Gadolinium-based nanoparticles to improve the hadrontherapy performances

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Abstract

Nanomedicine is proposed as a novel strategy to improve the performance of radiotherapy. High-Z nanoparticles are known to enhance the effects of ionising radiation. Recently, multimodal nanoparticles such as gadolinium-based nanoagents were proposed not only to amplify the effects of X-rays and \(\gamma\)-rays, but also to improve MRI diagnosis. For tumours sited in sensitive tissues, childhood cases and radioresistant cancers, hadrontherapy is considered superior to X-rays and \(\gamma\)-rays. Hadrontherapy, based on fast ion radiation, has the advantage of avoiding damage to the tissues behind the tumour; however, the damage caused in front of the tumour is its major limitation. Here, we demonstrate that multimodal gadolinium-based nanoparticles amplify cell death with fast ions used as ionising radiations. Molecular scale experiments give insights into the mechanisms underlying the amplification of radiation effects. This proof-of-concept opens up novel perspectives for multimodal nanomedicine in hadrontherapy, ultimately reducing negative radiation effects in healthy tissues in front of the tumour.

Key words: Nanomedicine; Gadolinium; Nano-sensitisation; Hadrontherapy; Theranostics

Background

Nanodrugs for cancer-therapy is a rapidly developing field of investigation, where new drug delivery vehicles, contrast agents and therapeutics are being processed with the goal of improving medical protocols.\textsuperscript{1–3} Recently, the use of nanomaterials was proposed as a promising way to enhance the performance of radiation therapies. Indeed, the limitation of conventional radiotherapy comes from the damage induced in the healthy tissues surrounding the tumour. In 2004, it was shown that the effects of X-rays can be amplified in tumours when gold nanoparticles are present.\textsuperscript{4} The \textit{in vivo} study demonstrated the high potential of using tumour-targeted nanomaterials to improve radiotherapies. Other studies performed on DNA and mammalian cells confirmed the properties of high-Z nanoparticles to amplify radiation effects.\textsuperscript{5,6}

On the other hand, fast ion-based radiation therapies (hadrontherapy and protontherapy) are considered superior approaches for the treatment of tumours located in highly sensitive tissues (brain, neck, eyes), paediatric cancers, and also tumours that are resistant to radiotherapy.\textsuperscript{7} The advantage of ions compared to photons stems from their property to induce maximum damage at the end of the track (called the Bragg peak). In operating conditions, the beam is tuned such that the Bragg peak is spread out and the maximum of the radiation effects coincides with the total volume of the tumour (mode of spread out Bragg peak). As a result, the damage induced behind the tumour is close to zero and the healthy tissues are preserved.\textsuperscript{8} Hence, hadrontherapy and protontherapy represent strong advances in cancer therapies. The major limitation of these techniques stems from the radiation effects that remain significant in front of the tumour (at the entrance of the track). It is thus a challenge to diminish the dose given to the patient and to enhance the biological effect of the treatment in the tumour. The use of tumour-targeted nanoparticles to amplify the radiation-effect of heavy ions in the tumour is a novel strategy, which has never been explored before.\textsuperscript{9}

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In this work we probed the effect of multimodal Gadolinium-based nanoparticles (GdBN) combined with carbon and helium ions radiations. This new type of multimodal nanoparticles, which behave not only as radiosensitisers but also as contrast agents, has been developed recently.9,10 This multimodality is very promising as it opens the perspectives to use a unique drug to improve simultaneous tumour monitoring and targeted therapy. This double modality, named theranostics, brings new issues in personalised medicine.11 It is already known that GdBN accumulate in tumours and present excellent properties as contrast agents in magnetic resonance imaging (MRI).12 In addition, in vitro and in vivo experiments demonstrated that GdBN are good radiosensitisers when gamma and x-rays are used.9,12 It is also important to mention that these nanoparticles are little toxic as demonstrated in our previous in vitro studies.13,14 Finally, we found that these nanoparticles accumulate in kidneys. We used a multi-scale approach to characterise the effects of GdBN at cellular and molecular scales. The efficiency of the nanoparticles to amplify cell death was evaluated using a Chinese hamster ovary cell line (CHO) because of its well-known and simple metabolism. CHO was previously used to probe the effects of Platinum Chloro 2,2':6',2" terpiridine, a well-known radiosensitiser.15 This model allows not only comparison of the biological impact of radiosensitisers combined with radiation, but also the avoidance of artefacts due to cell-specific biological functions. Indeed, human cell lines, which differ by their reaction to radiation (e.g. cell death pathways, radioresistance), could not be used as probes. We also quantified the yields of simple and complex (nano-size) damage using a molecular probe to distinguish and quantify the impact at the molecular level. In this perspective, pBr322 plasmid was used to quantify accurately and rapidly the induction of single strand breaks and double strand breaks that respectively correspond to simple and complex damages (see Supplementary Materials for a view of the plasmid conformations with simple and complex breaks). Plasmids and cells containing nanoparticles were irradiated with medical beams provided by the Heavy Ion Medical Accelerator Chiba (HIMAC, Japan), which is currently one of the most advanced hadrontherapy centres. In addition, the action sites of the nanoparticles in the cells were identified by two complementary methods of microscopy. Finally, this work is not only the first to highlight the amplification effects induced by multimodal nanoparticles combined with heavy ion radiation, but also the first evidence that these effects are initiated by nano and sub-nanoscale processes. We show that these processes take place in the cytoplasm, far from the nucleus.

