Mutagenesis vol. 23 no. 4 pp. 285–292, 2008
Advance Access Publication 18 March 2008
doi:10.1093/mutage/gen005

Radioprotective effect of sulfasalazine on mouse bone marrow chromosomes

Sudheer K. Mantena, M. K. Unnikrishnan* and P. Uma Devi[1]

Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal 576 104, Karnataka, India and [1]Department of Research, Jawaharlal Nehru Cancer Hospital and Research Centre, Idgah Hills, Post Box No. 32, Bhopal 462 001, Madhya Pradesh, India

Sulfasalazine (SAZ), a prescribed drug for inflammatory bowel disease, is a potent scavenger of reactive oxygen species. The present study was undertaken to ascertain its ability to protect against gamma radiation-induced damage. Acute toxicity of the drug was studied taking 24-h, 72-h and 30-day mortality after a single intraperitoneal injection of 400–1200 mg/kg body weight (b.wt.) of the drug. The drug LD_{50} for 24- and 72-h/30-day survival were found to be 933 and 676 mg/kg b.wt., respectively. The optimum time of drug administration and drug dose-dependent effect on *in vivo* radiation protection of bone marrow chromosomes was studied in mice. Injection of 30–180 mg/kg SAZ 30 min before gamma irradiation (RT) with 4 Gy produced a significant dose-dependent reduction in the RT-induced percent aberrant metaphases and in the frequency of micronucleated erythrocytes at 24 h after exposure, with a corresponding decrease in the different types of aberrations. The optimum dose for protection without drug toxicity was 120 mg/kg b.wt. At this dose, SAZ produced >60% reduction in the RT-induced percent aberrant metaphases and micronucleated erythrocytes. SAZ also produced a significant increase in the ratio of polychromatic erythrocytes to normochromatic erythrocytes from that of irradiated control. Injection of 120 mg/kg of the drug 60 or 30 min before or within 15 min after 4 Gy whole-body RT resulted in a significant decrease in the percent of aberrant metaphases and in the frequency of micronucleated erythrocytes at 24 h post-irradiation; the maximum effect was seen when the drug was administered 30 min before irradiation. These results show that SAZ protect mice against RT-induced chromosomal damage and cell cycle progression delay. SAZ also protected plasmid DNA (pGEM-7Zf) against Fenton's reactant-induced breaks, suggesting free radical scavenging as one of the possible mechanism for radiation protection.

Introduction

Development of effective and nontoxic radioprotective agents is of considerable interest for radiation medicine, space flights, nuclear industries and emergencies. A large number of chemical and biological agents have been screened and reviewed in this connection (1,2). Several chemicals have been found to provide good protection against gamma radiation (RT) in experimental animals, but their clinical utility is limited by the drug toxicity on repeated administration (2). The only drug approved for clinical use in cancer therapy is amifostine, a synthetic phosphorothioate compound, which also produces dose-limiting drug toxicity at the maximum effective dose (2). In view of this, search for newer more effective agents is inevitably continuing.

Discovery of new drug molecules is an extremely slow and a very expensive proposition with a high rate of failure. The strategy of identifying new uses for existing drugs is more feasible and rewarding. Sulfasalazine (SAZ), an existing prescribed drug for inflammatory bowel disease (3), was synthesized in 1942 to combine an antibiotic, sulfapyridine (SP), and an anti-inflammatory agent, 5-aminosalicylic acid (5-ASA) (4). About 30% of the orally administered SAZ is absorbed in its unaltered form, whereas the rest is subjected to degradation by colonic bacteria and azo reduction into SP and 5-ASA (5). Studies comparing equimolar doses of SAZ, 5-ASA and SP applied topically to patients with distal colitis suggested that the effects of SAZ and 5-ASA were equivalent and significantly superior to SP (6–8). Pruzanski *et al.* (9) reported that SAZ was more potent in inhibiting the formation of 5-lipoxygenase products, whereas its split products, 5-ASA and SP, were shown to be much less potent. Further, numerous pharmacological and biochemical effects have been described for SAZ, including immunosuppressive and immunomodulatory actions on lymphocytes and leukocyte functions (10–15). SAZ is also a known free radical scavenger (16,17) and is referred to as antioxidant in the treatment of inflammatory bowel diseases (18).

Radiation is known to produce oxygen-free radicals which are implicated in the process of DNA damage, cell killing, mutagenesis and carcinogenesis (2); hence, it is reasonable to assume that agents capable of scavenging free radicals would play a significant role in modulating these processes. The radioprotection of normal cells by a number of synthetic and natural compounds has been reported to be mediated through free radical scavenging activity (2). SAZ is also reported to decrease acute gastrointestinal complications due to pelvic radiotherapy (19–21). However, there are no preclinical reports on the effectivity of SAZ on RT-induced cytotoxicity. Hence, the present study was carried out in order to evaluate the radioprotective effect of SAZ injected intraperitoneally (i.p.) against the RT-induced genotoxicity in the mouse bone marrow.

