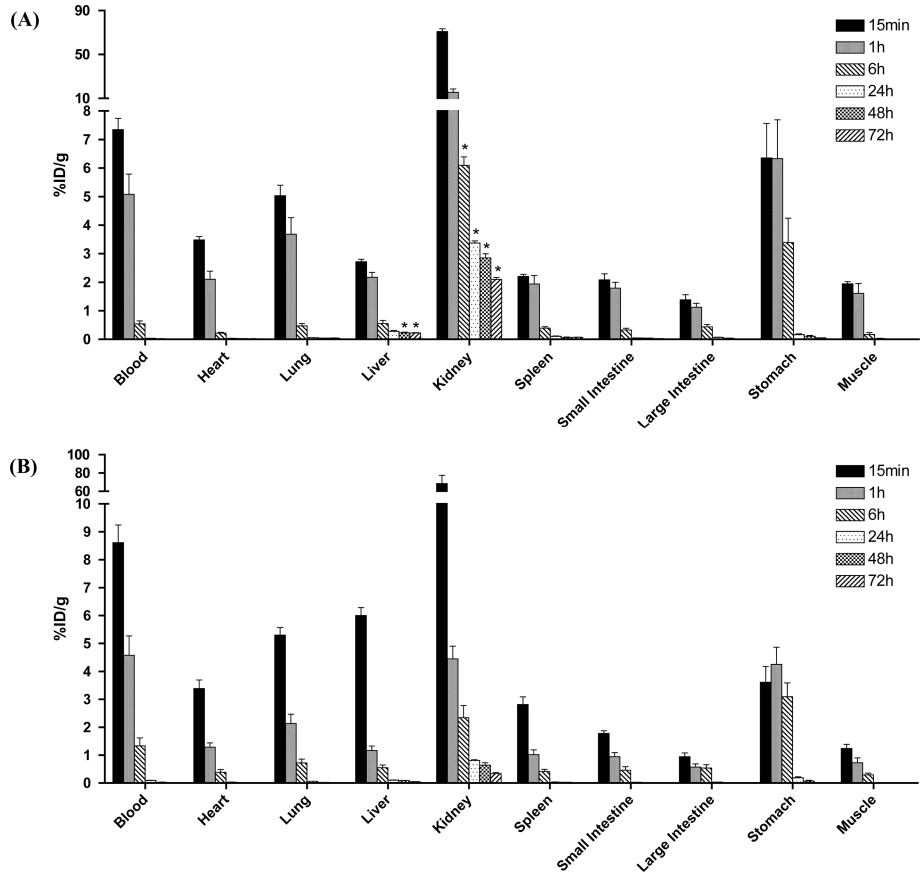

Figure 3. Biodistribution of ^{125}I-labeled HPMA copolymer conjugates P1 (panel A) and P2 (panel B) given at 60 mg/kg drug equivalent dose in non-tumor-bearing mice. Activity per organ is expressed as % injected dose per gram of tissue (% ID/g) following necropsy at 15 min, 1, 6, 24, 48, and 72 h postintravenous injection. Rapid decrease in blood is accompanied by decreasing activity in organs. Significantly higher prolonged activity of conjugate P1 occurs in kidney compared to the nontargeted polymer P2 (B) and is indicated on the graph as * $p < 0.001$. Data is expressed as mean ± SD.

