Pharm Res (2010) 27:2683–2693
DOI 10.1007/s11095-010-0267-7

RESEARCH PAPER

HPMA Copolymer-Aminohexylgeldanamycin Conjugates Targeting Cell Surface Expressed GRP78 in Prostate Cancer

Nate Larson • Abhijit Ray • Alexander Malugin • Daniel B. Pike • Hamidreza Ghandehari

Received: 25 June 2010 / Accepted: 30 August 2010 / Published online: 16 September 2010
© Springer Science+Business Media, LLC 2010

ABSTRACT

Purpose This study focused on the synthesis and *in vitro* characterization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugates for the delivery of geldanamycin to prostate cancer tumors. Conjugates were modified to incorporate WIFPWIQL peptide, which binds to cell-surface-expressed Glucose-regulated protein 78.

Methods HPMA copolymers containing aminohexylgeldanamycin with and without WIFPWIQL peptide were synthesized and characterized, and stability in pH 7.4 and pH 5.0 buffers, complete cell culture medium, and fetal bovine serum was evaluated. The comparative cell surface expression of GRP78 in DU145 and PC3 cell lines was assessed and competitive binding to cell surface expressed GRP78 evaluated. The ability of the conjugates to inhibit cell growth was also evaluated *in vitro*.

Results HPMA copolymer-aminohexylgeldanamycin conjugates were stable with maximal release observed in fetal bovine serum at 37°C of approximately 10% in 72 h. HPMA copolymers bearing WIFPWIQL peptide bound to cell surface expressed GRP78 with affinities comparable to free WIFPWIQL peptide and demonstrated increased cytotoxicity as compared to untargeted conjugates.

Conclusion HPMA copolymer aminohexylgeldanamycin conjugates bearing WIFPWIQL peptide have the ability to bind to cell-surface-expressed GRP78 and inhibit the growth of human prostate cancer cells, suggesting that the conjugates have the potential to target solid prostate cancer tumors.

KEY WORDS Geldanamycin · HPMA copolymers · GRP78 · targeted delivery · prostate cancer

ABBREVIATIONS

AH-GDM	aminohexylgeldanamycin
AIBN	N,N'-azobisisobutyronitrile
ANOVA	analysis of variance
DMSO	dimethylsulfoxide
EPR	enhanced permeability and retention
ER	endoplasmic reticulum
FBS	fetal bovine serum
GDM	geldanamycin
GFLG	Gly-Phe-Leu-Gly
GG	Gly-Gly
GRP78	glucose-regulated protein 78
HPLC	high performance liquid chromatography
HPMA	*N*-(2-hydroxypropyl)methacrylamide
Hsp70	heat-shock protein 70
Hsp90	heat-shock protein 90
HUVECs	human umbilical vein endothelial cells
MTD	maximum tolerated dose
ONp	p-nitrophenol
PEG	polyethylene glycol
RGD	Arg-Gly-Asp
SEC	size exclusion chromatography

N. Larson · A. Ray · A. Malugin · H. Ghandehari
Department of Pharmaceutics and Pharmaceutical Chemistry
University of Utah
Salt Lake City, Utah 84108, USA

N. Larson · A. Ray · A. Malugin · D. B. Pike · H. Ghandehari
Center for Nanomedicine, Nano Institute of Utah, University of Utah
Salt Lake City, Utah 84108, USA

D. B. Pike · H. Ghandehari
Department of Bioengineering, University of Utah
Salt Lake City, Utah 84108, USA

H. Ghandehari (✉)
Departments of Pharmaceutics & Pharmaceutical Chemistry
and Bioengineering, Utah Center for Nanomedicine
Nano Institute of Utah, University of Utah
383 Colorow Road, Room 343
Salt Lake City, Utah 84108, USA
e-mail: hamid.ghandehari@pharm.utah.edu

TLC	thin layer chromatography
VEGF	vascular endothelial growth factor
WIFPWIQL	Trp-Ile-Phe-Pro-Trp-Ile-Gln-Leu
WST-8	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt

INTRODUCTION

Prostate cancer remains the second largest cause of cancer-related deaths among men, and an estimated 27% of the new cancer cases presented in 2009 were prostate cancers (1). Geldanamycin (GDM), a benzoquinone ansamycin, is a naturally occurring inhibitor of heat-shock protein 90 (Hsp90) and has been widely studied as an anticancer agent (2). Hsp90 is highly expressed in a variety of cancers, including melanoma, leukemia, colon, lung, breast, and prostate cancers (3), and is thought to play an important role in regulating the folding and activity of its client proteins, which include growth-stimulating proteins involved in malignant transformation (4). As Hsp90 client proteins have also been implicated in prostate cancer progression (5), GDM naturally presents itself as an attractive therapeutic agent against this disease. However, the clinical use of GDM has been limited by several factors. It exhibits high hepatotoxicity at therapeutic doses in animal models (6), is poorly soluble in water, and is metabolically unstable (3). While GDM derivatives with improved tolerance, metabolic stability, and water solubility are currently under investigation, clinical response is still limited (7, 8).

The use of drug conjugates with water-soluble polymers such as poly ethylene glycol (PEG) and *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers are well suited to overcome these limitations (9, 10). First, polymeric carriers can increase the water solubility of poorly water-soluble drugs (11). Second, the use of polymeric conjugates can significantly alter drug pharmacokinetics and biodistribution (12, 13). This can result in a significant increase in the blood plasma half-life of the drug, which in turn results in an increased total drug exposure. Third, these polymeric conjugates accumulate in solid tumors by the passive enhanced permeability and retention (EPR) effect due to the leaky tumor vasculature and reduced lymphatic drainage within the tumor microenvironment (14). This translates to a higher maximum tolerated dose (MTD) of a given chemotherapeutic agent (15). The advantage of HPMA copolymers over other water-soluble polymers is that simple chemical modifications can be used to alter drug loading, targeting moiety content, and molecular weight (16, 17). Drug molecules can be attached to the polymer backbone via the lysosomally degradable peptide sequence Gly-Phe-Leu-Gly (GFLG) allowing intracellular release by lysosomal proteases (18) while remaining stable during systemic circulation (19). Several HPMA copolymer-drug conjugates have progressed to clinical trials for the treatment of a variety of solid tumor cancers (20, 21). However, clinical success has remained marginal (22). The inclusion of targeting moieties bound to the polymer backbone can further enhance accumulation in the target site while minimizing systemic exposure. For example, previous work in our laboratory has described anti-angiogenic HPMA copolymer conjugates bearing RGD (Arg-Gly-Lys) peptides as targeting moieties toward endothelial cells in the neovasculature of solid tumors (23–25). The success of anti-angiogenic therapy, however, is limited, as angiogenesis inhibitors can inhibit tumor growth in areas of neovascularization but have no direct effect on the survival of tumor cells in the regions of mature, non-proliferating vessels and do not exert cytotoxicity directly to tumor cells (26). An alternative strategy is to use a combination treatment of targeted drug delivery to both the tumor vasculature and tumor cells.