Materials and methods

Animals

All experiments were carried out on random-bred Swiss albino mice of both sexes from the animal colony of the Department of Radiobiology that were 6–8 weeks old and weighed 25 ± 5 g. The colony was maintained under controlled

*To whom correspondence should be addressed. Tel: +91 820 2572195; Fax: +91 820 2570061; Email: mkunnikrishnan@gmail.com

conditions of temperature (23 ± 2°C) and humidity (50 ± 5%) and a 12-h light–dark cycle. The animals were housed in sanitized polypropylene cages containing autoclaved paddy husk as bedding. They were fed on standard mice food, freshly prepared (composition given by Cancer Research Institute, Mumbai, India) and autoclaved, and filtered acidified water *ad libitum*. We maintained an equal number of male and female mice through out the study. In the present study, the number of micronuclei (MNs) in the polychromatic erythrocytes (PCEs) were found to be 77.4 ± 5.2 for males and 82.9 ± 6.9 for females, and the number of MN in normochromatic erythrocytes (NCEs) were 6.4 ± 0.1 for males and 5.9 ± 0.1 for females, in the mice exposed to 4-Gy RT. The MNs arise due to chromosome breaks and so the MN data obtained for male and females should reflect the chromosomal aberrations data as well; therefore, we did not collect the chromosomal aberration data based on sex. Other studies have suggested that susceptibility to RT may be sex dependent (22,23). However, in the present study, we found no differences in the induction of MN between the sexes; therefore, no further attempts were made to study sex-based differences in the susceptibility to RT-induced damage. Even if there were sex-dependent differences, the outcome of the present study would not have changed because same number of males and females were used for the control and treatment groups, and whatever error exist would be same in all the groups.

Drug

SAZ was procured from Sigma Chemical Co. (St. Louis, MO, USA). A suspension of SAZ in 1% sodium salt of carboxy methyl cellulose (CMC) was freshly prepared prior to use and injected i.p.

Irradiation

Mice were placed in well-ventilated Perspex boxes (23.5 × 23.5 × 3.5 cm, partitioned into 3 × 3 × 11-cm cells for each animal) and exposed to whole-body RT from a ^{60}Co Gammatron teletherapy unit (Siemens, Erlangen, Germany) at a dose rate of 1.66 Gy/min and an source to surface distance of 90 cm. The irradiation facility was provided by the Department of Radiotherapy and Oncology, Shridi Sai Baba Cancer Hospital, Manipal, Karnataka, India.

Experimental protocol

All the studies were conducted according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.

Determination of acute toxicity of the drug

Acute toxicity was determined in fasting mice (24). Animals were divided into groups of 10 each and injected i.p. with 1% CMC (Aldrich Chemical Co., Milwaukee, WI, USA) or 400, 600, 800, 1000 and 1200 mg/kg body weight (b.wt.) of SAZ. The mice were observed continuously for 2 h, then frequently up to 6 h and daily thereafter for 30 days, and mortality was recorded. The percentage mortality at 24 h, 72 h and 30th day was converted into probit values by referring to the appropriate table and the LD_{10} and LD_{50} were calculated (25).

Screening for radioprotection

Drug dose response Animals were divided into groups of five each and treated as follows:

1. 0.3 ml of 1% CMC per mouse, 30 min prior to sham irradiation.
2. 0.3 ml of 1% CMC per mouse, 30 min prior to RT (4 Gy).
3. 30–180 mg/kg b.wt. of SAZ, 30 min prior to RT (4 Gy).
4. 30–180 mg/kg b.wt. of SAZ, 30 min prior to sham irradiation.

Cytogenetic damage in the bone marrow cells was studied by chromosomal aberration analysis and MN assay 24 h post-irradiation. Based on this study, drug dose which gave the maximum protection (120 mg/kg b.wt.) was used in the following experiments.

Selection of optimum time of drug administration

Animals were divided into groups of five each and injected i.p. with 120 mg/kg (one-fifth LD_{50} approximately) b.wt. SAZ or 1% CMC 60 min before or at 15 min after whole-body RT with 4 Gy. The data from these experiments were compared to the data from the earlier experiment where animals were pretreated with 120 mg/kg SAZ 30 min before radiation. Chromosomal aberration analysis and MN assay were done 24 h post-irradiation.

Procedure for bone marrow studies

At 22 h after irradiation, all the animals were injected i.p. with 0.025% colchicine (0.01 ml/g b.wt.) and sacrificed 2 h later by cervical dislocation. Both femurs were dissected out, one of the femurs was used for chromosome preparation and the other for MN assay.

Chromosomal aberrations

Metaphase plates were prepared by the air-drying method (26). Briefly, bone marrow from the femur was aspirated, washed in saline, treated hypotonically (0.559% KCl), fixed in 3:1 methanol:acetic acid, dried and stained with 4% Giemsa (Sigma). Chromosomal aberrations were scored under ×800 total magnification with a light microscope (AO Reichert, Depew, NY, USA). A total of 500 metaphase plates were scored per animal. Different types of aberration like chromatid breaks, chromosome breaks, fragments, rings and dicentrics, as well as cells showing polyploidy and severely damaged cells (SDC, cells with 10 or more aberrations of any type) were scored. The aberrations were identified using the criteria given by Savage (27). When breaks involved both the chromatids, it was termed 'chromosome-type' aberration, while ''chromatid-type'' aberration involved only one chromatid. If the deleted portion had no apparent relation to a specific chromosome, it was called a fragment (28). The number of aberrant metaphases is expressed as percent aberrant metaphases and the different aberrations are expressed as aberrations per 100 cells. Data are presented as mean ± SE for five mice.

MN assay

The MN test affords a procedure for the detection of aberrations involving anaphase chromosome behavior utilizing the bone marrow erythroblast (29). The test is based on the formation of 'MNs' from particles of chromatin material that, due to chromosome breakage or spindle dysfunction, do not migrate to the poles during anaphase and are not incorporated into the telophase nuclei of the dividing cell. Such chromatin fragment or even whole chromosomes in the case of chromosome lag results in the formation of one or more small satellite nuclei in the cytoplasm of the daughter cells. When the treatment time with the mutagens or carcinogens is between 24 and 30 h, the majority of these MNs are found in the newly formed PCEs.

