PROTECTION OF FEMALE C3H MICE AGAINST WHOLE-BODY γ-IRRADIATION WITH 2,3-DIMETHYL-6-((2-DIMETHYLAMINO)ETHYL)-6*H*-INDOLO[2,3-*b*]QUINOXALINE (B220)

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Radioprotection by 2,3-dimethyl-6-(2-(dimethylamino)ethyl)-6*H*-indolo[2,3-*b*]quinoxaline (B220) on survival and growth of female C3H mice exposed to acute whole-body gamma-radiation was evaluated for 7.5-8 months following irradiation in two separate experiments. For adult (12 weeks old) mice, B220 administration increased median survival after 8 Gy by a factor of 1.27 when given within 24 h pre-irradiation, administration up to 24 h post-irradiation had a similar effect (1.20) but in addition resulted in 1 of 9 (11 %) mice alive after 32 weeks. For adult mice irradiated with 10-14 Gy, B220 had no significant effect on survival. For very young mice (4 weeks old), however, B220 administration within 24 h pre-irradiation protected from growth retardation at both 1 and 6 Gy, and from gray-hairing at 6 Gy. In conclusion, the well tolerated drug B220 offered radioprotection in both studies and its administration could be further optimized.

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INTRODUCTION

Ionising gamma (γ)-irradiation causes radiation damage in biological organisms. Reactive species are produced through radiolysis of water (e.g. $HO^{\bullet}$, $H^{\bullet}$, e_{aq}^{-}) and ionization of biomolecules such as proteins, DNA and lipids make them prone to reactions with molecular oxygen (O_2).[1,2] The damage (disintegration and oxidation of organic molecules) initiates an inflammatory response with attacking neutrophils (granulocytes, monocytes and macrophages) releasing various reactive oxygen species (ROS).[1,2]

Drugs initially found to protect from γ-irradiation, such as sulfhydryls[3] and WR-2721 (amifostine)[4] give side effects at high doses or offer limited protection in certain organs (e.g. the central nervous system).[1,5] More recently, however, some interesting examples have been reported including flavonoids,[6] caffeine,[7] α-TMG (vitamin E analog),[8] *Mentha arvensis* (mint),[9] 5-androstenediol,[10] Tempol,[11] molecular hydrogen (H_2) containing water,[5] Ex-Rad™,[12] JNJ7777120 (indole),[13] and particularly the recently studied indole 3,3'-diindolylmethane (DIM; derived from ingestion of indole-3-carbinol found in cruciferous vegetables such as cabbage, Brussels sprouts and broccoli) that offered astonishing radioprotection also at very high doses (up to 13 Gy) both *in vivo* and in cell culture.[14]

The synthetic indole-drug 2,3-dimethyl-6-((2-(dimethylamino)ethyl)-6*H*-indolo[2,3-*b*]quinoxaline (B220, structure shown in figure 1) has been found to be well tolerated, having preventative effects on growth of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) promoted skin tumours *in vivo* possibly by interfering with enzymes involved in the generation of ROS in the inflammatory response to TPA,[15] and was found to downregulate phagocyte NADPH-oxidase activity *in vitro*, inhibiting release of ROS, by affecting signalling downstream of protein kinase C (PKC).[16]

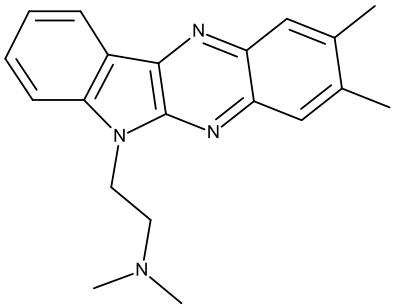

Figure 1. 2,3-Dimethyl-6-((2-dimethylamino)ethyl)-6*H*-indolo-[2,3-*b*]quinoxaline (B220).

