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Review Article

Gold Nanoparticles for Radiation Enhancement *in Vivo*

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Abstract

Enhancing the effect of radiation on tumors would be a significant improvement in radiation therapy. With radiation enhancement, less radiation could be used to achieve the same goals, lessening damage to healthy tissue and lessening side effects. Gold nanoparticles are a promising method for achieving this enhancement, particularly when the gold nanoparticles are targeted to cancer. This literature review discusses the properties of gold nanoparticles as well as existing *in vivo* radiation enhancement results using both targeted and non-targeted gold nanoparticles.

Keywords: Tumors; Radiation; Gold Nanoparticles; Enhancement; Targeted; Dose; Mice

Abbreviations:

GNP: Gold nanoparticles;

PEG: Polyethylene Glycol;

kVp: Kilovolt Peak;

Gy: Gray;

pHLIP: pH-Low Insertion Peptide;

IV: Intravenous;

CTR: Complete Tumor Regression;

DTPA: Diethylenetriamine pentaacetic acid;

BSA: Bovine Serum Albumin.

Introduction

In radiation therapy for cancer, radiation is delivered after precise calculations so that a maximum dose is given to the tumor and a minimum dose is given to healthy tissue. Despite these efforts, radiation still affects healthy tissue. This effect is especially dangerous when the tumor is located near important organs. Thus, it is important in radiation therapy to reduce the dose and the damage to healthy tissues and organs [1].

One of the current strategies to reduce radiation is the use of radiation enhancers, which can absorb and make tumor cells more susceptible to it. They are designed to improve tumor cell killing, since making a tumor more susceptible to radiation means that less radiation can be used. And if less radiation is used, there will be less adverse effects on normal tissues [2].

Radiation enhancers can include materials like nanoparticles, e.g. carbon nanotubes, gold nanoparticles and quantum dots. In this paper, we will focus on the use of gold nanoparticles (GNPs). Gold is a good radiation enhancer. The radiosensitization of biomolecules by GNPs can be caused by locally increased radiation absorbed energy. Gold, a high Z material, is capable of absorbing radiation at significantly higher rates than tissue. The advantage in absorption can grow to about a factor of 100 for certain keV photon energies (20 keV shown in [3], can be checked in a database [4]). Additionally, gold nanoparticles that interact with radiation can release a number of Auger electrons via the Auger effect. The Auger effect occurs when an excited atom (for example, an ionized gold atom) releases its extra energy in a form of an electron instead of a photon.

Radiosensitization can also be caused by modified sensitivity of targeted biomolecules to radiation [5]. The efficiency of chemical radiosensitization mechanism is significantly influenced by the strong binding of GNPs to the biological target as DNA [5]. Jain et al. [6] suggests a possible biological mechanism of radiosensitization by GNPs even in the absence of radiation, with GNPs potentiating the effect of bleomycin. The results showed that the GNPs caused chemosensitization to the radiomimetic agent bleomycin at a range of concentrations with a sensitizer enhancement ratio similar to that observed for the kilovoltage photons.

Auger electrons have comparatively low energies (approximately 80 keV or less in gold), and because of this they have a short range of action in tissues. It can result in the delivery of a precise and lethal dose in their immediate vicinity. However, this short range also indicates that gold nanoparticles need to be located within tumors, near the vital cellular structures, in order to maximize the radiation enhancement effect [7,8]. This suggests the need for the gold nanoparticles to be targeted to cancer cells.

In this paper, we will review the use of gold nanoparticles as a radiation enhancer *in vivo*. Specific topics include:

- Properties of gold nanoparticles
- Important Experimental Variables
- *In vivo* radiation enhancement results for non-targeted gold nanoparticles
- Nanoparticle Targeting
- *In vivo* radiation enhancement results for targeted gold nanoparticles

Properties of Gold Nanoparticles

Gold nanoparticles properties include the following:

i) Gold is an inert material and can be made to be biocompatible using surface modification, like surface coating of the GNPs [2,3,6-17].

ii) Gold nanoparticles can be linked to biomolecules, either via stabilizers (polyethylene glycol, maleimide) or directly to sulfhydryl (-SH) groups of moieties such as peptides, antibodies, small molecules or proteins. Tumor targeting can be achieved by conjugating the GNP surface to peptides, ligands, antibodies or drugs [2, 3, 6-9, 11-14, 16, 18].

iii) Gold nanoparticles, as any nanoparticle, have a large surface to volume ratio. The relatively large surface area provides opportunity for interactions with molecules. Having large number of surface ligands, gold nanoparticles allow flexible design and multi-functionality by incorporating mixed ligands [3, 12, 16] .

iv) The nanoparticles including GNPs exhibit preferential deposition at tumor sites due to the enhanced permeation and retention (EPR) effect. This makes them to be effective as drug carriers and radiation enhancers [1, 8, 16]. This is related to the small size of GNPs, and the leaky vasculature of tumors.

v) The size of GNPs can be tuned to a wide range (1-1000 nm) and various shapes [2, 3, 6, 9, 12, 16, 18].

Important Experimental Variables

The following variables are known to affect the amount of radiation enhancement that the gold nanoparticles are capable of delivering:

Concentration of gold: The concentration of gold nanoparticles

in the tumor sites (and thus the number of gold atoms) affects the radiation enhancement capability. Hainfeld et al. [19] doubled the concentration of non-targeted gold nanoparticles (from 1.35 to 2.7 grams of Au per kilogram mouse weight), and increased survival by 72% (from 50% to 86%).

