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Protective Efficacy of S-2(2-aminoethylamino) Ethyl Phenyl Sulphide (DRDE-07) and Its Analogues on Lung Toxicity Induced by Sulphur Mustard in Mice

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Abstract

Sulfur mustard (SM); is a lipophilic Chemical Warfare Agent and has been used from World War-I. SM is threat to military and civilian populations as there is no prophylactic and therapeutic treatments are available. Lung is one of the major remote target organs for dermal SM exposure. SM toxicity is mainly attributed to oxidative stress and inflammation. Aim of the study was to see the preventive effect of DRDE-07 and its analogues on SM induced lung toxicity in mice. The mice were pretreated with DRDE-07 and its analogues (0.2 LD50, oral), after 30min., SM (0.8 LD50) was applied on hair clipped area of mice. The lung bronchoalveolar lavage (BALF) and lung tissues were collected on day 3 and day 7. Cutaneous SM exposure significantly reduced the lung glutathione (GSH); level and activities of superoxide dismustase (SOD); Catalase and glutathion-S-transferase (GST); enzymes in BALF and lung tissue. The histopathological analysis also indicates inflammatory cells accumulation in lung tissue after SM exposure. The pretreatment of animals with DRDE-07, DRDE-30 and DRDE-35 protected the antioxidant enzyme activities, reduced the tissue melondialdehyde (MDA), reactive oxygen species (ROS) formation and inflammatory cells accumulation. The DRDE-30 demonstrated pronounced beneficial effects than other two molecules i.e., DRDE-07 and DRDE-30. The protection offered by these compounds is attributed to the present of amino and sulfur group and their possible role in scavenging of ROS. Since there is no approved antidotes are available for SM poisoning, these analogues may be considered as prototype candidate.

Keywords: Sulphur mustard; Oxidative stress; Inflammation; DRDE-07; Lung tissue; Antidote

Introduction

Sulphur mustard (SM; bis(2-chloroethyl)sulphide) is a cytotoxic Chemical Warfare Agent (CWA) with strong alkylating properties and it has been used since World War I. Due to lipophilic nature, SM rapidly penetrates target tissues and induces alkylation of proteins, lipids and nucleic acid which in turn leads to DNA damage and cytotoxicity [1]. Skin, eyes and respiratory systems are the major target organs for SM vapour and induce a dose and time dependent toxic effects. Lung is one of the primary target organs for SM toxicity through inhalation exposure and dermal exposure [2,3]. The SM exposure results in damage to the pulmonary epithelium, lung edema and injury to skin and eyes [4,5]. Oxidative stress and inflammations are the major contributors for SM induced pulmonary toxicity apart from the apoptosis and DNA damages [6,7]. SM is considered as activator of proteases, which results in proteolysis of vital intracellular enzymes and structural proteins [8,9]. Subcutaneous or intraperitoneal administration of half mustard induced oxidative stress as evidenced by increased levels of oxidized glutathione, lipid peroxidation and glutathione-S-tranferases in the mouse lung [10-12]. SM affects the lung by the generation of an ascorbyl radical within one hour and induces lipid peroxidation and membrane damages [13]. SM induced oxidative stress by over production of reactive oxygen species (ROS) and molecular damage of antioxidant systems are the hypothesized events in SM exposed patients [6]. Lung tissues are protected against oxidative damage by enzymatic and non-enzymatic antioxidant defense systems [14]. Reduced glutathione plays an important role in detoxification of exogenous and endogenous reduced oxygen radicals produced after SM exposure [1]. Recent study by Laskin et al. [15] demonstrated that both SM and nitrogen mustard deplete the GSH level and inhibit the antioxidant enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidise, and glutathione reductase. The in vitro models targeting oxidative stress, inflammation, apoptosis and DNA damages pathways using various therapeutic agents were tested with some success [16] Though various compounds have been tested for their prophylactic efficacy against SM both in vitro and in vivo, but none of them have been approved for treatment [17-20].