Materials and Methods

Gadolinium-based nanoparticles (GdBN)

The Gadolinium-based nanoparticles consist of a polysiloxane core surrounded by gadolinium chelates that are covalently grafted on the inorganic matrix.16 The procedure of synthesis and the characteristics of these nanoparticles are detailed elsewhere.16 Briefly, their size is 3.0 ± 1.0 nm diameter and their mass is about 8.5 ± 1 kDa. These nanoparticles, highly stable, can be lyophilised and are stored at 4 °C. They are found to be biocompatible and to efficiently enrich tumours.9

Cell culture

CHO cells grew in Minimum Essential Medium-alpha (MEM-a) supplemented with 10% foetal bovine serum, penicillin (100 mg/mL) and streptomycin (100 mg/mL).15 Exponentially growing cells (1.56 × 10⁵ cells) were plated in flasks (Nunc Slide Flask 170920, 25 cm³) at least 12 h before irradiation. Cells were maintained in 5% CO₂ incubator at 37 °C. GdBN was added to the cell medium 6 h before irradiation at a concentration of 1 mmol L⁻¹ in gadolinium. At this concentration, the nanoparticles are not toxic.9,17 The cells were irradiated under atmospheric conditions, at room temperature. The combined effect of radiation and nanoparticles on cells was quantified by clonogenic assay. After irradiation, cells were trypsinised and plated into 60 mm Petri dishes (Falcon 3002) at a density of 100 surviving cells per dish. The plating efficiency was close to 85%. After ten days of incubation, the colonies were treated with 10% formalin and stained with 1% methylene blue. The colonies were counted to determine the survival fractions.
Table 1

<table>
<thead>
<tr>
<th>Ion beam</th>
<th>Sample</th>
<th>$\alpha$ (Gy$^{-1}$)</th>
<th>$\beta$ (Gy$^{-2}$)</th>
<th>$\alpha/\beta$(Gy)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(±0.02)</td>
<td>(±0.003)</td>
<td>(±0.7)</td>
<td></td>
</tr>
<tr>
<td>He$^{2+}$ Control</td>
<td>0.17</td>
<td>0.044</td>
<td>4.0</td>
<td>0.94</td>
<td>t2.4</td>
</tr>
<tr>
<td>GdBN</td>
<td>0.22</td>
<td>0.044</td>
<td>5.0</td>
<td>0.94</td>
<td>t2.5</td>
</tr>
<tr>
<td>C$^{6+}$ Control</td>
<td>0.19</td>
<td>0.047</td>
<td>4.0</td>
<td>0.94</td>
<td>t2.6</td>
</tr>
<tr>
<td>GdBN</td>
<td>0.27</td>
<td>0.049</td>
<td>5.6</td>
<td>0.94</td>
<td>t2.7</td>
</tr>
</tbody>
</table>

Results

The survival curves of mammalian cells free (controls) and cells loaded with GdBN irradiated by C$^{6+}$ and He$^{2+}$ beams are presented in Figure 1.

The cell survival fractions decreased exponentially with the increase in radiation dose. More interestingly, this decrease was stronger in the presence of GdBN. These results are similar to the effects observed with GdBN activated by high-energy photons. 12,17,18 It also confirms results reported on the amplification induced by metal nanoparticles when light ions (protons) are used to irradiate the cells. 19 This is the first proof that tumour-targeted nanoparticles amplify cell death when fast heavy ions are used as ionising radiation.

