Nanoparticle Mediated Tumor Vascular Disruption:

A Novel Strategy in Radiation Therapy

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Supporting Information

Materials and Methods

Synthesis and characterization of targeted gold nanoparticles

Targeted gold nanoparticles (RGD:AuNP) were prepared by standard procedures. Briefly, colloidal gold was prepared by the reduction of gold salt (chloroauric acid) in the presence of a stabilizing/ reducing agent, tetrakis (hydroxymethyl) phosphonium chloride (THPC). THPC-stabilized gold nanoparticles were further PEGylated (by ligand exchange process) using a mixture of thiolated -SH-(PEG) derivatives. The PEG-stabilized gold nanoparticles were further subjected to EDC coupling at room temperature to conjugate *Arg-Gly-Asp* (RGD) and a near infrared dye (AF647). The nanoparticles were subsequently purified using membrane-filtration technique. RGD-AuNP was subjected to membrane-dialysis by using ≈12-14 kDa cellulose membrane against purified double-distilled water to remove any traces of un-reacted EDCI or other hydrolyzed products along with the un-reacted RGD molecules. The final solution consisted of purified RGD:AuNP nanoparticles. The targeted, optically viable RGD:AuNP was further characterized for particle size (using DLS and TEM), surface morphology (using TEM) and absorption/ excitation maximum read-outs (using spectrophotometry).

Scanning transmission electron microscopy

Tumor samples were excised from Panc-1 tumor-bearing mice treated -with and -without RGD:AuNP and subjected to irradiation at 24 h *post-i.v*. Soon after, the tumor samples were immersed in 10% neutral buffered formalin (≈4% formaldehyde) solution and further processed

for STEM imaging. Samples placed on the copper grids were covered with a carbon film prior to the analysis with JEOL 2100F in a STEM-HAADF (Scanning Transmission Electron Microscope- High Angle Annular Dark Field) mode at 200 kV. Optimal HAADF−STEM resolution was ≈1 nm. Energy dispersive x-ray spectroscopy (EDS) was performed with an Oxford X−max system, with an 80 mm^2 silicon drift detector (SDD) and energy resolution of 136 eV.

Transmission electron microscopy

High-resolution transmission electron microscopy (TEM EM 400T; Philips, The Netherlands) was carried out for the pancreatic tumor sections. Small tumor tissue fragments/ pieces (≈1-2 mm^3) were obtained from the dissected whole tumor, and fixed using a mixture of 2.5% glutaraldehyde in 0.2 M Sorensen buffer at pH: 7. Using ultracryotome, thin sections were sliced, and washed/ stained in aqueous uranylacetate (2%) for ≈2 h. The tissue was then placed in 4°C in dark conditions. The tissue was further dehydrated by 100% ethanol and propylene oxide to be embedded in liquid epoxy resin (Epon™). High-resolution electron microscopy was carried out at different magnifications ranging from 2500x - 30000x to visualize tumor blood vessels pre- and post-irradiations.

Laser-induced breakdown spectroscopy imaging

Laser-induced breakdown spectroscopy (LIBS) imaging was performed on paraffin-embedded tumor tissue sample. Briefly, a standard optical-imaging system is equipped with a LIBS laser injection line and a three-dimensional motorized platform for sample positioning. The LIBS experiment used fundamental Nd:YAG 1064 nm laser pulses of 5 ns, working at 10 Hz. The

measurements were performed at room temperature with accurate control of the focal distance between the objective and the sample. The laser pulses were controlled to deliver only one impulsion for each position in the sample. Each image was recorded with a resolution of 50 µm. The light emitted by the plasma plume was collected and guided using optical fibers through a Czerny-Turner spectrometer equipped with a 1200-L/mm grating blazed at 300 nm and an intensified charge-coupled device (ICCD) camera (Shamrock 303 and iStar, Andor Technology). The ICCD camera was synchronized with the Q-switch of the laser and the spectrum acquisition was performed with a delay of 250 ns, a gate of 4 µs and a gain of typically 90/255. The width of the entrance slit of the spectrometer was set to 35 µm. In this configuration, a spectral measurement range of 30 nm was accessible with a spectral resolution of ≈ 0.15 nm. Software developed in-house in the LabVIEW environment controlled the entire system and allowed for the performance of automated sequences to scan the region of interest of the tissue sample with a specific lateral resolution.

