Theranostic Gold Nanoparticles Modified for Durable Systemic Circulation Effectively and Safely Enhance the Radiation Therapy of Human Sarcoma Cells and Tumors\textsuperscript{1,2}

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Abstract

Radiation therapy (RT) is an integral component of the treatment of many sarcomas and relies on accurate targeting of tumor tissue. Despite conventional treatment planning and RT, local failure rates of 10\% to 28\% at 5 years have been reported for locally advanced, unresectable sarcomas, due in part to limitations in the cumulative RT dose that may be safely delivered. We describe studies of the potential usefulness of gold nanoparticles modified for durable systemic circulation (through polyethylene glycosylation; hereinafter “P-GNPs”) as adjuvants for RT of sarcomas. In studies of two human sarcoma-derived cell lines, P-GNP in conjunction with RT caused increased unrepaired DNA damage, reflected by approximately 1.61-fold increase in γ-H2AX (histone phosphorylated on Ser\textsuperscript{139}) foci density compared with RT alone. The combined RT and P-GNP also led to significantly reduced clonogenic survival of tumor cells, compared to RT alone, with dose-enhancement ratios of 1.08 to 1.16. In mice engrafted with human sarcoma tumor cells, the P-GNP selectively accumulated in the tumor and enabled durable imaging, potentially aiding radiosensitization as well as treatment planning. Mice pretreated with P-GNP before targeted RT of their tumors exhibited significantly improved tumor regression and overall survival, with long-term survival in one third of mice in this treatment group compared to none with RT only. Interestingly, prior RT of sarcoma tumors increased subsequent extravasation and in-tumor deposition of P-GNP. These results together suggest P-GNP may be integrated into the RT of sarcomas, potentially improving target imaging and radiosensitization of tumor while minimizing dose to normal tissues.

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Introduction

About 15,000 new diagnoses and 5000 deaths related to sarcoma are expected in the United States each year [1]. Multimodal treatment consisting of surgery and radiation therapy (RT) is the mainstay of clinical management of most sarcomas. RT has been shown to improve local control rates and overall survival in patients with advanced soft tissue sarcoma tumors of the extremity [2–4]. Furthermore, recent analysis demonstrated that the addition of RT to surgical resection for early-stage retroperitoneal soft tissue sarcomas significantly prolongs survival [5]. However, such benefits have not been observed in more advanced disease, potentially because of the inability to deliver effective doses of RT. Current RT treatment strategies favor relatively large margins on the radiation treatment field to address the invasive tendency of many sarcomas, but the risk of excessive radiation-induced damage to organs and surrounding tissue limits the dose of RT that can be given to achieve local control or eradication of residual tumor [6]. The use of adjuvant chemotherapy remains investigational because of the risk of side effects and lack of evidence for improving overall survival in operable disease and is thus often reserved for palliation of metastasis [7,8]. Inadvertent damage to surrounding normal tissue is minimized by optimizing radiation treatment planning and delivery, including incorporating contrast-enhanced diagnostic imaging modalities such as computed tomography (CT) or magnetic resonance imaging to help delineate critical structures and appropriate treatment margins [9]; despite these advances, however, local failure rates of approximately 10% to 28% at 5 years are still observed [10,11].

Nanoscale agents may provide an innovative strategy for improving the therapeutic index in treating sarcoma. In particular, gold nanoparticles (GNPs) represent a biologically safe class of materials that has attracted considerable attention in cancer imaging and therapy. From a diagnostic imaging perspective, gold can exhibit greater CT attenuation at clinically relevant imaging energies than typical iodine-based agents, rendering them especially suitable for CT-based radiation treatment planning [12,13]. GNP may also be tunable for custom characteristics (such as size, shape, surface chemistry, or electronic properties) and may further undergo multiplexing with other imaging agents (such as gadolinium or other magnetically active materials) [14,15]. Nanoparticles can accumulate passively through the enhanced permeability and retention (EPR) effect within tumors, because of the abnormal physiology and anatomy of tumors (i.e., leaky vasculature, large fenestrations in the endothelium, slow venous return, and poor lymphatic drainage) [16]. EPR can be enhanced by chemically “decorating” nanoparticles with organic molecules [such as polyethylene glycol (PEG)], which allow them to persist in systemic circulation for extended periods, augmenting buildup of GNP in tumor through EPR [17]. In addition to the prolonged systemic half-life, PEG-modified GNP (P-GNP) are characterized by uniform size, relatively uncomplexed synthesis, and stability under physiological conditions [18].

