Preclinical Evaluation of DMA, a Bisbenzimidazole, as Radioprotector: Toxicity, Pharmacokinetics, and Biodistribution Studies in Balb/c Mice

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ABSTRACT

Radiotherapy, a therapeutic modality of cancer treatment, non-selectively damages normal tissues as well as tumor tissues. The search is ongoing for therapeutic agents that selectively reduce radiation-induced normal tissue injury without reducing tumoricidal effect, thereby increasing the therapeutic ratio of radiation therapy. Our laboratory established 5-(4-methylpiperazin-1-yl)-2-[2’-(3,4-dimethoxyphenyl)-5’ benzimidazolyl] benzimidazole (DMA) as noncytotoxic radioprotector in mammalian cells. DMA showed an excellent radioprotection in mice at single nontoxic oral dose by a dose-reduction factor of 1.28. An oxygen radical absorbing capacity assay confirmed its free-radical quenching ability. Single bolus dose and 28-days of repeated administration of DMA in mice for toxicity studies determined an LD$_{50}$ of >2000 mg/kg body weight (bw) and 225 mg/kg bw, respectively, suggesting DMA is safe. Histopathology, biochemical parameters, and relative organ weight analysis revealed insignificant changes in the DMA-treated animals. The pharmacokinetic study of DMA at oral and intravenous doses showed its $C_{max}$ study of 1 hour, bioavailability of 8.84%, elimination half-life of 4 hours, and an enterohepatic recirculation. Biodistribution study in mice with Ehrlich ascites tumors showed that $^{99m}$Tc-DMA achieved its highest concentration in 1 hour and was retained up to 4 hours in the lungs, liver, kidneys, and spleen, and in a low concentration in the tumor, a solicited property of any radioprotector to protect normal cells over cancerous cells. We observed that the single-dose treatment of tumor-bearing mice with DMA 2 hours before 8 Gy total body irradiation showed an impressive rescue of radiation-induced morbidity in terms of weight loss and mortality without a change in tumor response.

Introduction

Radiotherapy, second only to surgery, is used with 80% of patients as one of the most effective modalities for the treatment and cure of neoplastic diseases (Nair et al., 2001; Parihar et al., 2007; Satyamitra et al., 2012). Ionizing radiation triggers a series of events starting from generation of reactive oxygen species (ROS). These ROS have a deleterious effect on macromolecules, living tissues, or cells, resulting in the transfer of radiation energy to biologic materials (Kalpana et al., 2011). Radioprotectors protect normal cells from radiation-induced damage. Radioprotective mechanistic approaches include damage protection by inhibiting free-radical generation or the hastening of free-radical scavenging, DNA and membrane repair enhancement, reconstruction of hematopoietic function, and stimulation of immune cell activity (Wang et al., 2013).

Through the adoption of conformal treatment techniques such as intensity-modulated radiation therapy and image-guided radiation therapy, the amount of radiation delivered to...
normal tissues surrounding the targeted tumor has been greatly reduced, but the irradiated normal tissue toxicity limits the dose that can be administered and contributes to treatment-related morbidity and thus decreased quality of life. Hence, normal tissue injury continues to be a limiting factor for radiation oncologists.

Several decades of preclinical and clinical research efforts have explored compounds of either natural or synthetic origin to investigate their effects in biologic systems to protect against radiation-induced damage. However, to date the only available prophylactic pharmacologic agent that has been approved by the U.S. Food and Drug Administration as a radioprotector is amifostine (Weiss and Landauer, 2009). Although amifostine and other compounds have shown good prophylactic effects, these compounds are limited to subcutaneous or intravenous routes of administration and are associated with in vivo toxicity (Bonner and Shaw, 2002) such as nausea, vomiting, and hypotension.

The continuous therapeutic need necessitates development of potent radioprotectors that can selectively protect normal tissues over tumor tissues with relevant tissue uptake and retention. Among the orally administered radioprotectants under investigation are melatonin, which has acute toxicity; genistein, which requires administration by a multiple-dosing regimen several days before irradiation; and 5-androstene-3β,17β-diol, which requires similarly high doses (1600 mg/kg) to provide significant survival advantage after radiation exposure (Whitnall et al., 2002; Landauer et al., 2003; Yildiz et al., 2006). Recently 3,3′-diindolylmethane, a natural product, has been shown to confer protection against ionizing radiation by stimulation of ataxia telangiectasia mutated signaling (Fan et al., 2013). Recently a Toll-like receptor 5 (TLR5) agonist (Burdelya et al., 2008) and TLR3 inhibitor (Takemura et al., 2014) have been shown to effectively ameliorate crypt cell death and gastrointestinal syndrome in animals.

The 2-(4-ethoxyphenyl)-6-[(4-methylpiperazin-1-yl)-1H-benzimidazol-2-yl]-1H-benzimidazole (Hoechst 33342) analog DMA (5-(4-methylpiperazin-1-yl)-2-[2′,3′,4′-trimethoxyphenyl]-5-benzimidazolyl[benzimidazole]), a DNA minor groove binder that binds preferentially to the AT-rich sequences, was synthesized and established as radioprotective in laboratory in vitro conditions. We have proved that DMA acts mechanistically as a radioprotector by reducing DNA damage and free-radical scavenging in mammalian cells. DMA was observed to be nonmutagenic and noncytotoxic to mammalian cells (BMG-1) in comparison with the parent analog (Tawar et al., 2003, 2007). It showed inhibitory action at micromolar concentrations in a human epithelial cancer cell line (HeLa), a human breast cancer cell line (MCF7), a human glioma cell line (U87), and a human embryonic kidney cell line (HEK293) (Singh and Tandon, 2011). A half-maximal inhibitory concentration (IC50) of DMA in primary human dermal fibroblasts and near-normal mammary epithelial cells (MCF 10A) was not achieved up to 72 hours, suggesting that it is less cytotoxic to normal cells in comparison with cancerous cells and can be developed as a safe therapeutice agent (Ranjan et al., 2013).

