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***In vivo* demonstration of enhanced radiotherapy using rare earth doped titania nanoparticles[†]**

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Abstract

Radiation therapy is often limited by damage to healthy tissue and associated side-effects; restricting radiation to ineffective doses. Preferential incorporation of materials into tumour tissue can enhance the effect of radiation. Titania has precedent for use in photodynamic therapy (PDT), generating reactive oxygen species (ROS) upon photoexcitation, but is limited by the penetration depth of UV light. Optimization of a nanomaterial for interaction with X-rays could be used for deep tumour treatment. As such, titania nanoparticles were doped with gadolinium to optimize the localized energy absorption from a conventional medical X-ray, and further optimized by the addition of other rare earth (RE) elements. These elements were selected due to their large X-ray photon interaction cross-section, and potential for integration into the titania crystal structure. Specific activation of the nanoparticles by X-ray can result in generation of ROS leading to cell death in a tumour-localized manner. We show here that intratumoural injection of RE doped titania nanoparticles can enhance the efficacy of radiotherapy *in vivo*.

1. Introduction

Nanoscale structures have the potential to radically change cancer therapies, providing non-fatal access to the interior of a living cell. One such application utilizes metallic nanoparticles as adjuncts to radiotherapy. The nanoparticles effectively provide dose enhancement and therefore increase therapeutic efficiency by selectively scattering and/or absorbing X-rays to cause localized damage to DNA and organelles within the cancer cells. The nanoparticles provide an enhanced interaction cross-section with the X-ray photons. Potential candidates include ZnS quantum dots,¹ gold particles,² or silicon nanoparticles.³ In this study, we sought to examine the *in vivo* efficacy of titania as a radiosensitizer.

Nanotitania has historically been used to induce cancer cell apoptosis and can be used as a photosensitizer in UV photodynamic therapy (PDT) for endobronchial and oesophageal cancers.⁴ Despite promising outcomes, PDT is however limited to superficial tumours since the UV light cannot penetrate deeply into human tissues.⁵ Since the photoelectrochemical

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reactions observed upon excitation with UV, are also promoted under X-ray irradiation with X-rays above 4.965 keV, which corresponds to the Ti–K edge,⁶ we proposed to optimize this reaction with X-ray excitation.

The absorption of energy excites electrons into states above the conduction band edge and excited electrons and holes are subsequently trapped at the particle surface to form unpaired electron species. In this excitation process, valence band electrons are also excited, leaving holes, just like in ultra-violet excitation. At the particle surface the holes are trapped by hydroxy groups and form hydroxyl radicals which can participate in subsequent chemical reactions and electrons can be trapped by adsorbed oxygen to form the adsorbed $O_2^{\bullet-}$.⁷ These reactive oxygen species (ROS) can destroy cell membrane structure^{8,9} and induce micronuclei¹⁰ and even cause DNA damage.^{9,11} The excited trapped electrons could also play a role in acting as a powerful local reducing agent. ROS production is the mechanism shared by all non-surgical therapeutic approaches for cancers, including chemotherapy, radiotherapy and photodynamic therapy, due to its implications in triggering cell death.^{12,13} Normal cells can tolerate a certain level of exogenous ROS due to a reserve antioxidant capacity, which can be mobilized to prevent cells from reaching a toxic threshold. Cancer cells are more reliant on the antioxidant system due to increases in ROS from metabolic abnormalities. This makes cancer cells more vulnerable to exogenous oxidative stress.^{14–17}

We have designed and synthesised titania nanoparticles doped with rare earth elements for ROS generation in tumours. The dopant profile was designed to absorb the maximum energy from a typical medical X-ray source which has a broad emission spectrum. The absorption, or mass attenuation, of the X-rays by the atomic shell electrons is “edge shaped”, and so we selected elements with a K edge below the peak of the X-ray emission. Elements were further combined to include smaller edge absorption features around the main absorption peak to mimic the X-ray emission spectrum using an amalgamation of K edges.¹⁸ This study showed good efficacy against immortalized cell lines, however spheroids and tumours have been reported to show increased resistance to ionizing radiation than monolayer cultures. The increased resistance appears to be due to the greater capacity of cells grown in contact to repair radiation damage.¹⁹ This stimulated the present study; to determine the efficacy of doped titania in three-dimensional cell culture and *in vivo* models.