Significant radiosensitization with GNP has been reported with orthovoltage RT [19–23] as well as megavoltage [24–26] and proton [27,28] radiation beams. Most cell culture studies have focused on a variety of carcinomas involving the cervix [19,23,29], prostate [20,22,24,27], colon [21], and breast [20,24,30], among others. Moreover, several previous studies with animal models have used intratumoral [30,31] and intraperitoneal [32] injections of GNP or employed RT delivery techniques that are not convenient or clinically feasible [23,33,34]. To our knowledge, the potential for GNP for enhancing the RT of sarcoma in both animal and cell culture models has not yet been explored. We therefore sought to investigate the potential use of GNP radiosensitizers for clinical treatment of sarcomas by employing translational models that closely recapitulate drug administration routes, radiation treatment planning, and the precise and focused targeting of RT routinely used in patients.

We therefore investigated the potential of P-GNP for sarcomas, both in cell culture and in a murine tumor model (schematic shown in Figure 1A). Our experimental techniques included intravenous (i.v.) administration of the P-GNP, CT imaging of mice with engrafted sarcoma tumors for RT treatment planning, with and without P-GNP administered, and precision-targeted RT delivered using the Small Animal Radiation Research Platform (SARRP), currently the most advanced method of delivering image-guided stereotactic radiotherapy to experimental animals (Figure W1). These experiments together enabled us to assess the potential of P-GNP for radiosensitizing sarcomas and improving diagnostic imaging for RT treatment planning, using techniques similar to those employed in clinical radiation oncology.

Materials and Methods

Synthesis and Characterization of P-GNPs

GNPs comprised of ~12-nm colloidal core of gold were synthesized using the Turkovich method [35]. Briefly, 60 mg of HAuCl₄ (Sigma-Aldrich, St Louis, MO) was dissolved in deionized water (200 ml), which was heated to boiling. Aqueous sodium citrate (15 ml, 55 mM) was then added to the boiling solution, at which point the solution turned from clear yellow to a deep wine red. After cooling and filtering the GNP solution through a 0.2-μm filter, methoxy-PEG-thiol [molecular weight (MW) ~ 5000; Laysan Bio Inc, Arab, AL] was added at a 4:1 weight ratio (PEG:HAuCl₄) and allowed to stir overnight. The resulting P-GNP solution was concentrated by centrifugal filtration, using 50K MWCO filters (EMD Millipore, Billerica, MA) and rinsed seven times with phosphate-buffered saline (PBS) to remove unreacted reagents. The gold concentration was determined using inductively coupled plasma mass spectrometry (ICP-MS) and confirmed with UV-Vis spectroscopy [36].

Transmission electron microscopy (TEM) images of P-GNP were obtained by diluting P-GNP in EMD Millipore water and depositing them on 200 mesh carbon-coated copper grids (Polysciences, Warrington, PA). Samples were imaged using a JEOL 1010 transmission electron microscope operating at 80 kV, which confirmed uniform dimensions (12.4 ± 1.3 nm; Figure 1B). For optical spectroscopy, dynamic light scattering (DLS), ζ potential measurements, and stock samples of P-GNPs were first diluted in PBS (pH 7.4). Optical spectra were recorded using a Cary Bio 100 UV/visible spectrophotometer (Varian; Agilent Technologies, Santa Clara, CA), which showed strong peaks near ~522 nm (Figure 1C) due to surface plasmon resonance. DLS measurements were performed with a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, United Kingdom) operating in noninvasive backscatter mode. DLS further confirmed the uniform dimensions of the P-GNP, with the gold cores conjugated to the PEG chains exhibiting hydrodynamic diameters of approximately 26 nm (Figure 1D). The low polydispersity index (a measure of nanoparticle size inhomogeneity) that was observed reflects the high degree of size control achieved in our synthesis (Figure 1D, inset). The ζ potential for the P-GNP after PEG modification was measured to be near-neutral (∼0.55 mV), indicating the effective shielding of the nanoparticle surface by the PEG groups. The physical and spectroscopic features described here are consistent with those reported in the literature [36].
Cell Culture

HT1080 human fibrosarcoma-derived cells were maintained in Dulbecco’s modified Eagle’s medium, and U2OS human osteosarcoma-derived cells were maintained in McCoy’s 5A medium (both from Invitrogen, Carlsbad, CA). Both cell lines were obtained from American Type Culture Collection and were tested free of mycoplasma contamination. All culture media were supplemented with 10% fetal calf serum (Invitrogen) and 1% antibiotics (Invitrogen). Cells were maintained in a humidified incubator that kept a 37°C and 5% CO2 environment.

