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Reissig, F.; Runge, R.; Naumann, A.; Kotzerke, J.;

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Cisplatin – A more Efficient Drug in Combination with Radionuclides?

Cisplatin – Größere Wirksamkeit bei Kombination mit Radionukliden?

Falco Reissig

[1] Institute of Radiopharmaceutical Cancer Research, Helmholtz-Zentrum Dresden-Rossendorf, Dresden, Germany (Ringgold ID: RIN28414)

Roswitha Runge

[2] Department of Nuclear Medicine, University Hospital/ Faculty of Medicine Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

Anne Naumann

[2] Department of Nuclear Medicine, University Hospital/ Faculty of Medicine Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

Jörg Kotzerke

[2] Department of Nuclear Medicine, University Hospital/ Faculty of Medicine Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

Abstract

The combination of conventional chemotherapeutic drugs with radionuclides or external radiation is discussed for a long period of time. The major advantage of a successful combination therapy is the reduction of severe side effects by decreasing the needed dose and simultaneously increasing therapeutic efficiency. In this study, pUC19 plasmid DNA was incubated with the cytostatic drug cisplatin and additionally irradiated with 99mTc, 188Re and 223Ra. DNA damages, such as single- and double strand breaks were determined by agarose gel electrophoresis. The threshold concentration value of cisplatin, which was tolerated by pUC19 plasmid DNA was determined to be 18-24 nM. Nevertheless, even at higher dose values (>100 Gy) and simultaneous incubation of cisplatin to 200 ng plasmid DNA, no significant increase in the number of induced single- and double-strand breaks was obtained, compared to the damage solely caused by the radionuclides. We thereby conclude that there is no direct dependence of the mechanism of strand break induction to the absence or presence of platinum atoms attached to the DNA. Reported increasing DNA damages in therapy approaches on a cellular level strongly depend on the study design and are mainly influenced by repair mechanisms in living cells. Nevertheless, the use of radioactive cisplatin, containing the Auger electron emitter 191Pt, 193mPt or 195mPt, is a bright prospect for future therapy by killing tumor cells combining two operating principles: a cytostatic drug and a radiopharmaceutical at the same time.

Introduction

Cancer Therapy using metal-containing pharmaceuticals is established for a long period of time. Especially, platinum compounds (such as cisplatin, carboplatin and oxaliplatin) play a big role when speaking about conventional and non-specialized tumor destruction [1-3]. The general use of platinum pharmaceuticals in cancer management focuses the rather unselective therapy of various tumor tissues, such as prostate, breast, non-small cell lung, squamous cell, head and neck and esophageal carcinoma [4-6]. By forming crosslinks within the strands of the DNA, those compounds inhibit cell repair and division, thus leading to a decrease of tumor volume [7].

In addition to their diagnostic and therapeutic potentials (depending on the physical properties of the radionuclide), the combination of a standard cytostatic agent and further irradiation with targeted or non-targeted radionuclides or external X-rays might be an advantage [8, 9]. Several radiosensitizing effects have been reported over the last years, providing promising prospects [10-13]. Moreover, increasing DNA damage was observed when combining cytostatic drugs and external radiation on single-strand DNA, plasmid DNA and on a cellular level [14-19]. Thereby, one of the major obstacles, the severe side effects of chemotherapy or drug resistances, will be overcome by enhancing the overall effect by parallel irradiation and coincident reduction of the chemotherapeutic dose [20].

The increasing efficiency regarding DNA damage caused by high LET and low energy Auger electron emitters, such as ^{99m}Tc, has been investigated in several studies [21-23]. For instance, some platinum isotopes are characterized as Auger electron emitters as well, thereby functioning as high efficiency therapeutic tools for nuclear medicine, when attached to a certain target [24-26]. In general, Auger therapy has been investigated and revealed a high potential for cancer treatment [27-29]. To give a concrete example, Auger therapy using ^{191}Pt-doped cisplatin is expected to be much more effective than monotherapy [30, 31]. Nevertheless, the production of ^{191}Pt, ^{193m}Pt and ^{195}Pt in appropriate qualities and quantities is challenging and still has to be discovered [25, 32-35]. Additionally, ^{99m}Tc-labeled cisplatin was already used for early-stage cancer diagnosis [36] An alternative way of inducing the emission of Auger

electrons is the excitation of platinum atoms and subsequent emission of electrons causing an Auger cascade and further enhancing therapeutic efficiency [37, 38].

