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Abstract

Radiation-induced DNA damage occurs as a consequence of both direct and indirect effects of ionizing radiation. The induction mechanism of DNA damage is mainly influenced by the physical characteristics of the radiation quality, especially the linear energy transfer. In general, hypoxia reduces the effect of irradiation treatment in tumor cells and leads to poor patient outcomes. Emitters with high linear energy transfer (alpha- or Auger-electron-emitters) can overcome this obstacle. Our aim is to demonstrate the influence of hypoxia on the interaction between different radiation qualities with DNA by using a cell free plasmid model modulated by the free radical scavenger dimethyl sulfoxide (DMSO).

Plasmid DNA was irradiated with ^{223}Ra, ^{188}Re, ^{99m}Tc and DNA-binding ^{99m}Tc-pyrene in the absence or presence of DMSO and either under normoxic or hypoxic conditions. The resulting DNA damage in form of single- (SSB) and double strand breaks (DSB) was analyzed by agarose gel electrophoresis. Applied radiation doses of up to 200 Gy of ^{223}Ra, ^{188}Re or ^{99m}Tc or 60 Gy of ^{99m}Tc-pyrene led to maximal yields of SSB (80%) in plasmid DNA. Irradiation with ^{223}Ra, ^{188}Re or ^{99m}Tc at 200 Gy induced 30%, 28% and 32% linear plasmid conformations, respectively, which are associated with DSB. Hypoxia had a minor effect on SSB and DSB induction from ^{223}Ra but a small enhancement in DSB for ^{188}Re and ^{99m}Tc. DMSO could prevent DSB completely and SSB DNA damage from the three “free” radionuclides to comparable levels. DNA-binding ^{99m}Tc-pyrene induced less SSB and DSB compared to free $^{99m}TcO_4^-$ due to its own radical scavenging properties. However, an additional incubation of DMSO could prevent the SSB and DSB induction only to a minor extent. Direct insults of Auger-electrons

from ^{99m}Tc-pyrene are more effective than high-energy electrons or alpha particles due to the minimal distance between the radionuclide and the DNA.

We conclude that hypoxia does not limit DNA damage in plasmids induced by ^{223}Ra, ^{188}Re, ^{99m}Tc and ^{99m}Tc-pyrene. Dose-dependent radiation effects were comparable for alpha-emitters and both high- and low-energy electron emitters. The radioprotection by DMSO was not influenced by hypoxia. Overall, the results indicate the contribution of mainly indirect radiation effects for ^{99m}Tc, ^{188}Re and ^{223}Ra. ^{99m}Tc-pyrene caused direct DNA damages. The direct participation of oxygen in cell-free plasmid DNA damage induction was not proven.

INTRODUCTION

The molecular nature of DNA damage is characterized by the physical properties of energy deposition and the chemical environment. Simple experimental systems for DNA damage allow the quantification of the dependence and relevance of these parameters. For this purpose, plasmid DNA models are frequently used to study the yields of single strand breaks (SSB) and double strand breaks (DSB) induced by α-particles and heavy ion beams *(1-5)*. Direct and indirect action can be differentiated by means of dimethyl sulfoxide (DMSO) which prevents radical-mediated, indirect DNA damage *(4, 6, 7)*.

Hypoxic regions of solid tumors have poor oxygenation levels due to insufficient blood supply *(8)*. In vivo, hypoxic cells are more radioresistant and resistant to chemotherapy than normoxic cells, leading to poor outcomes in cancer patients due to malignant progression and metastases *(9)*. In a pioneering study, Gray et al. showed that the presence of oxygen during irradiation induced an increase in radiosensitivity *(10)*. The radioresistance of hypoxic cells can be quantified by the oxygen enhancement ratio (OER), which is defined as the ratio of the radiation doses required to produce the same biological effects under hypoxic and normoxic conditions *(11)*. The radioresistance of hypoxic cells in tumor tissues is attributed to the so-called oxygen effect *(12)*. One reason that DNA damage is increased under normoxic conditions is that higher levels of reactive free oxygen species (oxygen radicals, ROS) are generated, leading to more DNA strand breaks *(13)*. Another reason is that under normoxic conditions, radiation-induced DNA lesions are irreparable due to rapid reactions with the free electrons from molecular oxygen *(14)*. However, in the absence of oxygen, ionizing radiation produces lower levels of DNA damage, and DNA radicals are reduced by hydrogen donation from sulfhydryl groups, leading to DNA repair *(15)*. The direct action of oxygen on the DNA insult was calculated using GEANT4 *(16)*. In addition to the chemical mechanisms, biological mechanisms (e.g. HIF-1α induction or DNA repair) are also important for the hypoxia-related tumor resistance.

