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In vitro inhibition of human neutrophil histotoxicity by ambroxol: evidence for a multistep mechanism

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1 Neutrophils are major culprits for the protease/antiprotease imbalance during various lung diseases, that is, chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary fibrosis and adult respiratory distress syndrome. Thus, these cells are presently considered an ideal target for the pharmacologic control of tissue injury during these diseases.

2 This study was planned in order to investigate if ambroxol and its precursor bromhexine are actually capable of preventing alpha-1-antitrypsin (A1AT) inactivation by stimulated neutrophils and possibly to look into the mechanisms underlying this event.

3 Ambroxol inhibited the production of superoxide anion by activated neutrophils, whereas bromhexine had no inhibitory effect.

4 Ambroxol decreased the production of hypochlorous acid (HOCl) from activated neutrophils with high efficiency, whereas bromhexine had a modest activity.

5 Ambroxol and bromhexine were capable of limiting the chlorination of monochlorodimedon by HOCl, displaying the capacity of directly scavenging the oxidant.

6 Ambroxol decreased the release of elastase and myeloperoxidase from activated neutrophils, whereas bromhexine was ineffective.

7 Ambroxol prevented the A1AT inactivation by neutrophils, whereas bromhexine was completely ineffective.

8 Among drugs currently available for *in vivo* use in humans, ambroxol is unique by virtue of its ability to prevent neutrophil-mediated A1AT inactivation *via* inhibition of HOCl production as well as HOCl scavenging. Also taking into account its capacity for curbing elastase release, the drug displays the potential to lessen the burden of oxidants/proteases and to increase the antiprotease shield at the site of inflammation. Thus, ambroxol appears to be a good candidate for raising attempts to develop new therapeutic histoprotective approaches to inflammatory bronchopulmonary diseases.
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Keywords: Alpha-1-antitrypsin; ambroxol; elastase; inflammation; neutrophils; oxidants; pulmonary diseases

Abbreviations: A1AT, alpha-1-antitrypsin; HOCl, hypochlorous acid; HBSS, Hanks' balanced saline solution; MCD, monochlorodimedon; fMLP, *N*-formyl-leucyl-phenyl alanine; PPE, porcine pancreatic elastase; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate; EIC, elastase inhibitory capacity; O_2^-, superoxide anion; MPO, myeloperoxidase

Introduction

Neutrophil inflammation, that is the inflammatory reaction characterized by tissue recruitment of neutrophils, is the hallmark of various respiratory tract diseases such as chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary fibrosis and adult respiratory distress syndrome (Dar & Crystal, 1999; Ware & Matthay, 2000; De Rose, 2002; Stockley, 2002). In fact, neutrophils are presently considered as a privileged target for the pharmacologic control of tissue injury at the bronchopulmonary level. Indeed, several new compounds, that is, phosphodiesterase type IV inhibitors, chemokine antagonists, protease inhibitors and antioxidants, proposed for the pharmacologic treatment of chronic obstructive pulmonary disease are thought to exert their activity by neutrophil-related mechanisms (Dallegri & Ottonello, 1997). In this regard, the growing knowledge about the pathophysiology of these conditions and the better understanding of neutrophil histotoxic pathways have led to the consideration of bronchopulmonary tissue injury as the result of an imbalance between proteases, largely neutrophil elastase, and protective antiproteases such as alpha-1-antrypsin (Ossanna *et al*., 1986; Stockley, 1999; MacNee, 2000). The recruitment and the activation of neutrophils at sites of inflammation are major determinants for the local loading of oxidants and proteases, responsible for the aforementioned imbalances (Stockley, 1999). Indeed, neutrophils are increased in the airways of patients with chronic obstructive pulmonary disease, and the sputum volume from patients with bronchiectasis strictly correlates with the content of neutrophil elastase in secretions (Ossanna *et al*., 1986). Moreover,

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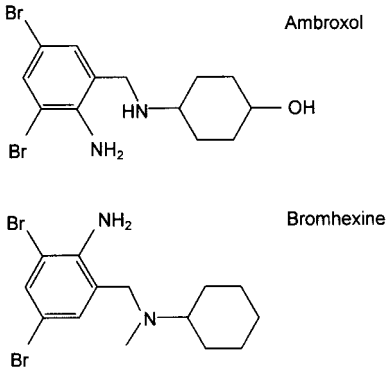

Figure 1 Chemical structure of ambroxol and bromhexine.

neutrophils are known to inactivate alpha-1-antitrypsin, the major endogenous inhibitor of serine proteases, through the generation of hypochlorous acid (HOCl) (Zaslow *et al*., 1983), in turn favoring the uncontrolled activity of elastase (Barnes, 1999).