Our in vitro results revealed that DMA is a potent radioprotector that is less cytotoxic than its parent analog Hoechst 33342, which demands further investigation to translate the results to an in vivo system. Therefore, we investigated the toxicity, pharmacokinetics, and tissue distribution of DMA in Balb/c mice. The dose-reduction factor (DRF), taken as a measure of radioprotection efficacy, was found to be 1.28. We performed acute and repeated toxicity studies as per Organization for Economic Cooperation and Development (OECD) testa number 423 and 407 guidelines; the lethal dose (LD50) could not be achieved after single-bolus oral administration of DMA up to 2000 mg/kg body weight (bw) with no observable toxic effects in biochemical estimation or histopathology. A mouse model bearing Ehrlich ascites tumors (EAT) was used to study the differential radioprotection of normal cells and tumor cells using DMA biodistribution studies; it revealed that uptake of DMA is higher in normal tissues than tumors, demonstrating its selective radioprotective action. The pharmacokinetic results showed that DMA undergoes enterohepatic recirculation with low bioavailability (8.8%). The tissue distribution of DMA is more important for its anti-irradiation activity, whereas bioavailability pertains to the blood distribution of any molecule.

Materials and Methods

Cell Culture

The HEK293 cell line was obtained from the National Centre for Cell Science (Pune, India). The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO2. Cultured cells were grown until 80% confluent, then trypsinized and seeded in 90-mm Petri dish 24 hours before the experiment. The experiments were performed at 50 μM DMA. All experiments were performed thrice in triplicate.

Animals

Male and female Balb/c mice weighing 23–25 g were obtained from National Institute of Nutrition (Hyderabad, India). Animals were housed in polypropylene cages in a ventilated room at 22 ± 2°C and 40–65% relative humidity, with a 12-hour light/dark cycle. They were provided with rodent rations and purified water ad labium. Animals were acclimatized for 5 days to the laboratory conditions before experimentation. The institutional animal ethics committee approved the protocols that were followed.

Chemicals

DMA was synthesized, characterized, and converted to its hydrochloride salt to make it water soluble and purified by high-performance liquid chromatography (HPLC) as per the procedure reported elsewhere (Tawar et al., 2003) (Fig. 1). The HPLC percentage purity of DMA was calculated to be 97.32%. Hereafter, DMA salt will be referred as DMA. Phosphate-buffered saline (PBS), EDTA, sodium bicarbonate, acetic acid, stannous chloride dihydrate (SnCl2.2H2O), Dulbecco’s modified Eagle’s medium, diethylenetriaminepentaaetic acid (DTPA), Hoechst 33342 trihydrochloride salt, fluorescein, 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH) and Trolox were purchased from Sigma-Aldrich (Bangalore, India), diagnostic kits from Siemens (Mumbai, India) for the estimation of serum biochemical parameters such as glucose, total cholesterol, triglycerides, total protein, albumin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase, total and direct bilirubin, creatinine, urea, uric acid and blood urea nitrogen were used. HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). 99mTc was procured from Board of Radiation and Isotope Technology (Navi Mumbai, India).
Formulation and Dosage

DMA at desired doses was administered to mice by dissolving it in sterile water (10 ml/kg bw) then feeding by oral gavage using a cannula; intravenous administration was given through the lateral tail vein in mice. The control mice were administered the same volume of saline (0.9% NaCl solution).

Irradiation

Balb/c mice with or without DMA treatment were subjected to total body irradiation (TBI) at 5, 6, 8, 9, or 10 Gray (Gy) in a single fraction, using a 60Co γ-ray source with a dose rate of 1.836 Gy/min and a surface-to-source distance of 80 cm.

Radioprotective Efficacy of DMA in Mice

Dose-Reduction Factor Analysis. For this study, mice were divided into 12 groups containing 10 animals each. Group 1 was the sham controls (saline treated); group 2 were treated with DMA (300 mg/kg bw); groups 3 to 7 were radiation controls (whole body exposure to 5, 6, 8, 9, and 10 Gy); groups 8 to 12 were treated with DMA (300 mg/kg bw) before TBI to 5, 6, 8, 9, and 10 Gy. Animals were observed for 30 days for their body weight, food, and water intake, signs of radiation sickness, and mortality. DRF was calculated by dividing the LD50/30 of mice treated with both DMA and radiation to the LD50/30 of mice treated with saline and radiation (Hosseinimehr et al., 2002; Saini and Saini, 2011). All the radioprotection work except toxicity and biodistribution was performed through oral gavage of DMA at the indicated dose unless as stated otherwise for the experiment.
Uptake and Efflux Study of DMA in HEK293T Cells. HEK293T cells were grown in Biotec Delta T-dishes (Lab-Tek chambered coverglass system; BiotecButler, PA) and maintained with 5% CO2 at 37°C. After 70% confluence, the medium was removed and washed with PBS. Cells were then incubated along with 50 μM DMA in complete medium for 3 hours. Subsequently, the cells were washed and imaged for DMA uptake after every 5-minute interval for 2 hours under a confocal laser scanning microscope (Nikon A1R; Nikon, Tokyo, Japan) using a 4,6-diamidino-2-phenylindole filter. After 2 hours, the medium was changed with fresh medium, and again the cells were washed and imaged for DMA efflux after every 5-minute interval for next 22 hours under a confocal laser scanning microscope. The images were then processed with the aid of Nikon A1R software.