In this recent study a certain amount of platinum-loaded pUC19 plasmid DNA was incubated with the alpha emitter ^{223}Ra, the therapeutic beta emitter ^{188}Re and the Auger (and gamma) emitter ^{99m}Tc. We aimed at understanding the emission of Auger electrons after excitation of Pt atoms by various radiation qualities. The induced DNA damages were therefore classified as direct and indirect damages. Strand breaks were characterized by separation of the three different plasmid DNA conformation states by agarose gel electrophoresis.

Methods

Radionuclides

^{99m}Tc was obtained as [^{99m}Tc]TcO_4^- by eluting a ^{99}Mo/^{99m}Tc radionuclide generator (Mallinckrodt) with 0.9% sodium chloride solution. ^{188}Re was obtained as [^{188}Re]ReO_4^- from a ^{188}W/^{188}Re radionuclide generator (ITM) by elution with 0.9% sodium chloride solution. ^{223}Ra was purchased as Xofigo® (Bayer) in its chemical form [^{223}Ra]$RaCl_2$ and used without further purification. Radioactivity measurements were carried out using Isomed 2010 dose calibrator.

Plasmid DNA

pUC19 plasmid DNA (2686 base pairs) was purchased from New England Biolabs. The DNA stock solutions were diluted in Tris-HCl buffer (10 mM Tris, pH adjusted to 7.5). Ethylenediaminetetraacetic acid was removed in advance using Amicon® centrifugal filter units (30 kDa, Merck). The plasmid DNA was washed with 10 mM Tris-HCl (pH 7.5) three times, diluted to a final concentration of 0.1 µg/µL and stored at -20°C. Only samples containing <5% open circular DNA fractions were used for experiments. Digestion of pUC19 plasmid to produce linear plasmid reference was performed using the restriction enzyme BamHI (Invitrogen) and the respective protocol.

Cisplatin incubation and irradiation procedure

Plasmid samples (200 ng) were incubated with different concentrations of cisplatin. A certain amount of a cisplatin stem solution was added to pUC19 and the volume was filled to 20 µL using bidest. H_2O. Irradiation experiments contained an additional amount of stem solutions of the respective radionuclide (^{99m}Tc, ^{188}Re, ^{223}Ra). All experiments were performed with and without 0.2 M DMSO in order to differ direct and indirect induced DNA damage. In every case, the final sample volume maintained 20 µL.

Measurement of DNA damage in pUC19 plasmid samples

All samples were incubated for 24 h. Blue juice loading buffer (2.5 µL, Invitrogen) was added to each sample and 10 µL sample solution were loaded in the wells of a 1.4% agarose gel in Tris-Acetate-EDTA buffer (Sigma Aldrich). The samples were separated using a voltage of 4V/cm for 120 min at 6°C. After electrophoresis, the three different DNA conformation states (supercoiled, linear, open circular) were identified based on their different electrophoretic mobility. The DNA bands were stained in an ethidium bromide bath (0.5 µg/mL) followed by visualization using a fluorescence imager (Diana, Raytest). The DNA damage was determined by quantification of fluorescence intensities of the three different DNA bands, whereby the open circular fraction represents SSB and linear plasmid fraction represents DSB (Software ImageJ). The percentage of SSB and DSB was calculated as partial intensity of one conformation with reference to the total intensity per band. All results were obtained from three independent studies. The averages and standard deviations were calculated.

Dosimetry

All dosimetry calculations were performed in accordance to the MIRD formalism as described before [43, 44]. Briefly, dose from a source to a target volume was calculated as the product of the time-integrated activity in source volume and source-target-specific S values. Every plasmid sample was irradiated in a total sample volume of 20 µL in micro tubes. The geometric shape was assumed as a spherical drop with a diameter of 1.68 mm. S values for ^{99m}Tc (1.29 E-10 Gy/(Bq*s)), ^{188}Re (2.15 E-9 Gy/(Bq*s)) and ^{223}Ra (2.17 E-7 Gy/(Bq*s)) were

estimated by Monte Carlo simulations using Geant4 to calculate mean dose in the whole volume assuming homogeneous activity distribution. Time-integrated activity, that corresponds to the total number of decays (N), was calculated using the following equation:

$$N = \frac{A * T_{1/2}}{\ln(2)} * (1 - \exp[-\ln(2) * \frac{t_{exp}}{T_{1/2}})$$

The parameters A (applied radioactivity), t_{exp} (exposition time) and $T_{1/2}$ (radionuclide's half-life) were used to calculate the overall dose in dependence of the experimental setup.

