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Interaction of low and high LET radiation in TK6 cells—mechanistic aspects and significance for radiation protection

Alice Sollazzo\textsuperscript{1}, Sara Shakeri-Manesh\textsuperscript{1}, Asal Fotouhi\textsuperscript{1}, Joanna Czub\textsuperscript{2}, Siamak Haghdoost\textsuperscript{1} and Andrzej Wojcik\textsuperscript{1,3,4}

\textsuperscript{1} MBW Department, Centre for Radiation Protection Research, Stockholm University, Sweden
\textsuperscript{2} Institute of Physics, Jan Kochanowski University, Kielce, Poland
\textsuperscript{3} Institute of Biology, Jan Kochanowski University, Kielce, Poland

E-mail: andrzej.wojcik@su.se

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Abstract
Most environmental, occupational and medical exposures to ionising radiation are associated with a simultaneous action of different radiation types. An open question remains whether radiations of different qualities interact with each other to yield effects stronger than expected based on the assumption of additivity. It is possible that DNA damage induced by high linear energy transfer (LET) radiation will lead to an opening of the chromatin structure making the DNA more susceptible to attack by reactive oxygen species (ROS) generated by the low LET radiation. In such case, the effect of mixed beams should be strongly expressed in cells that are sensitive to ROS. The present investigation was carried out to test if cells with an impaired capacity to handle oxidative stress are particularly sensitive to the effect of mixed beams of alpha particles and x-rays. Clonogenic cell survival curves and mutant frequencies were analysed in TK6 wild type (wt) cells and in TK6 cells with a knocked down hMYH glycosylase. The results showed a synergistic effect of mixed beams on clonogenic cell survival of TK6wt but not TK6MYH\textsuperscript{−} cells. The frequencies of mutants showed a high degree of interexperimental variability without any indications for synergistic effects of mixed beams. TK6MYH\textsuperscript{−} cells were generally more tolerant to radiation exposure with respect to clonogenic...
cell survival but showed a strong increase in mutant frequency. The results demonstrate that exposure of wt cells to a mixed beam of alpha particles and x-rays leads to a detrimental effect which is stronger than expected based on the assumption of additivity. The role of oxidative stress in the reaction of cells to mixed beams remains unclear.

Keywords: mutations, cell survival, low LET radiation, high LET radiation, mixed beams, risk assessment

(Some figures may appear in colour only in the online journal)

Introduction

Most environmental, occupational and medical exposures to ionising radiation are associated with a simultaneous action of different radiation types. In the environment, organisms are often exposed to a mixed field of gamma radiation (e.g. from $^{226}$Ra) and alpha particles (e.g from $^{222}$Rn) [1], whereby radon may form a significant part of the annual effective dose in radon-prone areas [2]. Occupations such as aircraft operation involve exposure to a complex field of gamma radiation, neutrons and protons [3]. Finally, during radiotherapy, patients may be exposed to gamma radiation plus neutrons, protons and neutrons [4] and carbon ions plus neutrons [5]. Radiations differ in the way that their energy is deposited along the tracks of charged particles [6] and it is common to differentiate between densely ionising radiations such as alpha particles and heavy ions and sparsely ionising particles such as electrons. Ionisation density is given in keV $\mu$m$^{-1}$ and defined as LET (linear energy transfer). Per unit dose, densely ionising radiations are more effective in damaging cells due to induction of more complex lesions. This is taken into account by assigning them high radiation weighting factors that, for purposes of radiation protection are used to calculate the effective dose [6]. In case of mixed beams, the effective doses from different radiations are simply added assuming that there is no interaction between different radiation types.

That this approach may lead to an underestimation of the effect was demonstrated by a number of reports suggesting that radiations of low and high LET may interact leading to biological effects beyond those expected from additivity [7–11]. It can be supposed that a synergistic (interacting) action of two radiation types can occur via various mechanisms. Firstly, it is possible that the action of both radiation types will lead to an increase of LET and, consequently, of DNA damage complexity. Such increased damage complexity observed at the level of chromosomal aberrations has been reported [10]. Secondly, it is possible that exposure to high LET radiation will engage the DNA damage response machinery to such a degree that the additional damage induced by the low LET radiation will not be repaired properly. Such an effect is indicated by the modified transition of small to large gH2AX foci that has been observed [9]. Thirdly, it is possible that damage induced by high LET radiation will lead to an opening of the chromatin structure making the DNA more susceptible to attack by reactive oxygen species (ROS) generated by the low LET radiation. In such a case, the effect of mixed beams should be strongly expressed in cells that are sensitive to ROS.