For the purpose of detecting genetic damage in the form of chromosome disrupting capacity, the PCE have some very useful characteristics. The expulsion of the mammalian erythrocyte nucleus follows the final mitotic division by several hours, but the resulting enucleated erythrocytes retain their cytoplasmic basophilia for ~24 h after nuclear expulsion. In addition, the MNs in the cytoplasm of these cells are not expelled with the nucleus. In view of this, if the bone marrow of a test animal is examined after 24 h after treatment, it is possible to discriminate between erythrocytes formed during the treatment and those formed prior to treatment. In this case, each animal could be considered to serve as its own control in as much as the frequency of MNs in the PCE formed during treatment may be compared with the frequency of MNs in the NCEs formed prior to treatment. Any mutagenic response during the 24-h time interval would result in an increase in the frequency of MNs only in the PCE population. Furthermore, the ratio between PCE and NCE under normal physiological conditions is ~1 (P/N = 1). Any significant decrease in the number of PCE and an increase in the number of NCE are considered as suggestive of bone marrow suppression.

The method of Schmid (30) was used with some modification. The animals of all the groups were killed at 24 h after irradiation. The femurs of each animal were dissected out, cleaned of adherent tissues and the bone marrow was flushed out into saline. The cell suspension was centrifuged and the pellet was resuspended in a few drops of fetal calf serum. The smears of cells were made on precleaned, precoded, dry slides, air dried and fixed in absolute methanol. The slides containing the cells were stained with 0.125% Acridine orange (BDH, Poole, UK) in Sorenson's buffer and observed under ×400 total magnification with a fluorescent microscope (Carl Zeiss Photomicroscope III, Oberkochen, Germany). For each animal, 10 000 erythrocytes were observed and the number of PCEs and NCEs were counted. The number of PCE and NCE with MNs [micronucleated polychromatic erythrocytes (MPCE) and micronucleated normochromatic erythrocytes (MNCE)] was recorded. The ratio of PCE to NCE (P/N) was also calculated.

DNA nicking assay

For *in vitro* studies, a 10-mM SAZ stock solution was prepared by dissolving the drug in 0.1 N NaOH plus phosphate-buffered saline (pH 7.2), followed by adjustment of pH to ~7.5 by slow addition of 1.0 N HCl. The reaction mixture containing SAZ (10–100 μM) was incubated for 1 h at 37°C with plasmid DNA (pGEM-7Zf) in the presence of ethylenediaminetetraacetic acid (EDTA) (30 mM)/hydrogen peroxide (30 mM)/ferrous sulfate (16 mM). After incubation, 2 μl of loading dye (bromophenol blue) was added and mixture was loaded onto an agarose gel well (31). Electrophoresis was conducted at 60 V in Tris-acetate–EDTA (0.04 M Tris-acetate and 1 mM EDTA, pH 7.4) buffer for 2 h. DNA bands were visualized and recorded in a gel documentation system.

Statistical analysis

Statistical evaluation of the data (selection of optimum time of drug administration; drug dose response) was done by one-way analysis of variance

(Graph PAD Instat Software). A value of $P < 0.05$ was considered to be significant.

Results

Drug toxicity

SAZ did not produce any death at 600 mg/kg, whereas 60% death occurred within 24 h at 800 mg/kg. No animal survived beyond 24 h at a dose of 1200 mg/kg. Twenty percent mortality was observed at 600 mg/kg at 72 h. In all dose groups, the animals which did not die within 72 h survived to 30 days. The LD_{50} for 24 h was calculated to be 933 mg/kg, which decreased to 676 mg/kg for 72 h and remained the same up to 30 days (Table I). The calculated LD_{10} dose was 500 mg/kg (72 h/30 days). Based on this study, we selected doses between 30 and 180 mg/kg to evaluate the radioprotective efficiency of SAZ.

Drug dose response

The bone marrow consists of asynchronous cell populations, and cells that are exposed in the G_2 and M phases of the cell cycle will manifest chromatid aberrations at 12 h after irradiation, while those which are in G_1 and early S phase of the cell cycle will contain chromosome breaks at 24 h, by which time the exposed cell would have divided. As a result, bone marrow cells will yield a higher number of chromosome breaks at 24 h post-irradiation. The cell cycle time of mouse bone marrow cells is 12 h (32) and scoring of chromosome aberrations at 24 h also takes care of the delay induced by ionizing radiation (33). Several authors have shown that chromosome aberrations decline at 48 h post-irradiation as the irradiated cell would already have divided at least once after the induction of aberrations, which results in a dilution of the observed chromosome aberrations (33,34). The chromosome aberrations have been reported to be reduced by half with successive cell divisions (35) and cells inflicted with lethal damage to DNA have been reported to be eliminated during successive divisions (1–4 division) after irradiation (36). Hence, in the present study, the metaphase spreads have been analyzed at 24 h post-irradiation.

