

Prevention of Granulocyte-mediated Oxidant Lung Injury in Rats by a Hydroxyl Radical Scavenger, Dimethylthiourea

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Abstract. Toxic, partially reduced metabolites of oxygen (toxic oxygen radicals) are increasingly implicated in acute leukocyte-mediated tissue injury. To further probe the roles of oxygen radicals in acute lung edema, I studied the effects of a recently described and very potent oxygen radical scavenger, dimethylthiourea (DMTU) (Fox, R. B., R. N. Harada, R. M. Tate, and J. E. Repine, 1983, *J. Appl. Physiol.*, 55:1456-1459) on polymorphonuclear leukocyte (PMN) oxidant function and on two types of lung injury mediated by oxygen radicals and PMN. DMTU (10 mM) blocked 79% of hydroxyl radical ($\cdot\text{OH}$) production by PMN in vitro without interfering with other PMN functions, such as O_2^- production, myeloperoxidase activity, chemotaxis, degranulation, or aggregation. When isolated rat lung preparations were perfused with PMN activated to produce $\cdot\text{OH}$, lung weights were increased from 2.3 ± 0.2 to 11.2 ± 0.8 g. DMTU (10 mM) prevented 70% of these increases (lung weights, 5.0 ± 1.1 g, $P < 0.005$). Finally, when intact rats were exposed to 100% O_2 for 66 h, lung weight:body weight ratios were increased from 5.78 ± 0.33 to 8.87 ± 0.16 g. DMTU (500 mg/kg) prevented 83% of this hyperoxia-induced lung edema in vivo (lung:body weight ratios, 6.05 ± 0.21 , $P < 0.001$). Pharmacokinetic

studies showed that DMTU diffused effectively into lung interstitial fluids and had a relatively long half-life (25-35 h) in the circulation. Because a variety of oxygen radicals, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), or $\cdot\text{OH}$ are produced by PMN, there is usually some uncertainty about which one is responsible for injury. However, in these studies, DMTU did not scavenge O_2^- and scavenged H_2O_2 only very slowly while scavenging $\cdot\text{OH}$ very effectively. Therefore, DMTU may be useful in the investigation of the roles of oxygen radicals, especially $\cdot\text{OH}$, in acute granulocyte-mediated tissue injury.

Introduction

Highly reactive, partially reduced metabolites of oxygen (toxic oxygen radicals) have been implicated in the pathogenesis of acute tissue injury, particularly in the lung (1-3). One likely source of oxygen radicals in tissues is the polymorphonuclear leukocyte (PMN), which produces large amounts of oxygen radicals in its phagolysosomes as a bactericidal mechanism (4). Activated PMN can cause acute lung injury by leaking these potent oxidants into lung tissues (5-8).

The contribution of PMN-derived oxidants to acute tissue injury has been demonstrated using antioxidant enzymes or chemicals to block PMN-induced injury, either in isolated perfused organs (3) or in whole animals (7-8). However, antioxidant enzymes, such as superoxide dismutase (SOD)¹ and/or catalase are large charged proteins and may not always penetrate nonperfused areas or provide protection inside cells (9). Furthermore, there are no known enzymatic scavengers of the most highly reactive oxygen radical, the hydroxyl radical ($\cdot\text{OH}$). While chemical scavengers of $\cdot\text{OH}$, such as ethanol and mannitol, are known and used, these agents may lack potency;

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1. Abbreviations used in this paper: AM, alveolar macrophage; DMSO, dimethylsulfoxide; DMTU, dimethylthiourea; HPO, horseradish peroxidase; MPO, myeloperoxidase; PMA, phorbol myristate acetate; SOD, superoxide dismutase; ZAS, zymosan-activated serum.

that is, they may not effectively scavenge $\cdot\text{OH}$ when used in concentrations which are not toxic in vivo (see Results).

To overcome these limitations, I used dimethylthiourea (DMTU) as an antioxidant to prevent oxidant lung injury in vitro and in vivo. DMTU is a recently described derivative of thiourea which does not cause the massive pulmonary edema characteristic of thiourea (10) and many other thiourea derivatives (11), but remains a highly effective $\cdot\text{OH}$ scavenger. Specifically, DMTU blocked $\cdot\text{OH}$ production by PMN but was not toxic to other PMN functions. It decreased PMN-mediated edema in the isolated-perfused rat lung and also protected intact rats against lung edema due to pulmonary oxygen toxicity, a process in which both oxygen radicals and PMN have been implicated (12–17). These findings suggest that DMTU may be a useful probe in the study of the contributions of oxidants in tissue injury.