The present investigation was carried out to test if cells with an impaired capacity to handle oxidative stress are particularly sensitive to the effect of mixed beams of high and low LET radiation. To this end, we used our x-ray and alpha particle mixed beam exposure facility [12] to irradiate two cell types: wild type (wt) human lymphoblastoid TK6 cells and TK6 cells with a knocked down hMYH glycosylase which is responsible for the removal of adenines or 2-hydroxyadenines misincorporated with template guanines or
7,8-dihydro-8-oxodeoxyguanines [13]. Clonogenic cell survival curves and mutant frequencies were analysed as the endpoints.

**Materials and methods**

**Cells and cell culture**

Experiments were performed with wt human lymphoblastoid TK6 cells and with TK6 cells that were transfected with shRNA against MYH. The former are referred to as TK6wt and the latter as TK6MYH. A detailed description of the transfection process and of the characteristics of the transfected cells can be found in [14]. Western blot analysis shows that TK6MYH cells express 7% of the MYH level found in TK6wt cells. TK6wt and TK6MYH cells were grown in suspension in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% defined bovine calf serum (DBS, Hyclone), 1% Pest (Invitrogen), and 10 mM HEPES. The cells were grown in 75 cm² flasks at 37 °C and 5% CO₂. The cell density was kept below 5 × 10⁵ ml⁻¹ during the duration of the whole experiment. Cell viability was monitored by trypan blue staining.

**Irradiation of cells**

Cells were irradiated and sham exposed in suspension on round polyamide (PA) disks (155 mm in diameter, custom-constructed in the Institute for Energy-JRC, Petten, Netherlands) as described earlier [10, 12]. Based on the measured LET and energy of alpha particles at the entrance into the cell layer, a dose of 0.2 Gy corresponds to, on average, 1 hit per cell. Prior to exposure, the discs were cleaned with 70% ethanol and warmed to 37 °C in an incubator. 0.25 ml of cell suspension (at a density of ca 2.5 × 10⁵ ml⁻¹) was positioned in the centre of the PA disc, covered with a 1.5 µm thick Mylar foil lid and spread out to an even layer. The dose-rate of alpha radiation was 0.265 Gy min⁻¹ (at the entrance to the cell suspension). The LET of alpha particles at the entrance to the cell suspension was between 100–172 keV µm⁻¹. It varied with the depth of the cell suspension from 100 to 238 keV µm⁻¹ [12].

The dose-rate of x-rays was 0.052 Gy min⁻¹ in the top table position and 0.068 Gy min⁻¹ in the bottom table position of the alpha irradiator. The energy of the x-rays was 80 keV [15]. The doses were: 0.2, 0.4, 0.6 and 0.8 Gy x-rays (X); 0.2, 0.4, 0.6 and 0.8 Gy alpha particles (α) and 0.1 α + 0.1 X, 0.2 α + 0.2 X, 0.2 α + 0.4 X and 0.2 α + 0.6 X Gy mixed beams. Controls were sham exposed. Assuming a nucleus diameter of 10 µm the alpha dose of 0.2 Gy corresponds on average to one hit per cell nucleus (calculated using the methodology described in [16, 17]).

**Clonogenic survival and envelopes of additivity**

For analysing clonogenic cell survival, a defined number of exposed cells (50–800 cells per well) were seeded in a well of a 6-well plate (3.5 cm in diameter, Thermo Scientific, Korea), mixed with 1.5 ml low melting agarose which was prepared in complete RPMI 1640 medium (final concentration 0.2%). The 6-well plate was placed for 12 min at 4–8 °C followed by incubation for 10 d at 37 °C for colony formation. Colonies were counted by eye. At least 4 independent experiments were performed.