Glucose-regulated protein 78 (GRP78), also known as immunoglobulin heavy-chain binding protein (BiP) was first discovered following glucose starvation in chicken embryo fibroblasts (27). GRP78 is a member of the Hsp70 protein family and is primarily found in the endoplasmic reticulum (ER) where it acts by facilitating protein folding and functions as a regulator of ER stress signaling (28). In the tumor microenvironment characterized by glucose deprivation, acidosis, and hypoxia, the accumulation of misfolded and underglycosylated proteins triggers the unfolded protein response, inducing the expression of GRP78 and migration of GRP78 to the cell surface. This presents an attractive molecular target with specific expression occurring presumably in cancer cells. In mouse models, GRP78 expression is selectively induced in cancer cells and cancer-associated macrophages but not in major adult organs (29). GRP78 is also detected on the surface of human prostate cancer cells and may play a role in promoting cell proliferation, survival, and metastasis (30, 31).

The octopeptide WIFPWIQL, selected by phage display, specifically binds to GRP78 and has shown binding to cell-surface-expressed GRP78 in human prostate cancer cells (32). Recent results demonstrate the ability of WIFPWIQL peptide-bearing liposomes to target DU145 prostate cancer cells as well as vascular endothelial growth factor (VEGF)-activated human umbilical vein endothelial cells (HUVECs) (33), an attractive target for anti-angiogenic therapy which may also be utilized by the conjugates described in this report, as GDM exhibits both antiangiogenic (34) and antitumor activity.

In the present study, the synthesis and *in vitro* characterization of HPMA copolymer-AH-GDM conjugates is described. AH-GDM was attached to the HPMA copolymer backbone via the lysosomally degradable GFLG linker as well as a non-degradable Gly-Gly (GG) linker for comparison in growth inhibition studies. Conjugates were further modified to incorporate the WIFPWIQL peptide to facilitate binding to cell surface expressed GRP78 in human prostate cancer cells lines.

MATERIALS AND METHODS

Materials

Geldanamycin (NSC 122750) was supplied by the National Cancer Institute Developmental Therapeutics Program (NCI DTP). The GRP78 targeting peptide WIFPWIQL was supplied and characterized by Anaspec, Inc. (San Jose, CA). *N*-(2-hydroxypropyl)methacrylamide (HPMA) (35), *N*-methacryloylglycylglycyl-p-nitrophenyl ester (MA-GG-ONp) (36), *N*-methacryloyl-glycylphenylalanylleucylglycine (MA-GFLG-OH) (37), *N*-methacryloyl-glycylphenylalanyl-leucylglycine-p-nitrophenyl ester (MA-GFLG-ONp) (37), and *N*-methacryloyl-tyrosinamide (MA-Tyr) (38) were synthesized and characterized according to previously described methods. Anti-GRP78 polyclonal antibody was obtained from Assay Designs, Inc. (Ann Arbor, MI). Na-^{125}I was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Iodogen reagent, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, was obtained from Thermo Fisher Scientific (Rockford, IL).

Synthesis and Characterization of Drug-Containing Comonomers

17-(6-aminohexylamino)-17-demethoxygeldanamycin (AH-GDM) and N-methacryloylglycylphenylalanylleucylglycl-17-(6-aminohexylamino)-17-demethoxygeldanamycin (MA-GFLG-AH-GDM) were synthesized according to previously described procedures with minor modifications (15, 39). Briefly, GDM (200 mg, 0.357 mmol) and 1,6-Diaminohexane (1.24 g, 10.7 mmol) were dissolved in anhydrous chloroform (30 mL) and stirred under N_2 gas for 2 h at room temperature. Product formation was monitored by thin layer chromatography (TLC) on silica gel with chloroform: methanol [9:1] as the mobile phase. Following product formation, the reaction mixture in chloroform was combined and washed 15X with 30 mL aqueous saturated sodium chloride to ensure removal of excess 1,6-diaminohexane. Complete removal of 1,6-diaminohexane was confirmed by its absence on silica gel eluted with chloroform: methanol [75: 25]. Organic layer was then dried over sodium sulfate, and solvent removed by rotary evaporation. Resulting dark purple solid AH-GDM was verified by mass spectrometry (MS). MA-GFLG-AH-GDM comonomer was synthesized by adding AH-GDM (223 mg, 0.346 mmol) to MA-GFLG-ONp (201 mg, 0.415 mmol) in 3 mL anhydrous dimethylsulfoxide (DMSO). N,N-Diisopropylethylamine (181 μL, 1.04 mmol) was added and the reaction mixture protected from light and stirred overnight at room temperature. Product formation was monitored by TLC on silica gel with chloroform: methanol [9: 1] and purified by silica gel column with ethyl acetate: methanol [9: 1] as the mobile phase. Solvent was removed, and resulting product MA-GFLG-AH-GDM was identified by MS. N-methacryloylglycylglycl-17-(6-aminohexylamino)-17-demethoxygeldanamycin (MA-GG-AH-GDM) comonomer was synthesized in a manner similar to MA-GFLG-AH-GDM utilizing MA-GG-ONp comonomer as a starting material instead of MA-GFLG-ONp. MA-GG-AH-GDM comonomer was similarly purified by silica gel column, and the resulting product was identified by mass spectrometry.

Synthesis and Characterization of HPMA Copolymer Conjugates

HPMA copolymers were synthesized via free radical precipitation copolymerization of comonomers in acetone: DMSO [9: 1] using N, N'-azobisisobutyronitrile (AIBN) as initiator. The feed composition of comonomers for all copolymers is given in Table 1, and a resulting HPMA copolymer structure is graphically represented in Fig. 1. The comonomer mixtures were sealed in a glass ampoule under N_2 gas and stirred at 50°C for 24 h. Following polymerization, product was obtained by precipitation into diethyl ether. p-Nitrophenol (ONp) content in the polymeric precursors was assessed by release of ONp from the copolymer in 1.0 N sodium hydroxide and quantification of released ONp by UV spectrophotometry at 400 nm. To obtain the final untargeted conjugates, ONp was similarly released from polymeric precursors and dialyzed against distilled water for 72 h in a 3.5 KDa molecular weight cut-off (MWCO) regenerated cellulose dialysis membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Apparent 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 x 30 cm) (GE Healthcare, Piscataway, NJ) using a Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare). The Superose 12 column was previously calibrated with fractions of known molecular weight HPMA homopolymers.

