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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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Running Title: Pharmacological Study of Bisbenzimidazole, DMA as Radioprotector

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NON- STANDARD ABBREVIATIONS: DMA, 5-(4-methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5' benzimidazolyl] benzimidazole; ROS, reactive oxygen species; HEK293, human embryonic kidney cell line; DRF, dose reduction factor; AST, aspartate aminotransferase; ALT, alanine aminotransferase; EAT, Ehrlich ascites tumor; ORAC, oxygen radical absorbing capacity.

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ABSTRACT

Radiotherapy, a therapeutic modality of cancer treatment, non-selectively damages normal tissues over tumor tissue as well. A continuous search is going on for the development of therapeutic agents, which selectively reduce radiation induced normal tissue injury without reducing tumoricidal effect, thereby increasing therapeutic ratio of radiation therapy. Our laboratory established 5-(4-methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5'benzimidazolyl] benzimi-dazole (DMA) as non-cytotoxic radioprotector in mammalian cells. DMA showed an excellent radioprotection in mice at single nontoxic oral dose by a dose reduction factor (DRF) of 1.28. ORAC assay confirmed its free radical quenching ability. Single bolus dose and 28-days repeated administration of DMA in mice for toxicity studies determined LD_{50} of >2000 mg/kg bw and 225 mg/kg bw respectively, suggesting DMA is safe. Histopathology, biochemical parameters and relative organ weight analysis revealed insignificant changes in DMA treated animals. Pharmacokinetic study of DMA at oral and intravenous doses showed its C_{max} = 1h, bioavailability of 8.84%, elimination half-life of 4h and an enterohepatic recirculation. Biodistribution study in Ehrlich Ascites Tumor (EAT) bearing mice showed that ^{99m}Tc-DMA achieved highest concentration in 1h and retained upto 4h in lungs, liver, kidneys and spleen, and in a low concentration in tumor, a solicited property of any radioprotector to protect normal cells over cancerous cells. We observed that single dose treatment of tumor bearing mice with DMA 2h prior to 8 Gy TBI, showed an impressive rescue of radiation induced morbidity, in terms of weight loss and mortality, without a change in tumor response.

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Introduction

Radiotherapy, second only to surgery, is utilized by 80% of the patients as one of the most effective modalities for the treatment and cure of neoplastic diseases (Satyamitra et al., 2012; Nair et al., 2001; Parihar et al., 2007). Ionizing radiation triggers a series of events starting from generation of reactive oxygen species (ROS). These ROS have deleterious effect on macromolecules, living tissues or cells resulting in the transfer of radiation energy to biological materials (Kalpana et al., 2011). Radioprotectors protect normal cell from radiation induced damage. Radioprotective mechanistic approaches include damage protection by inhibiting free radicals generation or hastening the free radical scavenging, DNA and membrane repair enhancement, reconstruction of hematopoietic function and stimulation of immune cell activity (Wang et al., 2013). Though after the adoption of conformal treatment techniques such as intensity modulated radiation therapy (IMRT) and image guided radiation therapy (IGRT), the amount of radiation delivered to normal tissue surrounding the targeted tumor has been greatly reduced, still 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 the normal tissue injury continues to be a limiting factor for radiation oncologists. During the past decades of preclinical and clinical research efforts several compounds either of natural or synthetic origin have been explored and investigated in biological systems to protect against the radiation induced damage. However, to date there is no approved prophylactic pharmacological agent available as radioprotector except amifostine which is an FDA approved agent (Weiss et al., 2009). Although amifostine and other compounds have shown good prophylactic effects, but 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. Among other radioprotectants under investigation given orally such as

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melatonin has acute toxicity, genistein requires administration by a multiple dosing regimen several days prior to irradiation, similarly high doses (1600 mg/kg) are needed for 5-androstene-3 beta, 17 beta-diol (AED) to provide significant survival advantage after radiation exposure (Whitnall et al., 2002; Landauer et al., 2003; Yildiz et al., 2006). Recently DIM (3, 3'- diindolylmethane) a natural product confers protection against ionizing radiation by stimulation of ATM signaling (Fan et al., 2013). Recently a TLR 5 agonist (Burdelya et al., 2008) and TLR 3 inhibitor (Takemua et. al.; 2014) effectively ameliorates crypt cell death and GIS in animals. The continuous need necessitates develop of potent radioprotector that can selectively protect normal tissues over tumor tissues with relevant tissue uptake and retention.

Hoechst33342 analogue, DMA (5-(4-methylpiperazin-1-yl)-2-[2'-(3,4-dimethoxyphenyl)-5'benzimidazolyl]benzimidazole]), a DNA minor groove binder, binds preferentially to AT rich sequences were synthesized and established as radioprotector in *in vitro* conditions in our laboratory. We have proved that mechanistically DMA act as radioprotector by reducing DNA damage and free radical scavenging in mammalian cells and was observed to be non-mutagenic, non-cytotoxic to mammalian cells (BMG-1) in comparison to parent analogue (Tawar et al., 2003; 2007). It showed inhibitory action at micromolar concentrations in 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 (HEK) (Singh and Tandon, 2011).The half maximal inhibitory concentration (IC_{50}) of DMA in primary human dermal fibroblasts (HDF) and near normal mammary epithelial cells (MCF 10A) was not achieved up to 72h suggesting that it is less cytotoxic to normal cells in comparison to the cancerous cells and can be developed as safe therapeutic agent (Ranjan et al., 2013). The above *in vitro* results revealed that DMA is a potent radioprotector less cytotoxic as compared to its parent analogue Hoechst 33342 demand its further investigation in order

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to translate the results in *in vivo* system. Therefore, in the present study we investigated toxicity, pharmacokinetics and tissue distribution of DMA in Balb/c mice. Dose reduction factor (DRF), taken as a measure of radioprotection efficacy was found to be 1.28. Acute and repeated toxicity studies were performed as per OECD guidelines 423 and 407 and lethal dose (LD_{50}) could not be achieved after single bolus oral administration of DMA up to 2000 mg/kg body weight (mg/kg bw) with no observable toxic effects in biochemical estimation and histopathology. Ehrlich Ascites Tumor (EAT) bearing mice model was used in order to study differential radioprotection of normal cells and tumor cells using DMA biodistribution studies revealed that uptake of DMA is higher in normal tissues than tumor demonstrating its selective radioprotective action. Pharmacokinetic results showed that DMA undergoes enterohepatic recirculation with low bioavailabilty (8.8%). The tissue distribution of DMA is more important for its anti-irradiation activity, whereas bioavailability is all about the blood distribution of any molecule.

Material and methods

Cell culture. The Human Embryonic Kidney cell line (HEK293) cell line was obtained from National Centre for Cell Science. (Pune, India). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) 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% CO_2. Cultured cells were grown until 80% confluent, trypsinized and seeded in 90-mm petri dish 24h before the experiment. Experiments were performed at 50µM DMA. All experiments were performed thrice in triplicate.

Animals. Male and female Balb/c mice weighing 23-25g were obtained from National Institute of Nutrition (NIN), Hyderabad, India. Animals were housed in polypropylene cages in a ventilated room at 22±2°C and 40–65% relative humidity, with a 12-h light/dark cycle. They were provided

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with rodent rations and purified water *ad labium*. Animals were acclimatized for 5 days to the laboratory conditions prior to experimentation. Institutional Animal Ethics Committee approved protocols 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 reported procedure (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), ethylenediamine tetraacetic acid (EDTA), sodium bicarbonate, acetic acid, stannous chloride dihydrate ($SnCl_2.2H_2O$), Dulbecco's modified Eagle's medium (DMEM), diethylenetriaminepentaacetic acid (DTPA), Hoechst 33342 trihydrochloride salt, DAPI (4',6-diamidino-2-phenylindole), Tris-HCl sodium chloride, sodium dodecyl sulfate (SDS), proteinase K, RNase, phenol, chloroform, agarose, ethidium bromide, boric acid, fluorescein, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) and trolox were purchased from Sigma-Aldrich (India), diagnostic kits from Siemens (India) for the estimation of serum biochemical parameters such as glucose, total cholesterol (TC), triglycerides (TG), total protein (TP), albumin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total and direct bilirubin, creatinine (CRE), urea, uric acid and blood urea nitrogen (BUN) were used. HPLC grade acetonitrile (ACN) was purchased from Merck (Germany). ^{99m}Tc was procured from Board of Radiation and Isotope Technology (BRIT) India.

