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Synergistic effects of cisplatin chemotherapy and gold nanorod-mediated hyperthermia on ovarian cancer cells and tumors

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Abstract

Aim—The synergistic effects of gold nanorod (GNR)-mediated mild hyperthermia (MHT; 42–43°C) and cisplatin (CP) activity was evaluated against chemoresistant SKOV3 cells *in vitro* and with a tumor xenograft model.

Materials & methods—*In vitro* studies were performed using CP at cytostatic concentrations (5 µM) and polyethylene glycol-stabilized GNRs, using near-infrared laser excitation for MHT.

Results—The amount of polyethylene glycol-GNRs used for environmental MHT was 1 µg/ml, several times lower than the loadings used in tumor tissue ablation. GNR-mediated MHT increased CP-mediated cytotoxicity by 80%, relative to the projected additive effect, and flow cytometry analysis suggested MHT also enhanced CP-induced apoptosis. In a pilot *in vivo* study,

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Supplementary material

Photographs of experimental setups and thermographic images; evaluation of polyethylene glycol-gold nanorod uptake by SKOV3 cells using TPL imaging; additional cell viability and flow cytometry data; changes in mean tumor volume over a 23-day period as a function of treatment; synergistic effects using a multidose regimen.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

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systemically administered polyethylene glycol-GNRs generated sufficient levels of MHT to enhance CP-induced reductions in tumor volume, despite their heterogeneous distribution in tumor tissue.

Conclusion—These studies imply that effective chemotherapies can be developed in combination with low loadings of nanoparticles for localized MHT.

Keywords

apoptosis; cisplatin; gold nanorods; hyperthermia; ovarian cancer; synergistic effects

The photothermal effects of plasmon-resonant gold nanorods (GNRs) on cells and tissues have been extensively studied [1–5]. GNRs can be engineered to be strongly absorbing at near-infrared (NIR) wavelengths, which penetrate more efficiently through biological tissues than visible or mid-infrared (IR) light. GNRs are also efficient at converting resonant absorption into heat, and have been the focus of numerous *in vitro* and *in vivo* studies based on localized hyperthermia [6–18]. Many of these studies involve moderate heating by tens of degrees, leading to irreversible damage of cells and tissues with subsequent necrosis. Significant reductions in tumor volume have been observed in rodent models inoculated with polyethylene glycol (PEG)-coated GNRs, then exposed to NIR laser irradiation [13–15]. The concentration of GNRs used for photothermal tissue ablation in these studies was 20 mg Au/kg tissue; while such loadings are currently being evaluated in clinical trials [19], recent *in vivo* studies have shown that rodents inoculated with PEG-coated nanoparticles (NPs) at several mg Au/kg can experience adverse foreign body responses, such as inflammation, reduced white blood cell count, and liver or kidney damage [20,21]. Au NPs can also stimulate the expression of gene products associated with systemic detoxification and lymphocyte production [22].

Hyperthermia at slightly elevated temperatures (42–43°C) has also been investigated as a form of adjuvant therapy. Anecdotes on the therapeutic effects of mild hyperthermia (MHT) date back as early as the second millennium BCE [23,24]; in the context of modern medicine, MHT has been shown to sensitize cells and tumors to drug action. Most studies on MHT-enhanced chemotherapies have been conducted with systemic heating [25–28], but recently the prospects of coupling chemotherapy with NP-mediated hyperthermia has been gaining attention [29–32]. Such studies raises several important practical issues, such as the minimum amount of NPs needed to generate MHT, the efficacy of NP-mediated MHT versus external heating sources, and reliable methods for distinguishing synergistic effects in MHT-enhanced chemotherapy from the effects of NP-mediated hyperthermia alone.

In this article, we assess the ability of GNR-mediated MHT to enhance the chemotherapeutic potential of cisplatin (CP) against human SKOV3 cells, which are intrinsically resistant to CP [33,34], using *in vitro* and *in vivo* models. CP is a DNA crosslinker that forms intrastrand lesions between adjacent guanine nucleotides, and interferes with vital nuclear processes, such as DNA replication and transcription [35]. However, CP-induced genotoxicity is reduced by various DNA repair pathways that remove structural aberrations from nuclear DNA. At higher concentrations, CP can also crosslink enzymes and other protein factors that could disrupt cell signaling pathways. Mechanisms

for CP resistance (in addition to elevated DNA repair) include changes in cellular uptake, drug efflux, increased production of detoxification enzymes and suppression of apoptosis.

The context for this study is based on several issues encountered during the development of NPs for photothermal therapy, described as follows:

- A high NP loading for tumor eradication by thermal ablation [13–15];
- The limited penetration and diffusion of NPs into tumor tissue, past the epithelial cells lining the tumor vasculature [36,37];
- An effective therapy for eradicating residual tumor cells following primary treatment.

The first two issues are challenges that are specific to nanomedicine – that is, the design of NPs for biomedical applications. In this regard, the use of GNRs and other energy-absorbing NPs for MHT has practical merit: the NP loadings needed to generate a mild thermal gradient are much lower than those used in thermal ablation, and heat diffusion into the surrounding tissue increases the effective range of the adjuvant effect. Furthermore, acute MHT presents little risk to healthy cells and tissues. The third issue is addressed by adjuvant chemotherapy, which is typical for any procedure involving surgical resection, ionizing radiation or other physical means of treatment. By establishing a positive synergy between MHT and chemotherapy, we aim to illustrate the potential value of NP-mediated hyperthermia in pre- and post-operative tumor treatment.

