Pyrrolidine dithiocarbamate is a potent antioxidant against hypochlorous acid-induced protein damage

Ben-Zhan Zhu, Anitra C. Carr, Balz Frei*

Linus Pauling Institute, Oregon State University, 571 Weniger Hall, Corvallis, OR 97331-6512, USA

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Abstract **The antioxidant potential of the dithiol compound pyrrolidine dithiocarbamate (PDTC) against protein damage induced by hypochlorous acid (HOCl) was investigated. The effects of PDTC were compared to those of reduced glutathione (GSH) and *N*-acetylcysteine (NAC). PDTC markedly and in a concentration-dependent manner inhibited HOCl-induced inactivation of α_1-antiproteinase, protein carbonyl formation on serum albumin and oxidation of human low-density lipoprotein. The direct scavenging of HOCl by PDTC was demonstrated by two quantitative methods, oxidation of ferrocyanide and chlorination of monochlorodimedon. In all assay systems, PDTC was two to three times more potent than GSH and NAC, while diethyldithiocarbamate was about as effective as PDTC. These data demonstrate that PDTC is a potent antioxidant against HOCl-induced protein oxidative damage, suggesting that PDTC might be useful in the prevention and treatment of inflammatory conditions.**

Key words: Pyrrolidine dithiocarbamate; Hypochlorous acid; α_1-Antiproteinase; Protein carbonyl; Low-density lipoprotein

1. Introduction

Activated neutrophils and monocytes generate substantial amounts of hypochlorous acid (HOCl) via the myeloperoxidase-catalyzed oxidation of chloride ions by hydrogen peroxide [1,2]. HOCl is a powerful oxidant capable of oxidizing many biologically important molecules [3–9], and has been implicated in emphysema, atherosclerosis and other inflammatory conditions [10,11].

Dithiocarbamates are low-molecular weight thiol compounds that possess a $(R_1)(R_2)N–C(S)–S–R_3$ functional group [12]. Pyrrolidine dithiocarbamate (PDTC) and diethyldithiocarbamate (DDTC) (Scheme 1) are two widely used dithiocarbamates in both basic and clinical research [12–26]. Many of the biological effects of dithiocarbamates are a result of their metal-chelating properties [12–16].

The biochemistry of dithiocarbamates is of interest because of their clinical use [12]. There have been a large number of recent experimental studies, particularly related to regulation of transcription factors and apoptosis, in which dithiocarbamates were used to modify the cellular redox environment [12,16–26]. Because PDTC can suppress activation of the transcription factor NFκB in response to diverse stimuli, including reactive oxygen species and HOCl, and is more potent than the thiol compound *N*-acetylcysteine (NAC), its mode of action was attributed to an antioxidant function [17–26]. However, the ability of PDTC to scavenge reactive oxygen species vis-à-vis its metal-chelating properties is incompletely understood.

PDTC is a stable pyrrolidine derivative of dithiocarbamate [27]. Since thiol compounds are generally considered to be efficient HOCl scavengers, the antioxidant effects of PDTC might be partly due to scavenging of HOCl. Therefore, in this study, the ability of PDTC to scavenge HOCl was evaluated by employing three qualitative, biologically relevant methods: α_1-antiproteinase inactivation, protein carbonyl formation, and low-density lipoprotein (LDL) oxidation; as well as two quantitative methods: ferrocyanide oxidation and monochlorodimedon chlorination. The effects of PDTC were compared to those of reduced glutathione (GSH) and NAC.

2. Materials and methods

2.1. Materials

PDTC, DDTC, GSH, NAC, bovine serum albumin (BSA), HOCl, α_1-antiproteinase, porcine pancreatic elastase, succinyl-$(ala)_3$-*p*-nitroanilide, 2,4-dinitrophenylhydrazine (DNPH), monochlorodimedon, dimethyl sulfoxide and ferrocyanide were purchased from Sigma (St. Louis, MO, USA). HOCl was obtained from Aldrich (Milwaukee, WI, USA). Due to the pK_a (7.5) of HOCl [28], it is present at physiological pH as both hypochlorite (OCl^-) and HOCl; thus, in this paper 'HOCl' is used to refer to this mixture of OCl^- and HOCl. The concentration of HOCl present in the diluted commercial NaOCl solution was determined either spectrophotometrically ($\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$) [28], or with the monochlorodimedon method (see below), assuming a 1:1 reaction stoichiometry between HOCl and monochlorodimedon [9].