This study aims at the determination of differences in DNA damaging mechanisms under hypoxic and normoxic conditions, as well as the dependence on different radiation qualities concerning their linear energy transfer (LET) using a simple, cell-free system. Plasmid DNA was irradiated with alpha-emitting ^{223}Ra, high- (^{188}Re) and low- (^{99m}Tc) energy electron emitters and a direct DNA binding, radiolabeled DNA intercalator ^{99m}Tc-pyrene. Additionally, all experiments were repeated in the same way but using the radioprotective agent DMSO to differentiate between direct and indirect (caused by ROS) radiation damage.

MATERIAL AND METHODS

Radionuclides and ^{99m}Tc-pyrene

The α-particle emitter ^{223}Ra ($^{223}RaCl_2$, Xofigo) was provided by Bayer Vital GmbH (Leverkusen, Germany) with an activity concentration of 1000 kBq/mL. ^{223}Ra (half-life 11.4 d) decays through a cascade of short lived α- and β-particle emitters. Every decay of ^{223}Ra consists of four α- and two β-decays, resulting in the emission of approximately 28 MeV with 95% of the energy caused by the α-emission. The mean LET of all alpha particles from ^{223}Ra and their decay products is about 112 keV/µm *(17, 18)*.

The β-emitter ^{188}Re ($^{188}ReO_4^-$) was obtained by elution of a 40 GBq alumina-based ^{188}W/^{188}Re generator (Isotope Technologies Garching GmbH, Germany). ^{188}Re owns the following physical characteristics: half-life of 17 h, maximal β-energy of 2.1 MeV, LET of 0.19 keV/µm, γ-emission of 155 keV *(19)*.

^{99m}Tc-pertechnetate ($^{99m}TcO_4^-$) was eluted from a ^{99}Mo/^{99m}Tc generator (Ultra-TechnekowTM DTE, Mallinckrodt Medical B. V., Le Petten, The Netherlands) using 0.9% saline. The radionuclide ^{99m}Tc (half-life 6 h) emits γ-rays with energies of 140 keV. Simultaneously, ^{99m}Tc statistically emits 5.1 electrons per decay; 94% of these having kinetic energies below 2 keV and a mean LET higher than 10 keV/µm *(20)*.

^{99m}Tc-pyrene was produced according to the literature *(21)*. In short: tricarbonyl kits for the chelation of ^{99m}Tc were prepared in-house. Every tricarbonyl kit consisted of the following components: 17 mg sodium tartrate, 3.5 mg sodium borate, 3.2 mg sodium carbonate and 8.1 mg potassium boranocarbonate. $^{99m}TcO_4^-$ (1 mL, ~ 5 GBq) was added to the tricarbonyl kit. Afterwards, the solution was mixed at 300 rpm for 30 min at 95°C and was used without further purification. The pH of the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ complex solution (~5 GBq/mL) was adjusted to 6-7 by adding PBS/1M HCl (190 µL, v/v, 1:1). For radiolabeling, 30 µg of the pyrene derivative were added to the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ complex solution and the resulting mixture was heated at 95°C and 500 rpm for 60 min. The resulting radiotracer was analysed and purified via HPLC (Merck Hitachi, Darmstadt, Germany) using a Merck Millipore Chromolith® Performance RP-18e (100 x 4.6 mm) column for analysis and purification. After purification, the final product exhibits a radiochemical purity of more than 95% and was used for the plasmid DNA experiments.

Plasmid DNA

The plasmid pUC19 (2686 base pairs, molar mass $1.75x10^6$ Dalton), originating from E.coli ER2272 was purchased from New England Biolabs (Ipswich, United Kingdom). The DNA stock solutions were diluted in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) to achieve a final concentration of 0.1 µg/µL and stored at -20 °C. Only samples containing > 95% closed circular DNA forms were used. Digestion of pUC19 to obtain linear plasmid reference was performed using the restriction enzyme BamHI (Invitrogen, Karlsruhe, Germany) to obtain linear DNA.