Amifostine (S-2-(3-aminopropylamino) ethyl phosphorothioic acid) designated as WR-2721 was developed during the era of World War II, by US Walter Reed Army Research Institute as an excellent radiation protector (Andreassen et al 2003). Since SM is a radiomimetic compound, DRDE-07 (S-2(2-aminoethylamino)ethyl phenyl sulphide) analogue of amifostine DRDE-07 was synthesises and tested in animal model against SM induced systemic toxicity. Pretreatment of DRDE-07 exhibited a significant protection than

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amifostine through oral route in mouse model [19]. Various analogues of DRDE-07 were also synthesized for achieving better protection in different animal models. Among the DRDE-07 analogues, DRDE-30 (S-2(2-aminoethyl amino)ethyl propyl sulphide) and DRDE-35 (S-2(2-aminoethyl amino)ethyl butyl sulphide) were given higher protection (27 fold in mouse) against SM toxicity and also have better safety in terms of LD50 by oral and intraperitoneal routes [21,22]. A number of studies have been reported on protective efficacy of DRDE-07 and its analogues (DRDE-30 and DRDE-35) on haematological, hepatic, renal and spleen toxicity induced by SM [23]. Few recent studies are available on pulmonary protection efficacy of DRDE-07 and its analogues (DRDE-30 and DRDE-35) against SM toxicity. Recently Kannan et al. [24] and Soni et al. [25,26] demonstrated that SM induced pulmonary biochemical and inflammatory alteration were significantly protected by DRDE-07 and its analogues. Sharma et al. [27] demonstrated that nitrogen mustard (HN_2) induced oxidative stress and leucopenia was significantly prevented by repeated dosing of DRDE-07 and its analogues in mice. The present study was under taken to evaluate the prophylactic efficacy of DRDE-07 and its analogues against SM induced alteration in the pulmonary endogenous antioxidant defense system such as the GSH level, antioxidant enzyme activities of SOD, CAT and GST and lipid peroxidation in female mice.

Materials and Methods

Chemicals

SM, DRDE-07 (S-2(2-aminoethylamino) ethyl phenyl sulphide), DRDE-30 (S-2(2-aminoethyl amino) ethyl propyl sulphide) and DRDE-35 (S-2(2-aminoethyl amino) ethyl butyl sulphide) was synthesised in the Synthetic Chemistry Division of DRDE, Gwalior and SM purity was found to be above 99% by gas chromatographic analysis. DRDE-07, DRDE-30 and DRDE-35 was characterized by elemental analysis, IR, and mass spectral analysis and the purity was checked by thin layer chromatography. All analytical grade chemicals were purchased from Sigma-Aldrich Corporation, St Louise, USA and Merck India Ltd.

Animals and experimental design

Swiss female mice (25-30 g) were obtained from Institute animal house and randomly divided into following groups and treated as follows:

Group I: Control (n=4)

Group II: SM, (0.8 LD50, pc.,) (n=8)

Group III: DRDE-07 (0.2 LD50 oral) + After 30min SM as in group II, (n=8)

Group IV: DRDE-30 (0.2 LD50 oral) + After 30min SM as in group II, (n=8)

Group V: DRDE-35 (0.2 LD50 oral) + After 30min SM as in group II, (n=8)

A day before percutaneous (p.c.,) SM application, hair on darsocaudal region was closely clipped using a pair of scissors. The SM (6.48mg/kg equal to 0.8 LD50 through percutaneous route) was diluted in PEG-300 and evenly applied on dorsal hair clipped area after 30 min of DRDE-07 and its analogues administration. DRDE-07, DRDE-30 and DRDE-35 were freshly prepared in distilled water and given by oral gavage. The animals were maintained in dust free paddy husk, food and water ad libitum. The animals were monitored for body weight and clinical symptoms for seven days. From the above groups half of the animals were sacrificed on day 3 and remaining on day 7. The above study was approved by establishment animal ethical committee. Animal care and maintenance was followed as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Ministry of Fisheries, Animal Husbandry and Dairying, Govt. of India).

Bronchoalveolar lavage fluid (BALF) and lung tissue collection

The animals were sacrificed on day 3 and 7 after SM exposure by cervical dislocation and the trachea was cannulated with a 20-guage needle in situ and secured with the suture. The lungs were lavaged using sterile saline (1.5ml/animal) and which was cycled for 5 times. The BAL fluid was immediately centrifuged at 800 g for 10 min at 4°C and supernatant (BALF) was collected for various biochemical assays. For histological evaluation, a part of lung tissues were fixed in buffered formalin. The tissue embedded in paraffin was sectioned (5μm) with semi-automated microtome (Microme, Walldorf, Germany) and stained with haematoxylin and eosin. The SM induced changes were assessed by a pathologist using Leica microscope fitted with camera (Leica, Wetzlar, Germany).

