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Biological efficiency of the therapeutic neutron on DNA and Cytogenetic Damage in the Presence and Absence of ^{10}B.

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Abstract

The aim of study is to investigate the induction of DNA damage and chromosomal aberrations (CA) by irradiation with mixed beam from Brookhaven Medical Research Reactor (BMRR) as well as alterations in biological effect due to boron pretreatment. Two different methods were applied to estimate the biological effect in normal and boron enriched cells: the single cell gel electrophoresis (SCGE) technique and the cytogenetic assay. In normal human lymphocytes and in the GS-L9 rat gliosarcoma cells, significant radiation dose-dependent increase of DNA and cytogenetic damage were observed. In boron-enriched cells, significantly higher level of damage was observed compared with the non-enriched cells for both cell types. Levels of the DNA damage detected in cells with and without boron enrichment were in a good agreement with a biological effectiveness of the beam evaluated on the base of the BNCT treatment planning procedure. However, the relatively high level of the DNA damage observed in the boron-enriched and non-irradiated gliosarcoma cells suggests that boric acid *per se* may also induce the damage in cells. Therefore, boron enrichment in cancer cells may be genotoxic and enhances the therapeutic effects of neutrons at low doses. Our data also indicates that biological efficiency of the boron neutrons capture therapeutic beam in spite of having a high impact from gamma rays (~73% of the dose) is 9.3 times more effective than X-rays alone.

Introduction

The boron neutrons capture therapy (BNCT) was proposed for treatment of cancer in 1940. The traditional fast neutron irradiation therapy applies neutrons of high energy, whereas BNCT requires either thermal or epithermal neutrons with energies 0.25 eV and up to 30 keV, respectively [1-4]. A key parameter for the successful application of BNCT is the delivery of sufficient amount of ^{10}B atoms to tumor cells. This treatment is based on selective concentration of boron-10 isotope (^{10}B) within or near the tumor cells to capture thermal neutrons effectively. Neutron capture by the ^{10}B atom results in a nuclear fission process [1, 3] as follows:

$$^{10}B + n_{th} \rightarrow {}^{4}He + {}^{7}Li^{*} + 2.31\ MeV$$
$$\downarrow$$
$$^{7}Li + \gamma + 0.48\ MeV\ (93.7\%)$$

Because the ranges of the high-LET (linear energy transfer) products of this reaction in tissue are comparable to the cell diameter, BNCT provides means to specifically kill those cells, hopefully tumor cells, that accumulate ^{10}B.

To elucidate the effectiveness of the BNCT, we have conducted a study with application of two types of cells – tumor and normal cells. The aim of our study was to compare dose response relationship curves for therapeutic radiation sources with and without

the presence of boron atoms in GS L-9 rat gliosarcoma cells and human lymphocytes, respectively. In our studies we applied the single cell gel electrophoresis assay (SCGE) and the classical cytogenetic assay for the assessment of the DNA breakage and chromosome aberrations (CA).

2. Materials and Methods

2.1 Cells and materials

GS-L9 rat gliosarcoma cells originated from an N-nitrosomethylurea-induced neoplasm in a Fisher 344 rats [4].

Fresh blood sample was collected from a healthy non-smoking male young donor, transferred to heparinized tube and then divided for various pre-treatments and exposures. Heparin was used as an anticoagulant for the collection of peripheral blood samples.

2.2. Boron pretreatment

In order to introduce boron ion into cells various concentration of boric acid were applied and than cells were incubated at 37^0C for two hours. Previous studies [4] have shown that intracellular concentration of boron, following incubation with boric acid, equaled the boron concentration in the medium the final concentration of boron atom's at cells was 10 ppm for GS-L9 rat gliosarcoma cells and 10 or 20 ppm for human lymphocytes.

2.3. Irradiation

In vitro irradiations were carried out at the epithermal port of the Brookhaven Medical Research Reactor (BMRR) operating at 3 MW power. The applied source is adopted for the boron neutron capture therapy (BNCT) of brain tumors [4 - 7].

