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Synergistic enhancement of cancer therapy using a combination of heat shock protein targeted HPMA copolymer-drug conjugates and gold nanorod induced hyperthermia

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Abstract

In the field of nanomedicine, selective delivery to cancer cells is a common goal, where active targeting strategies are often employed to increase tumor accumulation. In this study, tumor hyperthermia was utilized as a means to increase the active delivery of heat shock protein (HSP) targeted *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-drug conjugates. Following hyperthermia, induced expression of cell surface heat shock protein (HSP) glucose regulated protein 78 kDa (GRP78) was utilized for targeted drug therapy. Conjugates bearing the anticancer agents aminohexylgeldanamycin (AHGDM), docetaxel (DOC), or cisplatin and the GRP78 targeting peptide WDLAWMFRLPVG were synthesized and characterized. Binding to cell surface expressed heat shock protein GRP78 on the surface of human prostate cancer DU145 cells was evaluated. HSP targeted AHGDM and DOC conjugates demonstrated active binding comparable to native targeting peptide. They were then assessed *in vitro* for the ability to synergistically induce cytotoxicity in combination with moderate hyperthermia (43°C, 30 min.). HSP targeted DOC conjugates exhibited high potency against DU145 cells with an IC₅₀ of 2.4 nM. HSP targeted AHGDM and DOC conjugates demonstrated synergistic effects in combination with hyperthermia with combination index values of 0.65 and 0.45 respectively. Based on these results, HSP targeted DOC conjugates were selected for *in vivo* evaluation. In DU145 tumor bearing mice, a single treatment of tumor hyperthermia, induced via gold nanorod mediated plasmonic photothermal therapy, and intravenous administration of HSP targeted HPMA copolymer-docetaxel at 10 mg/kg resulted in maintained tumor regression for a period of 30 days. These results demonstrate the potential for tumor hyperthermia to increase the delivery of HSP targeted macromolecular chemotherapeutics.

Keywords

HPMA copolymers; hyperthermia; GRP78; gold nanorods; targeted drug delivery; prostate cancer

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Introduction

In the continual fight against cancer, chemotherapy remains a powerful tool for clinicians, particularly for patients with advanced or metastatic disease. Although chemotherapeutics may act through various molecular mechanisms, they share essentially the same goal of preventing the replication and growth of cancer cells. They are pharmacologically designed to act primarily on rapidly dividing cells present within cancerous tissue. Much recent attention has focused on targeted drug delivery[1], whereby specific delivery of chemotherapeutics to the tumor site is desired. By physically localizing drugs to only the region of interest, exposure to healthy tissues is minimized, resulting in decreased adverse effects. It is anticipated that this additional physical targeting, coupled with the already existing pharmacological targeting can result in improvements in the safety profile of chemotherapeutics. As the goal of the clinician in administering chemotherapy is to eradicate or reduce the size of cancerous tumors, these improvements in safety offer opportunities for more aggressive treatment, thereby increasing the odds of positive, clinical response.

One option to achieve enhanced delivery is via active targeting. This approach relies on the inclusion of cancer-specific targeting moieties, which are designed to enhance cancer cell binding and internalization.[2] For example, it was previously demonstrated that the attachment of cyclic Arg-Gly-Asp (RGD) peptides to the side chains of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers can facilitate binding to $\alpha_v\beta_3$ cell adhesion integrins of angiogenic blood vessels in the neovasculature of solid tumors. In this way, tumor accumulation and retention can be increased several fold as compared to their non-targeted counterparts.[3, 4]

The utilization of such active targeting strategies, while effective, does not address the issue of tumor heterogeneity.[5] Although it is well understood that a tumor is not a collective mass of cells with a single phenotype, tumors are often characterized as “positive” or “negative” for a particular cancer cell marker or target. In reality, however, tumors are composed of multiple cell populations whose dynamic nature is supported by both genetic instability and epigenetic diversity.[6, 7] Therefore, only a subset of cells within a tumor may be susceptible to a particular targeted therapy. While initial treatment often yields progress in terms of tumor reduction, selection occurs resulting in tumor adaptation, resistance and recurrence.[8]

Previously, we have described a strategy which seeks to in part overcome these limitations by increasing tumor accumulation and the efficiency of active targeting.[9-11] This was achieved using a combination of gold nanorod-induced tumor hyperthermia and heat shock protein targeted HPMA copolymer-drug conjugates. Following exposure to hyperthermia, increases in cellular stress induce the expression of heat shock proteins (HSPs), which act as molecular chaperones to help stabilize cellular protein structures and inhibit protein aggregation.[12] The vast majority of cancer research in this realm has focused on the inhibition of HSPs and the resulting client protein aggregation and subsequent induction of apoptosis.[13] A number of clinical trials evaluating HSP inhibition are currently in progress for a variety of different cancers.[14] Previously, we have demonstrated that glucose regulated protein 78 kDa (GRP78), a member of the HSP70 family of proteins, is induced following heat shock.[9, 15, 16] While much of this expression occurs within the cell, significant expression also occurs on the cell surface.[9, 17] We therefore utilize this induced cell surface expression as a specific cancer target to increase delivery. This is in contrast to other HSP related therapies, where the inhibition of HSPs results in tumor cell

death. Such a strategy is advantageous in that it does not rely on the inherent baseline expression of any particular cell receptor, but is directed instead towards this induced target.