Size, Shape and Surface Chemistry of GNPs: There are various sizes and shapes of gold nanoparticles available, and this affects their uptake by the cells. Also, surface chemistry is an important parameter, which affects biodistribution and cellular uptake of nanoparticles. Chithrani et al. [20] found that the cellular uptake of spherical GNPs of 14, 30, 50, 74, 100 nm in diameter is size dependent. Cells *in vitro* had a maximum uptake for 50 nm sized spherical GNPs. The rod shaped nanoparticles exhibited less uptake by cells compared to spherical particles. For example, cells took up 500 and 375% more 74 and 14 nm spherical gold nanoparticles than 74 × 14 nm rod-shaped gold nanoparticles, respectively. The authors noted that in addition to size and shape of GNPs the surface chemistry might also affect cellular uptake of nanoparticles. Non-homogeneous coating of nanoparticles with citric acid ligands and presence of cetyl trimethylammonium bromide (CTAB) molecules at the surface of rod GNPs could result in lower cellular uptake. In an *in vitro* study of GNPs as radiation enhancers in cancer therapy, 50 nm spherical GNPs showed the highest radiosensitization enhancement factor (REF) (1.42 at 220kVp) compared to gold nanoparticles of 14 and 74 nm (1.20 and 1.26 respectively) [10]. 12.1 and 27.3 nm size spherical GNPs coated with polyethylene glycol (PEG) showed high radiation enhancement compared to 4.8 and 46.6 nm size, both *in vitro* and *in vivo*, with accumulation of GNPs in the tumor with high concentration [21]. This is in contrast to the computational study performed by McMahon et al. [17] , which predicts an increase in radiation enhancement with decreasing size of spherical GNPs. Puvanakrishnan et al. [22] compared cellular uptake for gold nanoshells and gold nanorods. The results indicated a higher accumulation of smaller rod GNPs in tumor compared to the larger nanoshell GNPs . However, the accumulation of nanorods and nanoshells in the liver increased significantly for higher doses. This suggests that the particle shape and size significantly affects tumor targeting and confirms that the smaller particles have enhanced accumulation in tumors compared to larger nanoparticles. Huang et al. [23] found that GNPs smaller than 10 nm have unique advantages over GNPs greater than 10 nm in localization and penetration of breast cancer cells, multicellular tumor spheroids and tumors in mice. The *in vivo* results showed that 2 and 6 nm tiopronin-coated GNPs were distributed throughout the cytoplasm and nucleus whereas 15 nm samples became aggregated in the cytoplasm. Tumor bearing mice were intravenously injected with a dose of 5 mg of Au per kg of mice. After 24 hours the amount of gold in tumor was 2.93 micrograms per gram of tumor for 2 nm, 0.79 micrograms for 6 nm and 0.14 micrograms for 15 nm particles. Compared to 15 nm GNPs, the 2 nm and 6 nm GNPs were widely distributed in different organs of the body due to small structures. Histological analysis showed that GNPs had almost no effect on tissues including liver, spleen, kidney, lung and heart, indicating good tissue biocompatibility of the GNPs.

Cell Line Used in Studies with GNPs: Radiation enhancement by GNPs is cell line specific. They enhance the radiation when treated with some cells but not all. Significant radiosensitization occurred in MDA-MB-231 cells at 160 kVp. However, no significant radiosensitization was observed in DU 145 or L132 cells, even though there was uptake of GNPs in both of these cell lines. In an *in vitro* experiment, uptake of GNPs was greater in MDA-MB-231 cells than in DU 145 or L132 cells, and hence radiation enhancement was better in MDA-MB-231 cells [6].

Intracellular Localization of GNPs: The location of gold nanoparticles inside of the cells affects radiation enhancement; for example, a GNP attached to DNA (Deoxyribonucleic acid) will likely have a greater impact than a GNP in other locations (for example, the local effect model discussed in [24]). Typically, not targeted nanoparticles will enter cell via endocytotic pathway and will be trapped in endosomal/lysosomal compartments and might exit cell via the exocytosis process. The uptake and removal of particles depend on its size, shape and surface properties [25]. The use of pH Low Insertion Peptides (pHLIP® peptides) to target gold nanoparticles to cancer cells (*in vitro*) resulted in location of GNPs to the plasma and nuclear membranes [7,15] . Ultra-small Au@tiopronin nanoparticles (2 and 6 nm) were localized throughout the cytoplasm and nucleus of cancer cells *in vitro* and *in vivo*, whereas 15 nm nanoparticles were found only in the cytoplasm and were aggregated [23].