2.2. α_1-Antiproteinase inactivation

α_1-Antiproteinase (400 μg/ml) was treated with HOCl (10–200 μM) in the absence or presence of PDTC or GSH (5–200 μM) for 30 min at 37°C. α_1-Antiproteinase activity was determined following addition of an equal volume of porcine pancreatic elastase (60 μg/ml) and 0.1 volume of succinyl-$(ala)_3$-*p*-nitroanilide (20 mM in dimethyl sulfoxide) as the colorimetric substrate ($\varepsilon_{410} = 8800\ M^{-1}\ cm^{-1}$) [29]. Combination of equal volumes of α_1-antiproteinase and elastase at the concen-

*Corresponding author. Fax: (1)- 541-737 5077.
E-mail address: balz.frei@orst.edu (B. Frei).

Abbreviations: apoB, apolipoprotein B-100; BSA, bovine serum albumin; DDTC, diethyldithiocarbamate; DNPH, 2,4-dinitrophenylhydrazine; GSH, reduced glutathione; HOCl, hypochlorous acid; IC_{50}, concentration required for 50% inhibition; LDL, low-density lipoprotein; NAC, *N*-acetylcysteine; PDTC, pyrrolidine dithiocarbamate; REM, relative electrophoretic mobility

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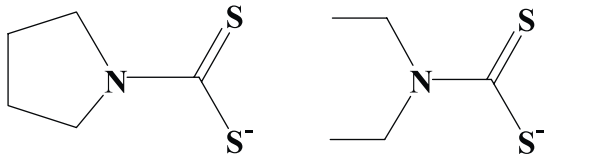

Scheme 1. Chemical structures of PDTC and DDTC.

trations stated above resulted in approximately 75% inhibition of elastase activity.

2.3. Protein carbonyl formation

BSA (1 mg/ml) was oxidized with HOCl in 0.1 M phosphate buffer (pH 7.4) at 37°C for 1 h. Protein carbonyls were assayed as described by Levine et al. [30]. Briefly, 0.5 ml of 10 mM DNPH in 2 N HCl was added to 1 ml of the incubation mixture containing BSA and HOCl, and incubated at room temperature for 1 h. Following addition of 0.5 ml 20% trichloroacetic acid, the samples were incubated on ice for 10 min and centrifuged in a bench-top centrifuge at 3000 rpm for 10 min. Protein pellets were washed three times with 3 ml ethanol:ethyl acetate (1:1, v/v) and dissolved in 6 M guanidine (pH 2.3). The peak absorbance at 370 nm was used to quantitate protein carbonyls, and the data were expressed as nmol carbonyl groups per mg of protein, using a molar absorption coefficient of 22 000 M^{-1} cm^{-1} for the DNPH derivatives [30].

2.4. LDL oxidation

LDL was isolated from fresh human plasma by sequential centrifugation [31], modified as described previously [32]. Isolated LDL ($1.019 < d < 1.067$ g/ml) was desalted by two sequential passages through PD-10 gel filtration columns (Pharmacia Biotech, Uppsala, Sweden). Total protein was estimated using a Lowry Micro Method Kit (Sigma P5656). For experiments, LDL was diluted to a concentration of 0.5 mg protein/ml ($\approx$1 μM LDL) in phosphate-buffered saline (10 mM phosphate, 140 mM NaCl, pH 7.4) containing the metal chelator diethylenetriaminepentaacetic acid (100 μM).

HOCl (100–500 μM) was added to LDL (0.5 mg protein/ml) with rapid, gentle mixing, and the samples were incubated for 30 min at 37°C. PDTC or NAC (25–200 μM) were added to LDL together with HOCl.

The change in the relative electrophoretic mobility (REM) of LDL was determined by agarose gel electrophoresis using a Paragon Lipoprotein Electrophoresis Kit (Beckman), as described previously [32]. LDL lysine residues were measured following derivatization with fluorescamine, as described previously [32].

2.5. Ferrocyanide oxidation

The oxidation of $Fe(II)(CN)_6$ (10 mM) by HOCl (0.2 mM) in 0.1 M phosphate buffer (pH 7.4) was employed as a reference reaction to investigate the stoichiometry of the reaction of HOCl with PDTC [33]. PDTC or GSH was incubated with HOCl for 5 min before the addition of $Fe(II)(CN)_6$. The absorbance of $Fe(II)(CN)_6$ was monitored at 420 nm ($\varepsilon = 1050$ M^{-1} cm^{-1}). Under these conditions, two molecules of $Fe(II)(CN)_6$ were oxidized by one molecule of HOCl within 5 min [33]. The inhibition of oxidation was calculated from the difference in absorbance in the presence and absence of various concentrations of PDTC or GSH.