BALF and tissue biochemical analysis

Total protein was estimated using Pierce BCA protein assay kit, Thermo Scientific, Rockford USA and absorbance was measured at 562nm and concentration was calculated from a standard curve obtained using bovine serum albumin supplied with kit. Tissue GSH level was determined by Ellman's method [28] and Malondialdehyde was estimated by method of Okhawa et al. [29]. Superoxide dismutase (SOD) activity was evaluated according to the method described by Marklund and Marklund [30] while Catalase was estimated using method of Aebi [31]. Glutathione -S- transferase activity was measured using the method of Habig et al. [32]. ROS level was assayed using dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Eugene, Oregon), which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. Reactive oxygen species activity was measured by determining fluorescence at 488 nm excitation and 525nm emissions using a fluorescence plate reader (Biotek Instruments, India).

Histopathological examination

The lung tissue samples were fixed in buffered formalin (10%), embedded in paraffin wax and 5 μm thick sections were prepared with semi-automated microtome (Microme, Walldorf, Germany). The sectioned specimens were stained with haematoxylin and eosin. The histological changes were assessed by a pathologist using Leica microscope fitted with camera (Leica, Wetzlar, Germany)

Statistical analysis

Data analysis were presented as mean ± SD for n=4 animals. The results were statistically analyzed by analysis of variance (ANOVA) and post hoc testing (Tukey test) using Sigma Stat statistical software (version 2.0). A p-value less than 0.05 was considered statistically significant.

Results

BALF biochemical changes have been used to determine the efficacy of tested therapeutic compounds against following SM exposure [33-35]. In the current study, percutaneous SM exposure caused a significant increase in BALF protein while GSH level decreased significantly on day 3 and day 7 compared to control group animals (Table 1). These changes suggest cellular injury and/or compromise in pulmonary endothelial/epithelial permeability caused by SM through increasing of oxidative stress and inflammation. Pretreatment of DRDE-07 and its analogues (DRDE-30 and DRDE-35) prevented the protein leakage and GSH depletion significantly ($p<0.05$) (Table 1). A significant decrease in BALF catalase , GST activities (on day 3 and 7) and SOD activity (on day 7) was observed in SM exposed group when compared to control group ($p<0.05$) (Figure 1-3). Though the BALF SOD activity increased by DRDE-30 and DRDE-35 compared to SM group, they are not statistically significant except by DRDE-30 on day 7 ($p<0.05$) (Figure 1). BALF Catalase activity was significantly protected by DRDE-07 and its analogues on day 3 and 7 ($p<0.05$) (Figure 2). The GST activity was completely restored by DRDE-07 and DRDE-30 on day 3 and 7 compared to DRDE-35 ($p<0.05$) (Figure 3).

Table 2 summarizes the protective efficacy of DRDE-07 and its analogues on lung tissue GSH and MDA altered by SM exposure. SM exposure depleted the GSH level while increased the MDA level on day 3 and 7 significantly compared to control group. Pretreatment of DRDE-07 and DRDE-30 prevented the SM induced GSH depletion compared to DRDE-35 on day 3 and 7. No significant increase of MDA level was observed on day 3 in DRDE-07 and DRDE-30 treated group of animals compared to DRDE-35 group. On day 7 DRDE-30 exhibited significant reduction of MDA level compared to SM and other treated group ($p<0.05$) (Table 2). Lung tissue antioxidant enzymes (SOD, Catalase and GST) were reduced significantly following SM exposure on day 3 and 7 ($p<0.05$) (Figure 4-6). DRDE-30 pretreatment increased the SOD activity on day 3 and 7 compared

Table 1: Prophylactic efficacy of DRDE-07 and its analogues on BALF enzymatic antioxidant system against SM induced toxicity

Group	Protein (mg/ml)		GSH (µmol/ml)	
	Day 3	Day 7	Day 3	Day 7
Control	0.94±0.018		30.66±1.09	
SM	1.25±0.083^{a}	1.47±0.229^{a}	26.3±0.94^{a}	18.8±1.49^{a}
DRDE-07	1.15±0.090^{a,b}	1.20±0.083^{a,b}	26.5±0.54^{a}	27.4±1.28^{a,b}
DRDE-30	0.98±0.110^{a,b}	0.99±0.115^{a,b}	29.1±1.28	28.8±1.37^{b}
DRDE-35	1.04±0.102^{a,b}	1.21±0.079^{a,b}	24.1±1.49^{a}	26.4±1.22^{a,b}

Values are expressed as mean ± SD (n=4); $^{a}p<0.05$ compared with control (no treatment); while $^{b}p<0.05$ compared with respective day SM group.