The targeting ligands: The targeting ligands enable nanoparticles to bind to cell surface receptors and enter the cells by receptor mediated endocytosis [16]. Nanoparticles accumulate at the tumor sites due to leaky, immature vasculature due to enhanced permeability and retention effect [8,13,16]. Chattopadhyay et al. [26] discusses the molecular targeting approach, which enables a larger amount of GNPs to cross the cellular membrane and accumulate in the cancer cell cytoplasm. The experimental result showed that the GNPs modified with trastuzumab for targeting HER-2 on breast cancer cells with 100kVp x-rays were more effective in decreasing the clonogenic cells survival as compared to the non-targeted GNPs [26]. Kong et al. [27] found that the local concentration of GNPs in target locations can be increased by localized delivery in comparison to the naked GNPs. 15nm GNPs with AET(cysteamine) were bound to the cell membrane when treated with MCF-7 cells whereas 15nm GNPs with Glu were distributed in the cytoplasm when treated with MCF-7 cells. More GNPs were taken up or bound to MCF-7 cells in case of Glu-GNPs and AET-GNPs than the naked GNPs. With the combined effect of 200kVp, 10Gy x-rays Glu-GNPs produced decreased cell survival compared to AET- GNPs [27]. Su et al. [16] used cyclic RGD conjugated with GNP, labeled with Iodine-125 as a radio-

sensitizer, for tumor targeting and enhanced radio-therapeutic efficacy. The results depicted consistent apoptosis and the volume loss, indicating effective suppression of tumor growth due to radiation therapy on the radio-labeled targeting ligand on GNPs compared to non targeted GNPs.

Biocompatibility of GNPs: Coating gold nanoparticles with polyethylene glycol (PEG) or bovine serum albumin (BSA) can increase the likelihood of each nanoparticle reaching the tumor, since chemically modifying the GNPs by organic molecules such as PEG or BSA helps GNPs to avoid reticuloendothelial system uptake and to increase circulation time in blood [3,8]. BSA capped GNPs are easy to synthesize, resulting in uniform size and stability under physiological conditions [8]. A non-exhaustive list of similar or related methods includes the following:

- Kim et al. [28] found that PEG-coated GNPs had a much longer blood circulation time (>4 h) than non-PEG-coated GNPs.
- PEG coated GNPs can accumulate in mouse sarcoma flank tumors to concentration 10 times that of muscle and 50 times that of brain [12].
- Puvanakrishnan et al. [22] investigated the effect of PEG-coated gold nanoshells and gold nanorods, and its tumor targeting efficiency on mice with a subcutaneous tumor. Mice received an IV injection of single and multiple doses of gold nanoshells and gold nanorods. The uptake of nanoshells and nanorods in the tumor was seen to increase for the multiple doses compared to the single dose. The particle accumulation in tumors for three consecutive doses was increased by 2 for gold nanoshells and 2.45 for nanorods, compared to the single dose. Similarly for five consecutive doses the particle accumulation in tumors was increased by 3 fold for nanoshell GNPs and by 1.6 fold for nanorod GNPs, in comparison to the single dose. The uptake of smaller PEG-coated gold nanorods was 12 times more compared to the uptake of larger PEG-coated gold nanoshells in the tumor after 24 hours. The results from this study suggest that multiple dosing might be an effective method to increase GNPs accumulation in tumors.

Method of Administration of GNPs to Animals: Direct injection of GNPs by intra-tumoral administration can aid tumor uptake [15] . Intravenous administration of gold nanoparticles still results in relatively large accumulation in tumor tissue, due to the enhanced permeability and retention effect discussed above, which is related to leaky vasculature within tumors (see for example [14]).

Radiation energy (for photon irradiation): Dose enhancement caused by GNPs has been observed in kilovoltage and megavoltage beams [6,10,19]. However, enhanced cell killing was monitored when cells and GNPs were irradiated with photons in the kilovoltage range [9,11,29-31]. Hainfeld et al. [3] found that dose enhancement factor depends on both radiation energy and the amount of GNPs.

The relative success of lower energy photons is likely due in part to the fact that, in general, lower energy photons have a higher absorption probability in gold than higher energy photons [4]. However, lower energy photons also come with the complicating factor that they are less penetrating, and may not be able to reach tumors deeper than skin depth. For most clinical purpose MeV photons are used due to the fact that for high energy photons , the energy is distributed over a wide range in soft tissue [9]. Chang et al. [30] used 6 MeV electrons to irradiate a 1-inch diameter tumor region of the leg of the mouse model. Chitrani et al. [10] used low energy kVp and high energy MVp for irradiating cells. The results showed that greater radiation sensitization was seen for kVp compared to MVp for the cell experiments. Further it was evidently found for the first time that radiation sensitization was enhanced even at the clinically relevant high X-ray energy of 6MVp. Also Jain et al. [6] showed a radiosensitization effect on cells at MV X-ray energies as well as at kV energies. The MDA-MB-231 cells were seen to be radiosensitized at MV X-ray energies. Popovtzer et al. [32] showed a radiosensitization effect when cetuximab coated GNPs were used for tumor targeting in a clinically relevant radiation treatment of 6MV energy. The results showed that there was no increase in tumor diameter at all for CTX-GNP+RT compared to 1.0 cm increase in tumor diameter for the control case.

If the mechanism of cancer destruction is primarily Auger electrons after the photoelectric effect, an intriguing photon energy to use would be an energy just above the k-shell energy of gold, 80.7 keV (see for example, the X-Ray Attenuation Database from the National Institute of Standards and Technology). Most interactions would occur with the k-shell electrons, and a photon energy just above the k-shell energy would take advantage of two factors: (1) the photoelectron released would be of low energy, and thus would be localized like the Auger electrons; (2) the photoelectric effect has a sharp spike in absorption coefficient at each shell energy, including the k-shell energy.

Radiation dose: Increasing the radiation dose from 30 Gy to 35 Gy increases the survival rate of the mice for the same KeV energy of 100 kVp x-rays [33]. However, at some point there must be a radiation dose of maximum effectiveness for a given experimental setup, since an extremely high dose of radiation would kill the experimental subjects.

