Advanced multimodal nanoparticles delay tumor progression with clinical radiation therapy

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Radiation therapy is a major treatment regimen for more than 50% of cancer patients. The collateral damage induced on healthy tissues during radiation and the minimal therapeutic effect on the organ-of-interest (target) is a major clinical concern. Ultra-small, renal clearable, silica based gadolinium chelated nanoparticles (SiGdNP) provide simultaneous MR contrast and radiation dose enhancement. The high atomic number of gadolinium provides a large photoelectric cross-section for increased photon interaction, even for high-energy clinical radiation beams. Imaging and therapy functionality of SiGdNP were tested in monkeys and pancreas tumor-bearing mice models, respectively. A significant improvement in tumor cell damage (double strand DNA breaks), growth suppression, and overall survival under clinical radiation therapy conditions were observed in a human pancreatic xenograft model. For the first time, safe systemic administration and systematic renal clearance was demonstrated in both tested species. These findings strongly support the translational potential of SiGdNP for MR-guided radiation therapy in cancer treatment.

1. Introduction

Radiation therapy is part of the clinical standard-of-care for more than 50% of cancer patients [1]. Although radiation is highly effective in killing cancer cells, it is often not possible to deliver a curative radiation dose without inducing collateral damage in adjacent healthy tissues. Radiation dose escalation can significantly improve local tumor control and thereby improve patient survival [2]. Intensity-modulated radiation therapy (IMRT), volumetric modulated arc therapy (VMAT) and image-guided radiation therapy (IGRT) are recent techniques that have substantially improved tumor-specific radiation dose delivery [3–5]. However, even with these progressing technical advancements, effective radiation treatment is elusive for many intransigent tumors because of the dose-limiting toxicity in healthy tissues.

Nanotechnology has great potential in cancer radiation therapy. Radiosensitizing and radiation dose enhancing agents (nanoparticles) can increase the effects of radiation within the disease site while maintaining the current clinical constraints on dose to healthy organs [6–8]. Nanoparticles made of high atomic number (Z) elements cause local amplification of radiation dose due to the emission of low energy photoelectrons and Auger electron interactions [9,10]. The interaction cross-section is highly dependent on the energy of the incident photons, with lower energy photons having a much higher probability of interaction. In clinical radiation therapy, ~95% of patient treatments are performed with high-energy X-ray beams produced by clinical linear accelerators with peak energy of 6 MV. This is in contrast to the preclinical X-ray irradiators with peak energies in the range of 200–300 kV. For this reason, many investigators have dismissed the potential for using clinical radiation therapy improvement with nanoparticles [11,12]. However, our data strongly refutes previous conclusions and shows that even with high-energy clinical beams, substantial DNA damage and tumor suppression can be invoked.

In addition to the physical dose enhancement induced by high-Z metallic nanoparticles, radiation-induced biochemical reactions may also contribute to the overall tumor damage. However, the essential mechanism is premature and still under investigation [13]. The radiosensitizing potential of gold (Au), gadolinium (Gd), hafnium (Hf), and bismuth (Bi) have been investigated preclinically [10,14–17]. Among these, Gd-based nanoparticles have the unique translational advantage of acting both as a radiation dose enhancement agent and MR imaging contrast agent, simultaneously.
2. Material and methods

2.1. Gadolinium-based nanoparticles

The gadolinium-based nanoparticles (SiGdNP) were synthesized and purified in compliance with GMP standards at Nano-H (Lyon, France). Structurally, SiGdNP is composed of an inorganic matrix of polysiloxane surrounded by covalently bound DOTAGA (Gd) ((1,4,7,10-tetra-azacyclododecane-1-glutaric anhydride-4,7,10-triacetic acid)-Gd^{3+}).

2.2. Characterization of the nanoparticles

Lyophilized nanoparticles were dispersed in PBS (7.4) for one hour at room temperature (Gd^{3+}: 100 mM). Hydrodynamic size and zeta potential measurements were carried out with a Zetasizer NanoS DLS (Malvern Instrument) at further diluted concentrations (Gd^{3+} = 10 mM). Average (diameter) size of 3.5 ± 1 nm and a zeta potential of 9 ± 5.5 mV was obtained for the SiGdNP formulation.