In this strategy, delivery of tumor hyperthermia is performed via gold nanorod (GNR) mediated plasmonic photothermal therapy (PPTT). Gold nanorods, having a well-defined size and shape exhibit a localized surface plasmon resonance (SPR) peak in the near infrared range.[18] When excited via a laser light source of a similar wavelength, hyperthermia can be induced in an efficient and extremely localized fashion. Using this technique, we have demonstrated that hyperthermia can increase the tumor accumulation and penetration of macromolecules via localized increases in blood flow and vascular permeability.[10, 11]

The current study is focused on the evaluation of heat shock protein (GRP78) targeted HPMA copolymer-drug conjugates in combination with hyperthermia in a prostate cancer model. Polymer-drug conjugates containing the following chemotherapeutics were evaluated: aminohexylgeldanamycin (AHGDM), an HSP90 inhibitor; docetaxel, a microtubule stabilizer; and cisplatin, a DNA crosslinker. The targeting peptide WDLAWMFRLPVG, previously selected by phage display[19], was conjugated to the side chains of the copolymers to facilitate binding to cell surface expressed GRP78. The relative binding affinity towards GRP78 for each conjugate was evaluated and *in vitro* cytotoxicity was assessed in combination with hyperthermia to determine if this strategy resulted in antagonistic, additive, or synergistic effects. Finally, utilizing GNR-mediated PPTT to deliver localized hyperthermia, this combination strategy was evaluated for efficacy and general tolerability *in vivo* in human prostate cancer bearing mice.

Materials and Methods

Materials

Geldanamycin (NSC 122750) was supplied by the National Cancer Institute Developmental Therapeutics Program (NCI DTP). Docetaxel was provided by AK Scientific (Mountain View, CA). Potassium tetrachloroplatinate ($\text{Cl}_4\text{K}_2\text{Pt}$) was obtained from Alfa Aesar (Ward Hill, MA). The GRP78 targeting peptide WDLAWMFRLPVG and the corresponding scrambled peptide RWLVVADPFLMG were synthesized via Fmoc chemistry using a Protein Technologies (Tucson, AZ) PS3 solid phase peptide synthesizer and identities verified by amino acid analysis and ESI/MS. For competitive binding studies, a rabbit anti-GRP78/BiP antibody (Enzo Life Sciences #ADI-SPA-826F) was radiolabeled with ^{125}I using a modified Iodogen method as previously described.[20] $\text{Na-}^{125}\text{I}$ was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and Iodogen reagent (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) was obtained from Thermo Fisher Scientific (Rockford, IL).

Synthesis and characterization of gold nanorods

Gold nanorods (GNRs) were synthesized using the seed-mediated growth method as previously described.[21] PEGylation was done by addition of methoxy-PEG-thiol (5kDa, Creative PEGWORKS, Winston Salem, NC) to the gold nanorod suspension. The average length and width of the GNRs were determined by transmission electron microscopy (TEM) to be 59×15 nm. The light absorption profile was evaluated by UV spectrometry, where a corresponding surface plasmon resonance peak of 800 nm was observed.

Comonomer synthesis and characterization

The comonomers *N*-(2-hydroxypropyl)methacrylamide (HPMA) [22]; *N*-methacryloylglycylglycyl-2-thiazolidine-2-thione (MA-GG-TT) [23]; *N*-methacryloylglycylphenylalanylleucylglycine (MA-GFLG-OH) [24]; and *N*-methacryloyl-tyrosinamide

(MA-Tyr-CONH₂) [25] were synthesized and characterized according to previously described methods. Conjugation of the anticancer agents docetaxel and AHGDM to produce the drug comonomers MA-GFLG-DOC and MA-GFLG-AHGDM were carried out and characterized by mass spectrometry as previously described [26, 27]. The precursor comonomer *N*-methacryloyl-glycylphenylalanylleucylglycyl-*N*-Boc-1,2-diaminoethane (MA-GFLG-NH-Et-NH-Boc), utilized later for cisplatin complexation, was synthesized by coupling MA-GFLG-OH to *N*-Boc-1,2-diaminoethane. Briefly, 1 mole eq. of MA-GFLG-OH and 3 mol eq. of *N*-Boc-1,2-diaminoethane were dissolved in anhydrous DMF. The solution was cooled to 4°C and 3 mol eq. of DIPC and DIPEA were then added dropwise. The solution was stirred overnight followed by additional stirring at room temperature for 1 hour. DMF was then removed and the resulting residue re-dissolved in diethyl ether and washed repeatedly with sodium phosphate buffer at pH 6.0. The final product was obtained after removal of diethyl ether and identity verified by mass spectrometry.

Synthesis and characterization of HPMA copolymer-drug conjugates

HPMA copolymers were synthesized via free radical copolymerization of comonomers in methanol acidified with glacial acetic acid using 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) as the initiator. The feed ratio of comonomers for each copolymer is given in Table I. The concentrations of comonomers and initiator in solution were maintained at 17.5 and 0.5 wt% to obtain conjugates with molecular weights ranging from 70 – 90 kDa. Polymerization was performed in a sealed ampule under N₂ gas and the temperature was maintained at 50°C for 24 h. Post polymerization, the polymers were precipitated and washed repeatedly in diethyl ether. Heat shock targeted conjugates were obtained by aminolysis of 2-thiazolidine-2-thione containing precursor copolymers with the GRP78 targeting peptide WDLAWMFRLPVG, and non-targeted conjugates were obtained by aminolysis with RWLWVADPFLMG (scrambled) peptide in anhydrous DMSO with 3 mole equivalents of DIPEA. For DOC conjugates, following aminolysis, reaction mixtures were diluted in 50 mM pH 6.0 sodium citrate buffer to prevent premature release of DOC via ester hydrolysis. For AHGDM conjugates, following aminolysis, reaction mixtures were diluted in DI water. Conjugates were then dialyzed against distilled water for 72 h using 3.5 kDa molecular weight cut-off (MWCO) regenerated cellulose dialysis membranes (Spectrum Laboratories, Inc., Rancho Dominguez, CA). The dialyzed solution was then lyophilized and stored at -20°C.

