RPS Research in Pharmaceutical Sciences, February 2014; 9(1): 23-29
Received: Jan 2012
Accepted: Dec 2012

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Radioprotective effect of sulphydryl group containing triazole derivative to modulate the radiation- induced clastogenic effects

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Abstract

Protection of biological systems against radiation damage is of paramount importance during accidental and unavoidable exposure to radiation. Several physico-chemical and biological factors collectively contribute to the damage caused by radiation and are, therefore, targets for developing radioprotectors. Chemicals capable of scavenging free radicals, relieving oxidative stress, promoting antioxidant activity and modulating immune response have been some of the radioprotectors extensively investigated with limited success. It has long been known that some of the most effective radioprotective agents are those which contain sulphydryl groups. 4-amino-5-mercapto-3-methyl-1, 2, 4-triazole (AMMT) is one of the well-known 1, 2, 4 triazole derivatives with functional sulphydryl group. The present study reports an evaluation of radical scavenging and radioprotective properties of sulphydryl group containing triazole derivative. The lethal dose of electron beam radiation (EBR) was studied by survival assay. The dose reduction factor (DRF) of AMMT was calculated using the ratio between LD_{50} of EBR with and without AMMT treatment. Radical scavenging property of AMMT was assessed by DPPH radical scavenging assay. The clastogenic effects of EBR were recorded by Micronucleus test in bone marrow cells and DNA fragmentation assay in mice hepatic cells. The survival assay results showed that the LD_{50} of EBR was 10 Gy. The calculated DRF for AMMT was found to be 1.2. DPPH radical scavenging assay showed a positive result when it was compared with the standard glutathione. Treatment of mice with 100 mg of AMMT for 15 days before irradiation significantly ($P<0.05$) reduced the frequency of micronucleus formation in bone marrow cells and also reduced the DNA fragmentation in hepatic cells. The results obtained in the present study indicated that AMMT has a protective effect against the EBR-induced mortality and clastogenicity.

Keywords: Electron beam radiation; DNA damage; Radioprotection; Triazole; Sulphydryl; Micronucleus

INTRODUCTION

Protection against ionizing radiation has practical applications in cancer radiotherapy and in the reduction of risk to exposed individuals. Many natural and synthetic/semi-synthetic chemicals have been investigated in the recent years for their efficacy to reduce adverse effects of ionizing radiation (1,2). However, at radioprotective concentration the inherent toxicity for some of the synthetic agents warranted further search of safer and effective compounds (3–5). Therefore, within these constraints, the strategy to evaluate the radiation protection ability of non-toxic and physiologically acceptable compounds seems promising and warrants investigation. It is widely accepted that most of the radiation induced biological damage arises from the interaction of free radicals with vital cellular biomolecules such as DNA, proteins and lipids (6).

It has long been known that some of the most effective radioprotective agents are those which contain sulphydryl groups, such as cysteine and cysteamine (7). Protecting against ionizing radiation by sulphydryl-containing compounds was first described by Patt and coworkers in the late 1940s (8). U.S. Walter Reed Army Institute of Research synthesized and screened about 4500 sulphydryl group containing compounds for this purpose. Among these only ‘amifostine’ which finds applications in radiotherapy was found suitable

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for human application while the rest were not suitable due to acute toxicities (9,10).

Triazoles are the important class of heterocyclic compounds having three nitrogen atoms. They are of two types, 1, 2, 3 triazoles and 1, 2, 4 triazoles. Various 1, 2, 4 triazoles and its derivatives are found to be linked with diverse pharmacological activities. Compounds containing 1,2,4-triazole ring have been reported to possess different biological activities such as antimicrobial (11), antifungal (12), antioxidant (13), anti-inflammatory (14), antiviral (15), anticancer (16,17), analgesic (18) and anti-convulsant (19) activities depending on the substituent in the ring system.

With this background, the aim of this study was to assess the radioprotective effect of sulphydryl group containing triazole derivatives to modulate the radiation induced clastogenic effects.

MATERIALS AND METHODS

Chemistry

4-amino-5-mercapto-3-methyl-1, 2, 4-triazole (AMMT- Fig. 1) was prepared according to the methods proposed in literature (20). The synthesized compound was then screened for its toxic dose and solubility. Melting points were taken in open capillary tubes and are uncorrected. The purity of the compounds was confirmed by thin layer chromatography using Merck silica gel 60F254-coated aluminum plates using ethyl acetate:n-hexane (3:7, v/v) as the solvent system.

DPPH radical scavenging assay

Free radical scavenging activity of compound was measured by DPPH using the method of Blois (21). Briefly, 1 mM solution of DPPH in ethanol was prepared, and this solution (1 ml) was added to sample solutions of AMMT in distilled water with concentration of 200-800 μg/ml.