HPMA copolymer-WIFPWIQL conjugate was synthesized via ONp ester aminolysis of polymeric precursors. HPMA copolymer precursor was reacted with WIFPWIQL peptide in anhydrous DMSO in the presence of pyridine

Table 1 Characteristics of HPMA Copolymer Conjugates

Polymer	Description	Feed composition (mol%)					Apparent M_w[a] (kDa)	M_w/M_n	AH-GDM content[b] (wt%)	WIFPWIQL content[c] (mmol/g)
		HPMA	MA-GG-ONp	MA-GFLG-AH-GDM	MA-GG-AH-GDM	MA-Tyr				
HPMA-(GFLG-AH-GDM)	AH-GDM conjugate utilizing lysosomally degradable GFLG linker	73	20	5	-	2	23.7	1.7	16.2	-
HPMA-(GG-AH-GDM)	AH-GDM conjugate having non-degradable GG linker	73	20	-	5	2	26.4	1.5	24.6	-
HPMA-(GFLG-AH-GDM)-WIFPWIQL	AH-GDM conjugate utilizing GFLG linker + WIFPWIQL peptide	73	20	5	-	2	23.7[d]	1.7[d]	16.2[d]	0.147

[a] Estimated by size exclusion chromatography

[b] Determined by UV spectroscopy

[c] Determined by amino acid analysis

[d] Values reported are for precursor polymer

for 24 h. The reaction was terminated, and unreacted ONp was released by slow addition of 0.1 N NaOH. Conjugates were dialyzed against distilled water in a 3.5 KDa MWCO regenerated cellulose dialysis membrane to remove low molecular weight compounds. Targeting peptide content of the conjugate was determined by amino acid analysis (University of Utah Core Research Facilities, Salt Lake City, UT). AH-GDM content of conjugates was determined spectrophotometrically at 340 nm.

Synthesis of AH-GDM Hydrochloride Salt (AH-GDM HCl)

The water-soluble hydrochloride salt of AH-GDM was synthesized for use as a control in stability studies. AH-GDM was dissolved in freshly prepared methanol hydrochloric acid and stirred overnight. The solvent was removed by rotary evaporation, and the resulting material was dissolved in deionized water, filtered through a 0.22 μm filter to remove insoluble AH-GDM, frozen and lyophilized. The resulting product was freely soluble upon reconstitution with deionized water.

Stability of HPMA Copolymer Conjugates

The release of free AH-GDM from the conjugates was assessed in 50 mM NaH_2PO_4 pH 7.4 buffer, 50 mM $NaH_2C_3O_2$ pH 5.0 buffer, complete DU145 cell culture media containing 10% FBS, and 100% FBS. AH-GDM equivalent concentrations were maintained below the aqueous solubility of free AH-GDM (< 300 ug/mL) in all test solutions to prevent saturation. Quantitative amounts of conjugates or AH-GDM·HCl control were dissolved in 1.5 mL of each test solution. One-hundred μL was removed at 0.5, 1, 2, 4, 8, 24, 48 and 72 h time points, extracted 3X with 100 uL dichloromethane and transferred to an HPLC vial. Solvent was removed by evaporation, and the resulting residue was reconstituted in HPLC mobile phase, and 20 μL

Table 2 Cell Growth Inhibition GI_{50} Values of HPMA Copolymer Conjugates in Comparison with Geldanamycin Compounds

Compound	Description	DU145		PC3	
		Mean GI_{50} (μM)	SD GI_{50} (μM)	Mean GI_{50} (μM)	SD GI_{50} (μM)
GDM	Geldanamycin	0.026	0.003	0.028	0.004
AH-GDM	Aminohexylgeldanamycin	2.7	0.2	3.2	0.2
HPMA-(GFLG-AH-GDM)	AH-GDM conjugate utilizing lysosomally degradable GFLG linker	2.6	0.2	2.8	0.3
HPMA-(GG-AH-GDM)	AH-GDM conjugate having non-degradable GG linker	19	2	15	1
HPMA-(GFLG-AH-GDM)-WIFPWIQL	AH-GDM conjugate utilizing GFLG linker + WIFPWIQL peptide	1.7	0.1	1.8	0.1

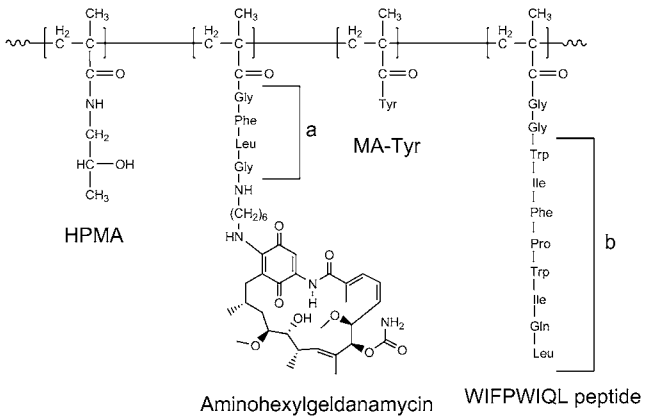

Fig. 1 Schematics of HPMA copolymer–AH-GDM-WIFPWIQL conjugates. [a] Lysosomally degradable GFLG linker represented as found in HPMA-(GFLG-AH-GDM) copolymer. HPMA-(GG-AH-GDM) polymer contains non-degradable GG linker. [b] WIFPWIQL peptide attached to HPMA copolymer backbone via non-degradable GG linker as shown. Untargeted HPMA-(GFLG-AH-GDM) copolymer does not contain WIFPWIQL peptide.

injected for analysis by HPLC. HPLC analyses were performed with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a photo diode array detector set at 350 nm for quantification using a Waters XBridge column (C18, 4.6 x 250 mm, 5 μm) and an isocratic mobile phase of 50 mM $NH_4C_2H_3O_2$: Acetonitrile [65: 35 v/v]. A calibration curve was generated by extracting and processing AH-GDM HCl as noted above. An extraction efficiency of 99.4±0.6% was obtained using this method. The cumulative percent AH-GDM released was calculated and plotted as a function of time. Conjugates dissolved in mobile phase alone were analyzed to determine concentrations at time zero.

Cell Culture

DU145 and PC3 human prostate cancer cell lines were obtained from ATCC (Manassas, VA). DU145 cell lines were cultured in Eagle's Minimum Essential Medium with Earle's Balanced Salt Solution (ATCC) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Thermo Scientific HyClone, Logan, UT). PC3 cell lines were cultured in F-12 K Medium (ATCC) supplemented with 10% (v/v) FBS. Cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2. For all procedures, cells were harvested using TrypLE^{TM} Express (Invitrogen, Carlsbad, CA), and cell lines were maintained in a logarithmic growth phase during the studies.

Radiolabeling of Anti-GRP78 Antibody

Anti-GRP78 antibody was radiolabeled with ^{125}Iodine using the Iodogen method with slight modification (40). Briefly, 20 μL of a 1 mg/mL solution of iodogen reagent in dichloromethane was added to a 1 cm x 7.5 cm glass tube and evaporated to dryness under N_2 gas. Ten μg of anti-GRP78 antibody and 1.0 mCi of Na-^{125}Iodine were combined in the glass tube and allowed to react at room temperature for 10 min with gentle mixing. The solution was transferred to another tube and diluted to 650 μL with phosphate-buffered saline (PBS) at pH 7.4. Radiolabeled anti-GRP78 antibody was purified using a Zeba Spin Desalting Column (Thermo Fisher Scientific) with a MWCO of 7 kDa.