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

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

Radioprotective efficacy of DMA in mice

Dose reduction factor (DRF) analysis. For this study, mice were divided into 12 groups containing 10 animals each. Group 1, sham control (saline treated);Group 2, DMA treated (300 mg/kg bw); Group 3 to 7, radiation control (whole body exposure to 5, 6, 8, 9 & 10 Gy); Group 8 to 12, treated with DMA (300 mg/kg bw) prior to whole body exposure to 5, 6, 8, 9 & 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 $LD_{50/30}$ of mice treated with both DMA and radiation to the $LD_{50/30}$ 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 indicated dose unless as stated in experiment.

Uptake and efflux study of DMA in HEK293T cells. HEK293T cells were grown in Bioptech Delta T-dishes (Lab-Tek Chambered Cover glass system) and maintained with 5% CO_2 at 37°C. After 70% confluence, the medium was removed and washed with PBS. Cells were then incubated along with 50μM DMA in complete media 3 h. Subsequently the cells were viewed and imaged for DMA uptake after every 5 min interval for 2 h under a confocal laser scanning microscope (Nikon A1R, Japan) using a DAPI filter. After 2 h, media was changed with fresh media and again cells were viewed and imaged for DMA efflux after every 5 min interval for next 22 h under a confocal laser scanning microscope. The images were then processed with the aid of Nikon A1R software.

Tumor studies. Ehrlich Ascites Tumor (EAT) cells were collected from the peritoneal cavity of the Balb/c harboring 8–10-days-old ascitic tumor. 10×10^6 EAT cells were injected intramuscularly in

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the Balb/c mice (n=20) on the right thigh. When tumors reached 0.5cm^3 in volume, mice were treated as described. The next day, animals were randomized and divided into 4 different groups of 5 animals each. The first test group was treated with 300 mg/kg, oral DMA, second test group subjected to total body irradiation of 8 Gy. Third group received 300 mg/kg, oral DMA and 8 Gy TBI both. The control group was similarly administered normal saline (0.2 ml, i.v.). On alternative day longest (l) and shortest diameter (b) of the tumors along with height (h) were measured with the help of a vernier calliper. Tumor volume of each animal was calculated using the formula l*b*h/2. Tumor bearing mice were euthanized when tumor sized more than 1cm^3 volume.

Toxicity Studies

Experimental design

Acute toxicity study. The acute toxicity study was done as per Organization for Economic Co-operation and Development (OECD) guidelines for testing of chemicals 423 (OECD, 2002). Briefly female mice (nulliparous and non-pregnant; 23-25 g bw) were divided into six groups containing four animals each. Control group I received saline at a dose volume of less than 10ml/kg bw while the test group II, III, IV, V and VI received single bolus dose of 300, 500, 750, 1500 and 2000 mg/kg bw of DMA by oral gavage. Mice weighing 25g received DMA concentration of 7.5, 12.5, 18.5, 37.5 and 50mg dissolved in 0.2ml sterile water for 300, 500, 750, 1500 and 2000 mg/kg bw doses. Animals were observed for 15-30 min after dosing for any physical symptoms and then checked after every 2-3 h for any physical changes in skin, fur, eyes, tail and 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, 100mg/kg bw and animals were observed for the mortality for 14 days to find out the lethal dose of DMA.

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28-days repeated dose oral toxicity study. 28-days repeated oral toxicity study was performed according to the OECD guideline 407 (OECD, 1995). In the present study, DMA was administered at three doses - 112.5 (2.81mg in 0.2ml sterile water or 24.33mM), 225 (5.62mg in 0.2ml sterile water or 48.66mM) and 450 (11.25mg in 0.2ml sterile water or 97.33mM) mg/kg bw/day. These doses were selected based on our observation that no Balb/c mice died, out of six animals used, after 15 days repeated oral administration of DMA (300mg/kg bw). Balb/c mice (23-25g) were divided into 6 groups with 6 animals (3 males + 3 females) each. Control group I received saline whereas group II, III and IV received DMA at 112.5, 225 and 450 mg/kg/day respectively for a period of 28 days. Animals were observed for mortality and morbidity twice a day and clinical observations were made once daily at particular time to detect signs of toxicity (1h after vehicle and/or DMA administration). The observations were the noted as described in acute toxicity study. On 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 serum was separated for biochemical analysis. Serum biochemical parameters were measured using diagnostic kits (Siemens, India). Organ perfusion was done by injecting the phosphate buffered saline in the left ventricle of the heart after cutting the renal artery. Tissues were removed, blotted and then weighed and relative organ weights were calculated by using formula.

$$Relative\ Organ\ Weight\ \% = \frac{Weight\ of\ the\ organ}{Weight\ of\ the\ animal} \times 100$$

Tissue histopathology. Tissues were then fixed in 10% neutral buffered formalin at 4°C and histopathological examination of vital organs such as brain, heart, kidney, liver, lungs, spleen and small intestine was performed by dehydration of tissues using alcohol, which was them removed in

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xylene and tissues were embedded in paraffin blocks for sectioning. Thin sections of tissues were stained with hematoxylin and eosin.

Oxygen Radical Absorbing Capacity-Fluorescein (ORAC-FL) Assay. The 485-P excitation and 520-P emission wave lengths were filters were used for this assay. DMA (20μL) and fluorescein (FL) (120 μL; 70 nM final concentration) in 200 μL final reaction volume were placed in 96-well microplates (Nunc, Denmark). The mixture was preincubated for 15 min at 37°C. AAPH solution (60 μL; 12 mM, final concentration) was added rapidly and fluorescence was recorded every minute for 80 min with automatic shaking prior to reading. A blank (FL + AAPH) using phosphate buffer (75 mM, pH 7.4) instead of DMA and ten calibration solutions using Trolox (1-10 μM, final concentration) as standard were also carried out. All reaction mixtures were prepared in triplicate. Fluorescence curves were first normalized to the corresponding blank curve of same assay by multiplying 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

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} fi/f0$$

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

Biodistribution study of DMA in EAT bearing Balb/c mice

Radiochemical synthesis of ^{99m}Tc-DMA. DMA was labeled with ^{99m}Tc using sodium pertechnetate, eluted in saline from molybdenum generator by the reported procedure (Panwar et al.,

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2004; 2007). DMA (23.5mg) was dissolved in water for injection (4 ml) in a shielded vial and stannous chloride (250µl: 1mg dissolved in N_2 purged 1ml 10% acetic acid) was added followed by addition of freshly eluted sodium pertechnetate. 6.15mCi radioactivity was added to the solution. The pH of the reaction mixture was adjusted to neutral by drop wise addition of 0.5 M Na_2CO_3 solution, filtered through 0.2 µ filter (4.4ml).

Radiochemical purity of ^{99m}Tc-DMA. The number of DMA involved in ^{99m}Tc complexation was determined by ascending thin layer chromatography (TLC) on ITLC-SG (Paul Gelman, USA) strips using 100% acetone and saline as developing solvent and simultaneously in pyridine: acetic acid: water (PAW) (3:5:1.5) and saline. Count of each component on developed TLC was used to calculate % of free Na $^{99m}TcO_4^-$, reduced ^{99m}Tc and the complex formed between ^{99m}Tc and DMA. ^{99m}Tc-DMA complex remain at origin and free technetium rose 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 1h in a humidified incubator maintained at 5% carbon dioxide, 95% air. Then the samples were centrifuged at 400 g and the serum was collected. To check for any dissociation of the complex in serum, radiolabeled DMA was incubated at 37˚C in human serum for 24 h at 1:1 ratio (50 vol.%). After different time points (0, 1, 2, 4 and 24h), samples were analyzed by ITLC-SG as described above. The percentage of free pertechnetate at a particular time point was estimated (Upadhyay et al., 2010).

