HSP70 Inhibition Synergistically Enhances the Effects of Magnetic Fluid Hyperthermia in Ovarian Cancer

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Abstract

Hyperthermia has been investigated as a potential treatment for cancer. However, specificity in hyperthermia application remains a significant challenge. Magnetic fluid hyperthermia (MFH) may be an alternative to surpass such a challenge, but implications of MFH at the cellular level are not well understood. Therefore, the present work focused on the examination of gene expression after MFH treatment and using such information to identify target genes that when inhibited could produce an enhanced therapeutic outcome after MFH. Genomic analyzes were performed using ovarian cancer cells exposed to MFH for 30 minutes at 43°C, which revealed that heat shock protein (HSP) genes, including HSPA6, were upregulated. HSPA6 encodes the Hsp70, and its expression was confirmed by PCR in HeyA8 and A2780cp20 ovarian cancer cells. Two strategies were investigated to inhibit Hsp70-related genes, siRNA and Hsp70 protein function inhibition by 2-phenylethanesulfonyl fluoride (PES). Both strategies resulted in decreased cell viability following exposure to MFH. Combination index was calculated for MFH treatment reporting a synergistic effect. In vivo efficacy experiments with HSPA6 siRNA and MFH were performed using the A2780cp20 and HeyA8 ovarian cancer mouse models. A significantly reduction in tumor growth rate was observed with combination therapy. PES and MFH efficacy were also evaluated in the HeyA8 intraperitoneal tumor model, and resulted in robust antitumor effects. This work demonstrated that HSP70 inhibition combination with MFH generate a synergistic effect and could be a promising target to enhance MFH therapeutic outcomes in ovarian cancer.

Introduction

Hyperthermia is the application of heat to tissues as a therapeutic tool using temperatures between 41°C and 47°C. Hyperthermia has been successfully employed as an adjuvant for the treatment of several cancers and is known to enhance the effects of chemotherapy and radiotherapy (1–3). Clinically, heat can be applied locally, regionally or in the whole-body, but current modalities are not site specific (1, 4). Challenges such as temperature homogeneity during application, achieving an efficient heat dose, patient discomfort, and hot spots limit the use of this therapy (2, 4). The use of nanotechnology could help overcome such challenges. Magnetic fluid hyperthermia (MFH) is an attractive alternative as it can deliver heat to the desired area using magnetic nanoparticles under the influence of a magnetic field (1, 2, 5).

There is evidence that MFH can deliver heat more efficiently than conventional hyperthermia (6–12). Aspects such as thermal chemosensitization, membrane permeabilization, and sensitization of drug-resistant cancer cells have been observed as responses to MFH in vitro (2, 7–9). These findings demonstrate that MFH has potential as an adjuvant cancer treatment, but there is still a dearth of knowledge in the field regarding the implications of MFH at the molecular level and how these can be exploited to enhance the effects of MFH in cancer treatment.

Previous work with conventional hyperthermia demonstrated that molecular and cellular responses depend on the cell line, thermal dose, and recuperation time (13–16). In the clinic, intraperitoneal administration of hyperthermia (HIPEC), in combination with chemotherapeutic drugs, have been investigated for more than three decades as an attractive adjuvant to cytoreductive surgery in ovarian cancer (17). Currently, there are more than 30 clinical trials that have recently started or concluded using this technology (18). A recent meta-analysis of clinical trials performed by Huo and colleagues (19), demonstrated that the combination of HIPEC, cytoreductive surgery, and chemotherapy showed significant patient survival. Still, a challenge remains: the ability to reach a therapeutic temperature without causing toxicity to healthy tissue and risk complications (20–24). Furthermore,
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intrinsic biological aspects such as the inherent thermo-tolerance properties of tumors are a challenging aspect that has been neglected to this point. Nanoscale heat generation systems such as iron oxide nanoparticles represent an attractive alternative as one could generate heat within the system, only under high-frequency and moderate amplitude magnetic fields that can be constrained to the tumor region. Therefore, the goal of this work was to investigate gene expression profiles after MFH in ovarian cancer cell lines to elucidate cellular response and select molecular targets to enhance its effect in vitro and in vivo. It was hypothesized that upregulated genes and proteins resulting from MFH treatment, when inhibited, could potentially enhance MFH outcome. Results indicated the upregulation of HSPA6, which encodes for heat shock 70 kDa protein 6 (Hsp70), several heat shock proteins (HSP), and ubiquitin C. Hsp70-related genes and proteins were selected as potential targets. Two strategies were investigated to inhibit Hsp70 genes and proteins (i) siRNA-based therapy; and (ii) HSP70 protein function inhibition by 2-phenylethylenesulfonamide (PES; ref. 25). Both strategies resulted in enhanced cell death in various ovarian cancer cell lines in vitro and in vivo.