The efficiency of GdBN to amplify radiation-induced cell death was evaluated by calculating the enhancing factor (EF):

$$EF = \frac{D^{50} \text{control} - D^{50} \text{nanoparticles}}{D^{50} \text{control}}$$  

$$D^{50} \text{ nanoparticles} \text{ and } D^{50} \text{ control} \text{ correspond to the radiation doses used to reach 50}\% \text{ of cell death in the samples loaded with GdBN and in the controls, respectively. The values are reported in Table 1. This analysis shows that the enhancement is more pronounced when carbon ions are used.}$$

To characterise the type of lesions amplified by the nanoparticles, we simulated the curves of cell survival (S) with a linear quadratic law (Eq. (2)).

$$S(D) = \exp(-\alpha D - \beta D^2)$$  

D is the dose of irradiation. The coefficient $\alpha$ corresponds to the contribution of lesions, which are directly lethal for the cell, whereas $\beta$ is attributed to the contribution of additive sub-lethal lesions. The values of $\alpha$ and $\beta$ determined by a fitting procedure are reported in Table 2. This analysis shows that the presence of nanoparticles induces an increase in $\alpha$ while $\beta$ remains nearly constant. The resulting increase of the $\alpha/\beta$ ratio indicates enhancement of the lethality of the radiation treatment when GdBN is added. The efficiency of GdBN is slightly lower than the effect of platinum salts which were previously tested under the same conditions. 15 However in the case of the salts, the increase of the biological effect was due to an increase of the two parameters $\alpha$ and $\beta$. With nanoparticles, the increase of $\alpha$ only opens the perspective to increase the dose fractions in the treatment because of the linear trend of the survival dose response curve. This is of great interest in the clinic.
The interaction of ionising radiation with biological targets leads to the rupture of covalent bonds in biomolecules (DNA, proteins and other biomolecules). Single bond breaks are easily repaired and have little consequence for living organisms. In contrast, complex damages (i.e. clustered bond breaking) are highly toxic for the cells. To identify the type of damage induced at the nanometric scale, experiments were performed with DNA plasmids used as molecular probes. Plasmids consist of supercoiled DNA rings (S). The induction of single strand breaks (SSBs) leads to the transformation of the native S shape in the relaxed circular form (C). The induction of double strand breaks (DSBs) corresponds to the rupture of two face-to-face bonds; their size is intrinsically superior to 2 nm, which is the distance between two DNA strands. In plasmids, DSBs lead to the transformation of the native S shape into the linear form (L). Hence, SSBs and DSBs in plasmids are indicative of the induction of simple and complex (nanometre size) damages, respectively. In this work, we quantified the efficiency of GdBN to amplify SSBs and DSBs. In some of the experiments, dimethylsulfoxide (DMSO), a commonly used radical scavenger was added (at a final concentration of 1 mol L$^{-1}$) to investigate the role of water radicals. The plasmids were irradiated under the same conditions as the living cells. The three conformers of the plasmid were separated by agarose gel electrophoresis (see Supplementary Materials). No significant artefacts due to the binding of GdBN to DNA were found in the electrophoresis. After quantification of the three band intensities corresponding to the three plasmid conformations, the respective yields of simple (SSB) and complex (DSB) damage were determined as follows. The supercoiled plasmids (S) bind 1.47-times less ethidium bromide than relaxed (R) and linear (L) conformations.

\[
\begin{align*}
\text{Total} &= 1.47 \times S + R + L \\
R' &= R/\text{Total} \\
S' &= 1.47 \times S/\text{Total} \\
L' &= L/\text{Total}
\end{align*}
\]

The induction of SSBs and DSBs per plasmid was determined using Poisson law statistics:

\[
\begin{align*}
\text{SSB yield (breaks per plasmid)} &= \ln \left( \frac{L'}{S'} \right) \\
\text{DSB yield (breaks per plasmid)} &= \frac{L'}{1 - L'}
\end{align*}
\]

The results obtained with pure plasmids (controls) and plasmids loaded with GdBN irradiated by helium or by carbon ions are presented in Figure 2.