The observed and expected levels of clonogenic cell survival after mixed beams exposure were compared using envelopes of additivity. This approach is based on constructing isobolograms [18] based on two assumptions: heteroadditivity and isoadditivity [19]. Constructing envelopes of additivity is one of the methods used to analyse whether two different radiation types, when given together, act in an independent way [20]. In this context, independent
means that a combined treatment leads to an effect expected from the addition of two treatments given independently. Envelopes of additivity should be applied when at least one of the dose-response relationships of the tested radiations is not linear [18, 20]. This applies to results of clonogenic survival presented here, where the survival curves following x-ray exposure were best fitted by a linear-quadratic function while the curves following alpha particle and mixed beam exposure were exponential.

**Mutant frequency**

TK6 cells are heterozygous in the thymidine kinase locus, hence the frequency of mutated cells can be measured by culturing in selection medium containing trifluorothymidine (TFT) [21]. Following exposure to radiation TK6\textsuperscript{wt} and TK6\textsuperscript{MYH-} cells were cultured in complete RPMI medium for 10 d at 37 °C allowing the expression of the mutated thymidine kinase. Every second day 0.5 × 10\textsuperscript{6} viable cells were sub-cultured. After 10 d cells from each treatment were transferred into a 6-well plate (3.5 cm in diameter, Thermo Scientific, Korea) and cultured in 1.5 ml of complete medium with 0.2% low melting agarose. 0.5 × 10\textsuperscript{6} cells were added to 2 wells and 1.2 × 10\textsuperscript{6} cells were added to 2 other wells. TFT at a final concentration of 5 µg ml\textsuperscript{-1} was added to these 4 wells. 50 cells each were added to the 2 last wells in order to estimate the clonogenic efficiency. The cells were incubated for 10 d and colonies were counted by eye. The frequency of mutants (MF) was calculated according to equation \( MF = \frac{M}{N \times CE} \), where \( M \) is the number of mutants observed in \( N \) cells grown in the presence of TFT and CE is the clonogenic efficiency, and always expressed per 10\textsuperscript{5} cells. 23 independent experiments were performed with non-irradiated cells, at least 8 experiments each with TK6\textsuperscript{wt} and 3 experiments with TK6\textsuperscript{MYH-} cells exposed to x-rays, alpha particles and a combination of both radiations.

**Statistical analyses**

Survival curves for single exposures to x-rays were fitted to the linear-quadratic equation

\[ S = e^{-aD - bD^2} \]

where \( a \) and \( b \) represent fitting coefficients and \( D \) is the absorbed dose in Gy. Fitting was performed with the help of Curve Expert 1.4 using the least-square method. For alpha particles and mixed beams, the \( b \) coefficient was omitted by fitting to a one-phase exponential decay according to the following equation

\[ S = e^{-aD} \]

where \( S \) represents the survival fraction, \( a \) the fit coefficient and \( D \) is the absorbed dose in Gy. The starting value \( S_0 \) was set to 1.

The effect of mixed beams of x-rays and alpha particles on clonogenic cell survival was analysed by the mathematical model of ‘envelope of additivity’ [18, 19]. Envelopes of additivity were constructed based on the fitted survival curves.

Mutant frequencies were analysed by Student’s \( t \)-test.

**Results**

First, it was interesting to compare the sensitivity of TK6\textsuperscript{wt} and TK6\textsuperscript{MYH-} cells to different types of radiation. The results of clonogenic survival assays are shown in figure 1. Overall, TK6\textsuperscript{wt} cells showed a lower level of survival than TK6\textsuperscript{MYH-}. The difference is minimal for
Figure 1. Clonogenic cell survivals of TK6\textsuperscript{wt} and TK6\textsuperscript{MYH} cells exposed to (A): alpha particles, (B): x-rays and (C): mixed beams. Graphs illustrate sensitivities of both cell lines to the different radiation qualities. Error bars: standard deviations.
alpha particles, pronounced for x-rays and strongest for mixed beams. The survival curves following x-rays were best fitted with a linear-quadratic function, while linear fits were most suitable for alpha particles and mixed beams. The a and b fitting coefficients were, respectively: TK6\textsuperscript{wt} alpha particles: 1.0, 2.14; TK6\textsuperscript{wt} x-rays: 1.29, 1.19; TK6\textsuperscript{wt} mixed beams: 1.0, 3.03; TK\textsuperscript{MYH}- alpha particles: 1.0, 1.81; TK\textsuperscript{MYH}- x-rays: −0.37, 2.64; TK\textsuperscript{MYH}- mixed beams: 1.0, 1.57.