Methods & materials

Synthesis of PEG-stabilized Au nanorods

All reagents were obtained from Sigma-Aldrich (MO, USA) or Fluka (MO, USA) and used as received unless otherwise stated. Methyl(PEG) thiol (mPEG-SH, 5 kDa) was obtained from Nanocs (NY, USA). Deionized water was obtained using an ultrafiltration system (Milli-Q[®]; Millipore, MA, USA) with a measured resistivity above 18 M Ω cm. GNRs were prepared with high-purity cetyltrimethylammonium bromide (cetyltrimethylammonium bromide [CTAB], SigmaUltra; >99%) using seeded growth conditions [38,39]. An aqueous solution (200 ml) of HAuCl₄ (0.5 mM), AgNO₃ (96 μ M), and CTAB (100 mM) was treated with ascorbic acid (0.54 mM) and the solution changed color from bright yellow to colorless. The solution was then treated with a freshly prepared Au NP seed solution (3–5 nm; 0.24 ml) and began to turn red within 20 min. The solution was allowed to stand at room temperature for 20 h to yield a 200-ml suspension of GNRs with a longitudinal plasmon resonance (LPR) centered at 825 nm and an optical density (OD) of 0.63. The GNRs were subjected to centrifugation at 6500 g for 45 min and separated from the supernatant, then redispersed in water to a final volume of 10 ml (OD: 10.9, based on 10 $\times$ dilution). The concentrated GNR solution was centrifuged again at 6500 g for 30 min and resuspended in water to a final volume of 2.8 ml (OD: 39.4, based on 10 $\times$ dilution), then treated with a 7 weight (wt)% mPEG-SH solution (28 mg in 0.4 ml). Excess mPEG-SH was removed 24 h later using five rounds of stirred membrane dialysis (molecular weight cut-off: 6000–8000 Da, 500 ml/h), followed by overnight dialysis. The mPEG-stabilized GNRs (PEG-GNRs) were centrifuged again at 6500 g for 30 min, then redispersed in deionized

water to a final volume of 10 ml (OD: 12.5, based on 20× dilution) with a LPR centered at 815 nm.

Particle characterization

Optical absorption spectra were recorded using a Cary® Bio50 (Agilent Technologies, CA, USA) spectrophotometer and quartz cuvettes. Transmission electron microscopy (TEM) images were obtained using a FEI/Philips CM-10 (OR, USA) with an accelerating voltage of 100 kV. Samples were prepared by depositing 10 µl of GNR suspension onto Formvar-coated copper grids (400 mesh) and allowing the droplet to sit for 25 min, followed by blotting the grid edge and drying the residual wetting layer in air. Zeta potential measurements were obtained using a Malvern Zetasizer Nano (Malvern, MA, USA), with GNR samples diluted in 10 mM phosphate buffered saline (PBS; pH 7.3) in a disposable microelectrode cuvette (DTS10603).

NP tracking analysis (NTA) was performed at room temperature using a Nanosight LM-10 system (Malvern). The NTA imaging flow chamber was cleaned with acetone and a microfiber cloth, then washed with commercial deionized water until no background particles were observed. The water was then removed from the imaging chamber with a sterile plastic syringe just prior to use. PEG-GNRs were diluted with deionized water to OD of 0.06 prior to loading in the NTA chamber; 50 µl of PEG-GNR solution was injected into the chamber in between each run to increase particle sampling. Seven videos were recorded and analyzed per sample at intermediate shutter speeds ($N_{\text{track}} > 500$ per run); the mean mode peak value was used as the hydrodynamic diameter.

Cell culture conditions

Cultures were maintained in a 5% CO₂ environment at 37°C. SKOV3 cells were obtained from American Type Culture Collection and cultivated in T-75 flasks (Becton-Dickinson Falcon, NJ, USA), using a standard culture medium (Roswell Park Memorial Institute: 1640, Gibco/Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (Fetal Bovine Serum Premium; Atlanta Biologicals, GA, USA), 1% glutamine and 1% penicillin–streptomycin (Invitrogen/Life Technologies, NY, USA). Cells between passages 14–24 were plated and grown to 90% confluence.

Multiphoton confocal microscopy

Two-photon excited luminescence (TPL) microscopy was performed using an inverted confocal laser scanning microscope (Nikon TiE A1R-MP; Nikon, NY, USA) equipped with a 60×/1.42 NA oil-immersion objective (PLAPON 60XO; Olympus, PA, USA), and a 810-nm pulsed laser (Mai Tai® DeepSee™, CA, USA) for TPL excitation. SKOV3 cells were incubated for 48 h at 37°C in glass-bottomed culture dishes (MayTek, 14 mm microwell, No.1 coverglass), then washed once with sterile PBS to remove nonadherent cells prior to treatment with PEG-GNRs dispersed in culture media (0.75 µg/ml). Cells were rinsed 24 h later with PBS, just prior to TPL analysis.