Cisplatin precursor copolymers were obtained by copolymerization of MA-GFLG-NH-Et-NH-Boc comonomers with MA-GG-TT, MA-Tyr-CONH₂, and HPMA in methanol. The comonomer feed ratio for each of the conjugates are given in Table I. The concentrations of comonomers and initiator (VA-044) in solution were maintained at 17.5 and 0.5 wt% respectively. Polymerization was performed in a sealed ampule under N₂ gas and the temperature was maintained at 50°C for 24 h. Post polymerization, the polymers were precipitated and washed repeatedly in diethyl ether. Following copolymerization, HSP targeted and non-targeted (scrambled peptide) conjugates were obtained via peptide aminolysis in DMSO/DIPEA in a similar manner as was performed for AHGDM and DOC conjugates. Following aminolysis, boc deprotection was performed in a cleavage cocktail which consisted of trifluoroacetic acid:ethanedithiol:triisopropylsilane:water [92.5:2.5:2.5:2.5 v/v], resulting in side chains bearing diamine groups for subsequent cisplatin complexation. Following deprotection, copolymers were dialyzed against distilled water using a 3.5 kDa MWCO dialysis membrane and lyophilized. Complexation of cisplatin to the resulting diamine side chains was then performed in distilled water using 5 mole equivalents of Cl₄K₂Pt. The resulting conjugates were again dialyzed against distilled water and lyophilized, followed by storage at -20°C.

Weight average molecular weight (M_w) and polydispersity (M_w / M_n) were determined by size exclusion chromatography (SEC) on a Superose 12 column (10 mm $\times$ 30 cm) (GE Healthcare, Piscataway, NJ) using a Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare). Samples were monitored for UV, differential refractive index (dRI), multi-angle laser light scattering (MALLS) and dynamic light scattering (DLS) detection using a DAWN HELEOS II light scattering instrument (Wyatt Technologies, Santa Barbara, CA). SEC coupled with dRI detection was used to confirm the absence of small molecular weight impurities. MALLS was used to determine molecular weight and polydispersity. For AHGDM conjugates, drug content was determined by UV spectrophotometry at 332 nm. For DOC conjugates, drug content was measured following enzymatic release of DOC by papain followed by quantification of free DOC by HPLC as previously described [26]. For cisplatin conjugates, Pt content was quantified by inductively coupled plasma mass spectrometry (ICP-MS) analysis (University of Utah Core Research Facilities, Salt Lake City, UT). Targeting peptide content for heat shock targeted and non-targeted (scrambled peptide) conjugates was quantified by amino acid analysis (University of Utah Core Research Facilities).

Cell culture

The DU145 human prostate cancer cell line was obtained from ATCC (Manassas, VA) and cultured at 37°C in a humidified atmosphere of 5% CO₂ in Eagle's Minimum Essential Medium (ATCC #30-2003) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone, Logan, UT). Cells were maintained in a logarithmic growth phase during all studies.

Competitive binding affinity of heat shock targeted conjugates to cell surface expressed GRP78

The comparative binding affinities of the GRP78 targeting peptide WDLAWMFRLPVG and copolymer-drug conjugates bearing the same to cell surface expressed GRP78 on the surface of DU145 cells were assessed using a competitive binding assay. Cells were harvested, washed with PBS, and re-suspended in binding buffer (PBS pH 7.4 + 1.0 % bovine serum albumin). Suspended cells were added to 1.2 μ m pore size 96-well Multiscreen HV filter plates (Millipore, Billerica, MA) at 100,000 cells per well. They were co-incubated at 4°C with 2 ng of ¹²⁵I-anti-GRP78 antibody and increasing peptide equivalent concentrations of copolymer conjugates or free WDLAWMFRLPVG peptide between 0 and 500 μ M. After 2 hrs, incubation was discontinued and media removed by vacuum filtration using a multiscreen vacuum manifold (Millipore). Retained cells were washed 5 times with PBS pH 7.4 + 1.0 % BSA. BSA was included in the medium to minimize non-specific binding to cells and surfaces during the assay. Filters were collected and radioactivity determined using a Cobra Auto-Gamma-counter (Canberra Industries, Inc., Meriden, CT). Each experiment was performed in triplicate, with 3 samples per experiment. Binding percentages relative to cells incubated with ¹²⁵I-anti-GRP78 antibody alone were calculated and non-linear regression analysis and determination of IC₅₀ values carried out using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Incubation with non-targeted copolymer conjugates (scrambled peptide) was also performed at equivalent peptide concentrations to serve as negative controls.