Radiation type: In most of the experiments with gold nanoparticles photon irradiation was used, but experiments have also been done using proton, electron and LET radiation. Kim et al. [34] found an increase in survival in mice treated with pro-

tons and either gold nanoparticles or magnetic nanoparticles. Chang et al. [30] found an increase in radiation enhancement from gold nanoparticles using electron radiation. Liu et al. [35] found that the survival fraction for HeLa cells when irradiated with high LET carbon ions was significantly less than when irradiated with low LET X-rays.

In vivo radiation enhancement results for non-targeted gold nanoparticles

Although many gold nanoparticle radiation enhancement studies have been done *in vitro*, only a few studies have been performed *in vivo*. No specific tumor targeting was utilized in the studies described in this section.

Hainfeld et al. [19] : The pioneer study of use of GNPs as a radiation enhancement was done in BALB/C mice bearing subcutaneous EMT-mammary carcinoma. In one experiment, the treatment group of mice received 1.9 nm GNPs at a concentration of 1.35 grams of gold per kg of mouse, injected intravenously into the tail, with irradiation started 2 minutes later. The animals received 30 Gy of radiation from a 250 kVp x-ray machine. These mice survived with only 1 of 10 mice having a visible tumor after 1 month, compared to no retardation of tumor growth for mice receiving only x-rays or gold.

In a second experiment, 50% of mice survived for one year after being given 1.35 grams of gold per kg of mouse and 26 Gy of radiation. In contrast, 86% of mice survived after being given 2.7 grams of gold per kg of mouse. 20% of mice survived with just radiation, and 0% of mice survived with just gold or with no treatment.

Other results showed that after injection of GNPs, many blood vessels became visible due to the gold absorption. Pharmacokinetics showed an early rapid rise followed by a slower clearance rate. Gold in tumor peaked at 7.0 ± 1.6 min and fell to half of its peak value at 41.2 ± 19.5 min; gold in muscle peaked at 5.3 ± 0.6 min and fell to half at 24.2 ± 2.6 min. The data showed that the GNPs cleared nearly twice as fast from normal muscle as from tumor. The injected gold solution was dark black/brown and at the periphery of some tumors was similarly dark. These tumor periphery results showed almost twice the gold concentration of the main tumor mass. The periphery of one tumor contained 6.5 mg Au/g, with a tumor to normal tissue ratio of 8.6. This leads to the fact that a targeting molecule, such as an antibody or peptide, attached to the gold nanoparticle would further improve the tumor specificity and distribution of GNPs within a tumor. The GNPs were shown to be non-toxic to the mice, based on preliminary toxicity testing.

Hainfeld et al. [36]*:* In this study, Hainfeld et al. tested the effects of radiation dose, radiation energy and a preheating strategy. C3H/HeJ mice were given subcutaneous highly radiation resistant SCCVII head and neck squamous cell carcinoma. 1.9 nm GNPs were found to be more effective at 42 Gy than at 30 Gy for the same radiation energy (68 keV median energy photons)**.** GNPs were also found to be more effective when used at 68 keV than at 157 keV for the same radiation dose (42 Gy). Further, GNPs were found to be more effective at 50.6 Gy, 157 keV than at 44 Gy, 157 keV.

The effect of preheating the mice was also investigated. Mice were preheated for 12-17 mins by submerging the legs of anesthetized mice containing tumor in 44°C water bath. GNPs were then injected (1.9 g/kg body weight) and the mice were heated again for 3 mins, and then irradiated a minute later with 30 Gy, 68 keV. As a result it was seen that the GNPs enhanced the synergy of hyperthermia and radiation therapy at sufficiently high radiation doses (30 Gy, compared to 15 and 23 Gy). The experimental results showed that there was not any damage in the leg of mice and that the tumor doubling time was 52 days for heat + radiation + gold compared to 45 days for radiation alone. The surviving fraction was 79% for heat + radiation + gold compared to 14% for radiation alone.

Hainfeld et al. [33]: In this study, Hainfeld et al. treated brain cancer in mice using gold nanoparticles. 50% long-term survival (>1 year) was found using B6C3f1 mice bearing Tu-2449 brain tumors. Irradiation (100 kVp x-rays, 30 Gy) occurred 15 hours after injection of 11 nm GNPs at a concentration of 4 grams of gold per kg of mouse. 0% long-term survival was found for mice given no treatment, GNPs only and radiation only.

Similarly, for a slightly higher radiation dose of 35 Gy, 56% long-term survival was found compared to 0% survival of mice with no treatment and 18% long-term survival for radiation only.

Further results showed that IV injected GNPs specifically localized in brain glioma in a 19:1 tumor to normal brain ratio. The micro CT measured by the tumor uptake of 1.5 ± 0.2% (weight by weight) gold, which was considered to be the highest gold concentration ever achieved in tumor by IV injection. Atomic absorption spectroscopy measured the uptake to be 1.5 ± 0.2% (weight by weight) gold. The GNPs were initially distributed throughout the tumor, very different from the subcutaneous tumors where GNPs of 15 nm were largely confined to the tumor periphery. The amount of gold delivered was high enough to multiply a radiotherapy dose of tumor by a calculated factor of approximately 300%. Hainfeld predicts this is an indication of difference in tumor and vasculature growth pattern, perhaps indicating the brain tumor cells are more migratory, thus not severely compressing central blood vessels limiting internal blood flow. No toxicity was seen for the concentration of gold used in this study.

