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Original article

Akt1/NFκB signaling pathway activation by a small molecule DMA confers radioprotection to intestinal epithelium in xenograft model

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ABSTRACT

Normal tissue protection and recovery of radiation-induced damage are of paramount importance for development of radioprotector. Radioprotector which selectively protects normal tissues over cancerous tissues improves the therapeutic window of radiation therapy. In the present study, small bisbenzimidazole molecule, DMA (5-(4-methylpiperazin-1-yl)-2-[2′-(3,4-dimethoxy-phenyl)-5′-benzimidazolyl]-benzimidazole) was evaluated for *in vivo* radioprotective effects to selectively protect normal tissue over tumor with underlying molecular mechanism. Administration of single DMA dose prior to radiation has enhanced survival of Balb/c mice against sublethal and supralethal total body irradiation. DMA ameliorated radiation-induced damage of normal tissues such as hematopoietic (HP) and gastrointestinal tract (GI) system. Oxidative stress marker Malondialdehyde level was decreased by DMA whereas it maintained endogenous antioxidant status by increasing the level of reduced glutathione, glutathione reductase, glutathione-s-transferase, superoxide dismutase and total thiol content in hepatic tissue of irradiated mice. Mechanistic studies revealed that DMA treatment prior to radiation leads to Akt1/NFκB signaling which reduced radiation-induced genomic instability in normal cells. However, these pathways were not activated in tumor tissues when subjected to DMA treatment in similar conditions. Abrogation of Akt1 and NFκB genes resulted in no radioprotection by DMA and enhanced apoptosis against radiation. Plasma half-life of DMA was 3.5 h and 2.65 h at oral and intravenous dose respectively and 90% clearance was observed in 16 h. In conclusion, these data suggests that DMA has potential to be developed as a safe radioprotective agent for radiation countermeasures and an adjuvant in cancer therapy.

1. Introduction

Radiotherapy targets cancerous cells during treatment but it has deleterious effects on surrounding normal tissues. Similarly, injury to hematopoietic (HP)/gastrointestinal tract (GI) system is major factor for acute radiation syndrome (ARS) associated death in organisms exposed to radiation [1,2]. Apart from radiotherapy, there are chances of radiation accidents such as Fukushima, Japan (2011), Tokaimura, Japan (1999), Goiânia, Brazil (1988), Chernobyl, Russia (1988), and Three Mile Island nuclear power station, United States (1979). Military and first responder which are employed to these fatal accidents areas for search-and-rescue purposes would also require radioprotectors as radiation countermeasures [3,4]. Thus, it is imperative to develop safe radioprotector for radiation countermeasures. Amifostine is only clinically approved radioprotector used for cancer patients undergoing radiotherapy [5]. It has dose limited toxicity and cause serious side effects such as nausea, vomiting and hypotension at its maximum effective doses [6]. Recently developed CBLB502 had shown an excellent radioprotection to healthy cells over cancerous cells [7]. CBLB502 has half-life < 20 min and will require repeated dosing for desired clinical effect by *i.v.* route of administration [8] whereas other radioprotectors such as methylproamine, PrC-210, ON01210/Ex-RAD® [9] and 3,3′-Diselenodipropionic acid (DSePA) [10] are at various stages of development.

Akt1 is known for cellular protection against ionizing radiation (IR)-induced apoptosis in germ cells [11]. Prostaglandin treatment reduces apoptosis of epithelial by Akt activation [12]. In response to IR stimuli, Nuclear factor-kappa binding (NFκB), a transcription factor, activates its downstream target genes which regulates cell survival, cellular proliferation and apoptosis [13,14]. Earlier studies suggested that radioprotectors like ON01210 and CBLB502 provides radioprotection by activation of PI3K/Akt [15] and NFκB [7] signaling pathway

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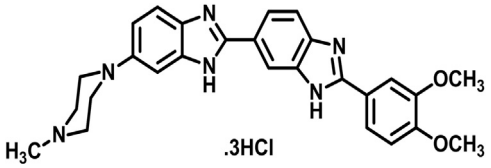

Fig. 1. Structure of DMA 3HCl.

respectively. DMA (Fig. 1), a bis-benzimidazole derivatives, has been shown as non-toxic, free radical scavenging radioprotector [16–18]. It has effective radioprotection at 1/7th dose of its maximum tolerable dose (MTD) of 2000 mg/kg [19]. DMA induces NIK mediated NFκB activation and modulates number of key regulatory pathways to overcome radiation-induced damage *in vitro* [18,20]. Here we have deciphered the molecular mechanism of DMA as a radioprotector in normal and tumor bearing Balb/c mice against total body irradiation (TBI). Single 200 mg/kg oral and 50 mg/kg intravenous (*i.v.*) DMA dose augments 80% and 100% survival respectively at 8 Gy radiation through activation of Akt/NFκB pathway, improves HP and GI conditions and maintains redox balance *in vivo*.

2. Materials and methods

2.1. Animals, cell lines and treatment conditions

Balb/c mice (25 ± 5 g) were obtained from National Institute of Nutrition, Hyderabad and experiments were conducted as per recommendation of Committee for the purpose of control and supervision of experiments on animals (CPCSEA), India and ARRIVE guidelines [19]. HEK293 cell line was obtained from National Centre for Cell Science, Pune, India whereas MRC5 cell line was kind gift from Prof. George Iliakis, University of Duisburg-Essen, Germany. Four experimental conditions were designed in all *in vitro* and *in vivo* experiments: control (untreated), DMA (50 μM for cells and indicated dose for mice), radiation (5 Gy for HEK293, 6 Gy for MRC5 and TBI in mice) and DMA + radiation (50 μM DMA + 5 Gy for HEK293, 6 Gy for MRC5 and indicated DMA + radiation dose for mice).