Tumor Studies. EAT cells were collected from the peritoneal cavity of the Balb/c harboring 8- to 10-day-old ascitic tumors. We injected 10 × 106 EAT cells subcutaneously in the Balb/c mice (n = 20) on the right thigh. When tumors reached 0.5 cm3 in volume, the mice were treated as described. The next day, the animals were randomized and divided into four different groups of five animals each. The first test group was treated with 300 mg/kg of oral DMA; the second test group was subjected to TBI of 8 Gy. The third group received 300 mg/kg of oral DMA and 8 Gy TBI both. The control group was similarly administered normal saline (0.2 ml, i.v.). On alternate days, the longest (l) and shortest (b) diameter of the tumors along with height (h) were measured with the help of a vernier caliper. The tumor volume of each animal was calculated using the formula: 

\[ V = \frac{4}{3} \pi (l \times b \times h)^{2/3} \]

Tumor-bearing mice were euthanized when the tumor was more than 1 cm3 in volume.

Toxicity Studies

Experimental Design. Acute toxicity study. The acute toxicity study was done as per OECD Test No. 423 guidelines for the testing of chemicals (http://ntp.niehs.nih.gov/iccvam/suppdocs/oecdtg/oecd_gl423.pdf). Briefly, female mice (nulliparous and nonpregnant; 23–25 g bw) were divided into six groups containing four animals each. Control group 1 received saline at a dose volume of less than 10 ml/kg bw; the test groups 2, 3, 4, 5, and 6 received single bolus dose of 300, 500, 750, 1500, and 2000 mg/kg bw of DMA by oral gavage. Mice weighing 25 g received a DMA concentration of 7.5, 12.5, 18.5, 37.5, and 50 mg dissolved in 0.2 ml of sterile water for 300, 500, 750, 1500, and 2000 mg/kg bw doses. Animals were observed for 15 to 30 minutes after dosing for any physical symptoms and then were checked after every 2 to 3 hours for any physical changes in skin, fur, eyes, and tail, and were also observed for their behavioral changes and thereafter twice a day for the next 14 days. On 15th day, the fasted (water allowed) animals were sacrificed for tissue collection. Similarly, intravenous dosing was given at 300, 200, 150, 125, and 100 mg/kg bw, and the animals were observed for mortality for 14 days to learn the lethal dose of DMA.

28-Day repeated dose oral toxicity study. The 28-day repeated oral toxicity study was performed according to the OECD Test No. 407 guidelines (http://ntp.niehs.nih.gov/iccvam/suppdocs/oecdtg/oecdtg07-2008.pdf). The DMA was administered at three doses: 112.5 (2.81 mg in 0.2 ml sterile water or 24.33 μM), 225 (5.62 mg in 0.2 ml sterile water or 48.66 μM), and 450 (11.25 mg in 0.2 ml sterile water or 97.33 μM) mg/kg bw per day. These doses were selected based on our observation that no Balb/c mice died out of the six animals used after 15 days of repeated oral administration of DMA (300 mg/kg bw). The Balb/c mice (23–25 g) were divided into six groups with six animals (three males + three females) each. Control group 1 received saline whereas groups 2, 3, and 4 received DMA at 112.5, 225, and 450 mg/kg per day, respectively, for a period of 28 days. The animals were observed for mortality and morbidity twice a day, and clinical observations were made once daily at a specific time to detect signs of toxicity (1 hour after vehicle and/or DMA administration). The observations were noted as described for the acute toxicity study.

On the 29th day, the fasted (water allowed) animals were sacrificed for tissue collection.

Estimation of biochemical parameters. Blood samples were collected by cardiac puncture in tubes, and the serum was separated for biochemical analysis. Serum biochemical parameters were measured using diagnostic kits (Siemens). Organ perfusion was performed by injecting the PBS into the left ventricle of the heart after cutting the renal artery. The tissues were removed, blotted, then weighed, and the relative organ weights were calculated with the following formula:

\[ \text{Relative Organ Weight} \% = \frac{\text{Weight of the organ}}{\text{Weight of the animal}} \times 100 \]

Tissue histopathology. Tissues were fixed in 10% neutral buffered formalin at 4°C, and the histopathological examination of the vital organs such as the brain, heart, kidney, liver, lungs, spleen, and small intestine was performed by dehydration of tissues using alcohol that was then removed in xylene; the tissues were embedded in paraffin blocks for sectioning. Thin sections of tissues were stained with hematoxylin and eosin.

Oxygen radical absorbing capacity–fluorescein assay. The 485-P excitation and 520-P emission wavelength filters were used for the oxygen radical absorbing capacity–fluorescein (ORAC-FL) assay. DMA (20 μl) and fluorescein (FL) (120 μl; 70 nM final concentration) in 200 μl of final reaction volume were placed in 96-well microplates (Nunc, Roskilde, Denmark). The mixture was preincubated for 15 minutes at 37°C. AAPH solution (60 μl; 12 mM, final concentration) was added rapidly, and fluorescence was recorded every minute for 80 minutes with automatic shaking before reading. A blank (FL + AAPH) using phosphate buffer (75 mM, pH 7.4) instead of DMA and 10 calibration solutions using Trolox (1–10 μM, final concentration) as the standard were also performed. All reaction mixtures were prepared in triplicate.

The fluorescence curves were first normalized to the corresponding blank curve of the same assay by multiplying the original data by the factor fluorescence blank, t = 0/fluorescence sample, t = 0. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as

\[ \text{AUC} = 1 + \int_{t=0}^{t=\infty} \frac{f_i}{f_0} dt \]

where \( f_0 \) was the initial fluorescence reading at 0 minutes and \( f_i \) was the fluorescence reading at time \( t \). The net AUC of a sample is the difference of AUC to the corresponding blank. Regression equations between the net AUC and the DMA concentrations were calculated. The ORAC-FL values were expressed as Trolox equivalents by using the standard curve calculated for each assay (Dávalos et al., 2004; Ou et al., 2001).

