Combining ultrasmall gadolinium-based nanoparticles with photon irradiation overcomes radioresistance of head and neck squamous cell carcinoma

Imen Miladi, PhD\textsuperscript{a,1}, Marie-Thérèse Aloy, PhD\textsuperscript{b,1}, Emma Armandy, Tech\textsuperscript{b}, Pierre Mowat, PhD\textsuperscript{a}, David Kryza, PharmD-PhD\textsuperscript{a,c}, Nicolas Magné, MD-PhD\textsuperscript{b}, Olivier Tillement, PhD\textsuperscript{a}, François Lux, PhD\textsuperscript{a}, Claire Billotey, MD-PhD\textsuperscript{a,c}, Marc Janier, MD-PhD\textsuperscript{a,c,2}, Claire Rodriguez-Lafrasse, PharmD-PhD\textsuperscript{b,d,2,*}

\textsuperscript{a}Institut Lumière Matière, UMR 5306 CNRS, Université de Lyon, Université Lyon 1, Villeurbanne cedex, France
\textsuperscript{b}Laboratoire de Radiobiologie Cellulaire et Moléculaire, EMR3738, Faculté de Médecine Lyon-Sud, Université de Lyon, Université Lyon 1, Oullins, France
\textsuperscript{c}IMTHERNAT, Hôpital Edouard Herriot, Hospices Civils de Lyon, Lyon, France
\textsuperscript{d}Unité Médicale d’Oncologie Moléculaire et Transfert, Hospices Civils de Lyon, Laboratoire de Biochimie et Biologie Moléculaire, Centre Hospitalier Lyon-Sud, Pierre Bénite, France

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Abstract

Gadolinium based nanoparticles (GBNs, diameter 2.9 ± 0.2 nm), have promising biodistribution properties for theranostic use in-vivo. We aimed at demonstrating the radiosensitizing effect of these GBNs in experimental radioresistant human head and neck squamous cell carcinoma (SQ20B, FaDu and Cal33 cell lines). Combining 0.6 mM GBNs with 250 kV photon irradiation significantly decreased SQ20B cell survival, associated with an increase in non-reparable DNA double-strand breaks, the shortening of G2/M phase blockage, and the inhibition of cell proliferation, each contributing to the commitment of late apoptosis. Similarly, radiation resistance was overcome for SQ20B stem-like cells, as well as for FaDu and Cal33 cell lines. Using a SQ20B tumor-bearing mouse model, combination of GBNs with 10 Gy irradiation significantly delayed tumor growth with an increase in late apoptosis and a decrease in cell proliferation. These results suggest that GBNs could be envisioned as adjuvant to radiotherapy for HNSCC tumors.

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Key words: Gadolinium nanoparticles; Radiosensitization; Radioresistance; Head and neck squamous cell carcinoma (HNSCC); Radiotherapy

Background

Radiotherapy is commonly used in cancer treatment but remains limited by intrinsic radioresistance of certain tumors such as sarcoma, glioblastoma, and head and neck squamous cell carcinoma (HNSCC). The current innovations to treat radioresistant tumors rely on either the delivery of a higher dose of radiation to the target volume by focused beams (three-dimensional conformational radiotherapy, intensity-modulated radiotherapy, or hadrontherapy)\textsuperscript{1} or the increase in the local effect of a given dose of radiation by radiosensitizing agents.

Thanks to advances in nanotechnology, it is now possible to propose the dose enhancement to the tumor using nanostructured radiosensitizers containing high-Z elements.\textsuperscript{2} High-Z elements have large photon-interaction cross sections and produce a large
variety of secondary emissions, all of which leading to the generation of reactive oxygen species (ROS). Since the pioneering works of Mc Carthy and Weissleder on multifunctional nanoparticles, different architectures have been developed such as doped hybrid silicas, quantum dots, carbon nanotubes, iron oxides, gold nanoparticles, FePt nanoclusters, and lanthanide oxide nanoparticles. However, only a few of these architectures can fulfill the double constraints of a size small enough (less 5 nm) for complete renal excretion but also large enough to gather all the species needed for theranostic purposes. Hainfeld et al proposed the use of metal nanoparticles, in particular gold nanoparticles (GNPs). Until now, GNPs are the most thoroughly studied nanoparticles used as radiosensitizers. However, recent studies have shown cytotoxic effects in cells exposed to GNPs. Although the abovementioned studies used uncoated GNPs, Hebert et al’s study showed that 5 nm GNPs coated with the chelating agent diethylene triamine-pentaacetic acid (DTPA) did not exhibit a radiosensitizing effect. Other compounds have been proposed as radiosensitizers such as hafnium and gadolinium.