Immunofluorescence for Phosphorylated Ser139 on Histone h2ax

Cancer cells grown within chamber slides were exposed to culture medium with 1 mM P-GNP for 24 hours and then irradiated with the SARRP (150 kVp, 15 mA) at a dose rate of 1.45 Gy/min through a wide circular field 11-cm collimator at a distance of 35 cm from beam to cells through a 0.15-mm copper filter. The cells were then fixed with 10% neutral buffered formalin (Sigma-Aldrich) for 10 minutes at the specified time point. Cells were then rinsed with PBS, and the nuclei were stained with Hoechst 33342 (25 μM) for 15 minutes. The slides were permeabilized with 0.5% Triton X-100 in PBS and then exposed to blocking buffer (PBS, 0.5% Triton X-100, 5% normal chicken serum, 1% BSA) for 30 minutes at room temperature and subsequently incubated overnight at 4°C with mouse monoclonal anti–phospho-histone h2ax primary antibody (JBW301; Upstate Biotechnology, Lake Placid, NY) at 1:1500 dilution in PBS (with 0.5% Triton X-100 and 1% BSA). Cells were washed with PBS and then incubated with chicken anti-mouse Alexa Fluor 594 secondary antibody (Molecular Probes, Eugene, OR) at 1:1000 dilution in PBS (with 0.5% Triton X-100 and 1% BSA) for 1 hour at room temperature. After rinsing with PBS, the slides were mounted with Prolong Gold Antifade Reagent (Invitrogen) and coverslips. Fluorescence imaging was performed using a Deltavision Deconvolution microscope (Applied Precision Inc, Issaquah, WA) equipped with a ×60 (1.42 NA) oil-immersion lens and thermoelectrically cooled 12-bit monochrome charge-coupled device (CCD) camera. Images were recorded as z-stacks (0.5-μm steps). Following reconstructive deconvolution, the maximum values of the pixels were used to assemble two-dimensional projections. Foci were counted in ImageJ after applying a top-hat filter and constant value threshold based on nonirradiated controls.

Assay for Clonogenic Survival

Cells were incubated for 24 hours in culture medium with or without 1 mM P-GNP in 60-mm dishes at predetermined densities and then irradiated with the SARRP (150 kVp, 15 mA) at the specified radiation doses. After replacing the culture medium, plates were kept in a humidified incubator that maintained 37°C and 5% CO2 environment for 10 to 14 days, when they were stained with crystal violet. A colony by definition had N > 50 cells. The surviving fraction was calculated as (number of colonies formed)/(number of cells plated × plating efficiency). Each point on the survival curve represents the mean surviving fraction from at least three replicates. The sensitizer
enhancement ratio (SER) was calculated on the basis of the radiation dose required to achieve a surviving fraction of 10% ($D_{SF0.1}$), in which $$\text{SER} = \frac{D_{SF0.1 \text{ of RT only}}}{D_{SF0.1 \text{ of P-GNP + RT}}}.$$ 

**Establishment of Human Sarcoma Tumors in Mice**

Athymic nude NCr (nu/nu) mice at 6 weeks old were obtained from National Cancer Institute (NCI). Before tumor implantation, mice were anesthetized with 140 and 10 mg/kg ketamine and xylazine, respectively. The mice were then injected subcutaneously with $2 \times 10^6$ HT1080 cells suspended in Dulbecco’s modified Eagle’s medium (50 μl) into the dorsal region near the thigh and then moved to a heated pad. Following recovery from anesthesia, the mouse was placed in its cage and given food and water ad libitum.

**In Vivo CT Imaging and RT**

The clinical RT of sarcoma commonly relies on treatment planning that includes CT imaging of the patients in appropriate immobilization devices and lying on “tables” similar to that used for treatment. For animal work, this is often best simulated with the SARRP. The SARRP is designed and created to emulate image-guided RT, with a gantry-mounted highly collimated treatment beam that can focus on a small treatment area, thus sparing normal tissue from inadvertent irradiation. Furthermore, the gantry also has a mounted cone-beam CT, enabling imaging, treatment planning, and treatment delivery without having to move the animal to a different device or table [37]. Cone-beam CT imaging was performed using the SARRP (Gulmay Medical, Inc, Camberley, United Kingdom). CT data were recorded at 50 kVp (0.5 mA), and 1440 projections were used to reconstruct the cone-beam images using the algorithm provided by the manufacturer. Mice were first imaged at baseline and then injected with 1.25 g of P-GNP per kg through the lateral tail vein, followed by imaging at the specified time points.

For radiotherapy treatment, anesthetized mice bearing HT1080 flank tumors were first imaged with the SARRP as described above, and then the CT image was used to determine the treatment isocenter for focused administration of RT (175 kVp, 15 mA) to the desired location, which was delivered through a 17-mm diameter collimator at a distance of 35 cm from beam to mouse through a 0.15-mm copper filter.