Biodistribution Study of DMA in EAT Bearing Balb/c Mice

Radiochemical Synthesis of 99mTc-DMA. DMA was labeled with 99mTc using sodium pertechnetate eluted in saline from a molybdenum generator by the procedure reported elsewhere (Panwar et al., 2004, 2007). DMA (23.5 mg) was dissolved in water for injection (4 ml) in a shielded vial, and stannous chloride (250 μl; 1 mg dissolved in N2 purged 1 ml 10% acetic acid) was added, followed by addition of freshly eluted sodium pertechnetate. We added 6.15 μCi radioactivity to the solution. The pH of the reaction mixture was adjusted to neutral by dropwise addition of 0.5 M Na2CO3 solution, filtered through 0.2 μm filter (4.4 ml).

Radiochemical Purity of 99mTc-DMA. The number of DMA molecules involved in 99mTc-complexation was determined by ascending thin-layer chromatography (TLC) on instant TLC silica gel (ITLC-SG; Pall Gelman, Port Washington, NY) strips using 100% acetone and saline as developing solvent and simultaneously in pyridine/acetic acid/water (3:5:1.5) and saline. The count of each component on developed TLC was used to calculate the percentage of free
Na$_{59m}$TcO$_4^-$, reduced $^{99m}$Tc, and the complex formed between $^{99m}$Tc and DMA. The $^{99m}$Tc/DMA complex remained at origin, and free technetium rise with the solvent front in acetone (Supplemental Fig. 1) (Hazari et al., 2010; Kumar et al., 2012).

**In Vitro Stability of Radiolabeled DMA in Human Serum.** Serum was separated by allowing the whole human blood to clot at 37°C for 1 hour in a humidified incubator maintained at 5% carbon dioxide, 95% air. Then the samples were centrifuged at 400 units (50 ml, 500 units) and incubating at 37°C for 1 hour. Plasma proteins were precipitated by adding 180 μl of acetonitrile (final volume was 250 μl each), and the samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. The clear supernatant (200 μl) was taken and was added to 200μl of distilled water to get a ~35% acetonitrile solution. The samples were injected onto the HPLC-MS/MS system (Gundala et al., 2014).

**Bioanalysis.** The stock solutions (each, 1 mg/ml) of DMA and Hoescht 33342 (internal standard) were prepared in acetonitrile/water (95%/5%v/v). A standard DMA calibration from 1–1000 ng/ml with internal standard (20 ng/ml) was quantified. The quantification standards were within ±15% of the nominal concentration, and the lower limit of quantification was within ±20% of the nominal concentration. Sample analysis was performed in positive ionization mode liquid chromatography tandem mass spectrometry with multiple reaction monitoring (m/z Q1/Q3) of DMA (m/z 235.1/412.0, retention time 5.3 minutes) and internal standard (m/z 227.1/396.0, retention time 6.5 minutes) (Agilent 6410 series; Agilent Technologies, Wilmington, DE). The ion spray voltage was set at 3000 V, and ionization temperature was 200°C with a drying gas flow rate of 20 l/min. Data acquisition and quantitation were performed using Mass Hunter software (Agilent Technologies). Separation was achieved using HP1100 series LC (Agilent Technologies) equipped with a photodiode array detector, using an Agilent Zorbax reverse-phase (C18, 2.1 × 50 mm, 5.0 μm) column. The column temperature was maintained at room temperature (25°C). A gradient method was employed to analyze the plasma samples using mobile phase A (0.1% formic acid in water) and mobile phase B (methanol). The B% was set as 30 (0 minutes), 90 (10–14 minutes), and 30 (14–24 minutes) with a flow rate of 0.3 ml/min. An injection volume of 10 μl was used for analysis.

**Pharmacokinetic Analysis.** Peak plasma concentration ($C_{max}$) of DMA and time to reach the $C_{max}$ ($t_{max}$) noted by observation of mean concentration-time profile. For the calculation of pharmacokinetic parameters, plasma concentration-time data were subjected to non-compartmental analysis using WinNonlin program, version 6.3 (Certara, Princeton, NJ) to calculate the pharmacokinetic parameters.

**Statistical Analysis.** Data are expressed as mean ± S.D. where applicable. The mean difference between the control and treated groups was analyzed by Student’s t test for acute toxicity and one-way analysis of variance followed by Tukey’s multiple comparisons as post hoc test for 28-day repeated toxicity using GraphPad prism 5.0 (GraphPad Software, San Diego, CA). *P* < 0.05 was considered statistically significant.

**Enzymatic Hydrolysis of DMA Conjugates.** Glucuronide conjugates were confirmed by treating plasma (200 μl) with β-glucuronidase (50 μl, 500 units) and incubating at 37°C for 1 hour. Plasma proteins were precipitated by adding 180 μl of acetonitrile (final volume was 250 μl each), and the samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. The clear supernatant (200 μl) was taken, and 200 μl of distilled water was added to get a ~35% acetonitrile solution. The samples were injected onto the HPLC-MS/MS system (Gundala et al., 2014).
Cellular Uptake and Efflux. DMA reached its highest level in 2 hours in cells (Fig. 1C). A further efflux study showed retention of DMA in cells for the next 22 hours. There was 65% DMA retained in HEK293 cells, which show higher affinity and binding of DNA to cellular DNA (Fig. 1D).