Single agent *in vitro* cytotoxicity

The ability of the HPMA copolymer-drug conjugates to inhibit the growth of DU145 human prostate cancer cells was evaluated *in vitro* using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazoliummonosodium salt (WST-8) cell viability assay (Dojindo Molecular Technologies, Inc., Rockville, MD). Media containing 0.5%

DMSO was used throughout to prevent precipitation of free drugs. This concentration of DMSO was previously determined to not affect cell viability over the time course of the experiments (data not shown). DU145 cells (3000 per well) were plated in 96-well plates for 24 h. Media was then removed and replaced with media containing treatments. Cells were exposed to treatments for 12 hours. For each treatment case, drug concentrations were varied to include data points ranging from approximately 100% to 0% cell viability. Following drug treatment, media was removed, cells washed with PBS, growth media replaced, and cells were allowed to grow for an additional 60 hours (72 hours total experiment duration). Media was then removed and cell viability quantified by WST-8 assay (modified MTT assay) using a SpectraMax M2 microplate UV spectrophotometer (Molecular Devices, Sunnyvale, CA). Each experiment was performed in triplicate, comprising assessment of viability at 8 different drug concentrations with 3 samples analyzed per concentration. Relative viability was calculated by normalization of UV absorbance against untreated cells. Relative viability as a function of log drug concentration was plotted and non-linear least-squares regression analysis and calculation of IC₅₀ values were performed using GraphPad Prism.

***In vitro* cytotoxicity of hyperthermia**

The cytotoxic effect of hyperthermia treatment alone was evaluated *in vitro* against DU145 using a method analogous to that used to determine single agent cytotoxicity. DU145 cells (3000 per well) were plated in 96-well plates for 24 h. Cells were then exposed to either 40, 43, or 46°C for various durations spanning 15 min to 48 hrs. Hyperthermia was induced via incubation in a humidified temperature controlled CO₂ incubator at 5% CO₂. (No GNRs were utilized in experiment performed *in vitro*). Cells incubated continuously at 37°C served as controls. Following hyperthermia treatment, cells were further incubated such that the total experiment duration was 72 hrs, followed by cell viability assessment as previously described. Each experiment was performed in triplicate, with 6 samples analyzed per treatment case.

***In vitro* combination treatment and combination index analyses**

The cytotoxic effects of the combination treatment of moderate hyperthermia and heat shock targeted conjugates were evaluated *in vitro*. DU145 cells (3000 per well) were plated in 96-well plates for 24 h. Media was then removed and replaced with media containing treatments. Cells were then immediately exposed to moderate hyperthermia (43°C, 30 minutes incubation) or control (37°C, 30 minutes incubation). Following hyperthermia treatment, cells were incubated for an additional 11.5 hours (12 hours total drug exposure) at 37°C. This sequence of treatments was chosen to mimic the scenario that may occur *in vivo*, where exposure to the conjugates occurs at the same time as hyperthermic treatment, followed by an additional period of systemic exposure. Following combination treatment, cells were washed and allowed to grow for an additional 60 hours. Cell viability was then assessed as previously described.

Thermal enhancement was defined as IC₅₀ of control (37°C) / IC₅₀ when combined with hyperthermia (43°C). Drug effect was defined as (1 - [% relative viability / 100]). Combination index analysis was performed using CalcuSyn combination index software version 2.1 (Biosoft, Inc., Cambridge, UK) based on the Chou-Talalay method.[28] Each experiment was performed in triplicate, comprising assessment of viability for each combination treatment at 8 different drug concentrations with 3 samples analyzed per concentration. Combination index was then plotted as a function of drug effect for each treatment case.

***In vivo* efficacy**

Efficacy of the combination treatment of tumor hyperthermia and heat shock targeted HPMa copolymer-docetaxel conjugates was evaluated in eight-week-old athymic (nu/nu) mice. Mice were obtained from Charles River Laboratories (Davis, CA) and used in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah. Under anesthesia, mice were subcutaneously injected with 10^7 DU145 cells on both the right and left flank of each animal. When the tumors had reached approximately 7 mm in diameter (2-3 weeks), the animals were administered PEGylated gold nanorods, synthesized and characterized as previously described[9], (9.6 mg/kg) via tail vein injection. After 48 hours, animals were then treated with a single intravenous dose of either saline, free docetaxel (formulated in polysorbate 80:EtOH:saline [20:13:67, v/v/v]), non-targeted (scrambled peptide) conjugate, or heat shock targeted HPMa copolymer-docetaxel conjugate at 10 mg/kg (DOC equivalent). Immediately following injection, tumors on the right flank received tumor hyperthermia via PPTT as previously described.[9] Briefly, animals were anesthetized and tumors were swabbed with 50% propylene glycol to enhance laser penetration.[10] Tumors on the right flank only were then radiated for 10 min using an 808 nm laser and intra-tumoral temperature monitored via a 33 gauge needle thermocouple. Tumor temperature was maintained between 42 and 43°C via minor adjustments in laser output power. Tumors on the left of each animal served as internal controls. Animals were then monitored twice a week for changes in tumor volume and animal weight. Eight animals were evaluated per treatment group. Tumor dimensions (length and width) were measured and tumor volume estimated as $\text{length} \times \text{width} \times \pi/6$. Normalized tumor volume (mean $\pm$ SEM) and changes in animal weight (mean $\pm$ SEM) were then plotted as a function of time.

Statistical analysis

Differences in relative binding affinity, IC_{50} values, combination index values, normalized tumor volumes, and changes in animal weight were determined by one-way ANOVA. Where differences were detected, Tukey's post-test was used to test for significance between groups. The default significance level was set at $\alpha=0.05$ for all statistical tests.