Photothermal heating & analysis

Laser-induced heating was performed using a NIR diode laser (808 nm, 0.76 W/cm²) with a collimating lens and a long-pass filter to remove adventitious higher-order emissions. For NIR irradiation in 96-well plates (0.2 ml/well), the laser beam was spread to a spot size of approximately 8 mm. For NIR irradiation in 24-well plates (0.5 ml/well), the laser beam was spread to a spot size of 2 cm. The temperatures of laser-treated wells were controlled by using neutral density (ND) filters (ND: 0.1–0.3) to limit the laser power. Surface temperatures were monitored by a thermographic IR imaging camera (FLIR SC305; FLIR, OR, USA) with a reported sensitivity of 50 mK at 30°C and an accuracy of 2%. The temperatures of control wells (on the same multiwell plate as laser-treated wells) were maintained between 35–38°C during laser irradiation using a metal heating block; the multiwell plates were immediately returned to the 37°C incubator after laser irradiation. All equipment and the surrounding area were sprayed with 70% ethanol before and after NIR irradiation to prevent bacterial infection.

Cell viability assay

Cell survival was quantified by the mitochondrial oxidation of methyl thiazolyl tetrazolium bromide (MTT assay) [40,41]. All concentration values during incubation are based on final volumes. In a typical experiment, SKOV3 cells were harvested after passage and plated at a density of 5,000 cells/100 µl in 96-well microtiter plates, then incubated at 37°C under a 5% CO₂ atmosphere for 24 h. Cells were treated with 100 µl CP and/or PEG-GNRs; the latter were also exposed to NIR irradiation and heated to 42–43°C for 30 min. Serial dilutions of CP were prepared from a stock solution in 0.9% NaCl with an initial concentration of 1 mg CP/ml; dilutions of mPEG-GNRs were prepared from a stock solution in deionized water with an initial OD of 12.5. Wells were incubated at 37°C for 36 h, exchanged with new media (190 µl) and freshly prepared 0.5% MTT (10 µl) and incubated at 37°C for another 4 h, then exchanged again and replaced with dimethyl sulfoxide (200 µl) and kept in the dark for 16 h. The production of purple formazan was quantified with an automated plate reader at 570 nm. Cell viability was normalized relative to control cells treated with media alone, prior to the MTT assay. All experiments were performed in triplicate.

Flow cytometry

Cells were assayed for apoptosis and necrosis using Annexin-V fluorescein isothiocyanate and 7-aminoactinomycin D (7-AAD) staining (Immunotech Beckman Coulter, IN, USA). Annexin-V binds to exposed phosphatidyl-serine headgroups on the outer membranes of cells undergoing apoptosis; 7-AAD is a DNA intercalator that can pass through the membranes of cells undergoing secondary apoptosis or necrosis. Flow cytometry and data analysis were performed using a Becton-Dickinson FAC-SCalibur and CellQuest Pro (Becton-Dickinson Biosciences). Typically, 25,000 SKOV3 cells were plated in 24-well plates and treated with CP and/or MHT the following day as described above, then incubated for 3 days at 37°C. Media containing floating cells was collected; wells were then treated with a trypsin solution (0.5 ml) for 5 min, and agitated with fresh Roswell Park Memorial Institute media (0.75 ml) to ensure complete cell detachment. All solutions were combined and centrifuged into pellets, which were redispersed in a binding buffer (100 µl)

and treated with solutions of 7-AAD (20 μ l) and Annexin-V fluorescein isothiocyanate (10 μ l), then kept on ice for 15 min before dilution with binding buffer (400 μ l). During flow cytometry analysis, the fluorescence gate was set so that 90% of the population in the control group (Ctrl[–]) occupied the lower left quadrant (Apop[–]/Necr[–]), based on a count of 10,000 cells. All experiments were performed in triplicate.

***In vivo* experiments**

All animal studies were performed in the Molecular Discovery and Evaluation Shared Resource, in the Purdue University Center for Cancer Research.

Female nude Balb/C mice were obtained from Harlan Laboratories (IN, USA) and housed for 10 days in a light-controlled environment. Subcutaneous tumors were prepared by implanting 10⁶ SKOV3 cells on the right flank, with tumors achieving an average volume of 212 mm³ after 21 days. PEG-GNRs were administered by tail vein injection (175 μ l at OD of 57.7; 5.7 mg Au/kg mouse) and allowed to circulate for 24 h. Three mice were euthanized for biodistribution studies; organs were harvested and stored at –80°C. Mice in the experimental groups (monotherapies and combined treatment) were anesthetized with isoflurane, then received an intratumoral dose of 5 μ M CP (0.1 ml) 3 min prior to a 10-min dose of NIR irradiation using a diode laser (808 nm, 0.72 W/cm²). Mice were monitored by thermographic imaging to ensure that the surface temperatures remained within MHT range (41–43°C). Tumor volumes and animal weights were monitored every 2–3 days for 23 days after treatment.

GNR biodistribution studies were performed using induced-coupled plasma mass spectrometry (ICP-MS). Excised organs were weighed prior to microwave heating in Teflon vials (<300 mg/vial) using a 1:1 mixture of 35% HCl and 70% HNO₃ (Ultrapure Aristar, IL, USA). Tissue digestion was performed in a CEM Mars 5 microwave (Matthews, NC, USA) operating at 600 W and an oven temperature of 130°C (10 min ramp time, 20 min hold time). All digestion samples were allowed to sit at room temperature overnight, then diluted 20- to 40-fold in 2% HNO₃ and 1% HCl prior to ICP-MS analysis. Liver samples were divided into two equal portions during digestion, then combined for analysis. ICP-MS measurements for Au were calibrated against internal standards at 5.0, 0.45, and 0.04 ppb. Injected dose (% injected dose) values are relative to an initial dose of mPEG-GNR (120 μ g/specimen).