DMA Does Not Protect Tumor Cells against Radiation. To evaluate whether DMA affected the radiosensitivity of the tumors, mice were injected with either PBS or DMA (300 mg/kg bw) 2 hours before each radiation treatment. The effect of DMA and radiation was monitored on tumor growth. On every alternate day the tumor growth was calculated after treatment with DMA or radiation or together in these animals. No significant reduction in tumor growth was observed in DMA-treated mice, but in radiation-treated and DMA + radiation–treated mice a significant reduction in tumor growth was observed (Fig. 1E). The control mice and those treated with DMA alone died by 16 day from the tumor whereas the death of all PBS-treated animals was from radiation toxicity by day 18. In contrast, the DMA-pretreatment mice survived until day 20, suggesting significant protection against radiation-induced mortality but no radioprotective effect on the tumors. Thus, DMA alone does not have any effect on tumor growth itself.

ORAC-FL Assay. The presence of fluorescence signal was constant over 80 minutes in the absence of AAPH, indicating its photostability under the excitation conditions used. When AAPH was added (blank), the fluorescence signal dropped with time. With increasing concentrations of Trolox (1–10 μM), the decay of the curve was delayed (Fig. 2, A and B). Similar results were observed for DMA (2–100 μM), showing that the decay of the curve was effectively delayed. The relative ORAC value (Trolox equivalents) was calculated as

\[ \text{Relative ORAC value} = \left( \frac{AUC_{\text{Sample}} - AUC_{\text{Blank}}}{AUC_{\text{Trolox}} - AUC_{\text{Blank}}} \right) \times \left( \frac{\text{molarity of Trolox}}{\text{molarity of sample}} \right). \]

The relative ORAC value (Trolox equivalents) was found to be 1.5 for DMA.

Toxicity Studies

Acute Toxicity Study. DMA did not cause mortality or toxicity symptoms such as abnormal demeanor and behaviors at oral doses of 300, 500, 750, 1000, 1500, or 2000 mg/kg bw, where 2000 mg/kg bw was taken as the limit dose as per the OECD test number 423 guidelines. The food and water intake was normal. Body weights did not change. The dose of 125 mg/kg bw of DMA was determined to be LD_{50} in cases of single intravenous administration (Fig. 3A). On 15th day when the animals were sacrificed, there were no significant change in the weights of the individual animals, and we found no change in organ appearance or organ coefficient (relative organ weight) except in the liver at the 500 mg/kg bw dose and the intestine at the 2000 mg/kg bw dose.

28-Day Repeated Dose Toxicity Study. There were dose-related toxicity signs and death observed in mice when given repeated doses for 28 days. The food and water intake were reduced. No mice survived when treated with DMA at a repeated dose of 450 mg/kg per day, and all were found dead within a 1 week period of DMA treatment. Fifty percent of the animals survived the DMA dose of 225 mg/kg per day for a period of 28 days, and no animals were found dead until the treatment duration was complete when treated with 112.5 mg/kg per day. So 225 mg/kg bw was considered to be LD_{50} in cases of repeated administration (Fig. 3B). Insignificant differences were found in organ appearance and the relative organ weights of the brain, heart, liver, lung, spleen, kidney, stomach, and intestine, recorded between the control and DMA-treated groups.

Estimation of Biochemical Parameters. Serum biochemical parameters levels (Table 1) were found to be insignificantly changed in DMA-treated animals in comparison with saline-treated control animals. Serum estimation of the biochemical parameters of the groups with doses of 225 mg/kg per day and 112.5 mg/kg per day showed no statistically significant differences between control and treated groups (Table 2).

Histopathology. No gross pathologic changes were observed in histopathologic sections of the brain, heart, liver, lung, spleen, kidney, or intestine in the acute and 28-day repeated studies. Examined organs showed no inflammation, necrosis, hemorrhage, or cellular abnormalities (deposits, degeneration, vacuoles, etc.) in any of examined organs.

In the heart, no inflammatory cell infiltration or myocardial fibrosis was observed, and the endocardium, epicardium, myocardium, and myocardial interstitial tissues were normal. In the liver, the hepatic lobule and sinusoidal, plate, and hepatic cell structures were also found unchanged; no inflammatory cell infiltration or necrosis was observed. In the kidneys, the structure of the renal cortex and medulla was normal; the renal corpuscles showed no hyperemia or exudation. The intestinal mucosa showed no change in intestinal villi or crypt of Lieberkühn, with normal villous architecture, submucosa,
and muscularies. Splenic lymphocytes were also observed to be normal in all animals (Supplemental Figs. 2 and 3).

**Biodistribution Study of DMA in Balb/c Mice**

**Radiochemical Synthesis and Purity of $^{99m}$Tc-DMA.** DMA was successfully labeled by $^{99m}$Tc. Radiolabeling efficiency was checked with ITLC strips, and it was found to be >98% and colloids <2%.

**In Vitro Stability of Radiolabeled DMA in Human Serum.** The radiocomplex stability in serum is of major concern, as it has to be used for intravenous administration to mice. We found that the radiocomplex was quite stable up to 24 hours; 3% of the radiolabeled DMA was dissociated in serum at 24 hours (Supplemental Fig. 4).

**Transchelation Assay.** Incubation of radiolabeled DMA with different concentrations (0.010, 0.030, 0.050, and 0.100 M) of DTPA for 1 hour at 37°C showed that binding strength was sufficient; even 0.1 M DTPA could dissociate only 3.34% free technetium from the radiocomplex, and the 99.96% that remained was labeled DMA (Supplemental Fig. 5). Thus, it strengthens the biodistribution of labeled DMA, which is mainly due to DMA and not free $^{99m}$Tc.

**Biodistribution of Radiolabeled DMA in EAT Tumor-Bearing Mice.** The biodistribution study suggested that the uptake of radiolabeled DMA was higher in the lungs, liver, spleen, and kidney. At 1 hour after injection, the percentage of the injected dose was found to be 14.96 ± 2.62, 17.78 ± 2.28, 10.14 ± 2.58, and 20.05 ± 0.29 (% ID/g ± S.D.) in the lungs, liver, spleen, and kidney, respectively, which dropped to 5.90 ± 0.06, 6.88 ± 0.98, 3.35 ± 2.36, and 6.03 ± 0.97 (% ID/g ± S.D.) with a ~3-fold decrease in the amount of the injected dose at the 24-hour time point. In comparison, the uptake in the blood, heart, stomach, intestine, muscles, brain, and tumor were rather low at all time points measured (Fig. 4A).