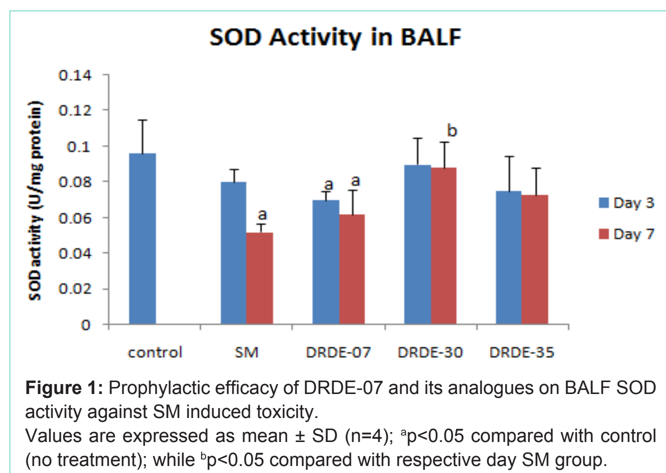

Figure 1: Prophylactic efficacy of DRDE-07 and its analogues on BALF SOD activity against SM induced toxicity.
Values are expressed as mean ± SD (n=4); $^{a}p<0.05$ compared with control (no treatment); while $^{b}p<0.05$ compared with respective day SM group.

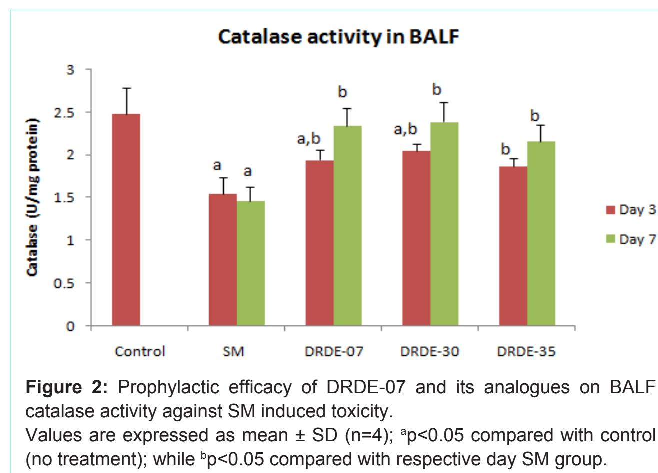

Figure 2: Prophylactic efficacy of DRDE-07 and its analogues on BALF catalase activity against SM induced toxicity.
Values are expressed as mean ± SD (n=4); $^{a}p<0.05$ compared with control (no treatment); while $^{b}p<0.05$ compared with respective day SM group.

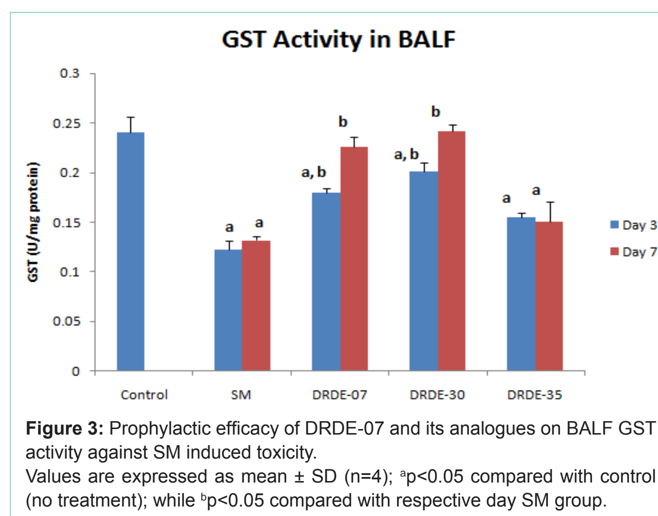

Figure 3: Prophylactic efficacy of DRDE-07 and its analogues on BALF GST activity against SM induced toxicity.
Values are expressed as mean ± SD (n=4); $^{a}p<0.05$ compared with control (no treatment); while $^{b}p<0.05$ compared with respective day SM group.