In all *in vitro* and *in vivo* experiments, DMA treatment at indicated dose was given 2 h before irradiation. Cells and mice were exposed to γ-irradiation using Co^{60} source (INMAS, Delhi, India) at 1.836 Gy/min.

2.2. Uptake and efflux of DMA

HEK293 cells (10^6cells/ml) were seeded and treated with complete media containing 50 μM DMA for 2 h. After 2 h incubation, cells were collected by centrifugation, resuspended in cold phosphate-buffer saline for subsequent flow cytometry analysis.

2.3. In vivo radioprotection by DMA

2.3.1. Balb/c mice survival by different mode of DMA administration against radiation

Mice were grouped into 4 groups each containing 10 animals depending upon the mode of DMA administration as indicated. Group 1, sham control (saline treated); Group 2, DMA treated {200 mg/kg by oral, 50 mg/kg by *i.v., i.p.* & *s.c.*}; Group 3, radiation (saline treated followed by radiation, TBI); Group 4, treated with DMA{200 mg/kg by oral, 50 mg/kg by *i.v.*, *i.p.* & *s.c.*} 2 h prior to radiation, TBI. Body weight, radiation sickness and mortality in animals were noted for 30 days. $LD_{50/30}$ (lethal radiation dose causing 50% mortality in 30 days) for radiation and DMA + radiation treated mice were calculated from these studies.

Dose reduction factor (DRF): 4 groups were created containing 10 mice each. Group 1, control; Group 2, DMA treated (200 mg/kg bw); Group 3, radiation control (5, 6, 8, 9 & 10 Gy TBI); Group 4, treated with optimum dose of DMA (200 mg/kg bw) prior to whole body exposure to 5, 6, 8, 9 & 10 Gy. Body weight, radiation sickness and mortality in animals were noted for 30 days. Ratio of $LD_{50/30}$ mice treated with both DMA and radiation to radiation was calculated as DRF.

2.3.2. Nude mice survival

4 groups of mice containing 5 animals each were created. Group 1, sham control; Group 2, DMA treated (50 mg/kg, *i.p.*); Group 3, TBI 7 Gy; Group 4, treated with DMA (50 mg/kg *i.p.*) prior to 7 Gy TBI. Body weight, radiation sickness and mortality in animals were noted for 30 days. Survival study was carried out for a period of 30 days.

2.3.3. Tumor xenograft generation

Melanoma model: 0.8×10^6 B16F10 cells were injected subcutaneously in the right flank of Balb/c mice (n = 24). When tumor volume reached 0.5 cm^3, mice were irradiated at 8 Gy as reported earlier [19].

2.3.4. Immunohistochemistry (IHC) of cell proliferation and IL-6 in tissue

Spleen cell proliferation was measured 3 days after irradiation using 5-bromo-2′-deoxyuridine (BrdU) (*i.p.*, 100 mg/kg) as labeling agent in each mouse 2 h before euthanasia. IL-6 expression was checked in small intestine through IHC as described previously [7].

2.3.5. Endogenous spleen colony forming assay

Group 1, sham control (normal saline treated); Group 2, DMA treated (50 mg/kg bw, *i.v.*); Group 3, radiation control (8 Gy TBI); Group 4, treated with DMA (50 mg/kg bw, *i.v.*) prior to whole body exposure to 8 Gy. Mice (6 animals each) were irradiated, 2 h post-administration of DMA. The mice were sacrificed on day 10 and spleens were recovered, cleaned for blood and weighed. [spleen index = (spleen weight/body weight) × 100] formula was used to calculate spleen index. Subsequently Bouin's fixative was used to fix spleens for 15 min and the number of macroscopic spleen cell colonies was counted manually [21].

2.3.6. Biochemical estimations for antioxidant enzymes and total protein in liver

Mice hepatic tissues were homogenised using REMI homogenizer in phosphate buffer and centrifuged at 10,000 × *g* for 15 min and aliquots of supernatant were separated. The supernatant was used for the biochemical estimations using standard spectrophotometric reported methods. ~100 mg of hepatic homogenate was mixed with 10 ml of 10% TCA and placed at 90 °C in a water bath for 30 min with stirring. Filtrate was dissolved with gentle warming in 0.1 mol/l NaOH. The total protein was determined by the Lowry method.

2.3.7. Total thiols estimation, lipid peroxidation

Briefly, 200 μl hepatic homogenate was mixed with phosphate buffer (pH 8.0), 40 μl of 10 mM DTNB and 3.16 ml of methanol. After 10 min incubation, absorbance was measured at 412 nm and total thiol content was calculated by standard method [22]. The amount of malondialdehyde (MDA) was done by reaction with thiobarbituric acid (TBA) at 532 nm by literature method [23].

2.3.8. Estimation of reduced glutathione

Glutathione was measured according to the Ellman's method [24].

2.3.9. Superoxide dismutase (SOD), Glutathione-S-transferase (GST) and Glutathione reductase (GR) activity

Superoxide dismutase (SOD) activity was assayed according to the Marklund and Marklund method [25]. CDNB was used as substrate to determine GST activity. The reaction mixture contained 1 mM of CDNB, 1 mM GSH in 0.1 M phosphate buffer (pH 6.5). GSH-CDNB conjugate formation was estimated at 340 nm and the activity was calculated by using $\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$ [26]. NADPH oxidation rate by GSSH was