**In Vivo Fluorescent Imaging**

Ten days following mock irradiation or 20-Gy RT, mice were injected with 0.15 nmol of fluorescent PEG contrast agent (IRDye...
800CW PEG; Li-COR Biosciences, Lincoln, NE) through lateral tail vein injection and imaged at the indicated time points using the Pearl Impulse Imaging System.

**Survival Analysis of Xenograft-Implanted Mice Treated with RT and GNP**

Mouse work was performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Sarcoma tumors implanted into mice were allowed to grow to a diameter of approximately 0.7 to 1 cm. Mice were monitored daily to ensure clean cages, adequate food and water, and good body condition. Tumor growth and mouse weight were measured every other day. Tumor dimensions were measured with calipers, and the volume ($V$) was approximated using the equation $V \approx \left(\frac{\pi}{6}\right) \times \text{length} \times \text{width} \times \text{height}$. Flank tumors were permitted to grow no larger than $V \approx 4.2 \text{ cm}^3$ (or a diameter of 2.0 cm) as per IACUC standards, corresponding to roughly 6 to 10 times the original tumor volume, at which point the animals were sacrificed. Mice were sacrificed if they exhibited excessive weight loss (>20%), tumor metastasis, lethargy, or other signs of distress consistent with IACUC standards. Survival time to this endpoint was calculated from date of treatment. Survival data were plotted using Kaplan-Meier techniques.

**Results**

**In Vitro Radiosensitization by P-GNPs**

We first tested the effects of P-GNP on the human sarcoma cell lines HT1080 (fibrosarcoma) and U2OS (osteosarcoma). Figure 2 shows the calculated density of DNA double-strand breaks (DSBs) in the HT1080 and U2OS cells, respectively, which were mock-irradiated (upper) or irradiated with 4-Gy RT (lower) either in the absence of (left columns) or preceded by exposure to P-GNP (right columns for each cell line). Cells treated with P-GNP consistently exhibited a higher density of persistent phosphorylated Ser139 on histone h2ax ($\gamma$h2ax) foci, indicating greater unrepaired DNA DSBs compared to those exposed to RT only. The density of $\gamma$h2ax foci increased 1.57-fold for HT1080 and 1.61-fold for U2OS cells compared to RT alone. Figure 2, C and D, show the calculated density of $\gamma$h2ax foci (number of foci per 100-µm$^2$ nuclear area) for over 60 nuclei in each treatment group for HT1080 and U2OS cells.

We subsequently tested the effects of P-GNP on the clonogenic survival of the sarcoma cells after exposure to ionizing radiation. The survival curves in Figure 3 show that the presence of P-GNP led to significantly reduced clonogenic survival for both HT1080 and U2OS cells compared to those treated with radiation alone. By fitting each of the survival curves to a linear-quadratic model, we calculated the SER to be 1.16 for HT1080 cells and 1.07 for U2OS cells (see Materials and Methods section).

**In Vivo Characterization of Circulating and Tumor-Localized P-GNP**

Mice i.v. injected with P-GNP were imaged with the on-board CT of the SARRP. The imaging indicated that the P-GNP is readily seen in the normal vasculature and is easily detectable even in smaller vessels such as the intralobar renal veins (Figure 4, A and B). We furthermore performed serial CT imaging to monitor the systemic persistence of P-GNP. Figure 4C shows representative coronal CT images of a mouse thorax at various time points after gold administration. P-GNPs exhibit robust systemic half-life, as shown by the ability to clearly delineate the chambers of the heart even at 24 hours after i.v. administration of a single dose of P-GNP (Figure 4C). The detectability (and therefore persistence) of the P-GNP in the blood then gradually diminished over time but still quantitatively showed CT hyperintensity at 48 hours ($I_B/I_M \sim 1$) compared to precontrast images ($I_B/I_M < 1$; Figure 4D); under normal contrast-free conditions, blood is slightly less radiopaque than muscle.

In animals with engrafted sarcoma tumors, CT scanning indicated that i.v. injected P-GNP accumulated within the tumor (Figure 4, E and F). The retention of the EPR-driven P-GNP within the tumor enhanced the distinction between tumor and normal tissues, including the depth of penetration into normal tissues by the tumor (the tumor/normal tissue interface indicated by the blue arrows in Figure 4, E and F). Computer-reconstructed imaging of this mouse in three dimensions further enabled us to visualize the differential distribution of P-GNP within the tumor (Figure 4G).