2. Materials and methods

2.1. Nanoparticle synthesis and characterization

Rare earth doped titania nanoparticles coated with silica (TiO_2 -mol%RE@SiO₂) were prepared according to Townley *et al.*,¹⁸ Rare earth metals (gadolinium(III) nitrate hexahydrate; europium(III) nitrate hydrate; erbium(III) nitrate pentahydrate [Sigma-Aldrich, Poole, United Kingdom]) were resuspended in 10 ml titanium isopropoxide at either 5 or 1 mol%, followed by the addition of 30 ml dry isopropanol (Sigma-Aldrich). This solution was then added dropwise to 500 ml of 50/50 (v/v) water/isopropanol mix while stirring vigorously. After 5 minutes, the precipitate was allowed to settle and then washed with 200 ml isopropanol, collected by filtration, and autoclaved. The slurry was dried and ground to a fine powder prior to firing at 700 °C for 3 hours. Nanoparticles were subsequently coated with a thin, discontinuous silica layer by hydrolysis of TEOS. Doped titania nanoparticles

(4.52 g) were resuspended in 200 ml Milli-Q water (pH 4.5) [solution I]. Next, 3-mercaptopropyltrimethoxysilane (1.89 ml; Sigma-Aldrich) was added to 50 ml Milli-Q water [solution II]. Twenty millilitres of solution II was then added to solution I and stirred. After 1 hour sodium silicate (40 ml; Sigma-Aldrich) was added. Samples were removed after 10 minutes, centrifuged, and washed with Milli-Q water, 3 times. Silica-coated particles were sonicated in water and passed through a 0.2 micrometre cellulose-acetate filter. Size distribution was analyzed using a CPS disc centrifuge (Model DC24000; CPS Instruments Inc., Oosterhout, The Netherlands). This system measures particle size distributions using centrifugal sedimentation within an optically clear spinning disc filled with fluid. Sedimentation is stabilized by a density gradient in the fluid, and accuracy is ensured through the use of known calibration standards before each test.

For high resolution TEM (HRTEM) the nanoparticle powders were dispersed in IPA, sonicated for 30 s and a drop of the liquid was deposited on a TEM grid with lacey carbon film coating. HRTEM microscopy experiments were performed on a JEOL 3000F field emission gun instrument.

Samples were further analyzed by energy-dispersive X-ray spectroscopy (EDX; JEOL 6480 LV SEM equipped with an Oxford Instruments INCA X-ray analysis system) for elemental composition, X-ray diffraction spectroscopy (XRD; Siemens D5000 powder diffractometer) for crystal phase, and X-ray photon spectroscopy (XPS; VG Microtech CLAM 4 MCD analyzer system) and Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) for analysis of the surface layers.

2.2. Spheroid preparation

Spheroids were prepared using a human liver hepatocellular carcinoma cell line (HepG2 cells; a gift from Dr Ricky Bogal, University of Birmingham). Cells were grown in Dulbecco's Modified Eagle's Medium – high glucose [DMEM; Sigma-Aldrich, Poole UK], supplemented with 10% FCS [Sigma-Aldrich], 2 mM L-Glutamine [Gibco Invitrogen, Paisley, United Kingdom], and 100 ml^{-1} Penicillin and 0.1 mg ml^{-1} Streptomycin [Gibco]) at 37 ° C in 5% CO₂ atmosphere. Cells were rinsed with phosphate buffered saline (PBS; Sigma) to remove non-adherent cells. Adherent cells were detached using trypsin–EDTA (Sigma) followed by neutralization with growth media. Sixteen-well plates were prepared by setting 250 μl of 1% (w/v) melted agarose in PBS in the bottom of each well, ensuring no air bubbles. The gel was allowed to set and then 200 μl DMEM media added to each well and allowed to soak in for 20 minutes. This was then repeated. Each well was then inoculated with 50 000 cells in 200 μl media. The cells were added carefully to the centre of each well and incubated overnight to form spheroids.