Small animal radiation research platform

A commercial small animal radiation research platform (SARRP, Xtrahl, Inc. Suwanee, GA) was used to perform the radiation studies in Panc-1 tumor xenograft bearing mice (6-8 mm^2) (n=3/cohort). Continuous isoflurane anesthesia (1-3%) was administered throughout each procedure. We irradiated at 10 Gy with a 220 kVp beam and 13 mA, using a 1.5 cm diameter collimator. The x-ray beams were exposed from two different orthogonal angles to minimize off-target irradiation. Furthermore, 3D dosimetry studies were carried out using Muriplan V.1.3.0, and the radiation dose distribution was measured for the tumor and the whole body. An ADCL-calibrated ion chamber was used to measure absolute dosimetry of the SARRP beam

and Gafchromic film (EBT3) was used to measure percentage depth dose and profiles. The full procedure for commissioning a low-energy X-ray source can be found in the American Association of Physicists in Medicine Report.

Inductively coupled plasma mass spectrometry

Samples (n=3) were digested using a combination of hydrochloric acid (HCl), nitric acid (HNO_3) and hydrogen peroxide (H_2O_2). All reagents used in the digestions were trace-element pure – either distilled in-house (HCl and HNO_3) or purchased at Optima grade (H_2O_2). Samples were initially digested in 3 ml HCl and 1 ml HNO_3 on a hot-plate at 100^{o}C overnight. Samples were removed from the hot-plate and 1 ml of H_2O_2 was added drop-by-drop (in order to prevent the vigorous reaction with organic matter), and placed back on the hot-plate at 100^{o}C overnight. The samples were dried and 2.5 ml of HCl was added, followed by another overnight heating on the hot-plate, which was then followed by the addition of 1 ml of H_2O_2. This step was repeated once. Finally, 0.3 ml of HCl and 0.25 ml of H_2O_2 were added and diluted to a final volume of 10 ml with milli-Q water (to make 3% HCl). Heart, bladder, spleen and tumor samples were further diluted a 100x with 3% HCl; whereas kidney, lung and liver samples required a 200x further dilution with 3% HCl to prepare for ICP-MS analysis. Gold concentrations were measured on a VG Plasma Quad Excell ICP-MS. The samples were introduced to the instrument in solution form, through a Meinhard-C concentric nebulizer at a flow rate of ≈1 ml/ min. Since gold is mono-isotopic, measurements were made on ^{197}Au. Instrumental drift was monitored and corrected for by analyzing a 1 ng/g Au standard at various times throughout the run (per 5 analysis items). A calibration curve was generated by analyzing Au standards of varying concentration (from 0.01 ng/g to 10 ng/g) interspersed throughout the analytical run,

and this curve had a r^2 value of 0.9999. Final Au concentrations were determined by comparing the signal intensity for the samples to the calibration curve.

Crystal violet assay

In vitro radiation effect of RGD:AuNP on human umbilical vein endothelial cells (HUVEC) was tested. HUVEC cells (≈10,000 cells/ well) were seeded on well plates and incubated with standard endothelial cell culture medium and allowed to grow for 1 week. After 4 h of incubation with RGD:AuNP (100 μgm/ well), irradiation (0 Gy, 5 Gy, 10 Gy) was performed. After irradiation, the cells were washed with PBS (7.4), and allowed to grow for an additional week. The cells were stained using 1% crystal violet and 10% ethanol dye solution. The plates were digitally scanned and the endothelial cells were observed in phase contrast microscopy before and after irradiation and the cells were counted. All measurements were performed in triplicates.