Irradiation procedure for plasmid DNA

Plasmid samples, containing 200 ng of plasmid DNA at the same concentration (0.1 µg/µL) and different amounts of radioactive solutions, were placed into 1.5 mL micro tubes (Eppendorf, Hamburg, Germany). One sample without any further containment than pUC19 DNA served as the control. Solutions of ^{223}Ra (1.5-30 kBq), ^{188}Re (0.42-6.25 MBq), ^{99m}Tc (3.3-

132 MBq) or ^{99m}Tc-pyrene (3.3-39.6 MBq) were added to the pUC19 DNA in a total volume of 20 µL. For the accumulation of the radiation dose (^{223}Ra, ^{188}Re and ^{99m}Tc: 0-200 Gy; ^{99m}Tc-pyrene 0-60 Gy) each sample was incubated for a period of 24 h in an incubator under either hypoxic or normoxic conditions. Hypoxic conditions were achieved in an incubator in which the oxygen concentration was set to 0.2%. The humidified atmosphere was balanced with nitrogen and the CO_2 concentration was set to 5%.

In the experimental setup four different incubation scenarios were considered and each set was subjected to various amounts of ^{223}Ra, ^{188}Re, ^{99m}Tc and ^{99m}Tc-pyrene:

1) without any modulation under normoxic conditions
2) without any modulation under hypoxic conditions
3) with the radical scavenger DMSO (0.2 M) under normoxic conditions
4) with DMSO (0.2 M) under hypoxic conditions

Measurement of DNA damage in pUC19 plasmids

After 24 h of incubation, 10 µL of each sample were mixed with 1.25 µL of blue juice electrophoresis loading buffer. The samples were pipetted in the wells of a 1.4% agarose gel in Tris-Acetate-EDTA buffer. The samples were run at 4 V/cm for 120 min at 6°C. After electrophoresis, supercoiled (SC), open circular (OC) and linear (L) forms of plasmid DNA were identified based on their mobility differences in the gel. The different DNA bands were stained with ethidium bromide (0.5 µg/mL) followed by visualizing of the resulting fluorescence (ChemiDoc XRS+, Bio-Rad Laboratories GmbH München, Germany). The DNA damage was determined by quantifying the relative fluorescence intensities of the three possible DNA bands (OC, L, SC) in each sample by using the ImageLab software (Bio-Rad).

Dosimetry

Dose calculations were performed for plasmid DNA experiments in accordance to the MIRD formalism *(22)* as described before *(19)*. In brief, the dose from a source volume to a target volume was calculated as the product of the time-integrated activity in the source volume and a source-target-specific S value.

As mentioned above, each plasmid DNA sample was irradiated in a total volume of 20 µL in micro tubes. The geometric shape of the radionuclide solution containing the plasmid DNA corresponds to a spherical drop with 1.68 mm radius. S values for ^{223}Ra (2.17E-7 Gy/(Bq s)), ^{99m}Tc (1.29E-10 Gy/(Bq s)) and ^{188}Re (2.15E-9 Gy/(Bq s)) were estimated by Monte Carlo simulations using Geant4 to calculate the mean dose in the sphere assuming an internal homogeneous activity distribution. The time-integrated activity, corresponding to the total number of decays in the micro tube N_{MT}, was calculated by

$$N_{MT} = \frac{A \cdot T_{1/2}}{\ln(2)} \left(1 - \exp\left[-\ln(2)\frac{t_{expo}}{T_{1/2}}\right]\right),$$

with A being the applied radioactivity, $T_{1/2}$ being the radionuclide's half-life, and t_{expo} being the exposition time. For example: 1 kBq ^{223}Ra, 1 MBq ^{188}Re and 10 MBq ^{99m}Tc accumulate within 24 h in a volume of 20 µL doses of 18 Gy, 118 Gy and 38 Gy, respectively.