Results and Discussion

Synthesis and characterization

A representative chemical structure of the HPMa copolymer-drug conjugates utilized in this study is given in Figure 1, and physicochemical characteristics are given in Table I. The macromolecular nature of the conjugates (via DLS detection) and the absence of small molecular weight entities (via dRI detection) were verified during SEC analysis (data not shown). All conjugates were synthesized to have a size above the renal threshold for HPMa copolymers (approximately 45 kDa)[29], thereby evading rapid renal filtration, and resulting in longer blood circulation times. This was of particular importance in our *in vivo* studies, as our combination strategy relies on two phenomena to increase tumor accumulation. First, tumor hyperthermia, induced immediately following intravenous administration of the conjugates, can increase both tumor blood flow and vascular permeability, thereby resulting in increased accumulation. Second, tumor hyperthermia can induce the expression of the cell surface heat shock protein GRP78. However, this expression does not occur immediately following exposure to hyperthermia, but requires between 8 and 12 hours before maximum expression in DU145 cells is observed.[9] Therefore, the utility of this heat shock targeting strategy requires that the conjugates still be available in circulation during this period of maximum expression. The conjugates were hence synthesized with molecular weights such that they exhibited these required long circulation times.

The current study utilizes HPMA copolymer-drug conjugates (AHGDM, DOC, or Pt) modified with the GRP78 targeting peptide WDLAWMFRLPVG in combination with tumor hyperthermia. For each drug that was evaluated (AHGDM, DOC, or Pt), precursor polymers bearing activated carboxyl groups (2-thiazoline-2-thione), were first synthesized. HSP (GRP78) targeting conjugates were obtained following aminolysis with the GRP78 targeting peptide WDLAWMFRLPVG. Previously reported work[9] utilized the GRP78 targeting peptide WIFPWIQL. However, the hydrophobic nature of this peptide limits the amount of drug loading that can be achieved while maintaining aqueous solubility. Conjugates bearing the WIFPWIQL peptide also demonstrated significant non-specific uptake in the liver, spleen, and kidneys.[9] The more hydrophilic alternative peptide (WDLAWMFRLPVG) was therefore used in this study. Non-targeted conjugates were prepared using a scrambled peptide (RWLWVADPFLMG) in a similar manner as GRP78 targeted conjugates. In this way, differences in behavior can be attributed to differences in binding affinity for GRP78, and not differences in copolymer physicochemical properties. The total amount of targeting peptide in each conjugate ranged from 15 – 20 wt% (Table I), which is consistent with levels previously shown to facilitate sufficient active targeting.[4, 30]

The amount of each drug that was incorporated into the copolymers was based on previous observations of what level could be achieved while maintaining sufficient aqueous solubility. While the percent conversion (% of drug incorporation based on feed amount) was high and ranged from 91 – 108% for AHGDM and Pt conjugates, lower conversions for DOC conjugates of 77 – 82% were observed (Table I). Since DOC is conjugated to the copolymer via ester linkages as compared to amide linkages for AHGDM and Pt conjugates, some hydrolysis of the ester bonds likely occurred post polymerization, most notably during attachment of the targeting peptides.

Competitive binding affinity of heat shock targeted conjugates to cell surface expressed GRP78

The relative binding affinities of each of the heat shock targeted conjugates towards cell surface expressed GRP78 was evaluated *in vitro* by a competitive binding assay. Previously, we have demonstrated that DU145 cells overexpress cell surface GRP78 to some extent, even without hyperthermic induction.[9] DU145 cells were co-incubated with a fixed concentration of ^{125}I -radiolabeled anti-GRP78 antibody and increasing concentrations of WDLAWMFRLPVG peptide, heat shock targeted conjugates, or controls (non-targeted conjugates). Incubation at 4°C was chosen to minimize non-specific cellular uptake of the antibody and conjugates. All heat shock targeted conjugates demonstrated some degree of binding towards GRP78, and all non-targeted conjugates demonstrated no evidence of competitive binding (Figure 2). For AHGDM and DOC conjugates, the binding affinities (on a molar peptide basis) for targeted conjugates were $1600 \pm 200 \mu\text{M}$ and $1080 \pm 70 \mu\text{M}$ respectively. These values were significantly different from that of free WDLAWMFRLPVG peptide ($96 \pm 14 \mu\text{M}$), signifying a decrease in the ability of the peptide to bind following conjugation. This anticipated result, however, is consistent with previous reports of HPMA copolymers utilizing peptides as targeting moieties, and we have previously demonstrated that such conjugates are effective in increasing tumor accumulation.[4, 26] Pt conjugates, however, demonstrated a significant shift in binding affinity to $230 \pm 40 \text{ mM}$. While this can possibly be explained due to conformational changes in the copolymer due to the presence of the cisplatin analogue, it most likely results directly from the synthetic strategy. Due to the highly reactive nature of the cisplatin analogue to nucleophilic substitution, it is required that the complexation of $\text{Cl}_4\text{K}_2\text{Pt}$ with -HN-Et-HN₂ side groups occur as the last synthetic step. However, during this step, it is possible that some complexation to Arg side chains of the heat shock targeting peptide WDLAWMFRLPVG occurs, thereby interfering with its ability to effectively bind cell

surface GRP78. This lack of effective binding could also be responsible for the lack of synergism observed for heat shock targeted Pt conjugates in combination studies (Figure 4).

Single agent *in vitro* cytotoxicity

The ability of each HPMA copolymer-drug conjugate to inhibit the growth of DU145 human prostate cancer cells was evaluated *in vitro*. In an effort to mimic a realistic *in vivo* exposure time, the cells were exposed to each treatment case (conjugates, free drugs, or controls) for 12 hours. This relatively short exposure time also provides an opportunity to allow differences in the kinetics or binding and uptake for different conjugates (i.e. non-targeted vs. targeted) to affect cytotoxicity.