SAZ up to a dose of 120 mg/kg did not produce any significant increase in the chromosomal aberrations in the sham-irradiated animals. An increase in the SAZ dose to 180 mg/kg resulted in the chromosomal aberrations, evidenced by a significant ($P < 0.001$) increase in the percent aberrant metaphases (Table II, Figure 1A). Irradiation with 4 Gy significantly increased the percent aberrant metaphases and the different types of aberrations. Pretreatment with SAZ produced a dose-dependent decrease in the percent aberrant metaphases (Table II, Figure 1A). The lowest dose used in the experiment, 30 mg/kg, reduced the percent aberrant metaphases from 53.26 to 47.80, which was not significant from the RT-alone group. Increase in the dose to 60 and 120 mg/kg gave a significant dose-dependent increase in the effect, with the percent aberrant metaphases cells falling to 28.60 for the SAZ dose of 120 mg/kg, which is about 50% less than that of the irradiated control. These results correspond to 0.286 aberrations per cell with SAZ (120 mg/kg) + RT treatment, which is significantly lower than RT-alone treated group (0.533 aberrations per cell). SAZ (120 mg/kg) significantly decreased breaks (chromosome and chromatid), fragments, rings and dicentrics ($P < 0.001$). This dose also significantly reduced cells with multiple aberrations and polyploids ($P < 0.001$). Further increase in dose to 180 mg/kg did not give additional protection; on the contrary, the effect was reduced (Table II, Figure 1A).

Similar results were observed in the MNs' study. SAZ alone did not produce any significant increase in the incidence of MNs in the sham-irradiated animals up to a dose of 120 mg/kg. However, 180 mg/kg, the highest dose used in this study caused the frequency of MPCE to increase significantly above normal in the sham-irradiated animals (Table III, Figure 1B). This dose also resulted in higher P/N ratio in the sham-irradiated animals (Table III, Figure 1B). Radiation (4 Gy) brought about a significant increase in the frequency of MPCE and MNCE and a significant decrease in the P/N ratio. All doses of SAZ, given 30 min (selected arbitrarily) before RT, produced a significant reduction in the number of MPCE compared to the irradiated control (Table III, Figure 1B) and this effect was dose dependent. The maximum decline was observed with 120 mg/kg SAZ. The MNs' frequencies (MPCE and MNCE) were 2-fold lower in the 120 mg SAZ + irradiated group than in the irradiated control. A similar effect was seen in increasing the P/N ratio, with 120 mg/kg producing the maximum effect (Table III, Figure 1C). Although pretreatment with 180 mg/kg produced a significant decrease in the number of MPCE and MNCE compared to the 4 Gy alone, the values were higher than those in the radiation +120 mg/kg group. Hence, dose of 120 mg/kg (SAZ) was chosen as the optimum dose for the subsequent experiments.

Selection of optimum time

Treatment with SAZ before or after RT resulted in a significant decrease in the percent aberrant metaphases. But the extent of protection varied with the time of drug administration. Drug treatment 30 min before RT produced the maximum decrease in percent aberrant cells (Table IV), MPCE and MNCE (Table V). This treatment also resulted in a significant decrease in all types of aberrations, as well as the polyploids and SDC (Table IV) and in a maximum increase in P/N ratio (Table V). The protection decreased as the time interval was increased from 30 to 60 min. However, pretreatment at 60 or 30 min was found to be more effective than 15 min post-radiation in reducing percent aberrant cells and the frequency of MPCE and MNCE. Based on these results, drug treatment 30 min before irradiation proved to be the optimum time of SAZ administration.

Plasmid DNA studies

Radicals generated by Fenton's reaction are known to cause oxidatively induced DNA strand breaks. Radical scavengers can protect DNA from such strand breaks. Hence, employing *in vitro* DNA nicking assay, we assessed the ability of SAZ to

Table I. Acute toxicity (24-h, 72-h and 30-day mortality) of SAZ in Swiss albino mice

Drug dose (mg/kg)	Log dose	24-h mortality		72-h/30-day mortality	
		Death (%)	Probit value	Death (%)	Probit value
400	2.6	0	0	0	3[a]
600	2.8	0	3[a]	20	4.10
800	2.9	60	5.25	80	5.80
1000	3.0	80	5.80	100	6.96[a]
1200	3.1	100	6.96[a]	100	6.96[a]

[a]Corrected formula for 0% dead: $100 \times (0.25/n)$; for the 100% dead: $100 \times [(n - 0.25)/n]$, $n = 10$. LD_{50} (24 h): 933 mg/kg; LD_{10} (72 h/30 days): 500 mg/kg; LD_{50} (72 h/30 days): 676 mg/kg.

Table II. Effect of various doses of SAZ on the induction of chromosomal aberrations in mouse bone marrow by whole body gamma - irradiation (4 Gy)

		Aberrations/100 cells					
Treatment SAZ (mg/kg)	Aberrant cells (%)	Chromatid breaks	Chromosome breaks	Fragments	Rings + Dicentrics	Polyploidy	SDC
RT (4 Gy)	53.3 ± 1.1	7.10 ± 0.48	1.80 ± 0.14	86.4 ± 0.8	3.90 ± 0.14	1.76 ± 0.12	7.16 ± 0.38
SAZ 30 + RT	47.8 ± 0.8[a]	6.04 ± 0.08	1.44 ± 0.06	77.6 ± 3.0	3.38 ± 0.24	1.64 ± 0.08	5.66 ± 0.08
SAZ 60 + RT	39.4 ± 1.3[c,f]	4.48 ± 0.30[c,e]	1.02 ± 0.04[c,e]	58.5 ± 2.3[c,f]	2.66 ± 0.06[c,d]	1.12 ± 0.10[a,e]	4.52 ± 0.06[b]
SAZ 120 + RT	28.6 ± 1.2[c,f,i]	3.16 ± 0.18[c,f,g]	0.72 ± 0.04[c,f]	37.5 ± 2.1[c,f,i]	1.80 ± 0.08[c,f,h]	0.70 ± 0.02[c,f,g]	2.42 ± 0.14[c,f,i]
SAZ 180 + RT	37.3 ± 0.1[c,f]	3.52 ± 0.40[c,f,g]	0.90 ± 0.08[c,e]	64.3 ± 1.7[c,e]	2.48 ± 0.08[c,d]	0.78 ± 0.16[c,f,g]	3.34 ± 0.04[c,e]
			SAZ alone (Sham-radiation)				
0 (1% CMC)	0.72 ± 0.08	0	0	1.28 ± 0.08	0	0	0
30	0.60 ± 0.06	0	0	1.22 ± 0.04	0	0	0
60	0.78 ± 0.02	0	0	1.34 ± 0.10	0	0	0
120	0.84 ± 0.12	0.06 ± 0.02	0	1.82 ± 0.10	0	0	0
180	3.28 ± 0.16[j]	0.28 ± 0.04[j]	0.14 ± 0.00	5.66 ± 0.28[j]	0	0	0