***Ex vivo* studies on GNR photothermal response in tumor tissue**

Harvested tumor samples stored at –80°C and thawed on ice for 30 min, prior to being brought to ambient temperature. Tumors were irradiated on two sides (defined as top and bottom) with a NIR diode laser, and monitored with a thermographic imaging camera. Several tumors were fixed for 24 h in a 0.1 M KNa₂PO₄ buffer (pH 7.3) containing 2.5 wt% glutaraldehyde (GA) and 2.5 wt% formaldehyde (FA). The fixed tissue samples were washed with PBS, blotted dry, then mounted on a wooden block. Tissue sections (100–300 μ m thick) were prepared with a vibratome slicer and stored at 4°C for 24 h in a buffered GA/FA solution, 1 wt% each), prior to measuring their photothermal response to the NIR heating laser.

Photoacoustic imaging

Tissue samples were characterized *ex vivo* using a reflection-mode photoacoustic (PA) system based on a novel design [42]. Briefly, laser pulses were generated from a wavelength-tunable laser (Surelite™ OPO PLUS; Continuum, CA, USA) at a wavelength of 815 nm with a repetition rate of 10 Hz and a pulse duration of 5 ns, pumped by a Q-switched Neodymium:YAG laser (SLII-10; Continuum; 532 nm). PA waves generated and detected using a spherically focused, single-element 10 MHz ultrasonic transducer (V322; Panametrics-NDT; General Electric, NY, USA). Conical lenses were used to create a toroidal beam that diverged around the transducer, which was submerged in a water tank sealed in a transparent polyethylene membrane for enhanced PA coupling, then refocused on the excised tumor tissues underneath. Imaging was performed by mechanical raster scanning of the PA transducer, controlled by LabView software (National Instruments, TX, USA); signals were amplified then transferred to a data acquisition system. The PA images and data were processed using Matlab™ (MathWorks, MA, USA).

Results

Preparation & characterization of PEG-stabilized Au nanorods

GNRs were prepared by the seeded growth method as previously described [38]. Absorption spectroscopy indicated the LPR peak to be centered initially at 825 nm (OD: 0.63). Excess CTAB was removed by two rounds of centrifugation and redispersion (C/R) in deionized water, to produce a highly concentrated GNR dispersion (OD: 39.4). We note that additional C/R cycles caused partial aggregation of CTAB-stabilized GNRs, as indicated by a broadening of the LPR baseline. The CTAB-stabilized GNRs were incubated overnight in a 0.9 wt% solution of 5-kDa mPEG-SH, followed by exhaustive membrane dialysis in deionized water to remove excess mPEG-SH and trace CTAB. These were subjected to an additional C/R cycle to yield a stable dispersion of PEG-GNRs (OD: 12.5).

Particle size analysis was performed using TEM and NTA; the mean GNR dimensions using the former was determined to be 46.2 ± 3.8 nm in length and 12.1 ± 1.2 nm in width, for a mean aspect ratio of 3.8 (Figure 1A). NTA analysis of PEG-GNRs (OD 0.06) indicated a mode peak corresponding to a hydrodynamic diameter (d_h) of 45 nm (Figure 1B), in accord with the TEM analysis [43]. The surface charge density of PEG-GNRs was close to neutral ($\zeta = -4.93$ mV), in accord with previous reports [16,18,20]. The LPR absorption peak of the final PEG-GNR dispersion was centered at 815 nm (Figure 1C). Using a molar extinction coefficient of $5 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$ determined for similarly sized GNRs [44], we estimate our PEG-GNR dispersions to contain 1.2×10^{11} particles/ml (12 $\mu\text{g Au/ml}$) at OD of 1.

Photothermal heating experiments were performed in 24- or 96-well plates, with serial heating by a NIR diode laser beam spread across the area of a single well (Supplementary Figure 1; see at www.futuremedicine.com/doi/suppl/10.2217/NNM.13.209). The addition of PEG-GNRs and/or CP to cells was performed 30 min prior to NIR irradiation; a baseline temperature near 37°C was maintained over the course of the experiment by placing wells on a heating block slightly above that temperature. The temperature of each well was monitored by a thermographic imaging camera during NIR laser irradiation (Supplementary

Figure 2). GNR-mediated MHT (42–43°C) was achieved in under 60 s using a laser power density of 0.72 W/cm², then maintained by attenuating the laser power with ND filters (ND: 0.1–0.3).

Well temperatures increased commensurately with GNR loadings at fixed laser powers (Figure 2A). Starting from room temperature, we observed that a PEG-GNR concentration of 1 µg/ml was sufficient for heating wells to the MHT range (Figure 2B), whereas 7 µg/ml raised the environmental temperature to that used for *in vivo* tumor ablation [13,14]. We also determined that GNR-induced heating provided much better thermal control than using a metal heating block: The ramp time to steady-state MHT using GNRs was under 60 s, whereas external heating required 10–20 min for stabilization (Figure 2C), and was easily perturbed by other ambient factors (e.g., convective air flow). Furthermore, temperature variations either within or between wells were much smaller with GNR-mediated heating (n = 9).