2.3. Cell death experiment

Spheroids were incubated overnight with 225 nanomoles of nanoparticles. Spheroids either received no treatment, nano-particles only, X-ray (3 Gy) only, or nanoparticle and X-ray treatment. After irradiation, the spheroids were returned to the incubator overnight.

2.4. Spheroid sectioning

Spheroids were collected, washed in PBS, and embedded in OCT (Tissue-Tek) and immediately placed on dry ice. Cryostat sections were cut at 8 μm and placed on glass slides.

2.4.1. Haematoxylin and Eosin staining—Sections were fixed with 4% paraformaldehyde for 5 minutes (50 μl per section) and then rinsed with water. Samples were immersed in Haematoxylin solution for 30 seconds and then rinsed with water followed by immersion in Eosin solution for 1 minute. Samples were rinsed with water and then immersed in 70% ethanol, 100% ethanol, and histoclear for 30 seconds each.

2.4.2. Immunohistochemistry—Sections were probed with cleaved PARP (Asp 214) antibody (Human specific; Cat. no.9541, Cell signalling technology, Danvers, MA, USA). The antibody detects endogenous levels of the large fragment (89 kDa) of human PARP1 produced by caspase cleavage and does not recognize full length PARP1 or other PARP isoforms. Immunohistochemistry was performed according to Walch *et al.*, 1994. Briefly, twenty microlitres of the primary antibody was added to the tissue sections, and the slides were incubated overnight at 4 $^{\circ}\text{C}$. Slides were then washed three times in 0.1% PBS–BSA and incubated with 3% donkey serum in PBS for 10 minutes. This was followed by washing with PBS–BSA. The secondary antibody was conjugated to Alexa Fluor 44 (anti-rabbit IgG (H&L), 2 mg ml^{-1} ; Invitrogen) and used at a 1/75 dilution in PBS. Following incubation for 1 hour, the slides were washed three times with PBS–BSA and mounted with Mowiol 4–88 (Sigma, Poole, UK). Slides were examined with a AE31 Motic fluorescence microscope using the appropriate filters.

2.5. Veterinary care

In vivo experiments were carried out at the Stanford Preclinical Oncology Laboratory at the Stanford Cancer Institute (SCI). All animal experimentation was approved by the Stanford University animal Care and Use Committee and was conducted in accordance with the Guide for Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996). The mice were housed at the animal care facility at Stanford University School of medicine. All mice were kept under standard temperature, humidity, and timed lighting conditions and were provided with mouse chow and water *ad libitum*. Animals were constantly monitored for signs of morbidity such as reduced feeding or grooming, decreased activity, decreased weight, the presence of a tumour larger than 1 cm diameter or evidence of laboured breathing. Sentinel animals were used for detailed serological testing to ensure that the colony remained pathogen free.

At the end of the trial, mice were euthanized using CO_2 .

2.6. *In vivo* experiments

2.6.1. Nanoparticle injection into xenograft—A549 lung adenocarcinoma cells were collected in exponential growth phase. Four million tumor cells were injected subcutaneously in the right hind limb of male, 7 week-old, SCID-Beige mice in a mixture of

1 : 1 serum-free medium and Matrigel (Becton Dickinson, NJ, USA). After one week mice were randomised into groups of five and treated.

Nanoparticles (50 microlitres of 0.05 mg ml⁻¹ and 1 mg ml⁻¹ TiO₂:5% Gd, 1% Er, 1% Eu@SiO₂) were injected intratumourally. Tumor width and length were measured using an electronic caliper. Tumor volume (mm³) was calculated using the equation $0.52 \times [\text{width (cm)}]^2 \times [\text{length (cm)}]$. The mice were also weighed before and during treatment.

2.6.2. X-ray irradiation—Radiation treatment was delivered using a 200 kV (Philips RT-250) X-ray irradiator at a dose rate of 0.586 Gy per min. (As a rule-of-thumb for the energy spectrum, the average Bremsstrahlung X-ray energy is assumed to be approximately $\frac{1}{3}$ of the maximum energy.)²⁰

Mice were immobilized in custom made lead jigs (Fig. 1). The jigs specifically exposed the dorsal flank (harbouring tumour xenografts) for irradiation without exposing non-tumour-bearing normal tissues. In the first trial, irradiated mice followed a regime of 5 days irradiation followed by 2 days break.