Materials and Methods

Cell culture

Ovarian cancer cell lines HeyA8, HeyA8 MDR, A2780, A2780 cp20, and SKOV3 were grown in RPMI1640 (Sigma-Aldrich), 15% FBS (Life Technologies), and 0.1% gentamicin sulfate (Sigma-Aldrich). Cells were maintained at 37°C, 95% relative humidity and 5% CO2. Cell lines were obtained from the institutional Cell Line Core Laboratory MD Anderson Cancer Center in 2012. Authentication was performed within 6 months of the current work by the short tandem repeat method using the Promega Powerplex 16HS Kit (Promega).

Magnetic nanoparticles

Carboxymethyl dextran coated iron oxide nanoparticles (CMDx-IO) were prepared using the co-precipitation method as previously described (8, 26). Briefly, an aqueous solution of ferric chloride hexahydrate, ferric chloride tetrahydrate, and ammonium hydroxide were mixed at a pH 8.0 under nitrogen at 80°C. Nanoparticles were peptizated with tetramethyl ammonium hydroxyl and dried. They were functionalized in dimethylsulfoxide, 3-amino propyltriethoxysilane, DI water, and acetic acid. Then washed with ethanol and dried. Later they were exposed to carboxymethyl dextran, N,N-[3-dimethylaminopropyl]-N’-ethyl carbodiimide hydrochloride, and N-hydroxysuccinimide, at pH 4.5–5.0. Particles were centrifuged and washed with ethanol and dried. Particles characterization is presented in Supplementary Fig. S1.

In vitro MFH

Attached cells. Cells were seeded in 35-mm cell culture dishes. New culture media and CMDx-IO nanoparticles (0.5 mgFe/mL) were added the following day. The dish was placed inside the magnetic induction coil of an Easy Heat 8310 LI (Ambrell) induction heater and exposed to a magnetic field intensity that ranged between 29 and 35 kA/m. The copper coil had 4 turns and an inner diameter of 2 cm. Cells were exposed to MFH for 30 minutes. After treatment, cells were seeded in a 25 cm² flask and placed in an incubator. After 48 hours, cell viability was assessed with Trypan Blue (Sigma-Aldrich).

Microarray analysis

Ovarian cancer cells were exposed to MFH for 30 minutes at 43°C. RNA was prepared after treatment using a mirVana RNA isolation labeling kit (Life Technology). Three hundred nanograms of RNA were used for labeling and hybridization on a Human HT-12 v4 Beadchip (illumina) following manufacturer’s protocols. The bead chip was scanned with an Illumina BeadArray Reader (illumina). Microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data (LIMMA) package using R language. The expression level of each gene was determined by the fold change in the untreated control group, and MFH treated group (43°C and 30 minutes) transformed into a log, base for analysis. The original microarray data were uploaded to GEO with accession number GSE92990.

Quantitative real-time PCR

RNA was extracted from cells and tumor tissue using Direct-zol RNA MiniPrep (Zymo Research). cDNA was synthesized from 1,000 ng of RNA with the Thermo Scientific Thermo Velo cDNA Synthesis Kit (Thermo Fisher). Quantitative Real-Time PCR was performed using SYBR Green (Thermo Fisher) on a 7500 Fast Real-Time PCR System and primers for HSPA6 and HSPA7 (Integrated DNA Technologies). Specific primers for HSPA6 F-CCGTCTGGGACTCATGGAATA, R-GAGATCTCGTCCATGGTGCT and HSPA7 F-CAACCTGCTGGGGCGTTTTGA, R-CCGGCCCTTGTACATTGCTGT ACTTT, 18s was used as an endogenous control.

