

DIFFERENTIAL MODULATION OF IgE-DEPENDENT ACTIVATION OF HUMAN BASOPHILS BY AMBROXOL AND RELATED SECRETOLYTIC ANALOGUES

B.F. GIBBS^{1,2}

¹*Department of Dermatology, University of Lübeck, Germany;* ²*Medway School of Pharmacy, University of Kent, United Kingdom*

Received June 15, 2009 – Accepted September 18, 2009

Ambroxol is a widely used secretolytic agent originally developed from vasicine, a natural alkaloid found in *Adhatoda vasica*, extracts of which have been used to treat bronchitis, asthma, and rheumatism. We previously reported that ambroxol inhibits IgE-dependent mediator secretion from human mast cells and basophils, key effector cells of allergic inflammation. Here, the mechanisms involved in the inhibitory properties of ambroxol were assessed in comparison to other secretolytic analogues (e.g. vasicine, bromhexine, sputolysin). The results show that, in comparison to ambroxol, which reduced IgE-dependent histamine release from basophils at 10 μ M–1 mM, the release of the amine was only moderately reduced by sputolysin and vasicine at 1 mM. In contrast, above 10 μ M, bromhexine was found to be toxic to basophils *in vitro* as evidenced by induction of histamine release and reduced cell viability. In contrast, the inhibitory actions of ambroxol at concentrations below 1 mM were not toxic and entirely reversible. Ambroxol was also more potent than either sputolysin or vasicine in attenuating basophil IL-4 and IL-13 secretions, whereas bromhexine-induced suppression of *de novo* cytokine synthesis was due to toxic effects. Additionally, ambroxol reduced IgE-dependent p38 MAPK phosphorylation in basophils, unlike bromhexine, sputolysin and vasicine. These results clearly show that ambroxol is both more potent and effective at inhibiting IgE-dependent basophil mediator release and p38 MAPK activity than the other secretolytic analogues employed. The therapeutic potential of ambroxol as an anti-allergic agent is further underlined by these data.

Basophils have been shown to contribute to the symptoms of allergic diseases, particularly during late-phase responses, due to their capacity to release histamine and leukotriene C₄ upon crosslinking of high-affinity IgE-receptors (Fc ϵ RI) with allergens (1-3). Moreover, basophils also support underlying atopy and Th2-type immunity by rapidly generating IL-4 and IL-13 (3-4), and may also support angiogenesis by virtue of producing vascular endothelial growth factor following IgE-dependent activation (5). Recent *in vivo* studies using basophil-

depleted mice have clearly demonstrated that basophils play a more prominent role than their tissue-fixed mast cell compatriots in orchestrating chronic allergic inflammation (6-7) and initiating Th2 differentiation in the lymph nodes (8). Although this cell type is usually present mainly within the circulation, during allergic inflammation, basophils migrate to affected tissues, such as the lungs of patients with allergic asthma (9-10). Given the above, basophils constitute a potentially important therapeutic target for the actions of new anti-allergic

Key words: ambroxol, bromhexine, vasicine, basophils, signal transduction, histamine, IgE

Mailing address: Dr. Bernhard F. Gibbs, PhD
Medway School of Pharmacy,
University of Kent at Medway,
Central Avenue,
Chatham Maritime, Kent, ME4 4TB, United Kingdom.
Fax: ++44 1634 883 927
e-mail: B.F.Gibbs@kent.ac.uk

0394-6320 (2009)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.
Unauthorized reproduction may result in financial and other penalties

drugs.

The secretolytic agent ambroxol may be considered as a novel anti-allergic drug, especially in the treatment of allergic asthma since we have previously shown that it inhibits IgE-mediated basophil and mast cell activation (11-12) in addition to its secretolytic properties (13). Since mucous production is also exacerbated by histamine, and constitutes a major obstructive component of serious asthmatic late-phase responses, the inhibition of basophil and mast cell histamine release by ambroxol may complement its current clinical application by reducing mucous production as well as reducing mucous viscosity.