2.3. Cell culture

Capan-1 (human pancreatic adenocarcinoma) cells were cultured in Iscove’s Modified Eagle Medium (IMEM), supplemented with 20% fetal bovine serum (FBS) (Sigma, USA) and 1% Penicillin-Streptomycin Glutamine (Invitrogen, USA). Optimal conditions of 37 °C and 5% CO₂ was observed in a humidified incubator.

2.4. Clonogenic assay

Capan-1 cells were incubated with 0.43 mg/ml of SiGdNP for 15 min prior to irradiation with an open field 220 kVp beam. Radiation dose levels of 2, 4, 6, 8, and 10 Gy were employed. The cells were further incubated for another 4 h after irradiation and afterwards washed with PBS, trypsinized and counted. The cells were seeded in 10 cm dishes at 300 cells per plate and allowed to grow for 10 days, before staining with a 1% crystal violet in 10% ethanol dye solution. The plates were digested, scanned, and automatically counted with an in-house developed software tool. Measurements were performed in triplicate. The effect of the SiGdNP is quantified by the calculation of the dose enhancement factor (DEF) using Matlab (v. R2013b). The DEF is the ratio of the area under the survival curves with and without nanoparticles.

2.5. Apoptosis assay

Apoptosis was quantified by Allophycocyanin Annexin V/7-Aminoactinomycin D staining (Biolegends, USA) followed by flow cytometry analysis according to the manufacturer’s instructions using BD FACS Canto II (BD Biosciences, USA). Measurements were performed with and without nanoparticles after incubating for 15 min at 0.43 mg/ml concentration. After irradiation, three different time points were analyzed: 15 min, 24 h, and 48 h.

2.6. In vitro experiments

Capan-1 cells were irradiated to 4 Gy and 10 Gy with 220 kVp beams, +/- SiGdNP (0.43 mg/ml). Cells were fixed with 4% v/v formaldehyde for 15 min at room temperature (RT), and then washed twice with PBS. Cells were permeabilized with BSA 1%, FBS 10%, 0.3% triton-100 for 1 h, RT. The cells were incubated overnight at 4 °C with 53BP1 antibody (H-300, Santacruz, USA), diluted 1 to 1000 in PBS containing 1% BSA, 0.1% triton x-100, then washed five times with PBS. Slides were mounted with Dapi Fluoromount-G (SouthernBiotech, USA). Fluorescence microscopy images were processed to visualize the foci. The DNA damage induced by the nanoparticles was determined by counting the number of cells with >10 foci.

3. Mice studies

All animal studies were approved and carried out according to the US and European animal ethical committee, after approval by local and governmental agencies.

3.1. Survival study

Immunocompromised CrTac: Ncr-Fox1tmn mice (Taconic biosciences, Inc.) were injected with 3 × 10⁶ capan-1 cells subcutaneously in the flank. A total of 8 mice per group were used for the preclinical irradiation (220 kV) and 5 mice per group for the clinical irradiation studies (6 MV). Tumors were allowed to reach a size of ~6 × 7 mm² before initiating in vivo experiments. The capan-1 tumor-bearing mice were intravenously injected with +/- SiGdNP (0.25 mg/g) and treated +/- irradiation (10 Gy). Mice were followed for 12 weeks after irradiation. A tumor >2.5 cm in any dimension was considered as terminal endpoint and sacrificed using standard institutional protocols. The Institutional Animal Care and Use Committee (IACUC) of the Dana-Farber Cancer Institute approved the in vivo experiments for this study.