Competitive Cell Receptor Binding Assay and Comparative Expression of Cell Surface-Expressed GRP78

The comparative affinities of free WIFPWIQL peptide and HPMA copolymer conjugates were assessed using a competitive binding assay to cell-surface-expressed GRP78 on DU145 and PC3 cells. DU145 and PC3 cells were harvested, washed with PBS, and re-suspended in binding buffer (20 mmol/L tromethamine, pH 7.4, 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L $MnCl_2$, 0.1% bovine serum albumin). Cell suspension was added in 1.2 μm pore size 96-well Multiscreen HV filter plates (Millipore, Billerica, MA) at 50,000 cells per well. They were then co-incubated at 4°C with 2 ng ^{125}I-anti-GRP78 antibody and increasing targeting peptide equivalent concentrations of copolymer conjugates or free WIFPWIQL peptide between 0 and 500 μM. Following 1 h incubation, media was removed from cells using a Multiscreen vacuum manifold (Millipore), and cells were washed 3X with binding buffer. Filters were collected and radioactivity determined using a Cobra Auto-Gamma-counter (Canberra Industries, Inc., Meriden, CT). Binding percentage relative to control wells containing only ^{125}I-anti-GRP78 antibody was calculated, and non-linear regression analysis and determination of IC_{50} values was carried out using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

The relative cell surface expression of GRP78 in DU145 and PC3 cell lines was estimated by calculating the absolute radioactivity recovered in wells incubated with 2 nm ^{125}I-anti-GRP78 antibody in binding buffer at 4°C for 1 h (control wells) from the preceding binding experiment.

Cell Growth Inhibition Studies

The ability of the conjugates to inhibit growth of DU145 and PC3 human prostate cancer cell lines was evaluated *in vitro* using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) cell viability assay (Dojindo Molecular Technolo-

gies, Inc., Rockville, MD). Due to the poor water solubility of the free drugs GDM and AH-GDM, stock solutions of conjugates, free drugs, and controls were prepared in DMSO and subsequently diluted, resulting in a final concentration of 0.5% (v/v) DMSO in complete growth medium. No significant toxicities were observed for DU145 or PC3 cells when exposed to 0.5% DMSO concentrations for 72 h. DU145 or PC3 cells (3,000 or 7,500 cells per well respectively) were plated in 96-well plates for 24 h. Cell culture medium was then replaced with media containing conjugates, free drugs, or controls, and cells were treated for 72 h. Following treatment, medium was removed, and wells were washed with 200 μL PBS. WST-8 reagent in complete growth medium (100 μL as 10% v/v) was added to each well, cells were incubated at 37°C/5% CO_2 for 120 min, and absorbance at 450 nm minus 630 nm was determined by UV spectrophotometry using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Relative viability was calculated by normalization of the absorbance of untreated cells. Non-linear least-squares regression analysis was performed using GraphPad Prism.

Statistical Analysis

Differences in relative cell binding affinity and *in vitro* growth inhibition IC_{50} values were determined by one-way ANOVA. Where differences were detected, a Bonferroni post-test was used to test for significance between groups. Differences in relative cell surface expression between DU145 and PC3 cells were evaluated using two-sided Student's *T*-test. The significance level was set at $\alpha = 0.05$ for all statistical tests.

RESULTS

Synthesis and Characterization of the Conjugates

Characteristics of HPMA copolymers synthesized are summarized in Table 1. AH-GDM containing copolymers with the degradable peptide linker GFLG, *i.e.*, HPMA copolymer-(GFLG-AH-GDM) and HPMA copolymer-(GFLG-AH-GDM)-WIFPWIQL had an apparent weight average molecular weight of 23.7 KDa, a polydispersity index of 1.67, and an AH-GDM drug content of 16.2% (wt/wt) based on evaluation of the polymeric precursor. The ONp content of the polymeric precursor was 0.639 mmol/g. Following attachment of WIFPWIQL peptide to the polymeric precursor, HPMA copolymer-(GFLG-AH-GDM)-WIFPWIQL had a peptide content of 0.147 mmol/g. The AH-GDM containing copolymer utilizing the non-degradable peptide linker GG had an apparent weight average molecular weight of 26.4 KDa, a polydispersity index of 1.50 and an AH-GDM drug content of 24.6% (wt/wt). No targeting peptide was attached to the AH-GDM containing copolymer utilizing the non-degradable linker.

Stability of the Conjugates

The release of AH-GDM as a function of time from copolymer conjugates was assessed in 50 mM NaH_2PO_4 pH 7.4 buffer, 50 mM $NaH_2C_3O_2$ pH 5.0 buffer, complete DU145 cell culture media containing 10% FBS, and in 100% FBS (Fig. 2). Release was minimal at pH 7.4 and pH 5.0 and in complete DU145 cell culture medium containing 10% FBS with less than approximately 1% release over 72 h, and no difference was observed for conjugates with and without WIFPWIQL peptide. In 100% FBS, release from conjugates was increased, with 6.6% and 11.3% released after 72 h from conjugates with and without WIFPWIQL peptide. The release from the conjugate with WIFPWIQL was less than the untargeted conjugate at 72 h ($p < 0.05$). Overall, the conjugates were stable and exhibited less than 5% release over 24 h in test solutions.

Comparative Cell Surface Expression of GRP78 and Competitive Binding

Competitive binding studies with DU145 and PC3 cells showed binding of copolymer-peptide conjugates to cell-surface expressed GRP78 with IC_{50} values of 1.2 ± 0.3 μM and 4 ± 1 μM, respectively, as shown in Fig. 3. Untargeted conjugates showed no competitive binding. At equivalent peptide concentrations, free peptide showed greater binding affinity ($p < 0.05$) as compared to polymer conjugates with IC_{50} values of 0.29 ± 0.07 μM and 1.1 ± 0.1 μM for both DU145 and PC3 cell lines. Comparative cell surface expressions of GRP78 as determined by ^{125}I-anti-GRP78 antibody binding and gamma counting were 2900 ± 300 and 1200 ± 200 cpm for DU145 and PC3 cell lines, respectively, indicating significantly higher cell surface expression of GRP78 in DU145 *versus* PC3 cell lines ($p < 0.0001$) (Fig. 4).