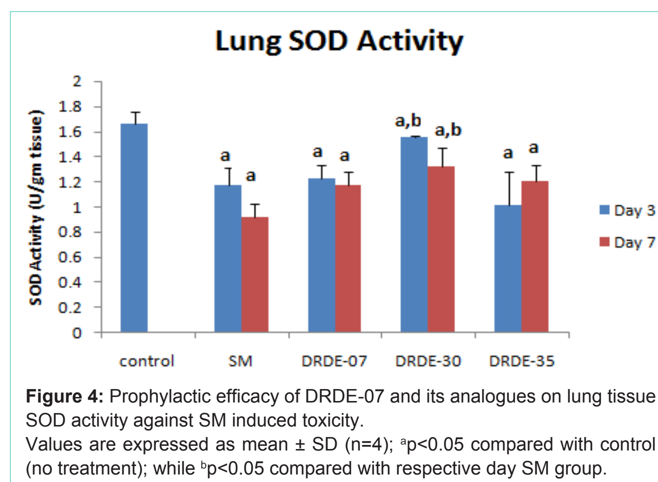

Figure 4: Prophylactic efficacy of DRDE-07 and its analogues on lung tissue SOD activity against SM induced toxicity.
Values are expressed as mean ± SD (n=4); $^{a}p<0.05$ compared with control (no treatment); while $^{b}p<0.05$ compared with respective day SM group.

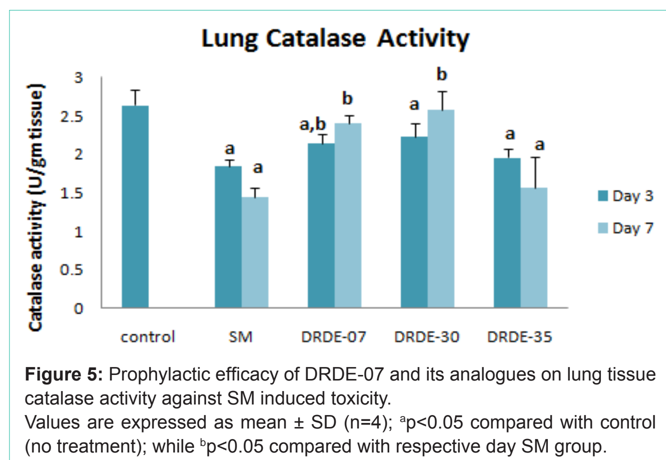

Figure 5: Prophylactic efficacy of DRDE-07 and its analogues on lung tissue catalase activity against SM induced toxicity.
Values are expressed as mean ± SD (n=4); [a]p<0.05 compared with control (no treatment); while [b]p<0.05 compared with respective day SM group.

Table 2: Prophylactic efficacy of DRDE-07 and its analogues on lung tissue GSH and MDA against SM induced toxicity.

Group	GSH (µmol/gm tissue)		MDA (nmol/mg protein)	
	Day 3	Day 7	Day 3	Day 7
Control	8.79±0.48		2.27±0.29	
SM	5.62±0.35[a]	4.43±0.30[a]	3.03±0.28[a]	8.08±0.65[a]
DRDE-07	6.89±0.29[a,b]	5.98±0.14[a,b]	2.64±0.18	3.45±0.28[a,b]
DRDE-30	7.43±0.44[a,b]	7.56±0.20[a,b]	2.69±0.31	2.81±0.22[b]
DRDE-35	6.76±0.51[a]	6.55±0.24[a]	2.82±0.26[a]	4.79±0.49[a,b]

Values are expressed as mean ± SD (n=4); [a]p<0.05 compared with control (no treatment); while [b]p<0.05 compared with respective day SM group.

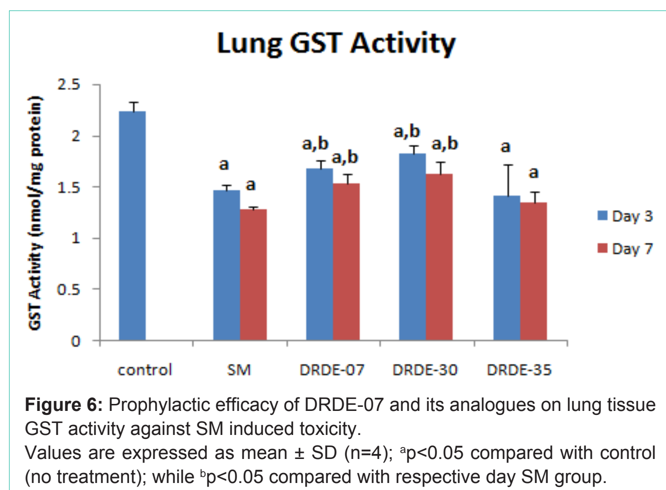

Figure 6: Prophylactic efficacy of DRDE-07 and its analogues on lung tissue GST activity against SM induced toxicity.
Values are expressed as mean ± SD (n=4); [a]p<0.05 compared with control (no treatment); while [b]p<0.05 compared with respective day SM group.