Sulfasalazine (SAZ) was administered 30 min before exposure to 4 Gy gamma-irradiation (RT). All the values are mean ± SE (n = 5).
[a]$P < 0.05$ compared to RT alone.
[b]$P < 0.01$ compared to RT alone.
[c]$P < 0.001$ compared to RT alone.
[d]$P < 0.05$ compared to RT + 30.
[e]$P < 0.01$ compared to RT + 30.
[f]$P < 0.001$ compared to RT + 30.
[g]$P < 0.05$ compared to RT + 60.
[h]$P < 0.01$ compared to RT + 60.
[i]$P < 0.001$ compared to RT + 60.
[j]$P < 0.001$ compared to 1% CMC alone.

protect plasmid DNA (pGEM-7Zf) from Fenton's reaction-induced damage. The hydroxyl radical generated by Fenton's reaction attack DNA guanosine residues, resulting in strand breakage and transformation from native supercoiled DNA (Form I) to nicked circular form (Form II). The radicals generated by Fenton's reaction totally disintegrated the DNA (Figure 2, lane 2). SAZ did not protect the plasmid in the native supercoiled form but protected DNA in the nicked circular form in a dose-dependent manner, without allowing complete degradation (Figure 2, lanes 6,7,8). These results confirm that free radical scavenging ability of SAZ might contribute for the protection of the DNA.

Discussion

Radiation produced a significant increase in the percent aberrant metaphases as well as in the number of different aberrations. Damage to the chromosomes is manifested as breaks and fragments, which appear as MNs in the rapidly proliferating cells (37). The increase in MNs observed in the RT-alone group thus confirms the radiation clastogenicity. Enhancement in the frequency of MNs and chromosomal aberrations has been reported earlier in the bone marrow of irradiated mice (38–45), which is supported by the present data. Pretreatment with all doses of SAZ decreased the RT-induced chromosomal aberrations as well as MNs, demonstrating that the drug protected against the clastogenic effect of radiation. Even though the protection increased with the drug dose above 30 mg/kg, the optimum dose that provided maximum protection without accompanying drug toxicity was 120 mg/kg, which is about one-sixth of its LD_{50} (676 mg/kg). At this dose, aberrant metaphases and micronucleated erythrocytes were reduced to 50–60% of the irradiated control values. The P/N ratio is an indicator of the rate of proliferation, and a decrease in the ratio at 24 h post-irradiation is an expression of the known early effects of radiation on cell cycle (38,44,45), indicating a suppression of erythropoiesis. There was a significant increase in the P/N ratio, suggesting that pretreatment of mice with SAZ protects against RT-induced cell cycle progression delay.

In our earlier study (46), we have observed that there is an optimum dose for protection by 5-ASA, which is a breakdown product of SAZ. An increase in dose above the optimum was accompanied by a decrease in radioprotective effect. A similar nonlinear response was observed with several hydroxyl radical scavengers by Grzelinska *et al*. (47). The protection against RT-induced changes, namely rigidity of erythrocyte membrane lipids, lipid peroxidation and the state of membrane proteins were maximum at lower concentrations, but decreased at higher concentrations of scavengers. A similar phenomenon may be applicable to SAZ, which itself is a very potent hydroxyl radical scavenger (48). Further, several studies suggest that SAZ can cause chromosomal damage and MNs' formation (49,50). We observed similar results in our study where we observed increased MNs and chromosomal aberrations in SAZ 180 mg/kg alone treated group. Hence, our data suggest that there is an optimum dose (120 mg/kg) beyond which the radioprotective activity decreases and the decrease may be a result of the contribution from the toxic effects of SAZ.

Cytogenetic studies showed that pretreatment with SAZ was highly effective in reducing both simple and complex chromosomal aberrations, indicating significant protection against single-strand breaks and double-strand breaks in the DNA. Simple chromosomal aberrations like breaks and fragments arise from unrepaired single-strand breaks while complex aberrations like rings and dicentrics require a more complex interaction, which involves rejoining of double-strand breaks in an error-prone manner (51). To further confirm the ability of SAZ to protect DNA, we employed *in vitro* DNA nicking assay where we used Fenton's reaction to produce the hydroxyl radicals. SAZ protected plasmid DNA (pGEM-7Zf) from Fenton's reaction-induced damage and the protection was concentration dependent (Figure 2) confirming the direct