Ambroxol was originally developed from the natural alkaloid vasicine which is found in *Adhatoda vasica*, of which extracts were used for centuries in India to treat bronchitis, asthma, and rheumatism (14). Several chemically-related analogues of ambroxol have also been used as secretolytic agents either in humans (e.g. bromhexine) or in veterinary medicine (e.g. sputolysin). However, to date there are no reports regarding their inhibitory actions on allergic effector cells such as basophils. Furthermore, the mode of action of ambroxol and related analogues is also unknown in terms of explaining the blockade of both degranulatory processes (e.g. histamine release) and *de novo* mediator generation (e.g. IL-4 synthesis). The aim of this study is therefore to compare the inhibitory actions of ambroxol with vasicine, sputolysin and bromhexine on IgE-dependent mediator generation from basophils and elucidate the mode of action of these agents on basophil signalling as well as assessing toxic mechanisms.

MATERIALS AND METHODS

The following reagents were purchased: anti-mouse Ig horse radish peroxidase, ECL+plus (Amersham, Buckinghamshire, UK); IL-4 ELISA (BioSource, Camarillo, CA, USA); SB203580, (Calbiochem, Bad Soden, Germany); IL-13 ELISA (Boehringer Ingelheim Bioproducts, Heidelberg, Germany); MACS basophil isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany); rabbit anti-p38 MAPK, rabbit anti-phospho p38 MAPK (New England Biolabs GmbH, Frankfurt am Main, Germany); Ficoll-Paque (Pharmacia Biotec, Uppsala, Sweden), anti-human IgE, 2 x concentrated Laemmli sample buffer, (Sigma, St. Louis, USA). All

other standard reagents were purchased at the highest grade obtainable.

Basophil purification

Human basophils were obtained from Buffy coats (leukocyte concentrate of 450 ml venous blood) prepared from healthy donors undergoing routine blood donation at the University of Lübeck (with ethical approval). Basophils were enriched by Ficoll density centrifugation followed by elutriation and purified close to homogeneity by negative selection using magnetic cell sorting (MACS basophil isolation kit, Miltenyi Biotec). In a previous study, we demonstrated that there are no functional differences between purified and unpurified basophils using this negative selection procedure (15). Following purification basophil purities ranged from 85 to 100% as determined by alcian blue staining, with a mean yield of 2.7×10^6 basophils per buffy coat donor and more than 95% viable cells as judged by Trypan blue exclusion.

Inhibitors

Ambroxol, vasicine, bromhexine and sputolysin were kindly donated by Boehringer Ingelheim Pharma KG, Germany. Ambroxol, bromhexine and sputolysin were dissolved in DMSO, all at 100 mM, and subsequently diluted in HEPES-buffered-Tyrode's solution immediately before use. Vasicine was dissolved in HEPES-buffered-Tyrode's solution and diluted accordingly. Solvent effects due to DMSO were observed at 1 mM drug concentration and the results were corrected for this. At other working dilutions of the compounds (100 μ M or less) no effects were observed due to DMSO in the absence of the drugs.

Cell stimulation and mediator assay

Basophils (1.2×10^5 nucleated cells per vial) were resuspended in HEPES-buffered-Tyrode's solution (400 μ l per tube), supplemented with 1 mM CaCl_2 and preincubated with various concentrations of the drugs for 15 min before stimulation with a sub-optimal dilution of anti-IgE (1/20,000 dilution). Controls consisted of cells incubated with the stimulus in the absence of the inhibitors and cells incubated in buffer alone. In mediator release studies, basophils were stimulated for either 30 min (for histamine release) or 5 h (for the secretion of IL-4 and IL-13). Histamine contents in the supernatants together with the cell pellets, which were diluted as required and lysed with perchloric acid (4%), were measured using a spectrofluorometric autoanalyser. Percentage histamine releases were determined from the total histamine content in the sum of pellet and supernatant tubes. Basophils incubated for 5 h were centrifuged and the respective release of IL-4 and IL-13 in the supernatants was assayed by ELISA, according to the manufacturer's instructions.