Prior to irradiation, DNA samples were more than 95% supercoiled, less than 5% circular, and contained no linear forms. The addition of GdBN did not damage DNA. In the controls and the GdBN-loaded plasmids, the number of SSBs and DSBs increased linearly with the dose, which indicates that simple and complex damages were induced.
complex damage is induced by single ionising events. In the presence of GdBN, the induction of molecular breaks (SSBs and DSBs) is significantly enhanced as compared to the controls. More interestingly, the induction of complex damage (DSBs) is more pronounced than the induction of simple breaks (SSBs). This has also been confirmed by similar molecular scale studies performed with metal nanoparticles. The efficiency of GdBN to amplify SSBs and DSBs is quantified using the amplification factors (AF), which are defined as follows:\textsuperscript{23}

\[ AF(X) = \frac{X_{\text{yield}_{\text{GdBN}}}}{X_{\text{yield}_{\text{control}}}} = \frac{Y_{\text{SSB}}}{Y_{\text{DSB}}} \]

The yield of SSB (respectively DSB) is defined as the number of breaks induced per plasmid and per gray. It corresponds to the slope of the dose response curves. The yields of SSBs and DSBs and the corresponding amplification factors (AF) are reported in Table 3. This quantitative analysis shows that, in the presence of GdBN, the induction of complex damage (DSBs) is enhanced by a factor of 45\% with helium ions, and 73\% with carbon ions. The variation of the simple breaks is less pronounced (24\% and 29\% with helium and carbon ions, respectively).

This experiment confirms that the addition of nanoparticles amplifies the molecular damage, in particular, the breaks of nanometre-size. It is worth mentioning that the amplification of this damage is more pronounced with the carbon ion beam, which is similar to the variation in cell death and to the induction of \textit{directly lethal} lesions. This multiscale study highlights the correlation between cell death and the induction of complex molecular damage. It demonstrates the importance of early stage mechanisms, which take place at the nano- and sub-nanometre scale.

SSBs and DSBs are commonly attributed to two types of elementary processes. The \textit{direct processes} correspond to the direct interaction of ions and/or electrons with biomolecules. The \textit{indirect processes} are attributed to the reaction of water radicals with surrounding biomolecules. In this work, we probed the role of water radicals and the effect of GdBN by adding a commonly used radical scavenger (DMSO) to some of the experiments.\textsuperscript{24,25} The results obtained in plasmids loaded or not with GdBN are presented in Figure 2 (A1 and A2) and summarised in Table 3. This study clearly shows that, when water radicals are scavenged, the molecular damage drops drastically. This is a clear indication that the action sites of the nanoparticles in the cells were identified by two complementary methods of microscopy. Confocal microscopy was used to observe the internalisation of GdBN functionalised with the cyanine 5.5, a near infrared fluorescent dye. Figure 3 corresponds to the superposition of the fluorescence and the optical images of living cells loaded with GdBN-cyanine.

This measurement indicates that the nanoparticles enter into the cytoplasm. This is in agreement with other results obtained with gold and gadolinium nanoparticles in living cells.\textsuperscript{27,28} Even after several hours of incubation, there was no fluorescence detected in the nucleus. The images recorded on living cells at different times of incubation show that the internalisation of GdBN-cyanine in CHO took less than ten minutes. Confocal microscopy is able to monitor the internalisation of the nanoparticles in living cells, but the addition of a fluorescent dye (here cyanine 5.5) at the surface of the nanoparticles can influence the uptake of the nanoparticles by the cells.\textsuperscript{29} Therefore, transmission electron microscopy experiments were performed to investigate the location of nanoparticles free of fluorophores.

An example of TEM and STEM measurements obtained with CHO incubated with nanoparticles free of cyanine is shown in Figure 4. Figure 4, A, corresponds to the image of transmitted electrons (TEM) through a region of a cell. This is the first indication of the presence of dense grains (black spots) in vesicles located in the cytoplasm of a cell. These grains are not specific of high-Z elements (gadolinium in this case). Therefore, dark field scanning transmission electron microscopy (STEM) was performed to collect at large angles the electrons transmitted and scattered by the heaviest elements (here gadolinium). This measurement, which is time consuming, was performed for limited regions only. Figure 4, B, shows the STEM image of the cell region delimited by the black frame in Figure 4, A. The white spots correspond to the electrons that were specifically scattered by gadolinium. This measurement confirms the presence of GdBN in the cytoplasmic vesicles.\textsuperscript{30} Images were collected for more than 40 cells in three different samples. They all demonstrate that GdBN does not penetrate the nucleus.