Western blot analysis

Cells were lysed with RIPA (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) incorporating the Protease Inhibitor sigmaFAST (Sigma-Aldrich). Protein concentration was determined with Bradford Reagent (Sigma-Aldrich). Equal amounts of protein were separated with 4% to 20% Mini-PROTEAN TGX Gel (Bio-Rad Laboratories) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was incubated with HSP70 antibody, 1:1,000 (Novus Biologicals) and β-actin rabbit mAb 1:1,000 (Cell Signaling Technology) overnight. Primary antibody was detected with anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology) and developed with SuperSignal WestPico Chemiluminescent Substrate (Thermo Scientific) in Chemidoc XRS (Bio-Rad Laboratories).
PES cytotoxicity in ovarian cancer cells
Cells were seeded in 96-well plates in 200 µL of media and allowed to attach overnight. Cells were exposed to a concentration of PES between 5 and 30 µmol/L for 24 and 48 hours. Cell viability was measured with EZUI4 assay (ALPCO) following the manufacturer’s protocol.

Determination of synergism
Combination index (CI) was evaluated using the proposed model of Chou–Talay with the CompuSyn software (27). CompuSyn uses the median–drug effect analysis. Concentration–effect curves were assumed to be sigmoidal for the single drug and the combination treatment. Calculation of doses was performed by the median–effect equation. The parameters used for the calculation were the fraction affected and PES and MFH exposure time. Equations are described in the Supplementary Material.

In vitro siRNA delivery and MFH
Cells were transfected with Lipofectamine RNAiMax (Invitrogen) at 2.5 µL of the reagent: 1 µg siRNA. HSPA6 siRNA from Rosetta Prediction Human (Sigma-Aldrich) was selected as target gene, sequence 1: SASI_Hs01_00244829, sequence 2: HSPA7 1 and HSPA7 2, sequence 3: SASI_Hs01_00244824 and sequence 4: SASI_Hs01_00244827 and SASI_Hs01_00244829 and control siRNA was MISSION siRNA Universal Control #1 (Sigma-Aldrich) at a concentration 60.2 or 100 nmol/L. A total 36 hours of transfection was performed. MFH was applied, and cell viability was measured following 48 hours of incubation as previously described.

Liposomal nanoparticle preparation (siRNA-DOPC)
HSPA6 and control SiRNAs for in vivo delivery were incorporated into 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes as previously described (28). DOPC and siRNA were mixed in the presence of excess tertiary butanol at a ratio of 1:10 (w/w) siRNA/DOPC. Tween 20 was added to the mixture in a ratio of 1:19 Tween 20:siRNA/DOPC. The mixture was vortexed, frozen in an acetone/dry ice bath and lyophilized. Before in vivo administration, this preparation was hydrated with PBS.

Subcutaneous MFH and HSPA6 siRNA delivery
Subcutaneous tumors were generated in an athymic nude mouse model. Studies were performed according to the protocol approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee. HeyA8 or A2780cp20 cells (1 × 10^6) suspended in 50 µL of Hank’s balance salt solution were injected subcutaneously in the mice right flank on day 0, to obtain subcutaneous tumors on day 7. Tumor volumes were 35–113 mm³. siRNA liposomes injection started on day 7, 5 µg of HSPA6 siRNA or control siRNA liposomes were injected intraperitoneally. Three MFH treatments and siRNA deliveries were performed. The first MFH treatment was applied on day 9. Tumor volume was calculated as (largest diameter) × (smallest diameter)^2 × π/6. MFH started with iron oxide nanoparticles injected directly into the tumor (5 mgFe/cm³ tumor volume). Nanoparticles were injected for 30 seconds. The tip needle was removed from the tumor after 5 minutes. MFH was applied 4 hours after nanoparticle injection to allow particle distribution (29). Mice were anesthetized and placed inside the magnetic induction coil of an EasyHeat 8310 LI (Ambrell) induction heater for 30 minutes at a magnetic field intensity of 23 kA/m. The tumor was positioned in the center of the coil. The copper coil consisted of 7.5 turns and an inner diameter of 3.16 cm. Warm water was circulated through a plastic tube to maintain the environmental temperature inside the coil at 37°C. Body temperature was measured in the anus to guarantee mice safety. The external temperature of the tumor and the surrounding wall was also measured with an NI-9211 thermocouple signal conditioner and acquisition board (National Instruments) and type T thermocouples (Omega Engineering).