Chang et al. [30]: Chang et al. used electron radiation with gold nanoparticles. C57BL/6 mice with B16F10 melanoma were injected (IV) with 13 nm GNP at a concentration of 1 gram gold per kg of mouse. 24 hours later, 25 gray of 6 MeV electron radiation was given using a Varian 2100C linear accelerator.

The results showed a retarded tumor growth and increase in survival of mice receiving GNPs followed by radiation compared to the radiation alone, GNPs alone and control groups of mice. Survival of mice treated with gold nanoparticles and radiation was 60% after 2 months, whereas survival was less than 20% for radiation treatment alone and 0% for gold nanoparticles alone or no treatment.

Biodistribution of GNPs 24 hours post IV injection of GNPs showed the accumulation of GNPs inside the tumor, with a tumor to tumor surrounding muscle gold ratio of 6.4:1. Also, higher concentrations of GNPs were found in the liver and spleen, indicating uptake of gold by the reticuloendothelial system.

The number of apoptotic cells detected in tumor by a TUNEL assay was significantly higher in mice treated with GNPs followed by radiation than in mice receiving only radiation, GNPs alone and control groups.

Compared to Hainfeld et al. [20], fewer GNPs were injected IV into the mice in Chang et al. (31)(2.7 g Au/kg versus 1 g Au/kg). Additionally, the irradiation was done 24 hours after injection, versus 2 minutes after injection in Hainfeld et al. [19].

Bobyk et al. [37]: Bobyk et al. studied the effect of gold nanoparticles and radiation in rats with brain tumors. Male Fischer rats bearing F 98 glioma cells were intracerebrally injected by 5 microliters of 15 nm GNP (25 mg/mL or 50 mg/mL) 20 minutes before irradiation using 88 keV x-rays with a dose of 15 Gy.

The untreated groups of rats had a mean survival time of 23.8 ± 1.6 days and the rats receiving GNPs alone had a mean survival time of 24.9 ± 0.8 days and 23.3 ± 0.7 days for 25 mg/mL and 50 mg/mL. This suggests that the GNPs alone did not improve the animal life span.

The group of rats that received radiation alone had a mean survival time of 33 ± 2.7 days, an increase of 38.8%. The group of rats receiving GNPs and x-rays had mean survival times of 34.9 ± 1.7 days (25 mg/mL) and 41.6 ± 3.2 days (50 mg/mL). Thus, the higher concentration of GNPs, combined with radiation, showed a 74% increase in mean survival time.

TEM results showed that GNPs are trapped by endosomes before being fused with lysosomes *in vitro*. *In vivo* results also showed the internalization of 15 nm GNPs by the endosomal pathway in cells on brain tissue biopsies but GNPs were not observed in the mitochondria, Golgi complex or nucleus. Additionally, 15 nm GNPs were observed in the healthy and tumor brain tissues by electron microscopy at all time points, up to 6 days after GNP injection. The clinical signs of toxicity was not seen during the observation period on any mice which received the lowest concentrations of GNPs. At the same concentration of GNPs for different sizes the smaller ones are found to be more toxic than the larger ones *in vivo*.

Joh et al. [38] coated 12 nm gold cores by PEG (polyethylene glycol) to make GNPs of hydrodynamic diameter of 23 nm. These GNPs were injected intravenously in female athymic mice bearing the most prevalent and aggressive primary brain tumor U 251. 48 hours post injection of pegylated GNPs (1.25 grams of gold per kg of mouse), the brain of the mice was given a radiation dose of 20 Gy from a 175 kVp small animal radiation research platform. The combined treatment of GNPs and radiation therapy increased the DNA damage to brain blood vessels *in vivo*, resulting in increased survival of mice and delayed tumor growth. Joh et al. interpret these results as suggesting that radiation-induced blood brain barrier disruption can be leveraged to improve the tumor-tissue targeting of GNPs, which would further optimize the radiation enhancement of brain tumors by GNPs. The GNP toxicity *in vivo* was very small in this study, as shown by the preliminary data.

Zhang et al. [21) studied size-dependent radiation enhancement using PEG-coated GNPs. PEG-coated GNPs of sizes 4.8, 12.1, 27.3 and 46.6 nm (concentration 4 mg of gold per kg of mouse) were injected intraperitoneally to female BALB/C mice bearing U14 tumors. The tumors were then irradiated by 5 Gy of gamma radiation. Mice were sacrificed after 24 days. The results indicated that all sizes of PEG-coated GNPs decreased the tumor volume and weight after 5 Gy of radiation, but 12.1 and 27.3 nm PEG coated GNPs induced appreciable decreases of tumor volume and weight, indicating that these sizes of particles have greater radiation enhancement effects compared to 4.8 and 46.6 nm particles. The toxicity *in vivo* was appreciably less on the basis of immune response and blood biochemistry. However the liver was slightly damaged. Also it was found that the GSH-protected GNPs had efficient clearance through the kidney.

Kim et al. [34]: Kim et al. studied the effects of proton radiation combined with gold and iron nanoparticles in mice. Gold nanoparticles (coated with DTPA, diethylenetriamine pentaacetic acid-cysteine conjugate) or iron nanoparticles (coated with alginate) of sizes 14 ± 1.2 nm and 10.6 ± 0.8 nm respectively were injected intravenously to Balb/c mice with CT26 tumors either on their leg or flank. The injected dose of particles was either 100 or 300 mg of metal per kg mouse. 24 hours after injection, proton irradiation was given with radiation doses of 10-41 gray. Two different strategies of proton radiation were employed: protons that were absorbed in the mouse (with the Bragg peak located on the tumor) and protons that traversed through the mouse.