**Scintigraphy in Tumor-Bearing Mice.** Animals’ images were captured at 2, 4, 8, 18, and 24 hours after administering the radiocomplex intravenously (100 μl, 0.140 mCu).

**TABLE 1**

Serum biochemical parameters of control (saline treated) and mice treated with DMA with single bolus oral administration at increasing dose of 300, 500, 750, 1500, and 2000 mg/kg bw.

Values are expressed as mean ± S.D., with n = 4 for control and treatment groups. Statistical analysis was performed by Student t test. P ≤ 0.05 was considered statistically significant. Samples were analyzed in triplicate.

<table>
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<tr>
<th>Biochemical Parameters</th>
<th>Dose</th>
<th>mg/kg bw</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
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<td>4.41 ± 0.07</td>
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<td>Albumin (g/dl)</td>
<td>2.67 ± 0.32</td>
<td>2.96 ± 0.22</td>
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<td>Cholesterol (mg/dl)</td>
<td>109.21 ± 16.4</td>
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<td>Triglyceride (mg/dl)</td>
<td>115.89 ± 23.29</td>
<td>105.89 ± 23.2</td>
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<td>Urea (mg/dl)</td>
<td>20.19 ± 4.71</td>
<td>19.83 ± 0.33</td>
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<td>Blood urea nitrogen (mg/dl)</td>
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<td>Uric acid (mg/dl)</td>
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<td>Creatinine (mg/dl)</td>
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<td>Total bilirubin (mg/dl)</td>
<td>0.25 ± 0.33</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>0.16 ± 0.26</td>
<td>0.18 ± 0.07</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>137.82 ± 28.47</td>
<td>123.76 ± 6.28</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>50.47 ± 9.34</td>
<td>61.42 ± 4.80</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>6.90 ± 1.36</td>
<td>6.99 ± 1.36</td>
</tr>
</tbody>
</table>
interest placed over areas to count the average counts per pixel showed that uptake of $\frac{[99m\text{Tc}]\text{DMA}}{\text{g tissue}}$ in tumors had a tumor-to-muscle ratio of $5.37 \pm 0.54$, $7.53 \pm 0.64$, and $3.95 \pm 1.5$ at the time points of 2, 4, and 24 hours, respectively. Scintigraphy images showed the time-dependent clearance of the radiocomplex from the organs and tumor (Fig. 4B).

**Pharmacokinetics of DMA in Balb/c Mice.** No additional peak of endogenous compounds was found in the blank plasma HPLC-MS/MS chromatogram (Supplemental Fig. 6A) at the retention time of DMA and internal standard. The DMA was spiked at different concentrations in plasma and processed, and the clear supernatant was injected onto HPLC-MS/MS. DMA and internal standard (Supplemental Fig. 7). The correlation coefficient ($r^2$) value was always greater than 0.999, indicating linearity at the concentration range of 1 ng/ml to 1000 ng/ml. The DMA plasma concentration-time profile and the pharmacokinetic parameters of DMA in Balb/c mice are shown in Fig. 5.

After intravenous administration, DMA was detected in plasma up to 24 hours. The elimination half-life, volume of distribution at steady-state, and systemic clearance were 4 hours, 36.7 l/kg, and 27.4 l/h per kg, respectively. After oral dosing, DMA exhibited two $C_{\text{max}}$ ($C_{\text{max1}}$: 303.6 ± 29.7 ng/ml and $C_{\text{max2}}$: 57.7 ± 5.8 ng/ml) at 1 and 4 hours, indicating that DMA is rapidly absorbed. The bioavailability (%F) was calculated from $\%F = \frac{(\text{AUC}_{\text{oral}} \times \text{Dose}_{\text{oral}})}{(\text{AUC}_{\text{intravenous}} \times \text{Dose}_{\text{oral}})} \times 100$ and was found to be 8.8%.

To delve further, a part of the plasma samples obtained after the 150 mg/kg oral dose was analyzed after $\beta$-glucuronidase hydrolysis. The hydrolyzed plasma samples showed a 1.5-times higher AUC (705.6 ng·h/ml) than those observed in unhydrolyzed plasma samples (Fig. 5).

**Discussion**

Ionizing radiation employed in radiotherapy is not exclusive—it does not protect the surrounding normal tissues over the tumor tissues. This creates a small molecular difference between normal and cancerous cells that renders it a non-selective therapy (Prabhakar et al., 2007). The main objective is to protect the normal tissues surrounding tumor tissues exposed to high doses of radiation; for this purpose, radioprotectors are in demand in clinical radiotherapy. Several naturally occurring or synthesized compounds have been investigated in biologic systems to protect against the damage...
induced by radiation exposure, but these molecules are associated with their own characteristic toxicities (Bonner and Shaw, 2002; Whitnall et al., 2002; Landauer et al., 2003; Yildiz et al., 2006).

Through microarray hybridization and protein expression analysis by two-dimensional PAGE followed by peptide finger printing, revealed large number of genes that are regulated in response to DMA and/or Infrared. Real time quantitation of the identified proteins and headers confirmed differential regulation of genes between three treatment conditions drug, radiation and drug + radiation treated cells as compared with control. We narrowed down to a unique mechanism of radioprotection of DMA. We observed that nuclear factor κB (NF-κB)–inducing kinase (NIK)/κB kinase α (IKKα)/IKKβ–mediated NF-κB activation by DMA alone or in combination with irradiation promotes a prosurvival pathway in cells (Kaur et al., 2012).