The bromhexine derivative ambroxol has been reported to interfere with the activation of neutrophils (Gillissen *et al*., 1997a; Cho *et al*., 1999) and with the ability of reagent HOCl and chloramine-T to inactivate alpha-1-antitrypsin (A1AT) (Cho *et al*., 1999). Nevertheless, no data are available regarding the ability of this drug to interfere with neutrophil-mediated A1AT inactivation directly. Consequently, the present research was planned in order to understand if ambroxol and its precursor bromhexine (Figure 1) are actually capable of preventing A1AT inactivation by stimulated neutrophils and possibly the mechanisms underlying this event.

Methods

Media and reagents

Hanks' balanced saline solution with 1 mg ml^{-1} glucose and without phenol red (HBSS, ICN Biomed, Milan, Italy) was used as an incubation medium. Catalase (bovine liver), superoxide dismutase (type I, bovine blood), taurine, L-methionine, *o*-dianisidine, monochlorodimedon (MCD), cytochalasin c, *N*-formyl-leucyl-phenyl alanine (fMLP), hypoxathine and xanthine oxidase were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. A1AT, porcine pancreatic elastase (PPE), *N*-succinyl-(1-alanyl)$_3$-*p*-nitroanilide and MeO-ala-ala-pro-val-pNA were purchased from Calbiochem Co., San Diego, CA, U.S.A. Heparin (Liquemin) was purchased from Roche, Milan, Italy, and Ficoll–Hypaque was purchased from Nyegaard & Co., Oslo, Norway. Phorbol-12-myristate-13-acetate (PMA, Sigma), stored at −20°C as a stock solution of 2 mg ml^{-1} in dimethylsulfoxide (C. Erba, Milan, Italy), was diluted in medium and used at a final concentration of 10 ng ml^{-1}. The reagent 5-thio-2-nitrobenzoic acid was prepared by reducing 5-5′-dithiobis (2-nitrobenzoic) acid (Sigma), as described by Aune & Thomas (1977). HOCl was generated by dissolving sodium hypochlorite (BDH Ltd, Poole, U.K.) into a solution buffered at pH 7.4. Sodium dodecyl sulfate (SDS), acrylamide and bisacrylamide were purchased from BRL, Gaithersurg, MD, U.S.A. Ambroxol and bromhexine were purchased from Sigma. Other reagent grade compounds were used as obtained from commercial suppliers.

Neutrophils

Heparinized (heparin 10 U ml^{-1}) venous blood was obtained from healthy volunteers. Neutrophils were isolated by dextran sedimentation, and subsequent centrifugation on a Ficoll–Hypaque density gradient, as described (Ottonello *et al*., 1995). Contaminating erythrocytes were removed by hypotonic lysis (Ottonello *et al*., 1995). Neutrophils were washed three times with HBSS and resuspended in HBSS. The final cell suspensions contained 97% or more neutrophils and more than 98% viable cells, as evaluated by the ethidium bromide–fluorescein diacetate test (Ottonello *et al*., 1995).

Inactivation of A1AT by neutrophils

The inactivation of A1AT by neutrophils was accomplished using 2.5×10^6 neutrophils, 125 μg A1AT and 10 ng ml^{-1} PMA in a final volume of 0.25 ml (Ottonello *et al*., 1995). The experiments were carried out in the absence and presence of appropriate doses of the drugs. The incubations (30 min, 37°C) were in Falcon Plastic tubes (10 × 100 mm, Falcon Plastic, Oxnard, CA, U.S.A.). At the end of the incubation period, methionine (500 nmol) was added to each tube to quench residual oxidants and the cell-free supernatants were isolated by centrifugation (500 × *g* for 5 min at 4°C). The capacity to inhibit PPE (elastase inhibitory capacity, EIC) by the A1AT present in the supernatants and its ability to complex with PPE (SDS–PAGE analysis) were then determined.