In electron microscopy, the kinetics of the internalisation process is lost because of the sample preparation (fixation and cut of the cells). However, the observation of vesicles is strongly indicative of the use of endocytosis to internalise the nanoparticles.\textsuperscript{31}

Finally, the two complementary microscopy tools, confocal microscopy and electron microscopy, confirmed that the nanoparticles are finally located in the cytoplasm of the cells. From these results, we can conclude that the early stage processes responsible for the amplification of cell death are initiated in the cytoplasm, far from the nucleus.
which also may dimerise into hydrogen peroxide (H₂O₂). Even at reactive oxygen species (ROS) such as hydroxyl radicals (HO°), less than 20 eV induce water radiolysis. It results in the production of electrons emitted by the nanoparticles with energies higher in the few nanometres surrounding the nanoparticle. The activation step is followed by several mechanisms of relaxation, which take place in the few nanometres surrounding the nanoparticle. The electrons emitted by the nanoparticles with energies higher than 20 eV induce water radiolysis. It results in the production of reactive oxygen species (ROS) such as hydroxyl radicals (HO°), which also may dimerise into hydrogen peroxide (H₂O₂). Even at low energies (<20 eV), electrons may dissociate water molecules into ROS via dissociative electron attachment. In addition to these effects, the water molecules may also be ionised due to their interaction with the positively charged nanoparticles. In this case, ionisation as a result of electron transfer from the water molecules to the positive site may lead to the fragmentation of the molecules into ROS. This cascade of processes takes place in the range of 10⁻¹⁵ to 10⁻¹² sec. Thus, the efficiency of the nanoparticles to amplify the radiation-induced damage comes not only from the enhancement of reactive oxygen species produced in the medium, but from the spatio-temporal distribution of these species in nano-size clusters (see Figure 5). It is the reaction of these highly reactive clusters with cytosolic molecules that is responsible for the induction of nano-size damage, directly lethal for the cells. This finding is in agreement with recent microbeam experiments, which shows that cytosolic radiation damage plays an important role in the cell death induced by radiation.

In conclusion, this study demonstrates for the first time the possibility to amplify the radiation effects of medical heavy ions (helium and carbon) using nanoparticles. The enrichment of tumours with nanoparticles allows enhancing radiation effects in its volume. Hence, the addition of nanoparticles during the treatment by fast ions would allow a reduction of the total radiation dose given to the patient, ultimately reducing negative radiation effects in healthy tissues in front of the tumour. The use of multimodal nanoagents such as Gadolinium-based nanoparticles opens the additional perspective to implement theranostics in hadrontherapy, a perspective to improve the...

Figure 3. Confocal microscopy image of CHO cells loaded with GdBN-cyanine 5.5. The nanoparticles (in red) are located in the cytoplasm.

Figure 4. (A) TEM image of gadolinium-based nanoparticles in CHO. (B) Dark field STEM image of the zone delimited by the black frame in the TEM image.
monitoring and therapeutic performances of the treatments. Besides the first proof-of-concept that nanomedicine can overcome the limitation of ion treatments, the multiscale approach adopted in this work shows that the effect of the nanoparticles is due to the activation of early nano- and sub-nanosize processes in the cytoplasm, far from the nucleus. This finding impacts the strategies to develop new nano-agents and treatment planning aimed at improving fast ion treatments.

Finally, the use of nanoparticles with radiation therapies will revolutionise clinical practices to treat cancer. The present findings demonstrate that this strategy can be used to improve not only the performances of conventional radiotherapy but also of other modalities such as the hadrontherapy. This strategy is at its start and requires many more tests. In the perspective of a full characterisation and classification of nanoagents, studies on human cell lines including the evaluation of the radiosensitising effects and the quantification of internalised nanoparticles will be processed. In vivo experiments with ion beams are also in the scope of our future studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.05.005.

References


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Gadolinium-based nanoparticles amplify the effects of medical ion radiation, which opens a new field of investigation dedicated to the implementation of theranostic (therapy and diagnostic) in hadrontherapy. Multiscale experiments on plasmids and living cells show that this amplification is due to the activation of nanoparticles in the cytoplasm and to the production of nano-size radicals clusters far from the nucleus.