These experiments yielded two primary observations. First, DOC treatments (free drugs and conjugates) were highly potent in inhibiting cell growth, with IC₅₀ values ranging from 2.47 to 15.7 nM (Table II, Figure 3b).

In comparison, AHGDM and Pt treatments were less potent with IC₅₀ values ranging from 1.81 to 2.76 μ M and 4.4 to 44 μ M respectively. The increased potency of DOC treatments, including that observed for the HSP targeted conjugate (1.8 nM), was a primary factor in choosing DOC therapy for *in vivo* evaluation (Table II). These observed values were consistent with previous reports for similar conjugates.[26, 27, 31] Second, increased cytotoxicity was observed for HSP targeted AHGDM and DOC conjugates in comparison with their non-targeted counterparts. For AHGDM conjugates, HSP targeting resulted in a reduced IC₅₀ of 1.85 as compared to 2.76 for the non-targeted conjugate ($p < 0.001$) (Table II and Figure 3b). For DOC conjugates, HSP targeting resulted in a reduced IC₅₀ of 1.86 as compared to 13.1 for the non-targeted conjugate ($p < 0.001$). These observations can be explained by the inherent cell surface expression of GRP78 on the surface of DU145 cells, which facilitates binding and internalization of HSP targeted conjugates, resulting in increased cytotoxicity. For the HSP targeted Pt conjugate, no difference in potency was observed as compared to the non-targeted conjugate. Again, this is possibly due to the loss of binding affinity of the HSP targeting peptide following cisplatin conjugation, as previously discussed.

In vitro cytotoxicity of hyperthermia

To ascertain the combined *in vitro* effects of the conjugates under investigation in combination with hyperthermia, it was first necessary to understand the effect hyperthermia alone has on cell viability. DU145 cells were exposed to hyperthermia (simple incubation) at varying temperatures (40°C, 43°C, and 46°C). Exposure time ranged from 15 min to 48 hours. Following exposure to hyperthermia, cells were incubated at 37°C for a total of 72 hours post initiation of hyperthermia, followed by assessment of cell viability. As anticipated, higher temperatures and increased exposure times were associated with decreased cell viability (Figure 3a). In terms of potency, the exposure times required to decrease cell viability to 50% (relative to unexposed cells) were 41 min, 2.7 hrs, and 18.2 hrs for 46°C, 43°C, and 40°C respectively (Figure 3a). It is important to note that for subsequent combination studies, this data indicates that exposure to hyperthermia (43°C, 30 min incubation) contributes only a 7.5% decrease in relative viability. The data obtained during the experiment for 43°C incubation was subsequently used for combination index analysis in combination studies.

In vitro combination treatment and combination index analyses

Previously, we reported that hyperthermia (43°C, 30 min incubation) induces an approximate 4-fold increase in the cell surface expression of GRP78 in DU145 cells, and demonstrated that this increased expression results in increased cellular uptake kinetics of

HSP targeted HPMA copolymers.[9] We therefore anticipated that this increased cellular uptake rate will translate into increased cytotoxicity of HSP targeted HPMA copolymer-drug conjugates. In this experiment, DU145 cells were exposed to a combination of hyperthermia (43°C, 30 min incubation) and 12 hours incubation with HSP targeted conjugates, non-targeted conjugates, or free drug controls. This timing was selected to be analogous to the exposure that occurs *in vivo* utilizing our combination strategy, where tumor cells are exposed to a brief period of simultaneous drug treatment and hyperthermia followed by a longer period of exposure to drug treatment alone. Cell viability was then assessed 72 hours following initiation of combination treatment. Combination index (CI) analysis was then performed to determine if the combined effects of drug treatment and hyperthermia were antagonistic (combination index > 1), additive (combination index = 1), or synergistic (combination index < 1). The combination index was evaluated as a function of effect, defined as $(1 - [\% \text{ relative viability} / 100])$.

All free drug controls (AHGDM, DOC, and cisplatin) were nearly additive (CI = 1) in combination with hyperthermia over the range of effects observed (Figure 4). At first glance, these observations appear to contrast other reports suggesting synergism of these compounds with hyperthermia.[15, 32] However, the combined effects and resulting combination index values are highly dependent on the sequence and timing of the combination therapy, and these experiments were performed to be analogous to potential *in vivo* exposure. These results are therefore not directly comparable with other studies and are best interpreted in a relative manner between the treatment groups in this study. All non-targeted conjugates were also additive in their combined effects with hyperthermia, with the slight exception of the non-targeted Pt conjugate (p-Pt), which was antagonistic at low effect levels. However, this sole observation was highly variable, and was not statistically significant. In combination with hyperthermia, both HSP targeted AHGDM and DOC conjugates demonstrated marked synergism with combination index values of approximately 0.65 and 0.45 respectively over a range of effect levels. The HSP targeted Pt conjugate demonstrated additive combined effects with hyperthermia, possibly due to its lack of binding affinity for GRP78 as previously discussed. For HSP targeted AHGDM and DOC conjugates, however, these results clearly demonstrate the potential for increased cellular delivery utilizing this heat shock targeted approach. These levels of synergism were not observed for the non-targeted AHGDM and DOC conjugates, which were prepared by addition of the scrambled peptide to same precursor polymer, suggesting this effect is primarily due to the HSP targeted conjugates' interaction with cell surface expressed GRP78.