Transchelation assay. Binding strength of ^{99m}Tc with DMA was checked by mixing 0.5 ml of the labeled preparation with different concentrations (0.010, 0.030, 0.050 and 0.100M) of diethylene triamine pentaacetic acid (DTPA) and incubated for 1h at 37˚C. The effect of DTPA on labeling efficiency was measured on ITLC-SG using acetone as the mobile phase, which separates the free

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pertechnetate and DTPA-complex (R_f = 0.8–1.0) from the ^{99m}Tc-DMA which remained at the point of application (R_f = 0) (Upadhyay et al., 2010).

Biodistribution of radiolabeled DMA in EAT (Ehrlich Ascites Tumor) bearing mice. Tumors were generated as described earlier and when tumors size reached approximately of 1cm^3 in volume, mice were divided in 8 groups of 4 animals each. An intravenous injection of ^{99m}Tc-DMA in a volume of 100µl (0.140 mCi) was injected through the lateral tail vein of each mouse. Mice were dissected and sacrificed at 1, 2, 4, 6, 8, 12, 18, and 24h post injection and different organs such as brain, heart, lung, liver, spleen, kidney, stomach, intestine, muscle and tumor were taken out in pre-weighed tubes after washing them with normal saline and drying. Organs were weighed and the corresponding radioactivity was measured using γ-scintillation counter along with an injection standard. Uptake of the radiotracer in organs was expressed as percentage injected dose per gram of the tissue (%ID/g) (Kaul et al., 2013).

Scintigraphy in tumor bearing mice. Tumor imaging was performed in EAT-bearing mice after injecting 100µl (0.140 mCi) of radiolabeled DMA intravenously in the lateral tail vein. Whole body images were captured using γ-camera at 2, 4, 6, 18 and 24h post injections. Semi quantitative analysis of the images acquired at different time points to calculate the lesion-to-background ratio of the radiotracer. A region of interest (ROI) was drawn around the lesion and around the contra-lateral thigh to calculate the background activity.

Pharmacokinetic study of DMA in Balb/c mice

Blood sampling and plasma samples extraction. Oral and intravenous pharmacokinetics was performed at a dose of 150 and 75 mg/kg bw, respectivly. Balb/c mice (n = 120, 60 males and 60 females) weighing 20-25 g consisting at least 6 mice per group. Blood samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 18 and 24h after oral and intravenous administration of DMA. Blood

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samples were collected by cardiac puncture after general anesthesia and stored into centrifuge tubes containing EDTA as anticoagulant. The blood samples were centrifuged at 3000 rpm for 10 min at 4°C, and the plasma was separated. Plasma samples were stored at -80°C until HPLC-MS/MS analysis. Plasma (50µl) was spiked with Hoechst 33342 (20 µl of 0.5 µg/ml water solution) as internal standard. Plasma proteins were precipitated by adding 180 µl acetonitrile (final volume was 250 µl each) and the samples were centrifuged at 14000 rpm for 10 min at 4ºC. Clear supernatant (200 µl) was taken and 200µl distilled water was added to get a ~35% acetonitrile solution. The samples were injected onto HPLC-MS/MS system (Gundala et al., 2014).

Enzymatic hydrolysis of DMA conjugates. Glucuronide conjugates were confirmed by treating plasma (200 µl) were treated with β-glucuronidase (50 µl, 500 units) and incubated at 37°C for 1h. Plasma proteins were precipitated by adding 180µl acetonitrile (final volume was 250 µl each) and the samples were centrifuged at 14000 rpm for 10 min at 4ºC. Clear supernatant (200µl) was taken and was added 200µl distilled water to get a ~35% acetonitrile solution. The samples were injected onto HPLC-MS/MS system (Gundala et al., 2014).

Bioanalysis. The stock solutions (each, 1 mg/ml) of DMA and Hoechst 33342 (internal standard) were prepared in ACN:water (95:5 %v/v). A standard DMA calibration from 1-1000 ng/ml with internal standard (20 ng/ml) was quantified. The calibration standards were within ±15% of the nominal concentration and lower limit of quantification was within ±20% of nominal concentration. Sample analysis was performed on positive ionization mode LC-MS/MS with multiple reactions monitoring (MRM, *m*/*z* Q1/Q3) of DMA (*m*/*z* 235.1/412.0, retention time (RT) 5.3 min) and internal standard (*m*/*z* 227.1/396.0, RT 6.5 min) (Agilent 6410 series). The ion spray voltage was set at 3000 V, ionization temperature was 200°C with drying gas flow rate of 20 L/min. Data acquisition and quantitation were performed using Mass Hunter software (Agilent Technologies, Wilmington, DE).

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Separation was achieved using HP1100 series LC (Agilent Technologies) equipped with a photodiode array detector, using an Agilent Zorbax reversed-phase (C18, 2.1 × 50 mm, 5.0 μm) column. The column temperature was maintained at the 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 min), 90 (10-14 min), 30 (14-24 min) with 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 Inc, Missouri, USA) to calculate the pharmacokinetic parameters.

Statistical analysis. Data are expressed as mean ± standard deviation (SD) where applicable. Mean difference between the control and treated groups were analyzed by Student's *t*-test for acute toxicity and one way ANOVA followed by Tukey's multiple comparisons as posthoc test for 28-day repeated toxicity using GraphPad prism 5.0. p value ≤ 0.05 was considered as statistically significant.

Results

Radioprotective efficacy of DMA in mice

Dose reduction factor analysis. A significant increase in percentage survival observed in group of animals treated with DMA prior to irradiation in comparison to those treated with radiation alone. Control mice given saline alone, showed 30-day survival rates of 60, 50, 40, 0 and 0%, when irradiated with 5, 6, 8, 9 and 10 Gy respectively. Pre-treatment with 300 mg/kg bw DMA increased the 30-day survival rates at each dose of γ-radiation with survival rates of 100%, 100%, 80%, 20% and 10% when

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irradiated with 5, 6, 8, 9 and 10 Gy, respectively (Fig. 1A). The Regression analysis of the radiation survival data showed $LD_{50/30}$ of 5.6 Gy in irradiated mice; pre administration of DMA before irradiation increased the $LD_{50/30}$ to 7.2 Gy, resulting in a DRF value of 1.28 (Fig. 1B). Further, the DMA treatment also delayed the appearance of radiation sickness (characterized by weight loss, irritability, lethargy, ruffling of hair, emaciation and epilation).

Cellular uptake and efflux study. DMA reached its highest level in 2h in cells (Fig. 1C). Further efflux study showed retention of DMA in cells for next 22 h. There was 65% DMA retained in HEK293 cells which show higher affinity and binding of DNA to cellular DNA (Fig. 1D).

DMA do 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 hour before each radiation treatment. 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 above animals. There was no significant reduction in tumor growth was observed in DMA treated, but in Radiation and DMA+ Radiation there was a significant reduction in tumor-growth (Fig 1 E). The control and DMA alone treated animal died by 16 day due to tumor whereas death of all PBS-treated animals was from radiation toxicity by day 18. On the contrary DMA pretreatment mice survived till day 20 suggesting significant protection against radiation induced mortality but had no radioprotective effect on the tumors. Thus DMA alone does not have any effect on tumor growth itself.

Oxygen Radical Absorbing Capacity-Fluorescein (ORAC-FL) Assay. The presence of fluorescence signal of fluorescein was constant over 80 min in the absence of AAPH, indicates its photostability under the excitation conditions used. When AAPH was added (blank) the fluorescence signal dropped with time at increasing concentrations of Trolox (1-10μM). (Fig. 2A & B). The

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similar results were observed for DMA (2-100μM) showing that the decay of the curve was effectively delayed. The relative ORAC value (Trolox equivalents) was calculated as

Relative ORAC value = $[(AUC_{Sample} - AUC_{Blank})/(AUC_{Trolox} - AUC_{Blank})]$ X (molarity of Trolox/molarity of sample).The relative ORAC value (Trolox equivalents) was found to be 1.5 for DMA.

