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Radiation dose enhancement of gadolinium-based AGuIX nanoparticles on HeLa cells

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Abstract

Radiation dose enhancement of high-Z nanoparticles is an active area of research in cancer therapeutics. When kV and MV energy photon beams interact with high-Z nanoparticles in a tumor, the release of secondary electrons can injure tumor cells, leading to a higher treatment efficacy than radiation alone. We present a study that characterizes the radiation dose enhancing effects of gadolinium-based AGuIX nanoparticles on HeLa cells. Our *in vitro* clonogenic survival assays showed an average dose enhancement of 1.54× for 220 kVp radiation and 1.15× for 6 MV radiation. The sensitivity enhancement ratio at 4 Gy (SER4Gy) was 1.54 for 220 kVp and 1.28 for 6 MV, indicating that these nanoparticles may be useful for clinical radiation therapy.

From the Clinical Editor: This study characterized the radiation dose enhancing effects of gadolinium-based AGuIX nanoparticles on HeLa cells, showing clear effects at 220kV as well as 6MV, suggesting that after additional studies, these nanoparticles may be beneficial in human radiation therapy.

Key words: Radiation oncology; Dose enhancement; Nanoparticles

Background

The use of nanoparticles in cancer research has enabled the design of diagnostic and therapeutic tools smaller than 1000 nm in size, on the length scale of cancer physiology.[1] Gold nanoparticles have been the subject of considerable therapeutic development because gold is biocompatible and has been approved for treatment in some diseases, such as rheumatoid arthritis.[2,3] The ability of gold nanoparticles to bind amine and thiol groups makes them useful for linking drugs to targeting agents, such as antibodies, for more precise tumor cell targeting. Heating gold nanoshells with near-infrared light in the vicinity of a tumor can selectively overheat the tumor tissue, causing cell death.[4] Furthermore, with a high atomic number and higher mass attenuation than iodine at energies above 80 keV, gold nanoparticles have been shown to be a promising radiocontrast agent, with 2.7 times more contrast per weight than iodine. In one study, researchers were able to distinguish blood vessels less than 100 μm in diameter by injecting 1.9 nm gold nanoparticles into mice intravenously. The uptake of these nanoparticles was 3.4 times higher into tumors than into muscle tissue 15 minutes after injection, making these nanoparticles especially useful for diagnostic and prognostic tumor imaging.[5] Another study reported 114% greater contrast to noise ratio with gold nanoparticles compared to iodine at 140 kVp.[6]

The high atomic number of gold (Z = 79) also provides dose enhancement during radiation therapy. When x-rays interact with gold nanoparticles, Auger electrons and photoelectrons are emitted, which have a range from the nanometer-scale to several micrometers. While these interactions have a higher cross-section at lower (kV) energies, conventional external beam radiation therapy is most often administered using higher energy (MV) photons.[2,7-12]

In vitro clonogenic survival assays have shown dose enhancement at 10% survival ranging from 1.43-1.66× for gold nanoparticle concentrations of 1 nM and 105-220 kVp beam energies.[13] Although Cho showed that Monte Carlo simulations predict a negligible dose-enhancement with MV radiation, ranging from 1-7% for 4 and 6 MV energy beams,[14] several *in vitro* studies have shown dose enhancement as high as 1.29× with 6 MV x-ray beams.[2,11,13,15] While it is widely

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We have no conflicts of interest to report.

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believed that these experimental results are due to the physical photoelectric and Auger processes, it has been suggested that, apart from their high-Z properties, gold nanoparticles may have additional biological effects that also cause radiosensitization.[2]

In *in vivo* studies, administering gold nanoparticles in combination with radiation treatments has been shown to increase the survival of mouse xenograft cancer models. In one study, 86% of Balb/c mouse tumor models that were administered 2.7 g Au/kg intravenously before 250 kVp radiation treatments survived one year after treatment, compared to 20% of mice that were administered radiation alone, and 0% of mice that received no treatment.[16] While many researchers have focused on gold's dose enhancing effects on tumor cells themselves, others have suggested that dose enhancement to the endothelium, which would cut off a tumor's blood supply, may be a more important mechanism for reducing tumor size.[8,17,18] Although the success of these initial studies indicates promising clinical potential for gold nanoparticles in radiation treatment of cancer, the cost of the raw materials (gold) could be prohibitively expensive. While the price varies by manufacturer, gold nanoparticles can cost nearly $200/mg (equivalent to about $39.4/μmol), while gadolinium nanoparticles, also a high-Z element, are currently less that $1/mg (equivalent to less than $0.16/μmol).