Animal tumor model and *in vivo* studies

All animal experiments were approved by the Institutional ethical committee, HMS IACUC (04903). Eight-week old NCrFox-nu/nu mice (Charles River Laboratories, Wilmington, USA) weighing ~25 g were fed with standard food pellets and water ad libitum. Mice were housed in ventilated cages and placed in clinically controlled room under customized conditions. Murine pancreatic adenocarcinoma cell line, Panc-1 (ATCC, USA) was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) and 1% Pen/ strep (10,000 U/mL penicillin; 10,000 μg/mL streptomycin, Invitrogen, USA), respectively. The culture was maintained at 37°C and

5% CO_2 and optimal humidity. Mice were inoculated with Panc-1 ($5x10^6$ cells / 100 µL) tumor cells by subcutaneous injection into the dorso-lateral right flank. A tumor size of ≈6-8 mm^2 was obtained in ≈1-2 months. Animals were conditionally inhalation anaesthetized during all experimental operations using isoflurane.

Confocal imaging

We visualized tumor blood vessels and nanoparticle accumulation 24 h post-IR using 3D-confocal imaging performed on a Zeiss 710 laser scanning confocal. FITC-dextran (70 kDa; 60 µL of 1 mg/ml) was injected into the mouse intravenously to label the blood vessels. The tumor was dissected carefully within 10-15 min after FITC-Dextran injection, attached to the bottom of a petri dish, immersed in PBS and imaged immediately on an upright Zeiss Examiner Z1 stand with a W-Plan Apochromatic 20x/NA 1.0 dipping objective. Excitation of FITC was performed with the argon laser at 488 nm and excitation of Alexa Fluor 647 tagged gold nanoparticles was performed with the 633 nm He-Ne laser. The emitted fluorescence signals were detected with photo multiplier tubes. Z-stacks of the tissue volume were collected and the 3D-reconstruction of tumor vasculature was carried out using Zeiss Zen software (Carl Zeiss Microscopy GmbH, Jena).

Histological examination

Multiple histological examinations were carried out to investigate the gold nanoparticle induced radiation effect on the tumor and tumor blood vessels. The harvested tumor tissue were fixed in 2% formalin (neutral buffered) and embedded in paraffin. Thin tissue slices (≈5µm) were cut, and the sections were mounted for antigen retrieval. Standard IHC steps of de-paraffinizing and

rehydrating was followed and the immunostaining (Leica Bond automated stainer) was carried out. Primary and secondary antibodies were used for CD34 (Abcam ab8158 / 1:100 and HRP) and phospho-histone γH2AX: ser139 from Cell signaling Technologies (#20E3 / 1:400) staining. The sections were also counterstained with Mayers hematoxylin. Following blocking and DAB steps, images were visualized using Zeiss (Axio Imager M2 microscope with a high-resolution Axiocam Mrm Rev.3 camera) at 20x and 100x magnification.

Statistical analysis

Results are presented as average ± standard deviation. Statistical analyses were performed using GraphPad Prism 5.01, employing the standard student's two-tailed t-test. $P<0.05$ was considered to represent statistical significance.