RESULTS

Dose-dependent DNA strand break induction was evaluated for the three unbound (non-chelated) radionuclides ^{99m}Tc, ^{188}Re, ^{223}Ra and the directly to the DNA binding ^{99m}Tc-pyrene derivative. All experiments were carried out under normoxic and hypoxic conditions with and without DMSO modulation to allow the differentiation between indirect and direct induced DNA strand breaks. Up to three different DNA bands were obtained after gel electrophoresis (OC,

L, SC). The classification of the DNA bands to the different conformation states is shown in Figure 1.

FIG. 1. Stained and excited agarose gel after irradiation of pUC19 plasmid-DNA with different dose to show the formation of SSB (OC conformation band) and DSB (L conformation band) as well as remaining SC plasmid DNA (dose increases from lane 1 to lane 5).

Irradiation with $^{99m}TcO_4^-$

Plasmid DNA was irradiated with non-chelated, low-energy Auger-electron-emitting $^{99m}TcO_4^-$ using different radioactivity amounts leading to total accumulated radiation doses of 5-200 Gy. Figures 2a and 2b show the results without and with DMSO modulation, respectively. All following data is expressed as average values calculated out of independent experiments with the respective standard deviations.

A clear decrease of SC plasmid fraction due to the formation of OC and L plasmid is shown for dose values up to 80 Gy where no SC plasmids are left. Higher doses up to 200 Gy led to a shift from in the distribution from OC to L plasmid formations. The L plasmid fraction, i.e. the amount of induced DSB, increased monotonously and roughly linearly with radiation dose. There was no substantial difference detected when comparing hypoxic and normoxic incubation conditions, although, it seems that hypoxia could have a small sensitizing effect. The additional incubation of 0.2 M DMSO led to a substantial reduction of the total amount of SSB by more than 50% and an entire loss of induced DSB (Figure 2b).

FIG.2. Comparison of the dose-dependent relative fluorescence intensity (RFI) to show the induction of single strand- (OC) and double-strand breaks (L) on pUC19 plasmid DNA after irradiation with Technetium-99m under hypoxic and normoxic conditions a) without and b) with DMSO as free radical scavenger.

Irradiation with $^{188}ReO_4^-$

Various amounts of high-energy electron emitting $^{188}ReO_4^-$ were incubated on plasmid DNA resulting in equivalent doses of 5-200 Gy after 24 h of irradiation. The dose-dependent induction of SSB and DSB is presented in Figure 3 (3a for experiments without, 3b with additional DMSO modulation).

Irradiation with ^{188}Re led to a clear decrease of intact SC plasmid DNA up to 80 Gy. Higher radiation dose values caused further induction of the L conformation which correlates with the number of DSB. Similar to the incubation of pUC19 with ^{99m}Tc-pertechnetate, there was no substantial difference detectable between the hypoxic and the normoxic experimental setup, although, there seemed to be a slight protective effect. By comparing Figure 3a to 3b, a clear radioprotective influence of 0.2 M DMSO can be observed and the effect is comparable to the ^{99m}Tc results.

FIG. 3. Comparison of the dose-dependent relative fluorescence intensity (RFI) to show the induction of single strand- (OC) and double-strand breaks (L) on pUC19 plasmid DNA after irradiation with Rhenium-188 under hypoxic and normoxic conditions a) without and b) with DMSO as free radical scavenger.

Irradiation with $^{223}Ra^{2+}$

Moreover, plasmid DNA was irradiated with the high-LET alpha emitter ^{223}Ra in the form of its commercially available $^{223}RaCl_2$. Different amounts of activity were incubated to accumulate equivalent doses of 10-200 Gy after 24 h of incubation under hypoxic and normoxic conditions. The results of the experiments are shown in Figure 4 (4a for experiments without, 4b with additional DMSO modulation).

Alpha particle-emitting ^{223}Ra induced similar dose-dependent SSB and DSB without DMSO compared to the other radiation qualities. There is practically no difference between the results obtained with the hypoxic and normoxic incubation procedure. A clear decrease of SC plasmid

DNA was noticeable until 80 Gy. Higher doses enhanced the formation of DSB. The efficiency of strand break induction by ^{223}Ra was strongly influenced by DMSO. The comparison of Figures 4a and 4b clearly shows that the induced DNA damage, both SSB and DSB, were drastically reduced and more than 80% of the plasmid remained in SC formation even at the highest dose of 200 Gy.