To quantify the increase in cytotoxicity observed for treatments in combination with hyperthermia as compared to treatment alone, the thermal enhancement ratio of each treatment case was evaluated (Figure 4b). The thermal enhancement ratio was defined as the IC₅₀ observed for the treatment alone (no hyperthermia) divided by the IC₅₀ observed for the treatment in combination with hyperthermia. These results support those from combination index analysis, with HSP targeted AHGDM and DOC conjugates exhibiting increased potency with thermal enhancement ratios of 2.2 and 3.9 respectively. These combined results demonstrate the ability of cell surface expressed GRP78 to facilitate increased delivery of HSP targeted HPMA copolymer-drug conjugates, resulting in an increase in overall cytotoxicity. The HSP targeted DOC conjugate was selected for further *in vivo* evaluation due to its combined high potency, significant thermal enhancement, and observed synergism in combination with hyperthermia.

***In vivo* efficacy**

Our previous work demonstrated that tumor hyperthermia, delivered via GNR mediated PPTT, and heat shock targeting can increase the tumor accumulation, penetration and

retention of HPMA copolymers.[9, 11] In these studies, combined HSP targeting and tumor hyperthermia resulted in an approximate 3.5 fold increase in tumor exposure as compared to controls. However, non-specific uptake in the liver, spleen, and kidneys was also observed, most likely due to presence of a highly lipophilic targeting moiety. Therefore, this study utilized the more hydrophilic WDLAWMFRLPVG peptide to target cell surface expressed GRP78. Building upon this previous work and the *in vitro* results demonstrating synergism of HSP targeted DOC conjugates in combination with hyperthermia, *in vivo* efficacy was evaluated. Docetaxel therapy in combination with tumor hyperthermia, induced via GNR mediated PPTT, was evaluated in DU145 human prostate cancer bearing nu/nu mice. Each animal bore two subcutaneous tumors (left and right flank). Tumors were approximately 7 mm in diameter at the time of treatment. Mice were injected intravenously with a single bolus dose of either free DOC, non-targeted (scrambled peptide) DOC conjugates, or HSP targeted DOC conjugates at 10 mg/kg DOC. Injection of saline solutions served as negative controls. Immediately following administration, tumor hyperthermia was performed via GNR mediated PPTT to the right tumor only as previously described[9], and tumor temperature was maintained between 42°C and 43°C for 10 min. These parameters were chosen based on previous observations, where increased delivery of macromolecules was observed [9-11] and this sequence of combination therapy was chosen to ensure that the conjugates were in systemic circulation during hyperthermic treatment, as well as during the period of elevated GRP78 expression (8-12 h post hyperthermia).[9] Tumors on the left served as normothermia controls. Mice were then monitored twice a week for changes in tumor volume and total body weight. No significant differences in animal weights between treatment groups were observed throughout the course of the study (Figure 5). This was anticipated, as therapy was given well below the maximum tolerated dose of docetaxel.[26] All treatments demonstrated some level of tumor reduction as compared to saline (Figure 5), and will be discussed in rank order of efficacy beginning with the least efficacious. Tumors treated with saline and hyperthermia showed minor tumor regression, with an average normalized tumor volume over 30 days of 393% as compared to 624% for saline alone (without hyperthermia). While *in vitro* experiments evaluating the effect of hyperthermia on cytotoxicity suggested minimal damage at this exposure level, the tumor regression observed indicates that some amount of tissue damage to the tumor occurred during hyperthermia treatment, most likely due to variations in the temperature distribution during heating. This is in agreement with previous work where we observed cellular damage by histology during similar treatments.[11] Treatments with free DOC and the non-targeted DOC conjugate were equally efficacious, with an overall growth of 321% observed. Combined treatment of free DOC or non-targeted DOC conjugates with hyperthermia were comparable to treatment with the HSP targeted DOC conjugate alone (no hyperthermia), with overall growths of 229%, 165%, and 198% observed respectively. Addition of hyperthermia to p-DOC treatment resulted in a 155% reduction in tumor size and addition of hyperthermia to p-DOC-HSP treatment resulted in a 149% reduction in tumor size over the course of the study. However, the p-DOC-HSP treatment without hyperthermia was more efficacious (198% on day 30) as compared to p-DOC treatment (320% on day 30). It is generally increasingly difficult to reduce tumor size completely to zero. Therefore, the similar reductions in tumor volume observed when hyperthermia is added to these treatments are anticipated. The only treatment group which maintained tumor reduction over the entire study period was hyperthermia in combination with HSP targeted DOC therapy, wherein tumor volume was maintained at 49% of initial volume after 30 days. These results demonstrate the efficacy of combination therapy and strongly support the proposed heat shock targeting rationale.

Conclusion

In this study, we demonstrated that tumor hyperthermia can be utilized as a tool to increase the active targeting of heat shock targeted HPMA copolymer-drug conjugates. The combined effects of hyperthermia and HSP targeted AHGDM and DOC conjugates were found to be synergistic in inducing cytotoxicity to DU145 human prostate cancer cells *in vitro*. We further demonstrated that a combination of tumor hyperthermia, delivered via GNR mediated PPTT, can increase the efficacy of HSP targeted HPMA copolymer-DOC conjugates in human prostate cancer bearing mice. These results demonstrate the potential for tumor hyperthermia to increase the delivery of targeted macromolecular chemotherapeutics.