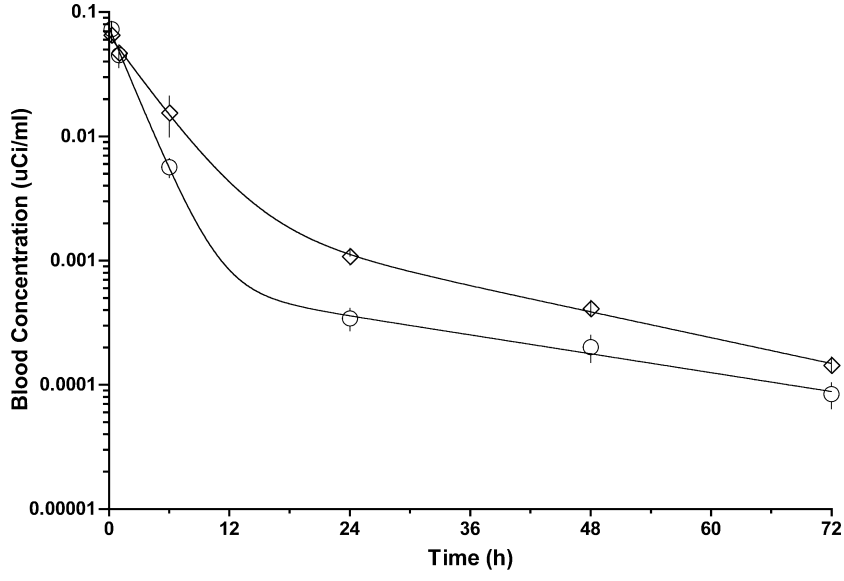

Figure 4. Pharmacokinetic profiles for HPMA copolymer conjugates in blood. Radioactivity measurements in blood expressed as μCi/mL for ^{125}I-labeled conjugates were analyzed by 2-compartment modeling. Circles: HPMA copolymer-AH-GDM-RGDfK conjugate (P1). Diamonds: HPMA copolymer-AH-GDM conjugate (P2). Data is expressed as mean ± SD.

Table 2. Pharmacokinetic Parameters of HPMA Copolymer Conjugates in Blood

sample	P1	P2
AUC (μCi/mL h)	0.190 ± 0.01	0.304 ± 0.02
$T_{1/2}$ (h)	1.748 ± 0.12	3.229 ± 0.25
CL (mL/h)	5.259 ± 0.33	3.286 ± 0.18
V_d (mL)	13.26 ± 1.29	15.31 ± 1.15

Kidney accumulation predominated for P2 with significantly higher accumulation than all other organs for the duration of the study after 1 h ($p < 0.001$).

Table 3 shows the tumor-to-background ratios for conjugates P1 and P2. An increasing trend for P1 is observed for most organs indicating prolonged tumor residence with clearance from background organs. In contrast, T/B ratios for P2 show generally consistent or decreasing numbers for which the majority are below one, indicating low tumor accumulation.

Tumor Concentrations of Free Drug. To study the relative concentrations of drug delivered to the tumor environment through targeted and nontargeted polymers as well as free drug, DU145 prostate tumor mice were injected with 30 and 60 mg/kg drug equivalent doses of targeted conjugate P1 and nontargeted conjugate P2. These doses were compared with the maximum tolerated dose of 30 mg/kg AH-GDM as previously determined.[21] The mice tolerated all doses administered as previously observed.[21] However, doses of 30 mg/kg AH-GDM and 60 mg/kg of drug equivalent copolymers caused a decrease in general movement and activity of the mice for approximately 5 min following injection.

Drug concentrations in prostate tumors at 4, 12, and 24 h p.i. are displayed in Figure 6. Free AH-GDM following in vivo polymer release was detected in tumors for all polymer treatments at all three time points sampled out to 24 h. AH-GDM in tumor following administration of AH-GDM hydrochloride at 30 mg/kg was detected at 4 and 12 h with no detectable concentrations in the tumor at 24 h. Significant differences existed in tumor drug levels for each time point. Most notably, targeted conjugate P1 at 60 mg/kg resulted in significantly higher tumor drug concentrations compared to all other treatments at all time points ($p < 0.001$). Following 12 h p.i., P1 30 mg/kg dose had significantly higher tumor drug levels than compared with 30 mg/kg drug equivalent doses of nontargeted conjugate P2 and AH-GDM ($p < 0.05$). Also observed in Figure 6 are increasing drug concentrations for the targeted conjugate (P1) at both dose levels between 4 and 12 h. This effect is not seen for the nontargeted conjugates and can be attributed to the prolonged accumulation of the targeted copolymers as shown in the biodistribution analysis (Figure 5).

Following 12 h p.i., tumor drug levels appear to reach a maximum for the targeted conjugates. At this time point, the amount of drug in tumor for P1 at 60 mg/kg dose was measured at 7.25 ± 1.64 μg AH-GDM/g tumor tissue. This is approximately 3 times higher than P1 at 30 mg/kg and 7.3 and 3.3 times higher than P2 at 30 and 60 mg/kg drug equivalent doses, respectively. Most importantly, it is 8.4 times higher than drug concentrations for 30 mg/kg AH-GDM. Finally, direct comparison of 30 mg/kg doses at 12 h shows that P1 resulted in 2.8 times higher tumor drug concentrations than AH-GDM.