***In vitro* studies on the photothermal sensitization of SKOV3 cells to cisplatin**

The synergistic effect of GNR-mediated MHT on CP cytotoxicity was assessed *in vitro* using SKOV3 cells and a viability assay based on mitochondrial oxidation (MTT assay). In a typical experiment, SKOV3 cells were plated at an initial concentration of 5000 cells/well and incubated for 24 h at 37°C, treated with CP and/or GNR-mediated MHT and incubated for 3 days, then evaluated by the MTT assay. Cells were plated at low density to encourage maximum growth over the course of the experiment, so that cells in the untreated group (Ctrl[–]) were not yet fully confluent by the fourth day. The IC₅₀ of acute CP treatment under these conditions was found to be 7.5 µM (Figure 3A), whereas the concentration for cytostasis was closer to 5 µM, as determined over the course of an 8-day study from an initial plating of 20,000 cells per well (Figure 3B). We note that these values are approximate, as the cytotoxic response can vary significantly between uncorrelated groups.

SKOV3 cells treated with PEG-GNRs (1 µg/ml) were subjected to acute GNR-mediated MHT by 30-min irradiation with the NIR heating laser, then incubated for 3 days at 37°C. GNR-mediated MHT had only a minor effect on cell viability, which remained above 80% relative to untreated wells (Figure 4). Two control experiments were performed to show that the photothermal effects were environmental in nature. First, SKOV3 cells without GNRs were placed on a metal block and heated externally for 30 min at 42–43°C, preceded by a 10–20 min induction period to reach steady-state MHT. Despite the additional preheating, cell viability 3 days after exposure was essentially the same as that after exposure to GNR-mediated heating. Second, cells exposed to PEG-GNRs at 37°C for 24 h were imaged by TPL microscopy, which revealed minimal PEG-GNR uptake in accord with earlier observations (Supplementary Figure 3) [45].

We then established that GNR-mediated MHT strongly enhanced CP cytotoxicity. Exposing SKOV3 cells to 5 µM CP and 30 min of GNR-mediated MHT with 1 µg/ml PEG-GNR increased cell death by over 100% after a 3-day incubation, relative to CP alone (over a twofold increase in CP potency). The synergy between CP and GNR-mediated MHT could be quantified by comparison with the so-called additive effect, defined as the product of normalized cell survival after separate monotherapies [25,29]. From the combined treatment

above, we obtained an 80% increase in cell death relative to the projected additive value ($p = 0.05$) (Figure 4). A similar but slightly lower synergy was also observed between 5 μM CP and MHT using an external heat source; however, shortening MHT exposure to 20 min reduced the synergistic effect to 20% (Supplementary Figure 4).

Both CP and MHT have a reputation for promoting apoptosis in cancer cell lines [34,46,47]. To determine whether the combined CP/MHT treatment of SKOV3 cells also produced a synergistic increase in apoptosis, we performed flow cytometry assays using Annexin-V fluorescein isothiocyanate and 7-AAD to measure the relative populations of healthy, apoptotic and necrotic cells exposed to 5 μM CP plus MHT from either PEG-GNRs (Figure 5) or an external heat source (Supplementary Figure 5). Data were normalized using cell populations from unheated controls (Ctrl[−]), then evaluated for differences in apoptosis (Apop⁺) and cell viability (Necr[−]). The synergistic effects in apoptotic response were mild: whereas MHT alone produced increases of 1.4–1.6-fold and exposure to 5 μM CP of over fivefold, the combined treatment using PEG-GNRs or an external heat source enhanced apoptosis by 7.3- or 10.4-times, respectively (Figure 6A). In the latter case, the synergy between MHT and CP was nearly 30% relative to the projected additive value ($p = 0.1$). Similarly modest results were obtained for cell viability: The synergy of combined treatments was 13 or 20% versus projected additive values (Figure 6B), less than that obtained using the MTT assay. Differences in these values can be attributed in part to their sensitivity to flow cytometry readout parameters, such as gate voltage settings.

***In vivo* study on the photothermal sensitization of SKOV3 cells to cisplatin**

Encouraged by the above results, we designed a pilot *in vivo* study to determine if the GNR-mediated MHT would translate to an increased reduction in tumor progression, in synergy with CP treatment. In order to keep experimental parameters closely aligned with those of the *in vitro* study, we elected to introduce PEG-GNRs by systemic administration (tail-vein injection) for uptake into tumor tissue via the enhanced permeation and retention effect [13–18], while perfusing tumors with a CP solution at a defined concentration a few minutes prior to NIR laser irradiation. All treatments were performed at a single time point, followed by tumor monitoring over a period of 23 days. We note that such a modest regimen should not be expected to generate clinically remarkable results; rather, our aim was to obtain evidence that synergistic MHT effects are operative *in vivo*, and should thus be implemented in the design of preclinical studies involving GNR-mediated photothermal therapy.

Groups of nude Balb/C mice bearing subcutaneous SKOV3 tumor xenografts (see '*In vivo* experiments') were divided into five groups: no treatment ($n = 5$); GNR-mediated MHT ($n = 3$); intratumoral injection of CP (single 50-nmol dose; $n = 4$); MHT plus 15 nmol CP ($n = 4$); and MHT plus 50 nmol CP ($n = 4$). Groups 2–5 were inoculated with PEG-GNRs via tail vein injection (120 μg Au/mouse), 24 h prior to CP and/or MHT treatment. Biodistribution analysis by ICP-MS ($n = 3$) indicated that the accumulation of GNRs in tumor tissue after 24 h was $6.8 \pm 1.3\%$ injected dose/g tissue, whereas that in blood was $3.7 \pm 1.7\%$ (Figure 7A). Not surprisingly, most of the GNRs were found in the liver ($45.7 \pm 10.5\%$) and spleen (not applicable due to high error), similar to that observed in previous animal studies [13–15].