Results

The basis for all combination experiments was the determination of a maximum concentration of cisplatin, which did not lead to significant DNA damage and thereby enabled the combination assays with radionuclides. Furthermore, the dose range for each radionuclide was determined, in order to ensure the detection of a potential enhanced damaging effect in form of increasing DNA SSB and DSB with or without additional incubation of DMSO as radical scavenger.

Determination of the maximum non-toxic cisplatin concentration

The influence of cisplatin on pUC19 plasmid DNA was examined over a wide concentration range starting at 600 pM to a maximum of 600 µM. A representative agarose gel showing the induced DNA damages is displayed in Fig. 1.

Fig. 1. Representative agarose gel after incubation of 200 ng pUC19 plasmid DNA with various concentrations of cisplatin for 24 h (600 pM - 600 µM). Gel was stained 30 min in ethidium bromide and fluorescence imaged afterwards. (lane 22 - non-affected pUC19 reference ; lanes 1 and 20 - size marker ; lane 9 - linear plasmid reference)

It is clearly shown that increasing concentrations of cisplatin lead to DNA damages of different extents. Starting at a concentration of 280 nM (lane 13), an intensified DNA smearing of DNA bands was detectable. Whereas a clear signal of intact supercoiled DNA was detected in lanes 2-8 and 10-12, the assignment of fluorescence intensity to a certain DNA band was not possible any more for higher cisplatin concentrations (lanes 13-19). Moreover, the overall fluorescence intensity of the stained DNA decreases in this range as well, indicating total DNA damage and destruction of the base pair structure.

To refine those results, a second cisplatin concentration range was investigated starting from 6 nM up to 660 nM, thereby covering the region of major interest in smaller concentration steps. A representative and stained agarose gel is displayed in Fig. 2.

Fig. 2. Representative agarose gel after incubation of 200 ng pUC19 plasmid DNA with various concentrations of cisplatin for 24 h (6 nM - 660 nM). Gel was stained 30 min in ethidium bromide and fluorescence imaged afterwards. (lane 22 - non-affected pUC19 reference ; lanes 21 and 40 - size marker ; lane 29 - linear plasmid reference)

In Fig. 2, the concentration values were verified by using a smaller concentration range. Starting from lane 28, smearing increases and fluorescence intensity decreases. Thus, a cisplatin concentration of (18-24) nM (lanes 27 and 28) was chosen to be the highest possible without inducing any substantial direct toxic effect by crosslink formation. These two concentrations were chosen for all later experiments with platinum-loaded plasmid DNA and simultaneous incubation of ^{99m}Tc and ^{188}Re to provoke increasing DNA disruption.

Moreover, platinum-containing DNA samples were analyzed by ICP-MS to determine the exact platinum amount that is actually bond to pUC19. The supernatant was separated from the DNA to remove unbound cisplatin. The incubation of 18-24 nM cisplatin (concentration in the sample solution) revealed an accumulation of approx. 10 % of the starting amount of cisplatin after 1 h. Consequently, the number of platinum atoms bond per plasmid molecule was 3-4 for the concentrations of major interest (18-24 nM).

Determination of a dose range for ^{99m}Tc, ^{188}Re and ^{223}Ra irradiation

As well as for the cytostatic drug *cisplatin*, the most suitable dose range had to be determined for each of the applied radionuclides. For this purpose, radioactive doses from 0 Gy up to 700 Gy (accumulated after 24 h of incubation; activity concentrations up to 2644 kBq/µL for ^{99m}Tc (200 Gy), up to 84 kBq/µL for ^{188}Re (200 Gy) and up to 2.0 kBq/µL for ^{223}Ra (700 Gy), respectively) were incubated with pUC19 plasmid DNA. All experiments were carried out with and without 0.2 M DMSO as free radical scavenger in order to differentiate direct and indirect induced strand breaks. The dose-dependent damaging profiles after incubation of ^{99m}Tc, ^{188}Re and ^{223}Ra, respectively, are displayed in Fig. 3 -5. The relative ratio of ESB was connected to

the percentage of open circular plasmid fraction, whereas the amount of DSB was connected to the percentage of linear plasmid fraction.

Fig. 3. Dose-dependent DNA damage caused by ^{99m}Tc; Samples were incubated for 24 h at room temperature to achieve the respective dose of (2.5-190) Gy.

Fig. 4. Dose-dependent DNA damage caused by ^{188}Re; Samples were incubated for 24 h at room temperature to achieve the respective dose of (2.5-190) Gy.