Intraperitoneal tumor model and MFH
The intraperitoneal tumor was generated in an athymic nude mouse model. Studies were performed according to the protocol approved by the M.D. Anderson Cancer Center Institutional Animal Care and Use Committee. 2.5 × 10^3 HeyA8 cells suspended in 200 µL of Hank’s balance salt solution were injected directly into the peritoneal cavity (IP) of mice. Treatment started 2 weeks after cells IP injection. PES was injected a 10 mg/Kg 2 days before the first MFH. CMDx-IO nanoparticles were injected intraperitoneally at 2 mg Fe/mouse in 200 µL of PBS the night before treatment. Next day mice were anesthetized with ketamine and MFH applied for 30 minutes, with the same coil configuration previously described. The temperature was also measured in the mouse belly.

Histology
Tumors were embedded in paraffin and cut (8-µm sections). Slides were deparaffinized, and rehydrated. The Prussian Blue, Iron Stain Kit (Sigma) was used following the manufacturer’s protocol.

Statistical analysis
Statistical analyzes were performed using the Student t test (two-tailed distributions, two samples with unequal variables). For values that were not normally distributed, the Mann–Whitney rank sum test was used. A P value of <0.05 was considered statistically significant.

Results
MFH in ovarian cancer cell lines
Four ovarian cancer cell lines, A2780 and HeyA8, and their corresponding drug resistant sub-lines were exposed to MFH at various temperatures (41°C, 43°C, and 45°C) and exposure times (30 or 60 minutes). Cell viability was assessed after 48 hours of recovery (Fig. 1A). No significant change in cell viability was observed at 41°C for all cell lines. However, at 43°C, a significant decrease in cell viability was observed for most cell lines. At 45°C, further reduction in cell viability was observed for all cell lines. The thermal dose for each case was calculated using cumulative equivalent minutes (CEM) as established by Dewey and colleagues (30). Supplementary Table S1 presents the CEM values for all conditions. Temperature and exposure time affected CEM values and cell viability. Higher CEM values resulted in enhanced cell death.

Gene expression in MFH in ovarian cancer cell lines
A gene array analyses was carried out to identify genomic changes resulting from exposure of ovarian cancer cells to MFH. A control group of untreated HeyA8 cells and an experimental group consisting of cells exposed to MFH for 30 minutes at 43°C...
were used. This temperature was chosen since the aforementioned results indicated a substantial decrease in cell viability when the temperature was increased from 41°C to 43°C. Compared with controls, 60 genes were upregulated (by ≥1.5-fold) in treated cells. The top 20 upregulated genes are presented in Fig. 1C. Supplementary Tables S2 and S3 present descriptions of each of the dominant upregulated and downregulated genes.

Data were further analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) and enrichment of Gene Ontology (GO) terms for significantly expressed genes. From this analysis, three main biological functions were found to be affected by MFH at 43°C, including response to unfolded proteins, response to protein stimulus, and protein folding (Table 1). Top genes related to the aforementioned functions affected by MFH were HSPs, Hsp70 (HSPA6/HSPA7, HSPA1A, HSPA1B, HSPA1L, HSPA4L), Hsp60 (LOC643300), Hsp40 (DNBAJ family), Hsp20 (CRYAB) and Hsp27 (SERPINH1), and BAG3 (modulator of Hsp70). Besides HSPs, the gene ubiquitin C (UBC) was also in the top ten upregulated genes after MFH. Figure 1B presents the connection of HSPA6 with upregulated genes from the microarray.

To validate the results from the microarray, qRT-PCR was performed for HSPA6 and HSPA7. Hsp70 was selected because it has a critical function in cancer cells and hyperthermia. Hsp70 is overexpressed in various human cancers, playing different roles as an inhibitor of apoptosis (31). HSPA6 and HSPA7 expression were confirmed by qRT-PCR, and expression was measured for up to 8 hours following heat shock treatment (Fig. 2A). A2780cp20 showed significant expression at 1 hour for both genes. In HeyA8 cells, gene expression was sustained for almost 4 hours at 41°C and 8 hours at 43°C.

Hsp70 protein level was also evaluated according to Western blot analysis. At 41°C, the highest expression level was observed after 6 hours of MFH treatment. At 43°C, protein expression for A2780cp20 cells increased after 1 hour of recovery time, and the highest expression was observed at 6 hours after MFH (Fig. 2B). HeyA8 cells showed no significant differences in Hsp70 protein expression via Western blot analysis (Supplementary Fig. S2C).