The results for tumor uptake showed that 15 minutes after injection of particles the tumor concentration was 137.4 ± 50.2 micrograms of gold per gram of tissue or 56.6 ± 18.2 micrograms of iron per gram of tissue, while the corresponding muscle concentration were 7.5 micrograms of gold per gram and 6.5 micrograms of iron per gram tissue. The tumor to normal tissue ratio was 18.3 for gold and 8.7 for iron after 15 minutes; after 24 hours it was 169.7 for gold and 88 for iron, thus

enabling enhanced tumor dose deposition. The ratio of tissue uptake to total injected dose was less than 1% after administration of both 100 and 300 mg of metal per kg of mouse.

In the irradiation experiment, mice receiving a gold or iron nanoparticle injection prior to various doses from the proton beam demonstrated 58% (absorbed protons) or 64-100% (traversing protons) long-term survival. All animals that were not given radiation died in 2-4 weeks. All proton radiation alone groups showed slowed tumor growth and resulted in only 13% (absorbed protons) or 11% (traversing protons) long-term remissions.

Complete tumor regression (CTR) in mice showed a direct dependence on proton and nanoparticle doses. Either 45 Gy proton alone or 21 Gy irradiation with 300 mg/kg magnetic nanoparticles injections produced 100% CTR in mice. *In vitro* experiments showed an increase in the generation of reactive oxygen species from the metallic nanoparticle and proton radiation treatment.

Chen et al. [8]: Chen et al. exploits the potential of BSA capped GNPs as an efficient sensitizer for glioblastoma, both *in vitro* and *in vivo*, on radiotherapy. Clonogenic assay was performed on U87 glioblastoma cells with or without BSA - GNPs of 28nm hydrodynamic diameter with a series of doses in between 0-8 Gy at 160kVp X- ray. Also 250 μL of 1.3mg mL^{-1} BSA-GNPs was injected intravenously to the mice model having U87 glioblastoma tumor of diameter 0.8-1 cm. The mice were then irradiated by 160kVp X rays with a dose of 3Gy after 2hr and 2Gy after 24 hr of treatment. The tumor volume was calculated every alternate day .All the mice were euthanized after 20 days of the treatment and the tumors were weighted.

The *in vitro* RT showed that the percentage of cell apoptosis was larger for BSA-GNPs + RT followed by RT alone then BSA-GNPs and the minimum percentage of apoptosis was for the control group. The *in vivo* RT showed that the relative tumor volume after 20 days of treatment was maximum for the control group and minimum for BSA-GNP + RT. The data obtained inferred that BSA-GNP + RT showed maximum tumor regression while X ray alone slowed down the tumor growth while BSA-GNP alone didn't affect tumor growth compared to control group of mice.

The weight of tumor for 4 different cases were in accordance with the results obtained for relative tumor volume, meaning that the weight of tumor was minimum for BSA-GNP-RT and maximum for the control group. There was rapid clearance of GNP level from the mice after the administration of BSA-GNPs and the *in vivo* toxicity of BSA-GNPs was determined by ICP-AES analysis of GNP level after the treatment of BSA-GNPs. This analysis showed no toxicity.

Nanoparticle Targeting

Successful targeting increases the likelihood that each gold nanoparticle will reach the tumor. Thus, there is the potential for targeted gold nanoparticles to improve the radiation enhancement effect. This is particularly true when the primary benefit from gold nanoparticles comes from Auger electrons, which have a short range, as discussed in the introduction. In addition to locating gold nanoparticles to cells, the resulting intercellular localization is also important, as discussed above. Targeting strategies can be divided into two categories: those that use cancer-targeting molecules, and other methods that do not.

Cancer cell targeting molecules

More specific tumor targeting can be done by surface conjugation (attachment) of antibodies, peptides and other tumor targeting molecules [12]. This can improve the therapeutic index (16, 40). Conjugating gold nanoparticles with targeting molecules enhances the interaction of the GNPs with the cell surface by enabling the GNPs to bind to the cell surface receptors and enter cells by receptor-mediated endocytosis [16]. A non-exhaustive list of targeting molecules used with gold nanoparticles (either *in vitro* or *in vivo*) includes the following strategies:

Glucose capped GNPs are designed to take advantage of an increased cancer cell requirement for glucose in order to target the cell cytoplasm [27].

pH-Low Insertion Peptide (pHLIP® peptide) conjugated 1.4 nm GNPs target tumor acidity, which is achieved in a result of membrane-associated folding of pHLIP® peptide . Peptides of the pHLIP® family can tether cargo nanoparticles to the surface of cells in diseased tissues, and it can move cell-impermeable cargo molecules across the membrane into the cytoplasm [40-42]. pHLIP® peptide has been shown to increase uptake of gold by a factor of approximately 5-10 in mouse tumors [15].

Antibodies such as trastuzumab have been successfully used to modify GNPs. Trastuzumab conjugated GNPs has been used for targeting MDA-MB-361 tumors in athymic mice, and combined with x-rays the tumors were reduced to half of their volume at 4 months compared with the treatment by x-rays alone [26].