Methods

Determination of $\cdot\text{OH}$ scavenging. $\cdot\text{OH}$ was quantitated by measuring its ability to decompose dimethylsulfoxide (DMSO) to methane, as previously described (18). $\cdot\text{OH}$ was generated by irradiating (^{60}Co , 700 rad) solutions (1 ml) consisting of 100 mM potassium phosphate (pH = 7.4) and 10 mM DMSO (Sigma Chemical Co., St. Louis, MO) in 4 ml rubber-stoppered glass tubes (Vacutainer, Becton-Dickinson Co., Rutherford, NJ), which were washed prior to use. DMTU (Alpha Products, Danvers, MA), thiourea (J. T. Baker Chemical Co., Phillipsburg, NJ), ethanol (U. S. Pharmacopeia), or mannitol (Sigma Chemical Co.) were added to some solutions. After stoppering, tubes were gassed with nitrous oxide (N_2O , U. S. Pharmacopeia) by means of injection needles placed through the stoppers and then tumbled for 15 min to equilibrate solutions with N_2O . Inclusion of N_2O in irradiated solutions increases yield of $\cdot\text{OH}$ and decreases yield of other radicals, such as e_{aq}^- (hydrated electron) and O_2^- , establishing a nearly one radical ($\cdot\text{OH}$) system (19). After irradiation, methane concentrations of the headspace gases were determined by standard gas chromatography (model GC-55 gas chromatograph, Beckman Instruments, Fullerton, CA), using authentic methane (Supelco, Inc., Bellefonte, PA) as standard, as previously described (18).

Determination of effect of DMTU on O_2^- and $\cdot\text{OH}$ production by PMN. Human PMN were isolated by Ficoll-Hypaque sedimentation and resuspended in Hanks' balanced salt solution containing Ca^{++} and Mg^{++} (HBSS, Gibco Laboratories, Grand Island, NY), as previously described (18). PMN production of O_2^- was measured by spectrophotometric determination of SOD (0.1 mg/ml, Sigma Chemical Co.) inhibitable reduction of cytochrome *c* (0.12 mM, Sigma Chemical Co.) by PMN stimulated with phorbol myristate acetate (PMA, 1 $\mu\text{g}/\text{ml}$, Consolidated Midland Corp., Brewster, NY), as previously described (18). PMN production of $\cdot\text{OH}$ was measured by PMN decomposition of DMSO to methane, as previously described (17). Solutions (1 ml) of DMSO (10 mM) in HBSS, which contained human PMN (1.6×10^7) stimulated by PMA (1 $\mu\text{g}/\text{ml}$), in rubber-stoppered glass tubes (as above) were incubated in air at 37°C with slow tumbling for 30 min. Reactions were then terminated by immersion in ice water and headspace methane concentrations determined as above.

Determination of effect of DMTU on peroxidase activity. Peroxidase activity was assayed by measuring its capacity to catalyze the oxidation of pyrogallol by H_2O_2 (20–21). Peroxidase activity was determined

from the change in absorbance at 420 nm of a solution of 0.5% pyrogallol, 1 mM H_2O_2 , and 0.05 U/ml horseradish peroxidase (HPO) (all from Sigma Chemical Co.) in 20 mM potassium phosphate (pH = 6.0). To determine whether DMTU affected this reaction by inhibiting HPO or by scavenging H_2O_2 , DMTU was added to some solutions after HPO but before H_2O_2 , while in other assays DMTU was added to the solution after H_2O_2 but 5 min before HPO. In additional studies, the effect of DMTU on myeloperoxidase (MPO) activity was also determined by the same method. Human PMN ($7.8 \times 10^6/\text{ml}$) were lysed with 15 $\mu\text{l}/\text{ml}$ of Nonidet P-40 (Sigma Chemical Co.), centrifuged at 500 g, and the supernatants recovered. Peroxidase activities of the supernatants, in the presence and absence of DMTU (5 or 10 mM), were determined from the change in absorption (420 nm) of solutions of supernatant (50 $\mu\text{l}/\text{ml}$), 0.5% pyrogallol, and 1 mM H_2O_2 in 20 mM potassium phosphate (pH 6.0).

Determination of effect of DMTU on PMN chemotaxis. Chemotaxis of human PMN in various concentrations of DMTU was determined by the agarose method (22). DMTU (2, 5, and 10 mM final concentrations) was incorporated into agarose (0.75%)-gelatin (0.25%) just prior to gelling so as not to oxidize the DMTU by heating. Three wells (2 mm) were cut in the agarose with an interwell distance of 2 mm. PMN were placed in the central well, zymosan-activated serum (ZAS) (18) in one peripheral well, and saline (control) in the other peripheral well. Plates were incubated at 37°C for 2 h, fixed with methanol (90%)-formalin (10%), and Wright's stained. The leading fronts of chemotaxis (ZAS) and chemokinesis (saline) were determined by microscopy and the chemotactic index [(chemotaxis-chemokinesis)/chemokinesis] calculated.

Determination of effect of DMTU on PMN degranulation. Degranulation of human PMN ($1 \times 10^7/\text{ml}$ in HBSS) was induced by opsonized zymosan particles (10:1 zymosan:PMN) in the presence or absence of 10 mM DMTU. Cell suspensions were incubated at 37°C for 2 h after addition of opsonized zymosan and then centrifuged (500 g). Granule enzymes were released from some cell suspensions by treatment with a detergent (Nonidet P-40). Activities in supernatants of β -glucuronidase (phenolphthalein glucuronic acid substrate, Sigma Chemical Co., 23, 24) and lactate dehydrogenase (25) were determined by standard methods. Vitamin B_{12} binding protein contents of supernatants were determined by a radioligand binding assay (26). ^{57}Co -labeled cyanocobalamin (5.25 ng) was incubated with supernatant (300 μl) and saline (450 μl) at room temperature for 5 min after which albumin-coated charcoal (500 μl) was added. After centrifugation (1000 g), the supernatants were counted in a gamma scintillation counter (Searle Diagnostics, Inc., Skokie, IL).