SM group and other treatment group (Figure 4). DRDE-07 and DRDE-30 exhibited the significant protection of Catalase activity compared DRDE-35 treated group (p<0.05) particularly on day 7. The lung GST activity inhibited significantly by SM exposure and pretreatment of DRDE-07 and DRDE-30 protected the GST activity on day 3 and 7 as compared to DRDE-35. The percentage of reactive oxygen species generation in lung tissue increased significantly by dermal SM application on day 3 (28%) and day 7 (57%) compared to control group (p<0.05) (Figure 7). Thirty minutes pretreatment DRDE compounds particularly DRDE-30 reduced the ROS generation significantly on day 3 and 7, while DRDE-07 and DRDE-35 on day 7 only.

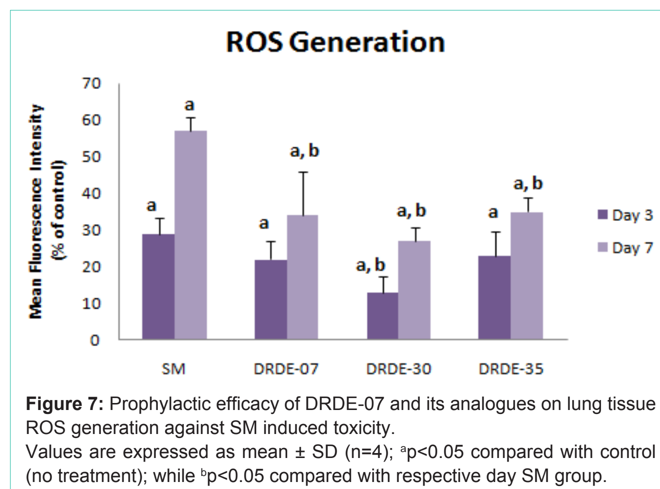

Figure 7: Prophylactic efficacy of DRDE-07 and its analogues on lung tissue ROS generation against SM induced toxicity.
Values are expressed as mean ± SD (n=4); [a]p<0.05 compared with control (no treatment); while [b]p<0.05 compared with respective day SM group.

Histological analysis of lung section revealed significant tissue damage and infiltration of inflammatory cells and blood vessel haemorrhage following SM exposure compared to control (Figure 8 B&C) on day 3 and 7 (Figure 8). The accumulation of inflammatory cells increased time dependently. Pretreatment of DRDE-30 significantly protected the tissue damage and prevented the infiltration of cells into lung tissue on day 7 (Figure 8, F&G), while DRDE-07 and DRDE-35 provided a moderate beneficial effect (Figure 8, H&I) on day 3 and 7.

Discussion

Several studies have indicated that lung is one of the remote target organs with greater pulmonary toxicity following SM exposure by the percutaneous (pc.,) or subcutaneous (sc.,) route [3,10]. Though the mechanism of action of SM is not clear, sulfonium ion formation, oxidative stress and inflammation events are attributed to the SM induced toxicity [4,36-38]. SM a pro-oxidant and SM induced oxidative stress increases the lipid peroxidation and decreased the antioxidant enzymes activities in the lungs of experimental animals [10,39]. In the current study SM induced significant alterations in BALF and lung tissue biochemical variables consistent with free radical induced oxidative stress. Our findings also support the theory of free radical mediated oxidative stress involved in the SM induced cellular injury, apart from DNA alkylation and inhibition of transcription and proteins synthesis [10,13,36,40].

SM triggers oxidative stress through glutathione depletion and altered expression of glutathione related enzymes [41]. GSH is a key survival antioxidant, oxyradical scavenger and protect the lung endothelial cells [42]. The Initiation and propagation of lipid peroxidation due to depletion of GSH is the main factor responsible for cytotoxicity[43]. Previously, we reported that SM exposure deplete the lung GSH and increases lipid peroxidation in mice [23-25]. The present study the MDA, which is an index of lipid peroxidation was significantly increased in mouse lung compared to control animal after SM application. In this study, SM significantly decreased tissue and BALF GSH level, followed by increase in tissue MDA level. Our findings are consistent with previous report which suggest that SM intermediates reacts with GSH, a tripeptide nucleophilic antioxidant and deplete the levels of GSH in serum and BALF [8,10,44]. DRDE-07 and its analogues pretreatment prevented the GSH depletion