Gadolinium nanoparticles offer many of the same therapeutic advantages as gold nanoparticles at a significantly reduced cost. Gadolinium-based compounds have been shown to be an effective MRI contrast agent, and are commonly used in clinical imaging.[19] Gadolinium-based complexes have also been shown to be effective agents for enhancing neutron-capture therapy in treating cancer.[20] B16F10 melanoma tumor xenograft mice that were treated with chitosan nanoparticles loaded with gadolinium survived over 40% longer than mice that received neutron irradiation alone.[21] However this potentially useful therapy is scarcely practiced, likely because the neutron sources required for neutron-capture therapy are uncommon.

Mowat et al have demonstrated that gadolinium nanoparticles may also have a dose enhancing effect when used with radiation.[22] By testing the effects of these nanoparticles on an *in vitro* radioresistant U83 cell line, the nanoparticles showed dose-enhancing effects with both kV and MV beam energies.

The goal of this article is to measure the cellular uptake of a gadolinium-based nanoparticle and quantify the dose enhancing effects at kV and MV energies in HeLa cells, a more general cancer cell model.

Methods

Gadolinium nanoparticles

Dehydrated, spherical, sub-5 nm gadolinium nanoparticles (AGuIX) were obtained from Nano-H (Lyon, France). The nanoparticles consist of gadolinium atoms attached to a polysiloxane shell via DOTAGA chelating agents. Nanoparticles were rehydrated in sterile, DEPC Treated Water (Invitrogen, USA) and stored at 4 °C until use per manufacturer's instructions.

Cell culture

HeLa cells (ATCC, USA) were cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% fetal bovine serum (Sigma, USA) and 1% penicillin-streptomycin-glutamine (Invitrogen, USA). Cells were stored in a humidified incubator at 37 °C and 5% CO_2.

Inductively couple plasma-mass spectroscopy

Cells were seeded in six-well plates at $4.0 * 10^4$ cells per well and allowed to grow for 18 hours. The cells were then incubated with 0.5 mM AGuIX, diluted in cell culture medium, for one hour, based on the experimental procedure by Mowat et al.[22] After incubation, the cells were washed with 1× PBS, trypsinized, counted, centrifuged, and then lysed with Ripa Buffer (Sigma, USA) for 30 minutes. The lysate was centrifuged, the supernatant was removed, and the lysate was resuspended in 1× PBS before processing.

Gadolinium concentrations were measured on a VG Plasma Quad Excell ICP-MS (Boston University, Department of Earth and Environment). The samples were introduced to the instrument in solution form, through a Meinhard-C concentric nebulizer at a flow rate of ~1 mL/min. The four highest abundance Gd isotopes (^{156}Gd, ^{157}Gd, ^{158}Gd and ^{160}Gd) were monitored, as well as ^{163}Dy to check for potential isobaric interferences on Gd from Dy (which were negligible). Instrumental drift was monitored and corrected for by analyzing a 1 ng/g Gd standard at various times throughout the run (every 5 analysis items). Calibration curves were generated by analyzing Gd standards of varying concentrations (from 10 pg/g to 500 pg/g) interspersed throughout the analytical run. These curves all had r^2 values of 0.999 or greater. Final Gd concentrations were determined from averaging the concentration data garnered from the calibration curves from all four Gd isotopes measured.