3.2. Magnetic resonance imaging

The biodistribution study with capan-1 tumor bearing mice (n = 3/group) was performed on a preclinical 7 Tesla Biospec 70/20 MRI scanner (Bruker BioSpin, United States). For the in vivo studies, a dose equivalent of 0.25 mg/g of SiGdNP was injected intravenously. A T1 RARE-VTR sequence using a repetition time of 9000 ms, echo time of 19.6 ms, and a flip angle of 180° was used. The acquisition matrix size and reconstructed matrix were 400 × 200 pixels, and the field of view was 200 × 200 mm², and slice thickness was 3 mm. Animals were pre-scanned, and then imaged at 2, 15, 30, and 45 min, and 1, 3 and 6 h post-SiGdNP injection. A region of interest was drawn across tumor and other vital organs and the T1-contrast was measured and correlated to its respective calibrations (ParaVision v. 5.1). For the phantom T1 relaxivity measurements, different dilutions of SiGdNP (0.01 mM to 0.5 mM) was prepared in de-ionized water and imaged using the exact set-up as above. Signal amplitudes were average and T1 relaxivity measurements calculated for absolute quantification of the nanoparticles.
4. Monkey studies

All animal studies were approved and carried out according to the European animal ethical committee guidelines.

4.1. Magnetic resonance imaging

Biodistribution study was carried out in cynomolgus monkeys (Macaca fascicularis) (n = 3/group) using a 3 T Prisma MRI scanner (Siemens, Germany) at 200 mg/kg of SiGdNP. Animals were anaesthetized with ketamine-xylazine (100–200 mg/kg). A T1 sequence using a repetition time of 3 ms, echo time of 1.12 ms, and a flip angle of 25° was used. The slice thickness was ~1 mm. The acquisition matrix size and reconstructed matrix were 352 × 286 pixels, and the field of view was 280 × 227.5 mm². Animals were scanned during the first 4 min and 30 min-p.i.

4.2. Pharmacokinetics and toxicity study

A total of 24 cynomolgus monkeys (Macaca fascicularis) (n = 6/group; 3 males and 3 females/group), were assigned to 4 groups: control-, low-, moderate-, and high-SiGdNP cohorts. The corresponding doses of 0, 150, 300, and 450 mg/kg/administration were administered once per week for 2 weeks (day 0 and day 7). Blood samples were collected following each administration at 5 and 30 min, 1, 2, 6 and 24 h. The blood plasma distribution kinetics was analyzed based on a non-compartment model (Kinetta 4.4.1, Thermo Fisher). All the animals were observed for mortality, clinical signs, ophthalmology, body weight, compartment model (Kinetica 4.4.1, Thermo Fisher). All the animals were observed for mortality, clinical signs, ophthalmology, body weight, food consumption, hematology, biochemistry, pathology, toxicokinetics and urinary parameters. Euthanasia was performed two weeks after the final injection.

4.3. Laser induced breakdown spectroscopy (LIBS) imaging

Paraffin-embedded tumor samples harvested 15 min after injection of the SiGdNP were prepared for LIBS imaging. Briefly, the instrumental setup was based on an optical microscope that combined a LIBS laser injection line, a standard optical imaging apparatus, and a 3D motorized platform for sample positioning. The LIBS experiment used Nd: YAG laser pulses of 1064 nm and 5 mJ energy, which were vertically focused onto the sample by a high-power 15× magnification objective to produce the laser-induced plasma. The pulse duration was 5 ns and the repetition rate was 10 Hz. During the experiments, the paraffin embedded sample was translated along two axes to image, pixel by pixel, the region of interest. During the sample scan, trigonometric surface positioning was used to compensate for any flatness anomalies and the laser energy was stabilized throughout the experiment by using a servo control loop. The optical signal was collected using a Czerny-Turner spectrometer equipped with a 1200-l/mm grating and an intensified charge-coupled device (ICCD) camera (Shamrock 303 and iStar, Andor Technology). In this configuration, a spectral range of 30 nm was accessible with a spectral resolution of about 0.15 nm. The spectral range covered in this case, from 282 to 317 nm, allowed lines originating from Mg (285 nm), Si (288 nm) and Iron (302 nm) to be detected.