Cell Growth Inhibition Studies

Cell growth inhibition activity of conjugates in DU145 and PC3 cell lines is shown in Fig. 5. Results demonstrate that modification of geldanamycin at position 17 with 1,6-diaminohexane reduced its ability to inhibit cell growth. All conjugates containing AH-GDM were capable of inhibiting cell growth. Conjugation of AH-GDM to HPMA copolymers did not have any significant effect on growth inhibition ($p > 0.05$), and attachment of WIFPWIQL peptide significantly reduced growth inhibition in both cell lines

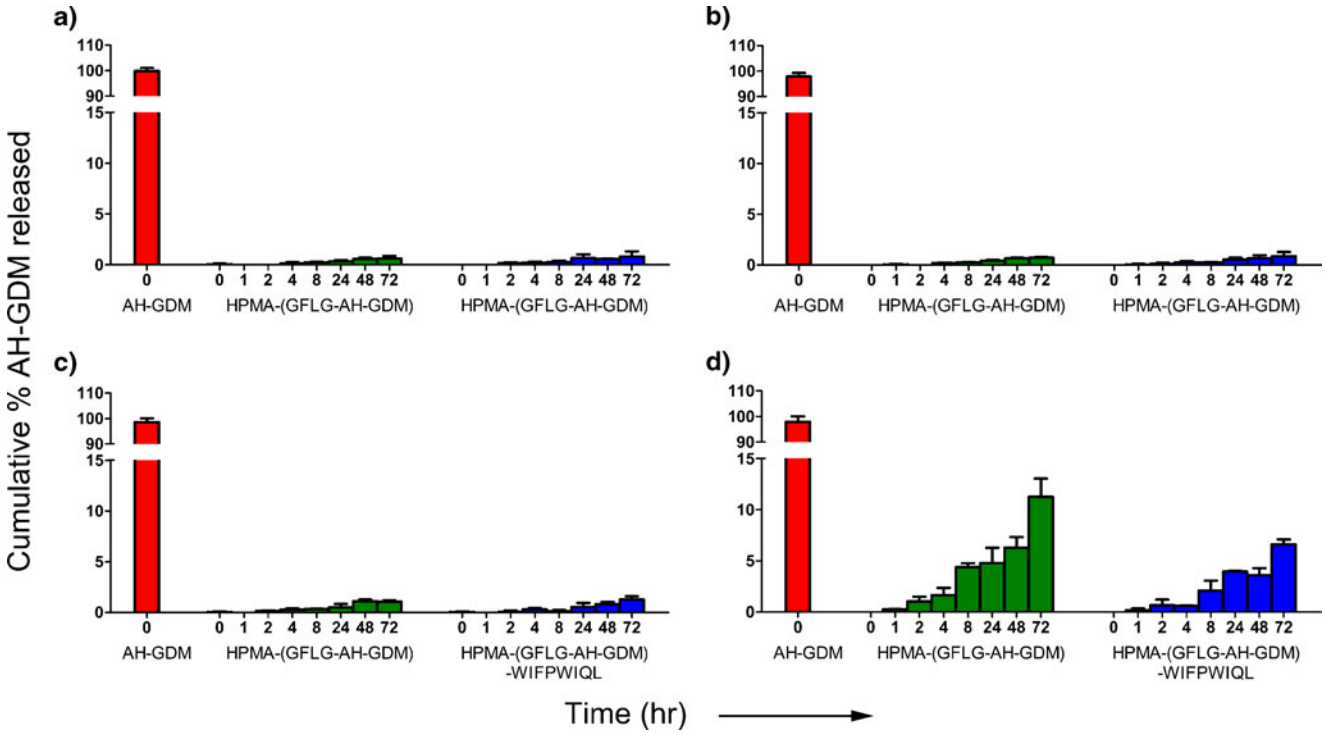

Fig. 2 Stability of HPMA copolymer–AH-GDM-WIFPWIQL conjugates in aqueous media. Release of free AH-GDM from HPMA copolymer-(*GFLG-AH-GDM*) (*green bars*) and HPMA copolymer-(*GFLG-AH-GDM*)-WIFPWIQL (*blue bars*) was assessed in **a** 50 mM NaH_2PO_4 pH 7.4 buffer, **b** 50 mM $NaH_2C_3O_2$ pH 5.0 buffer, **c** complete DU145 cell culture media containing 10% FBS, and **d** 100% FBS. AH-GDM · HCl salt (*red bars*) was included in each case as control. Data expressed as mean±SD.

with GI_{50} values of 1.7±0.1 μM and 1.8±0.1 μM as compared to 2.6±0.2 μM and 2.8±0.3 μM for untargeted conjugates in DU145 and PC3 cell lines, respectively ($p<0.05$). Attachment of AH-GDM to the HPMA backbone via the nondegradable GG linker resulted in a significant loss of growth inhibition with GI_{50} values of 19±2 μM and 15±1 μM, respectively, for DU145 and PC3 cell lines as compared to AH-GDM attached via the lysosomally cleavable GFLG linker ($p<0.001$). HPMA homopolymer and free WIFPWIQL peptide controls showed no significant reduction in cell viability over equivalent concentration ranges (data not shown).

DISCUSSION

The synthesis and *in vitro* characterization of a new HPMA copolymer-AH-GDM conjugate bearing prostate cancer-targeting WIFPWIQL peptide is described herein. It is anticipated that the therapeutic index of AH-GDM can be improved by selectively targeting these conjugates to the cell-surface-expressed GRP78 of metastatic prostate cancer cells. HPMA copolymers containing the geldanamycin derivative AH-GDM and bearing WIFPWIQL peptide were successfully synthesized. Modification of native GDM to AH-GDM is necessary to generate a reactive free amine, thus enabling conjugation to the HPMA copolymer backbone. AH-GDM was chosen over other amine geldanamycin derivates since they have previously demonstrated favorable stability and cleavage by the lysosomal enzyme cathepsin B (41). HPMA copolymers achieved an AH-GDM drug loading ranging from 16.2 to 24.6 wt% and were water soluble. To generate the targetable HPMA copolymer, WIFPWIQL peptide was successfully conjugated to the HPMA backbone via the non-degradable GG linker, and conjugation was verified and quantified by amino acid analysis. The molecular weight and molecular weight distribution of the conjugates were estimated by SEC and were comparable to similar conjugates previously reported (15, 24).

Competitive binding studies demonstrated binding of WIFPWIQL peptide-bearing conjugates to cell-surface-expressed GRP78 in DU145 and PC3 cell lines, and untargeted conjugates showed no evidence of competitive binding in either cell line as demonstrated in Fig. 3. Although a statistical difference in the binding affinity of free peptide in comparison with the targeted conjugate at equivalent peptide concentrations was detected, the binding affinities were similar and demonstrate that attachment of WIFPWIQL peptide to the HPMA copolymer backbone has little effect on the ability of WIFPWIQL peptide to actively bind GRP78. Cell surface expression of GRP78