2.3.1 Irradiation of GS-L9 rat gliosarcoma cells

Prior to irradiations, the GS-L9 rat gliosarcoma cells [5] used in this study were incubated for 2 hr in cell culture medium with or without boric acid at the concentration of 10 ppm. For irradiation, 10^5 -10^6 cells in 1.5 ml of growth medium were transferred into 1.5 ml polyethylene centrifuge tubes and inserted into a 14x14x14 cm^3 Lucite cube [6] at 3.5 cm depth along the center line of the cube. Radiation doses delivered to the cells were calculated using a BNCT treatment planning software [7] assuming for boronated cells a higher biological efficiency. The following dose rates were obtained for different BNCT dose components at the cells location in the cube:

- boron dose (the dose resulting from the $^{10}B(n,\alpha)^7Li$ reaction) – 0.69 cGy/min for each ppm of boron;
- gamma dose (the dose resulting from the $^1H(n,\gamma)^2H$ reaction and the gamma contamination of the neutron beam) – 7.16 cGy/min;
- non-boron, high-LET dose (the dose resulting from the recoil protons and the $^{14}N(n,p)^{14}C$ reaction) – 1.97 cGy/min.

The total radiation dose rates to the cells incubated with and without boric acid were:

- 15.98 cGy/min, (0 ppm of ^{10}B)
- 9.13 cGy/min, (10 ppm of ^{10}B).

The different irradiation times for boron pretreated and non-treated cells were adjusted so that the both groups of tumor cells received total radiation doses in the range from 0 to 5 Gy.

2.3.2 Irradiation of human lymphocytes

Blood samples untreated or pretreated one an hour earlier with various concentrations (10 and 20 ppm) of ^{10}B in boric acid were irradiated with mixed beam containing epithermal neutrons from the Brookhaven Medical Research Reactor. The times of irradiation (min) and total dose (Gy) for separate probes without boric acid pretreatment were as follow:

- 0 – 0Gy;
- 2.11 – 0.2 Gy;
- 5.29 – 0.5 Gy;
- 16.26 – 1.5;
- 21.55 – 2 Gy, respectively.

In case of samples pretreated with various concentrations of ^{10}B (10 and 20 ppm) the time of irradiation was 5.29 min and total dose 0.9 Gy, and 1.3 Gy, respectively.

2.4 DNA damage analysis (SCGE)

The DNA damage induced in GS-L9 rat gliosarcoma cells were estimated using single cell gel electrophoresis (SCGE) technique also known as a Comet assay (8, 9).

Slide preparation

Slides (two repetitions of each slide) were prepared according to the standard procedure described elsewhere [8, 9]. Slides were immersed for 1 h at 4^0C in a freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% sodium sarcosinate), with 1% Triton X-100 and 10% DMSO added just before use. Then the slides placed side by side in a horizontal gel electrophoresis tank filled with fresh electrophoresis buffer (1 mM EDTA, 300 mM NaOH) to a level of about 0.25 cm above the slides. After 20 min of unwinding of DNA, the electrophoresis was conducted at 4^0C for 30 min at 30 V, 300 mA. All of the steps after lysis were carried out under yellow light to prevent any induction of additional DNA damage. After electrophoresis, the slides were washed three times for 5 min with a Tris buffer (0.4 M Tris, pH-7.5) and stained with 60 µl of 17 µg/ml ethidium bromide dissolved in distilled water. Before analysis, the slides were stored in a light proof box containing moist PBS at 4^0C.

Slide analysis

Slides were analyzed with the method proposed by Anderson (8) at 200x magnification using an epifluorescence microscope equipped with an excitation filter of 515-560 nm from a 100-W mercury lamp, and a barrier filter of 590 nm. From each sample were analyzed 50 randomly selected cells (25 cells from each of two replicate slides). In order to estimate the critical biological effect induced by treatment we joined the high and total categories (this two class contain 60% of DNA damage), and calculated percent of damaged cells per each treatment {6).

2.5 Cytogenetic analysis in human blood lymphocytes

2.5.2 Culture procedure

After irradiation the standard culture procedure was performed [10, 11]. Whole blood samples of 1.4 ml were cultured in 20 ml Eagle medium supplemented with 20% bovine serum and 1.4 ml of LF-7 (polish equivalent of phytohemagglutinin), with penicillin and streptomycin. An addition of 0.075 µM 5-bromo-2-deoxyuridine to the cultures was done to distinguish between the first and subsequent mitosis. The cultures were incubated for 48 h. Two hours before the end of the culture, colcemid (0.1 µl/ml) was added to each culture to arrest mitosis. Then cells were harvested and stained using the Hoechst-Giemsa technique.