Under the preferred treatment, irradiation treatment comprised 10 fractions of radiation at 2.5 Gy, followed by 3 fractions of radiation at 2 Gy.

Arrows show time line (days) of administration of nano-particles (NP) and radiation treatment.

2.6.3. Organ harvesting and sectioning—Organs were collected at the end of the study and freshly obtained tissues were fixed in 4% paraformaldehyde and transferred to 70% ethanol after 24 h. Tissues were then embedded in paraffin, cut into 5 µm sections and mounted on glass slides. Tissue sections on the slides were deparaffinized with xylene, hydrated using a diluted alcohol series, immersed in 3% H₂O₂ in distilled water for 15 min to quench endogenous peroxidase activity and stained with haematoxylin.

3. Results

3.1. Design and synthesis of doped titania nanoparticles for generation of ROS in response to X-ray irradiation

Titania nanoparticles were doped with the rare earth elements gadolinium, europium and erbium. The effect of this dopant blend is to mimic the X-ray emission spectrum using an amalgamation of K-edges and therefore optimize localized energy absorption from a conventional medical X-ray.¹⁸ The energy from the X-ray is able to promote similar photo-electrochemical reactions to UV light, resulting in the generation of ROS.

Nanoparticles were silica coated to inhibit aggregation and to improve biocompatibility (ESI; Fig. 1a †). XPS-spectra and TEM showed the silica to be 1–2 nm thick. ToF-SIMS demonstrated the patchy nature of the silica coating (see Townley *et al.*, 2012 (ref. 18) for details). The thin and discontinuous silica provides desirable surface properties without restricting access of X-ray generated charge to the surrounding medium and maintaining the ROS flux.

The particles were synthesized to have a hydrodynamic diameter of 65 nm. A typical example shows a peak with a full-width half maximum of 0.05, and a polydispersity index of 2.072 (ESI, Fig. 1b †). Thus, the particles are a suitable size for cellular uptake, and ROS generation and have a bio-compatible surface. The nanoparticles were shown by XRD to be single-phase anatase and are polycrystalline and have a primary crystallite size of 3–10 nm (Fig. 2). The combinations of rare earths used in this study were chosen on the basis of previous experiments.¹⁸

3.2. Cell death is increased in multicellular spheroids after irradiation in the presence of titania nanoparticles

To investigate cellular response to the nanoparticle-augmented radiation, HepG2 cells were grown as three-dimensional spheroids. The multicellular tumour spheroids are composed of mixed cell populations and are representative of the avascular stage of tumour development.²¹ Spheroids were irradiated with either 0 Gy or 3 Gy in the presence or absence of TiO₂:1% Gd, 1% Eu, 1% Er@SiO₂ titania nanoparticles. Treated spheroids were frozen, sectioned and H&E stained. Cells treated with nano-particles only showed cells with a normal morphology and contiguous spheroid structure (Fig. 3a, A). After irradiation only, the cells appear damaged and the spheroid structure less compacted (Fig. 3a, B). After irradiation augmented with nanoparticles the cells appear morphologically similar to cells which are undergoing/have undergone cell death and there is very little contact between the cells of the spheroid (Fig. 3a, C).

3.3. Cells showed increased levels of the apoptotic marker cleaved-PARP after irradiation in the presence of nanoparticles

To further investigate the effect of the nanoparticles in increasing cell death after radiation treatment, staining was performed on treated spheroids using immunofluorescent detection for visualization of the apoptotic marker cleaved-PARP. PARP is a 116 kDa nuclear poly (ADP-Ribose) polymerase which is involved in DNA repair in response to environmental stress.²² PARP helps cells to maintain their viability; cleavage of PARP represents cellular disassembly and therefore serves as a marker of cells undergoing apoptosis.²³ Control samples which were neither incubated with TiO₂:1% Gd, 1% Eu, 1% Er@SiO₂ nanoparticles nor exposed to radiation showed no c-PARP expression (Fig. 3b, a and e). Nanoparticle incubated samples without irradiation showed very low levels of expression (Fig. 3b, b and f). Irradiation in the absence of TiO₂:1% Gd, 1% Eu, 1% Er@SiO₂ nanoparticles showed distinct points of expression which indicates limited expression of the c-PARP (Fig. 3b, c and g). This may be due to nuclei containing a limited part of labelled