4.4. Radiation therapy

Mice were imaged and irradiated using the Small Animal Radiation Research Platform (SARRP) (Xtahl, Inc.). Animals were anaesthetized with 1–3% isoflurane for the duration of each procedure (imaging or irradiation). Similar to the clinical workflow, a cone beam tomography (CBCT) was performed on each mouse to calculate the dosimetry and to determine the radiation beam arrangement (65 kVp, 1.5 mA). Treatment was performed using a 12 mm circular collimator (220 kVp, 13 mA). The radiation dose was delivered in one fraction of 10 Gy by two beams at 0° and 90° angles. The treatment planning system Muriplan (v.1.3.0) was used to calculate the dose distribution in the tumor and healthy organs prior to the irradiation. Four groups (−SiGdNP/−IR; +SiGdNP/−IR; −SiGdNP/+IR; +SiGdNP/+IR) of 8 mice each were used to characterize the effect of the SiGdNP as a radiosensitizer.

To perform clinical irradiation, animals were anesthetized and placed in a cage after intra-peritoneally injecting 0.1 mL/20 g mouse wt. of ketamine/xylazine mixture. 10 cm solid water (CIRS, Inc) was placed between the mouse and the radiation source and an additional 3% iso-wt. of ketamine/xylazine mixture. 10 cm solid water (CIRS, Inc) was placed between the mouse and the radiation source and an additional 2 cm of tissue equivalent clinical bolus material used for backscatter. The treatment planning system ECLIPSE (Aria V.11) which is routinely used for patients treated in the clinic, was used to calculate...
the dose distribution in tumor and healthy organs. The AAA calculation algorithm for a 5.5 × 10 cm² field size, gantry at 180°, and surface-skin distance of 90 cm was used. Simulation was performed for the standard and flattening filter free 6 MV irradiation beams (TrueBeam-Varian, CA). The capan-1 tumor-bearing mice was then injected with 0.25 mg/g of SiGdNP i.v. Fifteen minutes post-injection, radiation treatment was performed with 10 Gy. Four groups (−SiGdNP/−IR; +SiGdNP/−IR; −SiGdNP/+IR; +SiGdNP/+IR) of 5 mice each were used to characterize the effect of the SiGdNP as a radiosensitizer.

4.5. Tumor volume tracking

Tumor growth was tracked with volumetric CBCT images acquired with the SARRP. The volumes were measured using the 3D Slicer software (v. 4.3.1). The tumor was manually segmented to track its volume. In parallel, a caliper measurement was performed on a daily basis to confirm the results of the image-based volume study.

5. Histological studies

5.1. Mice studies

Animals were irradiated 15 min after injection of the SiGdNP following the same procedure as the survival study above. The tumor was harvested 30 min after irradiation and fixed in 2% formalin followed by paraffin embedding. Thin tumor sections of ~5 μm were cut and the tissue sections were counterstained for H&E staining and γH2AX staining. A similar procedure was performed to quantify the toxicity in the healthy organs using primary antibody, Abcam ab26350 and secondary antibody, ser139 (Cell signaling Technologies, USA). Images were analyzed using a Zeiss Axio microscope at 63× magnification.
5.2. Monkey studies

H&E staining was performed on tissue sections excised from the heart, lung, kidneys and liver to visualize the toxicity induced by the nanoparticles.

5.3. Statistical analysis

Statistics was performed with GraphPad (GraphPad Prism 5.0). The differences between the groups were evaluated by two-tailed unpaired t-test. Statistical tests for the in vivo survival studies were performed with a mantel-cox log-rank test (Kaplan-Meier).

6. Results and discussion

6.1. Gadolinium-based theranostic nanoparticles

SiGdNP was synthesized using earlier reported methods [22,28], wherein gadolinium atoms were chemically chelated with DOTA and covalently entrapped within a polysiloxane (-Si-O-Si-) network (Fig. 1a, Fig. S1a). With a hydrodynamic size of 3.5 ± 1 nm, a surface charge of 9 ± 5.5 mV and other optimal physicochemical properties (Fig. S1b–d), SiGdNP demonstrated substantial MR imaging capabilities in vitro (Fig. S2a–b). When administered in vivo, these nanoparticles permeate the (fenestrated) tumor blood vessels and are taken up by the