The minimum sensitivity of the ELISAs were 3 pg/ml for IL-4 and 1 pg/ml for IL-13. In some experiments, following termination of reactions and separation of supernatants for mediator assay, a portion of diluted and re-suspended cell pellets (20 μ l out of 1.5 ml) were subjected to Trypan blue exclusion analysis for cell viability. Statistical significances were assessed using a paired Student's t-test.

Western blot analysis

Highly pure basophil preparations (>95% pure, 2×10^5 /sample) were treated with the drugs, as described above, and stimulated at 37 °C for 10 min in order to assess their effects on p38 MAPK phosphorylation. SB203580, a p38 MAPK inhibitor, was employed as a positive control.

Reactions were then terminated rapidly by placing tubes in ice after which all tubes were immediately centrifuged and cell pellets lysed by vigorous mixing in 15 μ l lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 10 mM EGTA, 5 mM DTT, 1% Nonidet P-40, 1 mM PMSF, 100 μ g/ml aprotinin, 20 μ g/ml leupeptin and 10 mM benzamidine). The samples were then stored at -80 °C prior to Western blotting. An equal volume of 2 x concentrated Laemmli sample buffer was added to the lysed cell pellets which were then boiled for 3 min. Proteins were separated by 12% SDS-PAGE and blotted onto nitro-cellulose membranes. The molecular weight of the resolved proteins was visualized using prestained molecular weight rainbow markers which were also included for each SDS-PAGE run. Membranes were blocked for 4 h in 5% skimmed milk in TBST buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20) with gentle agitation. Following 3 x 5 min washes in TBST, membranes were incubated overnight (at 4°C) with specific rabbit antibodies to either phosphorylated or total p38 MAPK (as an indication of even cell loading). Following incubation with the primary antibodies, membranes were successively washed (4 x 5 min, TBST) and incubated with anti-rabbit Ig horse radish peroxidase for 2 h with gentle agitation. After washing, unbound secondary antibody proteins were visualized using autoradiography according to the manufacturer's instructions (ECL+plus, Amersham, Buckinghamshire, UK). Comparisons of phosphorylated (activated) signalling proteins were made, not only on the basis of equal cell numbers but also by observing the even signal intensity of total expression. After detection, membranes were stripped for 10-20 min using Re-blot plus reagent (Chemicon international), washed in TBST (4 x 5 min) and reprobed. SB203580, a p38 MAPK inhibitor, was employed as a positive control. Even cell loading was determined using antibodies against total p38 MAPK.