To examine more closely the localization of P-GNP within tumor tissue, we compared histologic sections of sarcoma tumors from mice injected with saline and 1.25 g of P-GNP per kg that were stained with both hematoxylin and eosin (H&E) and “silver enhancement” of gold,
the latter being a signal amplification technique that can improve detection of Ag onto the nanoparticles (Figure 5A) [38]. Compared to the tumor from the mouse injected with saline, the P-GNP–treated tumor showed clear deposition of gold throughout tumor tissue, including EPR-driven deposition of P-GNP especially marked in regions adjacent to tumor vasculature, as shown in Figure 5A. Next, by quantifying the total gold uptake in muscle, brain, and tumors using ICP-MS, we observed that sarcoma xenografts show approximately 10 and 80 times higher P-GNP deposition than, respectively, muscle and brain, with the markedly reduced levels of P-GNP within brain probably due to the protective effects of the blood-brain barrier (and consistent with the absence of neurologic abnormalities in animal subjects following administration of P-GNP; Figure 5B).

Figure 4. GNP-enhanced CT imaging in mice. (A and B) Coronal CT sections of the same mouse abdomen before (A) and 5 minutes after (B) injection of P-GNP (1.25 g/kg) i.v. into the lateral tail vein. The red arrow highlights the interlobar vessels. (C and D) Sequential CT images (transverse) of the thorax of the same mouse (C) and blood-to-muscle contrast ratio ($I_B/I_M$) at each time point (up to 48 hours after P-GNP injection) shown in the histograms (D). Columns in D represent mean ($n = 3$) blood-to-muscle CT intensity ratios ($I_B/I_M$) compared to precontrast images, with error bars indicating SD. (E and F) CT imaging of a representative mouse tumor engrafted with sarcoma cells (HT1080). The upper images show coronal views of the mouse before P-GNP injection (E) and 48 hours after injection (F) of P-GNP, whereas the lower images show the respective transverse views. In each case, the tumors are indicated by the blue arrows. Gold accumulation is distinguished by CT hyper-intensity at tumor site in F. (G) Three-dimensional reconstruction of the contrast-enhanced CT image in F illustrates the spatial heterogeneity of EPR-driven P-GNP accumulation in HT1080 tumors. P-GNPs were pseudocolored in gold based on intensity thresholding.

Figure 5. Histologic and ICP-MS analyses of nanoparticle EPR in mice. (A) Representative histologic sections of sarcoma tumors excised from mice 48 hours after i.v. injection with P-GNP (right) or saline (left) stained with H&E and silver enhancement. Blood vessels of comparable caliber are indicated by green arrows. (B) Quantification of P-GNP accumulation in brain ($n = 3$), muscle ($n = 3$), and tumor ($n = 6$) tissues measured by ICP-MS in mice sacrificed 48 hours after injection.
P-GNP Enhances the Efficacy of Radiotherapy in Mice Engrafted with Human Sarcoma

Having confirmed that P-GNP can accumulate in sarcomas as described above, we next sought to assess the ability to enhance the efficacy of RT. We therefore compared the effects of mock treatment, P-GNP alone, radiation alone, or the P-GNP followed by radiation in mice with sarcoma. The radiation was administered using the SARRP, which enabled accurate image-guided determination of treatment isocenter and field size and shapes to fully encompass the tumor yet spare normal tissue. Figure 6 shows serial caliper measurements of tumor volume for sarcoma-bearing mice receiving the respective treatments. Whereas P-GNP alone did not affect tumor growth compared to mock-treated mice, and RT alone modestly slowed tumor growth, the combination of RT after P-GNP led to maximal regression of tumor, with statistically significant differences against all other groups (Figure 6A). The marked inhibition of tumor growth provided by the combination of RT after P-GNP led to maximal regression of tumor, with statistically significant differences against all other groups (Figure 6A). The marked inhibition of tumor growth provided by the combination of RT after P-GNP led to maximal regression of tumor, with statistically significant differences against all other groups (Figure 6A). The marked inhibition of tumor growth provided by the combination of RT after P-GNP led to maximal regression of tumor, with statistically significant differences against all other groups (Figure 6A).

We compared the extravasation of a fluorescently labeled PEG dye (a bioimageable surrogate for vascular permeability) injected i.v. into healthy mice after 20-Gy RT versus mock irradiation in the rear flank. Figure 7A illustrates that RT 10 days before PEG dye injection leads to persistent fluorescence over background in the irradiated zone (indicated by the dashed circle) having the same pattern as the beam collimator, whereas the mice in the other treatment groups succumbed.

