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Enhanced efficacy of combination heat shock targeted polymer therapeutics with high intensity focused ultrasound

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ABSTRACT

Combination of polymer therapeutics and hyperthermia has been shown to enhance accumulation in selectively heated tumor tissue. The additional use of heat shock (HS)-targeting towards tumor tissues can further enhance accumulation and retention, and improve therapeutic outcomes. In this work, high intensity focused ultrasound (HIFU) was used to generate hyperthermia in prostate tumor tissue. Upregulation of the cell surface HS receptor glucose regulated protein 78 kDa (GRP78) was observed after treatment with HIFU hyperthermia which was then targeted by specific HS-targeting peptides. We used the peptide sequence WDLAWMFRLPVG attached to the side chains of water-soluble *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing docetaxel (DOC) conjugated via a lysosomally degradable linker. It was shown that HIFU-mediated HS-targeted copolymer–DOC conjugates improved treatment efficacy in a murine prostate tumor xenograft model. These results show that the use of HIFU hyperthermia in combination with HS-targeted polymer–drug conjugates has potential to improve therapeutic outcomes in prostate cancer treatment.

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The ultimate goal of targeted drug delivery is to selectively deliver therapeutics to the disease site and allow for increased dosages to be administered to the patient while simultaneously reducing off-target effects. Polymer therapeutics have been developed in an attempt to accomplish this goal for delivery of anticancer drugs to solid tumors.[1] Such constructs can extend blood circulation times of conventional drugs and increase accumulation within cancerous tissues through passive delivery by the enhanced permeability and retention (EPR) effect.[2] The use of these and other nanomedicines has led to improved therapeutic outcomes with altered biodistribution in certain cases minimizing side effects (e.g. Doxil reducing the cardiotoxicity of doxorubicin).[3] Still, in a majority of cases only moderately enhanced localization to the tumor tissue is observed, increasing from approximately 1% to 5% of injected dose (ID).[4] The impact of nanoscale delivery systems for treatment of solid tumors can be limited due to the variability of EPR effect depending on tumor type, size, location, and preclinical to clinical correlation.[5] Therefore, combination approaches must be considered including augmentation of the EPR effect.[6]

Methods to further enhance the delivery of nanomedicines through augmentation of the EPR effect include mild hyperthermia. At the tissue level, this mechanism can both increase blood flow and improve vascular permeability by vasodilation[7] leading to improvements in local delivery. Mild hyperthermia (41-43 °C) has been shown to enhance the delivery of nanomedicines to solid tumors.[8] At the cellular level, mild hyperthermia has the ability to upregulate cell surface HS receptor glucose regulated protein 78 (GRP78).[9] Specific peptide sequences have been developed by phage display which show a strong binding affinity towards the GRP78 receptors.[10] These peptides include WDLAWMFRLPVG (single letter amino acid abbreviations are used).[11]

Abbreviations: AIBN, Azobisisobutyronitrile; ANOVA, Analysis of variance; DOC, Docetaxel; EBD, Evans blue dye; ESI/MS, Electrospray ionization mass spectroscopy; EPR, Enhanced permeability and retention; FBS, Fetal bovine serum; FDA, Food and Drug Administration; Gd, Gadolinium; GNR, Gold nanorod; GRP78, Glucose-regulated protein-78; HIFU, High intensity focused ultrasound; HPMA, *N*-(2-hydroxypropyl)methacrylamide; IC_{50}, Inhibitory concentration of 50%; ID, Injected dose; MA-GFLG-DOC, *N*-methacryloylglycylphenylalanylleucylglycine-docetaxel; MA-GG-TT, *N*-methacryloylglycylglycyl-2-thiazolidine-2-thione; MFH, Magnetic fluid hyperthermia; M_n, Number average molecular weight; MRI, Magnetic resonance imaging; M_w, Weight average molecular weight; M_w/M_n, Polydispersity index; MRgHIFU, Magnetic resonance imaging-guided HIFU; PBS, Phosphate buffered saline; PDI, Polydispersity index; PPTT, Plasmonic photothermal therapy; RFA, Radiofrequency ablation; SEC, Size exclusion chromatography; Seg-EPI, Segmented-echo planar imaging; TE, Echo time; TR, Repetition time.

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Methods such as plasmonic photothermal therapy (PPTT), magnetic fluid hyperthermia (MFH), and radiofrequency ablation (RFA) can induce hyperthermic conditions.[8] We have previously demonstrated that mild hyperthermia by gold nanorod (GNR)-mediated PPTT enhances the delivery of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer–drug conjugates containing GRP78 targeting moieties in the side chains to solid tumors.[9] HS-targeted copolymer–docetaxel (DOC) conjugates showed enhanced efficacy when hyperthermia was applied in combination.[11] While results of this research are promising, PPTT in combination with polymer therapeutics requires a prior injection of nanoparticles delivered intravenously which then accumulate in tumor tissue by the EPR effect.[12] The accumulation of these particles in tumor tissue allows for laser energy to be locally absorbed.[13] However, after this injection, only a small fraction of the gold nanoparticles reach the tumor site leading to a large amount (>90%) of off-target accumulation in other organs such as the liver and spleen.[12] Additionally, in order to heat deep-seeded tumors, an optical fiber needs to be invasively placed in the body. These drawbacks limit the applications of this promising combination strategy. Alternative methods that are non-invasive and provide a higher depth of tissue penetration are needed to improve the clinical application of combination of mild hyperthermia and polymer therapeutics to treat solid tumors.