Figures

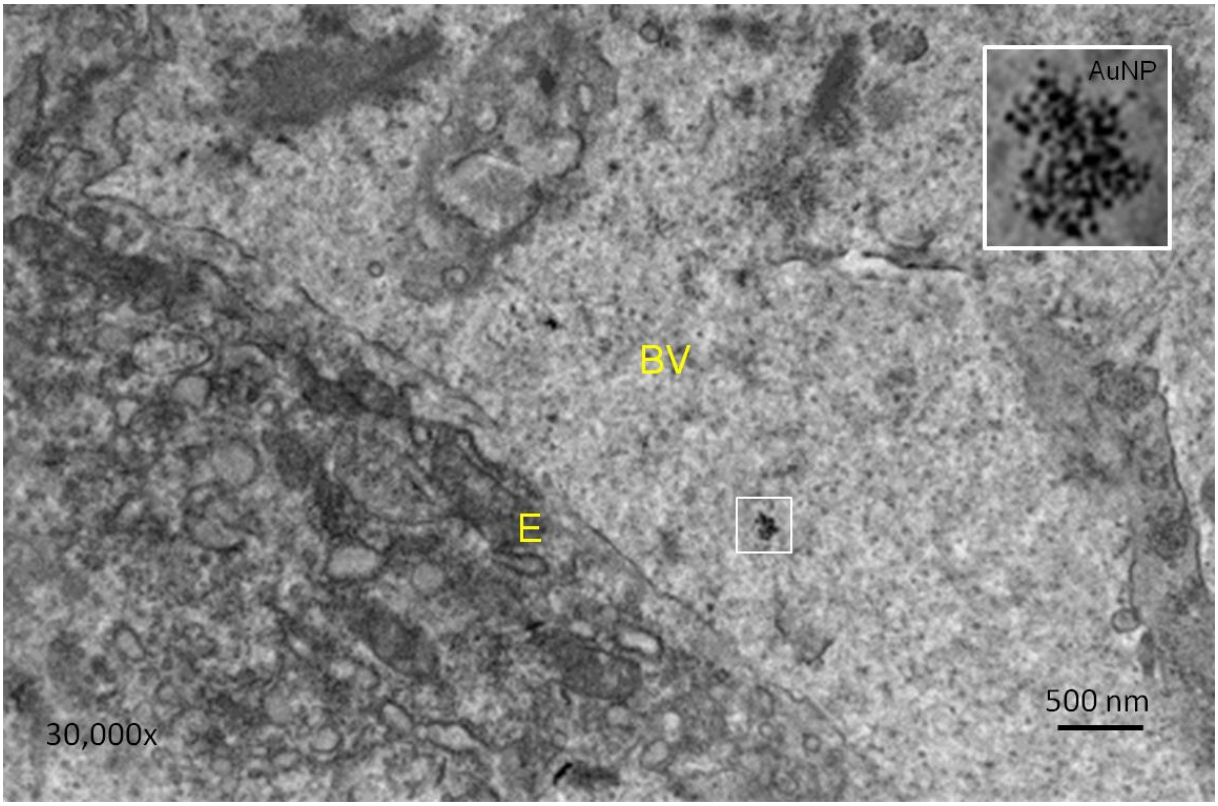

Figure S1: TEM imaging of Panc-1 tumor treated with (control) non-targeted gold nanoparticles (AuNP) at early time point (1 h). AuNPs were found to be distributed throughout the the blood stream without active uptake by the endothelial cells. Several clusters of gold nanoparticles were however noticed in the blood stream (see inset) E: Endothelium; BV: Blood vessel.