Discussion

In this study the in vivo biodistribution and tumor drug delivery of a previously described HPMA copolymer containing the geldanamycin (GDM) derivative aminohexyl-GDM (AH-GDM) and the $\alpha_v\beta_3$ integrin targeting peptide RGDfK were described.[21] The goal is to improve geldanamycin chemotherapy by reducing dose-limiting toxicities and improving tumor response through higher localized concentrations of drug.[15,17] Our laboratory has previously characterized HPMA copolymers bearing RGD peptides as having enhanced accumulation in solid tumors compared to nontargeted conjugates and peptide alone.[4−6] The $\alpha_v\beta_3$ integrin is expressed on the surface of endothelial cells of angiogenic blood vessels and is absent on the luminal surface of cells in established vessels.[8] Such angiogenesis-targeted copolymers are directed to the $\alpha_v\beta_3$ integrin expressed on angiogenic blood vessels through conjugation of cyclic RGD peptides (namely RGDfK) to the copolymer backbone that have increased affinity and stability as compared to linear RGD sequences.[35,36] Expression of the $\alpha_v\beta_3$ integrin on tumor cells can also contribute to the enhanced tumor accumulation of these copolymers in vivo.[9,21]

The enhanced tumor accumulation of these HPMA copolymer-RGD conjugates were exploited for the delivery of chemotherapeutic drugs and radionuclides.[7,21] The antitumoral effect of targeted conjugates containing a therapeutic radionuclide was previously demonstrated in mice.[7] HPMA copolymer-RGDfK conjugates bearing the chemotherapeutic agent AH-GDM were also evaluated and showed higher toxicity toward DU145 prostate cancer cells in vitro and increased tolerability of greater than 2-fold in non-tumor-bearing mice compared with free AH-GDM.[21]

In this study, HPMA copolymers were synthesized containing the geldanamycin derivative AH-GDM with and without the targeting moiety RGDfK. The geldanamycin derivative AH-GDM was necessary for covalent attachment to comonomers and subsequent copolymerization, and has shown favorable stability and in vitro activity compared with other amino-alkyl derivatives.[27] The estimated molecular weight was also similar and below the renal threshold for

(35) Bogdanowich-Knipp, S. J.; Chakrabarti, S.; Williams, T. D.; Dillman, R. K.; Siahaan, T. J. Solution stability of linear vs. cyclic RGD peptides. *J. Pept. Res.* **1999**, *53*, 530–41.

(36) Koivunen, E.; Wang, B.; Ruoslahti, E. Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. *Bio/Technology* **1995**, *13*, 265–70.