2.6. Monochlorodimedon chlorination

Monochlorodimedon chlorination was measured directly by monitoring absorbance at 290 nm ($\varepsilon = 19\,900$ M^{-1} cm^{-1}) [9] of a 50 μM monochlorodimedon solution containing 0.2 mM HOCl. HOCl was added slowly to 1 ml of monochlorodimedon in 0.1 M phosphate buffer (pH 7.4), which was continuously vortex-mixed during the addition. Absorbance of each solution was read 5 min later. The inhibition of chlorination was calculated from the difference in absorbance in the presence and absence of various concentrations of PDTC or GSH.

3. Results and discussion

3.1. α_1-Antiproteinase inactivation

HOCl is a strong oxidant released from activated phagocytes that readily reacts with amino acid residues in proteins, in particular methionine, cysteine and lysine. A prominent effect of HOCl at sites of inflammation is inactivation of the protease inhibitor α_1-antiproteinase. Due to this inactivation, the ability of α_1-antiproteinase to inhibit elastase is lost. The resulting higher activity of elastase has been implicated in tissue degradation underlying, e.g., lung emphysema. The extent of α_1-antiproteinase inactivation by HOCl in the presence of various scavengers has been used to quantify their antioxidant potency [29,34,35].

As shown in Fig. 1A, addition of HOCl (10–200 μM) to α_1-antiproteinase (400 μg/ml) dose-dependently impaired the ability of α_1-antiproteinase to inhibit elastase. This inactivation of α_1-antiproteinase by HOCl was markedly attenuated by PDTC (100 μM) (Fig. 1A). The dose dependence of the protective effect of PDTC was investigated using 50 μM HOCl, a concentration at which α_1-antiproteinase was inactivated by about 90% (Fig. 1A). As shown in Fig. 1B, PDTC (10–200 μM) effectively and dose-dependently prevented inactivation of α_1-antiproteinase. The concentration required for 50% inhibition (IC_{50}) of HOCl-induced inactivation of α_1-

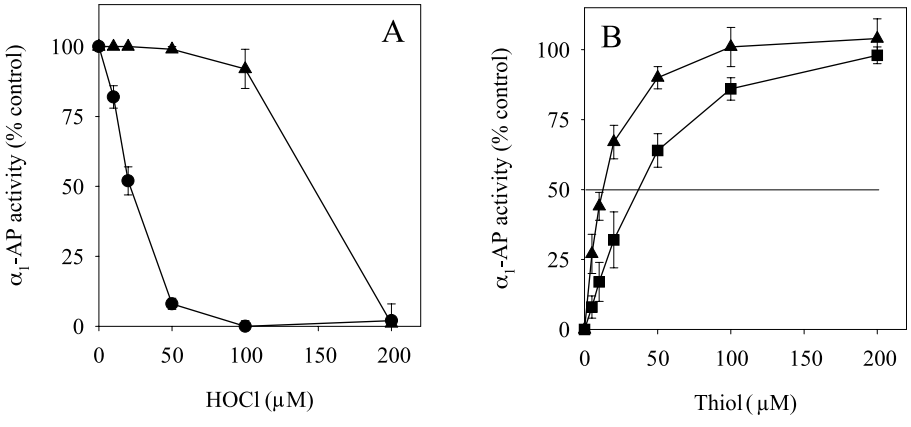

Fig. 1. A: Concentration-dependent inactivation of α_1-antiproteinase (400 μg/ml) by HOCl (●), and inhibition by 100 μM PDTC (▲). B: Concentration-dependent protection by PDTC (▲) and GSH (■) against inactivation of α_1-antiproteinase by 50 μM HOCl. α_1-Antiproteinase activity was measured as described in Section 2. The horizontal line in panel B indicates 50% inhibition. Data represent the mean ± S.D. of three separate experiments.