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Figure 1. Representative chemical structure of HPMA copolymer-drug conjugates

The chemotherapeutic agent aminoheylgeldanamycin (AHGDM), docetaxel (DOC), or cisplatin was conjugated to the copolymer side chain via the enzymatically degradable GFLG linker (a), and the glucose regulated protein 78 kDa (GRP78) targeting peptide WDLAWMFRLPVG (b) was conjugated via a GG linker.

Figure 2. Binding affinity of HPMA copolymer-drug conjugates to GRP78 expressed on the surface of DU145 human prostate cancer cells
 Strong binding affinity was observed for HSP targeted conjugates bearing AHGDM and docetaxel, and weak affinity was observed for the HSP targeted conjugate bearing cisplatin. For all conjugates, binding affinity was reduced as compared to native WDLAWMFRLPVG peptide. No evidence of binding was observed for non-targeted conjugates. Data expressed as mean $\pm$ SD of 3 experiments.

Figure 3. Cytotoxicity of hyperthermia (a) and single-agents (b) towards DU145 human prostate cancer cells

a) Increases in temperature and exposure time were associated with increased cytotoxicity. Exposure times of 18.2 hrs, 2.7 hrs, and 42 min were needed to reduce viability to 50% at temperatures of 40°C, 43°C, and 46°C respectively. **b)** Growth inhibition IC₅₀ values of free drugs (grey), non-targeted conjugates (blue), and HSP targeted conjugates (red) under normothermia conditions (37°C). DOC conjugates were significantly more potent than AHGDM or Pt conjugates, with IC₅₀ values in the nanomolar range. HSP targeted AHGDM and DOC conjugates were also significantly more potent than their non-targeted counterparts. Data expressed as mean ± SD or 3 experiments. *** indicates statistically significant differences (p<0.001).

Figure 4. *In vitro* combination of hyperthermia and drug therapy

a) Combination index values as a function of drug effect against DU145 cells. Treatments consisted of free drug (black), non-targeted (scrambled peptide) conjugates (blue), and HSP targeted conjugates (red). HSP targeted AHGDM and DOC conjugates demonstrated significant synergism in combination with hyperthermia. Drug effect was defined as $(1 - [\% \text{ relative viability} / 100])$. **b)** Thermal enhancement ratios ($IC_{50} \text{ normothermia} / IC_{50} \text{ hyperthermia}$) for each treatment case. **c)** Direct comparison of combination index values between treatments at 50% drug effect. All data expressed as mean $\pm$ SD of 3 experiments.

Figure 5. *In vivo* efficacy of tumor hyperthermia and heat shock targeted HPMA copolymer-docetaxel therapy

a) A combination of tumor hyperthermia, delivered via PPTT, and HSP targeted docetaxel therapy (p-DOC-HSP) results in maintained tumor regression over a period of 30 days following a single intravenous dose at 10 mg/kg DOC eq. **b)** No differences in animal weights were observed between treatment groups over the same time period. **c,d)** Treatments without and with hyperthermia. Data represented as mean $\pm$ SEM, with N=8 animals per treatment group.

Table I
Characteristics of HPMA copolymer-drug conjugates

Polymer	Description	Feed composition - (mol%)				M_w^a (kDa)	M_w/M_n^a	Drug content ^b wt % (% conversion)	Targeting peptide content ^c wt % (% conversion)
		HPMA	MA-GFLG-X*	MA-GG-TT	MA-Tyr-CONH ₂				
p-AHGDM	Non-targeted AHGDM conjugate	88	5	5	2	76	1.4	12.7 (106%)	17.6 (64%)
p-AHGDM-HSP	HSP targeted AHGDM conjugate	88	5	5	2	71	1.4	12.9 (108%)	17.0 (61%)
p-DOC	Non-targeted DOC conjugate	90.5	2.5	5	2	78	1.4	6.2 (77%)	16.7 (56%)
p-DOC-HSP	HSP targeted DOC conjugate	90.5	2.5	5	2	81	1.4	6.6 (82%)	15.4 (52%)
p-Pt	Non-targeted Pt conjugate	87	6	5	2	89	1.5	4.1 (91%)	19.7 (68%)
p-Pt-HSP	HSP targeted Pt conjugates	87	6	5	2	86	1.5	4.5 (100%)	18.8 (65%)

* where X is AHGDM, DOC, or HN-Et-HN-Boc (precursor for Pt conjugates)

^a as measured by SEC/MALLS

^b Determined by UV spectroscopy for AHGDM, enzymatic release/HPLC for DOC, and ICP/MS for Pt.

^c Determined by amino acid analysis. Scrambled peptide used for non-targeted conjugates.

Table II

In vitro cytotoxicity IC₅₀ values of HPMA copolymer-drug conjugates in combination with hyperthermia.

IC ₅₀	AHGDM (μM)		DOC (nM)		Pt (μM)	
	Control (37°C)	Hyperthermia (43°C)	Control (37°C)	Hyperthermia (43°C)	Control (37°C)	Hyperthermia (43°C)
Free drugs	1.81 ± 0.05	1.39 ± 0.06	2.47 ± 0.08	1.86 ± 0.06	4.4 ± 0.2	3.2 ± 0.1
Non-targeted conjugates	2.76 ± 0.05	2.52 ± 0.08	15.7 ± 0.6	13.1 ± 0.5	44 ± 2	42 ± 2
Heat shock targeted conjugates	1.85 ± 0.05	0.83 ± 0.03	9.4 ± 0.3	2.4 ± 0.1	43 ± 2	37 ± 2