Assay of the EIC of A1AT

The EIC of A1AT was used for the assessment of the activity of A1AT. The method was a modification of the standard spectrophotometric assay (Ottonello *et al*., 1995). Briefly, 40 μl of supernatant containing A1AT and 20 μl of 37 U ml^{-1} PPE were mixed with 100 μl of 2.0 mol l^{-1} Tris HCl buffer (pH = 8.0) in a microtiter tray (Titertek microtritation equipment, Flow). The tests were carried out in triplicate. The microplate was agitated for 30 min (37°C) in a microplate spectrophotometer reader (Titertek Twinreader Plus, Flow). Then, 20 μl of the reaction mixture was transferred into the wells of another microtiter tray containing 230 μl of the PPE substrate (6.6 mmol l^{-1} *N*-succinyl-(1-alanyl)$_3$-*p*-nitroanilide in 0.05 mol l^{-1} Tris-HCl, pH = 8.0, containing 0.3% dimethylsulfoxide). The tray was agitated for 10 s (25°C), the absorbance at the wavelength of 405 nm was read immediately and again every 1 min for 5 min and the change in absorbance was calculated. The percent loss of EIC was calculated by comparing the ability of equal amounts of control and test samples of A1AT to suppress PPE activity. The reproducibility of the assay, expressed as the coefficient of variation, was 11.02% ($n = 10$).

SDS–PAGE analysis

The capacity of A1AT to complex with PPE was examined by SDS–PAGE analysis (Dallegri *et al*., 1999). Briefly, 20 μg of native A1AT or neutrophil-treated A1AT was incubated alone or with 1.6 μg of PPE for 30 min at 25°C. The samples were

then heated at 100°C for 1.5 min in 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue and 65 mmol l^{-1} Tris-HCl (pH = 8.8). The samples were analyzed by slab gel electrophoresis using 3% polyacrylamide stacking gel (pH = 6.8) and a 7.5% polyacrylamide resolving gel (pH = 8.8). After electrophoresis, proteins bands were visualized with silver stain (Bio-Rad, Richmond, CA, U.S.A.).

HOCl assay

The generation of HOCl by neutrophils was measured by the taurine trapping technique (Weiss *et al*., 1982), as described (Ottonello *et al*., 1995). The incubations were carried out using 10^6 neutrophils in a final volume of 1 ml containing 20 mmol l^{-1} taurine in the absence and presence of different concentrations of the drugs. At the end of the incubation period (60 min, 37°C), the amount of HOCl trapped by taurine (yielding taurine monochloramine) in the cell-free supernatants was determined by measuring (OD = 412 nm, $\varepsilon = 13.6$ mmol l^{-1} cm^{-1}) the oxidation of 5-thio-2-nitrobenzoic acid spectrophotometrically. Control experiments were carried out to test the capacity of the drugs to react directly with taurine monochloramine. To this aim, neutrophils were stimulated with PMA in the presence of taurine. Then, appropriate doses of the drugs were added to the cell-free supernatants before the addition of 5-thio-2-nitrobenzoic acid.

Competition between drugs and MCD for HOCl

The addition of reagent HOCl to MCD ($\varepsilon = 20.2$ mmol $^{-1}$ cm^{-1} at 290 nm) results in the rapid chlorination of MCD with a consequent decrease in absorbance at 290 nm (Cuperus *et al*., 1985). To test the capacity of the drugs to compete with MCD for reagent HOCl, MCD (20 nmol) was mixed with various doses of each drug, and then 16 nmol of reagent HOCl was added (final volume = 1 ml, $t = 25$°C, pH = 7.4). The absorbance of the samples was measured at 290 nm after 1 min incubation.

Superoxide anion release assay

The release of superoxide anion (O_2^-) was studied by using a modification of the method of Babior *et al*. (1973), as previously described (Ottonello *et al*., 1999). Briefly, neutrophils (5×10^5) were incubated (20 min, 37°C, 0.5 ml final volume) with 80 μmol l^{-1} ferricytochrome *c* and 10 ng ml^{-1} PMA in the absence or presence of 300 U ml^{-1} superoxide dismutase. The reactions were then stopped by adding 2 ml of ice-cold 1 mmol l^{-1} *N*-ethyl-maleimide and the O_2^- production was determined in the supernatants from the OD_{550} of samples without superoxide dismutase minus OD_{550} of samples with superoxide dismutase using an extinction coefficient of 21.1 mmol l^{-1} cm^{-1}. The O_2^- production was also evaluated in a cell-free hypoxathine–xanthine oxidase system using 0.2 U ml^{-1} xanthine oxidase, 0.15 mmol l^{-1} hypoxanthine and 30 μmol l^{-1} ferricytochrome *c* in the absence or presence of the drugs.