Determination of effect of DMTU on PMN aggregation. Aggregation of human PMN was determined by aggregometry of PMN (4×10^6) in 1 ml of HBSS plus 0.5% human serum albumin in an aggregometer (model PAP-2A, Bio/Data Corp., Willow Grove, PA) after stimulation with zymosan-activated human serum (50 μl , 27).

PMN perfusion of isolated rat lungs. Human peripheral blood PMN were isolated as described above. Heart-lung preparations were isolated from 350–400 g Sprague-Dawley rats (Charles River Breeding, Inc., Wilmington, MA). After satisfactory anesthesia was obtained with pentobarbital, rats were anticoagulated with heparin. A tracheostomy was placed and rats were continuously ventilated (10 ml/kg) for the duration of the experiment with a rodent ventilator (Harvard Apparatus Co., South Natick, MA). After opening the chest by sternectomy and exsanguinating by transecting the inferior vena cava, a polyethylene catheter was placed through a right ventriculotomy into the main pulmonary artery and tied. A large bore polyethylene catheter was

then placed into the left ventricle through an apical left ventriculotomy and tied in place. Perfusion of the lungs then commenced at a rate of 9 ml/min with HBSS (Gibco Laboratories, Grand Island, NY) containing 4% bovine serum albumin (Sigma Chemical Co.). Dissection of the heart-lung unit was then completed and the preparation suspended in a warmed humidified environment. In some preparations, PMA (0.25 μ g/ml) was added to the perfusate reservoir and/or PMN (3×10^7) were injected slowly (over 2–4 min) into the pulmonary artery catheter. Pulmonary artery pressures were measured using Statham transducers and a multichannel recorder (both from Gilson Medical Electronics, Middleton, WI). After 45 min of perfusion, the lungs (minus heart) were weighed and then lavaged once with 10 ml of saline. Albumin concentrations of the lavages were determined spectrophotometrically by a brom-cresol green dye binding method (28, 29).

Determination of DMTU pharmacokinetics in rats and sheep. With pentobarbital anesthesia, heparinized polyethylene catheters were placed in the right common carotid arteries of rats (400 g) by cutdown, and tunneled subcutaneously to the backs of the necks and exteriorized there. After allowing 24 h for recovery from surgery, rats were injected intraperitoneally with DMTU at a dose of 500 mg/kg (200 mg in 4 ml of normal saline). Heparinized blood samples (1 ml) were drawn before dosing with DMTU and at 1, 4, and 8 h after dosing. Circulating blood volume was maintained by intra-arterial volume expansion (1.5 ml) with normal saline plus 4% bovine serum albumin (at 0 and 1 h) or heparinized whole rat blood (at 4 h). Additional pharmacokinetic studies were performed in sheep fitted with chronic lung lymph fistulas to determine the concentrations of DMTU in lung lymph as compared with plasma. 1–2-yr-old sheep were fitted with carotid and pulmonary artery catheters, and with lung lymph fistulas as previously described (30); care was taken to prevent any diaphragmatic contributions to lung lymph flow. DMTU in a dose of 1.0 g/kg was given by intravenous infusion in lactated Ringer's solution over 30 min. Lymph and blood samples were collected at 0, 1, 4, 6, and 24 h.

Plasma and lymph levels of DMTU were determined by high pressure liquid chromatography (HPLC) (31). Plasma was separated from blood by centrifugation (200 g). Samples were mixed vigorously with 2.5 vol of absolute ethanol and then centrifuged (500 g for 10 min). The resulting supernatants were dried under nitrogen. The product was dissolved in 1 ml of methanol and chromatographed on a C18 Radial-Pak HPLC column (Waters Associates, Millipore Corp., Milford, MA) with a mobile phase of 5% methanol:95% water at a flow rate of 3 ml/min and detection at 254 nm (model 441 absorbance detector, Waters Associates). Authentic DMTU (Alpha Products) was used to standardize the assay. Pharmacokinetic parameters of DMTU metabolism were determined from a standard one compartment open model (32).

Measurement of \cdot OH scavenging by serum from DMTU-treated rats. Sprague-Dawley rats (300 g, Charles River Breeding Laboratories, Inc.) were injected with DMTU (500 mg/kg intraperitoneally) or equal volumes of saline. 2 h later, rats were sacrificed in a carbon dioxide chamber. Blood was obtained by open chest cardiac puncture and sera isolated by centrifugation (500 g \times 10 min). Solutions (1 ml) consisting of 80% serum, 100 mM potassium phosphate (pH = 7.4), and 10 mM DMSO were placed in rubber-stoppered glass tubes (as above), gassed with N_2O , irradiated (700 rad), and headspace methane concentrations determined as above.

