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Tumor vascular permeabilization using localized mild hyperthermia to improve macromolecule transport

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Abstract

The abnormal tumor vasculature presents a major challenge to the adequate delivery of chemotherapeutics, often limiting efficacy. We developed a nanoparticle-based technique to deliver localized mild hyperthermia (MHT) used to transiently alter tumor vascular transport properties and enhance transport of macromolecules into tumor interstitium. The strategy involved administering and localizing accumulation of stealth gold nanorods (GNRs, 103 μg of GNRs/g of tumor), and irradiating tumor with a low-photon laser flux (1 W/cm^2) to generate MHT. The treatment increased vascular permeability within 24 h after treatment, allowing enhanced transport of macromolecules up to 54 nm in size. A mathematical model is used to describe changes in tumor mass transport properties where the rate of macromolecular exchange between interstitial and vascular region (R) and maximum dye enhancement (Y_{max}) of 23-nm dextran dye are analytically solved. During enhanced permeability, R increased by 200% while Y_{max} increased by 30% relative to untreated group in pancreatic CAPAN-1 tumors. MHT treatment also enhanced transport of larger dextran dye (54 nm) as assessed intravital microscopy, without causing occlusive cellular damage. Enhanced vascular transport was prolonged for up to 24 h after treatment, but reversible with transport parameters returning to basal levels after 36 h. This study indicates that localized mild hyperthermia treatment open a transient time-window with which to enable and augment macromolecule transport and potentially improve therapeutic efficacy.

Keywords

Intravital microscopy; macromolecular enhancement; mild hyperthermia; transport barriers; vascular permeabilization

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BACKGROUND

Ineffective treatment of cancer is partly due to impaired delivery of drugs to cancer cells¹ caused by tumor physical environment that presents multiple barriers to drug delivery. These barriers are considered mass transport differential characteristics and include (i) elevated tumor interstitial fluid pressure, (ii) poor tumor perfusion, (iii) disorganized and tortuous sluggish vasculature, (iv) low convective transport, and (v) dense extracellular matrix (ECM)^{1–3}. These factors hinder transvascular drug penetration across blood vessel walls and into the tumor interstitium^{2,4,5}. Therefore, achieving effective delivery of therapeutic agents requires that these biophysical barriers are successfully negotiated or overcome^{1,6}.

Several strategies have been used to improve drug delivery across vascular and stromal barriers including the use tumor vessels normalization strategies⁷ and mechanical stimuli⁸ to drive delivery of macromolecules into tumor interstitium. A recent use of tumor normalization strategy showed improved delivery and penetration of nanomedicines up to 23 nm in diameter, but there no appreciable increase in the delivery of larger particles (> 123 nm)⁷. Therefore enabling the delivery of larger macromolecules requires novel strategies needed to overcome these antagonistic barriers. Since efficacy of most chemotherapeutics is predicated on achieving adequate transport across the endothelial layer into the tumor interstitium⁹, loosening or creating gaps between endothelial cells presents an attractive strategy for increasing macromolecular transport (including drugs). This may have implications in overcoming transport limitations associated with cancer treatment¹⁰. As an example, increased vascular permeability using photodynamic therapy (PDT) treatment caused prolonged vascular leakage (~ 2 days) that improved therapeutic delivery and efficacy in patients ailing from age-related macular disorder (AMD)¹¹. However, it also led to complete loss of vascular barrier function and occlusion¹² where unmitigated increase in vascular permeability due loss of barrier function potentiates tumor cell extravasation, an early event in the metastatic cascade¹³.

Hyperthermia is widely used for cancer treatment¹⁴ in experimental animals¹⁵ and patients¹⁶. It can enhance cancer treatment by directly causing cell death, sensitizing cells to chemotherapy¹⁷ and radiotherapy¹⁸, and by promoting of tumor re-oxygenation¹⁹. It has also been used as external stimuli to modify behavior of cells and nanotherapeutics to achieve site-specific drug release, improve drug internalization, and synergize antitumor effect of therapeutics²⁰. Most of these photothermal therapy applications have used high photon flux of 5 – 48 W/cm² to raise tumor temperature to ~ 46°C¹⁷ and kill cancer cells by DNA denaturation²¹. While these ablative hyperthermia have somewhat improved tumor transport at the tissue periphery (regions of mild temperatures)²², the fact that it causes irreversible tissue damage (including vascular occlusion)²³ represents a major clinical drawback. Patients are often given chemotherapy infusions in multiple cycles for which intact vasculature is necessary. Hence, we reasoned that the use of mild hyperthermia (MHT, low flux, 42°C) would transiently permeabilize tumor vessels, improve macromolecule transport without causing damage that would occlude tumor vessels and hinder delivery.