To establish the clinical relevance of DMA as an anti-irradiation drug, we investigated its in vivo toxicity and efficacy parameters in an animal model. Acute toxicity values such as LD_{50} and toxicity symptoms are used as the basis to classify chemical compounds into toxicity categories and determine their further regulation (Zbinden and Flury-Roversi, 1981). A 2000 mg/kgbw was taken as the limit dose as per OECD test number 423 guidelines for acute toxicity. Mortality rate, serum biochemical parameters, and tissue histopathology were used as end points for the toxicity determination. Toxic effects on renal function were evaluated by serum urea, blood urea nitrogen, uric acid, and creatinine levels. Changes of albumin, triglycerides, alkaline phosphatase, ALT, and AST were used to identify acute hepatic injury, which must be investigated toward recognizing chemical-induced hepatotoxicity. Because the liver is the main organ involved in lipid metabolism, other parameters such as glucose, triglycerides, total proteins, and total cholesterol were measured to discern hepatic damage (Satyanarayana et al., 2001; Ramaiah, 2007; Antonelli-Ushirobira et al., 2010). We did not observe any significant changes in serum biochemical parameters such as AST, ALT, creatinine, blood urea nitrogen, uric acid, or urea in DMA-treated animals at different doses in comparison with the control group (Tables 1 and 2).

No gross pathologic changes in histopathology in any of the organs were observed; 0% mortality and no noticeable indicators of toxicity such as abnormal behaviors were observed up to the limit dose of DMA. The histopathologic analysis showed no vascular or inflammatory changes (Supplemental Figs. 2 and 3) such as centrilobular necrosis, congested sinusoids, or nuclear changes. No cellular infiltration or degeneration in the liver, kidney, brain, or lung tissues was found. In addition, no damage to crypt epitheliums and epithelial cells in the intestine was observed, and the megakaryocytes were normal in the spleen. No vascular congestion in the heart with inflammatory changes in the cardiomyocytes was found with DMA treatment.

As per the Globally Harmonized System of Classification and Labeling of Chemicals, the LD_{50} value for DMA could not be achieved up to 2000 mg/kgbw, so it can be classified as a chemical agent under category 5, in accordance with its nontoxic behavior after oral administration. In comparison, the LD_{50} of known radioprotectors in oral dose has been reported as 810 and 1510 mg/kgbw for WR2721 (amifostine) and NIT2011 (nitronyl nitroxyl radical) (Wang et al., 2013). After 28 days of repeated administration of DMA at 450 mg/kgbw per day, the dose was lethal, and all mice died within 7 days; however, at 225 mg/kgbw per day, 50% animals survived until the date of sacrifice. Although treatment-related physical changes were observed—reduction in food and water intake, diarrhea, loss of agility, and decrease in body weight—no significant change was observed in the biochemical parameters or histopathology. A significant change in body weight, along with diarrhea (a symptom of gastrointestinal syndrome), was observed in mice treated with repeated doses of DMA (225 and 450 mg/kgbw per day) (Fig. 3C). This might have been one of the reasons for mortality among the mice. No mortality was observed at 112.5 mg/kgbw per day dose administration.

DRF evaluates the quantitative capacity of the radioprotective agent to enhance the tolerance of tissues and its ability to reduce radiation-induced toxicity symptoms and death (Bonner and Shaw, 2002). The DRF of 1.28 was obtained with DMA for a range of radiation doses (5–10 Gy), which is comparable to the DRF value of known radioprotectors (Hahn et al., 1992; Weiss and Landauer, 2000; Krishna and Kumar, 2005; Lee et al., 2008; Mantena et al., 2008; Saini and Saini, 2011; Shrikant et al., 2012) (Table 3). It is not possible to rank these radioprotectors as they are administered by different routes; however, if we were to compare the orally administered synthetic molecules, DMA could be ranked second: it has shown good radioprotection at a single dose, and no toxicity was observed up to 2000 mg/kg dose. Whereas a subcutaneous injection of 500 mg/kgbw of the radioprotective agent Ex-RAD (ON01210.Na; 4-carboxystyryl-4-chlorobenzylsulfone, sodium salt) in mice 24 hours and 15 minutes before radiation showed a DRF of 1.16 (Ghosh et al., 2012). A single subcutaneous injection of 200 mg/kg of genestein given at 24 hours before radiation showed a DRF of 1.16 (Landauer et al., 2003). γ-tocotrienol, a tocot antioxidant and potent radioprotector, at an optimal dose of 200 mg/kg given subcutaneously 24 hours before irradiation had a DRF of 1.29 in CD2F1 mice (Ghosh et al., 2009). For drugs intended for prophylactic or...
therapeutic use—for example, to radiation casualties from an accident, such as civilians, military personnel, or first responders exposed to radiation-contaminated areas—the oral route is the most advantageous and practical way to administer treatment. It is, therefore, of great interest to determine the radioprotective efficacy of an agent after oral administration. The DRF for oral administration of 17-dimethylaminoethylamino-17-demethoxygeldanamycin, 24 hours before irradiation was 1.68 (Lu et al., 2013).