![Fig. 3. Whole body MR imaging and blood plasma kinetics in mice and monkeys. (A–B) T1-weighted MR imaging (7T, Bruker BioSpec, US) of SiGdNP (0.25 mg/g) injected in mice bearing capan-1-pancreatic tumors (n = 3/group) shows early tumor discrimination (at 1 min post-i.v.) followed by increasing accumulation. Maximum tumor accumulation of SiGdNP occurs at 15 min post-i.v. Some accumulation in the liver (~6 %ID) was also observed. (C–D) Biodistribution studies were carried out in cynomolgus monkeys (n = 3/group) after administration of a single bolus infusion of 100 mg/kg SiGdNP via external saphenous vein. Whole body MR imaging (T1 sequence) was carried out at different time points to visualize the early circulation and longitudinal renal clearance. The elimination of SiGdNP via the kidneys (collecting ducts) was clearly visible. (E) Blood samples were collected after administration of a single bolus injection of 150, 300 and 450 mg/kg of SiGdNP (2.5 mL/kg equiv.) to cynomolgus monkeys (n = 24; 12 male and 12 female) and pharmacokinetic parameters were analyzed. All data are represented as a mean ± SD.](image-url)
malignant tumor cells due to EPR effect [29,30]. Retention of nanoparticles within the tumor facilitated longitudinal (noninvasive) MR imaging and target-specific radiosensitization (Fig. 1b–c).

7. Radiosensitization potential of SiGdNP in vitro

The radiosensitization potential of SiGdNP (0.43 mg/mL) was tested in capan-1 pancreatic adenocarcinoma cells. The nanoparticles were endocytosed and prominently localized as clusters in the sub-cellular compartments (Fig. S2c) [9,31]. High degree of DNA damage was observed in the nanoparticle-treated cancer cells at the tested radiation doses of 4 and 10 Gy. The density of 53BP1-radiation induced foci formation is highest in the 10 Gy treated sample with >90% of the cells exhibiting specific DNA damage (Fig. 2a). Distinct quantitative and dose-dependent variations were found in the nanoparticle-treated vs. non-treated samples (78.1 ± 3.5% vs. 60.5 ± 4.1% at 4 Gy and 95.4 ± 2.3% vs. 79.4 ± 3.8% at 10 Gy) (Fig. 2b). FACS analysis was performed to quantitatively estimate the changes in early and late apoptosis for samples with/without radiation (+/−IR) and with/without nanoparticles +/−SiGdNP. Although capan-1 pancreatic cells are known to exhibit a slightly elevated basal DNA damage threshold [32], the combined SiGdNP and radiation experimental arm +SiGdNP/+IR demonstrated high early and late apoptosis (41.8% and 23% respectively) compared to the other controls (15.7% and 18.7%) (Fig. 2c). This trend remained consistent at 24 and 48 h post-IR (Fig. 2d). The clonogenic cell survival assay confirmed significant (P < 0.01) decrease in cell survival when SiGdNP and radiation were combined. The calculated radiation dose enhancement factor (DEF) for +SiGdNP/+IR samples was found to be 1.37 (Fig. 2e).