High intensity focused ultrasound (HIFU) is a non-invasive technique that can locally heat tissues and achieve a large penetration depth of up to approximately 20 cm through the tissue.[14] We have previously shown in pre-clinical mouse tumor models that MRI guided HIFU (MRgHIFU) can be used to non-invasively generate and maintain uniform hyperthermia in subcutaneous tumor tissue and that the resulting thermal effects can lead to enhanced delivery of HPMA copolymer–gadolinium conjugates in solid tumors.[15] It was shown that after 5 h post heating a significant increase in copolymer accumulation is achieved in heated tumors versus control non-heated tumors. The accumulation of these non-targeted systems enabled a transient increase in copolymer concentration in a mouse sarcoma model peaking at approximately 4-5 h post HIFU heating[15] as assessed by the longitudinal relaxation time (T1) measured in the tumor tissue and compared to the control tumor. To further build on the utility of HIFU mild hyperthermia in enhancing the delivery of macromolecular constructs, in this manuscript we have used a combination of non-invasive MRgHIFU hyperthermia with HPMA copolymer-WDLAWMFRLPVG conjugates containing docetaxel (DOC) in the side chains to improve the efficacy of the conjugates in a murine model of human prostate xenografts.

Methods

Synthesis and characterization of HPMA copolymer conjugates

Comonomers of HPMA,[16] *N*-methacryloylglycylglycyl-2-thiazolidine-2-thione (MA-GG-TT), and *N*-methacryloyl-glycylphenylalanylleucyl glycine-docetaxel (MA-GFLG-DOC)[17] were synthesized as described previously. DOC was provided by AK Scientific (Mountain View, CA). Free radical precipitation copolymerization using azobisisobutyronitrile (AIBN) as the initiator in methanol at 50 °C for 24 h was used to prepare the copolymers. The product was then precipitated and washed with diethyl ether followed by dialysis against deionized water to remove unreacted comonomers and initiator. The copolymers were lyophilized to obtain the final product. Weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (PDI) were calculated by the ratio of M_w/M_n and were estimated by size exclusion chromatography (SEC).

The GRP78 targeting peptide WDLAWMFRLPVG and corresponding scrambled peptide RWLWVADPFLMG were synthesized via Fmoc chemistry using a Protein Technologies (Tucson, AZ) PS3 solid phase peptide synthesizer, verified by amino acid analysis and electrospray ionization mass spectrometry (ESI/MS).

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% CO_2 in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS). Cells were maintained in a logarithmic growth phase during all studies.

In vitro efficacy of heat shock targeted copolymer–drug conjugates

DU145 cells (3000 per well) were plated in 96-well plates for 24 h. Medium was then removed and replaced with medium containing treatments. Cells were exposed to either heat shock targeted copolymers or untargeted copolymers for 12 h at varying concentrations between 0 and 1200 nM DOC concentration. One group was incubated at 37 °C while a second group was exposed to heat shock (HS) (43 °C for 30 min) and then incubated at 37 °C for the remainder of the 12 h. This thermal dose profile was chosen to be consistent with previous experiments[11] as this thermal treatment showed a 4-fold increase in cell receptors in vitro.[9] For each treatment case, drug concentrations were varied to include data points ranging from approximately 100% to 0% cell viability. Following drug treatment, medium was removed, cells washed with PBS, growth medium was replaced, and cells were allowed to grow for an additional 60 h (72 h of total experiment duration). Medium was then removed and cell viability was quantified via CCK-8 assay using a SpectraMax M2 microplate UV spectrophotometer (Molecular Devices, Sunnyvale, CA). Each experiment was performed in triplicate, comprising assessment of viability at 10 different drug concentrations with 4 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 inhibitory concentration of 50% (IC_{50}) values were performed using GraphPad Prism.

In vivo tumor model

In vivo experiments were carried out using nu/nu mice containing two DU145 human prostate cancer subcutaneous tumor xenografts, one on each flank. Inoculations were performed by injecting 200 μL of phosphate buffered solution (PBS) containing 10×10^6 cells subcutaneously and allowing tumors to grow for 28-30 days to reach a size of 7-11 mm in diameter. Tumor sizes were measured every 3 days using calipers. Once the tumors reached the desired size, they were then treated with MRgHIFU hyperthermia.

In vivo MRgHIFU heating

Prior to MRgHIFU treatment, the mice were anesthetized (2% isoflurane), a needle thermocouple was inserted into the center of the tumor and two minutes of temperature data was obtained to determine a baseline tumor temperature. The animal was placed on an agar mold on the MRgHIFU device with the tumor placed in an access hole that provided an acoustic window between the HIFU transducer and the tumor. The agar mold provided a large region to obtain stable MRI phase measurements to improve the MRI temperature measurement reconstruction. A custom two-channel radiofrequency coil was placed on top of the animal, and a small animal monitoring system was used to monitor the animal (respiration and temperature, SA Instruments, Inc.).

All heating was performed using an MRgHIFU small animal system (Image Guided Therapy, Inc., Bordeaux, France, 16-element annular transducer, f = 3 MHz, 1 × 1 × 3 mm full-width-half-maximum intensity focal spot size, 3.5 cm focal length) placed in a Siemens 3 T Trio MRI scanner. MR temperatures were monitored with the proton resonance frequency (PRF) method using a 2D segmented-echo planar imaging

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