Here, we report on a novel nanoparticle-based approach used to produce mild hyperthermia and enable transient and reversible vascular permeabilization that allow enhanced time-dependent delivery of large macromolecules (> 23 nm). Vessel damage by thermal ablation is reported to occur when temperature is raised to 43°C for 240 min²⁴ and hence our rationale to implement MHT below these threshold settings. The treatment was given for 20 min to overcome thermo-tolerance prevalent in tumor tissue²⁵. MHT treatment (41–42°C) has mostly been used to achieve site-specific delivery of thermally sensitive liposomes²⁶ and polymeric carriers²⁷ that can degrade and release its content, improving antitumor effect^{28,29}. These approaches have relied on using whole body and/or water-bath heating⁹ to achieve MHT, technologies that either pose deleterious effects to other organs or are slow to achieve desired temperature elevations. Here, we use GNRs that possess high conversion efficiencies, 10⁴–10⁶ times higher than common organic fluorochromes³⁰ and can rapidly convert energy into heat. This ensures that miniature amounts of GNRs would be required to achieve rapid selective MHT. We tested this hypothesis by first coating GNRs with polyethylene glycol (PEG) to produce stealth particles with long *in vivo* circulation half-life which increases propensity for passive tumor accumulation via Enhance Permeation and Retention (EPR) effect³¹. After GNRs localization, the effect of MHT treatment on mass transport properties in pancreatic cancer (CAPAN-1) models was evaluated. Intravital microscopy was used to quantify vascular and interstitial transport properties after MHT treatment. We further evaluated the time- and size-dependence using fluorescent dextrans which were confirmed by fluorimetric and histological analyses. Our efforts should open avenues towards using MHT therapies to synergize delivery of macromolecules, thermally sensitive or otherwise, and enhance drug localization, with the potential to reduce dosing and improve therapeutic efficacy.

METHODS

Animal models

Pancreatic subcutaneous (s.c.) tumors were generated in *nu/nu* mice (Charles River Laboratories, Wilmington, MA) by a one-time injection of 5×10^5 CAPAN-1 cells in the hind right flank (ATCC, Manassas, VA). Once tumors reached 5–7 mm in diameter (290–350 mm³), they were prepared for MHT treatment, intravital microscopy, and fluorimetric analyses. All MHT and intravital animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (AUP 1010-0029) at the Houston Methodist Research Institute.

Reagents

Gold nanorods used to generate localized hyperthermia were synthesized using the following reagents. Gold (III) chloride (99.9%), silver nitrate (99.99%), L-ascorbic acid (99%), cetyltrimethyl ammonium bromide (CTAB, 99%), benzylhexadecyldimethyl ammonium chloride (BDAC), sodium borohydride (96%), and formamide were purchased from Sigma-Aldrich (St. Louis, MO); 5kDa Methyl-PEG5K-thiol was purchased from Laysan Bio (Arab, AL). MTS viability assay kit was obtained from Promega (Madison, WI); Evans blue dye (980.81 g/mol) from Fisher Scientific while fluorescein isothiocyanate (FITC-) and tetramethyl rhodamine isothiocyanate (TRITC-) labeled dextran dyes (70kDa

and 2,000kDa) were purchased from Invitrogen (Carlsbad, CA). Rat anti-mouse CD31 was purchased from BD PharMingen (part # 550274, San Jose, CA) while Alexa Fluor-648 goat anti-rat was purchased from Molecular probes (Eugene, OR).

Preparation of PEG-coated gold NRs

Cetyltrimethylammonium bromide (CTAB)-coated GNRs were synthesized as described previously³² (Supplementary). The particles were functionalized with polyethylene glycol-thiol (PEG5K-thiol) to create stealth GNRs that are highly stable and can accumulate in tumor microenvironment by EPR effect³¹. After ligand exchange reaction, unbound CTAB were eliminated from solution by dialysis and evaluated for cell cytotoxicity by MTS viability assay. Cytotoxic effects of modified particles were compared to the effect of as-synthesized CTAB-coated GNRs in CAPAN-1 cells. *In vivo* bio-distribution characteristics and extent of accumulation in tumor were evaluated by ICP-MS and evaluated to determine whether it can create desired MHT temperature.