Myeloperoxidase (MPO) and elastase release assays

Neutrophils (5×10^5) were preincubated (5 min) with 5 μg ml^{-1} cytochalasin c. Then, 100 nmol l^{-1} fMLP was added and the incubation was carried out for 1 h at 37°C (final volume 0.5 ml) in the absence and presence of the appropriate doses of the drugs. These experimental conditions were chosen taking into account the fact that PMA is a poor activator of neutrophil primary granule exocytosis. The MPO activity in supernatants was determined by using 0.167 mg ml^{-1} *O*-dianisidine and 0.1 mmol l^{-1} hydrogen peroxide in 50 mmol l^{-1} phosphate buffer (pH 6.0), as previously described (Ottonello *et al*., 1999). One unit of enzyme activity was defined as that oxidizing 1 μmol of *O*-dianisidine min^{-1} at 25°C (OD_{550} extinction coefficient: 11.3 mmol l^{-1} cm^{-1}). Control experiments were carried out to test the capacity of the drugs to inhibit MPO directly. To this aim, neutrophils were stimulated with PMA. Then, appropriate doses of the drugs were added to the cell-free supernatants, and the MPO activity was tested by the *O*-dianisidine assay. The elastase activity in supernatants was determined with 1 mmol l^{-1} MeO-ala-ala-pro-val-pNA (dissolved in dimethyl sulfoxide) in 0.1 mol l^{-1} HEPES buffer at pH 7.5, containing 0.5 mol l^{-1} NaCl and 10% dimethyl sulfoxide at 25°C, as previously described (Ottonello *et al*., 1997). The cleavage of the substrate was monitored spectrophotometrically at 410 nm. The elastase activity was determined as nmol of substrate cleaved per hour (OD_{410} extinction coefficient: 8.8 mmol l^{-1} cm^{-1}).

Statistical analysis

The data were expressed as mean ± s.e.m. The differences were determined by the nonparametric Mann–Whitney test or ANOVA followed Dunnett's multiple comparison test using GraphPad InStat version 3.05 for Windows 95, GraphPad Software, San Diego CA, U.S.A. The differences were accepted as significant when $P < 0.05$.

Results

Effect of ambroxol and bromhexine on neutrophil O_2^- production

Human neutrophils (1×10^6), incubated with 10 ng ml^{-1} PMA for 20 min at 37°C, were found to produce 69.4 ± 4.9 (mean ± 1 s.e.m., $n = 10$) nmol of O_2^- as detected by superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Under the same experimental conditions, resting neutrophils generated 2.6 ± 3.3 (mean ± 1 s.e.m., $n = 10$) nmols of O_2^-. As shown in Figure 2, ambroxol inhibited O_2^- production by activated neutrophils in a dose-dependent manner. The dose of ambroxol able to induce 50% inhibition ($IC_{50\%}$) was 146.7 ± 39.9 μmol l^{-1} (mean ± 1 s.e.m., $n = 4$). In contrast, bromhexine had no inhibitory effect. In order to understand whether the inhibitory activity of ambroxol is related to an interference with the O_2^- assessment, the effect of the drug was studied using a cell-free hypoxathine–xanthine oxidase system generating O_2^-. This enzymatic system generated 5.9 ± 0.1 μmol min^{-1} of O_2^- (mean ± 1 s.e.m., $n = 3$) in the absence of ambroxol and 5.6 ± 0.1 nmol O_2^- min^{-1} in the presence of 1 mmol l^{-1} ambroxol. Therefore, the drug neither interferes with the O_2^- assay nor acts as a scavenger of O_2^-. In conclusion, ambroxol but not bromhexine, appears to have cell-directed inhibitory effects.

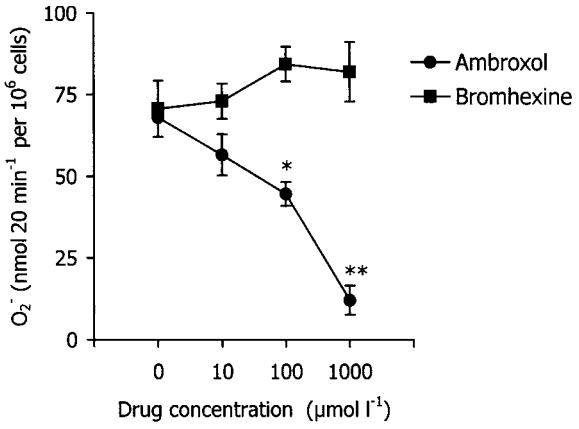

Figure 2 Effect of different doses (abscissa) of ambroxol and bromhexine on the production of superoxide anion (O_2^-) by neutrophils. Results are expressed as mean ± 1 s.e.m. (ambroxol: $n=4$, bromhexine: $n=5$). *$P<0.05$; **$P<0.01$ vs control without the drug (0); ANOVA followed Dunnett's multiple comparison test.