Toxicity Studies

Acute toxicity study. DMA did not cause mortality and toxicity symptoms such as abnormal spirit and behaviors at oral dose of 300, 500, 750, 1000, 1500 and 2000mg/kg bw where 2000mg/kg bw was taken as the limit dose as per the OECD 423 guidelines. Food and water intake was normal. Body weights did not change. The dose of 125mg/kg bw of DMA was determined to be LD_{50} in case of single intravenous administration (Fig. 3A). On 15^{th} day, when animals were sacrificed, there were no significant change in weights of the individual animals and it was found that there was no change in organ appearance and organ coefficient (relative organ weight) except in liver at 500mg/kg bw and intestine at 2000mg/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. Food and water intake were reduced. No mice survived when treated with DMA at a repeated dose of 450mg/kg/day, all were found dead within one week period of DMA treatment. Fifty percent animals survived the DMA dose of 225mg/kg/day for a period of 28-days and no animal was found dead till the complete duration of treatment when treated with 112.5mg/kg/day. So 225mg/kg bw was considered to be LD_{50} in case of repeated administration (Fig. 3B). Insignificant differences were found in organ appearance and relative organ weights of brain, heart, liver, lung, spleen, kidney, stomach, and intestine recorded between the control and DMA treated groups.

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Estimation of biochemical parameters. Serum biochemistry parameters levels (Table 1) were found to be insignificantly changed in DMA treated animals in comparison to saline treated control animals. Serum estimation for biochemical parameters of group with dose 225mg/kg/day and 112.5mg/kg/day showed insignificant between control and treated groups (Table 2).

Histopathology. No gross pathological changes were observed in histopathological sections of brain, heart, liver, lung, spleen, kidney and intestine in acute and 28-days 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 and myocardial fibrosis was observed and endocardium, epicardium, myocardium and myocardial interstitial tissues were normal. In the liver hepatic lobule, sinusoidal, plate and hepatic cell structure were also found unchanged and no inflammatory cell infiltration and necrosis was observed. In the kidneys, the structure of renal cortex and medulla was normal and in the renal corpuscles there was no hyperaemia or exudation. Intestinal mucosa showed no change in intestinal villi and crypt of Lieberkuhn with normal villous architecture, submucosa and muscularies. Splenic lymphocytes were also observed to be normal in all animals (Supplemental Fig. 2 & 3).

Biodistribution Study of DMA in Balb/c Mice

Radiochemical synthesis and purity of ^{99m}Tc-DMA. DMA was successfully labeled by ^{99m}Tc. Radio labeling efficiency was checked by ITLC strips and it was found to be more than 98% and colloids were less than 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 and it was found that the radio complex was quite stable up to 24 h, 3% of the radiolabeled DMA got dissociated in serum at 24 h (Supplemental Fig. 4).

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Transchelation assay. Incubation of radiolabeled DMA with different concentrations (0.010, 0.030, 0.050 and 0.100 M) of diethylenetriaminepentaacetic acid (DTPA) for 1 h at 37 ˚C showed that binding strength was sufficient enough that even 0.1 M DTPA could be able to dissociate only 3.34% free technetium from the radio complex and 99.96% was remained as labeled DMA (Supplemental Fig. 5). Thus it strengthens the biodistribution of labeled DMA which is mainly due to DMA and not by free ^{99m}Tc.

Biodistribution of radiolabeled DMA in EAT-tumor bearing mice. Biodistribution study suggested that the uptake of radiolabeled DMA was higher in lungs, liver, spleen and kidney. At 1h post injection, the percentage of 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 ± SD) in lungs, liver, spleen and kidney, which was dropped to 5.90 ± 0.06, 6.88 ± 0.98, 3.35 ± 2.36 and 6.03 ± 0.97 (% ID/g ± SD) with ~3 fold decrease in the amount of injected dose at 24h time point. In comparison, the uptake in 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, and 24 h after administering the radiocomplex intravenously (100µl, 0.140mCu). Semiquantitative analysis generated from region of interest (ROI) placed over areas counting average counts per pixel showed that uptake of ^{99m}Tc-DMA in tumor with tumor to muscle ratio of 5.37 ± 0.54, 7.53 ± 0.64 and 3.95 ± 1.5 at 2, 4 and 24h time points. Scintigraphy images showed the time dependent clearance of radiocomplex from the organs and tumor (Fig. 4B).

Pharmacokinetics of DMA in Balb/c mice. No additional peak of endogenous compounds was found in blank plasma HPLC-MS/MS chromatogram (Supplemental Fig. 6A) at the RT of DMA and internal standard. The DMA was spiked at different concentrations in plasma, processed and the clear supernatant was injected onto HPLC-MS/MS. DMA and Hoechst 33342 were eluted at 5.3 and

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6.5 min, respectively (Supplemental Fig. 6C). A calibration curve was plotted between DMA plasma concentration 1, 2, 4, 10, 20, 40, 100, 200, 400, 1000 ng/ml and the peak area ratio of DMA vs IS (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 plasma concentration-time profile and the pharmacokinetic parameters of DMA in Balb/c mice are shown in Fig. 5. Following intravenous administration, DMA was detected in plasma up to 24 h. The elimination half-life, volume of distribution at steady-state and systemic clearance were 4 h, 36.7 L/kg and 27.4 L/h/kg, respectively. Following per oral dosing, DMA exhibited two C_{max} (C_{max1}: 303.6 ± 29.7 ng/ml and C_{max2}: 57.7 ± 5.8 ng/ml) at 1 and 4h post dose indicating that DMA is rapidly absorbed.The bioavailability (%F) was calculated from $\%F_{p.o.} = \{(AUC_{peroral} * Dose_{intravenous})/(AUC_{intravenous} * Dose_{peroral})*100\}$ and was found to be 8.8%. To delve further, a part of the plasma samples obtained after 150 mg/kg oral dose was analyzed after β-glucuronidase hydrolysis. The hydrolyzed plasma samples showed 1.5-times higher AUC (705.6 ng*h/ml) than those observed in unhydrolyzed plasma samples (Fig. 5).

Discussion

Ionizing radiations employed in radiotherapy, are not exclusive, in protecting surrounding normal tissues over tumor tissues and this creates a small molecular difference between normal and cancerous cells render it as 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 and for this purpose radioprotectors are in demand in clinical radiotherapy. Several naturally occurring or synthesized compounds have been explored and investigated in biological systems to protect against the radiation exposure induced damage but these molecules are associated with their own

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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 2D PAGE followed by peptide finger printing, revealed large number of genes that are regulated in response to DMA and /or IR. 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 to control. We narrowed down to a unique mechanism of radioprotection of DMA. We observed that NIK/IKKα/IKKβ mediated NFκB activation by DMA alone or in combination with IR promotes prosurvial pathway in cells (Kaur et al., 2012). In order to establish clinical relevance of DMA as an anti-irradiation drug, it's *in vivo* toxicity and efficacy parameters were investigated in animal model. Acute toxicity values such as LD_{50} and toxicity symptoms are used as the basis to classify the chemical compounds into toxicity categories and their further regulations (Zbinden and Flury-Roversi, 1981). A 2000 mg/kg bw was taken as the limit dose as per the OECD 423 guidelines for acute toxicity. Mortality rate, serum biochemical parameters and tissue histopathology were used as end points for 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, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are used to identify the acute hepatic injury which is necessary to investigate and recognize chemical-induced hepatotoxicity. Liver is the main organ involved in lipid metabolism so in order to relate the hepatic damage, other parameters such as glucose, triglycerides, total proteins and total cholesterol were also measured to relate to the liver 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, BUN, uric acid, urea etc. in DMA treated animals at

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different doses in comparison to control group (Table 1 & 2). No gross pathological changes in histopathology in any of the organ was observed, 0% mortality and no noticeable toxicity such as abnormal behaviors upto limit dose of DMA were observed. The histopathological analysis does not show any vascular and inflammatory changes (Supplemental fig. 2 & 3) such as centrilobular necrosis, congested sinusoids and nuclear changes. No cellular infiltration and degeneration in liver, kidney, brain as well as lung tissues were reported. In addition, no damage to crypt epitheliums and epithelial cells in intestine was observed, megakaryocytes were normal in spleen. DMA treatment did not show any vascular congestion in the heart with inflammatory changes in the cardiomyocytes. As per GHS, for DMA, LD_{50} value could not be achieved up to 2000 mg/kg bw so it can be classified as chemical agent under category 5 and this depicts its nontoxic behavior following oral administration. In comparison, the LD_{50} of known radioprotectors at oral dose is reported, 810 and 1510 mg/kg bw for WR2721 (amifostine) and NIT2011 (Wang et al., 2013). 28-days repeated administration of DMA at 450mg/kg bw/day dose was lethal and all mice died within 7 days, however 50% animals were survived till the date of sacrifice with 225mg/kg bw/day. Although treatment related physical changes were observed i.e.; reduction in food and water intake, diarrhea, loss of agility, decrease in bw but no significant change was observed in biochemical parameters and histopathology. Significant change in body weights, along with diarrhea (a symptom of gastrointestinal syndrome) was observed in mice treated with repeated dose of DMA (225 & 450 mg/kg bw/day) (Fig. 3C). This might be one of the reasons of mortality of animals . No mortality was observed at 112.5mg/kg bw/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