Initiation of localized mild hyperthermia using gold nanorods

Gold nanorods are precisely tunable plasmonic nanomaterials with strong optical absorption with coefficients 10^4 – 10^6 times higher than those of conventional organic fluorochromes^{33,34}. They are used in combination with NIR irradiation to generate selective and localized heating. Mice bearing CAPAN-1 pancreatic tumors were injected with PEG-coated GNRs at a concentration at 10 mg/kg (200 μ L, 1.4×10^{11} particles/mL) by intravenous injection (*i.v.*). The particles were allowed to passively accumulate at the tumor site and then 72 h post-injection, mice were randomly allocated into several groups ($n = 5$). The NIR heating approach involved sweeping a 4-mm spot size laser hand-piece across entire tumor surface (~ 1 W/cm², 1.0 Hz, Delta 30, Angio-dynamics, UK) to generate MHT which was sustained by irradiation (1.0 Hz) for duration of treatment. For comparison, thermal ablation ($> 47^\circ\text{C}$) was generated using higher laser flux (~ 5 W/cm², 1.0 Hz). During irradiation, an IR thermographic camera (FLIR, Thermacam S60) was used to monitor tumor surface temperature distribution while microprobes (Oxford Optronics, Oxford, UK) were placed at the tumor bed and used to measure temperature changes.

Assessment of blood perfusion during heating

Having generated sustained focal tumor heating, we next probed for changes in tumor transport starting with alterations in tumor blood perfusion which was measured using laser Doppler microprobes (Oxford Optronics, Oxford, UK). Briefly, probes placed on superficial tumor vessels on anesthetized mice were used to monitor changes in perfusion during MHT treatment and also to measure changes in untreated tumors (as control). Stable baseline recordings were obtained before treatment and monitored until heating was terminated.

Assessment of tumor permeability by Evans blue

Changes in vascular permeability after MHT treatment in CAPAN-1 pancreatic tumors was determined as described previously¹⁰. Briefly, after treatment animals were *i.v.* injected with Evans blue dye (10 mg/kg, PBS) or with fluorescent dextran dye (10 mg/kg, $M_w = 70$ & 2,000kDa). At various times after treatment (i.e. $t = 0$ –36 h, $n = 8$), mice were perfused

with 50 mL of 0.9% saline to remove macromolecules in circulation and tumor tissues were excised and weighed. Tumors were homogenized with formamide solution (200 μ L per 1 mg of tissue) and digested at room temperature over 72 h as described previously¹⁰. Total dye concentration in tumor homogenate was quantified with a microplate reader (Synergy H4 hybrid reader, BioTek Instruments Inc., Winooski, VT) using the following fluorescent emission/excitation settings: 470/680 nm for Evans blue, 495/518 for 70kDa FITC-dextran, and 594/620 nm for 2,000kDa TRITC-dextran dyes. Homogenate fluorescence was compared to a standard curve, prepared by the same procedure using tumors from un-injected animals but spiked with known dye concentration.