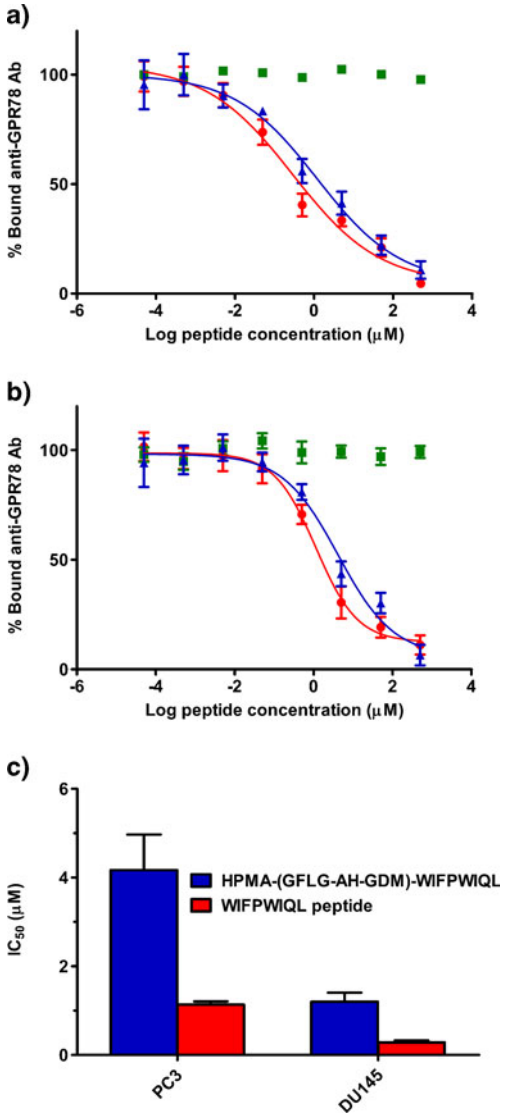

Fig. 3 Competitive binding of HPMA copolymer-AH-GDM-WIFPWIQL conjugates to DU145 and PC3 cells lines. **a** DU145 or **b** PC3 cells were seeded in filter plates and co-incubated at 4°C with ^{125}I-anti-GRP78 antibody and increasing targeting peptide equivalent concentrations of HPMA copolymer-(*GFLG-AH-GDM*) (*green squares*), HPMA copolymer-(*GFLG-AH-GDM*)-WIFPWIQL (*blue triangles*), or free WIFPWIQL peptide (red circles). Following 1 h incubation, media was removed and cells washed. Filters were collected and radioactivity determined by γ-counting. Binding percentage relative to control wells containing only ^{125}I-anti-GRP78 antibody was calculated and data analyzed by non-linear regression. **c** IC_{50} values for HPMA copolymer-(*GFLG-AH-GDM*)-WIFPWIQL (*blue bars*) and WIFPWIQL peptide (*red bars*) represented graphically for comparison. HPMA copolymer-(*GFLG-AH-GDM*) demonstrated no competitive binding to DU145 or PC3 cells. Data expressed as mean±SD.

was observed in both DU145 and PC3 cell lines and agrees with previous studies (32, 42).

Growth inhibition studies demonstrated the ability of all conjugates bearing AH-GDM to limit the proliferation of both DU145 and PC3 cells *in vitro*. The modification of geldanamycin at position 17 with 1,6-diaminohexane reduced its ability to inhibit growth *in vitro* and is consistent with previous reports. It is possible that alternative geldanamycin analogues having functionality allowing conjugation to polymeric backbones may need to be further investigated. However, previous animal studies (15) have demonstrated that HPMA copolymers bearing AH-GDM are tolerated at much higher doses than free AH-GDM, suggesting that efficacious levels can be delivered. It is interesting to note that conjugation of AH-GDM to the HPMA copolymer backbone did not result in a significant decrease in its ability to inhibit growth of DU145 and PC3 cell lines. An additional HPMA-copolymer bearing AH-GDM was synthesized to investigate whether release of AH-GDM from the HPMA copolymer backbone was critical for growth inhibition. In this case, the AH-GDM was attached to the copolymer backbone via the non-degradable GG linker, as compared to the lysosomally degradable GFLG linker. The ability of the HPMA copolymer containing the non-degradable GG linker to inhibit growth was reduced approximately seven-fold and five-fold for DU145 and PC3 cell lines, suggesting the necessity of release of free AH-GDM via lysosomal degradation as well as increased activity and binding to HSP90 of free AH-GDM as compared to HPMA copolymer bound AH-GDM. The ability of the non-degradable system to inhibit cell proliferation is, however, maintained, with a GI_{50} of 19 μM and 15 μM observed for DU145 and PC3 cell lines. A similar result has been reported for HPMA copolymer conjugates bearing adriamycin bound to the polymer backbone via GFLG and GG linkages (43). However, the exact mechanism of action for the non-degradable system requires further investigation.

The ability to target malignant tumors such as prostate cancer is a long-standing goal in oncology. Unfortunately, tumor-targeting approaches tend to suffer from lack of specificity and incomplete tissue penetration. By screening

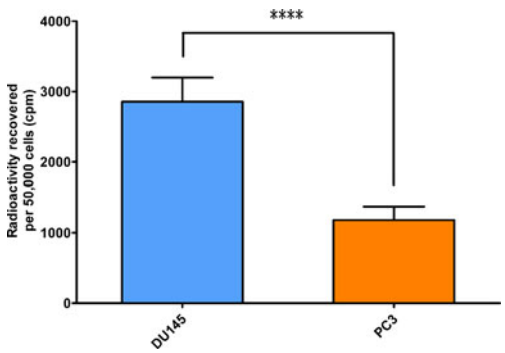

Fig. 4 Relative GRP78 cell surface expression in DU145 and PC3 cell lines. DU145 or PC3 cells were seeded in filter plates and incubated with 2 nM ^{125}I-anti-GRP78 antibody in binding buffer at 4°C for 1 h. Following 1 h incubation, media was removed and cells were washed. Filters were collected and radioactivity determined by γ-counting. Data expressed as mean±SD. **** Indicates significance at $p < 0.0001$.