Measurement of effect of DMTU on hyperoxia-induced lung edema. Rats were exposed to 100% O_2 (40 liter/min) at a barometric pressure of 730 torr. Some rats were treated with DMTU injected at the beginning of O_2 exposure (500 mg/kg intraperitoneally) and every 12

h thereafter (125 mg/kg intraperitoneally). Controls were injected with equal volumes of saline on a similar schedule. After 66 h of 100% O_2 exposure, rats were removed from the hyperoxia chamber. All rats survived at least 30 min after termination of O_2 exposure. Rats were then sacrificed with pentobarbital, weighed, and exsanguinated by open-chest cardiac puncture. Their lungs were removed, weighed, and lavaged five times with 10 ml of saline. Pleural effusion volumes were determined at the time of chest opening by aspiration into graduated syringes. Albumin concentrations of the first aliquot of lung lavage were determined as above. Leukocytes in lavages and cardiac blood were counted in a hemocytometer and differential counts were performed on Wright's stained cytocentrifuge preparations (lavages) or smears (blood).

Statistical methods. Significances of differences between and among groups were determined by Student's *t* test or analysis of variance, as appropriate, using two-tailed distributions and standard methods (33).

Results

\cdot OH radical scavenging effectiveness of DMTU. To determine the \cdot OH scavenging effectiveness of DMTU in vitro, the ability of DMTU to inhibit \cdot OH-mediated methane production from solutions of DMSO was measured. Solutions were exposed to \cdot OH generated by gamma irradiation of water in the presence of nitrous oxide. Inhibition of methane production from irradiated DMSO by DMTU ($85 \pm 2\%$) was similar or better than thiourea ($69 \pm 5\%$), and was more effective than two other commonly employed \cdot OH scavengers, ethanol ($56 \pm 6\%$) and mannitol (no inhibition), when the concentration of each scavenger was 10 mM (Table I).

Effect of DMTU on O_2^- and \cdot OH production by PMN. Because production of toxic oxygen radicals from PMN can cause acute lung injury (5–8), the effectiveness of DMTU in scavenging oxygen radicals, such as O_2^- and \cdot OH, produced by

Table I. Effect of \cdot OH Scavengers on Methane Production from Irradiated DMSO*

Additions	Methane produced (ppb)	Inhibition
		%
DMSO	2818 \pm 124 (8)‡	0
DMSO + DMTU (2 mM)	2709 \pm 227 (4)§	4 \pm 8
DMSO + DMTU (5 mM)	1776 \pm 189 (4)¶	37 \pm 7
DMSO + DMTU (10 mM)	427 \pm 58 (4)¶	85 \pm 2
DMSO + thiourea (10 mM)	822 \pm 144 (4)¶	69 \pm 5
DMSO + ethanol (10 mM)	946 \pm 330 (3)¶	56 \pm 6
DMSO + mannitol (10 mM)	4030 \pm 354 (4)§	

* ppb, parts per billion. DMSO (10 mM in sodium phosphate, 100 mM, pH = 7.4) was exposed to gamma irradiation (^{60}Co , 700 rad) in the presence of nitrous oxide.

‡ Mean \pm SEM (n).

§ Not significantly less ($P > 0.05$) than samples with no scavenger.

¶ Significantly less ($P < 0.05$) than samples with no scavenger.

PMN activated by PMA was determined. While DMTU (up to 10 mM) did not diminish PMN production of O_2^- , as measured by cytochrome *c* reduction, modest concentrations of DMTU (2 mM) significantly inhibited net $\cdot OH$ production by PMN, as determined by methane production from DMSO (Table II). This decreased net $\cdot OH$ production by PMN did not result from a generalized toxic effect of DMTU on PMN oxidative metabolism, as evidenced by no decrease in O_2^- production by PMN in the presence of concentrations of DMTU of up to 10 mM. Indeed, O_2^- production by PMN was significantly increased by DMTU.

H₂O₂ scavenging by DMTU. Because DMTU inhibited $\cdot OH$ production but not O_2^- production by PMN and because $\cdot OH$ is derived from O_2^- , it appeared that DMTU exerted its antioxidant effect on PMN function subsequent to the formation of O_2^- . Specifically, O_2^- can be metabolized to H_2O_2 which can then subsequently interact with additional O_2^- via the Haber-Weiss mechanism to form $\cdot OH$ (see Discussion). Therefore, while the studies above showed clearly the $\cdot OH$ scavenging activity of DMTU when $\cdot OH$ was generated directly (without O_2^- or H_2O_2 precursors) from radiation, it is important to consider the possibility that, in the PMN, $\cdot OH$ production could also be decreased by scavenging of H_2O_2 by DMTU. Earlier reports of reaction of thiourea with H_2O_2 supported this possibility (34).

Because the absorption spectrum of DMTU interferes with the standard spectrophotometric assays for H_2O_2 (35), the ability of DMTU to inhibit a H_2O_2 -dependent reaction, oxidation of pyrogallol by HPO and H_2O_2 , was determined.