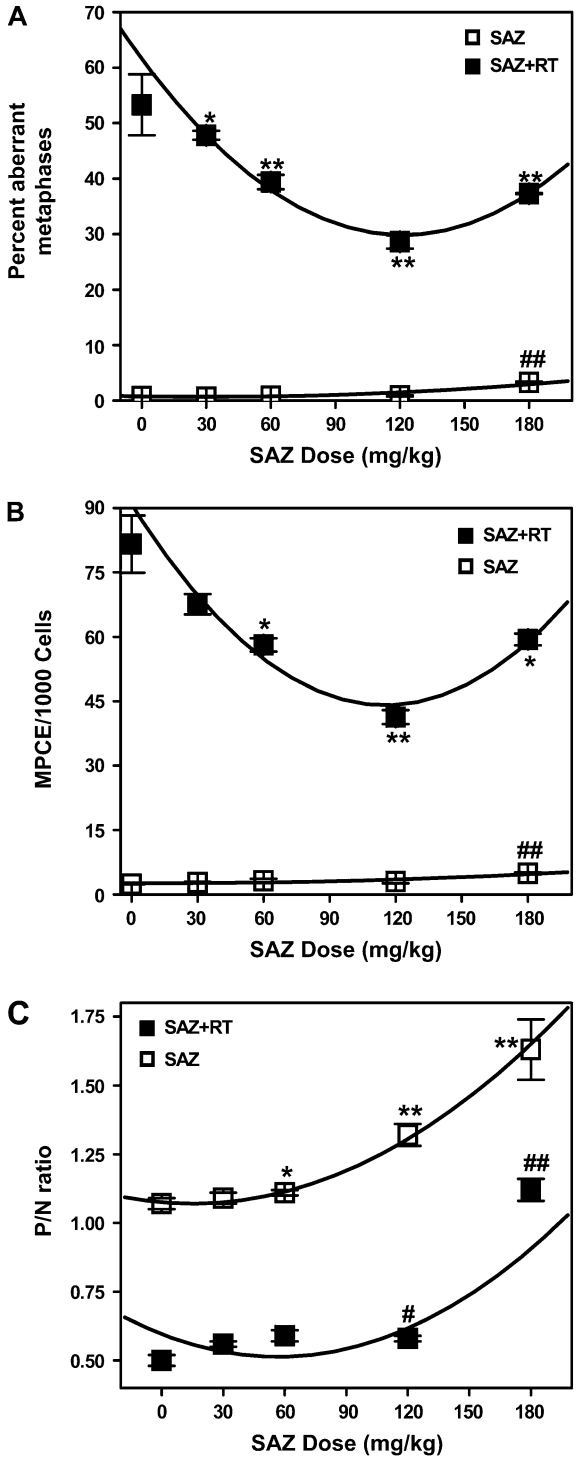

Fig. 1. Effect of different doses of SAZ (30–180 mg/kg b.wt.) on the percent aberrant metaphases; MPCE and P/N ratio in mouse bone marrow at 24 h after exposure to whole-body gamma and sham irradiation. Panel (**A**), percent aberrant cells metaphases; panel (**B**), number of MPCE per 1000 cells; panel (**C**), P/N ratio. (■) Gamma irradiation (RT) groups and (□) sham irradiation groups. Values are mean ± SE ($n = 5$). Vertical bars are 95% confidence intervals of mean. *$P < 0.05$, **$P < 0.001$ compared to RT alone; #$P < 0.05$, ##$P < 0.001$ compared to 1% CMC alone.

radical scavenging activity of SAZ. Several investigators have demonstrated the ability of free radical scavengers to protect cellular DNA against a significant proportion of indirect effects of ionizing radiation, where hydroxyl radicals are believed to be the primary active species responsible for the damage (52–54). Shimoi *et al.* (55) attributed the radioprotective effect of antioxidant plant flavonoids to their ability to scavenge free radicals. They concluded that the flavonoids play a role in scavenging free radicals such as hydroxyl radicals induced by RT. Free radical scavengers like chlorogenic acid, beta-carotene, vitamin C and E have been shown to have radioprotective effect against RT-induced MNs' induction in mice (56,57) and lymphocytes (58). In our earlier study, we have shown that SAZ to be effective against not only the chain-initiating and chain-propagating radicals in the lipid phase, but also against radicals generated in the aqueous phase (48). Hence, the free radical scavenging activity of SAZ appears to have a role in the protective effect against RT-induced clastogenicity.

The efficacy of any compound to act as an antioxidant is known to depend not only on its local concentration at the site of radical generation but also on its reaction rate constant with the free radicals. Our earlier studies on the kinetics of reaction of SAZ with various free radicals by pulse radiolysis (48) showed a relatively high rate constant for hydroxyl radical reaction with SAZ (6.7×10^9/M/sec) that was comparable with that of other well-known antioxidants. This rate is much faster than the rate of reaction hydroxyl radicals with DNA (rate constant—3 to 6×10^8/M/sec) (59), thus suggesting that SAZ will function as effective radical scavenger and protect DNA if present at the crucial sites at the time when the radicals are formed. This could explain our finding from the cytogenetic studies that shows protection by the SAZ is maximum when administered prior to RT. The protective effect of SAZ extended from 60 min before to 15 min after RT. But the maximum protection was obtained when the drug was administered 30 min prior to irradiation. This suggests that the drug must be present in the system at the time of irradiation. Many flavonoids and thiols are reported to be good protectors when they are present in the system at the time of RT (60,61). Michalowski (62) suggested that the prostaglandin inhibitors would be more effective if given before the cascade of oxidative stress is started by radiation insult rather than after the cascade is already established. Since SAZ is known to block prostaglandins, it can be used as a protector when the eventuality of the radiation exposure is known and it may not be suitable for unplanned exposures, e.g. accidents, spillage, etc.