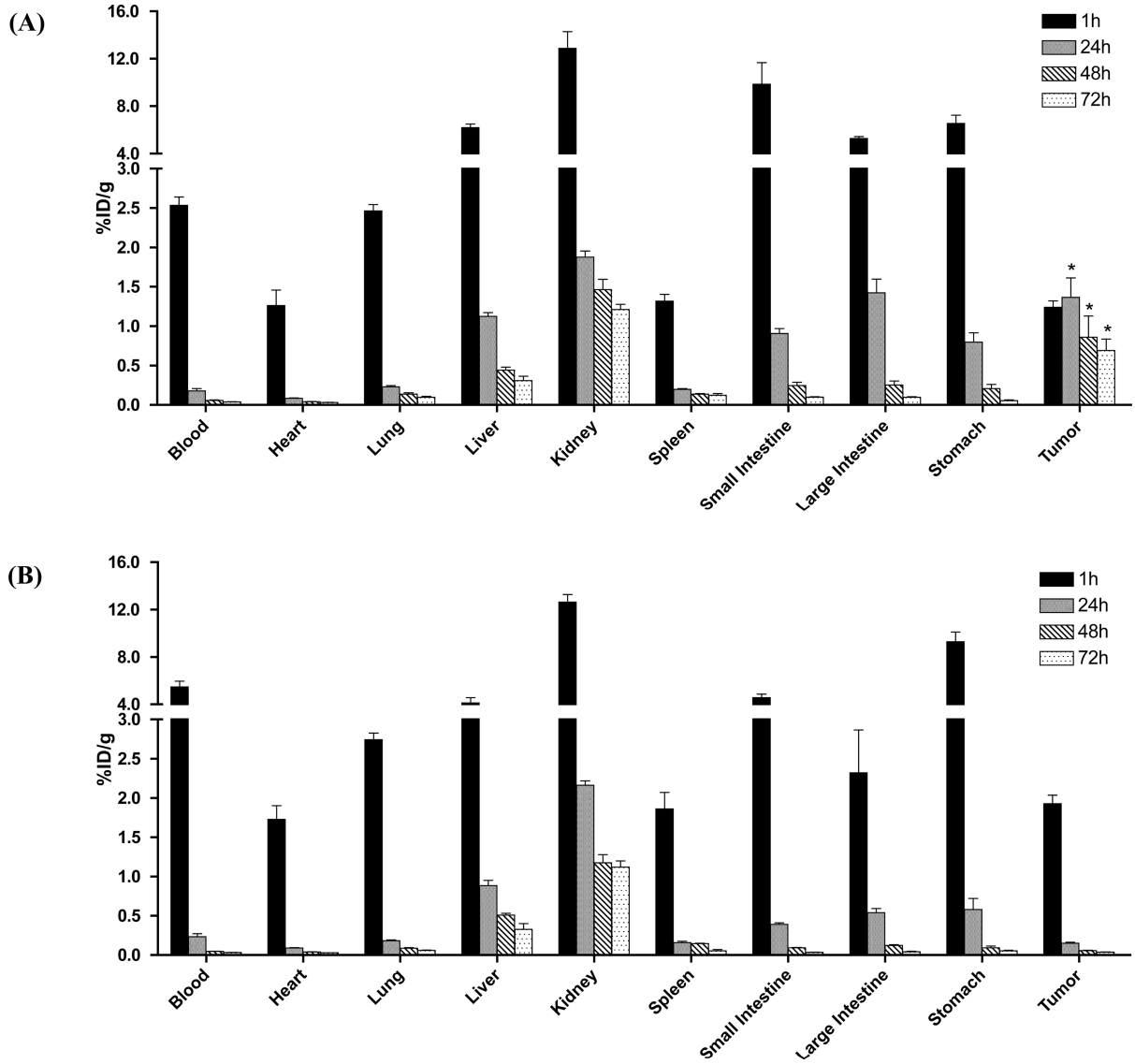

Figure 5. Biodistribution of ^{125}I-labeled HPMA copolymer conjugates P1 (panel A) and P2 (panel B) in prostate tumor-bearing mice. Activity per organ is expressed as % injected dose per gram of tissue (% ID/g) following necropsy at 1, 24, 48, and 72 h postintravenous injection. Rapidly decreasing blood concentrations are observed with no significant accumulation differences in normal organs. Significantly higher localization of the targeted conjugate P1 occurs in the tumor after 1 h compared with the nontargeted conjugate P2 as indicated on the graph as $*p < 0.05$. Data is expressed as mean ± SD.

HPMA (approximately 45 kDa[37]), allowing for the elimination of the copolymer conjugates predominantly by the kidneys. Active binding of the copolymer conjugate P1 containing RGDfK was demonstrated in vitro with HUVECs. Binding affinities of RGDfK and P1 are similar to those previously reported.[21,33] It was also shown that the presence of AH-GDM on the copolymer backbone does not influence binding because copolymers not containing RGDfK showed no radioligand displacement from the $\alpha_v\beta_3$ integrin on HUVECs.[21]

The biodistribution of copolymers P1 and P2 was evaluated using therapeutically relevant doses of polymer (60 mg/kg drug equivalent) in non-tumor-bearing mice to assess normal tissue distribution and evaluate the clearance of the conjugates from the blood and sensitive organs. Sequestration of the polymer was not observed in any organs with all showing clearance of the conjugate with time (Figure 3). Evaluating the distribution to and clearance from normal organs is essential to establishing the utility of the conjugates for future therapeutic applications. Renal accumulation of

(37) Seymour, L. W.; Duncan, R.; Strohalm, J.; Kopecek, J. Effect of molecular weight (M_w) of N-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal, and intravenous administration to rats. *J. Biomed. Mater. Res.* **1987**, *21*, 1341–58.

(38) Boswell, C. A.; Eck, P.; Regino, C. A.; Bernardo, M.; Wong, K.; Milenic, D.; Choyke, P.; Brechbiel, M. Synthesis, characterization, and biological evaluation of integrin alphaVbeta3-targeted PAM-AM dendrimers. *Mol. Pharmaceutics* **2008**, *5*, 527–39.