Irradiation setup

For 220 kVp irradiation, cells were irradiated using a 35 cm source-to-surface distance (SSD) and a dose rate of 270 cGy/min on a Small Animal Radiation Research Platform (SARRP) using a Varian x-ray tube source.[23] This energy was selected because our *in vivo* experiments are performed at the same energy. For 6 MV irradiation, cells were treated on a clinical linear accelerator (Varian Medical Systems, Inc. Palo Alto, CA) at a depth of 10 cm, 90 cm SSD. Doses ranged from 0 to 8 Gy for both setups. This energy was selected because of its direct clinical applications.

For both energies, absolute dose was measured using an ADCL-calibrated ion chamber. For the MV energies, the AAPM TG-51 procedure was used, and for the kV, the AAPM TG-61 procedure was used. For MV and kV dosimetry, daily uncertainty was +/− 3%.

Full scatter conditions were met using 30 × 30 cm slabs of energy-appropriate solid water (CIRS, Inc.) as well as tissue-equivalent bolus material surrounding the well plate. kV Irradiations were performed at 0 cm depth. MV Irradiations were performed at 10 cm depth.

Clonogenic survival assays

Cells were seeded in six-well plates at $4.0 * 10^4$ cells per well and allowed to grow for 18 hours. The cells were incubated with 0.5 mM AGuIX, diluted in cell culture medium, for one hour. The cells were then irradiated according to the specifications

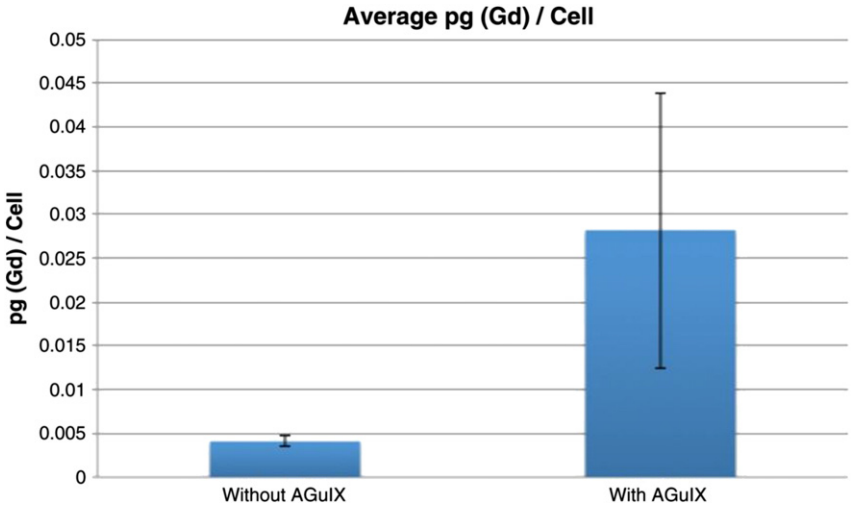

Figure 1. HeLa cells uptake AGuIX nanoparticles *in vitro*. Analyzing cell lysates of HeLa cells with ICP-MS shows that following one hour of incubation, HeLa cells uptake the AGuIX nanoparticles *in vitro*.

above. After irradiation, cells were incubated for another 4 hours. Afterwards, the cells were washed with PBS, trypsinized, and counted. The cells were then replated in 100 mm dishes at 400 cells per plate and allowed to grow for 14 days, before staining with a 1% crystal violet, 10% ethanol dye solution. The plates were allowed to dry overnight before being digitally scanned and manually counted for colonies. The cell counts from the replicates, four for the kV assays, and three for the MV, were fit with a linear-quadratic model by plotting the data on a log (% survival) vs dose plot, and using the MATLAB lsqcurvefit function to fit model parameters.

Results were reported as two measurements: dose enhancement and a sensitivity enhancement ratio at 4 Gy (SER4Gy). The dose enhancement is a measure of the gain in effective radiation dose caused by the nanoparticles. It is calculated as a ratio of the area under the curve between the log-linear plots of cell survival with and without AGuIX nanoparticles. The SER4Gy is a measure of the nanoparticles' cell killing enhancement at a specific dose, calculated as the ratio of cell survival without and with AGuIX at 4 Gy.