For groups 3–5, each specimen was anesthetized 24 h after GNR administration and injected with a solution of CP (0.15–0.50 nmol/tumor); groups 4 and 5 were then irradiated with a NIR heating laser for 10 min at a constant power of 0.72 W/cm², while monitored by IR thermography. The laser-irradiated tumors exhibited a steady-state surface temperature of 43°C within 3 min, whereas irradiation of tumors without GNRs (group 1) did not exceed 37°C (Figure 7B–D).

Tumors monitored over 23 days yielded strong evidence that GNR-mediated MHT enhanced the *in vivo* efficacy of CP treatment (Figure 8; for raw data see Supplementary Figure 6). The mean tumor volumes increased 5.2-fold in the absence of CP and PEG-GNRs (group 1; n = 5), whereas those treated with an acute dose of CP (0.50 nmol) grew 3.2-fold (group 2; n = 3). However, a 0.15 or 0.50 nmol dose of CP significantly retarded tumor growth when combined with 10 min of GNR-mediated MHT, with a mean volume increase of 2.3-fold after 23 days (group 5; n = 4). Despite the large variations in tumor volumes in this study, the combined CP–MHT treatment is clearly significant when compared with untreated tumors ($p < 0.01$; $df = 8$). The ability of MHT to enhance the potency of CP treatment is less dramatic in this study, but still strongly suggestive of a synergistic *in vivo* effect (Table 1).

Previous *in vivo* studies on the combined effects of CP and intraperitoneal MHT ($T = 4.5^{\circ}\text{C}$) in rat models have indicated related synergistic effects. CP combined with MHT resulted in a fourfold increase in CP uptake into peritoneal CC531 tumors [48] and a 3.2-fold increase in CP–DNA adducts in extracted tumor cells, as well as delays in tumor growth by as much as 6 weeks [49]. Similar *in vivo* results have been reported using combinations of carboplatin and MHT [50].

The heterogeneous nature of the tumor vasculature can give rise to an uneven GNR distribution, which raises some questions about the uniformity of tumor heating under MHT conditions. We partly addressed this issue by performing *ex vivo* thermographic and PA imaging analysis on tumors, harvested 24 h after systemic PEG-GNR administration (Figure 9). Thermographic imaging was recorded during NIR laser irradiation at a frame rate of 60 Hz, which yielded semiquantitative data on heat diffusion rates from regional ‘hot spots’. PA imaging was performed on sliced tumor tissue and compared with thermographic data to establish a relationship between GNR density (NIR absorption) and localized photothermal heating.

Thermographic imaging of excised whole tumors during NIR laser irradiation confirmed that the GNR distribution was heterogeneous (Figure 9A). The same analysis was also performed on sectioned slices from the same tumor, whose ‘hot spots’ correlated with those observed in whole tissue (Figure 9B). PA imaging of these tumor sections provided density maps of NIR absorption, which complemented those produced by IR thermography. Despite the unevenness of the initial heating response, time-lapsed thermographic recordings indicated rapid thermal diffusion in tumor tissue during NIR irradiation: Global temperature increases to MHT levels were achieved within seconds, at the same laser power used in the *in vivo* study (Figure 9C). From these observations, we conclude that the heterogeneous distribution of GNRs in tumors is unlikely to be a significant factor in MHT-enhanced therapies.

Discussion

The antiproliferative effect of cisplatin is generally attributed to its genotoxicity, with cell cycle arrest in the S-phase [51]. CP is also known to cause intrastrand crosslinking in mitochondrial DNA, and can react with cytoplasmic RNA and proteins at high doses [52,53]. These disruptions often lead to cell apoptosis; however, DNA crosslinking by CP can be reversed by the DNA damage response – that is, the recruitment of proteins for the removal and repair of DNA lesions or strand breaks [54,55].

There is growing evidence that mild hyperthermia can enhance CP-induced genotoxicity [56,57]. MHT can increase CP membrane permeability and subsequent penetration into the nucleosome [58], and accelerate the rate of CP hydrolysis to its active diaquo form [59]. The nuclear matrix is especially thermolabile: histone-binding proteins that govern DNA coiling are known to denature at temperatures as low as 40°C [47,60]. With respect to the DNA damage response, MHT has been shown to induce the thermal degradation of BRCA2, which supports homologous recombination in the repair of double-strand DNA breaks [61]. This suggests that BRCA-deficient cancers may be particularly susceptible to combined CP–MHT therapy.

In our *in vitro* studies, GNR-mediated MHT significantly enhances CP-induced cytotoxicity in SKOV3 cells. This is most evident in the loss of mitochondrial activity 3 days after treatment, with an effective doubling in potency relative to CP alone and a synergy factor as high as 80% relative to the additive effect (Figures 4 & 5). Our results are similar to those in the study by Hauck *et al.*, which featured intracellular GNRs for laser-induced MHT in combination with CP treatment against suspensions of leukemia cells [29]. However, GNR-mediated MHT is better suited for treating primary tumors at fixed locations, rather than disseminated cancer cells in the bloodstream. Our study also shows that a low concentration of extracellular GNRs is sufficient to support MHT-enhanced cytotoxicity.

The generation of localized MHT by GNRs offers at least two advantages over systemic heating by indirect sources, including hyperthermic intraperitoneal chemotherapy [62,63] and antenna-guided microwave heating [64]. First, GNR-mediated heating can reach steady-state temperatures more quickly: Photothermal MHT in microtiter wells was achieved within 1 min, whereas a heat block required up to 20 min to achieve steady-state MHT (Figure 2). Second, GNR-mediated heating offers greater precision and uniformity: The temperature range of wells heated by GNRs was less than 0.5°C, whereas that of externally heated wells was approximately 2°C.