Based on these protective roles of B220 on reducing ROS production, the aim of this study was to test if B220 would have any protective effect on γ-irradiation *in vivo*. Mortality, weight gain (growth) and other possible complications was investigated after whole-body γ-irradiation of C3H female mice given B220 intraperitoneally (*i.p.*) at a dose similar to those used in some previous radioprotector studies.[7,9]

RESULTS

Study I: adult (12 weeks old) mice exposed to 0-14 Gy

As shown in Table 1 and Figure 2, at high doses γ-radiation (10, 12, 14 Gy) all the 12 weeks old mice had died after 13 days and no effect of B220 was seen. A sharp threshold for survival was noticed between 6 and 8 Gy: nearly all mice exposed to 6 Gy survived (one animal in the

6 Gy control group died 31 weeks after irradiation) during the investigated period over 32 weeks (7.5 months) following irradiation, but most mice exposed to 8 Gy died within 25 days. At 8 Gy, pre-administration of B220 increased the median survival time by 27 % (from 15 to 19 days). B220 given post-irradiation increased the median survival time by 20 % (from 15 to 18 days) and in addition resulted in 11.1 % long-term survival as one mouse was still alive at the end of study (this mouse became gray-haired 10 weeks after irradiation, at age 23 weeks).

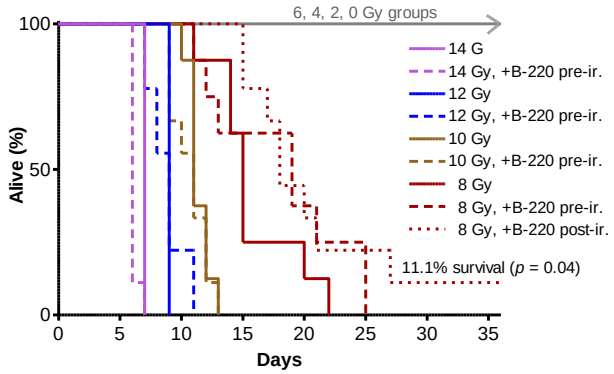

Figure 2. Survival curves as % alive for adult (12 weeks old) female C3H mice exposed to 0-14 Gy whole-body γ-radiation.

The Gehan-Breslow-Wilcoxon survival curve analysis test showed that administration of B220 post-irradiation significantly increased the survival compared to no B220 administration at 8 Gy (p=0.04). The 6 Gy mice developed gray-haired skin 11 weeks after irradiation, at age 24 weeks.

Table 1. Lethality and effect of B220 upon whole-body γ-irradiation of adult (12 weeks old) female C3H mice followed for 32 weeks.

	Median survival in days			Effect of B220
Gy	Control	+B220 pre-ir.	+B220 post-ir.	
14	7 (N=8)	6 (N=9)	-	none
12	9 (N=8)	9 (N=9)	-	none
10	11(N=8)	11(N=9)	-	none
8	15 (N=8)	19 (N=8)	18 (N=9)	pre-ir: 27 % increase post-ir.: 20 % increase and 11 % long time survival
6	one death after 31 weeks (N=8)	no death (N=9)	-	none
4	no death (N=8)	no death (N=9)	-	none
2	no death (N=8)	no death (N=9)	-	none
0	no death (N=8)	no death (N=8)	-	none

Mice in the 4, 2 and 0 Gy groups all survived and had not become gray-haired at end of the study. Mean body weights for the 17 groups at the beginning of the study (12 weeks old) lay in the range 23.3-26.5 g. At the end of study, mean body weights in surviving groups (6, 4, 2 and 0 Gy) lay in the range 34.7-41.8 g and no significant effect due to irradiation could be seen among groups not administrated B220. Also, B220 had no effect on weight gain in these groups.

Study II: 4 weeks old mice exposed to 0, 1 and 6 Gy

As shown in figure 3, both 1 and 6 Gy retarded growth for the very young mice compared to non-irradiated mice. On the day of irradiation, the six groups' mean weights were 12.6-14.6 g. After rapidly gaining weight, average weights were 29.0-38.9 g at week 36 (8 months after irradiation, at 9 months of age), reflecting a 209-285 weight % increase versus the day of irradiation depending on group (Figure 3A). The weight % data for both individual mice and as group means could be well fitted to two-phase association curves (Figure 3A).