Slide analysis

Unstable chromosome aberration analysis was done in all the cells with good metaphase spread in the first mitosis. Moreover, the population of the first and subsequent

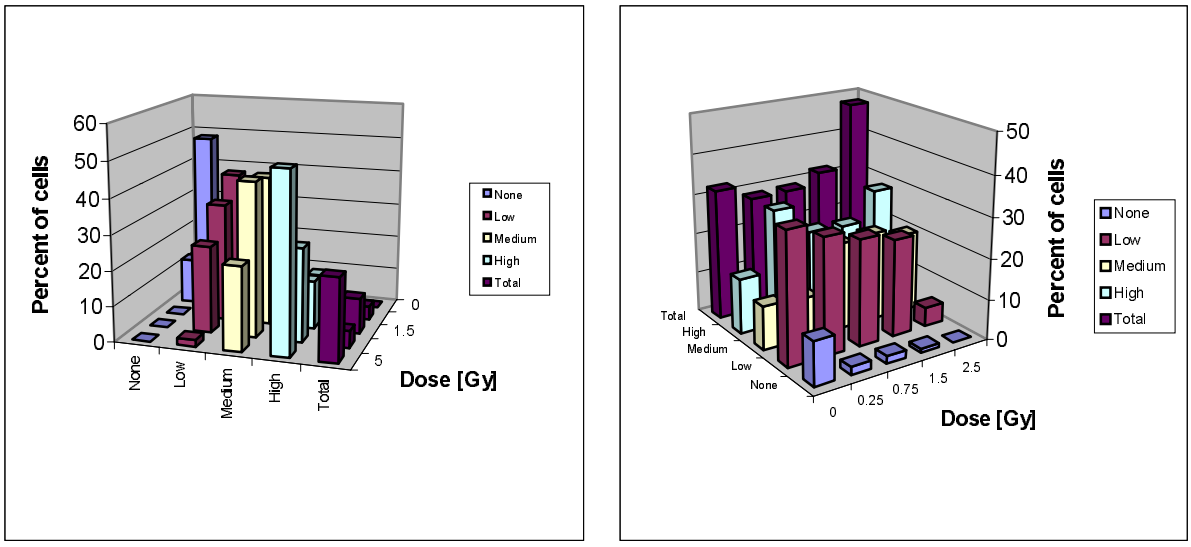
mitotic cells was also evaluated, whether cell cycling influenced the yield of unstable chromosome aberrations frequency detected after radiation exposure. The percentage of aberrant cells (%AbC), dicentric and ring frequencies (CAbF), total aberration frequencies including gaps (TAbF), aberration frequencies excluding gaps (AbF) were used as a measure of cytogenetic damage.

3. Statistics

The least square best fit for the dose-response curves and correlation coefficients were calculated with a use of Excel 5.0. Student's t test were applied to determine statistically significant differences between DNA damages due to irradiation or BSH pre-treatment.

4. Results

Figure 1a and 1b present changes in the distribution of the DNA damage in GS-L9 rat gliosarcoma cells irradiated with mixed beam at presence and absence of ^{10}B.



1a 1b

Figure 1a and 1b. Changes in the distribution of the DNA damage in GS-L9 rat gliosarcoma cells irradiated with mixed beam at presence and absence of ^{10}B.

The percentage of GS-L9 rat gliosarcoma cells with various degrees of DNA damage (none, low, medium, high and total) after irradiation with mixed of neutrons and gamma rays ranging from 0 to 5 Gy is presented at the Table 1.

Table1.
DNA damage (expressed as a percent of cells with specific class of DNA damage) detected in GS-L9 rat gliosarcoma cells after irradiation with therapeutic beam from Brookhaven Medical Research Reactor.