Table 3. Tumor-to-Background Ratios for HPMA Copolymer-AH-GDM Conjugates with and without RGDfK

	1 h	24 h	48 h	72 h
HPMA-AH-GDM-RGDfK (P1)				
blood	0.49	7.66	15.43	18.04
heart	0.98	16.50	21.94	23.04
lung	0.50	5.97	6.32	7.25
liver	0.20	1.21	1.95	2.24
kidney	0.10	0.73	0.59	0.57
spleen	0.94	6.94	6.26	5.61
small intestine	0.13	1.51	3.51	6.97
large intestine	0.24	0.96	3.41	7.25
stomach	0.19	1.72	4.13	12.53
HPMA-AH-GDM (P2)				
blood	0.35	0.64	1.21	1.18
heart	1.12	1.66	1.43	1.29
lung	0.66	0.82	0.65	0.65
liver	0.47	0.17	0.11	0.12
kidney	0.15	0.07	0.05	0.03
spleen	1.04	0.94	0.40	0.71
small intestine	0.42	0.38	0.62	1.13
large intestine	0.83	0.28	0.47	0.89
stomach	0.21	0.26	0.62	0.71

the RGDfK-bearing conjugate is higher than the conjugate without peptide. It has been demonstrated that RGD peptides show high kidney accumulation when administered as mono- and multimeric units alone or as part of a macromolecule.[38−41] Our previous studies demonstrated that compared to peptide alone renal uptake of HPMA copolymer-RGD conjugates was decreased through the conjugation of the peptide to the copolymer.[4,5] In the current study we demonstrated higher kidney uptake for the 60 mg/kg dose of RGDfK-bearing conjugate P1. This differential renal uptake was not observed when copolymer conjugates were administered at the lower dose (4 nmol, Figure 5). It is possible that RGDfK has mediated increased renal activity of the P1 conjugate only at higher doses. This phenomenon needs further investigation.

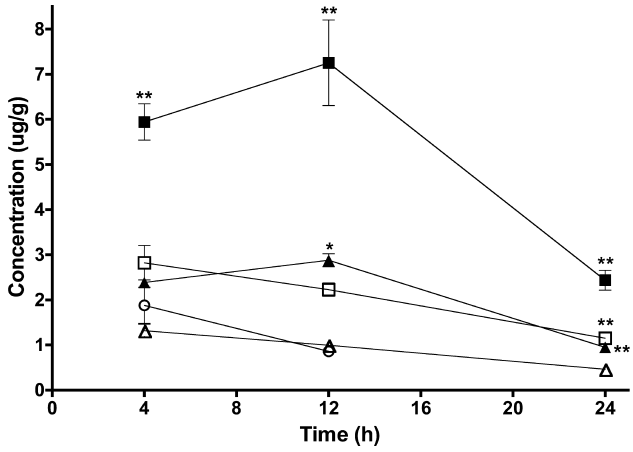

Figure 6. Tumor accumulation of free AH-GDM at 4, 12, and 24 h post intravenous administration of P1 and P2 copolymer conjugates at 30 and 60 mg/kg drug equivalent and 30 mg/kg AH-GDM hydrochloride. Data shows increasing tumor concentrations of drug for copolymer P1 treatments compared with nontargeted copolymer P2. Tumor concentrations following treatment with AH-GDM 30 mg/kg fall rapidly and are not detectable at 24 h. Closed triangles: P1 30 mg/kg. Closed squares: P1 60 mg/kg. Open triangles: P2 30 mg/kg. Open squares: P2 60 mg/kg. Open circle: AH-GDM 30 mg/kg. Data expressed as mean ± SD. Significant differences for polymer treatment groups compared with drug alone at each time point indicated as $*p < 0.05$, $**$ $p < 0.001$.

Slightly higher concentrations for P2 in blood at 6 h and beyond translate into a blood half-life that is approximately 1.5 h longer than P1 (Table 2). Accordingly, clearance is higher for P1. Aside from these differences, both conjugates have a rapid half-life and high clearance when compared with other HPMA copolymers.[42] Greater exposure of P2 in blood as reflected by the AUC values could presumably result in higher tumor concentrations of polymer or drug, however as shown in Figures 5 and 6 this is not the case.