8. Noninvasive MRI-based biodistribution studies in mice and monkeys

Whole body MR imaging (VTR-T1 sequence, 7 T, Bruker BioSpin, United States) was performed using capan-1-tumor bearing mice (−6 × 8 mm²) and the longitudinal SiGdNP accumulation was noninvasively visualized in tumor and healthy organs. As early as 1 min post-i.v. injection of SiGdNP (0.25 mg/g), delineated tumor margins were visible due to gadolinium based MR contrast (Fig. 3a). EPR-mediated progressive accumulation of SiGdNP in the tumor peaked at 15 min p.i. (2.27 ± 0.44 %ID). The MR signal from the kidneys and bladder were also prominent at 15 min (R. kidney: 13.85 ± 0.98 %ID; L. kidney: 11.92 ± 1.58 %ID; bladder: 14.85 ± 1.49 %ID) and gradually declined thereafter, implying early clearance of ultra-small SiGdNP. (Fig. 3b). Furthermore, comparably less accumulation (5.84 ± 1.04 %ID) was observed in the liver, which steeply declined after 15 min (2.26 ± 1.09 %ID at 45 min p.i.) (Fig. 3b). The minimal accumulation in peripheral organs is primarily attributed to the rapid RES uptake of SiGdNP by the circulating macrophages. However, the fate of SiGdNP after liver deposition has not been determined. Nominal late accumulation was observed in other secondary organs such as muscle and heart [26]. Biodistribution studies were carried out in cynomolgus monkeys (Macaca fascicularis) (n = 3) after administration of a single i.v. bolus infusion of 200 mg/kg of SiGdNP (via external saphenous vein). The early systemic circulation and longitudinal renal clearance of SiGdNP was
clearly visualized by whole body MR imaging. At 30 s post-i.v. injection, the nanoparticle-related MR signal ($T_1$) was observed in peripheral vessels near the heart (Fig. 3c). The MR images illustrate the early circulation of SiGdNP over the next 2 to 4 min (Movie 1). At 30 min post-i.v. administration, a substantial decline in the SiGdNP signal was observed with an overall reduction in the $T_1$-contrast in the kidneys (Fig. 3c). In agreement with the mice biodistribution profile, the monkey data confirmed the rapid renal clearance of SiGdNP. A magnified MR image of the posterior kidney section shows late-retention (30 min) of SiGdNP in the collecting ducts of the renal capillaries, indicating renal excretion (Fig. 3d).

In another set of experiments in cynomolgus monkeys ($n = 24$), SiGdNP was injected at three different doses: low, moderate and high (150, 300 and 450 mg/kg, respectively), to evaluate the dose-dependent blood plasma distribution kinetics. For all tested doses, after an initial peak in concentration, the SiGdNP signal rapidly declined from 0.5 to 6 h (Fig. 3e). By ~9 h post-administration, the total SiGdNP concentration in the blood had fallen to negligibly low amounts indicating that long-term toxicity events are unlikely in large animals; a factor that strongly supports its clinical translation.

9. Toxicity and pharmacokinetic profiling in monkeys

SiGdNP toxicity was determined with excised organs from cynomolgus monkeys. H&E staining was performed on the tissue specimens of vital organs treated (+/−) SiGdNP. Even at the highest tested dose (450 mg/kg), no apparent histological differences or toxicities were observed in the heart, lung, kidney and liver (Fig. 4a). Pharmacokinetic evaluations were carried out based on a non-compartmental model (Kinetica™ 4.4.1, Thermo Fisher) using 3 different doses: 150, 300, and 450 mg/kg, injected i.v. twice in a one-week time interval (Fig. 4b, Fig. S3). Based on the absence of any antemortem or postmortem findings, the no-observed-effect level (NOEL) in the present study was determined to be 450 mg/kg/administration in both female and male monkeys.

Fig. 5. Tumor localization and image-guided radiation therapy. (A) Intratumoral SiGdNP localization in capan-1 tumors at 15 min post-injection was imaged using laser-induced breakdown spectroscopy. (B) MR-guided tumor delineation ($T_1$-VTR acquisition) was performed after injection of 0.25 mg/g SiGdNP in capan-1-tumor xenografts. (C–D) Schematic depiction of the radiation setup in which tumor xenografts were irradiated with preclinical (10 Gy, orthogonal, 220 kV) radiation beams (brown dotted lines). The radiation dose distribution in the tumor and surrounding tissues were calculated on a cone-beam CT image. (E–F) Schematic depiction of the radiation setup in which tumor xenografts were irradiated with clinical (10 Gy, unilateral, 6 MV) radiation beams (blue dotted line). For each case, care was taken to deliver homogenous radiation to the tumor while sparing the healthy tissues (as performed in the clinics). The qualitative isodose distributions show maximum radiation dose in the tumor. (G–H) The dose-volume histograms shows that more than 95% of the tumor received 95% of the prescription dose in under preclinical conditions and 90% of the tumor received 90% of the dose under clinical conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
tested monkeys. At a dose of 150 mg/kg administration, systemic exposure (area under the curve) of SiGdNP on day 8 was calculated to be 3419 ng·h/mL in males and 2842 ng·h/mL in females. The nanoparticles exhibit a (biological) $T_{1/2}$ of ~2.2 h and a clearance rate of 187 mL/h/kg and 171 mL/h/kg in both males and females, respectively, indicating an effective elimination process (Fig. 4b). All pharmacokinetic parameters were independent of gender variations as evident from the M/F ratio (Fig. 4b, Fig. S3). By the end of day 1, there was negligible presence of SiGdNP in the systemic circulation.