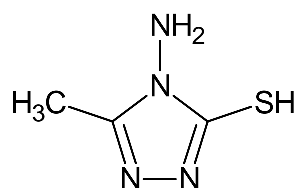

Fig. 1. 4-amino-5-mercapto- 3-methyl-1, 2, 4-triazole

The mixture was shaken vigorously and allowed to stand at room temperature for 20 min. Then the absorbance was measured at 517 nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = (A_0 - A_1/A_0) \times 100$$

where, A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the samples or standards.

Animal care and handling

Animal care and handling was carried out according to the guidelines set by WHO (World Health Organization; Geneva, Switzerland). The institutional animal ethical committee has approved this study. Swiss albino mice aged 6-8 weeks and weighing 30 ± 5 g, taken from an inbred colony, were used for this study. The mice were maintained under controlled conditions of temperature and light (light/dark 10/14 h). They were provided standard mouse feed and water *ad libitum.*

Toxicity studies

Toxicity study for AMMT was conducted according to the method suggested by Miller and Tainter (22). Male Albino mice weighing 30 ± 5 g were starved (with free access to water) for 18 h prior to the experiment. The compound was dissolved in water and administered orally at different concentration ranging 100, 500, 1200, 1400, 1600 mg/kg body weight and intraperitonialy at 100, 500, 900 and 1200 mg/kg body weight. Each group contained 6 animals.

Irradiation

The irradiation work was carried out at Microtron center, Mangalore University, Mangalore, Karnataka, India. The animals were restrained in well-ventilated perspex boxes and exposed to whole-body. The electron beam irradiated at a distance of 30 cm from the beam exit point of the Microtron accelerator at a dose rate of 72 Gy/min.

Survival assay

The male Swiss albino mice were used for the Survival assay. These animals were divided into 6 groups. These animals were irradiated to 4, 6, 8, 10, 12 Gy and 14 Gy

radiation dosages. The number of mice surviving 30 days after exposure against each dose will be used to construct survival dose response curve. The 7th and 8th groups of animals were treated with 100 mg/kg AMMT orally for 15 days (the required amount of AMMT was dissolved in distilled water). After the final dose administration, the animals were exposed to 10 Gy and 12 Gy electron beam radiation (EBR) respectively. The dose reduction factor (DRF) was calculated by the method of Miller and Tainter (22). DRF=$LD_{50/30}$ with treatment of AMMT/$LD_{50/30}$ without treatment of AMMT.

Experimental protocol

The following groups of animals were used for experimental study. The mice were divided into 4 groups (n=6 in each group). Group I served as control, treated with distilled water alone. Group II animals were received AMMT dissolved in distilled water, (100 mg/kg body weight) orally for 15 days. Group IV animals were also treated with the same dosage (100 mg/kg body weight) for 15 days. One hour after the final administration, group III (radiation control) and group IV animals were exposed to 6 Gy (sub-lethal dose) single dosage electron beam radiation at dose rate of 72 Gy/min.

Micronucleus assay

The animals that survived up to 15 days after exposure were scarified on day 16 and the bone marrow cells were used for micronucleus assay. The mouse bone marrow micronucleus test was carried out according to the method described by Schmidt (23) by evaluation of chromosomal damage in experimental animals. The bone marrow from femur was flushed in the form of a suspension into a centrifuge tube containing 5% bovine serum albumin (BSA). The cells were dispersed by gentle pipetting and collected by centrifuge at 2000 rpm for 5 min at 4°C. The cell pellet was resuspended in a drop of BSA and bone marrow smear were prepared. After air drying the smear were stained with May-Grunwald/Giemsa. Micronucleated polychromatic erythrocytes and normochromatic erythrocytes were observed under Microscope. The percentage of micronucleated polychromatic erythrocytes (MnPCEs), micronucleated normochromatic erythrocytes (MnNCEs) was calculated.

DNA fragmentation studies

The mice liver tissue (0.1 g) samples were homogenized with 1mlof lysis buffer (saline citrate buffer 20×, pH 7, Tri-sodium citrate 0.3 M, NaCl 3 M). The homogenate was centrifuged at 4000 rpm for 10 min. The pellet was mixed with double the volume of 2.6 M NaCl and centrifuged at 12000 rpm for 10 min. The supernatant was transferred to new microtubes where the DNA was precipitated with 700 µl of absolute cold ethanol and incubated later at -20°C for 2 h.

The DNA samples were centrifuged, washed with 700 µl ethanol 70% and resuspended in TE buffer (10 mM of Tris base pH 8.0 and 1 mM of EDTA). It was then treated with 30 µg/ml of RNAse, and incubated in water bath for 40 min at 37°C. The DNA obtained was kept at -20°C (24). The isolated DNA was subjected to electrophoresis by using 0.8% agarose gel at 75 v for 30 min. The gel was stained using ethidium bromide and observed under UV transilluminator.