The biodistribution of the copolymers was carried out in DU145 prostate tumor-bearing mice. This cell line has demonstrated higher $\alpha_v\beta_3$ expression in vitro[21] with effective accumulation of HPMA copolymer-RGD conjugates in vivo.[4,6] The DU145 mouse tumor model is also potentially useful for evaluating the efficacy of these conjugates because the cell line demonstrated greater susceptibility to HPMA copolymer-AH-GDM-RGDfK conjugates in vitro compared with AH-GDM alone.[21] The targeted conjugate P1 demonstrated effective tumor targeting when compared with the nontargeted conjugate P2 at time points beyond 1 h (Figure 5). The data show that the dose of polymer received by the tumor at 1 h was retained with modest increase between 1 and 24 h. However, the overall dose received by the tumor in the current study is lower than what our laboratory has previously demonstrated.[5] A study of HPMA copolymer-RGDfK biodistribution showed that accumulation in tumor reached a maximum of approximately 5% ID/g at 48 h with sustained activity of approximately 4% ID/g after 192 h. The

(39) Ahmadi, M.; Sancey, L.; Briat, A.; Riou, L.; Boturyn, D.; Dumy, P.; Fagret, D.; Ghezzi, C.; Vuillez, J.-P. Chemical and biological evaluations of an ^{111}In-labeled RGD-peptide targeting integrin alpha(V) beta(3) in a preclinical tumor model. *Cancer Biother. Radiopharm.* **2008**. DOI: 10.1089/cbr.2008.0528.

(40) Janssen, M. L.; Oyen, W. J.; Dijkgraaf, I.; Massuger, L. F.; Frielink, C.; Edwards, D. S.; Rajopadhye, M.; Boonstra, H.; Corstens, F. H.; Boerman, O. C. Tumor targeting with radiolabeled alphaVbeta3 integrin binding peptides in a nude mouse model. *Cancer Res.* **2002**, *62*, 6146–51.

(41) Wang, L.; Shi, J.; Kim, Y.-S.; Zhai, S.; Jia, B.; Zhao, H.; Liu, Z.; Wang, F.; Chen, X.; Liu, S. Improving tumor-targeting capability and pharmacokinetics of ^{99m}Tc-labeled cyclic RGD dimers with PEG_4 linkers. *Mol. Pharmaceutics* **2009**, *6* (1), 231–45.

(42) Pan, H.; Sima, M.; Kopečková, P.; Wu, K.; Gao, S.; Liu, J.; Wang, D.; Miller, S. C.; Kopeček, J. Biodistribution and pharmacokinetic studies of bone-targeting N-(2-hydroxypropyl)methacrylamide copolymer-alendronate conjugates. *Mol. Pharmaceutics* **2008**, *5*, 548–58.

dose of the polymer in the tumor at 1 h (approximately 1% ID/g) increased over 4 times after 48 h. The polymeric architecture of these conjugates was substantially different whereby they contained no drug comonomers and higher estimated molecular weight. Increased tumor accumulation of these conjugates may be attributed to extended polymer activity in the blood pool still above 1% ID/g at 48 h, allowing for further trafficking to the tumor and accumulation mediated by RGDfK and passively through the enhanced permeability and retention (EPR) effect.[43] One possible strategy to improve the overall extent of tumor localization of copolymers and maximize the tumor concentration of drug is to increase the molecular weight of the conjugates to allow for longer blood circulation time and improved EPR effect.[44] However, synthesizing these conjugates with higher molecular weight closer to the renal threshold is challenging because of the high percentage of the chain transfer agent MA-GG-ONp in the comonomer feed limiting polymer chain extension. The high feed ratio of this comonomer is intended to allow for sufficient conjugation sites and increased content of RGDfK peptide per polymer backbone. However, it may be necessary to sacrifice some peptide on the backbone to synthesize larger copolymers that do not undergo such rapid clearance.

To measure the tumor concentrations of AH-GDM achievable through copolymer localization of these conjugates, injections of 30 and 60 mg/kg drug equivalent polymer were administered and compared with 30 mg/kg free AH-GDM in DU145 prostate tumor-bearing mice. Free AH-GDM hydrochloride was administered at the maximum tolerated dose (MTD) as previously determined.[21] Doses of the copolymers were chosen for comparison with the MTD for free drug and double the MTD of the free drug (60 mg/kg drug equivalent). The high drug content in the copolymers aided formulation of the doses in saline preventing the solutions from becoming too viscous at high polymer concentration (approximately 40 mg/mL).