With regard to hyperthermic effects *in vivo*, MHT is inevitably generated during photothermal ablation, and can thus be useful in secondary therapies against residual tumor cells. There are numerous examples showing that PEG-GNRs extravasate into vascularized tumor tissue via the enhanced permeation and retention effect [13–18]. However, the high GNR loadings used for photo-thermal ablation increase the collateral necrosis of healthy tissue. MHT does not produce significant tissue necrosis by itself, and photothermal imaging shows that it can be generated up to several centimeters away from bioaccumulated GNRs (Figure 7) [14,65]. MHT also has the capacity to induce other physiological effects, such as

greater tissue permeability and blood vessel dilation with increased blood flow in the tumor microenvironment [47,66,67]. By contrast, temperatures above 43°C can cause a breakdown in vascular integrity that restricts blood flow, which reduces drug permeation and promotes hypoxia [68].

Last, we note that extravasated GNRs are very slowly eliminated from the body (on the order of weeks to months), and can continue to provide MHT near the tumor site long after primary treatment. To determine whether the cytotoxicity of a single CP dose could be further enhanced by multiple MHT exposures, we conducted a preliminary *in vitro* study using external heating to provide a 30-min daily regimen of MHT (Supplementary Figure 8). This was found to be the case: the synergistic effect of MHT increased to nearly 100% over 4 days, 250% over 6 days, and over 300% over an 8-day regimen. It is, therefore, reasonable to suggest that the efficacy of postoperative chemotherapy can be greatly improved by a daily regimen of MHT, based on a single dose of GNRs.

Conclusion

Significant synergistic effects are observed in the treatment of cisplatin-resistant SKOV3 cells and tumors with GNR-mediated MHT and cisplatin. Steady-state MHT can be achieved at PEG-GNR loadings of 1 µg/ml and a NIR laser diode operating at 0.72 W/cm². GNR-mediated heating is rapid, and offers greater control over temperature versus external heating sources. The MHT produced by PEG-GNRs is essentially environmental in nature, and has minimal impact alone on cell viability. However, the effective *in vitro* cytotoxicity of 5 µM CP more than doubles after a 30-min MHT session, based on viability assays 3 days post-treatment. The synergy in CP–MHT cytotoxicity can be quantified by comparison with the projected additive effect, which yields values as high as 80% depending on assay type and experimental variables such as incubation time and MHT exposure. A mildly synergistic effect in apoptotic response is also observed 3 days post-treatment, based on flow cytometry analysis. The *in vitro* results are matched by a pilot *in vivo* study, which reveals significant retardation in tumor growth relative to CP monotherapy. These studies suggest that therapeutic regimens may be developed using low dosages of CP and GNRs, with particular relevance toward postoperative cancer treatments.

Future perspective

The expectations of nanomedicine and its potential benefits are gradually being tempered, from highly optimistic notions of ‘magic bullet’ nanocarriers that precisely deliver therapeutic payloads to diseased cells, to more practical objectives of reducing dosage levels used in adjuvant therapies. Regardless of the goal, improved outcomes using NP-enhanced therapies can still have a significant impact on patient care and quality of life. With respect to NPs that convert NIR light [13–15] or alternating current magnetic fields [69,70] into localized hyperthermia, the extravasation of such NPs into vascularized tumors for tissue ablation remains promising, but the current loading requirements are high and raises concerns over foreign body reactions and other immunotoxic responses. The concurrent development of combination or adjuvant chemotherapies enhanced by daily MHT offers an avenue for increasing the efficacy of NP-mediated hyperthermia at reduced loadings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Executive summary

Background

- The clinical application of localized mild hyperthermia (MHT) is limited by currently available technologies for tissue heating. The delivery of near-infrared-active gold nanorods (GNRs) to vascularized tumor tissues offers a promising approach for developing adjuvant therapies based on MHT.
- Resistance to chemotherapy is a major concern in postoperative cancer care. MHT has the potential to increase the efficacy of chemotherapy against residual cancer cells, including chemoresistant strains.

Results

- Preparation and characterization of polyethylene glycol-stabilized Au nanorods:
 - The loading requirement for GNR-mediated MHT is several times lower than that used for tissue ablation.
- *In vitro* studies on the photothermal sensitization of SKOV3 cells to cisplatin:
 - A significant synergistic effect is observed *in vitro* when treating SKOV3 cells with 5 μ M cisplatin (CP) and 30 min of MHT, 3 days post-treatment. The efficacy of combined treatment is 80% greater than the projected additive effect of individual treatments.
 - Both CP and MHT increase apoptosis, although the synergy of the combined treatment is mild.
- *In vivo* study on the photothermal sensitization of SKOV3 cells to cisplatin:
 - The combined CP–MHT treatment significantly retards tumor growth in an SKOV3 tumor xenograft mouse model after 23 days, relative to CP or MHT monotherapies.

Discussion

- GNR-mediated MHT is environmental in nature, based on its similarity to external MHT and the minimal cell uptake of polyethylene glycol-GNRs.
- *In vitro* GNR-mediated MHT is more efficient and precise than external heating.
- It is desirable to use lower GNR loadings to reduce nonspecific effects related to the foreign body response and immunotoxicity.
- The synergy between GNR-mediated MHT and CP treatment may be even higher with multiple treatments. A multiday regimen of MHT from a single dose of GNRs may be a promising way to increase synergy with chemotherapy.