FIG. 4. Comparison of the dose-dependent relative fluorescence intensity (RFI) to show the induction of single strand- (OC) and double-strand breaks (L) on pUC19 plasmid DNA after irradiation with Radium-223 under hypoxic and normoxic conditions a) without and b) with DMSO as free radical scavenger.

Irradiation with DNA-binding ^{99m}Tc-pyrene

In addition to the three radionuclides which were not expected to directly interact with the DNA, different amounts of Auger-electron-emitting ^{99m}Tc-pyrene were incubated over 24 h leading to radiation doses of 5-60 Gy. The results of the normoxic and hypoxic experiments are shown in Figure 5 (5a for experiments without, 5b with additional DMSO modulation).

FIG. 5. Comparison of the dose-dependent relative fluorescence intensity (RFI) to show the induction of single strand- (OC) and double-strand breaks (L) on pUC19 plasmid DNA after irradiation with Technetium-99m-pyrene under hypoxic and normoxic conditions a) without and b) with DMSO as free radical scavenger.

Dose-dependent strand break inductions were detected for the complete evaluated dose range from 5-60 Gy. There was no difference between normoxic and hypoxic experiments provable. The additional incubation with 0.2 M DMSO did not have any further relevant influence on the results. The remaining amount of directly induced SSB at 60 Gy was 40% and 15% of DSB, respectively. Compared to the three “free” radiometals, this was the highest value of remaining direct DNA damage resulting from a dose of 60 Gy.

DISCUSSION

In the present work, plasmid DNA was used as a valuable tool to better understand the radiation effects of radionuclides with different LET by comparing hypoxic to normoxic incubation conditions. Damage by ionizing radiation has been divided into three categories: 1. The direct effect, in which the DNA is ionized itself. 2. The quasi-direct effect, which involves the ionization of water molecules that are tightly bound to the DNA. 3. The indirect effect, in which radiolysis of the solvent gives rise to highly reactive species which may subsequently react with the DNA *(23)*. The oxygen fixation hypothesis says that DNA lesions that are produced by x-rays with chemical participation of oxygen cannot be chemically restored to an undamaged state nor repaired by the enzymatic repair system *(12)*. The oxygen enhancement ratio (OER) is lowered from acute (1 Gy/min) to low dose range (1 Gy/h) *(11, 24)* and with an increasing LET *(25)*. However, Maucksch et al. could demonstrate that low-dose irradiation over a time period of 24 h is sufficient to demonstrate an oxygen enhancement ratio of 1.6 for ^{188}Re or ^{99m}Tc-HMPAO in A431 tumors cells while α-emitter ^{223}Ra yielded an OER of 1.08.

In isolated nuclei, OER for DNA damage decreases form about 3 in whole cells to 1 *(26)*. When isolated DNA is irradiated in solution the absence of oxygen does not lead to a decrease in damage unless low-molecular-weight thiols are also present *(27)*. Cellular and molecular repair of x-ray induced DNA damage depends not only on oxygen tension but on nutritional status, too *(28)*.

It was generally accepted that high-LET emitters predominantly interact with DNA by direct effects in which the target itself is ionized by the particle rather than by indirect effects of radicals which originated from the radiolysis of DNA-surrounding water *(2)*. Hence, it could be expected that the α-emitter ^{223}Ra (mean LET 112 keV/µm) may induce primarily a direct radiation response in contrast to the β- and Auger-electron-emitter. The free radical scavenger

DMSO can be used as a protective agent for the DNA by catching •OH radicals generated by the radiolysis of water *(29)*.

However, the quantification of DNA damage in plasmid DNA exposed to ^{223}Ra, ^{188}Re and ^{99m}Tc revealed, regardless of the largely different LET, a dose-dependent increase in open circular and linear DNA conformations with comparable effect size for similar doses of the three isotopes. This is in line with the data of Matsumoto et al. who observed that total •OH generation levels were identical at the same dose irrespective of whether X-ray or carbon-ion irradiation was used *(30)*. Studying the contribution of indirect action to radiation-induced mammalian cell inactivation, Ito et al. found that the efficiency of indirect action was much greater than that of direct action even at the highest LET of 440 keV/µm considered in their study *(31)*. Hirayama et al. concluded that the contribution of indirect action in cell killing decreases with increasing LET but indirect action by •OH radicals still contributes 30% or more of the cell killing in the very high-LET region of up to 2106 keV/µm *(32, 33)*.