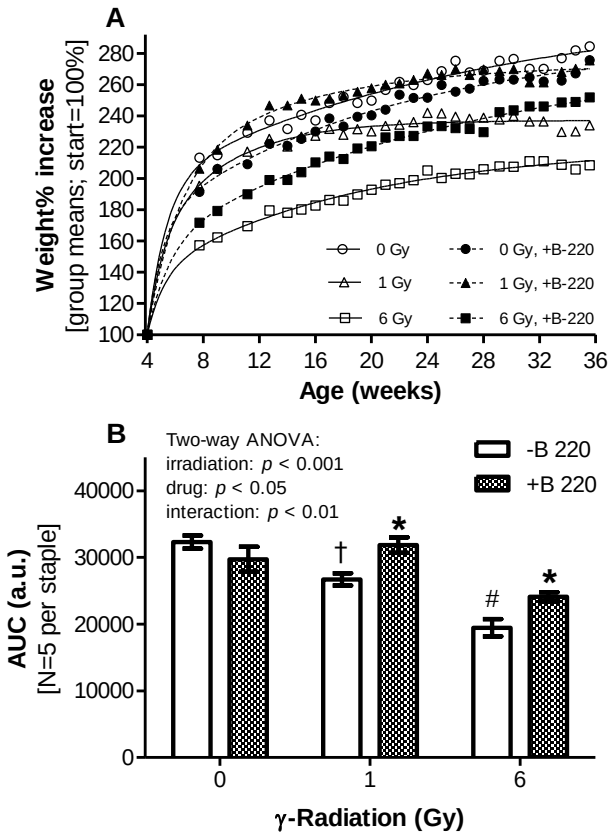

Figure 3. Effect of whole body γ-irradiation (0, 1 and 6 Gy) and B220 pre-administration on growth of 4-week-old female mice. Data shown as averages in A and means ± SEM in B.

Calculation of area under curve (AUC) values for the individual mice (N=5 per group) allowed statistical comparisons among groups. Analysis using two-way ANOVA (Figure 3B) determined that growth was overall retarded due to radiation ($p < 0.001$), that B220 exerted a protective effect ($p < 0.05$) and that there was an interaction between radiation and B220 ($p < 0.01$), thus that B220 counteracted growth retardation due to irradiation. The Bonferroni post test (*) identified protection by B220 at both 1 ($p < 0.05$) and 6 Gy ($p < 0.05$). At 0 Gy, however, B220 administration had no effect on weight gain. One-way

ANOVA analysis of groups not administered B220 showed that growth was retarded at 1 Gy ($p < 0.05$ (†)) and that 6 Gy affected growth even more ($p < 0.05$ (#)). At 6 Gy, the mice developed gray-haired skin about 18 weeks after irradiation (at age 22 weeks) and B220 reduced the degree of gray-hairing (visual observation). One mouse in each of the 6 Gy groups died 25 weeks after irradiation (for AUC calculations their weights were assumed constant until the end of study).

Toxicity of B220

No signs of clinical toxicity were seen after two i.p. administrations of B220 at 25 mg kg^{-1} body weight doses (in total 50 mg kg^{-1} body weight) with a gap of 22 h in between. Topical administration of 1 mg B220 alone twice a week for 20 weeks had previously not shown any apparent toxicity.[15]

DISCUSSION

This study provides detailed information regarding mortality and growth of female C3H mice after acute γ-irradiation. For adult mice (Study I), B220 showed protective effects at 8 Gy (Figure 2) when administrated 24 and 2 h before, or, 2 and 24 h after, irradiation. For 4 weeks old mice (Study II), B220 counteracted growth retardation at 1 and 6 Gy (Fig 3) and decreased gray hairing of skin at 6 Gy. For 4 weeks old mice, 1 Gy caused adverse developmental effects on growth, whereas further weight gain in adult mice was not affected by as much as 6 Gy. At 6 Gy, both the 12 and 4 week old mice became gray-haired at about the same age (at 24 and 22 weeks of age, respectively), thus the adult mice became gray-haired considerably sooner after irradiation.

Regarding drug administration pre- or post-irradiation for optimal protection from γ-radiation damage, studies by others have found that both ways can work. Dosing with DIM either before or up to 24 h after irradiation protected rats from lethal irradiation doses up to as high as 13 Gy.[14] The time-point of drug administration may indeed play a role. In a study on 7-8 week old C57BL/6 mice exposed to 8 Gy whole-body γ-irradiation, switching to a diet high in antioxidant supplements 24 h after the irradiation event significantly increased the survival and was considerably more efficient than when beginning the antioxidant administration 12 or 48 h after irradiation.[17] The reason for this is unclear. Also the dose and frequency of administration can play a role. For the two flavonoids orientin and vicenin, both worked better as radioprotectors at 50 than at 75 or 100 μg kg^{-1} body weight.[6] Maximum protection was achieved by administration 30 min before irradiation, injection at other time points (2 or 1 h before, or 30 min after) offered less protection.[6] DIM, however, offered better protection at 75 mg kg^{-1} body weight than at lower doses when administered on 14 consecutive days after irradiation to 13 Gy.[14]