HSPA6 (Hsp70) was selected as the target gene because it was the most upregulated (32). Two approaches were performed to inhibit Hsp70 including gene silencing by siRNA and protein function inhibition using PES. HSPA6 siRNA enhanced the effects of MFH A2780cp20 and HeyA8 cell lines were selected to further assess the efficacy of HSPA6 inhibition along with MFH (Fig. 2). A2780cp20 and HeyA8 were selected according to their capability to create subcutaneous tumor models. First, several siRNA sequences were investigated to evaluate their capacity to inhibit HSPA6 expression. Two siRNA sequences per cell line were established for HSPA6 inhibition with 50% to 60% of gene knockdown. For HeyA8 cell line, seq #1 at 60.2 nmol/L and seq #2 at 100 nmol/L were able to inhibit HSPA6. In the case of A2780cp20 cells, seq #3 at 60.2 nmol/L and seq. #4 at 100 nmol/L (Supplementary Fig. S2A) were also successful in inhibiting HSPA6. The in vitro siRNA effects were evaluated in each cell line (Fig. 2C and

Table 1. Biological functions after MFH treatment of HeyA8 cells (43°C and 30 minutes)

<table>
<thead>
<tr>
<th>Biological function</th>
<th>Number of genes</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to unfolded proteins</td>
<td>14</td>
<td>9.99E−17</td>
</tr>
<tr>
<td>Response to protein stimulus</td>
<td>15</td>
<td>8.43E−16</td>
</tr>
<tr>
<td>Protein folding</td>
<td>14</td>
<td>1.88E−11</td>
</tr>
</tbody>
</table>

Figure 1.
Gene expression after MFH in ovarian cancer cells. A, Cell viability when MFH was applied to ovarian cancer cells at 41, 43, 45°C for 30 and 60 minutes; (n = 3; error SE). B, Pathway of upregulated genes from IPA after MFH was applied to HeyA8 cell lines for 30 minutes at 43°C. C, Top 20 upregulated genes after MFH was applied to HeyA8 cell lines for 30 minutes at 43°C. Analysis revealed a significant upregulation of HSP70-related genes (one-way ANOVA, n = 3, P < 0.02).
D). Combination treatment (HSPA6 siRNA + MFH) resulted in a higher decrease in cell viability when compared to MFH or HSPA6 siRNA applied independently.

Antitumor effects of HSP70 inhibition and MFH in vitro
The effects of HSP70 inhibition using PES inhibitor were tested. PES cytotoxicity was measured (Fig. 3A) and results presented a reduction in cell viability as PES concentration was increased. Supplementary table S4 illustrates the IC_{50} values for PES when exposed to ovarian cancer cell lines. A2780cp20 cells showed lower IC_{50} values to PES when compared to HeyA8 and SKOV3 cells. Previous work by Granato and colleagues, revealed PES affected membrane permeability since HSP70 supports lysosome membrane integrity, especially in cancer cells (33). To confirm such mechanism in ovarian cancer cells lysosomal permeabilization was measured. Results indicated that lysosome permeabilization increased as the concentration of PES increased as depicted by the higher percentage of pale cells (Supplementary Fig. S3A). Lysosome permeabilization was also investigated by imaging the release of cathepsin B into the cytosol. Cathepsin B in control cells was located in lysosomes as small red points (Supplementary Fig. S3B). In PES-treated cells a diffuse pattern was observed, indicating the relocation of cathepsin B into the cytosol confirming lysosome permeabilization (Supplementary Fig. S3B).

The effect of in vitro combination treatment (MFH + PES) was assessed in ovarian cancer cells (Fig. 3B). PES concentrations were selected as to provide viabilities higher than 90% when used independently (Fig. 3A and Supplementary Fig. S3E), 5 μmol/L for A2780cp20 and 10 μmol/L for HeyA8 and SKOV3. Two MFH temperatures were selected, 41°C representative of mild hyperthermia, and 43°C representative of hyperthermia. Combination treatment decreased cell viability to approximately 40% at 41°C.
compared with MFH alone (Fig. 3B). Results indicated that the combination treatment generated a cytotoxic effect on cells, which could indicate synergy. For this purpose, combination index (CI; Fig. 3C) was calculated using the Chou–Talalay (27) and CompuSyn methods. Cell viabilities and dose response curves from PES and MFH (Supplementary Fig. S3E) were used to compute CI values. The median effect principle of the mass-action law was evaluated using parameters described in Supplementary Table S5. The degree of synergism defined by Chou and Marin was analyzed (27). According to this analysis, A2780cp20 and HeyA8 cells at 41°C (top) and 43°C (bottom) for 30 minutes after 48 hours of recovery. C, Combination index of MFH and PES for ovarian cancer cell lines.