Irradiation time [min]	Dose [Gy]	None [%] ± SE	Low [%] ± SE	Medium [%] ± SE	High [%] ± SE	Total [%] ± SE
0	0	48.0 ± 0.0	36.0 ± 0.0	10.0 ± 2.0	6.0 ± 2.0	0.0
5.29	0.5	13.0 ± 6.6	40.0 ± 4.9	33.0 ± 1.9	12.0 ± 1.6	4.0 ± 1.6
6.26	1.5	0.0	33.7 ± 5.7	42.5 ± 8.5	13.7 ± 3.0	10.0 ±1.2
32.52	3	0.0	24.8 ± 7.0	43.8 ± 1.5	26.7 ± 6.6	4.8 ± 2.8
54.46	5	0.0	1.9 ± 1.1	23.8 ± 7.2	50.5 ± 4.6	23.3 ± 4.0

Table 2 presents the percentage of ^{10}B enriched cells with various degrees of DNA damage which were irradiated with the same beam and in the same conditions.

Table 2.
DNA damage (expressed as a percent of cells with specific class of DNA damage) detected in GS-L9 rat gliosarcoma cells pretreated with boric acid and irradiated with therapeutic beam from Brookhaven Medical Research Reactor.

Irradiation time [min]	Dose [Gy]	None [%] ± SE	Low [%] ± SE	Medium [%] ± SE	High [%] ± SE	Total [%] ± SE
0	0	10.8 ± 6.6	32.3 ± 5.0	10.8 ± 5.5	13.7 ± 7.0	32.3 ± 4.2
3.8	0.25	1.9 ± 1.1	28.7 ± 8.9	10.1 ± 4.9	28.7 ± 5.1	28.7 ± 7.1
9.23	0.75	1.9 ± 2.0	26.2 ± 4.5	21.5 ± 5.1	21.5 ± 2.5	29.0 ± 2.4
18.46	1.5	1.0 ± 1.0	24.0 ± 5.9	22.0 ± 8.7	21.0 ± 3.0	32.0 ± 1.6
31.17	2.5	0.0	4.7 ± 2.3	19.8 ± 3.0	28.3 ± 3.6	48.1 ± 5.9

Figure 2 presents biological effect dependence on the total dose observed for the GS-L9 rat gliosarcoma cells with or without boric acid pretreatment. Biological effect in Figure 2 is expressed as a percent of cells qualified to the category of DNA damage as high and total.

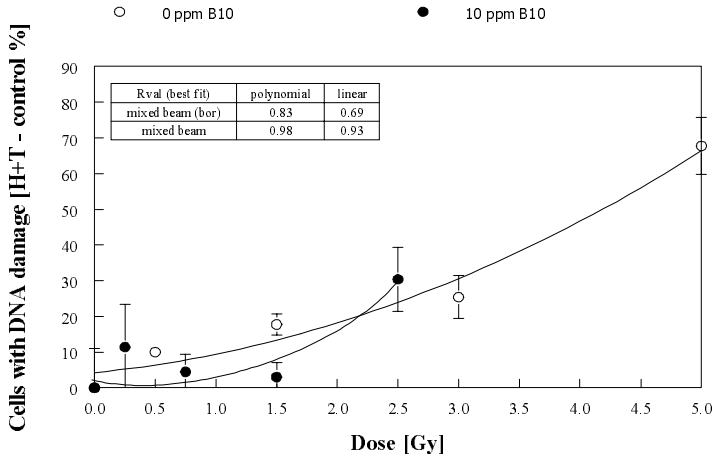


Figure 2. The dose response curves for DNA damage estimated in pretreated or nontreated with boric acid GS-L9 rat gliosarcoma cells after irradiation with mixed beam from BMRR Biological effect dependence on the total dose observed for the GS-L9 rat gliosarcoma cells with or without boric acid pretreatment.

The chromosome aberration frequency induced in normal and boron enriched human lymphocytes by mixed neutrons are presented in Table 3.

Table 3.
Chromosomal damage detected by classical cytogenetics in normal and boron enriched human lymphocytes after irradiation with neutrons from Brookhaven Medical Research Reactor.