GNPs functionalized with RGD peptide (Arg-Gly-Asp), NLS (Nuclear Localization signal) peptide (H-Cys-Gly-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Ala-Pro-OH) and pentapeptide (H-Cys-Ala-Leu-Asn-Asn-OH) were shown to enhance tumor uptake of GNPs [25].

The conjugation of RGD peptides to radiolabeled GNPs produced biocompatible and stable multimeric systems with target-specific molecular recognition. The properties listed below which are demonstrated by ^{177}Lu-GNP-RGD compared to the other radiopharmaceuticals make it suitable to be used as a molecular targeting radiotherapy agent. ^{177}Lu-GNP-RGD leads to significant reduction in VEGF gene expression, helps to reduce tumor metabolic activity, induces less tumor progression, fewer intratumoral vessel, yields more uptake and retention in tumor [43].

Choi et al. [44] modified the surface of 50 nm GNPs with PEG and transferrin (a tumor targeting ligand) to make particles of size 80 nm. $4.5x10^{11}$ particles were injected IV to female A/J mice containing subcutaneous Neuro2A tumors, and all organs were collected after 24 hours. The results showed that the GNP localizations within a particular organ are influenced by the transferrin content whereas the nanoparticle accumulations in the tumors and other organs are independent of transferrin.

Shah et al. [45] found that 30 nm PEG-coated GNPs interact with blood cells *in vivo*, which results in longer blood circulation that correlates strongly with tumor uptake. In tumors, accumulation was increased by 10 times using GNPs conjugated with a bioactive ligand (tumor necrosis factor) compared to untargeted GNPs.

In vivo radiation enhancement results for targeted gold nanoparticles

To the author's knowledge, there are only a few papers currently existing where targeted gold nanoparticles are used to enhance radiation effects on tumor *in vivo*.

Chattopadhyay et al. [26] used 30 nm GNPs conjugated with monoclonal antibody transtuzumab (AuT) to target the human epidermal growth factor receptor-2 (HER-2). Female athymic CD1nu/nu mice bearing MDA-MB-361 cells were intra-tumorally injected with 4.8 mg/g of AuT (0.8 mg of GNPs per gram of mouse) followed by 11 Gy, 100 KVp x-rays after 24 hours. They found a 46% reduction in tumor volume at 4 months as compared to treatment with x-rays alone (16% increase in tumor volume).The analysis of the body weight index curves for different mice groups revealed no normal tissue toxicity by the use of Au-Ts with RT.

Su et al. [16] used 20nm GNPs conjugated with clinically used therapeutic radionuclide Iodine-125 labeled to cRGD as a tumor targeted radiosensitizer. IV injection of 100uL (containing 1mg Au) was given to Balb/c mice bearing NCI-H446 lung tumors. Co-60 source, 5Gy γ rays were used to irradiate the tumor tissues. RT effect was assessed by IV injection of Tc-99m-Annexin V (18.5MBq/mouse) after 2 days of treatment for evaluation of apoptosis induced by radiosensitized RT and SPECT performed.

The degree of apoptosis which is shown in numerical value was measured for 5 different groups and the results showed that there was a significant difference between targeted radiosensitizer based RT (cRGD-GNP-RT) (9.8±2.7) and non-targeted radiosensitizer (GNP+RT) based RT (5.5±1.4) (P=0.011). Also, a significant difference was seen between radiolabeled (I-125) cRGD-GNP-RT (11.2±2.1) and non-targeted radiosensitizer based RT (5.5±1.4) (P<0.01). However, a significant difference was not seen between the treated and untreated cases between I-125- cRGD-GNP-RT (11.2±2.1) and cRGD-GNP-RT(9.8±2.7) (P=0.093). This showed that the radiosensitivity was enhanced by the targeting effect. The above results show that I-125 showed the therapeutic effect but the improvement compared to cRGD-GNP-RT was not statistically significant. However the proper choice of a more effective radionuclide like I-131 can heavily enhance the therapeutic effect.

After 21 days, the percentage volume increase in tumor for different groups of mice was also measured and found that it was maximum in control (312.1%±96.9%), then in RT alone (137.1%±35.5%), then in GNP+RT(85.5%±44.2%). However the increase in tumor volume was suppressed to 33.1%±17.1% for cRGD-GNP-RTand was even less increase (15.2%±17.8%) for the I-125- cRGD-GNP-RT.

Also, the functionalized PEG which was used in this research showed good stability and clearance avoiding the uptake by RES. The *in vivo* toxicity of PEG covered GNPs and cRGD-GNPs was found to be low which was verified because there was no obvious loss of weight of mice.

Popovtzer et al. *[32]* used 1 mg of cetuximab(CTX) alone or 200uL; 25mg mL^{-1} Au of 30nm with I_gG or CTX coating, injected IV into the tail vein of mice having a A431 head and neck cancer model of diameter 10mm, then irradiated after 24 hrs with 25 gray from a 6MV,x-ray. In contrary to the results obtained by Hainfeld et .al where there was the shrinkage of tumor, here the results showed that there was no increase in tumor diameter at all for CTX-GNP+RT compared to 1.0cm increase in tumor diameter for the control case, 0.4cm for CTX+RT, 0.6 for I_gG-GNP+RT, 0.3cm for RT only and 0.9cm for CTX only cases. This shows that the radiation is enhanced by the tumor targeted GNPs. A set of experiments to study the biological mechanism of radiosensitized GNPs was done. Decreased vasculatization in tumors was seen after 1 and 6 weeks of the treatment in CTX-GNP+RT group than control, RT only and CTX+RT groups. Also, tunnel assay results showed that apoptosis was higher after 1 week and that there was less apoptosis after 6 weeks of treatment in CTX-GNP+RT group compared to RT only group. And other results showed that the level of proliferation and tissue repair was reduced in the CTX-GNP+RT group compared to other groups.. Further, no cytotoxic effect was seen on the mice.