Monitoring of vascular function by intravital microscopy

Tumor vascular function and changes in flow dynamics were evaluated by intravital microscopy (IVM) immediately after MHT treatment (within 5 min) or at specified end-points. Tumors were exposed using a skin-ap procedure described previously³⁵. Live animals were imaged on an upright Nikon A1R MP-ready laser scanning confocal microscope platform equipped with a resonance scanner, isoflurane anesthesia system, heated stage, and custom coverslip mounts³⁶. Anesthetized mice were administered with fluorescent dextran by retro-orbital injection (10 mg/kg, 50 μ L PBS) and dye diffusion visualized by IVM for up to 60 min. The retro-orbital route was chosen over tail-vein injection due to its accessibility and reproducibility for quantitative imaging experiments. Fluorescent dyes were acquired with the following settings: FITC labeled dextran was excited at 488 nm while TRITC labeled dextran was excited at 561 nm collected at band-pass filters widths 30–50 nm centered at 525 nm for FITC- and 579 nm for TRITC-labeled dextran. 512 $\times$ 256 bit two-channel images of *s.c.* tumors were acquired over multiple field of views (FOVs) from the time of dye injection using x4 objective lens with a pinhole of 1.0 Airy units. To minimize local phototoxicity and compensate for tissue heterogeneity, non-overlapping FOVs were randomly selected and imaged at a 20-sec interval to reduce photobleaching. The camera and acquisition settings were kept constant between different treatment groups. Animals were sacrificed, tumor excised and prepared for histological and spectrofluorometric analyses to confirm the effect of hyperthermia on dye extravasation. The extent of dye extravasation was quantified by average dye intensity over 8 regions of interest (ROIs) of several FOVs (size: 0.25 μ m $\times$ 0.25 μ m). These data were used in a mathematical model³⁷ to yield parameters of mass transport that describe macromolecular delivery in tumor vasculature and interstitial regions. The rate of exchange of injected dye between vasculature and interstitial (R) and maximum enhancement of the vasculature ($Y_{\max}$) are analytical solutions of a first-order differential equation in the mathematical model recently described by Koay et al³⁷.

Persistence and timing of vascular permeability

To determine persistence and longevity of vascular permeability as well as inform of the optimal time-of-opportunity, we evaluated the amount of dye accumulation in animals receiving MHT treatment relative to untreated group. At each time-point after MHT treatment, animals were injected with dextran dye (50 μ L, 10 mg/kg in PBS) and allowed to circulate and extravasate into tumor for 1 h ($t = 0$ –36 h, $n = 8$). The duration of dye circulation between treatment groups was kept constant (i.e. dye was injected at 24 h time-

point and allowed 1 h for circulate). This was done to account for dye accumulation that may be transported into tumor parenchyma at earlier time-points after treatment. Animals were sacrificed and tumors excised, weighed, and homogenized in formamide (200 μ L/mg of tumor). The amount of dextran dye was quantified by spectrophotometric analyses where dye concentrations were derived from a standard curve, longevity and persistence data was based on the amount of accumulated dye.

Immunohistochemical analyses after hyperthermia

Histological analyses were performed to evaluate morphological effects from MHT treatment compared to the effects caused by thermal ablation. Following thermal treatment and IVM experiments, animals were euthanized and tumor tissues excised for histological analyses. Tumor samples were frozen in OCT compound and 10- μ m-thick tissue slices were obtained using a cryostat (Model HM 505 E, Microm International GmbH, Walldorf, Germany) and fixed in acetone. Histological assessment was performed by hematoxylin and eosin (H&E) staining and imaged using a light microscope (Nikon Eclipse 80i, Nikon, Carrollton, TX). For immunochemical staining of tumor vasculature, rat anti-mouse CD31 (1:200) and Alexa Fluor-648 goat anti-rat (1:1,000) secondary antibody were used. The slides were washed three times with PBS and mounted in Vectashield Mounting medium with DAPI. At least three images from representative microscopic fields were analyzed for each tumor sample.

Statistical analysis

Statistical analysis was performed using OriginLab statistical software (OriginLab Co, Northampton, MA) where Student's t-test was used to compare treatment groups to controls while one-way ANOVA test was used for multiple comparisons. A value of $p < 0.05$ was considered to represent a significant difference between groups.

RESULTS

Initiation of localized mild hyperthermia

Gold nanorods (50 $\times$ 11 nm, ~ 4.5 ratio) with high thermal absorption properties were prepared and PEGylated through ligand exchange reaction (Figure 1A) to reduce cytotoxic effects of CTAB. Modification was also performed in order to increase circulation half-life and allow passive GNR accumulation in tumor by EPR effect³⁸. Cell viability assays showed that PEG-coated GNRs were significantly less toxic to CAPAN-1 cells than CTAB-coated rods, suggesting that modified particles are amiable to cell-based and *in vivo* applications (Supplementary, Figure S1). Figure 1 shows photothermal properties of heat-absorbing GNRs used to induce selective and localized hyperthermia. GNRs were prepared using previously reported protocol³² and PEGylated³⁹ to make stealth particles with good heating capabilities, reaching 50°C in a 80 μ g/mL GNR solution (Supplementary, Figure S2). The circulation half-life for PEG-coated GNRs increased to ~18 h compared to 1 h for surfactant CTAB-stabilized GNRs. Diluted GNR solution caused a 25-degree temperature increase under NIR irradiation (~1 W/cm², 810 nm, 20 min) compared to saline solution alone. After *i.v.* injection and circulation, approximately ~ 103 μ g of GNRs/g of tumor (~ 10% of injected dose) accumulated in tumors after 72 h (Supplementary, Figure