Next, we compared the sensitivity of each cell line to the different types of radiation. The results of clonogenic survival assays are shown in figure 2. In both cell lines only a minor difference was seen between x-rays and alpha particles. With respect to mixed beams, only TK6\textsuperscript{wt} cells revealed a level of survival distinctly lower than for x-rays and alpha particles applied alone. In order to test whether the difference between the expected and observed level of survival is significant following exposure to mixed beams, envelopes of additivity were constructed. The results are shown in figure 3. For TK6\textsuperscript{wt} cells (top row of graphs) the
combined dose corresponding to the observed level of survival was always positioned to the left of isobologram envelopes indicating a synergistic (higher than additive) effect of mixed beams. This was not observed for TK6MYH- (bottom row of graphs). At surviving fraction of 0.6, the observed value was positioned inside the isobologram envelope indicating additivity. At surviving fractions of 0.4 and 0.2, the observed survival values were positioned to the right of isobologram envelopes indicating an antagonistic (lower than additive) effect.

The mutant frequencies observed in both cell lines are shown in figure 4. In TK6wt cells, the base line mutant frequency was $0.14 \pm 0.16$ per $10^5$ cells. The frequency increased linearly with dose, but we observed very large interexperimental variabilities. Results are restricted to dose ranges of 0–0.6 Gy because no meaningful mutant frequencies were seen after doses of 0.8 Gy (not shown). The highest radiation-induced mutant frequency was $0.53 \pm 0.50$ per $10^5$ cells following 0.4 Gy of x-rays. No significant differences between the dose response curves were observed. The base line mutant frequency in TK6MYH- was $0.86 \pm 0.45$ per $10^5$ cells. No clear dose response was evident. Indeed, elevated mutant frequencies were only observed following an x-ray dose of 0.6 Gy ($2.14 \pm 1.0$ per $10^5$ cells) and a mixed beam dose of 0.6 Gy ($2.12 \pm 0.35$ per $10^5$ cells).

**Discussion**

The concept that radiations of different LET may interact when applied together is not new. Already in 1960 Barendsen et al [22] tested the effect of combined exposures of alpha particles
and x-rays but concluded that the action of both radiation types is independent. Similar results were published by others [12, 23, 24]. However, some studies indicated that the survival probability of cells exposed to mixed beams is lower than expected based on an independent action [7, 8, 25–29]. Interestingly, McNally et al observed that the interaction between radiations of different quality is observed irrespectively of whether both radiation are given simultaneously or in sequence, provided that the time between the irradiations is shorter than 3 h [29, 30]. We have been involved in this line of research since several years and obtained results suggesting both additivity of high and low LET radiation [12] and synergistic effects [9–11]. The current results obtained with TK6\textsuperscript{wt} cells also suggest that alpha particles and x-rays interact in a way leading to a lower than expected level of clonogenic cell survival.

This result is important from the perspective of radiation protection because it suggests that the health effects per unit dose of mixed radiation beams may be stronger than expected based on a simple sum of single doses coming from radiations of various qualities, even if these are multiplied by appropriate radiation weighting factors. A good example of the problem is the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Mutant frequencies per 10\textsuperscript{5} cells in A: TK6 wt cells and B: TK6\textsuperscript{MYH} cells exposed to alpha particles, x-rays and mixed beams. Error bars: standard deviations.}
\end{figure}
use of risk factors derived from the Hiroshima and Nagasaki survivors to predict cancer risk in other cohorts. The atomic bomb explosions generated a mixed beam of gamma radiation and neutrons [31]. The high biological effectiveness of neutrons is taken into account by multiplying the neutron dose component by a radiation weighting factor [32]. The derived risk factors per unit weighted dose are then applied to predict lifetime attributable risks among people living in areas contaminated by the pure gamma emitter $^{137}$Cs which was dispersed in consequence of the Chernobyl or Fukushima Daiichi disasters [33, 34]. What is not taken into consideration is the possibility of an interaction of neutrons and gamma radiation in inducing biological effects. The presence of such interaction would mean that the risk per unit gamma dose calculated for Chernobyl and Fukushima victims is overestimated. Needless to say, the possible mechanisms of synergism and the conditions under which it occurs need to be studied in greater detail before this effect can be seriously considered in radiation protection.