Results

Inductively coupled plasma-mass spectrometry (ICP-MS)

Using ICP-MS, we quantified the uptake of AGuIX in HeLa cells at an extracellular concentration of 0.5 mM (Figure 1). This concentration was chosen based on the results of previous work.[22] Assuming an average HeLa cell volume of 2600 μm^3,[24] the intracellular concentration of gadolinium after one hour of incubation, minus the background detection from the control, was 0.059 mM.

kV Irradiation

Next, we irradiated the cells with doses between 2 and 8 Gy after incubating the HeLa cells with or without the nanoparticles, and characterized their reproductive viability using a clonogenic assay. The cell survivals were plotted and a trend line was fit to the results using a linear-quadratic model. The results from experiments with 220 kVp x-ray radiation show that cells incubated with AGuIX nanoparticles are more sensitive to radiation than control cells (Figure 2). The log-linear plots in these studies show the mean percent survival across the experimental replicates (four replicates for the kV assays and three for the MV). For each replicate, the irradiated cells were plated in three separate dishes, and the percent survival was calculated as the mean survival of the three plates, relative to unirradiated control plates. The error bars represent the standard deviation of the calculated means for all of the replicates. The sensitivity enhancement ratio at 4 Gy (SER4Gy) for this study, calculated as the ratio of cell survival without and with AGuIX at 4 Gy, was 1.54. The SER4Gy measures the increase in cell killing by the nanoparticles at a specific dose.

MV Irradiation

Using the same method as for kV irradiation, we used a linear-quadratic model to plot our clonogenic assay results (three replicates) for HeLa cells treated with 6 MV x-ray radiation with and without AGuIX (Figure 3). The SER4Gy for these cells was 1.28.

Discussion

ICP-MS

Our results show that after one hour of incubation, the intracellular concentration of gadolinium was within an order of magnitude of the extracellular concentration (0.059 mM compared to 0.5 mM, respectively). However in Mowat et al's published study, they found that the concentration of gadolinium inside U87 cells was 12 times larger than in the media following a one-hour incubation. Similar variations in uptake of Gd-based nanoparticles between cell types have been observed in studies

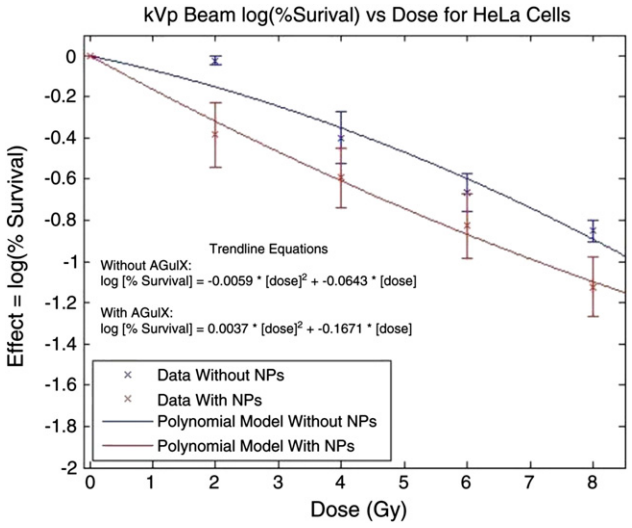

Figure 2. AGuIX causes 1.54× average dose enhancement on HeLa cells irradiated with 220 kVp x-rays between 0 and 8 Gy *in vitro*. The plot shows the linear-quadratic cell survival curves for HeLa cells incubated with and without AGuIX nanoparticles. Regression analysis was used to fit the data to the linear-quadratic model log [% Survival] = α * [dose] + β * [dose]^2.