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antigenic sites.²⁴ Spheroids treated with both nanoparticles and radiation show a higher number of cells with a larger expression of c-PARP and a much brighter signal showing increased cell death (Fig. 3b, d and h). Comparison between Fig. 3b, g and h insets clearly shows the difference between the punctate expression in the radiation only sample, and the more extensive expression after nanoparticles augmented radiation. This shows that after irradiation in the presence of nanoparticles, cells show increased levels of death as shown by the apoptotic marker cleaved-PARP.

3.4. Mice xenograft models showed no toxicity due to nanoparticle treatment

To investigate the efficacy and safety of the nanoparticles fully *in vivo*, a xenograft model was used. Human non-small cell lung cancer xenografts were established by injecting A549 cells subcutaneously into the hind limb of a SCID Beige mouse. Tumours were excised post-sacrifice and sectioned and H&E stained. The tissue showed secretory type cells with glands and spaces which is consistent with the xenograft tumours being derived from adenocarcinoma (Fig. 4).²⁵ Lung, liver, and bone marrow samples were taken from the shielded parts of the mice to establish toxicity in the mice which could arise from the *in vivo* presence of nanoparticles but not as a direct result of irradiation (as would be found in the unshielded exposed hind limb). Fig. 5a shows H&E stained sections of the paraffin-embedded tissues.

The sections do not show any differing histology for tissues taken from mice which have X-ray activated nanoparticles, or non-activated particles, nor are there any differences seen for the different nanoparticle loadings of the tumour.

Fig. 5b shows a comparison of the irradiated tumour-bearing limbs (c and d) with the shielded contralateral limbs (a and b). The exposed limb shows mild to severe bone marrow depletion and intramedullary haemorrhage in the long bones (tibia and distal femur). It should be noted that the entire limb was exposed to radiation and not just the tumour. Bone marrow in the contralateral limb was within normal limits (Fig. 5a and b). The presence of intermedullary haemorrhage indicates that the blood supply to both trabeculae and bone marrow has been interrupted. A close relationship has been reported between intra-medullary haemorrhage and trabecular necrosis and bone marrow necrosis.²⁶ These types of lesion are typical of post irradiation treatment. The presence or absence of nanoparticles did not affect the histology.

The liver and heart were also removed from experimental animals and examined visually (data not shown). There were no lesions in the organs to indicate any systemic effect or toxicity due to the nanoparticles. A few incidental lesions were noted (alveolar haemorrhage, tiny inflammatory foci in the liver and a thymic lymphoma in one mouse and a keratin cyst in the tongue of one mouse, data not shown). These were considered to be due to techniques such as cardiac blood draw and associated haemorrhage in the lung, or strain background lesions (Dr Donna Bouley, Dept. Comparative Medicine, Stanford School of Medicine, personal communication).

3.5. Nanoparticle-augmented radiation therapy reduces tumour growth compared to radiation treatment alone

For this study we followed a fractionated radiotherapy protocol which would be analogous to standard clinical radiotherapy. One reason for the success of fractionated radiotherapy is this reoxygenation of the tumour. Also, exposure to radiation triggers a number of reactions inside a tumour cell. The sudden death of a part of the cell population changes the conditions for the surviving cells. After each fraction of radiation, well-oxygenated cells are killed leading to a re-oxygenation of hypoxic survivors which are more likely to be destroyed in the next fraction.²⁷ This reoxygenation of the tumour is particularly important since the potential for the generation of ROS is increased in regions with higher oxygen concentrations.