Table II. Effect of DMTU on Net Production of O_2^- and $\cdot OH$ by PMN In Vitro*

Test conditions	Cytochrome <i>c</i> reduced†	Methane produced (ppb)§
	nmol	
PMN	1.6±0.2 (4)	
PMN + PMA¶	74.7±5.3 (4)	2208±161 (5)
PMN + PMA + DMTU (2 mM)	103.0±3.1 (4)	440±277 (5)**
PMN + PMA + DMTU (5 mM)	99.9±1.3 (4)	387±287 (5)**
PMN + PMA + DMTU (10 mM)	98.9±1.8 (4)	466±242 (5)**

* "Net Production" refers to the amount of radical detectable in the presence of scavenger.

† Nanomoles SOD inhibitable cytochrome *c* reduced per 2×10^6 PMN/30 min.

§ Parts per billion (ppb) of methane in headspace gas produced from DMSO per 1.6×10^7 PMN/30 min.

^{||} Mean±SEM (*n*).

¶ PMA (1 µg/ml).

** Significantly less ($P < 0.001$) than samples without DMTU.

Under the test conditions employed, 10 mM DMTU did not significantly inhibit this reaction (Table III). To determine whether DMTU scavenged H_2O_2 , DMTU was added to the H_2O_2 -containing substrate 5 min before the HPO was added to allow time for prior reaction of DMTU with H_2O_2 . Under these circumstances, 10 mM DMTU showed a slight but nonsignificant tendency to inhibit the reaction. Moreover, since the reaction velocity 5 min after combining DMTU with H_2O_2 was at least 90% of that seen immediately after combining them, the second order rate constant could be estimated to be on the order of $10^{-1} M^{-1} s^{-1}$ or less, which is considerably less than that for thiourea, $1.2 \times 10^{10} M^{-1} s^{-1}$ (19), which is about that of DMTU as shown in Table I.

Because MPO contributes to the bactericidal activities of PMN (36) and thus could contribute to PMN-mediated lung injury, additional studies of the effects of DMTU on MPO activity were performed. DMTU (up to 10 mM) did not significantly inhibit MPO activity (Table III).

Effect of DMTU on PMN chemotaxis. Since locomotion is an important PMN function which may contribute to PMN-mediated lung injury, the effects of DMTU on PMN chemotaxis (directed migration) and chemokinesis (random migration) were determined. While there was no significant effect of DMTU on chemokinesis, there was a significant ($P < 0.01$) increase in chemotaxis in response to ZAS in the presence of DMTU in concentrations of 2, 5, and 10 mM (Table IV).

Effect of DMTU on PMN degranulation. Since PMN contain a variety of proteolytic enzymes stored within two types of intracellular granules (specific and azurophilic granules) which could contribute to tissue injury, the effect of DMTU on PMN degranulation was determined. DMTU (10 mM) did not significantly diminish degranulation of either a specific granule-associated enzyme, vitamin B₁₂ binding protein (37), or an azurophilic enzyme, B-glucuronidase (Table V). Control studies also showed that DMTU did not interfere with the assays for these enzymes as evidenced by a lack of effect of DMTU on the activities of enzymes released from PMN by detergent (Nonidet P-40). In additional control studies, lactate

Table III. Effect of DMTU on Peroxidase Activities of HPO and MPO

Test conditions	Change in absorbance $_{420}/min$
HPO	0.709±0.004 (5)*
HPO + DMTU (10 mM)	0.715±0.008 (4)
HPO + DMTU (10 mM)‡	0.689±0.026 (4)
MPO§	0.106±0.005 (4)
MPO + DMTU (5 mM)	0.098±0.002 (5)
MPO + DMTU (10 mM)	0.095±0.002 (3)

* Mean±SEM (*n*).

‡ DMTU combined with H_2O_2 5 min before addition of HPO.

§ From PMN lysates ($4 \times 10^5/ml$).

Table IV. Effect of DMTU on PMN Chemotaxis

Test conditions	Chemotactic index*	Absolute chemotaxis	Chemokinesis
PMN + ZAS‡	3.50±0.05 (18)§	2.79±0.03 (18)	1.13±0.016 (18)
PMPN + ZAS			
+ DMTU (2 mM)	3.98±0.04 (30)	3.49±0.05 (30)	1.17±0.012 (30)
PMN + ZAS			
+ DMTU (5 mM)	3.82±0.05 (26)	2.95±0.03 (26)	1.05±0.015 (26)
PMN + ZAS			
+ DMTU (10 mM)	4.00±0.06 (27)	3.37±0.07 (27)	1.12±0.012 (27)

* Chemotactic index = (chemotaxis - chemokinesis)/chemokinesis; absolute chemotaxis = chemotaxis - chemokinesis.

‡ ZAS (100%)

§ Mean±SEM (n).

^{||} Significantly greater ($P < 0.05$) than chemotaxis without DMTU.

dehydrogenase release into supernatants was similar with either unstimulated or stimulated cells and either in the presence or absence of DMTU, indicating that cell lysis did not contribute to the differences between the groups.

Effect of DMTU on PMN aggregation. Because PMN aggregation within the pulmonary vasculature has been proposed to contribute to lung injury (38), the effects of DMTU on PMN aggregation in response to ZAS were determined. DMTU (10 mM) did not significantly inhibit PMN aggregation (Fig. 1).