The aim of the present investigation was to analyse the effect of alpha particles, x-rays and mixed beams of both radiation types on clonogenic cell survival and mutant frequency in two sublines of TK6 cells differing in sensitivity to oxidative stress. Experiments were carried out with wt TK6 cells and TK6 cells with knocked down hMYH glycosylase. The results show a synergistic effect of mixed beams on clonogenic cell survival of TK6 wt but not TK6MYH- cells. The frequencies of mutants showed a high degree of interexperimental variability without any indications for synergistic effects of mixed beams. As expected, the level of mutants was higher in TK6MYH- cells as compared to TK6 wt, however TK6MYH- cells were generally more tolerant to radiation exposure with respect to clonogenic cell survival.

We used envelopes of additivity to compare the expected with observed levels of clonogenic cell survival. The method is based on constructing isobolograms assuming two different forms of interaction of cytotoxic or genotoxic agents for which the dose response relationships are not linear [18, 19]. A significant deviation from the expected level of damage is given when the combined dose corresponding to the observed level of survival is above (lower than additive) or below (higher than additive) the envelope. We saw a higher than additive effect of mixed beams at all levels of survival in TK6 wt cells. This was not observed for TK6MYH-, pointing towards the important role of oxidative stress in the reaction of cells to radiations of different qualities. A few words should be written about the fact that calculation of isobolograms does not take into account errors associated with fitting the survival curves. The reason for this is that the isobolograms are calculated assuming two modes of interaction: the heteroadditivity mode and the isoadditivity mode [18] and the resulting envelope of additivity encompasses an area of uncertainty, anywhere in which the measured experimental point must lie in order to be counted as resulting from additivity. Hence, adding uncertainty regions to the isobolograms appears superfluous.

Mechanistically, it can be supposed that high and low LET radiations interact in several ways: by an increased ionisation density within a target volume leading to an amplified damage complexity [10], by engaging the DNA damage response machinery to such a degree that the additional damage induced by the low LET radiation will not be repaired properly [9] and by modifying the chromatin structure in consequence of high LET radiation damage making the DNA more susceptible to attack by ROS generated by the low LET radiation. In such a case the effect of mixed beams should be strongly expressed in cells that are sensitive to ROS.

We tested this hypothesis by analysing the effect of mixed beams in TK6MYH- cells with an impaired ability to identify and remove adenines or 2-hydroxyadenines misincorporated with template guanines or 7,8-dihydro-8-oxodeoxyguanines [13].

Among DNA bases, guanine is the most vulnerable DNA target for oxidation by ROS [35]. Upon exposure to ionizing radiation, one of the most abundant types of oxidative damage produced is the guanine adduct 8-dihydro-8-oxo-guanine or 8-oxo-G [35]. If not readily removed from DNA, 8-oxo-G can mispair with adenine, leading to a G:C→T:A transversion
OGG1 and MYH, components of the base excision repair (BER) mechanism, are responsible for preventing this type of mutation. While OGG1 readily eliminates 8-oxo-G from the DNA, MYH acts post-replicatively by removing adenine when mispaired with oxidized guanine. Once adenine is removed, DNA polymerase λ can insert cytosine restoring the original sequence [38]. TK6MYH− cells have a low level of the MYH protein and therefore they do not remove mispaired adenines. Somewhat surprisingly, our results clearly show that, contrary to TK6wt cells, TK6MYH− cells do not show higher sensitivity to mixed beams than expected from an additive action of alpha particles and x-rays. On the contrary, at doses related to surviving fractions of 0.2 and 0.4 the effect of mixed beams appeared antagonistic. However, since the result was not consistently seen at all levels of survival, we conclude that the effect of mixed beams in this cell line is additive. We have seen a similar results and drew a similar conclusion in our previous study [12]. Another surprising result was that the TK6MYH− cells generally showed a higher level of survival than did the TK6wt cells. This difference was minimal after alpha particles, pronounced after x-rays and strongest after mixed beams. At the same time, the spontaneous MF was 6 times higher in TK6MYH− cells as compared with TK6wt cells, clearly indicating the impact of the low level of MYH on accumulation of oxidative DNA lesions. So how can the survival resistance of TK6MYH− cells be explained? Oka and Nakabeppu [39] suggest that a high activity of MYH may push damaged cells into cell death. Under elevated oxidative stress, 8-oxo-G accumulates and the low fidelity polymerases β and κ may reintroduce adenine opposite to 8-oxo-G leading to so called futile BER cycles. The futile short-patch BER results in accumulation of MYH-generated SSB leading to activation of poly(ADP-ribose) and cell death. This process is mediated by p53 [40]. Given the fact that p53 is active in TK6 cells [41], the low level of MYH in TK6MYH− cells protects them from apoptosis at the cost of a higher G:C→T:A mutation rate as compared to TK6wt cells. Interestingly, the obtained results substantiate the role of oxidative DNA damage in the cellular effect of mixed beams. 