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comparable to the DRF value of known radioprotectors (Hahn et al., 1992; Landauer et al., 2000; Lee et al., 2008; Krishna and Kumar, 2005; Mantena et al., 2008; Saini et al., 2011; Shrikant et al., 2012) (Table 3). Although it will not be correct to rank radioprotectors as they had been given by different routes, but if we compare among orally administered synthetic molecules DMA can be ranked second which has shown good radioprotection at single dose and no toxicity was observed upto 2000 mg/kg dose. Whereas, a subcutaneous injection of 500 mg/kg bw of a radioprotective agent, Ex-RAD or ON01210.Na (4-carboxystyryl-4-chlorobenzylsulfone, sodium salt) in mice, 24 h and 15 min prior to radiation showed DRF of 1.16 (Ghosh et al., 2012). Single subcutaneous injection of 200 mg/kg genestein, given at 24h before radiation showed DRF of 1.16 (Landauer et al., 2003). Gamma-tocotrienol, a tocol antioxidant and a potent radioprotector, at an optimal dose of 200mg/kg given subcutaneously 24 h before irradiation had a DRF of 1.29 in CD2F1 mice (Ghosh et al., 2009). For the drugs meant for prophylactic or therapeutic use, oral route is a most advantageous and practical way for administration to treat radiation casualties from a radiation accident in civilians and military personnel, or first responders exposing to radiation contaminated areas. It is, therefore, of great interest to determine the radioprotective efficacy of an agent after oral administration. The DRF for oral administration of 17-dimethylamino ethylamino-17- demethoxygeldanamycin, 24 h before irradiation was 1.68 (Lu et al., 2013). Reactive oxygen species cause DNA damage and induce cellular toxicity by inducing 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, DNA damage reduction and free radical quenching capacity of DMA by gel electrophoresis and ORAC assay was analyzed. The free radical quenching and DNA binding property of DMA increased prevention of DNA damage by two fold. DMA almost retained cellular genomic DNA integrity which, was not observed in case of irradiated conditions. ORAC- FL assay was

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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-amidino-propane) dihydrochloride (AAPH). 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), serves as positive control inhibiting fluorescein decay in a dose dependent manner. It confirmed free radical scavenging property of DMA responsible for marked reduction in radiation-induced DNA damage (Fig. 2A & 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 less than 1.5 for vitamin C (0.95) and glutathione (0.62) and more than 1.5 for chlorogenic acid (3.14) and genistein (5.93). This shows free radical scavenging and antioxidant property of DMA by inhibiting the decrease in fluorescence of fluorescein. *In vitro* cellular uptake and efflux study showed 65% DMA was retained in HEK293 cells upto 24h which show higher affinity and binding of DMA to cellular DNA. DMA had no effect on the growth of unirradiated or irradiated tumors (Fig. 1E) DMA do not protect the tumor cells as it enters less in tumor cells.

An efficient radioprotector reaching in high concentration in normal tissues than in tumor could demonstrate its selective protection. EAT, a murine breast cancer cells can develop ascites, if ascites fluid injected intraperitoneally, but if it is given subcutaneously, a solid form is obtained (Mehmet et al., 2011). Therefore to measure the *in vivo* tissue uptake, biodistribution of ^{99m}Tc-DMA was performed in EAT bearing mice. ^{35}S-labeled WR-2721 biodistribution and blood clearance studies in C3H mice bearing RIF-1 tumors after intraperitoneal injection were reported to identify those tissues and post injection times which would suggest a high level of radioprotection, because the ability to protect late-responding normal tissues of clinical relevance against radiation damage was of more interest. It was reported that the concentration of protector (milligram per gram tissue) in various

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organs at 30 min postinjection ranked in the order: kidney> liver = lung > gut > heart> blood > skin> tumor > brain (Rasey et al., 1984). Biodistribution of [^{125}I]iodo Hoechst 33342 in LS174T tumor bearing mice following 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 biodistribution of ^{99m}Tc-DMA in EAT bearing mice showed that major organs for DMA uptake are lungs, liver, kidneys and spleen than tumor. The high uptake and accumulation of ^{99m}Tc-DMA in liver and kidney at each time point might be due to its hepatobiliary and renal excretion. Pharmacokinetics showed that after oral administration, DMA was quickly absorbed, distributed and exhibited a multiple peak phenomenon in mice. However, its large volume of distribution (Vss) 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 liver. Some of the glucuronides are secreted via biliary route into the intestine and have poor reabsorbtion 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 1.5 times higher AUC (705.6 ng*h/ml) than those observed in unhydrolyzed plasma samples. The volume of distribution (V_{ss}, 36.7 L/kg) of DMA is larger than the total blood volume of mouse (0.085 L/kg) (Davis and Morris, 1993) indicating that the compound is readily distributed in extravascular system. However, the systemic clearance of DMA is higher than the hepatic blood flow of the mouse (5.4 L/h/kg) which indicates an extrahepatic elimination of the compound.

In summary, cellular uptake, efflux study, biodistribution and pharmacokinetics suggest longer retention and tissue affinity and thus sustained free radical scavenging for longer period of

time that led to better radioprotection with DMA by preventing DNA damage. Moreover its selective uptake in normal tissues than tumor could be taken as relevant observation in the development of radioprotectors against normal tissue injury. Although, TBI affects multiple organs, causing death due to GI syndrome and hematopoietic syndrome , which causes death within 30d after exposure to 3-8 Gy. DMA improved survival over a wide range of radiation doses 5-10 Gy, suggesting that it can protect both GI and hematpoietic injury.

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Authorship Contributions

Participated in research design: Nimesh, Tiwari, Tandon, Chuttani, Hazari, Mishra, Katyal, Gundala, Aneja.

Conducted experiments: Nimesh, Tiwari and Tandon.

Performed data analysis: Yang, Lal, Sharma, Nimesh, Tiwari, Hazari, Tandon.

Wrote or contributed to the writing of the manuscript: Nimesh, Tiwari and Tandon

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Footnotes

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Figure Legends

Fig. 1. (A) % Survival of Balb/c mice after total body irradiation to 5, 6, 8, 9 and 10 Gy irradiation, with or without oral 300mg/kg bw DMA pre-treatment; (B) Plot of % survival of Balb/c mice irradiated at different doses (5, 6, 8, 9, and 10 Gy) to radiation after oral 200mg/kg bw DMA treatment (n = 6 in each group) for DRF calculation; (C) Graphical representation of DMA uptake in HEK293 cell line (from 3 independent experiments); (D) Graphical representation of DMA efflux from HEK293 cell line (from 3 independent experiments); (E) Effect of 300mg/kg bw oral DMA on radiation treated EAT growth in Balb/c mice. Tumor bearing mice (n=5) treated with saline, DMA alone, 8 Gy TBI alone and DMA+Radiation (8 Gy TBI). Data is nonsignificant between groups receiving 8 Gy TBI. Tumor volumes were measured until animals died.

Fig. 2. (A) Effect of trolox concentrations on Fluorescein (FL) fluorescence decay curve; (B) Effect of DMA concentrations effect on Fluorescein (FL) fluorescence decay curve.

Fig. 3. (A) % survival of Balb/c mice after single intravenous injection of DMA at different doses (300, 200, 150, 125 and 100 mg/kg bw) (n = 6 in each group); (B) % survival of Balb/c mice after 28-days repeated oral administration of DMA at three doses (450, 225 and 112.5mg/kg bw) (n = 6 in each group) ; (C) % Body weight loss in mice after repeated administration of DMA at 225 and 450 mg/kg bw, mice. n=6, results are means±SD. We did not observe any death at bolus oral dose upto 2000mg/kg bw (Data not shown).