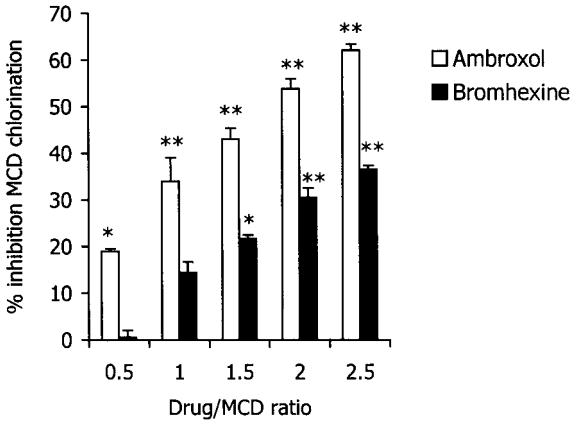

Figure 4 Inhibitory effect of various concentrations of ambroxol and bromhexine on the chlorination of MCD by reagent HOCl. The abscissa shows the ratio between the concentration of each drug and that of MCD. Results are expressed as mean ± 1 s.e.m., $n=3$. *$P<0.05$; **$P<0.01$ vs control without the drug; ANOVA followed Dunnett's multiple comparison test.

Effect of ambroxol and bromhexine on the HOCl production by neutrophils

Hydrogen peroxide (H_2O_2), derived from dismutation of the generated O_2^-, is transformed into HOCl by MPO released by neutrophils undergoing activation. As shown in Figure 3, ambroxol decreased the production of HOCl with high efficiency whereas, in comparison, bromhexine had a modest activity. On the contrary, ambroxol did not react with taurine monochloramine (taurine monochloramine in the absence of ambroxol: 33.3 ± 0.5 nmol l^{-1} h^{-1} per 10^6 cells, taurine monochloramine in the presence of 1 mmol l^{-1} ambroxol: 34.9 ± 0.9 nmol l^{-1} h^{-1} per 10^6 cells, mean ± 1 s.e.m., $n=3$). Similar results were obtained in the presence of different doses of ambroxol ranging from 1 to 100 μmol l^{-1} (data not shown). The dose of ambroxol capable of inducing 50% inhibition (IC_{50}) was 9.7 ± 0.7 μmol l^{-1} (mean ± 1 s.e.m., $n=5$). This value is significantly lower ($P=0.0159$) than the IC_{50} for O_2^- production mentioned above. Therefore, it appears that the ability of ambroxol to inhibit oxidant production (O_2^- and in turn H_2O_2) is only in part responsible for the observed effect on HOCl bioavailability in the neutrophil surroundings.

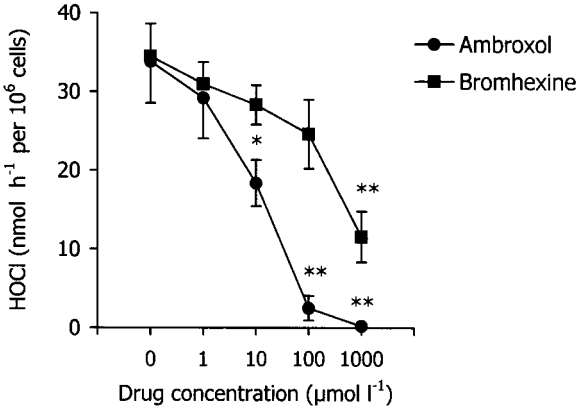

Figure 3 Effect of different doses (abscissa) of ambroxol and bromhexine on the production of HOCl from neutrophils. Results are expressed as mean ± 1 s.e.m., $n=5$. *$P<0.05$; **$P<0.01$ vs control without the drug (0); ANOVA followed Dunnett's multiple comparison test.

Consistently, ambroxol, and bromhexine as well, were capable of directly inactivating HOCl. Indeed, both the drugs were found to limit the chlorination of MCD by reagent HOCl (Figure 4).

Effect of ambroxol on neutrophil primary granule exocytosis

Neutrophil primary granule exocytosis was studied in slightly different experimental conditions than those used for oxidative assays. Indeed, taking into account the fact that PMA is a poor activator of primary granule exocytosis, neutrophils were triggered with the chemoattractant fMLP after cytochalasin c pretreatment. In these conditions, neutrophils were found to release 15.3 ± 3 mU MPO h^{-1} per 10^6 cells (mean ± 1 s.e.m., $n=3$) and 293.0 ± 32.5 nmol elastase activity h^{-1} per 10^6 cells (mean ± 1 s.e.m., $n=6$). As shown in Figure 5, ambroxol was found capable of reducing the release of both the enzymes, whereas bromhexine was ineffective (not shown). On the