An intriguing question remains as to why the synergistic effect of mixed beams seen at the level of clonogenic cell survival in TK6wt cells was not detectable at the level of mutations. We do not have a clear answer but it appears likely that there are two contributing factors. The first factor is the high interexperimental heterogeneity of the results. We carried out 23 independent repeats to determine the baseline MF and 8 independent repeats to determine the mutant frequency after irradiation. Although a weak dose-response relationship could be observed, the large error bars possibly masked any differences between the treatments. The second factor may be related to the futile BER cycles discussed in the previous paragraph. A consequence of the cycles is death of cells with a high level of oxidative damage. Indeed, the lowest level of cell survival per unit dose was observed in cells exposed to mixed beams. Hence, the lack of mixed beam effect on the level of mutations could be due to selective elimination of cells carrying oxidative damage which could potentially lead to mutations. Similar overkill effects are known in toxicology, where excessive cell death following exposure to a mutagen results in a reduced frequency of chromosomal aberrations [42]. Also, a similar inverse relationship between cell killing and mutant induction was observed between TK6wt cells and WTK1 cells with an impaired apoptosis pathway [41]. In TK6MYH− cells, a very poor dose response was seen for mutant frequency and this result was also surprising. Again, we do not have a ready explanation for this, but it is worth pointing out that the baseline level of mutants is already much higher than that observed in TK6wt cells even following the highest radiation dose. It is thus possible that also in this cell line selective cell death prevented the expression of radiation-induced mutants. 

In addition to what is written above, it is interesting to note that the problem of a lack-
clonogenic cell survival has already been observed a long time ago [43]. Tikvah Alper distinguished between type N damage, which represents the mutagenic action of radiation on the DNA and type O damage, which represents some other damage not directly related to the DNA. Based mainly on results with bacteria, Alper postulated that the level of type O damage is influenced by the presence of oxygen while the level of type N damage is not [43]. Given the limited level of knowledge about cell biology during her time, it is difficult to infer the cellular mechanisms behind type N and O effects that Alper had in mind, but our observation that the synergist effect of mixed beams is evident at the level of clonogenic cell survival in TK6\textsuperscript{wt} cells only suggests that it is associated with type O damage.

An issue that needs to be discussed is the lack of a clear difference in survival between alpha particles and x-rays. Per unit dose, radiation of high LET usually has a stronger killing effect than x-rays [44] which is explained by the induction of the strongly deleterious clustered lesions [45]. Using TK6 wt cells, Evans et al [46] observed an RBE of 1.5 for $^{56}$Fe-particle radiation. We only observed a weak difference between radiation types in the two studied cells lines, with linear-quadratic survival curves in cells exposed to x-rays and linear survival curves in cells exposed to alpha particles (and mixed beams). We do not have a ready explanation for this but a contributing factor could be that, contrary to Evans et al [46], we exposed cells on disks covered by a Mylar foil. Such exposure condition is associated with a mechanical stresses that forces many cells into premature death and could mask the differential effect of high and low LET radiation.