HSPA6 silencing and MFH reduced tumor growth in vivo
To identify the in vivo effect of HSP70 inhibition during MFH, HSPA6 siRNA was used to silence HSPA6 in a subcutaneous ovarian tumor model. siRNA was delivered with DOPC nanoliposomes. Mice were divided into four groups, control siRNA-DOPC, control siRNA-DOPC and MFH treatment, HSPA6 siRNA-DOPC and combined HSPA6 siRNA-DOPC with MFH treatment. The efficacy of siRNA delivery and gene silencing was confirmed
Research demonstrated that reduction in tumor growth can be enhanced by the number of MFH treatments (34, 35). For this purpose, three MFH treatments were scheduled 48 hours apart. CMDx-IO nanoparticles were injected intratumorally, 4 hours before treatment, to allow them to distribute in the tumor. The siRNA-DOPC was administered according to previous publications, two times per week and injected intraperitoneally (28). Tumor growth was slower in groups that were treated with MFH when compared with those not treated with MFH (Fig. 4B and Supplementary Fig. S4B). Supplementary Table S7 presents the statistical values of the final tumor weight. By the end of the treatment, relative tumor volume for the control groups and control siRNA-DOPC with MFH presented a 3- to 4.4-fold increase when compared to their initial value whereas HSPA6 siRNA-DOPC was only a 1.4-fold. Final tumor weight for HSPA6 siRNA-DOPC and MFH was 65% lower than control siRNA-DOPC and MFH (Fig. 4C). The temperature profile is exhibited in Supplementary Fig. S4A. Tumor temperatures of 43°C were reached.

A second tumor model was evaluated using the A2780cp20 cells under a similar treatment schedule (Fig. 4D and E). Tumor volume and weight were measured. Tumor weight for the combination treatment, HSPA6 siRNA-DOPC and MFH, was approximately 65% smaller when compared with MFH and control siRNA-DOPC groups. For the control groups and control

![Timeline for MFH in vivo. A, Relative tumor volume in function of time of HeyA8 tumor model. B, Ex vivo HeyA8 tumor weight. Number of mice 4–5 per group. C, Relative tumor volume of A2780cp20 tumor model. D, Ex vivo A2780cp20 tumor weight. E, Prussian Blue staining to determine distribution of iron oxide nanoparticles from collected tumor from A2780cp20 and HeyA8. (*, P < 0.05; **, P < 0.01).](image-url)
siRNA-DOPC MFH, an increase in relative tumor volume was observed with a fold increase between 2 and 6.6. HSPA6 siRNA-DOPC and MFH presented a relative 0.7-fold change in volume. HSPA6 inhibition improved the outcome of MFH treatment in subcutaneous models. The biological effect of MFH was assessed for apoptosis. Caspase-3 activity was observed in MFH-treated tumors (Supplementary Fig. S4B). The distribution of CMDx-IO nanoparticles was evaluated in both tumors models using Prussian Blue (Fig. 4F). Iron oxide nanoparticles remained in the tumor tissue of both subcutaneous tumor models until the tumor was collected at the end of the experiment.

MFH was also tested in the HeyA8 intraperitoneal tumor model in combination with PES. Treatment started when PES was injected IP at day 12 after cells injection (Fig. 5A). PES dose was selected from previous reports where it was administered every 5 days (25, 36, 37). The following day, CMDx-IO nanoparticles were injected intraperitoneally, the technique allows the internalization of nanoparticles in intraperitoneal tumors as described elsewhere (38). At day 14 mice were exposed to MFH for 30 minutes. A total of three MFH treatment and PES injection were performed. Mice were sacrificed when controls appeared moribund. Tumor weight of PES and MFH treated mice (Fig. 5B), was 65% smaller when compared with the control MFH group. Mice were monitored and no sign of sickness or pain were observed days after MFH. Body weight and feeding habits remained unchanged. Results agreed with in vitro outcomes of synergy between PES and MFH.