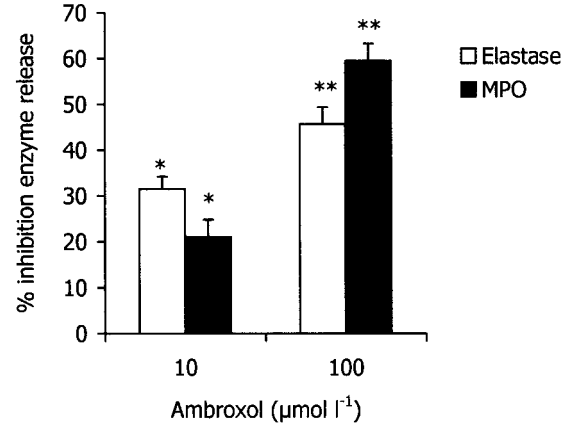

Figure 5 Effect of ambroxol on neutrophil primary granule exocytosis. Results are expressed as percent inhibition of MPO (mean ± 1 s.e.m., $n=3$) and elastase (mean ± 1 s.e.m., $n=6$) release from fMLP-activated neutrophils. *$P<0.05$; **$P<0.01$ vs control without the drug; ANOVA followed Dunnett's multiple comparison test.

contrary, ambroxol was completely ineffective on MPO activity, determined as the capacity of oxidizing *O*-dianisidine (MPO activity in the absence of ambroxol: $16.5 \pm 2.5\,mU\,h^{-1}$ per 10^6 cells, MPO activity in the presence of $1\,mmol\,l^{-1}$ ambroxol: $16.1 \pm 1.9\,mU\,h^{-1}$ per 10^6 cells, mean $\pm$ 1 s.e.m., $n=3$). Similar results were obtained testing MPO activity in the presence of different doses of ambroxol ranging from 1 to $100\,\mu mol\,l^{-1}$ (data not shown).

Ambroxol prevents the inactivation of A1AT by neutrophils

When exposed to stimulated neutrophils, $25\,\mu g$ of A1AT was completely inactivated during the 60 min incubation period. Conversely, resting neutrophils were ineffective. As summarized in Figure 6, ambroxol prevented the A1AT inactivation by neutrophils in a dose-dependent manner. Bromhexine was completely ineffective (Figure 6). In order to confirm the activity of ambroxol, the effect of the drug on the ability of A1AT to bind elastase was studied. As shown in Figure 7, native A1AT (lane 1) formed an SDS-stable complex with elastase (lane 2), whereas A1AT exposed to activated neutrophils (lane 3) did not complex with elastase. In the presence of ambroxol, A1AT was protected from inactivation by stimulated neutrophils and consequently a band that comigrated with the A1AT-elastase complex (lane 4) could be detected.

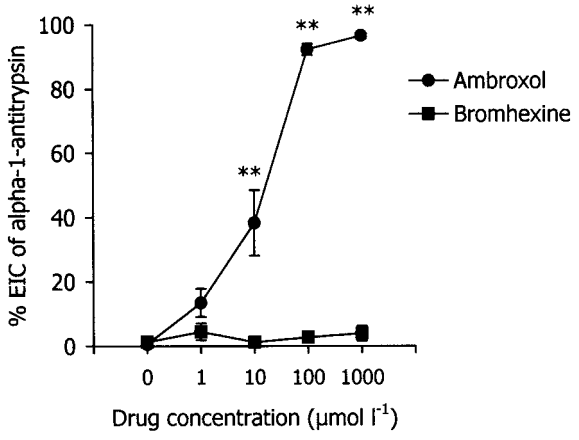

Figure 6 Effect of different doses (abscissa) of ambroxol and bromhexine on the inactivation of A1AT by neutrophils. After incubation with neutrophils, the activity of A1AT was measured as EIC using PPE. The percent EIC of A1AT (ordinate) was calculated by comparison of control samples of A1AT incubated in the absence of cells. Results are expressed as mean $\pm$ 1 s.e.m. (ambroxol: $n=5$, bromhexine: $n=3$). $^*P<0.05$; $^{**}P<0.01$ *vs* control without the drug; ANOVA followed Dunnett's multiple comparison test.

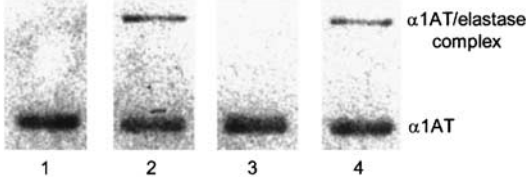

Figure 7 Analysis of the interaction of PPE with A1AT, incubated with neutrophils in the absence or presence of ambroxol. Lane 1 = native A1AT; lane 2 = A1AT plus PPE; lane 3 = neutrophils-exposed A1AT plus PPE; lane 4 = A1AT exposed neutrophils in the presence of $100\,\mu M$ ambroxol plus PPE. The lanes shown are from a representative experiment of three experiments**.