Results indicating the existence of an interaction between high and low LET radiation leading to a higher than additive detriment also have implications for chemical toxicology. The reason for this is that, similarly to low LET radiation [47], the majority of chemical toxins generate an oxidative stress which contributes to DNA damage [48–50]. Exposure to such compounds together with high LET radiation, as for example in radon-prone areas, may lead to cell damage beyond the level expected from additivity. The difference between chemotoxin-radiation and radiation–radiation interactions is that exposure thresholds may exist in case of the former, below which no interaction is to be expected. This is because some chemical toxins are only detrimental above a certain exposure level when, for example, they pass cell membranes, overwhelm the cellular detoxification system or become activated [51]. No such threshold is believed to exist for radiation [6]. Hence, studies of chemotoxin-radiation interactions must encompass dose-response relationships going down to low exposure levels in order to detect possible thresholds.

Coming back to radiation–radiation interactions, it is interesting to speculate how the present study could be expanded in order to obtain a deeper understanding of the mechanisms and magnitude of the synergistic interaction between high- and low-LET radiation. A problem with the TK6 cell system used by us is the high variability of the results and the lack of a clear RBE for alpha particles. We have chosen TK6 cells because they are suitable for analysing mutant frequencies and we have previously constructed the TK6\textsuperscript{MYH} subline which allowed to test if an impaired capacity to handle oxidative stress renders cells particularly sensitive to the effect of mixed beams. However, as all lymphatic cells, these cells easily undergo premature cell death due to harsh experimental conditions as applied in the present experiments, possibly masking the genotoxic effect of radiation. Also, they grow in suspension and are round which, unlike adherently growing cells, gives rise to dosimetric uncertainties in an exposure scenario with short-ranged alpha particles. Hence, it would be advantageous to carry out experiments with an adherently growing cell line which shows larger RBE values for the two radiation types to better address whether any synergy existed. Such experiments are currently under way in our laboratory.

A further question is related to the future research agenda on synergistic interactions between high and low LET radiations, and between ionising radiations and chemotoxic
agents. Investigations should aim at elucidating the mechanisms of interaction and its magnitude in relation to health risk. With respect to mechanisms we believe that there are three possible ways how high and low LET radiations can interact. Firstly, it is possible that the action of both radiation types will lead to an increase of LET and, consequently, of DNA damage complexity. This possibility can be tested by applying Monte Carlo simulations of the quantity, quality and spatial distribution of initial DNA damage under mixed beam exposure scenario [52]. Secondly, it is possible that exposure to high LET radiation will engage the DNA damage response machinery to such a degree that the additional damage induced by the low LET radiation will not be repaired properly. This possibility can be investigated by live analysis of DNA repair proteins that participate in the formation of radiation induced foci [53]. Thirdly, it is possible that damage induced by high LET radiation will lead to an opening of the chromatin structure making the DNA more susceptible to attack by ROS generated by the low LET radiation. This possibility could be studied using methods to measure chromatin condensation [54]. With respect to health risk, research should focus on such stochastic effects as cancer and cardiovascular effects after mixed beam exposure. Since epidemiological studies are difficult to carry out, an interesting approach is to run cell experiments with focus on selected key events in critical pathways to the development of the effect (so called adverse outcome pathway—AOP), as is being used for chemical carcinogenesis [55]. The AOP strategy was recently recommended for studying the dose and dose rate effect [56, 57] and could be applied to study the effects of mixed beams. Animal experiments on the effects of combined exposure would also yield very useful results. Appropriate experimental data is probably available from extensive animal studies carried out in the USA and Europe after the second world war [58–60] war but would need analysis from the perspective of combined exposures.

In summary, the study confirms our earlier results showing that exposure of cells to a mixed beam of alpha particles and x-rays leads to a detrimental effect which is stronger than expected based on the assumption of additivity. Although the mechanism of this phenomenon remains unclear, it does suggest that simple extrapolation of radiation risks from populations exposed to mixed beams of radiation, such as the Hiroshima and Nagasaki survivors, to populations exposed to pure gamma radiation, such as residents of areas contaminated by $^{137}$Cs, may not be correct.

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The authors declare no conflicts of interest

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