Fig. 4. (A) Biodistribution of ^{99m}Tc-DMA at different time points after injection in EAT bearing mice. Data are represented as mean % injected dose/g (% ID/g) ± SD; (B) Scintigraphic images of EAT-bearing mice showing the uptake and clearance of radiolabeled DMA at 2, 4 and 24h postinjection.

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Fig. 5. Pharmacokinetic parameters and mean plasma concentration-time profile of DMA after intravenous (75 mg/kg bw) and oral (150 mg/kg bw) dose (before and after hydrolysis with β glucuronidase) in Balb/c mice. Bar represents SEM. Each value represents the average of six mice dosed orally[a] (150 mg/kg bw) and intravenously[b] (75 mg/kg bw); Values of C_{max} are mean ± SEM; AUC_{0-t} = area under the plasma concentration-time curve upto last sampling time, C_{max} = plasma peak concentration, t_{max} = time to C_{max}, $t_{1/2}$ = elimination half-life, MRT_{last} = mean residence time up to last sampling time, V_{ss} = volume of distribution at steady-state

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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.

Biochemical Parameters	Dose (mg/kg bw)					
	Control	300	500	750	1500	2000
Total protein (g/dL)	5.01±1.07	4.41 ±0.07	5.51±1.87	5.55±0.31	5.75±0.34	5.48±0.37
Albumin (g/dL)	2.67±0.32	2.96±0.22	2.87 ±0.39	2.53 ±0.08	2.59±0.1	2.69±0.18
Cholesterol (mg/dL)	109.21± 16.4	86.75±10.28	97.13±8.99	92.09±13.6	93.28±16.4	82.79±18.4
Triglyceride (mg/dL)	115.89± 23.29	105.89±23.2	120.58± 33.1	79.56±26.9	71.42±16.3	94.38±17.1
Urea (mg/dL)	20.19±4.71	19.83±0.33	28.50 ±3.25	29.76 ±5.24	37.15±5.33	32.22±6.8
Blood Urea Nitrogen (mg/dL)	9.41±2.17	9.26±0.15	13.20±1.52	13.71 ±2.71	12.34±2.87	15.09±3.2
Uric acid (mg/dL)	2.19±0.30	2.8±0.98	2.18±0.32	2.88 ±0.70	2.36±0.24	1.99±1.03
Creatinine (mg/dL)	1.90±0.36	1.96±0.18	2.20±0.29	2.24±0.60	1.90±0.17	1.79±0.14
Total bilirubin (mg/dL)	0.25±0.33	0.23±0.03	0.28±0.18	0.22±0.19	0.14±0.04	0.27±0.09
Direct bilirubin (mg/dL)	0.16±0.26	0.18±0.07	0.08±0.08	0.10±0.08	0.24±0.06	0.11±0.02
Glucose (mg/dL)	137.82± 28.47	123.76± 6.28	132.53± 7.88	143.23± 6.55	153.77± 28.98	145.22± 34.38
Alkaline Phosphatase (U/L)	50.47±9.34	61.42±4.80	56.76±19.15	53.08±24.68	43.95±5.11	49.92±2.11
AST (U/L)	26.85±4.72	29.81±5.67	24.33±7.22	27.33±5.19	32.98±16.21	30.33±3.05
ALT (U/L)	6.90±1.36	6.99±1.36	7.10±1.98	7.40±2.36	7.55±1.70	7.89±3.22

Values are expressed as mean ± standard deviation (mean ± SD) with n = 4 for control and treatment groups. Statistical analysis was performed by Student t-test. p value ≤ 0.05 was considered as significance. Samples were analyzed in triplicates.

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TABLE 2; Serum biochemical parameters of control (saline treated) and mice treated with DMA with repeated administration at 112.5 and 225 mg/kg bw for 28-days.

Biochemical Parameters	Control (Saline treated)	112.5mg/kg bw	225mg/kg bw
Total protein (g/dL)	32.25±8.8	39.91±0.92	35.89±6.23
Albumin (g/dL)	4.31±1.20	3.37±0.31	3.93±2.01
Cholesterol (mg/dL)	56.87±9.48	62.01±12.73	57.10±0.97
Triglycerides (mg/dL)	143.15±4.47	161.84±54.28	157.81±39.87
Urea (mg/dL)	59.24±12.49	63.91±5.24	71.72±8.39
Blood Urea Nitrogen	27.66±5.83	29.84±2.45	33.49±3.92
Uric acid	4.16±1.41	5.07±1.82	3.62±1.26
Creatinine	2.30±0.34	2.10±0.10	1.83±0.02
Total bilirubin	0.25±0.13	0.27±0.23	0.29±0.20
Direct bilirubin	0.33±0.19	0.25±0.13	0.26±0.23
Glucose (mg/dL)	174.39±42.35	188.82±49.97	190.25±77.07
Alkaline Phosphatase (U/L)	25.38±1.46	27.46±4.67	28.48±12.48
AST (U/L)	37.25±2.55	36.12±4.21	40.25±3.88
ALT (U/L)	29.22±4.01	28.78±2.33	33.45±2.98

Values are expressed as mean ± standard deviation (mean ± SD) with n = 6 for control and treatment groups. Statistical analysis was done by one way ANOVA followed by Tukey□s multiple comparisons as posthoc test using Graph Pad prism 5.0. p value ≤ 0.05 was considered as significance. Samples were analyzed in triplicates.

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TABLE 3; List of known radioprotectors with rank on the basis of their DRF values.

Radioprotector	DRF/Route of Administration	Radioprotector	DRF/Route of Administration
Amifostine	1.8/i.p.	Thymol	1.25/i.p
Glutathione	1.7/i.p	Fucoidan	1.20/i.p.
Aloe gel	1.47/Oral	4-carboxystyryl-4-chlorobenzylsulfone, sodium salt	1.16/s.c.
Rajgira	1.36/Oral	Rutin	1.14/Oral
DMA	1.28/Oral	CBLB502	1.3/s.c.
δ-tocopherol	1.27/s.c.	5-Aminosalicylic acid	1.08/i.p.