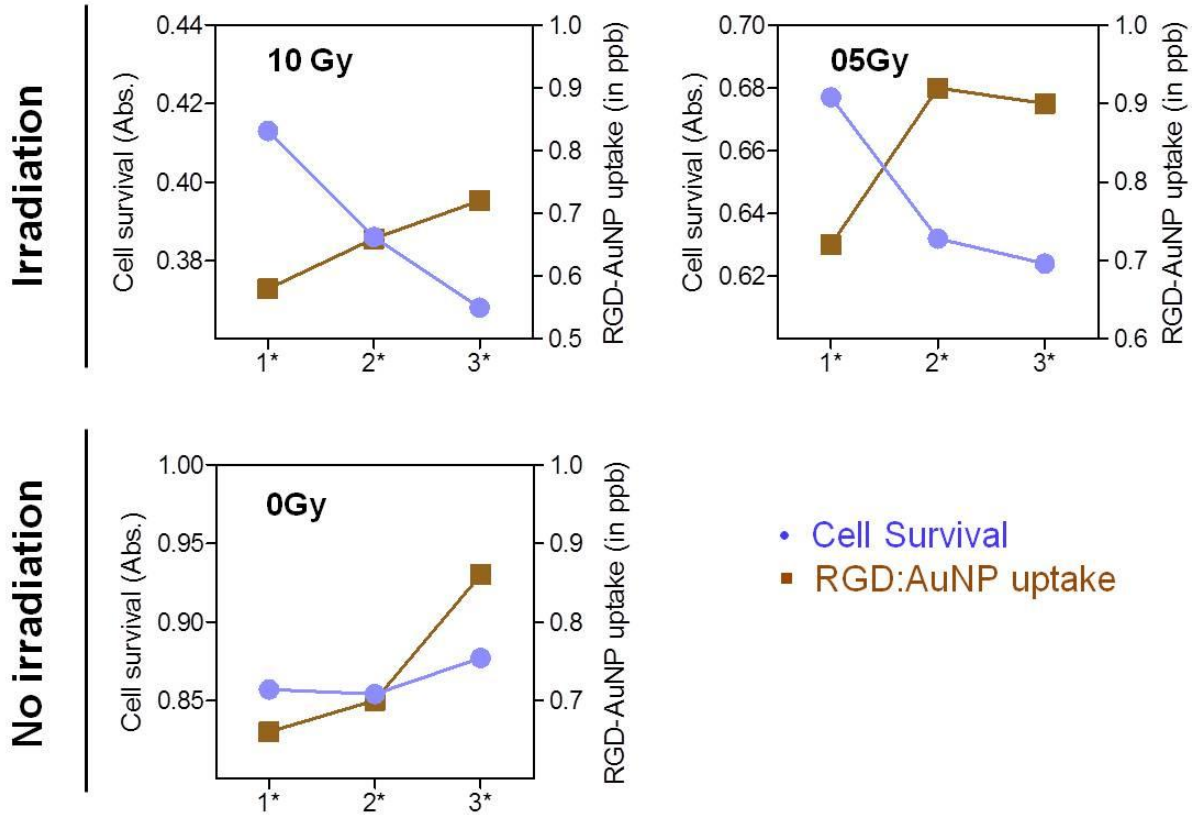

Figure S2. Cell survival *versus* uptake study. Analysis of the cell survival *versus* gold nanoparticle uptake in a well-to-well crystal violet assay revealed an inverse relationship (i.e. higher uptake is associated with less survival in irradiated samples). In the case of non-irradiated samples (0 Gy), cell survival showed no relationship to the particle uptake. These results demonstrate the impact of gold nanoparticle uptake on radiosensitization in the treated samples.

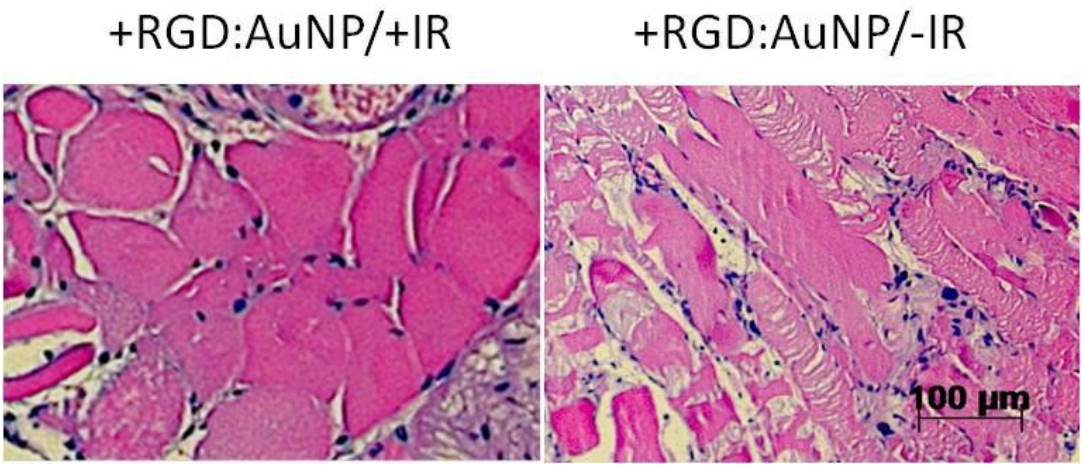

Figure S3. Radiation effect in muscle tissue. H&E staining of muscle tissue and its respective control excised from within close proximity to an irradiated tumor. No qualitative damage to the muscle tissue was apparently seen in both samples. Blue: Nucleus; Pink: cytoplasm.