Figure 1. Characterization of polyethylene glycol-gold nanorods

(A) Transmission electron microscopy of polyethylene glycol-gold nanorods (46×12 nm);

(B) size analysis by nanoparticle tracking analysis (d_h : 45 nm); (C) optical absorption spectrum ($\lambda_{\max}$: 815 nm).

Figure 2. Photothermal heating with polyethylene glycol-gold nanorods

(A) Increases in solution temperature as a function of mPEG-gold nanorod concentration.

(B) Steady-state mild hyperthermia in polyethylene glycol-gold nanorod dispersion at 1 µg/ml using near-infrared laser irradiation at maximum power (0.72 W/cm^2) for 3 min, then maintained by attenuating the beam with a neutral density filter (neutral density: 0.2). (C)

Heating profile of plate placed on a prewarmed heating block.

Figure 3. *In vitro* cytotoxic response of SKOV3 cells to cisplatin

(A) IC₅₀ bar graph of cisplatin for SKOV3 cells after a 3-day exposure to cisplatin (initial plating of 5000 cells/well); (B) viability of SKOV3 cells as a function of cisplatin concentration over an 8-day time course (initial plating of 20,000 cells/well; n = 3).

Figure 4. Viability of SKOV3 cells 3 days after treatment with GNR-mediated MHT (42–43°C, 30 min), CP (5 μ M) or both

The combined cytotoxic effect of CP and GNR-mediated MHT was significantly greater relative to the projected additive effect, indicative of synergy (** $p = 0.05$). MHT produced by external heating is shown for comparison. CP: Cisplatin; GNR: Gold nanorod; MHT: Mild hyperthermia.

Figure 5. Flow cytometric analysis of SKOV3 cell populations, 3 days after treatment with CP and/or gold nanorod-mediated MHT
(A) Cells exposed to gold nanorods without MHT (Ctrl[-]); **(B)** cells treated with 5 μ M CP; **(C)** cells exposed to mild hyperthermia (43°C) for 30 min; and **(D)** cells with combined CP–gold nanorod-MHT treatment. Cells testing positive for Annexin-V (right quadrants) and/or 7-AAD (upper quadrants) are assigned as apoptotic (Apop⁺) and/or nonviable (Necr⁺), respectively, with percentage cell populations listed in each quadrant (n = 3). 7-AAD: 7-aminoactinomycin; CP: Cisplatin; Ctrl: Control group; FITC: Fluorescein isothiocyanate; MHT: Mild hyperthermia.

Figure 6. Histograms based on flow cytometry data for (A) apoptotic response (Apop⁺) and (B) viability (Necr⁻)

Bars represent percentages based on 10^4 cells, for studies involving GNR-mediated MHT (dark: $n = 3$) or external MHT (light: $n = 2$). Projected additive effects presented at right (* $p = 0.1$). Ctrl: Control group; GNR: Gold nanorod; MHT: Mild hyperthermia.

Figure 7. *In vivo* distribution and localized mild hyperthermia of systemically administered polyethylene glycol-gold nanorods

(A) Select GNR biodistribution data, 24 h after tail vein injection (n = 3). **(B & C)**

Thermographic images of mice during NIR irradiation of tumor xenografts, inoculated with or without GNRs. **(D)** Tumor surface temperature of representative specimen as a function of NIR irradiation time; control animal injected with 0.1 ml saline instead of polyethylene glycol-GNRs. GNR: Gold nanorod; ID: Injected dose; NIR: Near-infrared.

Figure 8. Relative increases in tumor volume, 23 days after a single treatment of CP (0.15 or 0.50 nmol) and/or GNR-mediated MHT

The combined CP–MHT treatment (groups 4 and 5) produces a significant reduction in tumor growth compared with group 1 (Ctrl[–]), even when using a reduced CP dose (** $p < 0.01$). Comparison of group 5 with group 2 (* $p < 0.25$) suggests that MHT directly enhances *in vivo* CP potency. Ctrl: Control group; CP: Cisplatin; GNR: Gold nanorod; MHT: Mild hyperthermia.

Figure 9. *Ex vivo* thermographic imaging of fixed tumor tissue, resected 24 h after systemic administration of gold nanorods (see facing page)

(A) Still images ($30 \times 30 \text{ mm}^2$) of a whole tumor acquired 10 s after exposure to NIR irradiation (800 nm, 0.71 W/cm^2). (B) Left: thermographic images of sectioned tumor tissue (200–300 μm thickness) before and during NIR irradiation (800 nm); right: corresponding PA images of tumor slices using a 815-nm probe laser. (C) Still images from a thermographic recording of temperature changes in a tumor section upon NIR irradiation. NIR: Near infrared; PA: Photoacoustic.

Table 1

Tests of significance for reduction in tumor growth 23 days post-treatment.

Control	Experiment	t-test [†]	p-value [‡]
Group 1 (Ctrl[-])	Group 4 (0.15 nmol CP + MHT)	2.161	0.005
	Group 5 (0.50 nmol CP + MHT)	3.772	0.013
Group 2	Group 4 (0.15 nmol CP + MHT)	1.761	0.116
(0.50 nmol CP)	Group 5 (0.50 nmol CP + MHT)	1.214	0.259

[†]Degrees of freedom: 8.

[‡]Two-tailed.

Ctrl: Control group; MHT: Mild hyperthermia.