S2). Subsequent thermographic surveillance of tumor during MHT treatment showed localized temperature increase only in mice pre-injected with PEG-coated GNRs (Figure 1C). Selective MHT was rapidly generated upon irradiation with tumor temperature reaching 42°C within 90 sec and was sustained for 20 min by pulsed laser irradiation. Invasive temperature probes further revealed that MHT treatment permeated through the tumor, reaching the tumor base (~ 7 mm) (Figure 1D). In previous studies, prolonged heat treatments have been given to overcome thermo-tolerance commonly associated with cancer cells²⁵. Overall temperature readings from thermal camera corresponded surprisingly well (within ~ 0.2°C) with microprobe readings at the tumor base.

Increased tumor blood perfusion and permeability

Changes in tumor perfusion and permeability were assessed after MHT using laser Doppler microprobes. Alteration in tumor perfusion may influence the amount of therapeutic agents transported into tumor microenvironment and thus improve chemotherapeutic delivery and efficacy. We found that blood perfusion increases (red curve) concomitantly with rising temperature (dotted curve) and peaks at a 11-fold increase relative to baseline (Figure 2A). Furthermore, elevated perfusion persists (~3-fold enhancement) after heating is terminated. We then assessed changes in vascular permeability after MHT in CAPAN-1 tumors using Evans blue dye, as previously described¹⁰. After heating, Evans blue dye extravasated and accumulated faster in the tumor interstitium in a time-dependent manner as compared to unheated control, suggesting that vascular permeabilization is a persistent phenomenon (Figure 2B).

Immediate and transient changes in vascular function after mild hyperthermia

The effects of MHT on transport of larger macromolecules were evaluated using IVM to visualize dynamic extravasation of dextran dyes (70kDa dextran (~ 23 nm)) from the tumor vasculature into the interstitium. Fluorescent intensities were extracted from randomly selected ROIs and used to characterize dextran dye transport at 1 and 5 h after hyperthermia. Analyses of acquired IVM images showed an immediate, discernable increase in extravascular dye concentration 1 h post hyperthermia (Figure 3A). At 5 h, the effect of hyperthermia was still discernible and significant when compared to unheated controls (Figure 3B). Average fluorescent intensity acquired over several FOVs and fitted into a mathematical model showed striking differences in mass transport parameters between hyperthermia and untreated groups. Analyses of these fluorescent intensities revealed that the average rate of dye exchange, R , ranged from 0.05 to 0.20 min⁻¹ for untreated group and 0.25 to 0.35 min⁻¹ for MHT treated group (Figure 3C), indicating that dye extravasation is more rapid in tumors receiving MHT. When measured in real-time, MHT-mediated dye accumulation was more rapid, resulting a larger overall increase in tumor fluorescence intensity at 1 h post-treatment (Figure 3D). The transport characteristics of dye accumulation injected 5 h after MHT ($Y_{\max} = 3661 \pm 297$, $R = 0.458 \pm 0.10$ min⁻¹) comparable with those at 1 h after MHT ($Y_{\max} = 3816 \pm 189$, $R = 0.325 \pm 0.18$ min⁻¹; Figure 3E), consistent with previous observations of persistence in permeability. The higher R value suggested that MHT treatment enhanced the rate at which dextran extravasate into the interstitium and yields a higher $Y_{\max}$, which is the total accumulated dye. To ascertain that the enhanced transport and dye influx did not result from the destruction of tumor microenvironment,

H&E staining was performed to assess histological integrity of the tumor after treatment. H&E staining analyses showed that MHT did not cause significant damage to the tumor cells (morphologically similar to the untreated group). In contrast, tumors exposed to thermal ablation showed significant cellular damage characterized by regions of thermonecrosis, corrupt extracellular matrix, damaged or enlarged cell nuclei, and coagulation (Figure 3F). These are some of the common observations in photothermal ablative therapies⁴⁰. As further evidence that MHT caused no vascular hindrance by vascular damage and shutdown, dye flow was observed 24 h after treatment (Supplementary, Figure S3); unlike previous studies in which vascular occlusion was observed as early as 24 h after hyperthermia treatment²³.