We developed gadolinium-based nanoparticles (GBNs), with a mean hydrodynamic diameter of 2.9 ± 0.2 nm, that demonstrated an ideal biodistribution with a rapid renal excretion after intravenous administration, and radiosensitizing properties. In-vivo, when GBNs were combined with monoenergetic synchrotron X-rays, we demonstrated an improved survival of rats bearing an orthotopic murine gliosarcoma. In vitro, we observed on U87 glioma cells a limitation of cell proliferation associated with an increase in single DNA breaks when a 660 keV (137Ce source) or a 6 MV irradiation was combined with nanoparticles. Rima et al reported in HNSCC SQ20B cells the mechanisms of internalization of GBNs. Using different microscopy techniques, it was demonstrated that GBNs penetrate into cells by passive diffusion and macropinocytosis. It gave also the basis for the current paper by showing that a 0.6 mM concentration in GBNs induced the highest clonogenic cell death combined with 2 Gy irradiation.

In the current work, we considered the in vitro and in vivo radiosensitizing effects of these GBNs in combination with conventional photon radiation in models of human radioresistant HNSCC. This was achieved using SQ20B cells and their associated stem cells, and confirmed in two other HNSCC cell lines. To support the potential clinical application in radiotherapy, in vivo studies were performed on a nude mouse model with SQ20B xenografts irradiated with photons after intratumoral injection of GBNs.

Methods

Gadolinium-based nanoparticles

GBNs consist of a polysiloxane core surrounded by gadolinium chelates (DTPA) covalently grafted to the inorganic matrix (Figure S1 in Supplementary Materials). GBNs were synthesized as described previously. For confocal and intra-tumor distribution studies, cyanine 5.5 (Cy5.5)-labeled GBNs were used.

Cell lines and culture conditions

The HNSCC SQ20B cell line, established from a patient with recurrence of a squamous cell carcinoma of the larynx after radiation therapy, was cultured as described previously. A SQ20B/SP/CD44+/ALDHhigh cell population, considered to be cancer stem cells, was isolated (Supplementary Materials) from the SQ20B cell line after two consecutive cell sorting runs on a BD-LSRII-FACScan flow cytometer (BD Biosciences, Le Pont de Clai, France). FaDu, a human hypopharyngeal HNSCC cell line obtained from the ATCC, was cultured in Minimum Essential Medium with Earle’s salts (PAA, Cölbe, Germany) supplemented with 1% non-essential amino acids (NEAA), 1% sodium pyruvate and 10% fetal bovine serum (FBS) (PAA). Cal33 cell line, derived from a squamous cell carcinoma of the tongue, was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (PAA) containing 15% FBS. All experiments were performed with exponentially growing cells.

GBN treatment and irradiation

Cells were seeded at a density of 40 000 cells/cm², 24 h before treatment with GBNs. Before treatment, cells were washed twice with prewarmed Hanks balanced salt solution (HBSS), and then incubated for 1 h at 37 °C with 0.1-2 mM GBNs suspended in HBSS. After incubation, cells were washed twice with PBS and were immediately irradiated in fresh medium. X-ray irradiation was performed at 250 kV delivered at a dose rate of 2 Gy/min using a X-Rad 320 irradiator (Precision X-ray Inc., North Branford, CT).

Visualization of GBNs by confocal laser scanning microscopy

After treatment with 0.1-2 mM Cy5.5-labeled-GBNs, cells were incubated with the subunit B of cholera toxin conjugated to Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR) used as plasma membrane marker. After fixation, cells were incubated with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) for nuclear counterstaining. The subcellular localization of GBNs was observed on a Leica TCS SP II confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) at 63 × magnification (Supplementary Materials).

Measurement of the intracellular concentration of gadolinium (Supplementary Materials)

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) analysis was performed as described previously on a Varian 710-ES ICP-OES instrument (Varian, Les Ullis, France).