RESULTS

Ambroxol suppressed IgE-induced basophil

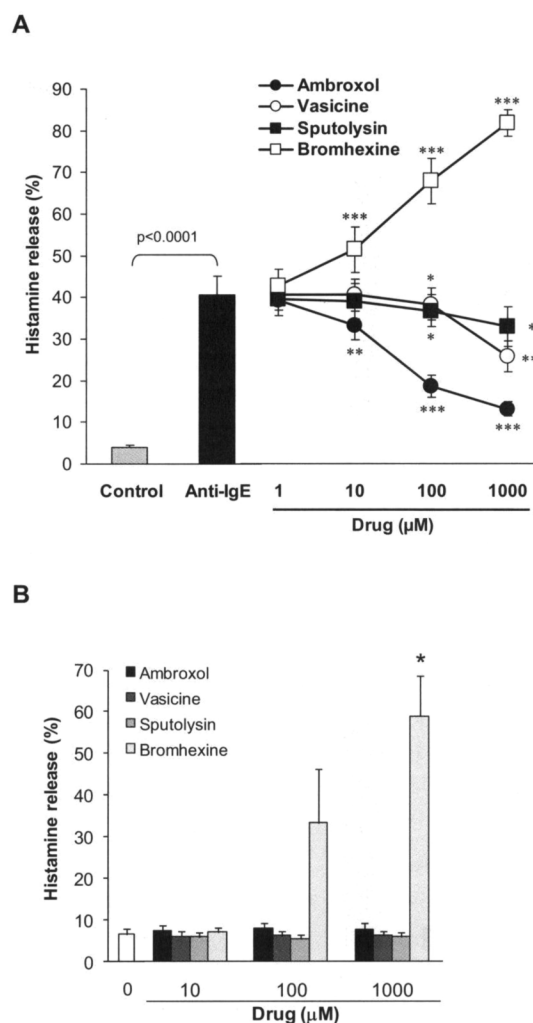


Fig. 1. *A)* Effects of ambroxol, vasicine, sputolysin and bromhexine on histamine release from human basophils stimulated with anti-IgE (1/20,000 dilution). Control represents basophils incubated with buffer alone. *, ** and *** represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) differences between cells stimulated with anti-IgE alone and in the presence of the compounds. Results represent mean histamine releases $\pm$ SEM for 12 separate experiments. *B)* Effect of the above agents on basophil histamine release in the absence of anti-IgE stimulation. Results represent mean histamine releases $\pm$ SEM for 5 separate experiments, where * denotes significant release above spontaneous secretions.

histamine release to a far greater extent than the other analogues employed, producing significant inhibition at concentrations at 10 μ M or higher (Fig. 1a). Vasicine and sputolysin were poor inhibitors of histamine release in comparison to ambroxol,

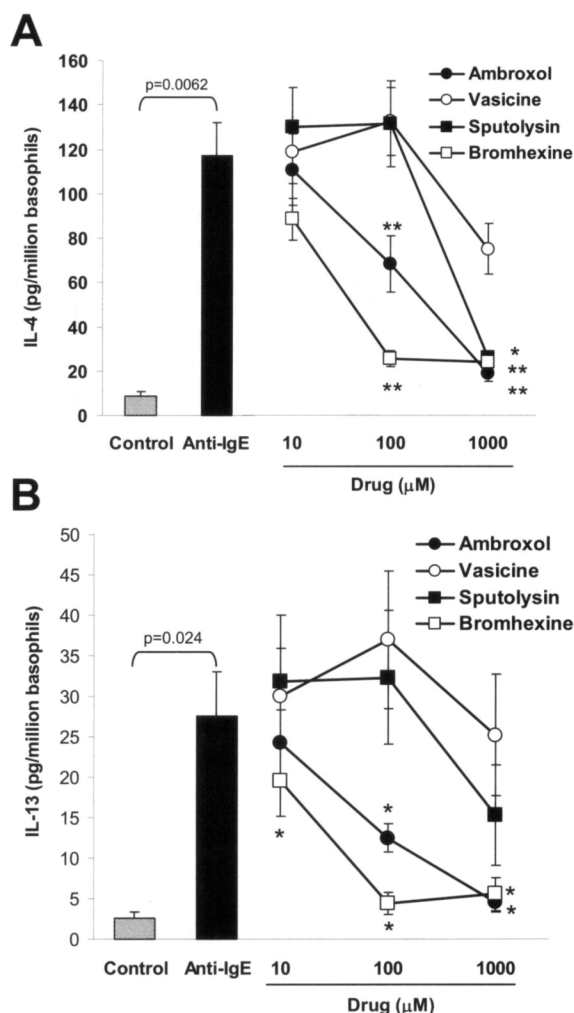


Fig. 2. Effects of ambroxol, vasicine, sputolysin and bromhexine on IL-4 (panel A) and IL-13 (panel B) secretion from human basophils stimulated with anti-IgE (1/20000 dilution). The results are shown as mean cytokine releases $\pm$ SEM for 5 separate experiments. *, ** and *** represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) differences between cells stimulated with anti-IgE alone and in the presence of the compounds.

while bromhexine failed entirely to block histamine release, and even caused significant enhancement above 10 μ M. This enhanced release was, in part, observed as direct stimulatory effects on basophils (Fig. 1b) that had been incubated with the drug for 30 min without anti-IgE stimulation. However, these effects of bromhexine were only statistically significant at 1 mM. At 100 μ M bromhexine alone

caused substantial basophil degranulation in some, but not all, donors while having no effect at 10 μ M or less, despite significant enhancement of IgE-mediated secretion shown in Fig. 1a. Conversely, neither ambroxol, nor vasicine, nor sputolysin caused histamine release from basophils, regardless of the concentration employed.