Hyperthermia-induced vascular permeability is lasting and reversible

A comprehensive study on the timing and duration of induced vascular permeability could help inform optimal time-of-opportunity at which hyperthermia would most improve the transport of macromolecules (chemotherapeutics). Persistence of hyperthermia-induced vessel permeability was determined by evaluating the amount of dextran dye accumulating in tumor at different time-points after MHT treatment where dye was allowed to circulate for 1 h for each study group. Spectrophotometric analyses of tumor homogenates showed increasing amounts of accumulated dye peaking at 24 h and returning basal level after 36 h. At 36 h, the difference in the total dextran dye concentration became insignificant, suggesting that the effect of hyperthermia in enhancing macromolecule extravasation had abated (Figure 4).

Enhanced extravasation of macromolecules up to 54 nm in size

The transport of larger macromolecules (i.e. ~ 54-nm dextran), similar to that of commonly used chemotherapeutic agents, was measured following MHT. The extent of dye diffusion 1 h after MHT treatment was measured and then quantified with IVM (Supplementary, Figure S3). While there was a discernible increase in the amounts of extravasated 2,000kDa dextran 1 h after MHT treatment and dye circulation, the difference was statistically insignificant. However, when the dye was allowed to circulate for 5 h, the amounts of dye in tumor interstitium significantly increased for tumor receiving MHT treatment compared to controls (Figure 5A). Spectrofluorimetric analyses showed statistically significant increase in the amount of accumulated dye 5h after injection which became more pronounced 24 h post heat (Figure 5B). At 24 h after hyperthermia, total fluorescence in tumor homogenate was 2-fold higher in the treated group than in the controls (Figure 5B). However, the pronounced difference in tumor dye concentration between the MHT-treated group and control became insignificant at 36 h. This observation suggested a time-dependent and transient effect of MHT which is consistent with the previous observation using 70-kDa dextran (Figure 4). This illustrates that the effect of localized MHT on vascular permeabilization increases with time, reaching an optimal after 24 h and abates 36 h later (Figure 4). The effect of hyperthermia was also confirmed by immunohistochemical staining where extravasated dextran dye (red) is observed outside of the vasculature (CD31 staining represented in green) (Figure 6). In the untreated control, the extent of dye extravasation was drastically reduced where dye co-localization was observed, suggesting that majority of these dye molecules were trapped in the vasculature or cleared out of circulation (Figure 6, no heat).

DISCUSSION

Efficacy of most chemotherapeutics is predicated on achieving adequate drug transport across the endothelial layer and into the tumor interstitium. Therefore, loosening or creating gaps between endothelial cells to improve interstitial transport is an attractive strategy. For example, improvements in delivery and therapeutic efficacy by increasing vascular permeability using photodynamic therapy treatment have shown significant clinical benefit in patients ailing from age-related macular disorder¹¹. This strategy, however, suffers a major limitation in that it causes permanent vascular damage and unmitigated increase in vascular permeability that can potentiate cell migration, an early event in the metastasis cascade¹³. Hyperthermia treatment, as external actuator of tumor transport, is attractive strategy that can also achieve similar transport enhancements but on a more controlled fashion²⁰.

In this report, we aimed to establish the use gold nanorod-mediated MHT treatment as a novel means to achieve vascular permeabilization and thereby enhance transport of larger molecules while avoiding negative effects associated with thermal ablation. Gold nanorods were selected because they possess high-energy conversion coefficients²⁹ and when used in combination with NIR light can penetrate through several centimeters into cancer tissue⁴¹. This nanoparticle-based approach was implemented in order to rapidly produce site-specific MHT treatment which was attained within 90 sec of treatment (Figure 1). Compared to water-bath heating, it is more rapid and selective and can be used to accurately actuate tumor vascular properties.