Discussion
In this work, we investigated the imaging and radiosensitizing effects of GNP s in cellular and animal models of human sarcoma. The radiosensitivities of fibrosarcoma (HT1080) and osteosarcoma (U2OS) cells were first characterized using assays of DNA damage and clonogenic survival. By themselves, the P-GNP had no effect on unrepaired DNA DSBs detected by the γH2AX [39] but markedly potentiated γH2AX phosphorylation in sarcoma cells that were subsequently irradiated with a sublethal dose of ionizing radiation (Figure 2). These findings are consistent with the decreased clonogenic survival of both cell lines treated with P-GNP + RT compared to RT alone (Figure 3). Previous studies have found in vitro radiosensitization consistent with the results shown here [23,24,30], but the observed SER has also been shown to vary (Table W1) [21,23,40] depending on the cell line, radiation source, field size, and also the size and shape of P-GNP (see Table W1 for a more thorough description of SERs reported in the literature). While RT directly damaging nuclear DNA is thought to be most predictive for cell killing (with DSBs being the most lethal), GNP s may also radiosensitize through alternative mechanisms such as increasing damage to cell membrane and mitochondria, enhancing dynamic chemical effects to further catalyze reactive oxygen species (ROS) formation and G2/M accumulation [22,41–44]. These latter

Figure 6. GNP-enhanced RT of sarcoma improves survival in mice. (A) Tumor volumes of mice with established sarcoma tumors mock-treated (n = 5), treated with P-GNP alone (n = 5), RT alone (n = 7), or P-GNP + RT (n = 6) were measured by calipers. Volumes were estimated using \( V \approx \frac{\pi}{6} (l \times w \times h) \); each point represents the mean tumor volume ± SEM. Asterisk indicates that statistical significance (\( P < .05 \)) between the RT versus GNP + RT groups was reached (\( \alpha = 0.05 \)) beginning 21 days after treatment. (B) Kaplan-Meier survival analysis of the mice in A. Only mice treated with combined GNP + RT noted enduring survival; mice in all other treatment groups succumbed.

Figure 7. P-GNP extravasation through tissue pretreatment with RT. We then used CT contrast enhancement imaging as an indicator of P-GNP extravasation in engrafted sarcoma tumors. The upper panel of Figure 7B shows representative CT intensity maps of tumors from mice receiving i.v. injection of P-GNP 10 days after mock irradiation (left column) or 20-Gy RT (right column). The tumor receiving RT before gold administration exhibited higher contrast enhancement compared to the nonirradiated control, with roughly two times greater overall brightness for the irradiated mouse compared to the nonirradiated control, and this was consistent with the ICP-MS measurements of P-GNP uptake for each treatment (1.8-fold increase in gold deposition; Figure 7C).
“nontargeted” mechanisms of enhancement by P-GNP can all be directed at tumor tissue in vivo when combined with physiological accumulation by EPR, which is potentiated by durable half-life (and further enhanced by the addition of targeting ligands) as discussed below.

Our animal studies show that circulating P-GNPs can serve as robust CT contrast agents that allow visualization of both vasculature and EPR-driven deposition of gold in sarcoma tumors and by directly exerting antitumor effects when used together with RT. Regarding the “diagnostic” attributes of P-GNP, our investigations build on previous studies that have suggested the value of P-GNPs as vascular imaging agents in experimental animals [12,45]. PEG modification enables prolonged circulation time; for example, Kommareddy and Amiji showed that the addition of PEG ligands increased the nanoparticle plasma half-life from 3 to 15 hours in mice [46]. Our own in vivo imaging experiments exhibited similar durable pharmacokinetics with the P-GNP (Figure 4, C and D), which favors greater accumulation in tumors through EPR. These advantages of P-GNP together potentially facilitate gross tumor volume contouring and RT targeting, which in turn may help minimize inadvertent RT to normal organs and surrounding tissues. The CT and histologic observations described here suggest that the accumulation of P-GNP within a tumor may be dependent on the density, location, or permeability of the tumor neovasculature.

P-GNP may provide a means to locally potentiate RT-induced damage to sarcoma tumors. In this work, tumor-localized accumulation of P-GNP further improved the efficacy of RT by enhancing radiation-induced damage to sarcoma tumors in vivo, as shown in Figure 6. These observations were achieved using the SARRP, one of the most currently advanced means of delivering tightly collimated, accurate radiation to animals and which more closely reflects the gantry-mounted RT given to patients. Finally, we provide evidence that RT through its ability to permeabilize the vasculature may enhance the passive extravasation of P-GNP, thus comprising a strategy that could be incorporated into the fractionated treatment commonly used in treating patients. Clinical radiotherapy protocols for sarcoma implement RT fractions of approximately 1.8 to 2 Gy (~60-Gy cumulative dose), and hypofractionated RT is currently being considered as a therapeutically effective and logistically favorable alternative for elderly patients [47]. As a proof of concept, our study used a fairly large radiation dose (20 Gy) before P-GNP administration to ensure a relatively rapid response. Translating these results to the clinical setting requires optimization of the radiation dose and timing between RT fractions and nanoparticle administration. While greater cumulative RT dose increases likelihood of subsequent permeability to P-GNP extravasation, administering nanoparticles too late in the fractionation course can detract from the potential benefits of radiosensitization. This mutually enhancing characteristic of radiation enhancing P-GNP uptake that, in turn, sensitizes the sarcoma to subsequent RT fractions suggests that P-GNP may be usefully integrated into radiation oncology in the clinical setting.