Though SAZ gave maximum protection when administered prior to RT, it did show significant radiation protection when administered 15 min post-irradiation, although the effect is much less pronounced than that observed when animals are pretreated with SAZ. Therefore, the response of SAZ might be similar to drugs such as the 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol (water-soluble analog of vitamin E), ascorbic acid and caffeine that are reported to be efficient radioprotectors both when administered pre- and post-irradiation (57,63,64). While free radical scavengers are effective in situations where the treatment compounds are present in the system during exposure to clastogenic agents (pre-irradiation treatments), they are ineffective when administered post-irradiation. Hence, we associate the reduction in chromosomal damage with SAZ treatment post-irradiation to an enhanced immune response or enhanced recovery of bone marrow. However, there is need to undertake further studies to elucidate the possible mechanisms.

Our results indicate the beneficial effects of SAZ against RT-induced genotoxicity in mouse bone marrow. Kilic *et al.* (20)

Table III. Effect of various doses of SAZ on the induction of micronuclei in mouse bone marrow by whole body gamma - irradiation (4 Gy)

Treatment SAZ (mg/kg)	MPCE/1000 cells				MNCE/1000 cells	P/N ratio
	One	Two	Three	Total		
RT (4 Gy)	64.6 ± 4.47	6.0 ± 0.92	1.7 ± 0.13	81.6 ± 6.68	6.2 ± 0.12	0.50 ± 0.02
SAZ 30 + RT	57.8 ± 3.09	3.8 ± 0.57	0.8 ± 0.24	67.6 ± 2.34	4.7 ± 0.32[b]	0.56 ± 0.01
SAZ 60 + RT	50.9 ± 1.94[a]	3.1 ± 0.24[a]	0.3 ± 0.19[c]	58.1 ± 1.57[a]	2.5 ± 0.24[c]	0.59 ± 0.02[a]
SAZ 120 + RT	36.6 ± 1.26[c]	2.1 ± 0.16[c]	0.1 ± 0.12[c]	41.1 ± 1.62[c]	2.1 ± 0.26[c]	0.78 ± 0.01[c]
SAZ 180 + RT	51.4 ± 2.14[a]	3.4 ± 0.09[a]	0.4 ± 0.33[b]	59.4 ± 1.36[a]	3.4 ± 0.30[c]	1.12 ± 0.04[c]
			SAZ alone (Sham-radiation)			
0 (1% CMC)	2.4 ± 0.02	0	0	2.4 ± 0.02	0.3 ± 0.01	1.07 ± 0.02
30	2.8 ± 0.15	0	0	2.8 ± 0.15	0.2 ± 0.02	1.09 ± 0.02
60	3.2 ± 0.46	0	0	3.2 ± 0.46	0.3 ± 0.03	1.11 ± 0.01
120	3.0 ± 0.39	0	0	3.0 ± 0.39	0.4 ± 0.12	1.32 ± 0.04[d]
180	4.9 ± 0.12[e]	0	0	4.9 ± 0.12[e]	0.9 ± 0.24[d]	1.63 ± 0.11[e]

Sulfasalazine (SAZ) was administered 30 min before exposure gamma-irradiation (RT). All values are mean ± SE (n = 5).
[a]$P < 0.05$ compared to RT alone.
[b]$P < 0.01$ compared to RT alone.
[c]$P < 0.001$ compared to RT alone.
[d]$P < 0.05$ compared to 1% CMC alone.
[e]$P < 0.001$ compared to 1% CMC alone.

Table IV. Effect of 120 mg/kg SAZ administered at different intervals on the induction of chromosomal aberrations in mouse bone marrow by whole body gamma - irradiation (4 Gy)

Treatment	Time Pre-/Post–RT (min)	Aberrant cells (%)	Aberrations/100 cells					
			Chromatid breaks	Chromosome breaks	Fragments	Rings +Dicentrics	Polyploidy	SDC
RT (4 Gy)		53.3 ± 1.1	7.10 ± 0.48	1.80 ± 0.14	86.4 ± 0.8	3.90 ± 0.14	1.76 ± 0.12	7.16 ± 0.38
SAZ + RT	− 60	36.4 ± 0.9[b]	4.80 ± 0.18[b]	1.02 ± 0.06[b]	54.7 ± 3.3[b]	2.64 ± 0.10[b]	1.12 ± 0.06[b]	3.68 ± 0.12[b]
SAZ + RT	− 30	28.6 ± 1.2[b,e]	3.16 ± 0.16[b,d]	0.72 ± 0.04[b]	37.5 ± 2.1[b,e]	1.80 ± 0.08[b,e]	0.70 ± 0.04[c,d]	2.42 ± 0.14[b,d]
RT + SAZ	+ 15	39.5 ± 1.3[b,g]	5.48 ± 0.20[a,g]	0.84 ± 0.08[b]	64.8 ± 2.6[b,c,g]	3.24 ± 0.06[a,d,g]	1.24 ± 0.08[a,f]	4.84 ± 0.04[b,d,g]
SAZ alone		1.24 ± 0.12	0.16 ± 0.01	0.04 ± 0.02	1.82 ± 0.04	0	0	0
1% CMC (Control)		0.72 ± 0.08	0.08 ± 0.06	0	1.28 ± 0.08	0	0	0

(−), Pre-RT; (+), post-RT (+). All values are mean ± SE (n = 5).
[a]$P < 0.05$ compared to RT alone.
[b]$P < 0.001$ compared to RT alone.
[c]$P < 0.05$ compared to SAZ (60 min pre) + RT.
[d]$P < 0.01$ compared to SAZ (60 min pre) + RT.
[e]$P < 0.001$ compared to SAZ (60 min pre) + RT.
[f]$P < 0.01$ compared to SAZ (30 min pre) + RT.
[g]$P < 0.001$ compared to SAZ (30 min pre) + RT.