Statistical analysis

All values were expressed as Mean ± SD. Comparison between the control and treated groups were performed by analysis of variance (ANOVA) with Bonferroni. In all these tests, criterion for statistical significance was $P<0.05$.

RESULTS

The compound AMMT showed very good radical scavenging property when compared to standard glutathione (Fig. 2). This indicates the antioxidant property of the study compound.

The animals were observed continuously for 1 h after drug administration for any changes in their behavior, movements, gait, writhing reflex, etc. The calculated i.p. LD_{50} was 900 ± 50 mg/kg for AMMT. After oral administration no mortality found up to 1600 mg/kg body weight.

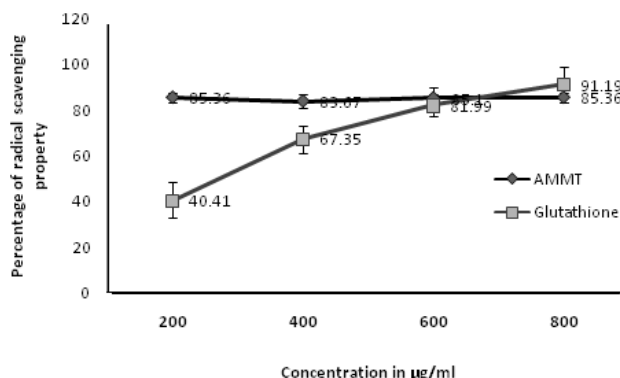

Fig.2. DPPH radical scavenging property of 4-amino-5-mercapto-3-methyl-1,2,4-triazole at different concentration ranging 200-800 μg/ml. The results were compared with the standard glutathione. The results are expressed as mean ± standard deviation. The results were statistically significant (P<0.05).

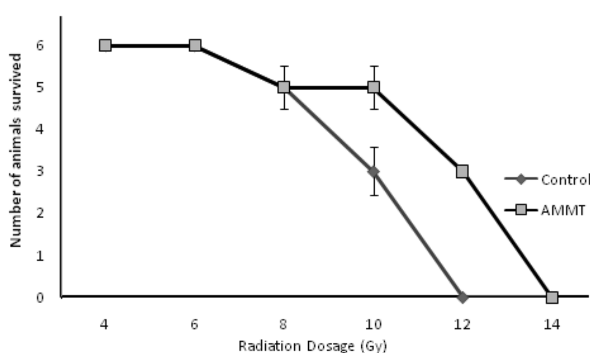

Fig. 3. Effect of 100 mg/kg of AMMT on the radiation induced mortality on day 30 post-irradiation in mice exposed to different doses of electron beam radiation. The results are expressed as mean ± standard deviation. The results were statistically significant (P<0.05).

Table 1. Effect of treatment with AMMT on protection against Micronucleus formation induced by electron beam radiation.

Groups	Control	100 mg/kg AMMT	6 Gy irradiation	100 mg/kg AMMT+6 Gy irradiation
PCE	48.72 ± 1.80	43.70 ± 0.50	27.10 ± 1.30	39.21 ± 0.90
MnPCE	0.0	0.19 ± 0.09	6.00 ± 0.20	4.27 ± 0.10
NCE	51.27 ± 3.40	50.15 ± 2.80	72.90 ± 3.90	60.78 ± 2.80
MnNCE	0.0	0.58 ± 0.08	12.00 ± 0.98	2.43 ± 0.19

Data expressed as average percentage (%) of cells. *P<0.05. PCE: polychromatic erythrocytes, NCE: normo-chromatic erythrocytes, MnPCE: micronucleated polychromatic erythrocytes, MnNCE: micronucleated normo-chromatic erythrocytes.

The radiation dose was found to be non-toxic up to a dose of 6 Gy, where no radiation induced mortality was observed. A further increase in the electron beam dose to 8 Gy led to 16% mortality. An increase in radiation dose to 10 Gy caused a 50% reduction in the mice survival. 100% mortality was recorded when the electron beam dose was increased to 12 Gy and 14 Gy. The LD_{50} of electron beam for acute radiation inducing mortality was 10 Gy (Fig. 3). The treatment of mice with AMMT before radiation exposure reduced the mortality rate up to 16% in 10 Gy and up to 50% in 12 Gy EBR. The calculated DRF was found to be 1.2 for AMMT.

The effect of electron beam irradiation with and without AMMT on the induction of micronucleus in bone marrow cells is shown in Table 1. The frequency of micronuclei was increased in group III, but micronuclei formation was declined significantly (P<0.05) in animals treated with AMMT (group IV).