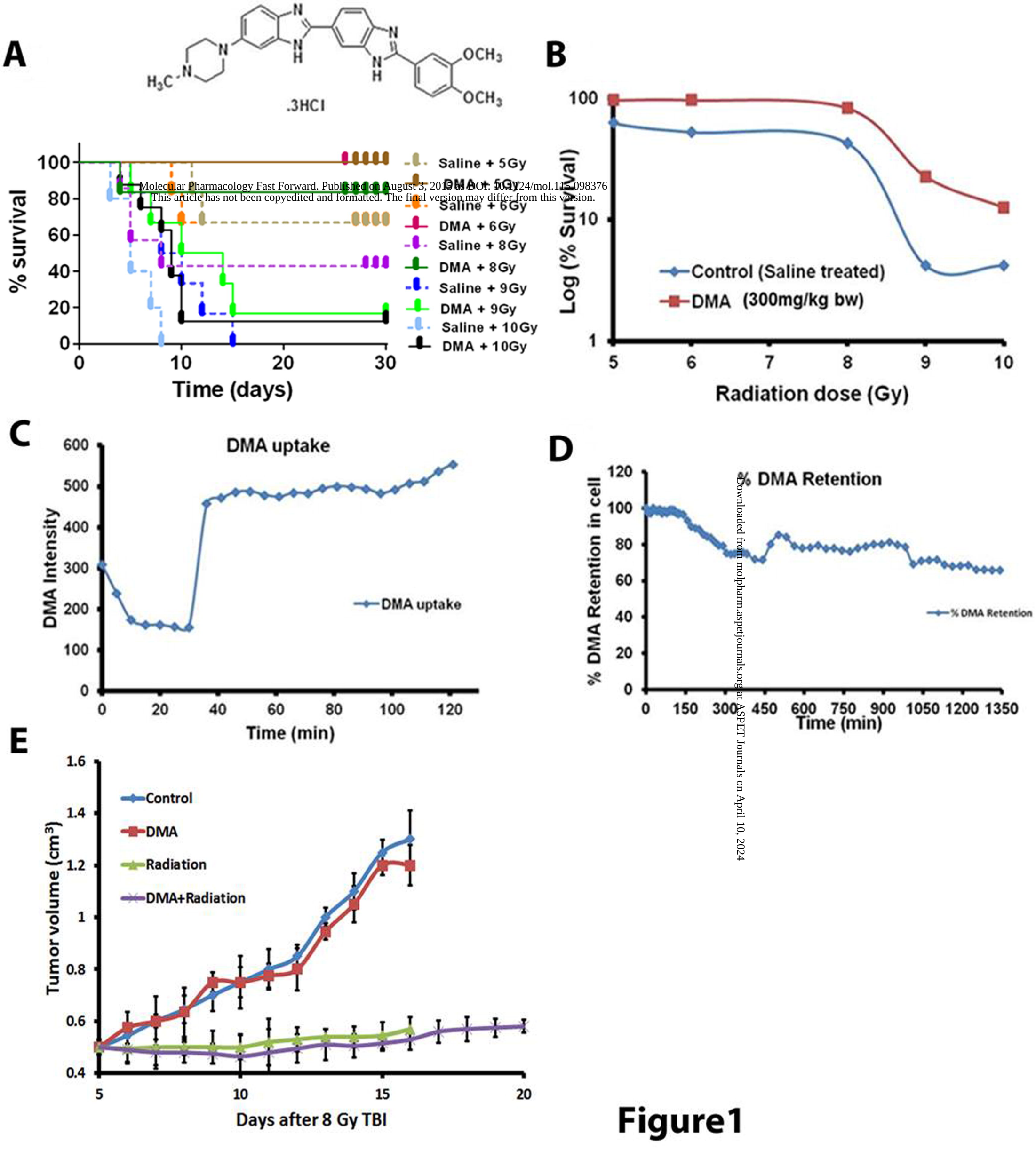


Figure1

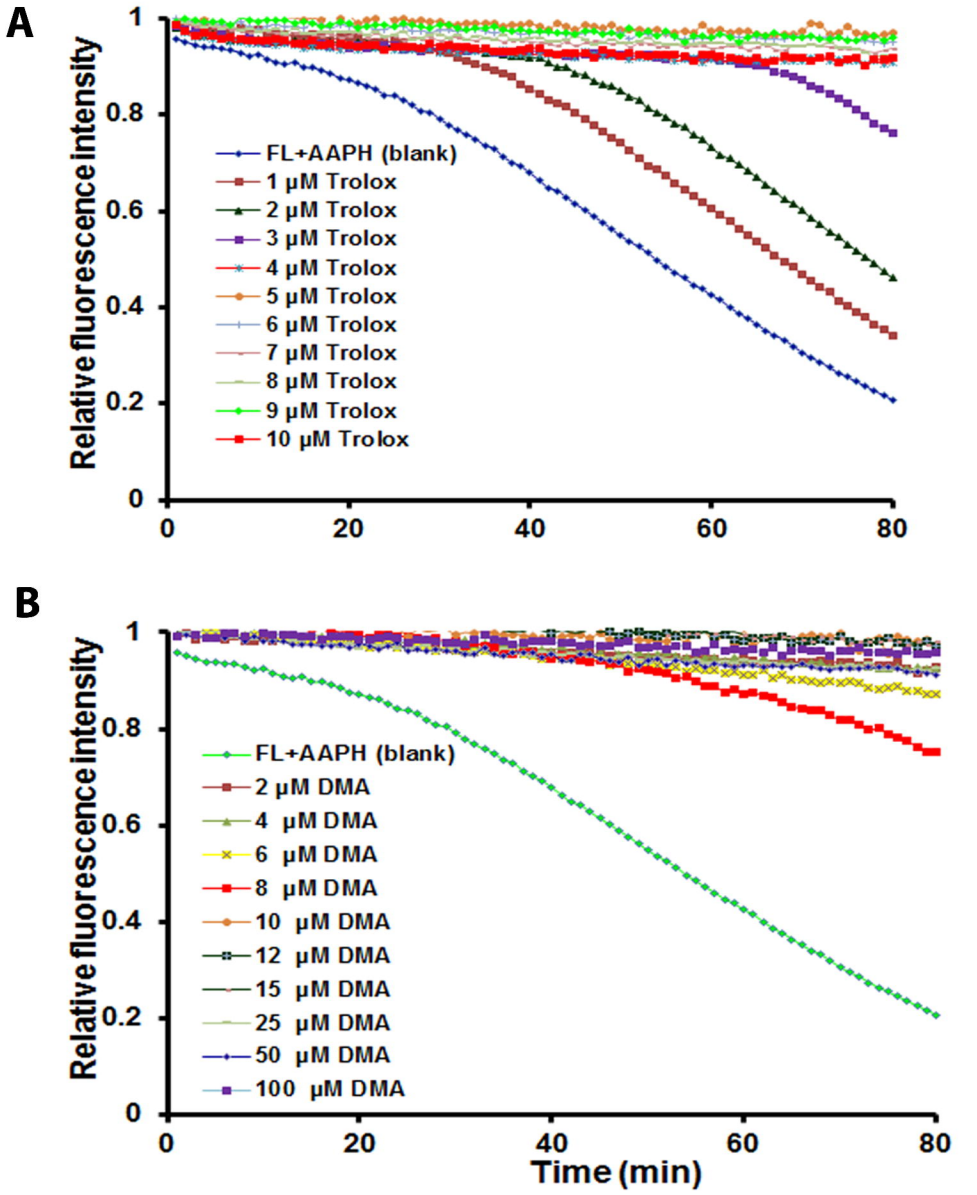

A
Relative fluorescence intensity
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
FL+AAPH (blank)
1 μM Trolox
2 μM Trolox
3 μM Trolox
4 μM Trolox
5 μM Trolox
6 μM Trolox
7 μM Trolox
8 μM Trolox
9 μM Trolox
10 μM Trolox
B
Relative fluorescence intensity
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
Time (min)
FL+AAPH (blank)
2 μM DMA
4 μM DMA
6 μM DMA
8 μM DMA
10 μM DMA
12 μM DMA
15 μM DMA
25 μM DMA
50 μM DMA
100 μM DMA