Higher tolerated doses of drug-equivalent targeted copolymer P1 successfully increased tumor drug concentrations compared with doses of free drug. The 60 mg/kg dose of targeted copolymer−drug conjugate P1 resulted in over eight times higher drug concentrations in the tumor at 12 h. The goal of polymer therapeutics is to increase the therapeutic index of a given drug through the ability to administer higher tolerated doses.[1] In the context of effective therapy this comparison is valid because we are assessing two equally tolerated doses of drug or drug-equivalent polymer. However, to make a direct comparison 30 mg/kg drug equivalent P1 still resulted in nearly 3 times higher tumor drug concentrations than free AH-GDM at the same dose at 12 h. Doses of P1 at 30 and 60 mg/kg also had 2.4 and 3.3 times higher tumor drug concentrations compared to P2 at respective doses. Both P1 targeted conjugate doses increased drug concentration between 4 and 12 h compared to decreasing drug concentrations at each time point for P2 conjugates and AH-GDM alone. This is most likely a result of the sustained accumulation of P1 in the tumor and the time required for tumor localization, cellular uptake and intracellular drug release. HPMA copolymers of higher molecular weight containing 5-fluorouracil (5-FU) have also shown the ability for tumor drug delivery.[45] These copolymers showed higher amounts of drug in tumor at earlier time points and concentrations below 1 μg/g at 24 h. This is unlike what we see for our targeted conjugates where the highest tumor drug concentrations occur after 12 h. Similarly observed between the previously mentioned and current study is the rapid decrease of tumor drug concentrations following free drug administration (Figure 6). Drug accumulation in tumors can be increased with larger molecular weight polymers especially at earlier time points.

The increase in tumor concentrations of free AH-GDM through polymer delivery is further impacted by considerations of polymer behavior and analytical detection. AH-GDM was attached to the copolymer backbone via the enzymatically degradable peptide spacer GFLG, and conjugates were previously shown to release the drug in vitro.[21] Some studies evaluating tumor drug concentrations following HPMA copolymer−drug treatments utilizing the same drug release mechanism have employed analytical hydrolysis techniques to measure the total drug (free plus polymer-bound) in tumors.[45−47] The method employed in the current study did not utilize such hydrolysis techniques for concern over AH-GDM degradation. Therefore, the analytical method used to detect drug in the tumor was only capable of measuring passively absorbed drug from AH-GDM administration or the free drug released from copolymers following cellular internalization and subsequent drug release. As a result the reported tumor drug concentrations in polymer treatments are probably an underestimation of total drug in tumor that would also include the fraction still bound to the copolymer backbone. Emerging evidence indicates that

(43) Maeda, H.; Wu, J.; Sawa, T.; Matsumura, Y.; Hori, K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Controlled Release* **2000**, *65*, 271–84.

(44) Seymour, L. W.; Miyamoto, Y.; Maeda, H.; Brereton, M.; Strohalm, J.; Ulbrich, K.; Duncan, R. Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier. *Eur. J. Cancer* **1995**, *31A*, 766–70.

(45) Yuan, F.; Qin, X.; Zhou, D.; Xiang, Q. Y.; Wang, M. T.; Zhang, Z. R.; Huang, Y. In vitro cytotoxicity, in vivo biodistribution and antitumor activity of HPMA copolymer-5-fluorouracil conjugates. *Eur. J. Pharm. Biopharm* **2008**, *70*, 770–6.

(46) Fraier, D.; Frigerio, E.; Pianezzola, E.; Strolin Benedetti, M.; Cassidy, J.; Vasey, P. A sensitive procedure for the quantitation of free and N-(2-hydroxypropyl) methacrylamide polymer-bound doxorubicin (PK1) and some of its metabolites, 13-dihydrodoxorubicin, 13-dihydrodoxorubicinone and doxorubicinone, in human plasma and urine by reversed-phase HPLC with fluorimetric detection. *J. Pharm. Biomed. Anal.* **1995**, *13*, 625–33.