A 3D dose calculation was performed with the clinical treatment planning system (Aria V. 11, Varian Medical Systems, Inc). The calculated isodose levels show maximum radiation dose (10 Gy) in the tumor (Fig. 5e−f). No histological damage was observed in the muscle tissue excised from the proximity of the irradiated tumor (Fig. S4). The dose-volume histograms (DVH) for each setup confirmed that more than 95% of the tumor received ~95% of the prescribed dose for preclinical irradiation and ~90% for the clinical irradiation (Fig. 5g−h).

### 10. Tumor localization and image-guided radiation therapy

To precisely measure the microscopic distribution of SiGdNP within the tumor, laser induced breakdown spectroscopy (LIBS) imaging was performed. To this end, capan-1 tumors were excised at 15 min post-i.v. administration of SiGdNP (0.25 mg/g). Silicon (Si) served as surrogate markers for SiGdNP localization in the tumor due to the chemically-coupled polysiloxane construct (−Si-O-Si−) in the nanoparticle design (Fig. S1a). Heterogeneous distribution of ultra-small nanoparticles in the tumor was clearly evident from the Si LIBS signal (Fig. 5a). Higher vascularity was demonstrated in the periphery of the tumor compared to the core (as inferred from the Fe signal) and SiGdNP accumulation was slightly more prominent in the periphery (Fig. 5a).

A targeted image-guided radiation therapy procedure was implemented preclinically on a common clinical workflow wherein the tumor was delineated based on an MR image for treatment planning and the 3D radiation dose calculations were performed (Fig. 5b). For preclinical irradiation (220 kV), the treatment plan consisted of a pair of orthogonal beams with the isocenter at the center of the tumor (Fig. 5c). The calculated isodose levels show maximum radiation dose in the tumor while substantially sparing the surrounding healthy tissues (Fig. 5d). To perform the clinical irradiations (6 MV), the tumor was positioned in the treatment beam with the body of the mouse protected by the collimator of the clinical linear accelerator. In order to simulate clinical photon scattering conditions, 10 cm of solid water (CIRS, Inc.) was placed between the source and the mouse and the tumor was wrapped in 1 cm of tissue-equivalent bolus material, providing some backscatter.

### 11. Survival studies and radiation-induced DNA damage

To test the therapeutic radiosensitization efficacy of nanoparticles in reducing aggressive tumor growth and improving overall animal survival in combination with preclinical and clinical radiation beams, we injected intravenously ~0.25 mg/g of nanoparticles to capan-1 tumor xenograft models (n = 8) and performed image-guided radiation therapy at 10 Gy using both preclinical and clinical beams. The measured tumor volumes (using 3D slicer) demonstrated significant differences in the tumor burden in the treated vs. non-treated groups. The preclinical beam (220 kVp) produced a ~3-fold difference ($P < 0.002$) in the tumor size for nanoparticle with radiation compared to radiation alone (+SiGdNP/+IR compared to −SiGdNP/+IR) (Fig. 6a). The survival of non-treated cohorts +SiGdNP/−IR and −SiGdNP/−IR was ~30 days (Fig. 6b), compared to ~45 days with radiation alone and ~85 days ($P < 0.0001$, Mantel-Cox test) for nanoparticle combined radiation (+SiGdNP/+IR). With clinical irradiation (6 MV), a statistically significant reduction in tumor size was measured for nanoparticles with radiation versus radiation alone, i.e. the +SiGdNP/+IR group compared to the −SiGdNP/+IR group (2.69 ± 0.16 vs. 5.32 ± 0.19 cm³) by day 50 ($P < 0.0001$, Mantel-Cox test) (Fig. 6c). Survival was extended by more than 60 days when compared to −SiGdNP/−IR and +SiGdNP/−IR, and almost 40 days compared to the −SiGdNP/+IR (Fig. 6d). This is the first study that demonstrates the therapeutic efficacy of silica-based gadolinium nanoparticles under clinical radiation therapy conditions.