Cytotoxicity assay

After GBN treatment and trypsinization, cells were reseeded into 96-well plates in fresh medium. Cell toxicity was analyzed between 24 and 72 h after treatment using a Cell Counting Kit-8 (CCK8) proliferation assay kit (Interchim, Montluçon, France) according to the manufacturer’s instructions. The results are expressed as the percentage increase in optical density at 48 and 72 h after treatment compared to 24 h.

γ-H2AX immunofluorescence assay

The detection of γ-H2AX foci was assayed by immunohistochemistry. Briefly, cells were fixed in 4% paraformaldehyde for 20 min, and immunodetection was performed as described previously. Foci were visualized on an Axio Imager Z1
fluorescence microscope (Carl Zeiss S.A.S., Le Pecq, France) and γ-H2AX staining was quantified using ImageJ software. The results are expressed as mean ± SD of a minimal counting of 100 nuclei per slide performed in duplicate.

Clonogenic survival assay

Cell survival curves were generated using a standard colony-formation assay as reported previously with minor modifications. After incubation with or without GBNs and irradiation in fresh medium at doses varying from 1 to 4 Gy, cells were trypsinized and reseeded at different concentrations into 25 cm² flasks (Supplementary Materials). Clonogenic survival curves were fitted according to the linear quadratic equation (SF = e^{-[α * D + β * D * D]}) where SF is the surviving fraction, α and β represent the initial slope (probability of lethal events) and the terminal slope (sublethal events) constants respectively; and D represents the irradiation dose. The SF at 2 Gy (SF2) was used as an index of radiosensitivity. D$_{10}$, D$_{37}$, and D$_{50}$ are the doses corresponding to 10%, 37%, and 50% of surviving cells, respectively.

Isobolographic method for assessment of the effects of radiation with and without GBNs

Dose–response interactions between radiation and GBNs were evaluated using the classical isobolographic method of Steel and Peckham. This method allows to evaluate if GBNs and radiation combination gave rise to antagonistic, additive, or synergistic effects, respectively (Supplementary Materials).

Flow cytometry quantitative analysis

The distribution of cells in the cell cycle was analyzed as reported previously with DAPI used instead of propidium iodide for DNA staining. Briefly, cells were fixed in 70% ethanol, incubated with 5 μg/ml DAPI, and analyzed at 460 nm on a BD LSR II FACSScan flow cytometer (BD Biosciences, Le Pont de Claix, France).

Total caspase activation was measured using the CaspACE FITC-VAD-FMK in Situ Marker kit (Promega, Charbonnières, France). Mitochondrial transmembrane potential (∆Ψ_m) was measured with the lipophilic JC1 probe (Molecular Probes, Eugene, OR), and intracellular ROS content was quantified with hydroethidine. The proliferative capacity of cells

Figure 1. Cellular uptake of GBNs and cell viability after a 1 h incubation with GBNs. (A) Representative confocal microscopy images of SQ20B cells incubated 1 h with increasing concentrations of Cy5.5-labeled GBNs. The nucleus shown in blue, cytoplasmic membrane green, and Cy5.5-GBN red; 63× magnification. (B) Quantification of gadolinium uptake assessed by ICP-OES. (C) Cell viability after GBN treatment. Each value represents the mean ± SD of three experiments in duplicate. (D) Time course of intracellular ROS production. Each value represents the mean ± SD of two experiments performed in triplicate.
was determined using an FITC BrdU Fow kit (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions.

**Western blot analysis of caspase 2**

Caspase 2 content was measured using the experimental procedure described previously and an anti-caspase-2 monoclonal antibody (Cell Signaling Technology, Danvers, MA) and anti-glyceraldehyde-3-phosphate-dehydrogenase (Bio-design International, Saco, ME) as a loading control. Proteins were revealed on the Intelligent Dark-box LAS-3000 (Fujifilm, Tokyo, Japan), and densitometric analysis was performed using Multi Gauge V3.0 software (Fujifilm).

**In vivo experiments**

Animal experimentation was performed according to the French legislation and approved by the local animal ethics committee of University Claude Bernard Lyon 1. A suspension of \(3 \times 10^6\) SQ20B cells in 150 μl of PBS was inoculated subcutaneously into the right flank region of 4-week-old female athymic nude mice (Charles River Laboratories, L’Arbresle, France) under gaseous anesthesia with 1%-2% isoflurane in a 25%/75% O₂/N₂ mixture.