Irradiation time [min]	Total dose [Gy]	Boron pretreatment	NM	CAbF ± SE	AbF ± SE	TAbF ± SE	AbC ± SE
0	0	-	203	0.0	0.005 ± 0.0003	0.015 ± 0.001	0.0
2.1	0.2	-	313	0.025 ± 0.001	0.077 ± 0.004	0.10 ± 0.006	2.55 ± 0.14
5.29	0.5	-	219	0.068 ± 0.004	0.17 ± 0.01	0.2 ± 0.01	6.85 ± 0.46
16.26	1.5	-	204	0.44 ± 0.03	0.79 ± 0.05	0.83 ± 0.06	44.12 ± 3.08
21.55	2	-	84	0.63 ± 0.07	1.14 ± 0.12	1.25 ± 0.14	63.09 ± 6.88
0	0	20 ppm	247	0.0	0.02 ± 0.001	0.07 ± 0.004	0.0

5.29	0.5	10 ppm	205	0.12 ± 0.008	0.21 ± 0.01	0.25 ± 0.02	12.19 ± 0.85
5.29	0.5	20 ppm	174	0.19 ± 0.01	0.28 ± 0.02	0.34 ± 0.03	18.96 ± 1.43

In normal lymphocytes there is a dose-dependent increase of CA reported as: dicentrics and rings frequency (CAbF), aberration frequency excluding gaps (AbF) and including gaps (TAbF), and percent of aberrant cells (AbC). In the cells pretreated with various concentration of boric acid and then irradiated for the same time with the same therapeutic beam (mixed of neutrons and gamma rays), an enhancement of the effect is observed. The data is also presented in Figure 3 to demonstrate the dose response curve.

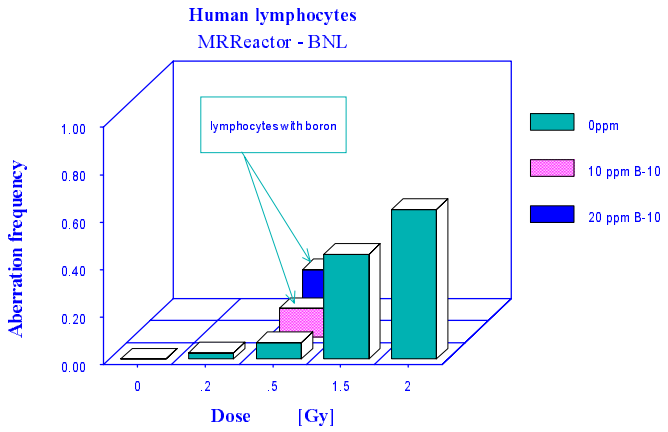


Figure 3. Aberation frequency evaluated in hyman lymphocytes pretreated or nontreated with boric acid and irradiated with mixed beam from BMRR.

Figure 4 compares dose response curves for X- rays, obtained in our previous study [12] and obtained in recent studies for the mixed beam from BMRR. There is clearly seen that boron enriched lymphocytes show lower efficiency than expected from dose response curve. There is also presented the dose response curve evaluated for epithermal and fast neutrons only, by subtracting the part due to the gamma rays contamination.

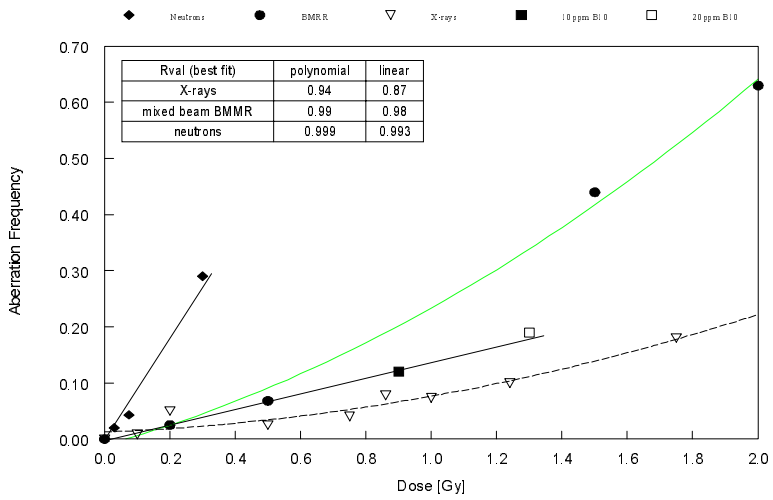


Figure 4. The dose response curves for human lymphocytes irradiated with X-rays, mixed beam from BMRR and neutrons – calculated effects after subtracting of the part due to the gamma rays contamination.