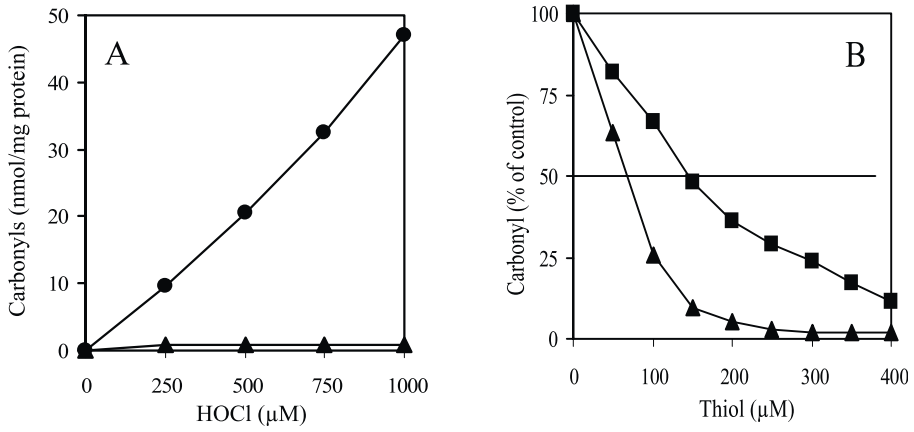

Fig. 2. A: Concentration-dependent formation of protein carbonyls in BSA (1 mg/ml) by HOCl (●), and inhibition by 500 μM PDTC (▲). B: Concentration-dependent protection by PDTC (▲) and GSH (■) against protein carbonyl formation in BSA by 1 mM HOCl. BSA carbonyl content was measured as described in Section 2. The horizontal line in panel B indicates 50% inhibition. Data represent the mean of three separate experiments, with S.D. less than 5%.

antiproteinase was used to compare the scavenging potency of PDTC with GSH, a major intracellular, water-soluble antioxidant. Based on this measure, PDTC was about three-fold more potent than GSH (IC_{50} = 13 μM PDTC and 38 μM GSH) (Fig. 1B).

The protection by PDTC was not due to regeneration of α_1-antiproteinase after it had been inactivated by HOCl, since addition of 100 μM PDTC to inactivated α_1-antiproteinase did not restore the inhibitory effect of α_1-antiproteinase on elastase (data not shown). Therefore, the most likely mechanism by which PDTC exerts its protective effect is direct scavenging of HOCl before it can react with and inactivate α_1-antiproteinase.

3.2. Protein carbonyl formation

Oxidative damage to proteins results in the formation of protein carbonyl groups [30]. Increased tissue protein carbonyls have been detected in numerous human diseases, such as rheumatoid arthritis, myocardial ischemia-reperfusion injury and Alzheimer's disease [30]. The amount of carbonyl groups formed on proteins reflects the extent of oxidative damage. Therefore, we measured carbonyl formation on BSA, used as a model protein, to test the ability of PDTC to scavenge HOCl.

As shown in Fig. 2A, the carbonyl content of BSA (1 mg/ml) increased linearly upon incubation with increasing concentrations of HOCl (250 μM to 1 mM). PDTC (500 μM) completely inhibited HOCl-induced carbonyl formation (Fig. 2A). As above, a fixed concentration of HOCl (1 mM) was used to determine the IC_{50} for PDTC and GSH. As shown in Fig. 2B, PDTC was about 2.3-fold more potent in scavenging HOCl than GSH (IC_{50} = 70 μM PDTC and 160 μM GSH).

3.3. LDL oxidation

Oxidation of LDL and increased negative surface charge due to modification of its protein moiety, apolipoprotein B-100 (apoB), have been implicated in increased macrophage uptake of LDL and formation of lipid-laden foam cells in human atherosclerotic lesions [36,37]. Exposure of LDL to increasing concentrations of HOCl (100–500 μM) resulted in a dose-dependent increase in its electronegative charge, as measured by REM, with a greater than 200% increase at