Conclusion

The papers reviewed in this article demonstrate the potential effectiveness of gold nanoparticles in the enhancement of radiation of tumors. Major results and methodologies are summarized in Table 1. Future experiments with gold nanoparticles and radiotherapy will likely involve the following areas: trials in humans, experiments using targeted gold nanoparticles and different radiation energies/types.

The eventual goal of gold nanoparticle treatments is to become viable for use in humans. One potential roadblock is that treatment with kilo-voltage x-rays is only capable of penetrating human tissue to a shallow depth. Perhaps trials using this treatment could be done starting with melanoma or other tumors, which could be accessed via catheterization, and future advances in engineering could help to eliminate this roadblock.

Table 1. Summary of major *in vivo* experimental results.

First author	Year	Animal model	Tumor model	Coating of GNPs	Size GNPs, nm	Time to RT	Radiation	Dose, Gy	Group	Route and Dose of Administration
Hainfeld	2004	Balb/C mice	EMT-6: murine mammary carcinoma		1.9	2 m	x-rays 250 kVp	30 26	Control Au only RT only Au+RT	IV injection 1.35 g Au/kg 2.7 g Au/kg
Chang	2008	C57BL/6 mice	B16F10: murine melanoma		13	24 h	electrons 6 MeV	25	Control Au only RT only Au+RT	IV injection 1 g Au/kg
Hainfeld	2010	C3H/HeJ mice	SCCVII: head and neck squamous carcinoma		1.9	1 m	x-rays 68 keV 157 keV	30 42 44 50.6	RT only Au+RT	IV injection 1.9 g Au/kg
Chattopadhyay	2012	CD1 nude mice	MDA-MB-361: human breast adenocarcinoma	Trastu-zumab	30	24 h	x-rays 100 kVp	11	Control Au only RT only Au+RT	Intratumoral (IT) injection 0.8 mg Au or 4.8 mg/g tumor
Zhang	2012	Female BALB/c mice	U14: murine cervical carcinoma	PEG	4.8 6.6 12.1 27.3	Exact time not given (soon after the injection of GNP)	Γ-rays	5	Control Au only RT only Au+RT	Intraperitoneal(IP) injection 4 mg/kg
Bobyk	2013	Fischer rats	F98: rat glioma		9 15	20 m	x-rays 88 keV	15	Control Au only RT only Au+RT	Intracerebral infusion (5 μL) 25 mg/mL 50mg/mL
Hainfeld	2013	B6C3f1 mice	Tu-2449: murine highly malignant brain tumor		11	15 h	x-rays 100 kVp	30 35	Control Au only RT only Au+RT	IV injection 4 g Au/kg
Joh	2013	nude female athymic mice	U251: human glioblastoma	PEG	23	48 h	x-rays 175 kVp	20	Control Au only RT only Au+RT	IV injection 1.25 g Au/kg
Kim	2012	Balb/c mice	CT26: murine colon carcinoma	DTPA+ cysteine FeNPs+ alginate	14 10.6	24 h	proton 45 MeV	10 - 41	Control Au only RT only Fe only Au+RT Fe+RT	IV injection 100 mg/kg 300 mg/kg

Chen	2015	Nude athymic mice	U87:glioblastoma	BSA	28	2h 24h	X-rays 160kVp	3 2	Control BSA+Au RT only BSA+Au+RT	IV Injection 250uL, 1.3mgmL^{-1}
Su	2015	Balb/c mice	NCI-H446:human lung carcinoma	Iodine-125+cRGD	20	4h	Γ-rays	5	Control RT only Au+RT cRGD+Au+RT ^{125}I+cRGD+Au+RT	IV Injection 100uL, 1mg Au 50mg/kg
Popovtzer	2016	Nude mice	A431: squamous head and neck carcinoma	I_gG CTX	30	24	X-rays 6MV	1.4/min	Control RT only CTXonly CTX+RT I_gG-Au+RT CTX-Au+RT	IV Injection 200uL; 25mg mL^{-1}

The roadblock mentioned in the paragraph above may also inspire more work with different radiation energies and radiation types. For example, the result of Kim et al. [35] with protons seems particularly promising. Additionally, *in vitro* studies [6,10] have shown radiation enhancement with gold nanoparticles and higher energy photons, although the enhancement is generally somewhat less than kilo-voltage photon results.

Regardless of the radiation type, it appears that tumor targeting will be of great use in this type of therapy. To conclude, we can say that GNPs modified with tumor targeting agents as pHLIP, cetuximab, cRGD and trastuzumab successfully enhanced the radiosensitization of GNPs. In the future, this could lead to more effective clinical radiotherapy with less toxicity [7, 16, 26,32]. Additional trials with other targeting methods would be beneficial and important.

In summary, gold nanoparticles are a promising research area with the potential to reduce the amount of radiation necessary in cancer treatments. Successful experimental work has already been done in this area, including work in mammals. More work is needed, and this future work has the potential of pushing the field into clinical relevance.

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