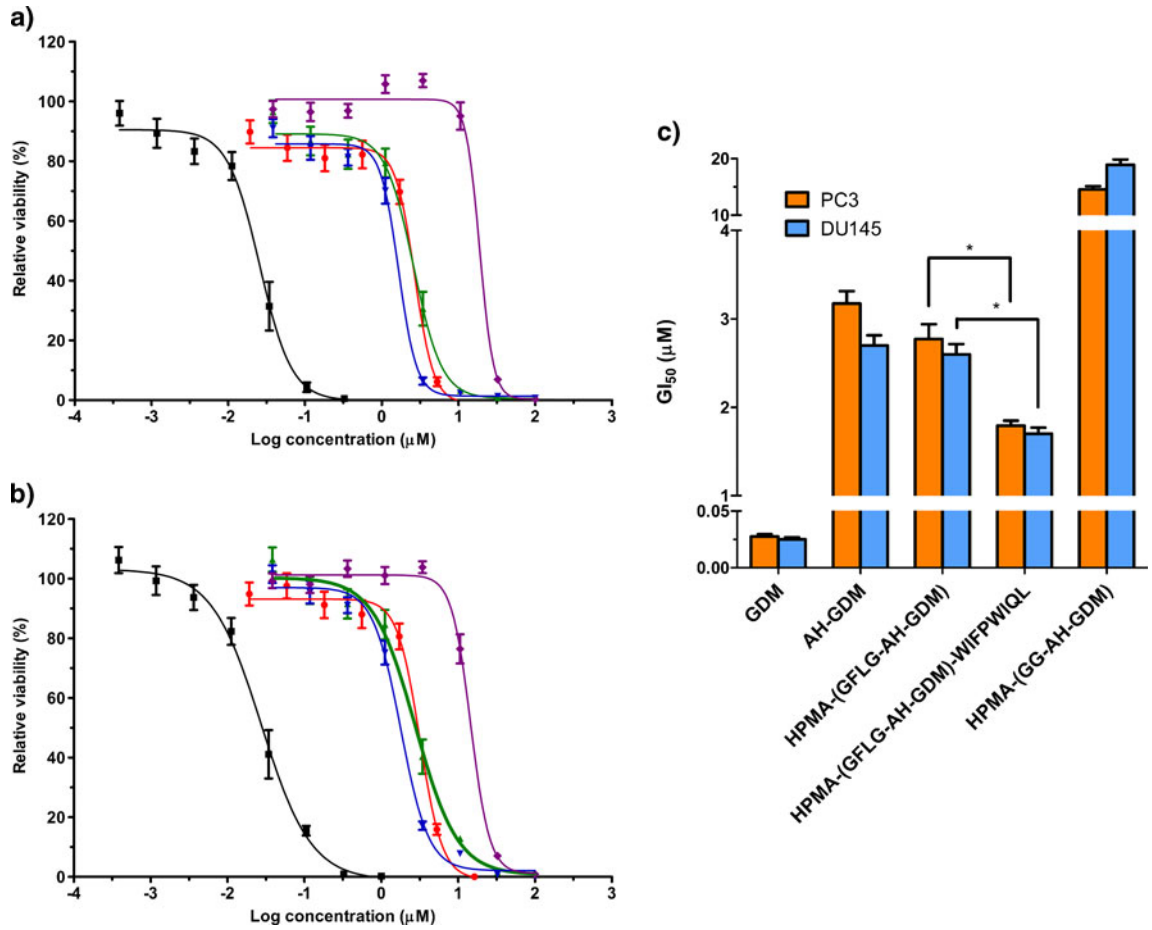

Fig. 5 Growth inhibition of HPMA copolymer–AH-GDM-WIFPWIQL conjugates. **a** DU145 or **b** PC3 cells were treated for 72 h with increasing drug equivalent concentrations of GDM (*black squares*), AH-GDM (*red circles*), HPMA copolymer-(*GFLG-AH-GDM*) (*green triangles*), HPMA copolymer-(*GFLG-AH-GDM*)-WIFPWIQL (*blue reverse triangles*), or HPMA copolymer-(*GG-AH-GDM*) (*purple diamonds*). Following treatment, cell viability was assessed by WST-8 assay. **c** GI_{50} values were determined in DU145 (*blue bars*) and PC3 (*orange bars*) by analysis using GraphPad Prism. * Statistical difference detected ($p < 0.05$) between untargeted and targeted conjugates. Other statistical differences exist between other groups and are not explicitly shown.

combinatorial libraries of peptides and antibodies using phage display, unique targeting ligands have been identified. This approach directly selects, *in vivo*, for circulating probes capable of preferential homing into tumors. As a result, new markers have been uncovered, providing a means for selective targeting of therapies and new insights into normal prostate and prostate cancer vasculature and tumor cell specificities.

Application of this technology has led to isolation of GRP78. This is a chaperone heat-shock protein which has been isolated by fingerprinting the circulating repertoire of antibodies from cancer patients (44) and has emerged as an excellent cancer target. GRP78 receptors (a) are abundant and functional on the tumor cell surface, (b) can confer tumor selectivity on specific inhibitors, and (c) regulate multiple signaling pathways related to apoptosis, immune response, and drug resistance. Given the promising therapeutic data in tumor models and the presence of the receptor in patient-derived samples, this system provides an ideal platform for targeted drug development. Studies on several ligand-receptor systems based on the tumor cell membrane expression of GRP78 have resulted in identification of a lead peptide motif, namely WIFPWIQL (32), which was used in this study. This peptide has been shown to specifically target tumor cells *in vitro*, *in vivo*, and in human cancer specimens *ex vivo*. Moreover, synthetic chimeric peptides composed of GRP78-binding motif WIFPWIQL, fused to a programmed cell death-inducing sequence promoted tumor suppression in xenograft and isogenic mouse models of prostate and breast cancer (32). Collectively, these preclinical data validate GRP78 on the tumor cell surface as a functional molecular target and WIFPWIQL as a useful targeting peptide that show potential for translation into clinical applications.

In this study, the attachment of the WIFPWIQL peptide to the side chains of biocompatible HPMA copolymers containing AH-GDM increased the ability of the conjugate to inhibit the growth of both DU145 and PC3 cell lines. Combined with the results from the competitive binding studies which show active binding of the targeted conjugate to both cell lines, this increased potency is possibly due to a

combination of both increased binding and cellular uptake. This result, coupled with our previous observations that HPMA copolymer AH-GDM conjugates terminated in cyclic RGD peptides substantially increase the localization of drug in solid tumors and improve safety (15, 24), bodes well for combination delivery of AH-GDM to both angiogenic and tumor cells for a more effective and less toxic treatment of prostate cancer.

CONCLUSIONS

HPMA copolymer conjugates containing the geldanamycin analogue AH-GDM and WIFPWIQL peptide for targeting cell-surface-expressed GRP78 were synthesized and characterized. Conjugates were stable under physiological conditions. The HPMA copolymer conjugate containing AH-GDM and WIFPWIQL peptide showed binding affinity to cell-surface-expressed GRP78 in both DU145 and PC3 prostate cancer cell lines as compared to the untargeted conjugate. The targeted conjugate also exhibited greater ability to inhibit the growth of prostate cancer cells *in vitro* as compared to the untargeted conjugate. This conjugate shows promise as a therapeutic agent in combination strategies for delivery of aminohexylgeldanamycin to solid tumors.

ACKNOWLEDGEMENTS

This research was supported by the National Institutes of Health grant R01 EB007171 and the Utah Science Technology and Research (USTAR) initiative.