Figure 2

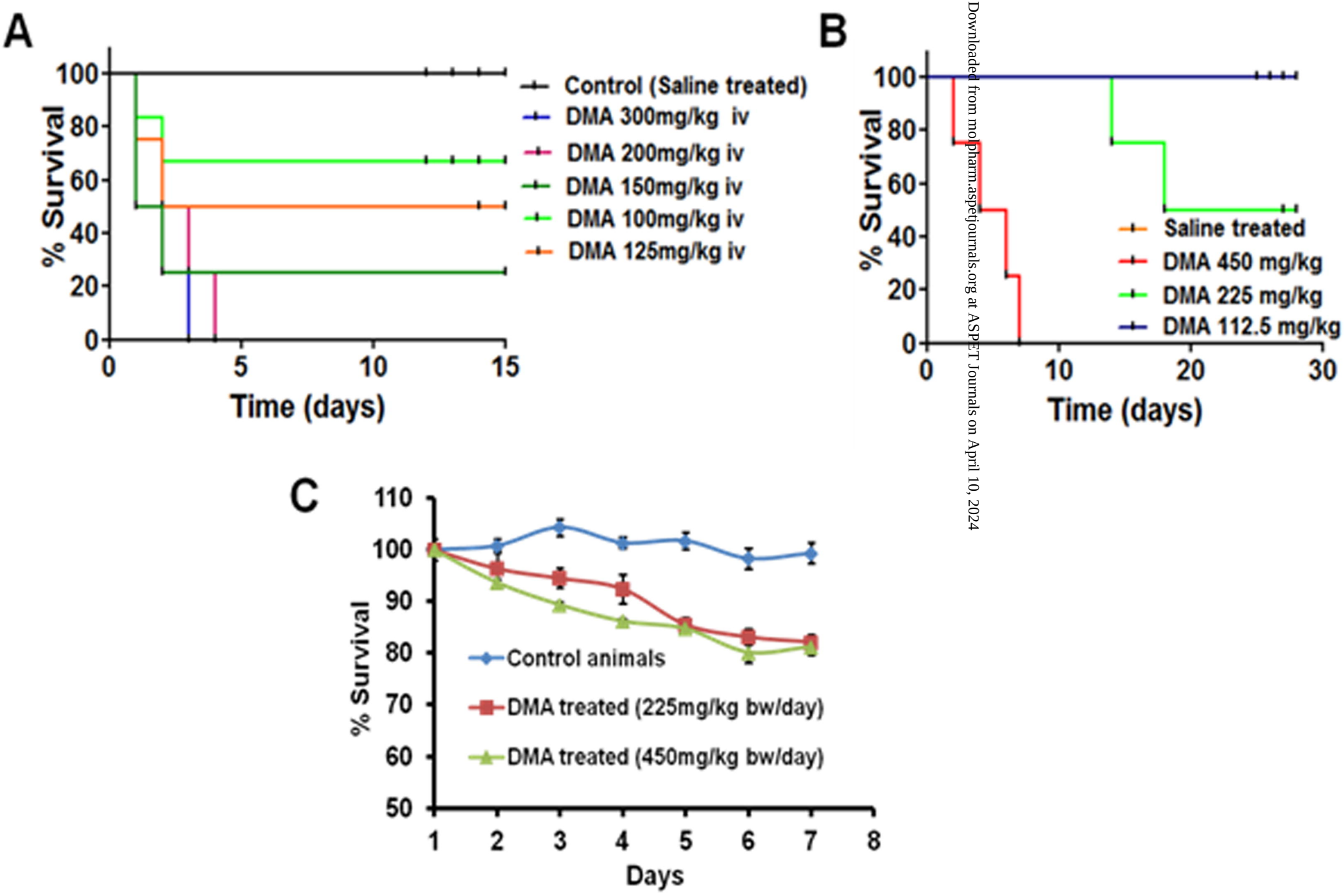


Figure 3

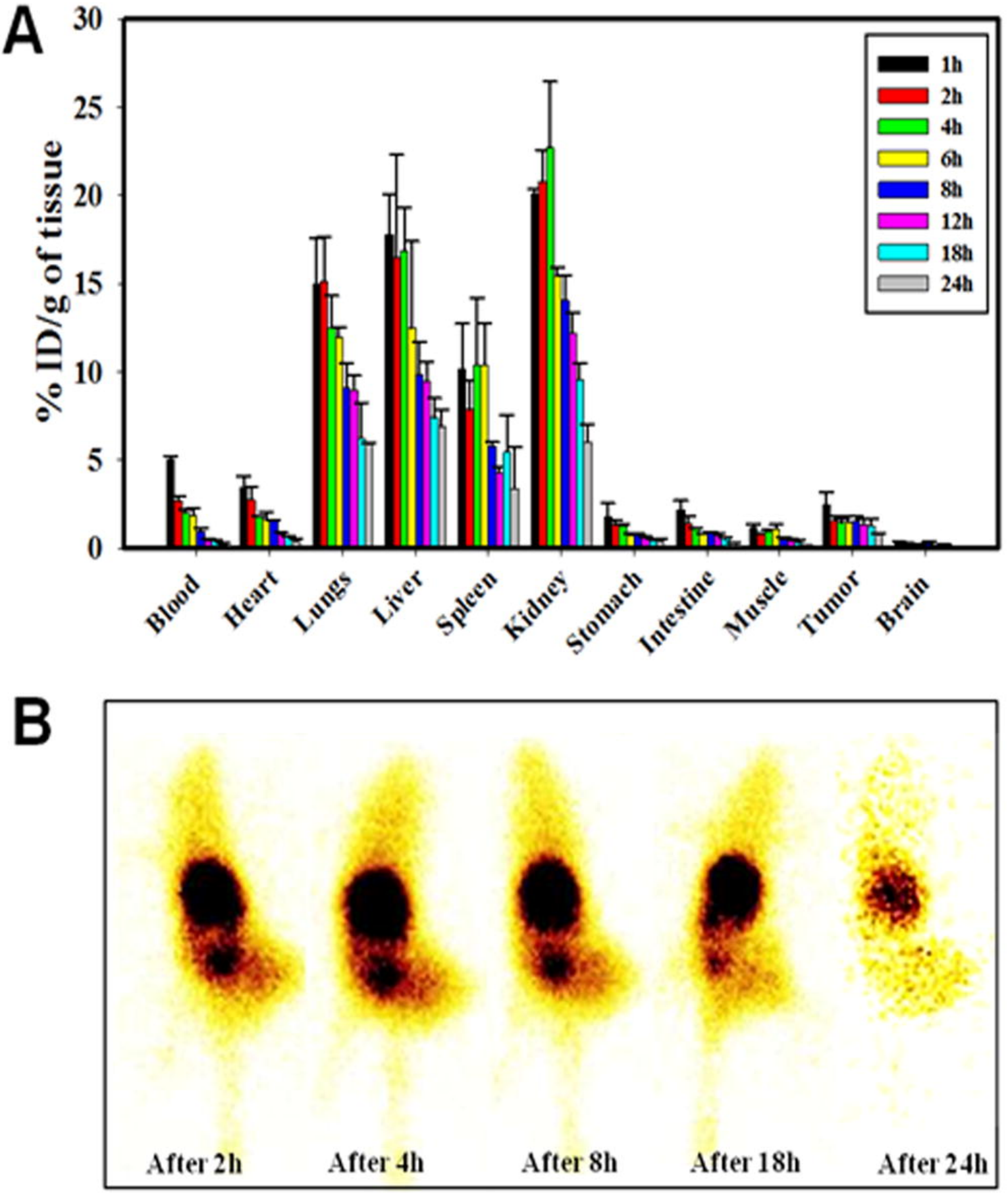
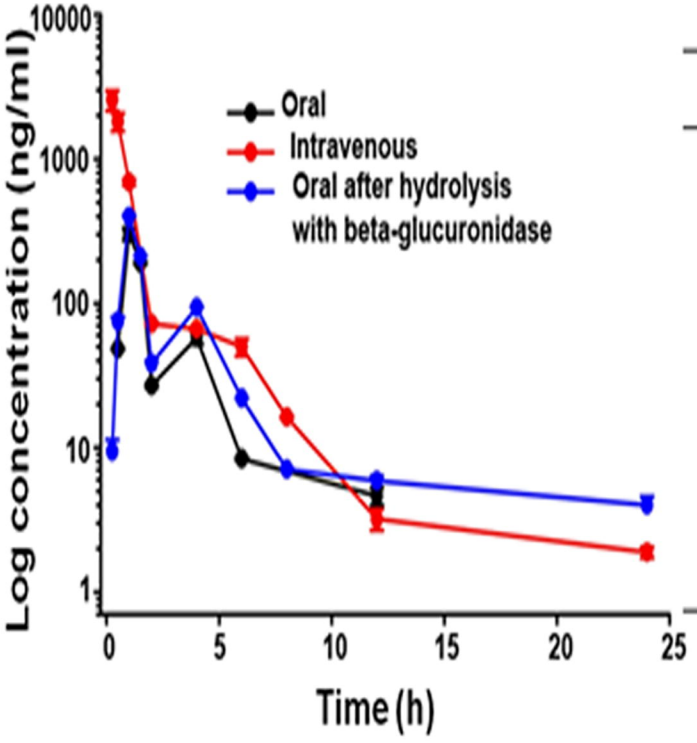

A
30
25
20
15
10
5
0
% ID/g of tissue
1h
2h
4h
6h
8h
12h
18h
24h
Blood
Heart
Lungs
Liver
Spleen
Kidney
Stomach
Intestine
Muscle
Tumor
Brain
B
After 2h
After 4h
After 8h
After 18h
After 24h

Figure 4



Parameters	Oral[a]	Intravenous[b]
C_{max} (ng/ml)	303.6 ± 29.7	-.
t_{max} (h)	1	-
AUC_{0-t} (ng h/ml)	467.5	2723.2
$t_{1/2}$ (h)	-	4
MRT_{last} (h)	2.6	1.2
V_{ss} (L/kg)	71.6	36.7
Clearance (L/h/kg)	27.5	27.4
Bioavailability (%)	8.84	-

Figure 5