Fig. 5. Dose-dependent DNA damage caused by ^{223}Ra; Samples were incubated for 24 h at room temperature to achieve the respective dose of (2.5-700) Gy.

Dose-dependent DNA damage was obtained for all radionuclides. An increasing amount of SSB was observed for dose values up to 150 Gy for ^{188}Re and ^{99m}Tc. Moreover, a further increase of the applied radiation dose did not lead to an increase of damage of the same extent compared to the 0-150 Gy. This kind of plateau is caused by a maximum percentage simple DNA damage (SSB) which was obtained at this dose values. Further damage will occur when applying much higher dose values and complex SSB as well as cluster damage, leading to DSB in consequence. In contrast, ^{223}Ra induces unexpected low DNA damage up to 150 Gy. Anyway, higher dose values consequently lead to an increased amount of SSB and DSB, respectively. The range of 40-130 Gy was chosen for the combination experiments with ^{99m}Tc and ^{188}Re to ensure a detection of increasing DNA damage. For the reason of less damage at dose points < 200 Gy ^{223}Ra, higher dose values were used for the irradiation of cisplatin-incubated plasmid DNA with ^{223}Ra.

In general, the additional incubation of 0.2 M DMSO led to a total reduction of DSB and reduced the SSB percentage to <5% for all experiments and radionuclides.

Irradiation of platinum-containing plasmid DNA with ^{99m}Tc and ^{188}Re

Plasmid samples, containing 18 nM and 24 nM cisplatin, were irradiated with ^{99m}Tc and ^{188}Re, accumulating dose values of 40 Gy to 110 Gy (350 - 1454 kBq/µL for ^{99m}Tc and 17 - 46 kBq/µL for ^{188}Re, respectively) over 24 h. An overview of the percentages of induced SSB after irradiating platinum-containing DNA versus DNA without cisplatin is displayed in Fig. 6 and 7. It has to be mentioned, that the number of DSB was almost zero and the additional incubation

of DMSO did not bring any further information. Therefore, these data are neglected in the following passages.

Fig. 6. Dose-dependent DNA damage caused by ^{99m}Tc in combination with 18 nM and 24 nM cisplatin; Samples were incubated for 24 h at room temperature to achieve the respective dose of (40-110) Gy.

Fig. 7. Dose-dependent DNA damage caused by ^{188}Re in combination with 18 nM and 24 nM cisplatin; Samples were incubated for 24 h at room temperature to achieve the respective dose of (40-110) Gy.

For both the combination of 18 nM and 24 nM cisplatin with both radionuclides ^{99m}Tc and ^{188}Re, no increasing DNA damage (concerning SSB induction) was observed. Within the standard deviations, all observed values of SSB are very similar. The number of induced DSB is negligible, with and without the additional incubation of DMSO as radical scavenger.

Irradiation of platinum-containing plasmid DNA with ^{223}Ra

For the reason of less induced DNA damages by ^{223}Ra alone, higher dose values (100-700 Gy; 0.3-2.0 kBq/µL) and higher cisplatin concentration values were examined in the combination experiments. An overview of the dose-dependent induced SSB by ^{223}Ra irradiation of platinum-containing plasmid DNA is displayed in Fig. 8.

Fig. 8. Dose-dependent DNA damage caused by ^{223}Ra in combination with (24–600) nM cisplatin; Samples were incubated for 10 d at room temperature to achieve the respective dose of (100–700) Gy.

^{223}Ra alone induced similar DNA damages as shown in Fig. 5. Both, at the dose points of 500 Gy and 700 Gy the percentages of SSB decreases whereas the fractions of DSB increase indicating the effect of ^{223}Ra at such high dose values. However, the increase of cisplatin concentrations in combination with ^{223}Ra did not enhance the percentages of SSB.

To sum up, even at higher dose values (applying ^{223}Ra as a high LET emitter) and higher cisplatin concentrations, no enhanced DNA damage in form of SSB or DSB was observed.