Mechanistically, the various radioprotectors have been suggested to act very differently. Dephosphorylation of WR-2721 produces the active cell-permeant thiol metabolite WR-1065, which has been reported to suppress the reactivity of intralysosomal iron.[18] Flavonoids, α-TMG, H_2, and Tempol presumably react with or modify free radicals and ROS.[5,6,8,11] Ex-Rad™ was reported to manifest its protective effects through up-regulation of PI3-Kinase/AKT pathways in cells exposed to radiation.[19] A suggested protective mechanism for DIM proceeds through activation of the nuclear kinase at axiateleangiectasia mutated (ATM) regulating responses to DNA damage and oxidative stress, and NF-κB activation.[14] The indole JNJ7777120 acts as a potent and selective antagonist of the histamine H4 receptor,[13] a member of the G protein-coupled receptor super family. The mechanism for the observed protection of B220 is unclear but is possibly due to its observed ability to down-regulate the secondary release of ROS by neutrophils,[16] preventing further oxidative stress after irradiation, and is not likely due to $OH^{\bullet}$-scavenging as B220 has been reported to poorly scavenge $OH^{\bullet}$.[16] B220 is also a DNA intercalator.[20]

In conclusion, B220 was found to be well tolerated at the dose tested and somewhat radioprotective in whole-body γ-irradiated mice. As no toxicity from B220 was observed this raises the chance for optimisation of B220 administration in terms of quantity and number of doses over time, something that likely will enhance the protective effects of B220 further.

MATERIALS AND METHODS

Animals and experimental design

Dark female C3H mice were ordered from M&B A/S (Denmark) and were allowed to acclimatize at the animal facility for a minimum of 5 days. Two separate whole body γ-irradiation studies were performed: (I) evaluation of B220's effect on survival of adult (12 weeks old) mice exposed to 0–14 Gy (0, 2, 4, 6, 8, 10, 12, 14 Gy; n = 8–9 mice per group) ± pre-administration of B220 at all irradiation doses (for 8 Gy, a separate group given B220 post-irradiation was also included), and, (II) evaluation of B220's effect on growth of very young mice (4 weeks old) irradiated with 0, 1 and 6 Gy (n = 5 mice per group). Animals were randomly allocated to groups. In both studies, surviving mice were carefully examined (physical appearance, body weight, etc.) weekly for at least 7.5 months after irradiation when they were humanely euthanized. The groups were kept in separate cages at the animal facility at Huddinge Hospital under controlled conditions with food and water available before and after irradiation. Cage size was adjusted to group size. The ethical permit for the study was S141/96, approved by Stockholm South Ethical Committee on Animal Research, and all experiments were performed in accordance with relevant guidelines and regulations.

Preparation of B220

B220 base was synthesised according to an earlier published method.[21] B220 was dissolved in acetone, then fresh corn oil was added and the solution stirred until all acetone had evaporated giving a final concentration of 1.7-3.2 mg B220 mL^{-1}, depending on the study. The solutions were stored protected from light at room temperature.

Administration of B220

After recording body weights, B220 was administrated. Animals received two 200 µl doses (*i.p.*) of corn oil containing B220 at a concentration giving 25 mg kg^{-1} of body weight each (in total 50 mg kg^{-1} of body weight), administered either before or after irradiation. Controls received corn oil. For pre-administration, the first dose was given 24 h before and the other dose 2 h before irradiation. For post-administration, one dose was given 2 h and the other 24 h after irradiation.

Irradiation of mice

On the day of γ-irradiation the mice were transported to the Department of Radiation Biology, Stockholm University. Irradiation was carried out using ^{137}Cs at 0.65 Gy min^{-1} inside a routinely dosimetry checked apparatus from Instrument AB Scanditronix (Uppsala, Sweden). Animals were whole-body exposed in round well-ventilated compartmentalised plastic boxes (taking up to 10 mice simultaneously).

Observation

The mice were carefully monitored daily the first month after γ-irradiation and then once a week for at least 32 weeks (7.5 months), checking signs of clinical toxicity (survival, weight, physical appearance, etc.). Survival curves were plotted using the Kaplan-Meier method.

Statistical analyses

Statistical significance was set to $p<0.05$ and analyses conducted using GraphPad Prism 5 (San Diego, California). Survival curves were compared using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. After normalizing body weights to the time-point of irradiation (set to 100 %), the areas under the growth curves were integrated (gives AUC values) for statistical comparison using one- and two-way ANOVA analyses.

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