Protection of isolated-perfused rat lungs from PMN-mediated edema by DMTU. Since the preceding studies indicated that DMTU scavenges [•]OH produced by PMN without impairing other important PMN functions (including O₂ production), it appeared that DMTU might be effective in preventing granulocyte-mediated lung edema if this injury results from [•]OH. Because PMN activated by PMA to produce toxic oxygen radicals can cause edema in isolated-ventilated rabbit lungs (6), this system was employed to investigate the ability of DMTU to block lung edema from granulocyte oxidants. As shown, perfusion of isolated ventilated rat lungs with PMA-activated PMN caused significant lung edema (Table VI). Neither PMN nor PMA alone caused significant edema. DMTU (10 mM) added to the perfusates reduced this edema by nearly 70%. Peak pulmonary artery pressures were similar in lungs perfused with PMA (66±9 mmHg), PMA plus PMN (75±10 mmHg), and PMA plus PMN plus DMTU (71±2 mmHg),

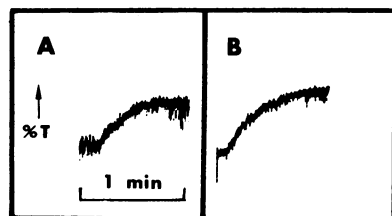


Figure 1. Similarity of PMN aggregation responses to ZAS in either the absence (A) or presence (B) of DMTU (10 mM). Aggregation responses were quantitated from the increases in light transmittance

(%T) of PMN-containing solutions as measured by an aggregometer.

Table V. Effect of DMTU on Release of Specific and Azurophilic Granule-associated Proteins from Human PMN

Test conditions	B-Glucuronidase*	Vitamin B12 binding‡	LDH§
HBSS	0 (2)	0 (2)	0 (2)
HBSS + PMN	0.31±0.06 (4)	136±14 (4)	7.47±1.93 (4)
HBSS + PMN + OPZ	0.57±0.05 (6)	381±23 (6)	6.47±1.12 (6)
HBSS + PMN + OPZ			
+ DMTU (10 mM)	0.65±0.08 (6)	350±18 (6)	1.93±0.40 (6)
HBSS + PMN + DMTU	0.32±0.01 (2)	153±5 (2)	3.34±0.11 (2)
HBSS + OPZ	0 (2)	0 (2)	0 (2)
HBSS + DMTU	0 (2)	0 (2)	0 (2)
HBSS + PMN lysate¶	26.0±0.04 (3)	25,608±1901 (4)	251±15 (2)
HBSS + PMN lysate			
+ DMT	26.2±0.02 (3)	25,769±1786 (4)	270±3 (2)

LDH, lactic dehydrogenase; OPZ, opsonized zymosan.

* Micrograms of phenolphthalein produced/50 µl sample.

‡ Counts per minute of ⁵⁷Co-labeled vitamin B₁₂ bound/300 µl of supernatant.

§ Change in absorbance at 340 nm/min × 10³.

^{||} Mean±SEM (n).

¶ 10⁵ PMN per milliliter lysed with 1% Nonidet P-40.

while PMN alone did not increase pressures (20±2 mmHg) above base line.

Pharmacokinetics of DMTU in rats. While the [•]OH scavenging potential of DMTU appeared promising in these in vitro studies, equivalent success in vivo would necessitate the achievement of similar concentrations in serum of animals treated with DMTU. Therefore, the pharmacokinetics of DMTU were investigated by injecting rats intraperitoneally with DMTU (500 mg/kg) and collecting samples of blood from indwelling carotid artery catheters at 1, 4, and 8 h. The average plasma concentration of DMTU was 3.64 mM (382 µg/ml) at 1 h and fell very slowly thereafter with an apparent half-life of ~34 h (Table VII). To determine the ability of DMTU to penetrate the interstitial fluid compartment, the concentrations of DMTU in serum and lung lymph from sheep treated with DMTU (1000 mg/kg intravenously) were also determined. Although DMTU concentrations in lung lymph (5.47±1.25 mM) were slightly less than serum concentrations (7.74±0.62) at 1 h after treatment, serum concentrations then dropped below lymph concentrations by 4 h. The serum half-life in sheep was about 25 h.

Hydroxyl radical production in sera of DMTU-treated rats. To determine whether DMTU retained its [•]OH scavenging activity in vivo, rats were injected intraperitoneally with DMTU (500 mg/kg) and exsanguinated 2 h later. The sera were then assayed for [•]OH-scavenging potential by measuring methane production from irradiated DMSO in the presence of these sera as described earlier. Methane production, which was 2530±168 parts per billion in the presence of serum from saline-treated rats, was decreased to 1904±158 parts per billion in the presence of serum from DMTU-treated rats ($P < 0.05$).

Effect of DMTU on hyperoxia-induced lung edema. Because the oxidant effects of PMN in vitro could be blocked by

Table VI. Effect of DMTU on PMN-mediated Edema in Isolated Rat Lungs

Test conditions	Lung weight	Lavage albumin
	g	mg/100 ml
HBSS + 4% BSA*	2.3±0.2 (13)‡	80±10 (13)
HBSS + 4% BSA + PMN§	2.6±0.4 (3)	119±70 (3)
HBSS + 4% BSA + PMA	2.6±0.4 (5)	142±60 (5)
HBSS + 4% BSA + PMN + PMA	11.2±0.8 (7)	923±80 (7)
HBSS + 4% BSA + PMN + PMA + DMTU (10 mM)	5.0±1.1 (6)	421±144 (5)

* HBSS + 4% bovine serum albumin (BSA).