With the exception of bromhexine, similar effects were observed regarding the actions of the drugs on IgE-mediated IL-4 (Fig. 2a) and IL-13 (Fig. 2b) release from basophils, where ambroxol was a more effective inhibitor of the release of these cytokines than vasicine or sputolysin. However, in contrast to histamine release, bromhexine produced a striking inhibition of the secretion of both cytokines from basophils stimulated by anti-IgE.

Since bromhexine caused blockade of *de novo* generated cytokine release but release of histamine this may possibly be attributed to toxic effects. Trypan blue exclusion assays, shown in Fig. 3, confirmed a toxic mechanism of action for bromhexine which was observed at 100 μ M and above for both stimulation periods used in this study (30 min and 5 h, in addition to a 15 min pre-incubatory period without anti-IgE stimulation). Cell viability was also slightly, but significantly, reduced for basophils incubated with bromhexine for 5 h at 10 μ M. In contrast, the other drugs were largely non-toxic over a wide range of concentrations following short (30 min) incubation with the cells, although viability was reduced to $<60\%$ with a high concentration (1 mM) of ambroxol after 5 h incubation. Sputolysin also gave rise to a slight reduction of cell viability at 1 mM.

To rule out any potential toxic and/or irreversible effect of ambroxol on basophils the reversibility of inhibition of basophil histamine release caused by anti-IgE was assessed. However, the inhibitory actions of ambroxol, in terms of blocking histamine release at 100 μ M were clearly observed to be entirely reversible, where cells pre-incubated with the drug for 15 min followed by washing out the drug led to no subsequent inhibitory effects (Fig. 4). Furthermore, the inhibitory actions of ambroxol were seen to involve partial blockade of p38 MAPK phosphorylation (Fig. 5a), a kinase which we have previously shown to be associated with the generation of various basophil-derived inflammatory mediators

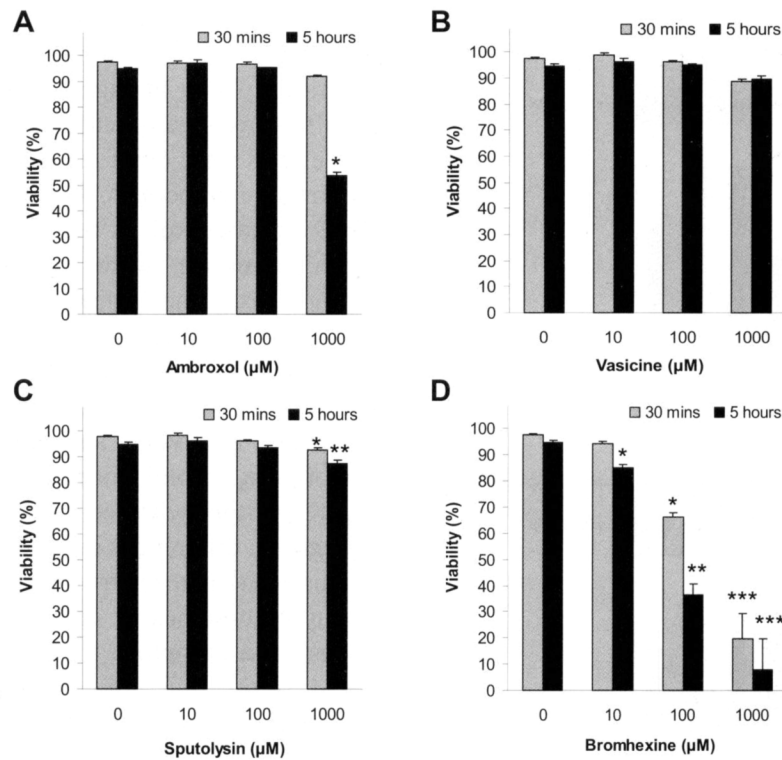


Fig. 3. Effects of ambroxol (A), vasicine (B), sputolysin (C) and bromhexine (D) on basophil viability as judged by Trypan blue exclusion. Cells were incubated with the drugs for 15 min before stimulation with anti-IgE, as described before, for the times shown. The results are depicted as mean viability $\pm$ SEM for 5-6 separate experiments. *, ** and *** represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) reduction in viability between cells stimulated with anti-IgE alone and in the presence of the compounds.