Preliminary optical imaging was performed in 3 mice after intratumoral injection of \(1 \mu\)mol of Cy5.5-labeled GBNs to determine the residence time of GBNs in the tumor. The signal intensity was visualized with a cooled back-illuminated charge-coupled device camera (ORCA II, Hamamatsu Photonics, Massy, France).

When the tumor reached 300-400 mm³ in volume, mice were then randomly selected for treatment and assigned into four groups: vehicle-alone (\(n = 11\)), vehicle + 10 Gy (\(n = 20\)), GBN (\(n = 12\)), and GBN + 10 Gy (\(n = 21\)). GBNs (1 μmol) or vehicle was injected into the tumor under anesthesia, and for the irradiated groups local irradiation was applied immediately at a single dose of 10 Gy (320 kV, 2 Gy/min). Clinical observations were performed daily and body weight and tumor volume were measured weekly. Tumor volume was calculated according to the formula: \(0.5236(L \times W^2)\) where \(L\) and \(W\) were the length and width, respectively. For each group, histological analyses were performed on tumors collected after 7 weeks of treatment. Tumors were fixed in 4% formalin for 24 h and then embedded in paraffin.
Euthanasia was performed as soon as an animal showed a weight loss >20% in 1 week, tumor volume >1000 mm³, or ulceration of the tumor.

**Immunohistochemical analysis of tumors**

TUNEL analysis was performed on 5 μm tissue sections to identify apoptotic cell death using a Dead End Fluorometric TUNEL kit (Promega) according to the manufacturer’s instructions. Digital images were obtained using an Axio Imager Z2 Zeiss fluorescence microscope. Apoptosis was quantified using ImageJ software. At least 150-200 nuclei per field were scored to calculate the average number of TUNEL-positive nuclei.

Immunodetection of Ki67 with mouse monoclonal anti-human Ki67 (clone MIB-1-Dako) antibody was performed on tumor slices using an Envision™ Flex +, High pH kit (Dako, Glostrup, Denmark) and a Dako autostainer. Slides were visualized on an Axio Imager Z2 Zeiss microscope at 20× magnification, and images were analyzed using Metafer4 Image Analyzer software (MetaSystems Hard & Software GMBH, Altusheim, Germany). The results are expressed as the average percentage of Ki67-positive nuclei (two fields for each slide in duplicate).

**Statistics**

For *in vitro* assessments, the results are expressed as the mean ± SD. Statistical significance was tested using Student’s *t* tests (Excel, Microsoft, Courtaboeuf, France). *P* < 0.05 was considered significant.

The results obtained *in vivo* are expressed as mean ± SEM. Statistical significance was tested using a nonparametric Mann–Whitney test (StatEL Integrated in Excel, Microsoft).

**Results**

**Cellular uptake of GBNs and cell viability**

Nanoparticle internalization was followed by confocal microscopy analysis after 1 h incubation of SQ20B cells at 37 °C with different concentrations of Cy5.5-labeled GBNs (Figure 1, A). At concentrations of 0.2, 0.4, and 0.6 mM, GBNs were distributed throughout the cytoplasm without colocalization with DAPI nuclear dye. Large aggregates were observed outside the cell at the vicinity of the plasma for a GBN concentration of 0.8 mM or higher. For subsequent *in vitro* experiments, we used 0.4 and 0.6 mM concentrations, as a clonogenic assay at 2 Gy showed that increasing clonogenic death occurred after incubation with these 2 concentrations.

At these concentrations, the intracellular concentration of gadolinium measured by ICP-OES was directly proportional to that of the incubation medium (Figure 1, B), and no cytotoxicity was observed 72 h following incubation with GBNs (Figure 1, C). The lack of generation of ROS from 0.5 to 240 h after treatment with GBNs confirmed their chemical stability in the biological media (Figure 1, D).