‡ Mean±SEM (n).

§ 3 × 10⁷ PMN/lung.

^{||} PMA (1 µg/ml).

^{||} Significantly less (*P* < 0.005) than PMN-PMA treated lungs.

concentrations of DMTU achievable in vivo, it appeared possible that DMTU might protect against oxidant lung injury in vivo. The injury chosen for these studies was pulmonary oxygen toxicity since evidence suggests that this injury results from oxygen radicals produced by lung cells exposed to hyperoxia (12–14), perhaps augmented by oxidants produced by influxes of PMN (15–17). Treatment of rats with a loading dose of DMTU (500 mg/kg intraperitoneally) followed by repeat treatments every 12 h (125 mg/kg intraperitoneally) very significantly reduced lung edema when measured after 66 h of exposure to 100% O₂ (Table VIII). DMTU treatment prevented 83% of increases in lung weight:body weight ratios, 100% of pleural effusions, and 73% of increases in lung lavage albumin concentrations (*P* < 0.01 in each case).

Table VII. Pharmacokinetics of DMTU in Rats and Sheep

Species	Time after dose	DMTU concentration (<i>mM</i>)	
		Plasma	Lymph
	<i>h</i>		
Rats	1	3.64±0.39 (4)*	ND
	4	3.49±0.48 (3)	ND
	8	3.16±0.43 (3)	ND
		Half-life: 34 h	
Sheep	1	7.74±0.62 (5)	5.47±1.25 (4)
	4	6.30±0.40 (4)	6.58±0.76 (3)
	6	6.07±0.60 (3)	6.66±0.47 (3)
	24	3.87 (1)	4.51 (1)
		Half-life: 25 h	

ND, not determined.

* Mean±SEM (n).

Since phagocytes such as PMN and alveolar macrophages (AM) may be involved in lung injury due to hyperoxia (15–17, 39), the numbers of PMN and AM in lung lavages as well as numbers of PMN in blood were determined. Exposure of rats to hyperoxia for 66 h caused increased numbers of both PMN and AM in lung lavages and increased numbers of PMN in blood (Table VIII). DMTU treatment blunted the increases in AM in lavage and PMN in blood but did not significantly (*P* > 0.05) decrease the influx of PMN seen in lavages. In control studies, DMTU treatment of rats exposed to normoxia did not significantly affect numbers of PMN or AM in lavages or PMN in blood.

Discussion

In the present investigations, DMTU effectively prevented lung edema in vitro and in vivo. Several findings support the conclusion that this protection resulted from scavenging of [•]OH. First, DMTU effectively scavenged [•]OH produced by gamma irradiation of water, as evidenced by inhibition of demethylation of DMSO. DMTU also scavenged [•]OH production from PMA-activated human PMN in vitro without evidence of impairment of other PMN functions, such as MPO activity, chemotaxis, degranulation, or aggregation. The concentrations of DMTU which inhibited [•]OH production by PMN in vitro were also effective in blocking edema in isolated rat lungs perfused with PMA-activated PMN, an injury known to be caused by PMN-derived oxidants (6). Finally, when rats were treated with DMTU in vivo to achieve concentrations in serum comparable with those in the in vitro studies, [•]OH scavenging activity was present in sera and the rats were protected against pulmonary oxygen toxicity, a lung injury involving toxic oxygen radicals (12–14) and PMN (15–17).

These studies are important because they suggest that DMTU may be a useful probe of the role of toxic oxygen radicals, particularly [•]OH, in tissue and organ injury. Such a probe would be important because (a) there are no known enzymatic scavengers of [•]OH and (b) previously described nonenzymatic chemical [•]OH scavengers, such as mannitol and ethanol, are so relatively low in their reactivity with [•]OH (19) that their effective use in vivo is precluded by their toxicities. Indeed, even the [•]OH scavenger DMSO, which is several times more reactive than either mannitol or ethanol (19) and relatively nontoxic (40), must be used in concentrations of 280 mM to give the same degree of protection to bacteria against [•]OH as is afforded by 5 mM DMTU (41).