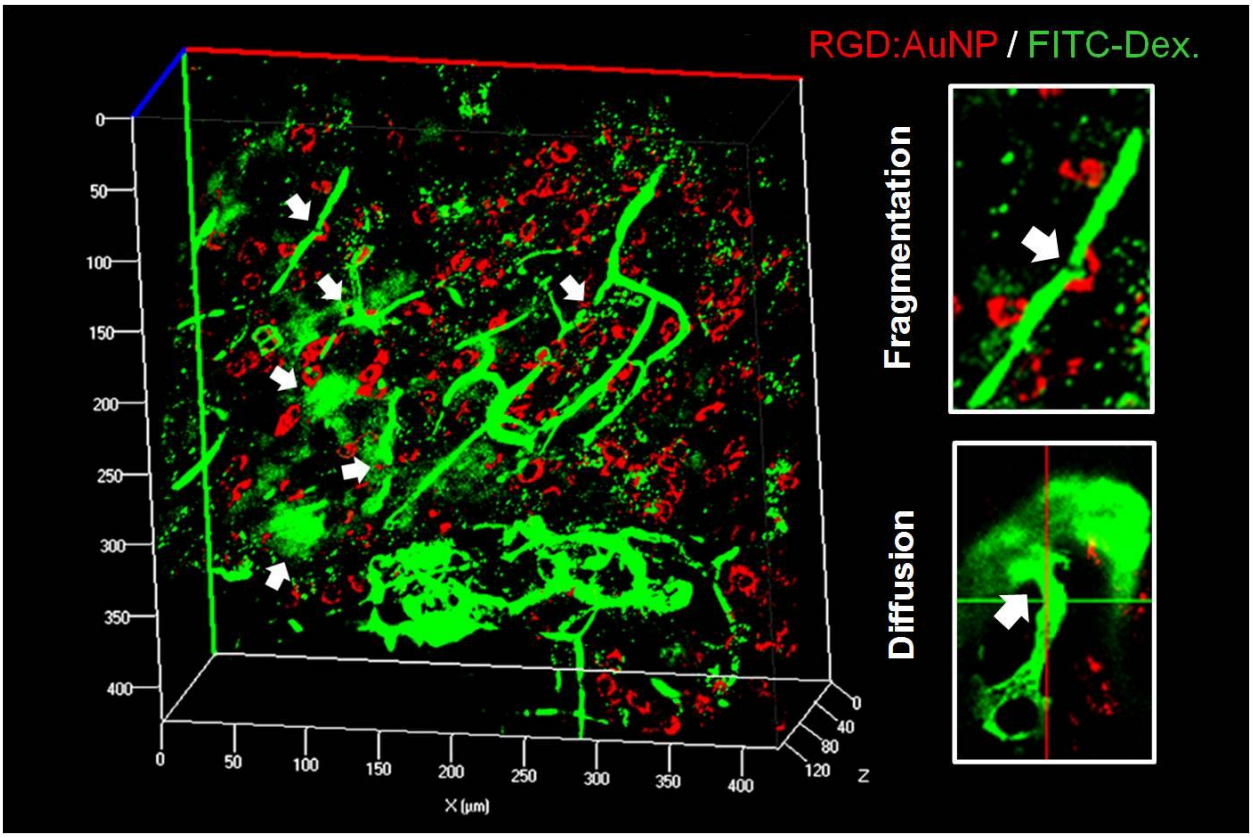

Figure S4. Imaging tumor vascular disruption. The vascular damage caused by RGD:AuNP upon irradiation was imaged by 3D confocal imaging. Distinct damage to the vessels resulting in the 'fragmentation' of microvessels was randomly observed. Following this, 'diffusion' of FITC-Dextran can be clearly noticed. Magnification: 20x. A z-series of confocal images was collected on a Zeiss 710 laser scanning confocal using an upright Examiner Z.1 and 20x NA1.0 W-Planapochromatic dipping lens and the images were 3D-reconstructed.