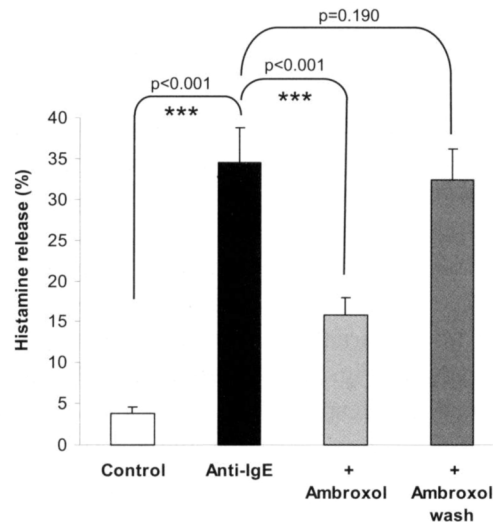


Fig. 4. Ambroxol (100 μ M) inhibits IgE-dependent basophil histamine release via reversible mechanisms. Basophils were incubated with or without ambroxol for 15 min before washing with warm (37°C) Tyrode's buffer (containing 1 mM calcium chloride) several times. Thereafter, ambroxol was added to a proportion of cells before stimulation with anti-IgE (1/20,000 dilution) to all tubes except buffer controls. The results are shown as mean histamine releases $\pm$ SEM for 7 separate experiments.

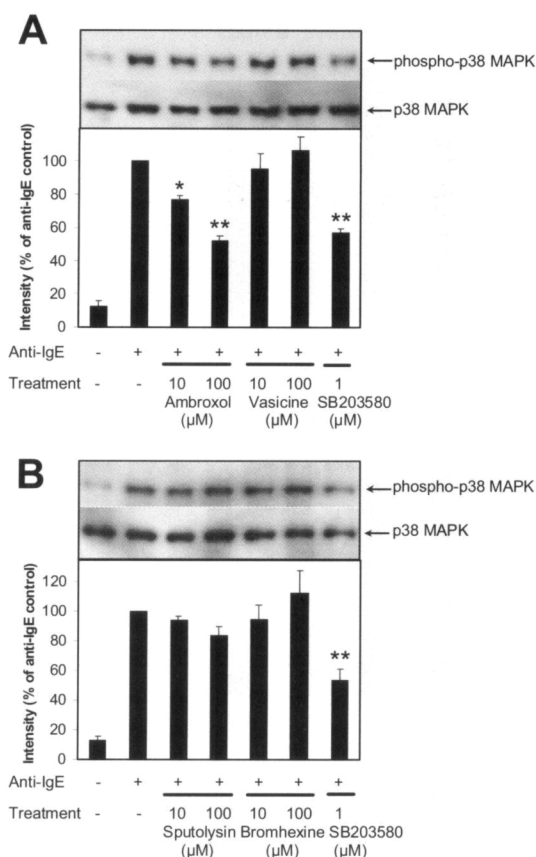


Fig. 5. Effect ambroxol and vasicine (panel **A**) as well as sputolysin and bromhexine (panel **B**) on IgE-induced p38 MAPK phosphorylation in human basophils. Lower panels show densitometric analysis of pooled intensities from 4-6 separate experiments and are expressed as percentage of intensity relative to anti-IgE controls for phospho-p38 MAPK. * and ** represent significant ($p < 0.05$, and $p < 0.01$, respectively) reductions comparing cells stimulated with anti-IgE alone and in the presence of the compounds/inhibitor.

(16). Vasicine, in comparison, had no discernable inhibitory effects on p38 MAPK activation (Fig. 5a) and similar negative data were obtained with respect to sputolysin and bromhexine (Fig. 5b).