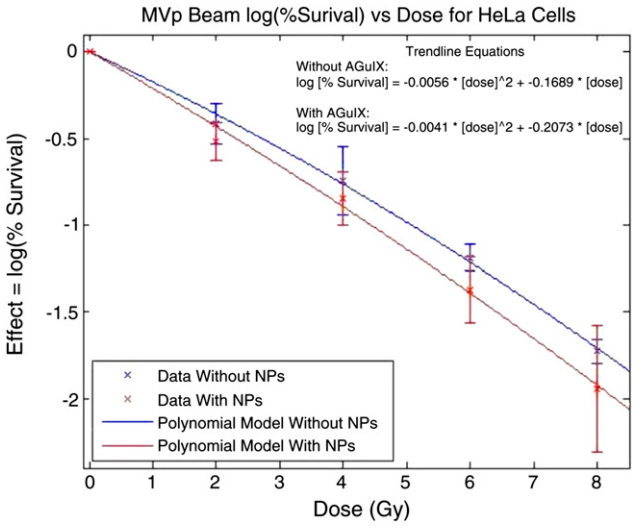

Figure 3. AGuIX causes 1.15× average dose enhancement on HeLa cells irradiated with 6 MV x-rays between 0 and 8 Gy *in vitro*. The plot shows the linear-quadratic cell survival curves for HeLa cells incubated with and without AGuIX nanoparticles. Regression analysis was used to fit data points to the linear-quadratic model log [% Survival] = α * [dose] + β * [dose]^2.

by the same group,[22,25] suggesting that uptake efficiency is cell type dependant. A similar effect is seen in gold nanoparticles, with the uptake efficiency and location of absorbed nanoparticles varying between cell lines.[2] More studies are necessary to determine the mechanism of AGuIX uptake and its efficacy across cancer types.

kV Irradiation

To estimate the average dose enhancement for the doses tested, we calculated a ratio between the area under the curve for the AGuIX and control survival curves. The area under the curve estimates the average survival for each condition across the doses used; a ratio between the average survival of cells with and without AGuIX allows us to calculate the average dose enhancement of the nanoparticles between 0 and 8 Gy. Using this method, we determined that the nanoparticles confer a 1.54× average dose enhancement to the HeLa cells with 220 kVp x-ray radiation. However the radiosensitization varied between radiation doses. For instance at a survival of 48%, an approximately 1.85× dose enhancement was observed. These findings suggest that AGuIX could be an effective dose-enhancer for low energy radiation therapies.

MV Irradiation

In HeLa cell clonogenic assays with 6 MV irradiation, we observed that the AGuIX nanoparticles cause an average dose enhancement of 1.15×. This is roughly the same magnitude as has been observed by others with gold nanoparticles under similar experimental conditions.[2,13,15] Furthermore at a survival of 90%, the AGuIX dose enhancement for HeLa cells was 1.22×. The unexpected similarity to the gold nanoparticle results may be due to an inherent uptake advantage of the AGuIX platform. The similarity may also be due to the relatively high concentration of nanoparticles used, compared to studies with gold nanoparticles. This hypothesis will be the subject of further study.

Other studies with gold nanoparticles, such as Rahman et al (2009), reported dose enhancement factors at 90% survival as high as 2.7-4.0 at 6 MeV, depending on the concentration of nanoparticles used.[11] While this seems significantly larger than our average dose enhancement, this study was done with a different radiation source (electrons, as opposed to x-rays) and a different cell line, which multiple studies, including Jain et al (2011), have shown can affect the dose enhancement by as much as 3-4×.

The observed difference in the dose enhancement between the kVp and MV energies supports our hypothesis that dose enhancement is primarily caused by photoelectrons. The probability of photoelectric interaction is inversely proportionate to the cube of the photon energy. While both the 220 kVp and clinical 6 MV photon beams studied in this work are composed of a spectrum of photon energies, the 220 kVp photon beam has substantially more low energy (high photoelectric interaction probability) photons compared to the clinical 6 MV photon beam.[2,11,13,15]

The results of this study suggest that gadolinium-based AGuIX nanoparticles could be an attractive alternative to gold nanoparticles for improving the efficacy of radiation cancer treatments. Furthermore, *in vivo* toxicology studies with tracheal administration of 50 mM Gd-based contrast agents in Balb/c mice have also shown favorable pharmacokinetics, low inflammatory cell recruitment at the site of administration, and negligible retention in the reticuloendothelial system, suggesting low nanoparticle toxicity.[26] Future studies will be required to confirm the nanoparticles' therapeutic effects *in vivo*.

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