Discussion

The different behavior of ^{223}Ra-induced DNA damage, compared to ^{99m}Tc and ^{188}Re might be caused by statistical issues of an alpha particle hitting the plasmid molecules. Since ^{223}Ra is characterized as high LET emitter, a higher percentage of DSB is assumed, but was not observed. The effect of less induced SSB as well might be due to the less ionization events in the sample environment and thus leading to less radiolysis and less SSB in consequence. It is rather unlikely that ^{223}Ra is less effective compared to ^{99m}Tc and ^{188}Re - it is more or less a matter of the used model and the different physical half-lives (<1 d vs. >11 d) and less decay events over 24 h of incubation time. Since the majority of DNA damage was induced indirectly and preventable by DMSO-incubation, ^{223}Ra seems to produce less or/and different reactive oxygen species and thus induced less damage compared to ^{99m}Tc and ^{188}Re. In the past, we reported findings in terms of unexpected similar DNA damage efficacy of ^{223}Ra in comparison to ^{188}Re or ^{99m}Tc [39]. Otherwise Ushigome et al. found a clear dependence between DSB induction and LET for helium ions (2.2 keV / µm) and ^{241}Pu (148 keV keV / µm) [40]. DNA damage in general was prevented to a high extent by the additional incubation of 0.2 M DMSO. Thereby, we consider the majority of DNA damage as indirect and radiolysis-mediated strand breaks, even though ^{188}Ra and ^{223}Ra are characterized as medium and high LET emitters. In consistence to our findings Peak et al. supposed that the SSB and DSB caused by the high-LET neutrons are, to some extent, due to DNA breaks initiated by indirect effects [39].

Concerning the experiments when combining cisplatin with radiation, we assume, that the mechanism of the already reported sensitizing effect of radiation to cisplatin therapy or vice versa is not connected to the bare mechanism of DNA strand break induction. Whereas the toxic characteristic of cisplatin alone and irradiation alone was proven in our DNA model, the interaction of both DNA toxic substances did not lead to an increasing number of strand breaks in these certain experiments. Recently, the DNA damaging effect of Auger electrons induced by radiolabeled cisplatin (^{189,191}Pt) was evaluated [41]. In plasmid DNA, most DNA damage was likely due to radicals induced by radio-cisplatin due to the slow binding between platinum and DNA. On the other hand, the authors suggest that radio-cisplatin also caused direct

plasmid breakage, because the fractions of linear plasmid were not prevented using the radical scavenger DMSO. Usage of plasmid-bound ^{99m}Tc-HYNICDAPI induced both direct SSB and direct DSB underlining the impact of inducing Auger electrons in the close proximity to DNA [21].

Indeed, cell repair mechanisms play a very important role in cancer therapy. Thus, cisplatin most likely blocks cell division and repair systems after intercalation and enables radiation to kill cells much more efficient, since tolerable damage thresholds are already overridden [5, 42]. Furthermore, drug resistances can be overcome, which do not matter to a plasmid DNA model [5].

Moreover, an induced Auger cascade has not been observed. If this would have been the case, severe DNA damage had to be detected, even towards isolated plasmid DNA. Neither the low LET emitter ^{99m}Tc, nor the medium-high LET emitter ^{188}Re emitted suitable energies to provoke this effect. ^{223}Ra most likely induces solely indirect damage, when it is not directly bound to the target [39].

Furthermore, ionization density is not high enough to induce any Auger electron emission or any efficiency enhancement of cisplatin in the considered DNA model.

Future experiments will cover the repetition of the experimental setup on a cellular level. The aim is to generate information about the influence of cell repair mechanisms and cell response after combination therapy with cisplatin and irradiation with different radionuclides. When in vitro experiments were performed successfully, new insights for endoradiotherapy with ^{177}Lu and/or ^{225}Ac can be generated and the potential clinical application might be adjusted by additional incubation of cisplatin to enhance the therapeutic efficiency.

Conclusion

An increasing DNA damage, when combining cisplatin with radiation (alpha, beta and gamma emitters), was not provable in the cell-free system. The mechanism of interaction between cisplatin and additional irradiation most likely depends almost exclusively on cell repair mechanisms that are irrelevant in an isolated DNA model. The principle of strand break induction was not influenced by presence or absence of platinum atoms in the DNA. A dose-dependent increase of DNA damage in form of SSB was observed for all tested radionuclides and the free radical scavenger DMSO was applied and consequently reduced SSB and DSB to a minor extent. Furthermore, the induction of Auger electron emission was not observed at all, since no increasing direct DNA damage was detected. Cisplatin, radiolabeled with ^{99m}Tc or any radioactive platinum isotope could overcome those obstacles and increase diagnostic and therapeutic performance, thereby enabling a new application field by using radioactive cisplatin in nuclear medicine.

Abbreviations

SSB – single strand break
DSB – double strand break
LET – linear energy transfer

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Competing Interests

All authors declare that there are no conflicts of interest.

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