While our cell culture and animal studies together with those published by others have described the safety, lack of toxicity,

![Figure 7.](image_url) Targeted radiotherapy enhances subsequent P-GNP accumulation. (A) Serial optical imaging of fluorescently labeled PEG dye administered i.v. in normal mice 10 days after mock irradiation (left) or irradiation with 20-Gy RT (right). The dashed orange circle outlines the irradiated area. (B) Upper: CT intensity landscape of P-GNP contrast for sarcoma tumors mock-irradiated (left) or irradiated with 20-Gy RT (right) 10 days before gold injection. Lower: Corresponding enhancement in tumor CT intensity ratio before and after gold injection ($E_{TM}$). (C) ICP-MS analysis of tumors excised from mice treated as in B. Data represent the means ± SE (n = 3 mice per group). Assessing whether RT(+) group had greater P-GNP uptake than RT(−) group implemented a one-tailed $t$ test ($\alpha = 0.05$).
pharmacokinetics, and radiosensitizing potential of nanoparticles, these clinically important parameters are not yet well established in humans, and thus, more extensive characterization is still required. Reassuringly, however, gold has been used in human medicine throughout history safely and is used today for other conditions such as rheumatoid arthritis [48], and several formulations of GNP s for anticancer treatment have entered clinical trials, such as CYT-6091 [49] and AuroShell particles (clinicaltrials.gov identifier No. NCT00848042). The results of these and future studies will help identify the optimal integration of P-GNP into standard radiation oncology practice.

In conclusion, we have investigated the therapeutic and diagnostic value of P-GNP combined with RT for sarcoma. The experiments outlined here used the SARRP to develop an integrated approach combining both imaging and therapy that resembles clinical treatment paradigms. Deposition of nanometer-scale agents to tumors occurred in a tumor-specific manner through EPR. Our data suggest that P-GNP, in conjunction with RT, enhances CT imaging and RT-induced tumor tissue damage and provides greater mean survival times in animal studies.

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References


Supplementary Materials

Mouse Irradiations with the SARRP

The SARRP (Gulmay Medical, Inc) is currently the most advanced method to deliver RT to translational animal models. Cone-beam CT imaging and on-board software allow detailed RT planning, and the radiation dose can be delivered with high precision (field size as small as 0.5 mm). For our experiments, CT data were recorded at 50 kVp (0.5 mA), and 1440 projections were used to reconstruct the cone-beam images using the algorithm provided by the manufacturer. Mice were first imaged at baseline and then injected with 1.25 g of P-GNP per kg through the lateral tail vein, followed by imaging at the specified time points. Irradiations (175 kVp, 15 mA) guided by CT imaging were administered through a circular field 1.7-cm collimator at a distance of 35 cm and through a 0.15-mm copper filter (for more information, see Wong et al. [37] or visit the manufacturer’s website at http://www.xstrahl.com/sarrp.htm; Figure W1).

Histologic Examination of Tumor Xenografts

Histologic examination of tumor xenografts is shown in Figure W2.

Weight of Treated Mice over Time

In addition to serial tumor volume measurements, we also recorded the weight of mice over time as a surrogate marker for their ability to thrive. We found that control mice that were mock-irradiated lost weight much more quickly than those who received RT due to the rapid progression of tumor growth. Furthermore, we also found that mice receiving dual modality treatment (RT + P-GNPs) exhibited greater weight gains over time after therapy compared to RT-only group. These data together with the tumor volume measurements shown in Figure 5 are suggestive of the therapeutic efficacy of dual modality treatment and also the low toxicity of P-GNPs (Figure W3).

Enhanced X-ray Absorption by P-GNPs

Enhanced X-ray absorption by P-GNPs is shown in Figure W4.

Distribution of Nanoparticles in Other Tissues

Distribution of nanoparticles in other tissues is shown in Figure W5.

P-GNP and Cell Proliferation

The effects of P-GNP on tumor cell proliferation are shown in Figure W6.

Comparison of RT Enhancement from the Literature

Comparison of RT enhancement from the literature is shown in Table W1.