We also show that mild temperature elevation strategy caused reversible changes to the vasculature where it increased perfusion (11-fold) during heating and 3-fold increase after cessation (Figure 2). This is consistent with previous observations where 12-fold increase in normal tissue⁴² and a 2-fold increase in tumor perfusion was observed with heating²³. While previous studies have shown temporal increases in blood perfusion, the treatments have also led to irreversible vascular disruption and shutdown²³ with negative implications in drug transport. Clinically, patients are typically given multiple rounds of drug infusions where functional vascular network is necessary. Here, we show that MHT treatment can enhance tumor transport properties while limiting damage to existing vessel network.

By intravital microscopy analyses, we have also demonstrated that mild hyperthermia caused a time-dependent increase in vascular permeability (Figure 2A) that led to a striking enhancement in the measured transport parameters and dye accumulation (Figure 3A). Lasting but reversible effects of this strategy was confirmed spectrofluorometrically which peaks at 24 h after treatment and returns to basal level 36 h later (Figure 4). This result is consistent with other reported observations using parallel heating modalities such as MR-guided High-Intensity Focus Ultrasound (HIFU) which have shown transient and reversible permeabilization over 72 h⁴³. The transiency of vascular permeability ensures that enhanced transport can be achieved within a narrow time-window while minimizing the possibility of increasing tumor invasiveness¹³. MHT treatment also enhanced the accumulation of larger molecules (~ 54 nm dextran) as evaluated by IVM analyses and corroborated by qualitative fluorescence analyses of tumor homogenate (Figure 5). Further

confirmation by histological analyses showed increased dye accumulation in the interstitial space compared to untreated group where majority of dextran dye were trapped in vessels (Figure 6).

The rate of macromolecule extravasation and accumulation between 23-nm (70kDa dextran) and 54-nm (2,000kDa dextran) dyes were significantly different with notable accumulation occurring at 1 h for smaller dye and at 5 h after MHT treatment for the 54-nm dextran. This suggested that larger macromolecule would require sufficiently prolonged time-of-opportunity to achieve enhanced accumulation. Therefore, an ideal time window that persists long enough to allow accumulation of macromolecules with long systemic circulation half-lives would be required, i.e., ~ 6 h for most PEG-coated particles ⁴⁴.

The use of MHT treatment has previously been used to achieve site-specific and targeted delivery of thermally sensitive drug carriers including liposomes ²⁹. Upon treatment, these carriers degrade and release their drug payload, leading to improved delivery and efficacy ²⁸. In this report, we capably produced sustained MHT while avoiding ablative conditions which allowed us to separate out and study contributions of vascular changes to overall drug delivery. The contributions from vascular changes to the overall improvements in drug and efficacy have been overlooked in many hyperthermia/drug studies ^{15,17} despite the evidence that increases in vascular permeability occur around tissue periphery (regions with mild hyperthermia) during thermal ablation ²². This effect may contribute to overall increase in drug and efficacy. So, our work has demonstrated that MHT treatment can alter vascular permeability and improve delivery without necessarily causing vessel occlusive damage associated with thermal ablation.

Localized MHT treatment enhanced interstitial transport, probably due to the perturbation of vascular endothelium that permitted increased extravasation into the interstitium without causing irreversible damage, unlike thermal ablation. The effect was transient on the endothelium whereas thermal ablation cause irreversible protein denaturation that ultimately leads to vascular occlusion. The use of NIR irradiation for thermal therapy in combination with chemotherapy ¹⁷ is becoming a mainstay technology in preclinical studies ⁴⁵ and our findings add another layer of understanding that can be leveraged to improve drug delivery. We envision that MHT treatment may be used to enhance the delivery of both thermally and non-thermally sensitive drugs and carriers.

While we have demonstrated the effectiveness of mild hyperthermia towards increasing tumor transport and dye accumulation, the study did not account for dye removal/re-entry that occurred during analyses. We addressed this shortcoming by keeping the animal-to-animal dye circulation time same. Additionally, inherent tumor heterogeneity may hinder uniform delivery and distribution of gold nanorods and could limit the applicability of NIR heating particularly in large tumors where vascular delivery is limited. This makes it difficult to localize sufficient amounts of GNRs to generate mild hyperthermia using low laser photon flux. In these instances, other parallel heating modalities such as HIFU may be explored as a means to produce mild heating.