DISCUSSION

These results clearly demonstrate that ambroxol is a more potent and effective inhibitor of basophil mediator release compared to both vasicine and its chemical analogues bromhexine and sputolysin,

despite considerable structural similarities of the latter (Fig. 6). Although ambroxol displayed moderate toxicity at high concentrations (1 mM), and after prolonged incubation with basophils, no toxic effects were observed at lower concentrations and for shorter periods. Given that the inhibitory effects of ambroxol, at least in terms of blocking histamine release, were reversible also indicates that non-toxic mechanisms primarily govern its inhibitory properties. The actions of ambroxol were contrasted by bromhexine, which led to considerable loss in cell viability even at low concentrations and after relatively short incubation periods. Furthermore, bromhexine failed to reduce p38 MAPK phosphorylation, unlike ambroxol, further highlighting differences in action *versus* ambroxol.

This is the first report regarding *in vitro* cellular toxicity of bromhexine and its histamine-releasing actions from basophils, the latter of which include direct stimulation of histamine release and enhancement of release of the amine following IgE-dependent stimulation. However, since maximum plasma levels of bromhexine following a 32 mg oral dose do not reach micromolar concentrations (and are usually below 90 nM) (17), it is questionable whether the observed effects are clinically relevant following standard application of standard doses. Nevertheless, these findings do potentially raise concerns regarding the use of bromhexine at high doses, or by direct application, especially in patients with allergic asthma or other allergic diseases. Indeed, several studies have reported reactions to bromhexine, resembling the actions of histamine, in both the gut and the skin (18). However, these reports are relatively few, involving a small number of patients, and possibly reflect both the relatively low concentrations reached using standard therapeutic doses of the drug and that bromhexine is rapidly metabolised to ambroxol.

In comparison to other secretolytic agents, such as the analogues used in the present study, and N-acetylcysteine (11), ambroxol is unique in displaying potential anti-allergic properties in addition to its known therapeutic attributes that support lung function (e.g. by increasing surfactant production, mucokinetic properties) and anti-inflammatory effects (19). Several studies have indicated a role for this drug in reducing bronchial hyper-reactivity

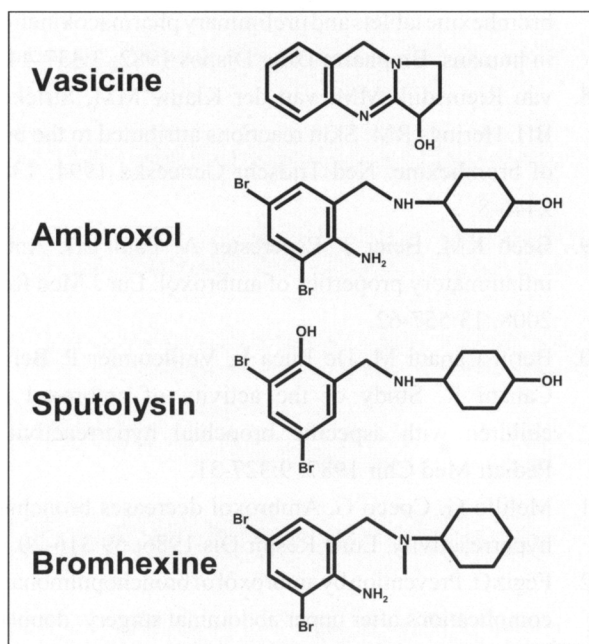


Fig. 6. Overview of the chemical structures of the secretolytic agents employed in this study. (The structures are based on the formulae given on the PubChem website from the National Library of Medicine.)

(20-21). However, the data presented here suggest that higher doses than normally given to decrease mucous viscosity would be necessary in order to achieve the inhibitory effect on basophil activity. In this regard, using higher doses of ambroxol may be feasible given its comparatively low toxicity and side effects, where daily doses of up to 1 g/day have been reported as being well tolerated (22-24). Ambroxol has also been shown to display greater affinity for lung tissue where concentrations are more than 10-fold higher than serum levels (24). Thus, due to its enrichment in lung tissues, ambroxol may be capable of exerting anti-inflammatory activities *in situ* by blocking effector functions of basophils and mast cells.