Figure W1. Diagram of the SARRP and irradiation schematic.
Table W1. Comparison of GNP Radiotherapy Enhancement Selected from the Literature.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Paper Citation</th>
<th>Cell Line</th>
<th>Radiation</th>
<th>GNP Concentration</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 MV</td>
<td>12 μM</td>
<td>SER = 1.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 MV</td>
<td>12 μM</td>
<td>SER = 1.16</td>
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<td></td>
<td></td>
<td></td>
<td>6 MeV electrons</td>
<td>12 μM</td>
<td>SER = 1.04</td>
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<tr>
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<td></td>
<td></td>
<td>16 MeV electrons</td>
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<td>SER = 1.35</td>
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<td></td>
<td></td>
<td>DU145</td>
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<td>SER = 0.92</td>
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<td></td>
<td></td>
<td>6 MV</td>
<td>12 μM</td>
<td>SER = 1.13</td>
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<tr>
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<td></td>
<td></td>
<td>6 MeV electrons</td>
<td>12 μM</td>
<td>SER = 1.12</td>
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<td></td>
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<td>L132</td>
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<td>SER = 1.05</td>
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<td>6 MeV electrons</td>
<td>12 μM</td>
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<tr>
<td></td>
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<td>100 kVp</td>
<td>2.4 mg/ml</td>
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<td>1 nM</td>
<td>DEF = 1.66</td>
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<td>6 MV</td>
<td>1 nM</td>
<td>DEF = 1.17</td>
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<td>Cs-137 (662 keV)</td>
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<tr>
<td>Liu et al.</td>
<td><em>Phys Med Biol</em>, 2010, 55, 931–945</td>
<td>CT-26, EMT-7</td>
<td>160 kVp</td>
<td>0.4–1 mM</td>
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<td>CT-26, EMT-8</td>
<td>6 MV</td>
<td>DEF ∼ 1</td>
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<tr>
<td>Joh et al.</td>
<td><em>PLoS One</em>, 2013, 8, e62425</td>
<td>U251</td>
<td>150 kVp</td>
<td>1 mM</td>
<td>SER ∼ 1.3</td>
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<td>Zhang et al.</td>
<td><em>Biomaterials</em>, 2012, 33,6408–33,6419</td>
<td>HeLa</td>
<td>Cs-137 (662 keV)</td>
<td>0.05 and 0.1 mM</td>
<td>DEF = 1.41–1.65</td>
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</table>

Enhancement can vary on the basis of several factors, including cell line and radiation source used.
Figure W2. Histologic examination and immunofluorescent staining of HT1080 xenografts. (A) H&E staining of HT1080 tumors excised from mice receiving the indicated treatments. (B) Cluster of differentiation 31 (CD31)/\gamma h2ax costaining of HT1080 tumor tissue, where CD31 stains green for endothelial cells. RT and RT + GNP samples were excised from mice 24 hours after radiotherapy. In the absence of radiotherapy, there was significantly lower expression of \gamma h2ax foci, irrespective of GNP administration. Twenty-four hours after RT, the immunofluorescence data show that there is more “red” signal in the RT + GNP sample compared to the RT sample, suggesting a greater persistence of unrepaired DNA DSBs in the former. The bottom panel (RT + GNP (60 days)) shows tissue from a mouse showing full regression of tumor 60 days after treatment, in which the tissue appears collagenous, fibrotic, and presumably absent of tumor.
Figure W4. CT contrast enhancement with P-GNPs. (A) Mass attenuation coefficients of gold compared to iodine and water. Gold exhibits increased attenuation (μ/ρ) compared to iodine and water within the X-ray energies at diagnostic imaging photon energies. Data compiled from the National Institute of Standards and Technology (NIST). (B) *in vivo* CT imaging with the SARRP (50 kVp, 0.5 mA) with and without P-GNPs; i.v. injection (1.25 g of Au per kg) of nanoparticles showed enhanced CT contrast of mouse vasculature 5 minutes after injection, in comparison to the control mouse.

Figure W3. Serial measurements of mouse weight. Curves represent the average of mouse weights for each treatment group.
Figure W5. Distribution of P-GNPs in other tissues after 48 hours. The uptake of P-GNPs in the liver, kidney, heart, lung, and brain is plotted as percent injected dose (%ID) per gram tissue, measured by ICP-MS. The liver shows relatively high P-GNP uptake (comparable to that of tumor), and this behavior is consistent with the CT hyperintensity observed in Figure 4B.

Figure W6. The effects of P-GNP on tumor cell proliferation. (A) Representative photograph of clonogenic survival experiment (10 days) of nonirradiated HT1080 cells treated with vehicle (control) and with P-GNPs. While the colony density of both plates are comparable, we observed that the control plates typically showed marginally higher colony density than those treated with nanoparticles (as shown here). (B) A 24-hour 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of U2OS cells treated with vehicle (control) or 1 mM P-GNPs. Similar to A, both treatment groups showed comparable viability.