Discussion

The present work investigated the molecular and cellular responses of ovarian cancer cells to MFH. We and others have attempted to elucidate the cellular and molecular features of MFH; however, there are still aspects at the molecular level that need to be understood (6–12). Results described herein indicated that there is overexpression of HSPs, in particular, Hsp70 as a result of MFH treatment. The resulting genomic analysis provided the means to design targeted combination therapies using direct-ed nanoscale heat delivery with magnetic nanoparticles. The inhibition of Hsp70 by RNA interference and the novel Hsp70 inhibitor, PES, enhanced the effect of MFH both in vitro and in vivo in various ovarian cancer cell lines and tumor models (subcutaneous and intraperitoneal). Furthermore, in vitro observations with PES demonstrated, for the first time, that the combined treatment resulted in a synergistic effect at mild hyperthermia temperatures. Findings of this work also indicated that the intraperitoneal injection of iron oxide nanoparticles is a feasible alternative to nanoparticle delivery to peritoneal tumors and that MFH treatment can be successfully administered without damaging healthy tissue.

To further understand such cellular responses, gene expression and pathway analyses were conducted. An experimental temperature of 43°C was selected because an inflection point in cell viability is observed for conventional hyperthermia (1, 39). Such behavior was also observed when MFH was applied as a sole treatment in vitro for various ovarian cancer cell lines as described herein.

Results are supported by prior work demonstrating that HSP-related genes, including Hsp70, Hsp40, Hsp105, Hsp110, Hsp47, and BAG3 are upregulated when conventional hyperthermia was applied to cancerous and normal human cells (13, 15, 16, 40–44). However, such experiments have not been previously conducted for MFH-treated ovarian cancer cells or any other cancer cell line. The genetic analysis of the response of ovarian cancer cells exposed to MFH revealed that several HSPs were upregulated as well as ubiquitin C (UBC). These findings demonstrated that MFH upregulated the expression of HSPs genes in ovarian cancer cell lines similar to conventional hyperthermia. Interestingly, MFH also overexpressed UBC, which has not been previously reported as a top overexpressed gene in microarray for conventional hyperthermia in treatments where the range in thermal dose was between 7.5 and 60 cumulative equivalent minutes (CEM; refs. 15, 40–42). At higher CEM (~300) ubiquitin genes expression have been reported (45, 46). In this particular work MFH treatments with a thermal dose of only 30 cumulative equivalent minutes significantly upregulated UBC. These results may provide further evidence that the extent of MFH damage in the cell might be more extensive than conventional hyperthermia as reported by our group and others (6, 7, 12).

After studying the gene expression resulting from MFH at 43°C, Hsp70 was selected as it was the most upregulated gene of the microarray, as confirmed by qPCR and Western blot analysis. In addition, Hsp70 is overexpressed in various cancer models, and high levels are associated with poor prognosis (31). Hsp70 plays a significant role in cancer, suppressing apoptosis and participate in tumor development. Under stress conditions, such as hyperthermia, Hsp70 avoids accumulation of unfolded proteins and refold aggregated proteins (31, 47). Several Hsp70 inhibitors have been designed with favorable results in vitro, but have failed efficacy in pre-clinical or clinical trials, with limitations as toxicity and reduced antitumor effect (11, 31, 48, 49). An alternative of two approaches were investigated in this work, inhibition of Hsp70 by RNA interference and, inhibition of protein function using PES.

Previous reports have demonstrated that Hsp90 inhibition in combination with MFH therapy increased cell susceptibility to hyperthermia and in vivo tumor regression (50–52). Hsp90 inhibition is commonly used in hyperthermia because it plays a central role in cancer signaling molecules, but research established that Hsp90 inhibition also upregulates Hsp70 production.
The goal of this work was to demonstrate that only Hsp70 inhibition will improve the antitumor effect of MFH. Hsp70 knockdown with siRNA has been achieved in vitro in human colon cells, prostate and the ovarian cancer cell line A2780 (37, 53, 54). The combination of Hsp70 RNA interference with MFH has not been previously reported in vitro or in vivo. Results demonstrated that HSPA6 inhibition with siRNA in combination with MFH enhanced treatment outcomes both in vitro and in vivo. Data confirmed that Hsp70 inhibition and MFH appear as an effective selective alternative, resulting in enhanced MFH therapeutic effects. The second approach was to inhibit the Hsp70 protein function. An alternative to this approach was to employ PES because it has been identified as a novel Hsp70 inhibitor with direct specificity (25, 36). In this work, PES was able to reduce cell viability in various ovarian cancer cells by similar mechanisms as those proposed in the literature (53–56). The combination of MFH and PES resulted in a synergistic effect in ovarian cancer cells. This is the first time that synergy is demonstrated from the combination treatment between PES and MFH. Sekihara and colleagues (37) demonstrated that when PES and conventional hyperthermia were combined in prostate cancer cells the number of cells in early apoptosis and necrosis and late apoptosis were increased, concluding that the cell death mechanism of the combination treatment partially depended on the caspase pathway. Furthermore, they demonstrated that the combination increased the number of cells in the G2–M phase and decreased the expression of cell cycle related molecules such as c-Myc and cyclin D1 causing cell growth arrest. These findings validated that Hsp70 protein function inhibition is enough to enhance the therapeutic outcome of MFH.