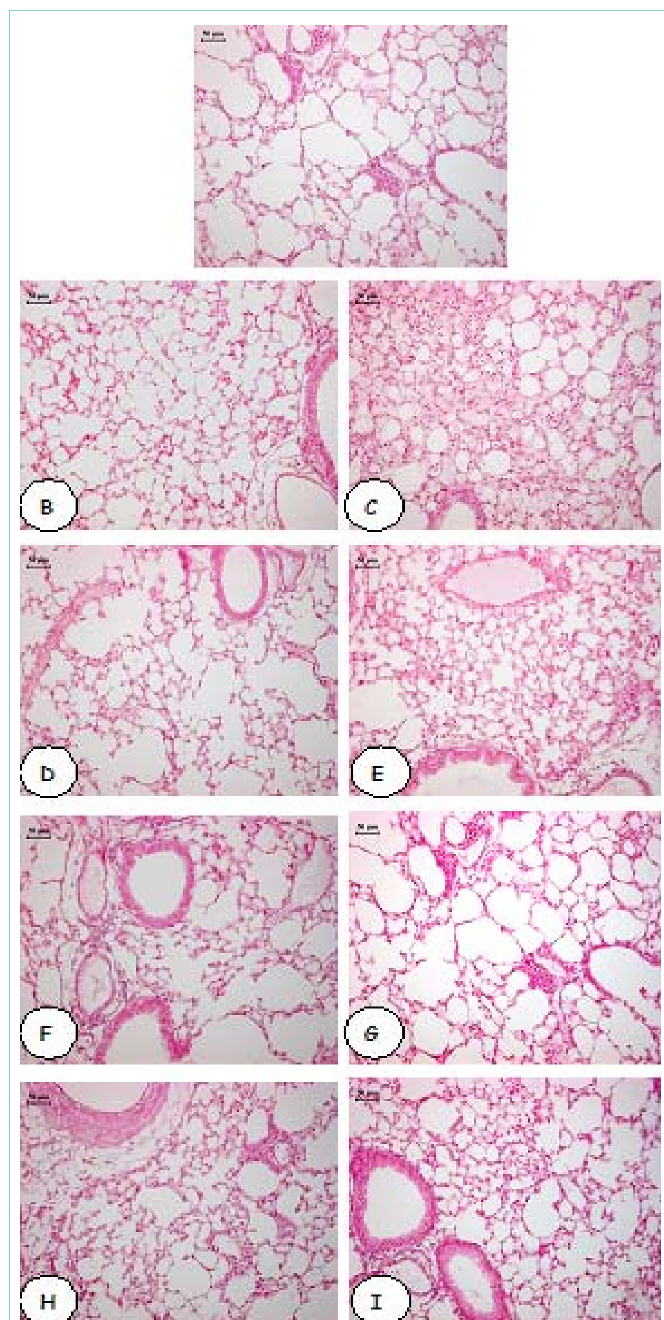

Figure 8: SM induced lung histological changes and protection by DRDE-07 and its analogues in female mice. After SM exposure lung tissue samples were collected on day 3 and 7, fixed in buffered formalin, processed sectioned stained with H&E and analyzed by a pathologist. Control animal tissues exhibited normal alveoli without any inflammatory cells (A). SM exposure induced accumulation of inflammatory cells in the alveolar spaces and epithelial cells damages were also noticed (B&C). Pretreatment of animals with DRDE-30 significantly reduced the inflammatory cells compared to DRDE-07 and DRDE-35.
A: Control; B&C: SM; D&E: DRDE-07; F&G: DRDE-30; H&I: DRDE-35 day3 and day 7 respectively.

and reduce the MDA formation in lung tissues. The increase in lung MDA and ROS level upon SM exposure is in agreement with the earlier report of Pohanka and Sobotka [45] and attenuated by DRDE compounds. Mustard exposure induces a significant lung histopathological changes which includes alveolar epithelial damage, increase in BALF protein level and accumulation of inflammatory cells in rodents [35,46,47]. Our experimental results also confirm the above significant changes and these changes were attenuated by the pretreatment of animals with DRDE compounds (Figure 8). The reduction of inflammatory cells in the lung by the DRDE compounds were attributed to the anti-inflammatory properties [23-25].