DNA double-strand breaks in tumor cells is a principal indicator of specific biological radiation response. In our initial qualitative analysis...
(by γH2AX staining), the in vivo treated tumors confirmed massive DNA damage for the +SiGdNP/+IR cohort compared to the −SiGdNP/+IR (Fig. 7a). No significant DNA damage was observed in healthy organs such as kidney, lung, heart and liver. Interestingly, both liver and kidney— the organs that were proximal to the irradiation site, were largely unaffected by the tumor-targeted irradiation and local dose enhancement. Further analysis of the tumor tissue demonstrated more than 80% DNA damage in the nanoparticle plus radiation treated cohort +SiGdNP/+IR. The magnitude of damage was ~60% for the radiation only group −SiGdNP/+IR and <10% for other controls −SiGdNP/−IR and +SiGdNP/−IR (Fig. 7b). These results clearly validate and confirm the dual-targeting concept wherein both targeted-radiation therapy and EPR-driven tumor accumulation of high atomic number nanoparticles combine to amplify the radiation response specifically in cancer cells while largely sparing surrounding healthy tissues.

Fig. 7. Preclinical radiation-induced DNA damage studies. (a) Radiation-induced DNA double strand breaks in the tumor and other vital organs are shown with γH2AX staining. The damaged tumor cell nuclei are stained in ‘brown’ (γH2AX+) and the viable cells in ‘blue’ (γH2AX−). Magnification: 100×. (b) γH2AX+ nuclei were counted across multiple image planes (n = 50) and further quantified. The values represent average ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Nanotechnology has great potential in cancer drug delivery, imaging and radiation therapy [33.34]. The ability to design and modulate multifunctional nanoparticles has paved the way for hybrid strategies to image and treat cancer [35–37]. In this regard, high atomic number ferromagnetic and paramagnetic nanoparticles can facilitate noninvasive imaging and concurrent radiation dose amplification for more accurate and effective treatment of cancer. With clinical radiation therapy practices incorporating more and more MR imaging, the clinical rationale for SiGdNP is only increasing.

While gadolinium-based nanoparticles have been used for image-guided drug delivery applications in previously reported in vivo studies [9,38,39], the current study focuses on using them as both radiation dose amplification and imaging agents. This is the first study demonstrating the safety and therapeutically beneficial administration of SiGdNP for image-guided radiation therapy using clinical 6 MV radiation beams. This is a major advancement in this field and provides a strong rationale for clinical translation. To this end, we have been careful to design and demonstrate a procedure that is highly compatible with current and emerging clinical workflows.

The combination of imaging contrast and radiosensitization without increased toxicity in healthy organs makes SiGdNP formulation an excellent candidate for future clinical translation in radiation therapy [6]. Long-term elimination from the tissues, degradation kinetics, and toxicity of SiGdNP has been previously tested using PET and MR imaging [40]. Regulatory toxicity studies in non-human primates showed that the high atomic materials can partly explain the biological effects, theoretically increased toxicity in healthy organs makes SiGdNP formulation an excellent candidate for future clinical translation in radiation therapy [6].

12. Conclusion

The experimental data in mice and nonhuman primates shown in this study clearly substantiates and justifies the clinical translational potential of SiGdNP. Intravenous administration of nanoparticles fits current clinical workflows for radiation therapy based on pre-treatment MR imaging as well as the emerging practice of real-time MR guidance on combined MR-linac devices. In either scenario, SiGdNP will serve as a valuable clinical nanomedicine for safe and effective imaging and therapy of cancer.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2016.07.021.

Competing financial interests

O. T. has one patent (WO2011135101) protecting the gadolinium nanoparticle (AGuIX®) design. The authors have no other relevant affiliations or financial interests with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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