4. Discussion

The relative biological effectiveness (RBE) of neutrons depends on the physical characteristic of the neutron beam and on the biological endpoint chosen for the assay. *In vitro* studies can play an important role in estimation of suitability of boronated substances for enhancing the BNCT application. In the case of GS-L9 rat gliosarcoma cells without ^{10}B pretreatment, there is a linear increase of the DNA breakage observed after irradiation with mixed beam (predominantly gamma rays, epithermal and fast neutrons) from BMRR. A higher level of damage is induced in cells with ^{10}B, although, the observed effect is statistically insignificant. Taking in to account that boron pretreated cells were irradiated much shorter time the simulation of the effect with BNCT treatment planning software [7] shows the good adjustment of the quality factors in simulation of BNCT procedure. Consequently, no synergism was observed (Fig. 2). Higher dispersion observed in boron pretreated cells might be explained by the variability in the boron uptake.

The cytogenetic analyses reveal in non-enriched cells a significant and dose-dependent increase of unstable CA throughout dose ranges under the study. Pre-treatment with boric acid (10 and 20 ppm of ^{10}B) and irradiation with dose of 0.5 Gy causes a significantly higher cytogenetic damage than observed in non-enriched cells. The maximum value of the experimentally determined RBE is the value that operates at low doses and is defined as:

$$RBE_0 = \alpha_n/\alpha_\gamma,$$

where α are slope coefficients from the dose response curves for tested and standard radiation (gamma) [12]. The maximal value (RBE_0) is therefore the parameter which is of direct relevance to the Quality Factor (Q). Table 4 presents values of the α coefficient from the dose-response curve for chromosomal damage evaluated after irradiation of the lymphocytes with neutrons from Brookhaven Medical Research Reactor and X-rays in our previous study [12] and the maximal RBE values of the BMRR beam under the study versus X-rays.

Table 4. The RBE and α values for chromosomal aberrations.

	Radiation	α	RBE_{max}
Chromosomal Aberrations	X- rays	0.035	-
	Mixed beam MRR	0.326	9.3

The relative biological effectiveness for cytogenetic endpoints (RBE) was calculated as the ratio of the fitted linear coefficients of dose-response curves for dicentric and rings induced with neutrons from Brookhaven Medical Research Reactor (0.326) and X-rays from our previous study (0.035) [12]. The RBE value (9.3) estimated from CA in present study does not seem to be low, particularly, that it contains a low percentage of fast and epithermal neutrons in the mixed beam (~15%). Obtained RBE_{max} seems to be in good agreement with the expectation based on the biological efficiency dependence on the neutron energy. The evaluated RBE value is similar to the RBE = 10, that had been reported for more energetic neutrons (5.6 MeV) [13-15] and lower than values reported for most effective energies. Estimated RBE_{max} value is even higher than the RBE value (5.99) obtained previously by Cebulska-Wasilewska et all [15] for gene mutations in Trad-SH assay for the same beam.

From the results presented in this paper, assuming the composition of the investigated beam and knowing the efficiency of X- rays in the induction of chromosome aberration evaluated with the same procedure, we can evaluate biological impact from neutrons. Table 5 shows, fractions (of the total doses) delivered to biological sample by neutrons and corresponding to them the biological effects, estimated after subtraction from the total effects

detected, those due to the gamma component. On Figure 3 are shown calculated effects corresponding to the neutrons doses. Table 5 presents α_{CA} coefficients, and maximal RBE values (28.3) estimated for the fraction of neutrons from the investigated beam. These values are significantly higher than those measured by us for fast neutrons from U-120 and for ^{252}Cf [9, 12]. Biological effectiveness of the investigated beam in boron enriched lymphocytes have shown enhancement (Fig. 3) but lower than expected from micro dosimetric procedure. Furthermore, the relatively higher level of the DNA damage observed in boron enriched than in non-enriched cells suggests that boric acid *per se* may induce the damage in these cells.

Table 5. The maximal RBE values and α values for chromosomal aberrations.

	Radiation	α	RBE_{max}
Chromosomal Aberrations	X- rays	0.035	-
	neutrons	0.99	28.3

So, our results show that mixed beam from BMRR prepared for BNCT protocol has high biological effectiveness compared to γ-rays, especially in the presence of ^{10}B. It appears that cancer cells are sensitive to boron enrichment, which enhances the therapeutic effectiveness of BNCT particularly at low doses. Although our results are preliminary, they strongly suggest that further investigations might be interesting.

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