REFERENCES

1. Cancer Facts & Figures 2009, American Cancer Society, Atlanta, Georgia, 2009.
2. Porter JR, Ge J, Lee J, Normant E, West K. Ansamycin inhibitors of Hsp90: nature's prototype for anti-chaperone therapy. Curr Top Med Chem. 2009;9:1386–418.
3. Fukuyo Y, Hunt CR, Horikoshi N. Geldanamycin and its anticancer activities. Cancer Lett. 2010;290:24–35.
4. Sharpand S, Workman P. Inhibitors of the HSP90 molecular chaperone: current status. Adv Cancer Res. 2006;95:323–48.
5. Banerji U. Heat shock protein 90 as a drug target: some like it hot. Clin Cancer Res. 2009;15:9–14.
6. Supko JG, Hickman RL, Grever MR, Malspeis L. Preclinical pharmacologic evaulation of geldanamycin as an antitumor agent. Cancer Chemother Pharmacol. 1994;36:305–15.
7. Ronnen EA, Kondagunta GV, Ishill N, Sweeney SM, Deluca JK, Schwartz L, *et al.* 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.
8. Banerji U, O'Donnell A, Scurr M, Pacey S, Stapleton S, Asad Y, *et al.* Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. J Clin Oncol. 2005;23:4152–61.
9. Kopecek J, Kopeckova P. HPMA copolymers: origins, early developments, present, and future. Adv Drug Deliv Rev. 2010;62:122–49.
10. Davis FF. The origin of pegnology. Adv Drug Deliv Rev. 2002;54:457–8.
11. Vicent MJ, Manzanaro S, de la Fuente JA, Duncan R. HPMA copolymer-1, 5-diazaanthraquinone conjugates as novel anticancer therapeutics. J Drug Target. 2004;12:503–15.
12. Seymour LW, 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.
13. Mitra A, Nan A, Ghandehari H, McNeill E, Mulholland J, Line BR. Technetium-99 m-labeled N-(2-hydroxypropyl) methacrylamide copolymers: synthesis, characterization, and in vivo biodistribution. Pharm Res. 2005;21:1153–9.
14. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release. 2000;65:271–84.
15. Borgman MP, Ray A, Kolhatkar RB, Sausville EA, Burger AM, Ghandehari H. Targetable HPMA copolymer-aminohexylgeldanamycin conjugates for prostate cancer therapy. Pharm Res. 2009;26:1407–18.
16. Nan A, Nanayakkara NP, Walker LA, Yardley V, Croft SL, Ghandehari H. N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers for targeted delivery of 8-aminoquinoline antileishmanial drugs. J Control Release. 2001;77:233–43.
17. Nan A, Croft SL, Yardley V, Ghandehari H. Targetable water-soluble polymer-drug conjugates for the treatment of visceral leishmaniasis. J Control Release. 2004;94:115–27.
18. Subr V, Kopecek J, Pohl J, Baudys M, Kostka V. Cleavage of oligopeptide side-chains in N-2(hydroxpropyl)meth-acrylamide copolymers by mixtures of lysosomal enzymes. J Control Release. 1988;8:133–40.
19. D. Putnam and J. Kopeček. Polymer conjugates with anticancer activity. Biopolymers II, Vol. 122, Springer Berlin, 1995, pp. 55–123.
20. Seymour LW, Ferry DR, Kerr DJ, Rea D, Whitlock M, Poyner R, *et al.* Phase II studies of polymer-doxorubicin (PK1, FCE28068) in the treatment of breast, lung and colorectal cancer. Int J Oncol. 2009;34:1629–36.
21. Duncan R. Development of HPMA copolymer-anticancer conjugates: clinical experience and lessons learnt. Adv Drug Deliv Rev. 2009;61:1131–48.
22. Duncan R, Vicent MJ. Do HPMA copolymer conjugates have a future as clinically useful nanomedicines? A critical overview of current status and future opportunities. Adv Drug Deliv Rev. 2010;62:272–82.
23. Mitra A, Coleman T, Borgman M, Nan A, Ghandehari H, Line BR. Polymeric conjugates of mono- and bi-cyclic alphaVbeta3 binding peptides for tumor targeting. J Control Release. 2006;114:175–83.
24. Borgman MP, Aras O, Geyser-Stoops S, Sausville EA, Ghandehari H. Biodistribution of HPMA copolymer-aminohexylgeldanamycin-RGDfK conjugates for prostate cancer drug delivery. Mol Pharm. 2009;6:1836–47.
25. Pike DB, Ghandehari H. HPMA copolymer-cyclic RGD conjugates for tumor targeting. Adv Drug Deliv Rev. 2010;62:167–83.
26. Benjamin LE, Keshet E. Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc Natl Acad Sci U S A. 1997;94:8761–6.
27. Shiu RP, Pouyssegur J, Pastan I. Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins

in Rous sarcoma virus-transformed chick embryo fibroblasts. Proc Natl Acad Sci U S A. 1977;74:3840–4.
28. Hendershot LM. The ER function BiP is a master regulator of ER function. Mt Sinai J Med. 2004;71:289–97.
29. Dong D, Dubeau L, Bading J, Nguyen K, Luna M, Yu H, *et al.* Spontaneous and controllable activation of suicide gene expression driven by the stress-inducible GRP 78 promoter resulting in eradication of sizable human tumors. Hum Gene Ther. 2004;15:553–61.
30. Misra UK, Deedwania R, Pizzo SV. Activation and cross-talk between Akt, NF-kappaB, and unfolded protein response signaling in 1-LN prostate cancer cells consequent to ligation of cell surface-associated GRP78. J Biol Chem. 2006; 281:13694–707.
31. Zhang Y, Liu R, Ni M, Gill P, Lee AS. Cell surface relocalization of the endoplasmic reticulum chaperone and unfolded protein response regulator GRP78/BiP. J Biol Chem. 2010;285:15065–75.
32. Arap MA, Lahdenranta J, Mintz PJ, Hajitou A, Sarkis AS, Arap W, *et al.* Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. Cancer Cell. 2004;6:275–84.
33. Y. Katanasaka, T. Ishii, T. Asai, H. Naitou, N. Maeda, F. Koizumi, S. Miyagawa, N. Ohashi, and N. Oku. Cancer antineovascular therapy with liposome drug delivery systems targeted to BiP/GRP78. Int J Cancer (Epub 2010/02/24).
34. Wu WC, Kao YH, Hu PS, Chen JH. Geldanamycin, a HSP90 inhibitor, attenuates the hypoxia-induced vascular endothelial growth factor expression in retinal pigment epithelium cells in vitro. Exp Eye Res. 2007;85:721–31.
35. Strohalm J, Kopecek J. Poly N-(2-hydroxypropyl) methacrylamide: 4. Heterogenous polymerization. Angew Makromol Chem. 1978;70:109–18.
36. Rejmanova P, Labsky J, Kopecek J. Aminolyses of monomeric and polymeric p-nitrophenyl esters of methacryloylated amino acids. Makromol Chem. 1977;178:2159–68.
37. 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 Control Release. 2000;64:63–79.
38. Lee JH, Kopeckova P, Kopecek J, Andrade JD. Surface properties of copolymers of alkyl methacrylates with methoxy (polyethylene oxide) methacrylates and their application as protein-resistant coatings. Biomaterials. 1990;11:455–64.
39. Kasuya Y, Lu ZR, Kopeckova P, Minko T, Tabibi SE, Kopecek J. Synthesis and characterization of HPMA copolymer-aminopropylgeldanamycin conjugates. J Control Release. 2001;74:203–11.
40. Walker JM. The protein protocols handbook. Towota: Humana; 1996.
41. Kasuya Y, Lu ZR, Kopeckova P, Tabibi SE, 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.
42. Misra UK, Deedwania R, Pizzo SV. Binding of activated alpha2-macroglobulin to its cell surface receptor GRP78 in 1-LN prostate cancer cells regulates PAK-2-dependent activation of LIMK. J Biol Chem. 2005;280:26278–86.
43. Malugin A, Kopeckova P, Kopecek J. Liberation of doxorubicin from HPMA copolymer conjugate is essential for the induction of cell cycle arrest and nuclear fragmentation in ovarian carcinoma cells. J Control Release. 2007;124:6–10.
44. Guerriero R, Testa U, Gabbianelli M, Mattia G, Montesoro E, Macioce G, *et al.* Unilineage megakaryocytic proliferation and differentiation of purified hematopoietic progenitors in serum-free liquid culture. Blood. 1995;86:3725–36.