A small preliminary trial (each treatment, $n = 3$) was performed to establish the optimal nanoparticle concentration (Fig. 6a). Sample concentrations of 0.05, 1, and 5 mg ml⁻¹ TiO₂:5% Gd, 1% Er, 1% Eu@SiO₂ were injected intratumourally. This clearly shows that during the five days of treatment, irradiated tumours showed a decreased change in volume, as would be expected. Tumours which were irradiated in the presence of nanoparticles showed variation in the volume but there was far more variation in tumour growth in non-irradiated samples.

A second trial was then performed over a longer time period with a higher radiation dose and larger sample size (each treatment, $n = 5$) (Fig. 6b). It can be seen that there is no significant difference between the tumour growth in the PBS control and the nanoparticle injected tumours in the unirradiated mice. In the irradiated mice, there is a slight, although not statistically valid, decrease in tumour volume. Irradiated tumours which have been injected with nanoparticles show a dramatic decrease in tumour growth; tumours injected with 1 mg ml⁻¹ TiO₂:5% Gd, 1% Eu, 1% Er@SiO₂ nanoparticles are approximately half the size of those treated with radiation alone. Comparison of tumours which had been irradiated in the absence of nanoparticles or in the presence of 1 mg ml⁻¹ TiO₂:5% Gd, 1% Eu, 1% Er @SiO₂ have a highly significant difference ($P = 0.0025$).

As an indication of the health of the mice, their weight was monitored. Those mice which were exposed to radiation showed minor weight loss over the course of the treatment *i.e.* less than 20%,²⁸(control: 93.1% $\pm$ 0.01%, 0.05 mg ml⁻¹ nanoparticles: 87.2% $\pm$ 0.03%, 1 mg ml⁻¹ nanoparticles: 80.19% $\pm$ 0.02%). Mice in the control group gained weight, irrespective of the presence or absence of nanoparticles (control: 105.1% $\pm$ 0.03%, 0.05 mg ml⁻¹ nanoparticles: 106.9 $\pm$ 0.04%, 1 mg ml⁻¹ nanoparticles: 106.7% $\pm$ 0.01%).

A further experiment examined the effect of lowering the radiation dose since this could be beneficial to patients in a clinical setting. Mice tumours were therefore irradiated with 1.5 Gy after weekly intratumoural injection with 1 mg ml⁻¹ TiO₂:5% Gd, 1% Eu, 1% Er@SiO₂. However, at such a low dose there was only a small improvement in decreasing tumour growth in comparison to irradiation treatment alone (increase in size compared to start of treatment: untreated 639.7% $\pm$ 41.4%, irradiation only 325.48% $\pm$ 33.38%, nanoparticles only 392.05 $\pm$ 81.18%, irradiation and nanoparticles together 259.41 $\pm$ 16.93%; each treatment $n = 7$).

4. Discussion

We have shown in this study that doped titania nanoparticles can be specifically activated by X-ray to slow the growth of xenograft NSCLC tumours.

The particulate nature of the nanoparticles permits direct intratumoural administration. It would be expected that the nanoparticles would be well retained within the tumour due to the lack of efficient drainage from solid tumours. However, any movement of the particles is likely to move into the lymphatic vessels that drain from the solid tumour, to the sentinel lymph node and other lymphatics. Therefore, they would not only treat the primary tumour but also the lymph nodes which may contain metastases.²⁹

The nanoparticle system used in this study generates ROS following excitation of valence band electrons to the conduction band by absorbed X-ray energy. ROS are generated by the energetic photons liberated from the de-excitation process in the doped titania nanoparticles. This is very similar to the mechanism commonly used in photodynamic therapy. ROS are unstable and highly reactive compounds that can strip electrons from cellular macromolecules rendering them dysfunctional,³⁰ or damage cell membranes by oxidatively modifying fatty acid components of the phospholipid bilayer.³¹ They can also damage DNA and proteins, compromise cellular repair mechanisms, and trigger apoptotic processes.³² The short lifetimes and diffusion distances of ROS would enable cancerous cells to be targetted with very limited damage to neighbouring healthy cells. Similarly if excited ‘‘hot’’ electrons are responsible for cell damage due to chemical reduction, these too have short lifetimes and small diffusion distances.³³