In conclusion, our findings demonstrate a novel use of gold nanorods to produce mild hyperthermia to increase macromolecule delivery into tumor. It provides a rationale for combining MHT treatment as an additional and complimentary adjuvant with nanoparticle drug delivery. The ability to generate site-specific hyperthermia means that the strategy can be targeted to the intended tumor site. By creating fenestrations, this strategy could be applied directly to improve transport of chemotherapeutics, including nanomedicines that are thermosensitive or otherwise. The use of mild hyperthermia may open up the enhancement of a broader regime of therapeutic sizes (up to 54 nm) beyond what has been reported previously. Such efforts would synergize targeting, transport, and efficacy of various therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Photothermal properties of PEG-coated GNRs used to induce selective and localized hyperthermia; A) schematic representation of functionalization strategy used to make PEG-coated GNRs using 5kDa methyl-PEG-thiol through ligand exchange; B) heating curves of dilute solution GNRs (80 µg/mL) showing selective 25-degree temperature elevation, control, PBS solution; C) thermographic images showing selective heating of s.c. tumor actuated by localized GNRs heating with minimal temperature rise in control group; D) temperature profile at tumor bed during hyperthermia treatment showing elevated and sustained at ~42°C which corresponded well with thermographic readings.

Figure 2. Nanoparticle-assisted MHT treatment enhances on tumor perfusion and permeability. A) Graph showing relative perfusion change as function of time, where perfusion rates quickly rise 11-fold compared to initial baseline and 3-fold higher after cessation; B) Amounts of Evans blue dye in tumor homogenate 1 & 3 h after hyperthermia as quantified fluorometrically at 470/680 nm excitation/emission wavelengths, respectively. Both time points demonstrated a significant effect in dye extravasation over the unheated control (2-

fold increase at 1 h and 2.5 fold increase at 3 h) with statistical significance denoted by * $p < 0.02$, ** $p < 0.004$ (n = 5).

Figure 3.

Enhanced extravasation of 23-nm dextran in CAPAN-1 pancreatic tumors resulting from MHT treatment as studied by IVM imaging and analyses; (A, B) time lapse IVM images showing increased dextran tumor accumulation in treatment groups 1 & 5 h post heat versus untreated groups; C) mean average fluorescent intensities with distinct R values between the treated and untreated group; D) average intensity acquired over 20 min (1 h post-heat) and fitted to a mass transport model with a higher Y_{max} and R for treated group; E) comparison of treatment groups (1 & 5 h post-treatment) showed similar average fluorescent intensities and transport enhancement properties with $Y_{max} = 3816$ & 3661 fluorescent units (FUs) and ($R = 0.325$ & 0.458 min^{-1}), respectively; F) Histological analyses confirm that MHT caused little morphological alterations to tumor microenvironment while thermal ablation caused cellular damage.

Figure 4.

MHT treatment causes persistent but reversible vascular effects. CAPAN-1 tumors were heated and evaluated at different times after MHT ($t = 0 - 36$ h) by injecting 70kDa dextran dye and quantifying fluorescence intensity in tumor homogenate and compared to unheated controls. The effects of localized MHT were measureable immediately treatment and persisted for 24 h and but returns to basal levels after 36 h with statistical significance denoted by * for $p < 0.025$, ** for $p < 0.04$, *** for $p < 0.020$, and **** for $p < 0.01$ ($n = 8$ per group).

Figure 5.

MHT treatment enhances macromolecule extravasation up to 24 h which abates at 36 h after treatment; A) (top panel) IVM images show enhanced dye extravasation (red) in interstitial space at 5 h after MHT and circulation of 2,000kDa dextran dye; (bottom panel) show reduced dye accumulation in unheated control with majority of dye in vascular region with little in interstitial space; FITC dextran dye was injected immediately (~ 1 min) and used as vessel tracer; B) total amount of 2,000kDa dextran in tumor homogenate after MHT treatment and quantified by spectrofluorimetrically at 561/595 nm emission/ excitation wavelengths. Statistical significance is denoted by * $p < 0.046$, ** $p < 0.004$, *** $p < 0.002$.

Figure 6.

Representative histological tumor specimens demonstrating increased extravasation of 2,000kDa dye after hyperthermia treatment and 5h of circulation compared to untreated group; Top panel) shows extravasated dye away from the vasculature into interstitium (CD31 staining showed in green) enabled by hyperthermia that caused little damage to tumor microenvironment where H&E staining revealed intact cell morphology; bottom panel) shows dye accumulated in the vasculature but hardly any in the tumor interstitium.