(47) Julyan, P. J.; Seymour, L. W.; Ferry, D. R.; Daryani, S.; Boivin, C. M.; Doran, J.; David, M.; Anderson, D.; Christodoulou, C.; Young, A. M.; Hesslewood, S.; Kerr, D. J. Preliminary clinical study of the distribution of HPMA copolymers bearing doxorubicin and galactosamine. *J. Controlled Release* **1999**, *57*, 281–90.

release from backbone is not always necessary for efficacy[48] and drug not measured in the tumor through this assay may be present potentially increasing the efficacy of the conjugates. However, if in fact AH-GDM requires polymer release to exert a therapeutic effect, then results of the current study comparing only free drug concentrations are more appropriate. In either case, it appears that the increase in localized dose of drug delivered by the conjugates can effectively address dose-limiting cytotoxicity issues that exist for current 17-AAG and 17-DMAG therapies. Additionally, when exploring the tumor drug concentrations achieved for the targeted conjugate P1 versus the nontargeted conjugate P2, the increase resulting from targeted conjugates is further emphasized because a previous drug release study demonstrated that the targeted conjugate containing RGDfK had overall lower drug release capacity.[21]

Administration of two increasing polymer doses was also designed to provide initial information on the possibility of reaching maximum drug concentrations in the tumor, effectively saturating drug delivery processes. Results following the single i.v. injections possibly indicate that tumor drug saturation was not achieved and the potential exists for higher tumor drug concentrations by increasing the polymer dose. Up to 80 mg/kg drug equivalent polymer formulation suitable for i.v. injection in mice was achieved and tolerated in vivo.[21] An alternative to increasing the dose of a single injection would be to administer multiple 60 mg/kg drug equivalent doses. The current study shows that repeated administration of the conjugates every 24 h may be warranted to further increase the tumor drug concentration. However, consideration of multiple doses must also include how this may affect potential toxic accumulation in sensitive normal organs, especially the liver and kidney.

The current study demonstrates the utility of targeted HPMA copolymer-RGDfK conjugates for the delivery of geldanamycin and other chemotherapeutic agents. Important attention must be paid to select drug candidates that are highly potent upon release from the copolymer backbone. Testing the polymeric nanomedicines in sensitive tumor models expressing the $\alpha_v\beta_3$ integrin on angiogenic blood vessels and/or tumor cells is necessary to demonstrate their full potential. It is easily conceivable that under the correct therapeutic conditions the ability to simultaneously increase tolerated drug doses as well as localized delivery of an agent is certain to bolster the chemotherapeutic armamentarium currently used for cancer therapy.

(48) Rihova, B.; Strohalm, J.; Hovorka, O.; Subr, V.; Etrych, T.; Chytil, P.; Pola, R.; Plocova, D.; Boucek, J.; Ulbrich, K. Doxorubicin release is not a prerequisite for the in vitro cytotoxicity of HPMA-based pharmaceuticals: in vitro effect of extra drug-free GlyPheLeuGly sequences. *J. Controlled Release* **2008**, *127*, 110–20.

Conclusions

Targeted HPMA copolymer-AH-GDM-RGDfK conjugates showed higher accumulation in tumor than nontargeted conjugates. Administration of the conjugates resulted in increased tumor concentrations of free drug. Together, these copolymers have demonstrated an increased tolerability and a higher amount of drug delivered to the tumor. These properties can be used to surmount the dose limiting cytotoxicity issues present for current geldanamycin therapy while at the same time improving efficacy through increased localized concentrations of the drug.

Abbreviations

17-AAG, 17-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin; AH, 6-aminohexylamino; AH-GDM, 17-(6-aminohexylamino)-17-demethoxygeldanamycin; EtOAc, ethyl acetate; GDM, geldanamycin; HPLC, high performance liquid chromatography; HPMA, *N*-(2-hydroxypropyl)methacrylamide; MA-GFLG-AH-GDM, *N*-methacryloylglycylphenylalanylleucylglycl-17-(6-aminohexylamino)-17-demethoxygeldanamycin; RGDfK, cyclo-(arginine-glycine-aspartic acid-D-phenylalanine-lysine); SEC, size exclusion chromatography.

Acknowledgment. The authors would like to thank the Translational Core Laboratory at the University of Maryland Greenebaum Cancer Center including Dr. Joseph Bryant, Dr. Mariola Sadowska, Dr. Eugene Ateh and Lanea George for assistance with drug accumulation studies. The authors would also like to thank Dr. Natalie Eddington and Dr. Hazem Hassan for their assistance with pharmacokinetic analysis. This research was supported by the National Institutes of Health Grant R01 EB007171. M.P.B. was supported in part by the American Foundation for Pharmaceutical Education.

MP900134C