Discussion

The present results suggest that: (A) ambroxol prevents the inactivation of A1AT by stimulated neutrophils with high efficiency; (B) the drug decreases the bioavailability of HOCl in the microenvironment where neutrophils undergo activation; (C) ambroxol is capable of reducing the release of elastase by activated neutrophils. In other words, in the presence of ambroxol, neutrophil oxidative and elastolytic activities are significantly inhibited. This, in turn, may result in the lessened burden of neutrophil-derived histotoxins at sites of inflammation.

In agreement with previous observations (Clark *et al*., 1981; Ossanna *et al*., 1986), the present results confirm that the inactivation of A1AT is mediated by neutrophil-derived HOCl or a compound having similar characteristics. Various studies have indeed shown that A1AT exposed to activated neutrophils undergoes HOCl-mediated oxidation of its reactive center, resulting in at least a 2000-fold reduction of its inhibitory/binding capacity to elastase (Zaslow *et al*., 1983). Consistent with these observations, A1AT exposed to activated neutrophils was found incapable of forming SDS-stable complex with elastase and, on the other hand, ambroxol prevented such a type of A1AT inactivation. Thus, ambroxol is endowed with the capacity of reducing the elastolytic burden of activated neutrophil by curbing elastase release and by protecting the activity of its main inhibitor, that is A1AT.

The mechanism whereby ambroxol exerts the effects on HOCl bioavailability seems to be of interest. In fact, various drugs such as certain anti-inflammatory agents (Cuperus *et al*., 1985; Dallegri *et al*., 1990; Ottonello *et al*., 1995) and selected antibiotics (Ottonello *et al*., 1991; Cantin & Woods, 1993; Dallegri *et al*., 1999) have been shown to scavenge HOCl generated by neutrophils. Consequently, they potently protect A1AT and cells from oxidative injury. As shown herein, ambroxol appears to act primarily by decreasing the production of HOCl. Indeed, the decreased production of HOCl is due to both the ability of ambroxol to inhibit the generation of HOCl precursors, that is O_2^- and H_2O_2, and to diminish the amounts of MPO released by neutrophils, that is the enzyme devoted to the transformation of the generated H_2O_2 into HOCl. Consistent with the concept of ambroxol as an agent capable of directly downregulating neutrophil functional responsiveness, the drug was shown to attenuate phagocyte respiratory burst in response to various stimuli, including modified IgG (Gibbs *et al*., 1999; Park *et al*., 1999), formylpeptides (Gibbs *et al*., 1999; Park *et al*., 1999) as well as opsonized phagocytosable targets or tumor necrosis factor-alpha (unpublished observations).

Furthermore, in competition experiments with MCD for reagent HOCl, ambroxol was shown herein to be also capable of directly inactivating the oxidant. These data confirm previous observations from other authors, carried out in various experimental settings (Lapenna *et al*., 1994; Nowak *et al*., 1994; Gillissen *et al*., 1997a, b). Nevertheless, such a type of activity does not imply a drug ability to prevent A1AT inactivation by HOCl generated by neutrophils. For instance, in the present setting, bromhexine was capable of scavenging

HOCl similar to ambroxol, but was completely incapable of preventing A1AT inactivation by neutrophils. This is therefore consistent with the concept that the reaction of a drug with HOCl is biologically significant only if it is fast enough to prevent the interaction of HOCl with a relevant biological target (Wasil *et al*., 1987). Furthermore, studies performed in cell-free systems to investigate the properties of a drug to prevent HOCl-mediated A1AT inactivation do not strictly reflect the results obtained in the presence of activated neutrophils. For example, Cho *et al*. (1999) have previously reported that A1AT inactivation by an MPO-halide cell free system can be inhibited by high doses of ambroxol. Nevertheless, here we provide the first evidence that ambroxol is actually capable of preventing A1AT inactivation by activated neutrophils at 1–2 log lower concentrations than those used by Cho, which is well within the concentrations detectable in lung tissue after intravenous infusion (Felix *et al*., 1996). It is noteworthy that in a cell-free system, the neutrophil elastolytic activity can be downregulated by HOCl in a manner partially preventable by ambroxol (Cho *et al*., 1999). This would raise the possibility that ambroxol can favor the elastolytic activity of neutrophils by preventing the inhibitory effects of HOCl on the enzyme. On the contrary, the present data show that ambroxol actually prevents the elastolytic activity of neutrophils by inhibiting the release of the enzyme. Consequently, in order to prove the relevance of drug-mediated effects, the activity of the drugs should be tested in complete neutrophil assay systems.