**GBN treatment sensitized HNSCC and stem-like cells to radiation**

We first investigated the radiosensitizing effect of GBNs on SQ20B radioresistant cells by quantifying the initial (30 min) and residual (24 h) double-strand breaks (DSB) as shown by γ-H2AX foci (Figure 2, A). Untreated or GBN-treated cells exhibited 2.3 ± 0.2 foci/cell at 30 min; exposure to a 2 Gy irradiation induced the formation of 17.9 ± 0.1 foci/cell in untreated cells. This number increased by 41% and 53% (to 25.8 ± 2.2 and 27.4 ± 1.5 foci/cell) when irradiation was combined with 0.4 and 0.6 mM GBN treatment, respectively (*P* < 0.001 compared with untreated cells). As expected 24 h after irradiation without GBNs, DSBs returned to basal levels given the radioresistance of SQ20B, while DSBs persisted in cells treated by the combination of GBNs and irradiation (about 30% of the initial DSB, *P* < 0.001). Survival curves were established for the different conditions (Figure 2, B), and cell radiosensitivity parameters were deduced from the linear quadratic equation (Tables 1 and 2). Treatment with 0.4 or 0.6 mM GBNs shifted the survival curve downward compared with irradiated-only cells. The *α* parameter increased with GBN concentration, while *β* did not vary, demonstrating that an increase in the number of directly lethal lesions led irrevocably to cell death. The radiation doses inducing 50% and 10% survival, the median

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**Table 1**

<table>
<thead>
<tr>
<th>Radiation response variables of SQ20B cells untreated or treated with GBNs.</th>
<th>α (Gy⁻¹)</th>
<th>β (Gy⁻²)</th>
<th>D50 (Gy)</th>
<th>D57 (Gy)</th>
<th>D10 (Gy)</th>
<th>SF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQ20B</td>
<td>0.04</td>
<td>0.05</td>
<td>3.4</td>
<td>4.1</td>
<td>6.5</td>
<td>0.72</td>
</tr>
<tr>
<td>SQ20B + 0.4 mM GBN</td>
<td>0.15</td>
<td>0.05</td>
<td>2.2</td>
<td>3.0</td>
<td>5.2</td>
<td>0.60</td>
</tr>
<tr>
<td>SQ20B + 0.6 mM GBN</td>
<td>0.5</td>
<td>0.03</td>
<td>1.4</td>
<td>2.0</td>
<td>4.1</td>
<td>0.35</td>
</tr>
<tr>
<td>SQ20B/SP/CD44+/ALDHhigh</td>
<td>0.02</td>
<td>0.05</td>
<td>3.6</td>
<td>4.4</td>
<td>7.7</td>
<td>0.82</td>
</tr>
<tr>
<td>SQ20B/SP/CD44+/ALDHhigh + 0.6 mM GBN</td>
<td>0.19</td>
<td>0.03</td>
<td>2.6</td>
<td>3.4</td>
<td>5.8</td>
<td>0.60</td>
</tr>
</tbody>
</table>

SF2: survival fraction at 2 Gy; D50, D57 and D10: doses of radiation corresponding to 50%, 37% and 10% of survival.

**Table 2**

<table>
<thead>
<tr>
<th>Isobolographic analyses of radiation and GBNs in SQ20B and SQ20B/SP/CD44+/ALDHhigh human head and neck squamous cell lines.</th>
<th>GBN (mM)</th>
<th>D (Gy)</th>
<th>At 10% SF</th>
<th>At 50% SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQ20B</td>
<td>0.4</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Syn</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Syn</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1</td>
<td>Syn</td>
<td>Syn</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Syn</td>
<td>Syn</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Syn</td>
<td>Syn</td>
<td></td>
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<tr>
<td></td>
<td>4</td>
<td>Syn</td>
<td>Syn</td>
<td></td>
</tr>
</tbody>
</table>

SQ20B/SP/CD44+/ALDHhigh 0.6 | 1 | Syn | Syn |
| | 2 | Syn | Syn |
| | 3 | Syn | Syn |
| | 4 | Syn | Syn |

10% SF, 50% SF: 10% and 50% of surviving cells; Syn: synergistic effect; Ant: antagonistic effect; +: additive effect.
lethal dose $D_{37}$, and the SF2 decreased with increasing concentrations of GBNs (Table 1). As expected, SQ20B/SP/CD44+/ALDH$^{high}$ cells were more radioresistant than SQ20B cells, the SF2 value and calculated parameters of SQ20B/SP/CD44+/ALDH$^{high}$ cells treated with 0.6 mM GBNs and irradiation (Table 1) were similar to those obtained in SQ20B cells treated with 0.4 mM GBNs and irradiation. These results demonstrated that GBNs overcame the resistance of SQ20B stem-like cells.