ROS cause DNA damage and induce cellular toxicity by inducing a number of lesions in DNA, including oxidized bases, abasic sites, DNA strand breaks, and cross-links between DNA and proteins (Mansour et al., 2008). In order to understand the mechanism involved for in vivo radioprotection, an ORAC-FL assay was performed to study the antioxidant capacity of DMA to cement the already proven free radical scavenging property of DMA (Tawar et al., 2003). This assay measures the loss of fluorescein fluorescence over time due to the peroxyl-radical formation by the breakdown of 2,2′-azobis(2-aminodipropyl)dihydrochloride (AAPH). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) serves as the positive control, inhibiting fluorescein decay in a dose-dependent manner. It confirmed the free-radical scavenging property of DMA responsible for the marked reduction in radiation-induced DNA damage (Fig. 2, A and B). The relative ORAC value (Trolox equivalents) was found to be 1.5 for DMA. In comparison, the relative ORAC values of known antioxidants were found to be <1.5 for vitamin C (0.95) and glutathione (0.62) and >1.5 for chlorogenic acid (3.14) and genistein (5.93). This shows the free-radical scavenging and antioxidant properties of DMA by inhibiting the decrease in the fluorescence of fluorescein. The in vitro cellular uptake and efflux study showed 65% DMA was retained in HEK293 cells up to 24 hours, which shows the higher affinity and binding of DMA to cellular DNA. DMA had no effect on the growth of unirradiated or irradiated tumors (Fig. 1E). DMA does not protect tumor cells because less of it enters tumor cells.

An efficient radioprotector that reaches higher concentrations in normal tissues than in tumor cells could demonstrate its selective protection. EAT, a murine breast cancer cell, can develop ascites when the ascites fluid is injected intraperitoneally; however, when it is given subcutaneously, a solid form is obtained (Mehmet et al., 2011). Therefore, to measure the in vivo tissue uptake, we performed a biodistribution study of [99mTc]DMA in EAT-bearing mice. The capability of protecting late-responding normal tissues of clinical relevance against radiation damage is of great interest, and 35S-labeled WR2721 biodistribution and blood clearance studies in C3H mice with RIF-1 tumors after intraperitoneal injection have been reported to identify the tissues and postinjection times that suggest a high level of radioprotection. Rasey et al. (1984) reported that the concentration of protector (milligram per gram of tissue) in various organs at 30 minutes after injection ranked in the following order: kidney > liver > lung > gut > heart > blood > skin > tumor > brain. The biodistribution of [125I]Hoechst 33342 in LS174T tumor-bearing mice after intravenous injection via lateral tail vein indicated that maximum uptake of injected radioactivity was taken up by the kidney followed by the liver and spleen (Harapanhalli et al., 1996). Similarly, the biodistribution of [99mTc]DMA in EAT-bearing mice showed that major organs for DMA uptake are the lungs, liver, kidneys, and spleen rather than the tumor. The high uptake and accumulation of [99mTc]DMA in liver and kidney at each time point might be due to its hepatobiliary and renal excretion. Pharmacokinetics studies showed that after oral administration, DMA was quickly absorbed and distributed, and that it exhibited a multiple peak phenomenon in mice. However, its large volume of distribution (V∞) and long half-life in mice indicate an extensive affinity with the tissues.

Glucuronidation is a major detoxifying reaction in metabolism and converts a large number of xenobiotics and endogenous molecules into highly water-soluble metabolites in the liver. Some of the glucuronides are secreted via the biliary route into the intestine and have poor reabsorption into the bloodstream and are efficiently eliminated from the body if not hydrolyzed by intestinal β-glucuronidase enzymes. The β-glucuronidase activity increases the enterohepatic circulation of the compounds (Beaud et al., 2005). The hydrolyzed plasma samples showed a 1.5-times higher AUC (705.6 ng*h/ml) than those observed in unhydrolyzed plasma samples. The volume of distribution (V∞) of DMA is larger than the total blood volume of mouse (0.085 l/kg) (Davies and Morris, 1993), indicating that the compound is readily distributed in the extravascular system. However, the systemic clearance of DMA is higher than the hepatic blood flow of the mouse (5.4 l/h per kg), which indicates an extrahepatic elimination of the compound.

In summary, cellular uptake, efflux, biodistribution, and pharmacokinetics studies suggest longer retention and tissue affinity and thus sustained free radical scavenging for a longer period of time, leading to better radioprotection with DMA by preventing DNA damage. Moreover, its selective uptake in normal tissues rather than in tumors could be taken as relevant observation in the development of radioprotectors against normal tissue injury. TBI affects multiple organs, causing death within 30 days after exposure to 3–8 Gy due to gastrointestinal syndrome and hematopoietic syndrome. DMA improved survival over a wide range of radiation doses 5–10 Gy, suggesting that it can protect against both gastrointestinal and hematopoietic injury.

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Preclinical Evaluation of DMA, a Bisbenzimidazole as Radioprotector: Toxicity, Pharmacokinetics and Biodistribution Studies in Balb/c Mice


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Supplemental Figure 1. Instant thin layer chromatography (ITLC) showing the point of application and the distance traveled by $^{99m}$Tc-labeled compound and free Tc.
Supplemental Figure 2. Tissue histopathological picture of mice treated with the DMA at increasing doses (300, 500, 750, 1500 and 2000mg/kg bw) (H&E, magnification ×200); Kidney, liver, heart, lungs, spleen, brain and intestine of control animals and DMA treated animals.
Supplemental Figure 3. Tissue histopathological picture of mice treated with the DMA at 225mg/kg bw dose for 28-days (H&E, magnification ×200); Kidney, liver, heart, lungs, spleen, brain and intestine of control animals and DMA treated animals.
Supplemental Figure 4. *In vitro* stability of $^{99m}$Tc-DMA at 0, 1, 2, 4 and 24h in serum.

Supplemental Figure 5. Effect of DTPA on radiolabeling efficiency of $^{99m}$Tc-DMA
**Supplemental Figure 6.** (A) Blank plasma LC-MS chromatogram; (B) Blank plasma spiked with IS (Hoechst 33342) LC-MS chromatogram; (C) LC-MS chromatogram of the plasma sample spiked with DMA and IS.
Supplemental Figure 7. Calibration curve plotted after injecting the DMA at different concentrations spiked in blank plasma.