The great reactivity of the DMTU molecule toward [•]OH is due to the sulfhydryl group as shown below on the right:

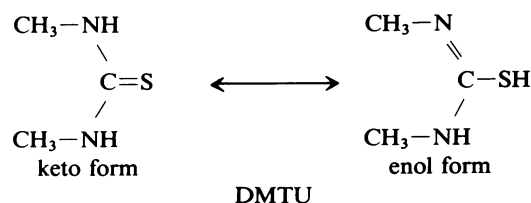


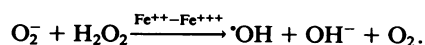
Table VIII. Effects of DMTU Treatment on Lung Edema and Lung Phagocyte Numbers of Rats Exposed to 100% Oxygen for 66 h

Treatment	Animal weights	Lung weight:body weight ratios ($\times 10^3$)	Lung lavage albumin	Pleural effusions	Blood PMN ($\times 10^3/\mu\text{l}$)	Lung lavage PMN (10^6)	Alveolar macrophages ($\times 10^6$)
	g		mg/100 ml	ml			
Exposed to 100% O ₂							
Saline*	332 \pm 6 (11)‡	8.87 \pm 0.16 (11)	133 \pm 33 (11)	2.1 \pm 0.8 (11)	3.32 \pm 0.49 (11)	1.78 \pm 0.64 (11)	28.2 \pm 2.9 (11)
DMTU§	341 \pm 5 (11)	6.05 \pm 0.21 (11)	44 \pm 7 (11)	0.1 \pm 0.1 (11)	2.43 \pm 0.48 (11)	1.49 \pm 0.41 (13)	18.8 \pm 1.8 (13)
Exposed to air							
Saline	302 \pm 4 (6)	5.78 \pm 0.33 (6)*	10 \pm 5 (6)	0 \pm 0 (6)	0.72 \pm 0.31 (5)	0 \pm 0 (6)	27.6 \pm 3.3 (6)
DMTU	307 \pm 5 (6)	5.23 \pm 0.05 (6)	20 \pm 5 (6)	0 \pm 0 (6)	1.17 \pm 0.21 (6)	0.05 \pm 0.05 (6)	30.6 \pm 4.9 (6)

* 4 ml, then 1 ml every 12 h \times 5. ‡ Mean \pm SEM (n). § 500 mg/kg intraperitoneally initially and 125 mg/kg intraperitoneally every 12 h thereafter. ^{||} Significantly less ($P < 0.01$) than saline-treated rats. ^{||} Not significantly different than saline-treated rats ($P > 0.05$).

The extremely favorable electron-donating characteristics of the sulfhydryl group may be due to the electron density around the carbon atom and the resonance stability inherent in the two enol and one keto forms. The parent compound, thiourea, shows similar $\cdot\text{OH}$ scavenging properties (19) for the same reasons, but cannot be used in vivo because it causes fatal pulmonary edema (11, 42). We previously showed that this was due to the oxidation in vivo of the primary amino groups of thiourea to a toxin, possibly cyanamide (10). From this, we reasoned that substitution of all the primary amino groups on the thiourea molecule with nonreactive substituents, such as the methyl groups in DMTU, would block the toxicity of the molecule without diminishing its antioxidant effectiveness. The present studies and previous studies (10) support this conclusion.

Several toxic oxygen radicals, such as O_2^- , H_2O_2 , and $\cdot\text{OH}$, could possibly contribute to lung injury. The present studies point toward $\cdot\text{OH}$ as being the primary cause of oxidant injury in the PMN-perfused isolated lung and in hyperoxic lung injury. First, O_2^- does not appear to be primarily involved since DMTU did not scavenge O_2^- from PMN in the present studies. Nevertheless, O_2^- may be secondarily involved, since SOD has been associated with protection against both PMN-induced lung damage (8) and pulmonary oxygen toxicity (12–14). Such secondary protection by SOD may result from the contribution of O_2^- to the production of $\cdot\text{OH}$. Specifically, in biological systems, much $\cdot\text{OH}$ may arise from electron exchange between O_2^- and H_2O_2 by the Haber-Weiss mechanism (43):



Recent studies have supported the role of this mechanism in acute inflammatory lung injury. For example, treatment of animals with the iron chelators, apolactoferrin or deferoxamine, protects against PMN-mediated tissue injury (7). Furthermore, protection of rats against pulmonary oxygen toxicity required both SOD and catalase (14).

Second, DMTU reacted relatively slowly with H_2O_2 , making

it less likely that scavenging of H_2O_2 was a major mechanism of protection in the present studies. Furthermore, while H_2O_2 can cause lung injury (2, 3), there are reasons to suspect that these effects are mediated principally by the reduction of H_2O_2 to $\cdot\text{OH}$ (7–14) by means of the Fenton reaction (44):



Specifically, it has been shown that the ability of H_2O_2 to damage bacteria (41, 45) and DNA (46) is primarily dependent on its reduction to $\cdot\text{OH}$ because prevention of $\cdot\text{OH}$ formation from H_2O_2 by eliminating iron from the system prevents damage.

Since the first preliminary communications of the $\cdot\text{OH}$ scavenging effectiveness of DMTU (47), there have been several preliminary reports of the use of DMTU as a probe for oxidant effects. DMTU prevented damage to AM exposed to hyperoxia in cell culture (48). DMTU also prevented lung edema in isolated rabbit lungs perfused with either PMN (49, 50) or an O_2^- generating system (purine-xanthine oxidase) (3). DMTU or SOD prevented ischemic injury in the kidney (51), providing further support for the hypothesis that ischemic tissue injury may be mediated by oxygen radicals (52). Finally, the failure of DMTU to significantly decrease lung edema in sheep treated with intravenous endotoxin has suggested that $\cdot\text{OH}$ may not contribute to this process (53).

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