Results of the isobolographic analysis are summarized in Table 2. For SQ20B cells, the effects for 0.4 mM of GBNs were either additive or more often synergistic. At 0.6 mM of GBNs, the combination was always synergistic regardless of the final cytotoxic efficiency (50% or 10% survival) and the applied radiation dose (1-4 Gy). The resulting effects for the 0.6 mM GBN dose on SQ20B/SP/CD44+/ALDH$^{high}$ cells were also synergistic regardless of the final survival and radiation dose.

Similarly, SF2 values of FaDu and Cal33 decreased significantly (Figure 2, C and Table 1), confirming that GBN treatment overcame radioresistance.

GBNs combined with radiation inhibited cell proliferation, modified the distribution of cells in the cell cycle, and induced mitotic catastrophe leading to apoptotic death

Considering the effects of combined treatment on cell survival, we next focused on cell proliferation. As shown in Figure 2, D, 85%-92% of non-irradiated cells, both GBN-treated and untreated cells, incorporated bromodeoxyuridine at the different times, demonstrating that GBN treatment did not impact cell proliferation and confirming the lack of toxicity. As expected, irradiation alone or combined with GBNs decreased the proliferative capacity of SQ20B cells between 24 and 240 h. At 24 h, when G2/M phase arrest is less important, the combined treatment caused a smaller decrease in proliferation than irradiation alone ($P < 0.005$) (Figure 3, A). From 72 to 240 h, this response was reversed; proliferation was lower in cells treated with GBNs and irradiation ($P < 0.005$) compared with radiation alone.

We next investigated the cell cycle distribution of cells (Figure 3). At 24 h, 10 Gy irradiation induced maximum transient G2/M phase arrest (43% of cells), whereas GBN treatment alone did not influence cell cycle distribution. The combined treatment significantly decreased the percentage of cells in G2/M phase to 33% and 28% after treatment with 0.4 and 0.6 mM of GBNs, respectively ($P < 0.005$) (Figure 3, A). The decrease in the percentage of cells in G2/M phase arrest observed at 48 h in cells irradiated with or without GBNs was concomitant with the appearance of a polyploid cell population ($>4n$ chromosomes) (Figure 3, B). This population became maximal (Figure 3, C) at 72 h after irradiation in the presence of 0.4 and 0.6 mM of GBNs (20% ± 0.4% and 23% ± 0.7% of polyploid cells, respectively, compared with 13% ± 0.3% in irradiated cells; $P < 0.001$) and decreased further at 240 h. A gradual increase in the percentage of cells in sub-G1 phase was noted from 48 to 240 h. GBNs plus irradiation caused greater accumulation of cells with a sub-G1 DNA content: 50% ± 3% and 57% ± 6% at 240 h after 0.4 and 0.6 mM GBN treatment, respectively, compared with 24% ± 3% for irradiated cells ($P < 0.001$) (Figure 3, D).

The decrease in polyploid cell percentage between 72 and 240 h following the combined treatment coupled with an increase in hypoploid cell percentage could be explained by mitotic catastrophe followed by apoptotic cell death. To confirm this, we examined cell morphology and quantified the activation of caspase-2 and total caspases as respective markers of mitotic catastrophe and apoptosis. Multinucleated cells and anaphase bridges, typical features of mitotic catastrophe, were observed at 120 h following irradiation (Figure 4, A). These morphological changes were associated with the activation of the caspase-2-dependent pathway: 70% decrease in procaspase-2 expression in 0.6 mM GBN-treated and irradiated cells compared with 40% decrease in irradiated cells without GBNs (Figure 4, B), confirming mitotic catastrophe. Few apoptotic bodies were also visualized, confirming the beginning of apoptosis (Figure 4, A). Activation of total caspases at 240 h (Figure 4, C).
confirmed the induction of late apoptosis following the combined treatment (150% greater than in the control cells) \( (P < 0.001) \). Significant production of ROS was observed from 24 h after irradiation and GBN treatment \( (P < 0.005) \) and was maximal at 240 h \( (P < 0.001) \), indicating a change in the redox status of the cells \( (Figure 4, D) \). \( \Delta \Psi_{m} \) decreased from 72 h after irradiation and this effect was intensified by GBN treatment, indicating impairment of mitochondrial function \( (Figure 4, E) \).
Combined treatment of GBNs with irradiation increased the regression of SQ20B xenografted tumors

Figure 5. A shows in vivo optical images acquired after intratumoral injection of 1 μmol of Cy5.5-labeled GBNs. The whole tumor became fluorescent immediately and for 15 min after injection, confirming that, at the time of irradiation (5 min after injection), most of the injected GBNs was within the tumor and therefore potentially effective.