During hypoxia, the formation of open circular and linear DNA conformations was similar in comparison to normoxia regarding the irradiation by ^{223}Ra and slightly increased concerning ^{188}Re as well as ^{99m}Tc. This means, that the hypoxic conditions during the irradiation had no important influence on the primary interaction of radiation with DNA, independent of the applied LET. Differences became only evident when repair unveils DNA lesions of different complexity.

The corresponding yields of SSB and DSB were markedly decreased by the addition of 0.2 M DMSO, with a similar efficiency for each radiation type (Fig. 2, 3 and 4 a .vs. b). This is in agreement with the study of Runge et al. using the same set of radioisotopes *(19)*. It was demonstrated, that •OH radicals are much more important than reactive free oxygen species for the induction of SSB and DSB in plasmid DNA. Moreover, the direct effect is less important than expected, especially for the high-LET emitters. Apparently, the distance between the radionuclide and the DNA was still too large. Therefore, the ionization density was insufficient to result in larger amounts of directly induced strand breaks.

The comparison of ^{99m}Tc and DNA-binding ^{99m}Tc-pyrene without DMSO revealed less DNA damage for ^{99m}Tc-pyrene for identical dose up to 60 Gy despite its DNA binding. This difference is explainable by the scavenger characteristics of the pyrene itself which was demonstrated by Reissig et al. *(21)*. Due to the applied production method of the radioconjugate the final product concentration was limited. In consequence, no higher doses could be achieved for direct DNA-binding ^{99m}Tc-pyrene. Furthermore, it was more complicated to keep constant reaction and experiment conditions for ^{99m}Tc-pyrene compared to the non-chelated, free radionuclides. Hypoxia did not influence the formation of DNA damage for the DNA-bound Auger-electron-emitter ^{99m}Tc. Remarkably, the addition of DMSO could prevent DNA damage only to a very small extent. As a consequence, a substantial amount of directly induced SSB and DSB was unpreventable. The direct DNA damage by Auger-electron-emitting ^{99m}Tc was also demonstrated earlier using DNA-binding ^{99m}Tc-labeled HYNIC-DAPI *(20)*.

While the DNA damage caused by high-LET alpha emission in plasmid DNA was preventable by DMSO to a large extent, the direct DNA damage by DNA-binding ^{99m}Tc-pyrene was much less influenced by DMSO. However, these data cannot be transferred to the intact cell or even tissue due to further barriers in cell systems like a different DNA conformation, the cell membrane itself, signal cascades and several repair mechanisms, respectively *(34)*. Regarding (tumor)tissues these influences are getting even more complex.

The observation of the oxygen effect in intact cells could not be confirmed in naked plasmid DNA. This means that other cellular mechanisms are to a large extent responsible for the reduced radiosensitivity in hypoxia, most important repair machinery. Furthermore, the heterodimeric transcription factor HIF-1 (hypoxia-inducible factor 1) plays an important role in adaption to hypoxia *(35)*. The oxygen-dependent α-subunit of HIF-1 (HIF-1α) is stabilized under hypoxic conditions and interacts with the constitutively expressed α-subunit of HIF-1 (HIF-1α). The dimeric protein HIF-1α regulates the expression levels of approximately 100 target genes that are associated with erythropoiesis, glycolysis, angiogenesis and apoptosis

in hypoxic tissues *(36, 37)*. HIF-1α activation leads to an increased resistance to radiotherapy. And these biological processes seem to be much more important than the formation of ROS due to irradiation in the primary interaction of irradiation and DNA.

CONCLUSION

Dose-dependent induction of SSB and DSB was found for high-LET emitters as well as for low-LET emitters to the same extent in the plasmid DNA model. The effect of hypoxia was minor in the cell-free system which points out that oxygen effects in the cellular system are primarily caused by biological effects rather than the formation of ROS for DNA damage. DMSO as free radical scavenger had radioprotective effects even regarding a high-LET alpha-emitter but not regarding the DNA-binding Auger-electron-emitter ^{99m}Tc-pyrene. Direct DNA damage could be demonstrated by the DNA-bound Auger-electron-emitter ^{99m}Tc-pyrene but not by the α-emitter ^{223}Ra.

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