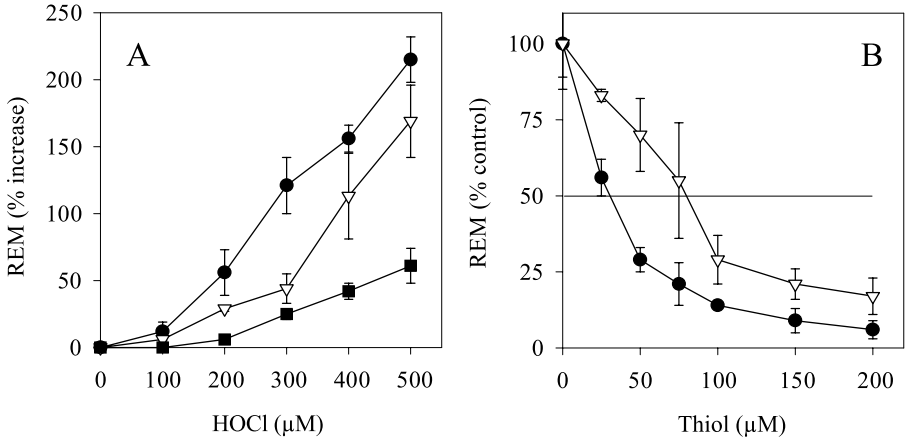

Fig. 3. A: Concentration-dependent increase in REM of LDL incubated with HOCl (●), and inhibition by 50 μM PDTC (■) and NAC (∇). B: Concentration-dependent protection by PDTC (●) and NAC (∇) against increased REM of LDL incubated with 500 μM HOCl. REM of LDL was determined as described in Section 2. The horizontal line in panel B indicates 50% inhibition. Data represent the mean ± S.D. of three separate experiments.

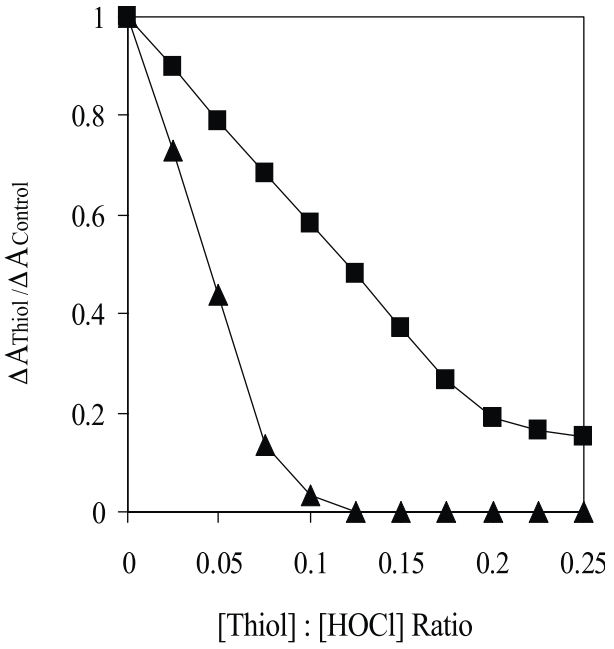

Fig. 4. Concentration-dependent inhibition by PDTC (▲) and GSH (■) of HOCl-induced oxidation of $Fe(II)(CN)_6$. Ferrocyanide oxidation was measured as described in Section 2. Relative absorbance changes at 420 nm $\Delta A_{Thiol}/\Delta A_{Control}$ are plotted against the concentration ratio of [Thiol]:[HOCl]. $\Delta A_{Thiol} = A_{Thiol}$ (with HOCl) $- A_{Thiol}$ (without HOCl). Data represent the mean of three separate experiments, with S.D. less than 5%.

500 μM HOCl (Fig. 3A). The extent of LDL oxidation measured by REM was not linearly correlated with HOCl concentration (Fig. 3A), most likely because HOCl can react with a number of different amino acid residues with different affinities, and oxidation of these amino acids results in different effects on the electronegative charge of the lipoprotein particle. In the presence of 50 μM PDTC or NAC, 500 μM HOCl caused only a 60% or 170% increase in REM, respectively (Fig. 3A). The increase in REM correlated with the loss of LDL lysine residues: 500 μM HOCl caused oxidation of 76% of the lysine residues in apoB, whereas in the presence of 50 μM PDTC or NAC only 34% and 58%, respectively, of the lysine residues became oxidized (data not shown). Exposure of LDL to 500 μM HOCl in the presence of increasing concentrations of PDTC or NAC (25–200 μM) resulted in a dose-dependent decrease in REM, with IC_{50} values of 31 μM PDTC and 80 μM NAC (Fig. 3B). These IC_{50} values were similar to those for inhibition of lysine oxidation, viz. 41 μM PDTC and 80 μM NAC (data not shown). Thus, PDTC is at least twice as effective as NAC in protecting against HOCl-mediated apoB modification in LDL.