Hsp70 inhibition and MFH were further evaluated in intraperitoneal tumor models with peritoneal administration of nanoparticles. The reason to select this model is that intratumoral injections are not practical in clinical settings. Besides, other methods such as nanoparticle targeting for intravenous administration still face significant challenges achieving the required levels of nanoparticle accumulation in tumors. Administration in the peritoneal area of antineoplastic agents is currently an alternative for treating small tumors located in the area. High drug concentrations and longer half-life can be achieved with intraperitoneal injections (37). Previous work has reported the intraperitoneal administration of iron oxide nanoparticles for MFH treatment in a mouse ovarian cancer model with peritoneal tumors but did not assess tumor growth, focusing solely on demonstrating cell death through histological analysis (38). In this work, we administered nanoparticles intraperitoneally to the HeyA8 intraperitoneal model and applied MFH. Results demonstrated that iron oxide nanoparticles when injected intraperitoneal in combination with MFH on intraperitoneal ovarian tumors showed an antitumor effect. We were also able to enhance MFH therapeutic outcome with the inhibition of HSP70 function using PES.

In summary, this work introduces the use of microarray gene expression to rationally design targeted combination therapies using directed nanoscale heat delivery with magnetic nanoparticles. Results demonstrated that MFH gene expression in ovarian cancer was dominated by HSPs and ubiquitin C. Hsp70 was found to be the most upregulated gene and its combination enhanced the effect of MFH in vitro and in vivo. In vitro combination treatments demonstrated for the first time the synergistic behavior between MFH and PES. The intraperitoneal delivery of iron oxide nanoparticles revealed that tumors were significantly affected when the combined treatment of MFH and HSP70 inhibition was applied. Therefore, results presented herein suggested that iron oxide magnetic nanoparticles under the influence of an alternating magnetic field in combination with heat response impairment drugs are a significant milestone for the translation of nano-based adjuvant therapies for ovarian cancer into the clinic.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.A. Court, H. Hatakeyama, C. Rodríguez-Aguayo, L. Ju-Seog, E.J. Juan, A.K. Sood
Writing, review, and/or revision of the manuscript: K.A. Court, H. Hatakeyama, S.Y. Wu, M.S. Lingegowda, G. López-Berestein, C. Rinaldi, E.J. Juan, A.K. Sood, M. Torres-Lugo

Acknowledgments
The authors are grateful to Dr. Camilo Mora and Dr. Marisel Sánchez for their support in confocal imaging and flow cytometry.

Grant Support
This work was supported by grants from the following: HHIS (NIH, CA 963000/CA 96297; to M. Torres-Lugo); P01CA03639 and CA016672, A.K. Sood), PR Institute for Functional Nanomaterials (EPS-100241, to Madeline-Torres-Lugo), Nanotechnology Center for Biomedical, Environmental and Sustainability Applications (HRD-1345156, to Madeline-Torres-Lugo) Ovarian Cancer Research Fund (OCRF, RP101502/RP, to S.Y. Wu), and Cancer Prevention and Research Institute of Texas training grants (RP101489, to S.Y. Wu, RP105959 and RP120214, to A.K. Sood). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 10, 2016; revised September 6, 2016; accepted February 4, 2017; published OnlineFirst February 21, 2017.


Molecular Cancer Therapeutics

HSP70 Inhibition Synergistically Enhances the Effects of Magnetic Fluid Hyperthermia in Ovarian Cancer

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