Multicellular tumour spheroids (MTS) are often used as an experimental system for the study of initial avascular tumour growth. In this study we inoculated MTS with nanoparticles prior to irradiation. We observed an increase in cellular damage when the spheroids were irradiated in the presence of nano-particles, and loss of cell contiguity. We also found increased levels of cleaved-PARP; an indicator of apoptosis. While it might be expected that all spheroids have a necrotic core which would be resistant to irradiation, it should be recognized that a cluster of cells does not necessarily contain a (radiobiologically) hypoxic core.³⁴ Cell lines such as the HepG2 do not spontaneously form spheroids in suspension culture and it is thought that spheroids that form in gel or agar underlay grow as relatively ‘loose packed’ clusters. This means that the diffusion gradients of metabolites and oxygen present in such spheroids may well be very different than those in tightly packed, spontaneously occurring spheroids in suspension culture.³⁵ Therefore, while the results obtained were highly suggestive of increased efficacy of radiation treatment in the presence of the nanoparticles, we further tested the technology in xenografts of human non-small cell lung cancer.

While the outlook for patients with tumours of small cell origin has been dramatically improved by chemotherapy, non-small cell tumours have been treated less successfully.³⁶ This is despite the fact that non-small cell lung cancer (NSCLC) is the most common form of lung cancer, accounting for about 80% of all lung cancer cases, and is the leading cause of cancer death for both men and women in the UK (Cancer Research UK: Lung cancer

incidence statistics). For the majority of patients with NSCLC, the extent of the disease makes the prospect of cure by any available treatment method unrealistic. The primary curative treatment for non-small cell lung cancer is surgery. However, the results of surgical treatment of lung cancer have essentially been unchanged during the past two decades, which is why a lot of interest has been focussed on adjuvant therapy, such as radio-therapy, given either pre- or postoperatively.³⁶

In this study we used fractionated radiotherapy. This is a technique used clinically to overcome radioresistance. Tumour cells which are hypoxic can be 2–3 times more radioresistant than normoxic cells.³⁷ This is because radiosensitization requires the presence of oxygen at the time of irradiation. The application of small, frequent doses results in the death of well-oxygenated peripheral cells and leads to the oxygenation of the next layer of tumour cells, enabling cell death in the next fraction of radiotherapy.

4.1. Toxicity (bone marrow)

Blood and bone marrow is one of the largest organs in the body,³⁸ and therefore an important potential target for any toxic effects. We examined bone marrow sections from the shielded limb and the exposed contralateral limb bearing the nanoparticle injected tumour. All of the exposed limbs had similar damage due to the irradiation, irrespective of whether the treatment was augmented with nanoparticles. The radiation damage was as expected since the bone marrow is a highly radiosensitive organ and reacts to ionizing radiation almost immediately after irradiation.³⁹ Bone marrow toxicity is often the dose-limiting factor for radioimmunotherapy in cancer patients, such that doses over 2 Gy can cause significant acute and long-term toxicity.⁴⁰ The bone marrow from the shielded limbs were all within normal limits (Fig. 5a). Due to the increase in efficacy of the nano-particle-augmented radiotherapy it would also be expected that the total radiation dose could be reduced to decrease damage to bone marrow. In addition there was no damage to visually inspected organs, or organ sections (Fig. 5a) showing that the nanoparticles are biocompatible and it could be extrapolated that the nanoparticles are inactive in the absence of irradiation.

NSCLC is a very relevant disease model, both clinically and economically. Using *in vivo* models, A549 cells have been shown to be relatively resistant to radiation, for example, Toth *et al.*⁴¹ did not observe cell death after irradiation, and even a dose of 12 Gy caused only growth arrest. Our study, in common with most other studies to date, used kV radiosensitization. However, megavoltage X-rays are often used for radical radiotherapy regimes, to provide both skin sparing and adequate dose deposition to central tumours.⁴² Monte Carlo modelling predicts much lower physical dose enhancements for metal nanoparticles but recent research suggests significant radiosensitization with clinical megavoltage X-ray sources.^{43,44} It would be interesting to determine the efficacy of the nanoparticle augmented radiotherapy under such a treatment regime. Further experimentation could also examine the effect of fewer, large doses of radiation, and assessment of a variety of different tumour types since the tumour type will determine the radiosensitivity and growth rate.