Table V. Effect of 120 mg/kg SAZ administered at different time intervals on the induction of micronuclei in mouse bone marrow by whole body gamma - irradiation (4 Gy)

Treatment	Time Pre-/ Post-RT (min)	MPCE/ 1000 cells				MNCE/1000 cells	P/N ratio
		One	Two	Three	Total		
RT (4 Gy)		64.6 ± 4.47	6.0 ± 0.92	1.7 ± 0.13	81.6 ± 6.68	6.2 ± 0.12	0.50 ± 0.02
SAZ + RT	− 60	46.1 ± 1.05[c]	2.2 ± 0.23[c]	0.3 ± 0.13[c]	51.7 ± 1.08[c]	3.8 ± 0.34[c]	0.61 ± 0.01[a]
SAZ + RT	− 30	36.6 ± 1.26[c,d]	2.1 ± 0.16[c]	0.1 ± 0.12[c]	41.1 ± 1.62[c]	2.1 ± 0.26[c,e]	0.78 ± 0.01[c,f]
RT + SAZ	+ 15	54.5 ± 0.89[a,h]	3.5 ± 0.33[a]	0.6 ± 0.14[c,g]	63.8 ± 0.75[a,g]	4.8 ± 0.29[b,h]	0.56 ± 0.03[h]
SAZ alone		3.0 ± 0.39	0.00	0.00	3.0 ± 0.39	0.4 ± 0.12	1.32 ± 0.04
1% CMC (control)		2.4 ± 0.02	0.00	0.00	2.4 ± 0.02	0.3 ± 0.01	1.07 ± 0.02

(−), Pre-RT; (+), post-RT (+). All values are mean ± SE (n = 5).
[a]$P < 0.05$ compared to RT alone.
[b]$P < 0.01$ compared to RT alone.
[c]$P < 0.001$ compared to RT alone.
[d]$P < 0.05$ compared to SAZ (60 min pre) + RT.
[e]$P < 0.01$ compared to SAZ (60 min pre) + RT.
[f]$P < 0.001$ compared to SAZ (60 min pre) + RT.
[g]$P < 0.01$ compared to SAZ (30 min pre) + RT.
[h]$P < 0.001$ compared to SAZ (30 min pre) + RT.

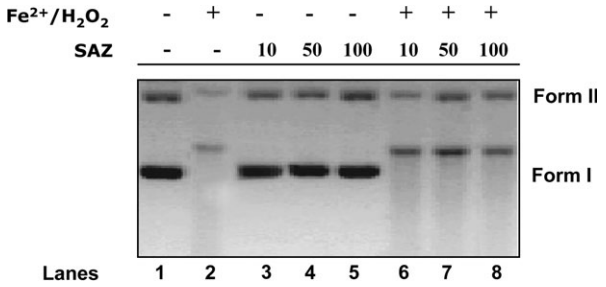

Fig. 2. Agarose gel electrophoresis pattern (reverse photonegative) depicting Forms I and II of DNA. Protection by SAZ against Fenton's reaction-induced strand breaks in plasmid (pGEM-7Zf) DNA is shown as reduction of Form II. Lane 1, pGEM-7Zf control; lane 2, DNA exposed to Fenton's reaction products; lane 3, 4 and 5, SAZ alone at the concentration of 10, 50 and 100 μM (drug control); lane 6, 7 and 8, SAZ at concentration of 10, 50 and 100 μM before addition of Fenton's reaction products, respectively. Form I = supercoiled DNA and Form II = nicked circular DNA.

reported that SAZ when administered orally was found to be effective in decreasing the symptoms of acute radiation enteritis and radiation-induced acute gastrointestinal toxicities. But on oral administration, only about 30% of SAZ is absorbed in its unaltered form, whereas the rest is subjected to degradation by colonic bacteria and azo reduction into SP and 5-ASA (5). In the present study, SAZ was administered by i.p. route, an approach used by several investigators to avoid the degradation of SAZ in the gut (65–67). SAZ when administered by oral route, along with 5-ASA, the 30% SAZ absorbed into systemic circulation on oral administration might contribute for the protection against radiation toxicity. So the ideal strategy for oral administration would be to increase the dose of SAZ, but this will also result in an increase in the 5-ASA concentrations *in vivo* that may not be very favorable, owing to the prooxidant properties of 5-ASA (68). Our earlier study showed that 5-ASA is effective against radiation-induced bone marrow cytotoxicity in mice exposed to gamma radiation, but the selection of dose is crucial as there is an optimum protective dose for 5-ASA and at doses above this there was a decrease in the protection owing to the pro-oxidant activity of 5-ASA (46). Therefore, parenteral route of administration might be the ideal route for a clinical study as the drug directly enters the systemic circulation. Alternatively, the degradation of SAZ can also be substantially reduced by using oral administration of SAZ in combination with antibiotics. However, if SAZ is to be used in cancer therapy, it is to be seen if it gives preferential protection to normal tissue without significant tumor protection. Further studies are warranted to exploit the clinical advantage of this compound.

Funding

Council of Scientific and Industrial Research, Government of India (1(11)131-2K3/1 to S.K.M. and 27/102/2000/EMR-II to M.K.U.).

Acknowledgements

The authors are grateful to Prof. M. S. Vidyasagar and Prof. J. G. R. Solomon of S.S.B. Cancer Hospital, Manipal, for providing the irradiation facility and help in dosimetry.

Conflict of interest statement: None declared.

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Received on June 12, 2007; revised on September 12, 2007; accepted on January 7, 2008