The disturbances in the lung antioxidant systems in response to SM exposure are characterized by alteration in enzymatic and non-enzymatic antioxidant parameters. The lung epithelial lining fluid contains catalase, superoxide dismutase, glutathione peroxidase, and large amount of glutathione [48,49]. Recent study by Tahmasbpour et al. [50] indicates that SM alter the oxidants/antioxidants system through the over expression of free radical producing related genes in human mustard lung. The cytosolic antioxidant controlling enzymes are important target for SM and its analogues. Our current study result confirms that percutaneous SM exposure decreases the BALF and lung SOD, CAT and GST activities. The inhibition of antioxidant enzymes activities by SM leads to increase in endogenous superoxide anion, H_2O_2 and lipid peroxides followed by Ca2+ influx which is responsible for cellular injury [51,52]. Our results are consistent with previous reports that show the inhibition of antioxidant enzyme activities in blood cells and body tissues of rats following percutaneous SM exposure [38,39]. The inhibition antioxidant enzyme activities may be because of a) changes in the expression of enzymes protein due to SM induced alkylation, b) decreases the de novo synthesis of enzyme proteins and/or c) irreversible inactivation of enzyme proteins by free radicals during the SM metabolism [53,54]. Superoxide dismutase (SOD) is an important antioxidant and serve as primary defence of human lung against oxidative stress and free radicals and attenuate mustard analogue induced lung toxicity [38,55]. The current study results shows that increased generation of ROS leads to reduction of antioxidant enzymes SOD and confirms selective elimination of superoxide radicals in dismuation reaction. The reduction of SOD activity also caused by increased level of peroxides which directly alters its activity [56]. Catalase is a heme protein which equilibrate and detoxifying superoxide anion and hydrogen peroxide in cells [57]. Catalase facilitates the breakdown of H_2O_2 in to H_2O and O_2. The Catalase activity was significantly inhibited in lung tissue and BALF of mice exposed to sulphur mustard. The catalase activity inhibition results in accumulation of H_2O_2 in lung tissue and that can undergo iron-catalyzed decomposition, leading to the production of hydroxyl radicals. The extremely reactive natures of hydroxyl radicals have been shown to induce lipid peroxidation, DNA damage, inactivate enzymes and cell death [58,59]. GST protects human airways by detoxification of various electrophilic molecules such as carcinogens, mutagens and several therapeutic drugs by conjugation with GSH60 GST is a thiol protein and its isoenzymes are sensitive to oxidative stress by thiol group modification [61,62]. The role of GSTA1-2 and GSTP1 is well reported that they terminate lipid peroxidation chain reactions by removing hydrogen peroxide and aldehydes generated during oxidative stress. Pretreatment of DRDE-07 and analogues attenuated the inhibition of SOD, CAT and GST activity in BALF and lung tissues by SM.

Amifostine is a prodrug which exhibited significant protection against radiation injury through scavenging of free radicals [63].

The beneficial effect of amifostine is attributed to the presence of -SH group which helps in the scavenging of superoxide anions and peroxyl radicals and increases the GSH levels and thereby protecting the alkylation of DNA [4,64,65]. Any drug to be orally effective against SM should have optimum lipophilicity, so that it can penetrate the cell and protect from alkylation induced by SM. Increased protection was observed by DRDE-30 and DRDE-35 when replacing the phenyl group of DRDE-07 with propyl and butyl groups as in DRDE-30 and DRDE-35 and it is attributed to increased lipophilicity [21,22]. In our study pretreatment of animals with DRDE-07 and its analogues significantly prevented the SM induced inhibition of SOD, Catelase and GST activity. The antioxidant, anti-inflammatory and analgesic properties of DRDE-07 and its analogues have already been reported against SM toxicity. Though there is no –SH group in DRDE-07 and its analogues, their protective efficacy against SM is attributed to the presence of functional amino and sulphide group which may help in scavenging SM, thereby reducing the toxicity. From the study results we assume that the protective efficacy of DRDE compounds may due to scavenging of ROS and stabilization of antioxidant enzymes by the amino and sulfur group present in the DRDE compounds. The above hypothesis needs further experiments in this direction.

Conclusion

Dermal SM exposure disturbs the lung antioxidant and oxidant balance through ROS generation and inflammation. The non-enzymatic and enzymatic antioxidant levels decreased significantly followed by lipid peroxidation and lung tissue structural changes. The pretreatment of DRDE and its analogues (DRDE-30 and DRDE-35) prevented the lung biochemical alterations. DRDE-30 exhibited more pronounced effects than DRDE-07 and DRDE-35. The preventive efficacy of DRDE compounds is because of their increased lipophilicity and able to reduce the SM induced biochemical alterations. Since there is no effective treatment available, the above tested compounds represents as potential prototype candidates for oral prophylactic and therapeutic treatment for SM poisoning.

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