5. Conclusions

Our study showed that under one treatment regime (Fig. 6b), the tumour volume could be decreased by around half when nano-particle augmented radiotherapy was used, compared to radiation alone. This was demonstrated in a lung adenocarcinoma line since lung cancer is the leading cause of cancer related deaths for both men and women worldwide.⁴⁵ Current treatment options for a patient with locally advanced lung cancer include chemotherapy, radiotherapy, and/or surgery.⁴⁷ These conventional therapies lead to a poor outcome, with a 5-year survival rate for NSCLC remaining at 15%.⁴⁶ The improvements that we have seen in this study using nanoparticle-augmented radiotherapy in mice models suggest that there is potential to translate this technology to the clinic, and to improve survival rates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Lead jigs were used to immobilize the mice during irradiation. The tumour-bearing dorsal flank was exposed through the slit without exposing normal tissues to irradiation.

Fig. 2. High Resolution Transmission Electron Microscopy image showing crystalline structure of doped anatase titania nanoparticles. The primary crystallite size is 3–10 nm.

Fig. 3.

(a) Nanoparticle augmented radiation damage to cells in HepG2 spheroids. Samples were incubated with TiO_2 :1% Gd, 1% Eu, 1% Er@SiO_2 and irradiated with 3 Gy. Representative images of H&E stained spheroid sections incubated with (A) nanoparticles only, not irradiated (B) no nanoparticles, irradiated (C) nanoparticles, irradiated. Scale marker shows 100 μm . (b) Nanoparticle augmented radiation increases expression of cell death marker. Spheroids were incubated with nanoparticles for 24 hours prior to treatment and then frozen and sectioned 24 hours after irradiation. Sections were taken from HepG2 spheroids after varying treatments and incubated with anti-cleaved PARP (green; e–h) and DAPI nuclear stain (blue; a–d). (a and e) control samples (b and f) nanoparticles no irradiation (c and g) 3 Gy irradiation only (d and h) incubated with nanoparticles and irradiated with 3 Gy. Scale marker shows 100 μm .

Fig. 4.
Typical section of H&E stained xenograft tissue showing morphology consistent with being derived from adenocarcinoma.

Fig. 5.

(a) Comparison of shielded tissues from animals containing X-ray activated and control non-activated nanoparticles. Representative images are shown of H&E stained sections paraffin-embedded sections of lung, liver, and bone marrow. Tumours were directly injected with 50 μ l of nanoparticles at the concentration indicated. Mice containing X-ray activated particles were irradiated with 2 Gy, daily, for 5 days, and then 2 days with no treatment. (b) Representative image showing H&E stained sections of paraffin-embedded bone marrow tissue showing (a and b) control (shielded) limb and (c and d) irradiated limb.

Fig. 6.

(a) *In vivo* nanoparticle therapy with single intratumoural injection. Human non-small cell lung cancer xenografts were established by injecting A459 cells subcutaneously into the right hindlimb of a SCID mouse. Tumours were grown for one week prior to treatment. Tumours were injected with 50 microlitres of 0.05, 1 or 5 mg ml⁻¹ TiO₂:5% Gd, 1% Eu, 1% Er@SiO₂. Mice were shielded from radiation except for the exposed tumour bearing flank. Mice were either irradiated with 0 Gy or 2 Gy, daily for five days. (b) *In vivo* nanoparticle therapy with multiple intratumoural injections. Nanoparticles (50 microlitres of 0.05 mg ml⁻¹ and 1 mg ml⁻¹ TiO₂:5% Gd, 1% Er, 1% Eu@SiO₂) were injected intra-tumourally on days 0, 13 and 20 of treatment. Mice were shielded from radiation except for the exposed tumour bearing flank. IR treatment comprised 10 fractions of radiation at 2.5 Gy, followed by 3 fractions of radiation at 2 Gy. The results are provided as tumour volume after 22 days; mean $\pm$ SE ($n = 5$).