Figures 5. B as well as Figure S2 in Supplementary Materials show the tumor evolution in the four groups of mice. Compared with the progressive and massive increase in tumor volume in the
vehicle-alone and GBN control groups, 10 Gy radiation delayed growth during the first week after irradiation. Combining GBNs with 10 Gy radiation strongly limited tumor growth. At the end of week 7, the mean tumor growth was 5 fold smaller in tumors that received combined treatment compared with irradiated-only tumors \((P < 0.05)\) and 11 fold smaller compared with control tumors. A complete tumor response was observed in three mice from the GBN + 10 Gy group. Administration of GBNs alone did not exert any antitumor effect on tumor-bearing mice.

**Intratumoral injection of GBNs before irradiation increased apoptotic cell death and altered tumor proliferation**

TUNEL staining (Figure 5, C) of tumor sections confirmed the results obtained in vitro. No induction of apoptosis was displayed in the untreated SQ20B tumors or in those treated with GBNs alone. Radiotherapy led to a slight increase in the number of apoptotic cells, whereas the combined treatment produced large fields of apoptosis. Apoptosis increased by 208% in GBN-treated and irradiated tumors compared with irradiated-only tumors \((P < 0.001)\). A decrease in tumor proliferation was observed in tumors treated with GBNs and irradiation compared with irradiation alone, whereas GBN treatment alone did not influence tumor proliferation (Figure 5, D). Quantitative analysis showed no significant difference in the percentage of Ki67-positive cells between vehicle-alone and GBN control groups and the vehicle + 10 Gy group. Ki 67 staining decreased significantly by 46% in tumors from the combination therapy group \((P < 0.005)\).

**Discussion**

In the present study, we demonstrated that small size nanoparticles, radiosensitized in vitro SQ20B cells and their corresponding stem cells, as well as 2 other HNSCC radioresistant tumor cell lines, showing a synergistic effect of GBNs combined with a 250 kV energy irradiation. We also demonstrated that cell death induced in response to the combined treatment was characteristic of mitotic catastrophe followed by late apoptosis, secondary to massive production of ROS and alteration of mitochondrial functions. These in vitro studies were confirmed in vivo in a xenografted SQ20B model on a photon irradiator specifically dedicated to animals at energies compatible with clinical transfer.

In SQ20B cells, 24 h after irradiation the combination of GBNs with photon irradiation induced a high level of irreparable DSBs, the most pertinent determinants of induction of cell death by radiation. Because GBN-treated cells did not accumulate in the G2/M phase following irradiation, DSB repair was inhibited. The incomplete DNA repair induced several disturbances during mitosis, leading to anaphase bridges and multinucleated cells. These morphological features are typical of mitotic catastrophe,\(^{28}\) a preliminary step to late apoptosis.\(^{29,31}\) Our findings are consistent with those of Morgan et al.\(^{32}\) showing that abrogation of the G2/M checkpoint by a Chk1 inhibitor radiosensitizes pancreatic tumor cells.

The interactions of GBNs with photons, which produce Auger electrons, ROS, and other species, increased the killing effect of irradiation, as shown by the high level of clonogenic cell death compared with irradiation alone. As observed by Townley et al.\(^{33}\) with gadolinium-doped titanium nanoparticles, we found significant ROS generation 24 h after GBN treatment combined with irradiation. These species are determinants of the induction of radiation-induced apoptosis,\(^{34}\) implying a marked increase in both mitochondrial dysfunction and caspase cleavage coupled to changes in redox status. These results confirm that overcoming radioresistance in HNSCC is linked to cell entry into apoptosis.\(^{20,34–36}\) The radiosensitizing properties of GBNs appear comparable to those of carbon ions, which are known to induce complex and irreversible DNA lesions, since the \(\alpha\) parameter becomes predominant in the linear quadratic equation.\(^{31}\) The combination of GBNs and carbon therapy may be of potential interest and may improve patient outcomes.