In summary, the present results demonstrate that ambroxol is endowed with the capacity to interfere with oxidative and proteolytic histotoxic activities of neutrophils by acting at multiple levels. In particular, ambroxol is able to inhibit the activation of respiratory metabolism producing O_2^- and HOCl, to curb the exocytosis of elastase- and MPO-positive primary granules, to impair the production of HOCl by decreasing the availability of MPO, to scavenge HOCl directly and to protect A1AT from neutrophil-mediated inactivation, and in turn to restore the capacity of the antiprotease to complex and inactivate elastase (Figure 8). To our knowledge, among drugs currently available for *in vivo* use in humans, ambroxol is unique by virtue of its ability to prevent neutrophil-mediated A1AT inactivation *via* inhibition of HOCl production as well as HOCl scavenging.

The present model system, which includes activated neutrophils and A1AT, appears to be a sensitive method to identify drugs having potential histoprotective activity in neutrophilic inflammatory processes. The inactivation of A1AT by neutrophils, with consequent uncontrolled elastase activity, is presently thought to be a major determinant of tissue injury during various neutrophilic inflammatory diseases, such as chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary fibrosis and adult respiratory distress syndrome (Dar & Crystal, 1999; Ware & Matthay, 2000; De Rose, 2002; Stockley, 2002). Consistent with this concept, oxidized A1AT, neutrophil elastase and MPO have been recovered in fluids from inflamed tissues in these conditions (McGuire *et al*., 1982; Cochrane *et al*., 1983; Hill *et al*., 1999; Van Der Vliet *et al*., 2000; Aaron *et al*., 2001; Stockley *et al*., 2001). Ambroxol was previously shown to inhibit both the locomotion of neutrophils and the production of cytokines relevant for cell tissue migration (Stokley *et al*., 1988; Bianchi *et al*., 1990; Pfeifer *et al*., 1997). Therefore, it can limit the excessive recruitment of neutrophils in inflamed bronchopulmonary tissue. These findings, coupled with the present results, suggest that the drug has the potential to lessen the burden of oxidants/proteases and to increase the antiprotease shield at the site of inflammation. Thus, ambroxol appears to be a good candidate for raising attempts to develop new therapeutic histoprotective approaches to inflammatory bronchopulmonary diseases.

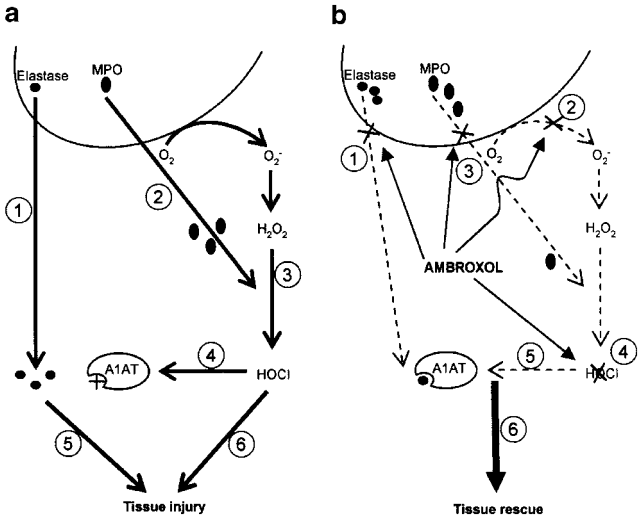

Figure 8 Proposed model for histoprotective properties of ambroxol. (a) Pathways of neutrophil-mediated tissue injury: extracellular release of elastase (1); chlorinated oxidant production by H_2O_2/MPO pathway (2,3); inactivation of A1AT by HOCl (4). These pathways converge to proteolytic (5) and oxidative (6) tissue injury. (b) Inhibitory effect of ambroxol of histotoxic pathways of neutrophils. The drug is capable of inhibiting the release of elastase (1); to diminish the bioavailability of HOCl by impairing the production of the oxidant precursor O_2^- (2), by curbing the release of the catalytic enzyme MPO (3) and by directly scavenging HOCl (4); to restore the antielastase activity of A1AT by neutrophil-mediated inactivation (5). Taken together, these activities result in ambroxol-mediated tissue rescue from neutrophil histotoxicity (6).

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