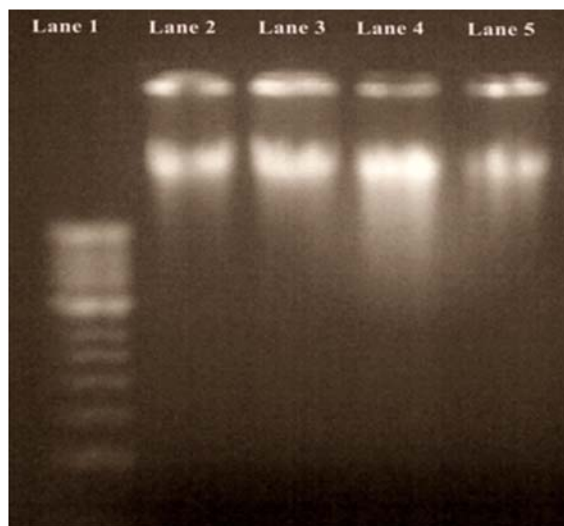

Fig. 4. DNA fragmentation assay. Lane1-DNA ladder, lane 2: Control, lane 3: drug control (100 mg/kg AMMT), lane 4: 6 Gy irradiation, lane 5: AMM treatement (100 mg/kg AMMT + 6 Gy irradiation).

DNA fragmentation is the biochemical hallmark of apoptosis. The apoptosis in hepatic cells due to electron beam radiation exposure was measured by DNA fragmentation assay. Cell genomic DNA showed the typical formation of DNA fragments in 6 Gy irradiated group (lane 4), but there was no fragmentation observed in AMMT treated group (lane 5) and it was almost similar to control (lane 2) and drug control (lane 3) group (Fig. 4).

DISCUSSION

Interaction of ionizing radiation with the biological system results in the generation of many highly reactive short-lived reactive oxygen species (ROS), mainly due to the hydrolysis of water (25). These ROS can attack cellular macromolecules like DNA, RNA, proteins, membrane constituents etc., causing dysfunction and damage (26).

Radioprotection may include scavenging of free radicals, protection of cellular and sub-cellular entities especially against oxidative damage, repair of target molecules like DNA, protein, etc., and restoration of cell proliferation. Compounds having such properties can offer protection against radiation damage (27). Aminothiols have been proved as the best radioprotectors with respect to their activity, tolerance and duration of action. However many of them have severe side effects, such as nausea, vomiting and hypertension (28,29). Therefore, present study evaluated non toxic and physiologically acceptable compound.

The antioxidant properties of thiol compounds are responsible for the prevention of radiation induced micronucleus formation and DNA damage (30). The effect of antioxidants on DPPH radical scavenging has been thought to be due to their hydrogen donating ability (31). Due to thereaction between antioxidant molecules and free radical, the absorbance of DPPH radical decreased. This will result in the scavenging of the radical by hydrogen donation. In this study, antiradical activities of triazole compounds and standard antioxidant such as glutathione were determined by using DPPH method. The data obtained indicate that triazole compounds showed very good activity as a radical scavenger by acting as hydrogen donors. This property is the reason for prevention of DNA damage in bone marrow and liver cells of mice.

However the present study focuses more on the sulphydryl group present in the AMMT derivatives, which can act as an antioxidant (32). The present study also shows insignificant amount of DNA fragmentation in drug control group when compared to control, but it clearly shows a reduction of DNA fragmentation in treated group when compared to irradiated group.

Triazole derivatives [2-(5-mercapto-4-phenyl-4H-(1,2,4) triazole-3-yl]-cyclohexa-1,5-dienol) also are known for their antitumor activity (33) against Ehrlich Ascites Carcinoma (EAC) bearing mice. The triazole derivative used in this study showed a toxic effect on mice (LD50; 60 mg/kg), but present study proved for its non toxicity on Swiss albino mice (no mortality up to 1600 mg/kg body weight; orally).

The micronucleus test and DNA fragmentation assay have been used as established methods to indicate radiation induced genetic damage. Therefore, DNA damage due to radiation was determined by micronucleus frequency in bone marrow cells and DNA fragmentation assay in hepatic cells of mice. The results obtained in these

parameters showed that AMMT helped in reducing the micronucleus formation and also it protects DNA strand against breakage in liver cells of mice.

CONCLUSION

In conclusion, our results demonstrate that triazole derivative with sulphydryl group gives significant protection to bone marrow and liver cells of mice against the clastogenic effects of electron beam radiation.

ACKNOWLEDGMENT

The authors are greatly thankful to Board of Research in Nuclear Science, Government of India for the financial support [2009/34/18/BRNS] and also we would like to thank all the staff members of Microtron center, Mangalore University, Mangalore, India for providing the radiation source.

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