Radiation therapy remains the most effective component after surgery in the treatment of HNSCC. However, many tumors invariably recur after radiation therapy, suggesting the presence of a radioresistant subpopulation of cells. Cancer stem cells are involved in cancer relapse and metastasis, and contribute to the resistance of tumors to treatments.\(^{21}\) We have shown that GBNs combined with irradiation can overcome radioresistance of SQ20B stem-like cells, and our strategy may therefore help increase patient survival.\(^{37}\)

We confirmed in vivo the radiosensitizing effects of GBNs in conjunction with irradiation in SQ20B-bearing nude mice. After a single 10 Gy dose, tumor growth was delayed because of decreasing cell proliferation associated with increased apoptosis. These effects were consistent with the results from in vitro studies of GNP radiosensitization with ionizing radiation.\(^{5,7,10,38,39}\) Maggiorella et al.\(^{40}\) showed that hafnium oxide nanoparticles have radiosensitizing effects in vitro and in vivo. These results indicate the clinical potential of GBNs in improving treatment outcomes of radioresistant tumors.

Intratumoral injection may not be the most pertinent route in association with radiotherapy, but its use before radiotherapy has recently been proposed for large-diameter nanoparticles such as hafnium nanoparticles in phase 1 clinical trials of soft tissue sarcomas (NCT01433068), and in a recent clinical trial in patients with locally advanced squamous cell carcinoma (HNSCC) of the oral cavity or oropharynx (NCT0194667).\(^{2}\)

In our study, since GBNs were not yet designed to specifically target HNSCC tumor cells, we preferred to use intra-tumoral injections to better control the local concentration of GBNs, even though an homogeneous distribution of GBNs in the tumor cannot be certain, leading to treatment variability, which may explain the variability in the GBN + 10 Gy group. Three recently published studies by Morlidas et al.\(^{41–43}\) reported GBNs functionalized with 3 different ligands targeting tumors rich in melanin, proteoglycans or \(\alpha\)v\(\beta\)3.

Regarding a potential clinical transfer, our data to date have not shown any adverse effects of GBNs. We have previously evaluated the compromise between a possible radiosensitizing effect and the absence of cytotoxicity in vitro by incubating SQ20B cells with increasing concentrations of GBNs. As in these previous studies,\(^{13,17,18,44}\) we found that GBNs were internalized in the cytosolic region of SQ20B cells but did not penetrate into the nucleus. As reported by Rima et al.\(^{18}\) GBNs...
were internalized by passive diffusion and macropinocytosis at concentrations less than 0.8 mM in the culture medium, and the irradiation efficiency decreased at concentrations higher or equal to 0.8 mM because GBNs formed aggregates around the cells. In our study, GBN alone did not influence SQ20B viability, cell cycle distribution, number of DSBs, or ROS production in vitro. These findings corroborate those of Townley et al.13 who reported the absence of a decrease in cell proliferation and ROS production in cells treated with titanium nanoparticles doped with up to 10% gadolinium. Our in vitro study strengthened the in vitro findings since no difference between the vehicle-alone and the GBN groups was found. No toxicity was observed by Le Duc et al.16 in their study of orthotopic gliosarcoma-bearing rats injected intravenously with 60 μmol of GBNs. In addition, no complementary activation of the immune system has been observed in vivo in the presence of GBNs.14,15

A large-scale reproducible synthesis has been set up, and the produced nanoparticles had excellent stability in highly diluted biological media and a complexation constant very close to that of the Dotarem® gadolinium-containing contrast agent. The degradation products of these nanoparticles in diluted medium have been studied extensively, and no direct toxicity on cultured cells was shown.

Another advantage of GBNs relies on their imaging properties because gadolinium is detected using MRI,45 whereas after specific labeling GBNs can be detected by single-photon emission computed tomography, positron emission tomography, and fluorescence imaging.46 GBNs offer, then, the possibility of verifying the delivered dose before treatment.

In conclusion, this study provided strong evidence, for the radiosensitizing effect of ultrasmall gadolinium based nanoparticles, which could be envisioned as passive or targeted compounds for theragnostic purposes.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.06.013.

References