3.4. Ferrocyanide oxidation and monochlorodimedon chlorination

The ability of PDTC to scavenge HOCl was further evaluated by two quantitative methods. The oxidation of ferrocyanide, $Fe(II)(CN)_6$, by HOCl (200 μM) was used to determine the stoichiometry of the reaction of HOCl with PDTC [33]. Based on this quantitative method, one molecule of PDTC was able to scavenge up to 11 molecules of HOCl, while one molecule of GSH was able to scavenge only four molecules of HOCl (Fig. 4). Identical stoichiometries for the reaction of HOCl with PDTC and GSH were obtained by employing the monochlorodimedon chlorination assay [9] (data not shown). Thus, PDTC appears to be a very potent HOCl scavenger. At present we cannot explain why one molecule of PDTC was able to scavenge up to 11 molecules of HOCl. Further studies are needed to explain this extraordinary result.

Virtually identical dose-dependent effects were observed with another dithiocarbamate, DDTC, against ferrocyanide oxidation and monochlorodimedon chlorination, as well as α_1-antiproteinase inactivation, BSA carbonyl formation and LDL oxidation (data not shown).

3.5. Direct interaction between PDTC and HOCl

To better understand the mechanism by which PDTC scavenges HOCl, the direct interaction between these two compounds was studied. PDTC possesses two distinct UV absorbance peaks at 254 and 278 nm, which correspond to the dithiocarbamate group. Addition of HOCl (100–600 μM) to PDTC (100 μM) led to a dose-dependent decay and disappearance of these two peaks (Fig. 5). Similar effects were also observed with DDTC (data not shown). These results indicate that the dithiocarbamate group of PDTC is responsible for its HOCl scavenging ability.

At low HOCl:PDTC ratios (≤ 2), PDTC was oxidized by HOCl to form a white precipitate, which was tentatively identified as its corresponding disulfide based on mass spectrometric analysis (data not shown) and previous studies on other dithiocarbamates [38]. The precipitate could be further oxidized by excess HOCl to form several as yet unidentified products (Fig. 5).

3.6. Summary

In this study, the ability of the dithiocarbamates, PDTC and DDTC, to scavenge HOCl was carefully evaluated by employing three qualitative, biologically relevant methods: α_1-antiproteinase inactivation, protein carbonyl formation and LDL oxidation; and two quantitative methods: ferrocyanide oxidation and monochlorodimedon chlorination. Although these five test systems use different parameters to assess the ability to scavenge HOCl, PDTC and DDTC were consistently found to be two to three times more potent than

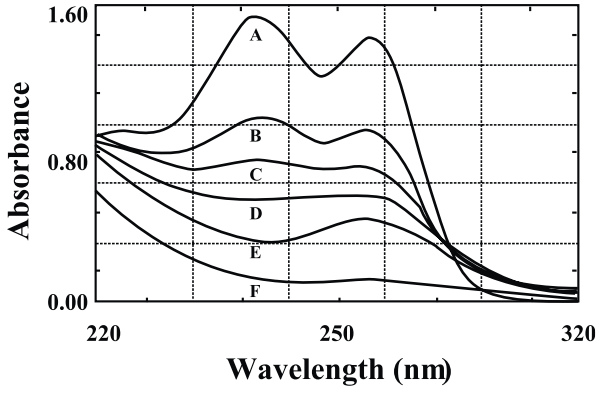

Fig. 5. Destruction of the dithiocarbamate group of PDTC by HOCl. PDTC (100 μM) was incubated without (A) or with HOCl (B–F) in 0.1 M phosphate buffer (pH 7.4) at room temperature for 5 min, and absorbance was recorded from 220 to 320 nm. HOCl concentrations (μM) were as follows: curve B, 100; curve C, 200; curve D, 300; curve E, 400; and curve F, 600.

GSH or NAC. As far as we know, this is the first report to show that these dithiocarbamates are potent HOCl scavengers. PDTC has been shown to suppress NFκB activation after HOCl stimulation in a T-lymphocytic cell line [25]. This effect was attributed to the general antioxidant activity of PDTC [25]. Based on our new findings, we propose that the protection by PDTC is through direct scavenging of HOCl. Because HOCl has been implicated in emphysema, atherosclerosis and many other inflammatory-immune injuries [1,2] and dithiocarbamates are already used clinically [12,39–41], in vivo studies in humans of the potential of dithiocarbamates to prevent or treat inflammatory conditions appear warranted.

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