The mode of action of ambroxol on basophils and mast cells is not well understood. This study however, shows for the first time that ambroxol affects p38 MAPK-dependent signal transduction. The p38 MAPK enzyme is associated with IgE-dependent and -independent basophil activation and appears to participate in the generation of all basophil mediator types (16, 25). Several reports have recently shown that ambroxol also moderately affects ion flux

through Na⁺ channels and Cl/HCO₃⁻ exchangers in human epithelial cell lines (26-27), alongside its known antioxidant and free radical scavenging properties (28-29). However, there is at present no clear evidence to show that these properties account for the mode of action of ambroxol on basophils. Nonetheless, the present study highlights the unique inhibitory effect of ambroxol on basophils compared to closely related chemical analogues already in clinical use. Moreover, these observations may serve to generate more potent analogues which retain ambroxol's inhibitory actions and low toxicity.

ACKNOWLEDGEMENTS

The author would like to thank Prof. Helmut Wolff and other former colleagues at the University of Lübeck for general support and to Dr. Alison Edwards (University of Kent) for helpful advice.

REFERENCES

1. Lichtenstein LM, Bochner BS. The role of basophils in asthma. *Ann NY Acad Sci* 1991; 629:48-61.
2. Atkins PC, Schwartz LB, Adkinson NF, von Allmen C, Valenzano M, Zweiman B. *In vivo* antigen induced cutaneous mediator release: simultaneous comparisons of histamine, tryptase, and prostaglandin D2 release and the effect of oral corticosteroid administration. *J Allergy Clin Immunol* 1990; 86:360-70.
3. Falcone FH, Zillikens D, Gibbs BF. The 21st Century Renaissance of the Basophil? Current insights into their role in allergic responses and innate immunity. *Exp Dermatol* 2006; 15:855-64.
4. Devouassoux G, Foster B, Scott LM, Metcalfe DD, Prussin C. Frequency and characterization of antigenspecific IL-4- and IL-13-producing basophils and T cells in peripheral blood of healthy and asthmatic subjects. *J Allergy Clin Immunol* 1999; 104:811-9.
5. de Paulis A, Prevete N, Fiorentino I, et al. Expression and functions of the vascular endothelial growth factors and their receptors in human basophils. *J Immunol* 2006; 177:7322-31.
6. Mukai K, Matsuoka K, Taya C, et al. Basophils play a critical role in the development of IgE-mediated

- chronic allergic inflammation independently of T cells and mast cells. *Immunity* 2005; 23:191-202.
7. Obata K, Mukai K, Tsujimura Y, Ishiwata K, Kawano Y, Minegishi Y, Watanabe N, Karasuyama H. Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* 2007; 110: 913-20.
 8. Sokol CL, Barton GM, Farr AG, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol* 2008; 9:310-8.
 9. Kepley CL, McFeeley PJ, Oliver JM, Lipscomb MF. Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. *Am J Respir Crit Care Med* 2001; 164:1053-8.
 10. Nouri-Aria KT, Irani AM, Jacobson MR, O'Brien F, Varga EM, Till SJ, Durham SR, Schwartz LB. Basophil recruitment and IL-4 production during human allergen-induced late asthma. *J Allergy Clin Immunol* 2001; 108:205-11.
 11. Gibbs BF, Schmutzler W, Vollrath IB, Brosthardt P, Braam U, Wolff HH, Zwadlo-Klarwasser G. Ambroxol inhibits the release of histamine, leukotrienes and cytokines from human leukocytes and mast cells. *Inflamm Res* 1999; 48:86-93.
 12. Gibbs BF, Wolff HH, Grabbe J. Ambroxol inhibits IgE-dependent mediator secretion from human skin mast cells. *Inflamm Res* 2000; 49(S):17-18.
 13. Malerba M, Ragnoli B. Ambroxol in the 21st century: pharmacological and clinical update. *Expert Opin Drug Metab Toxicol* 2008; 4:1119-29.
 14. Claeson UP, Malmfors T, Wikman G, Bruhn JG. *Adhatoda vasica*: a critical review of ethnopharmacological and toxicological data. *J Ethnopharmacol* 2000; 72:1-20.
 15. Haisch K, Gibbs BF, Körber H., Ernst M, Grage-Griebenow E, Schlaak M, Haas H. Purification of morphologically and functionally intact human basophils to near homogeneity. *J Immunol Methods* 1999; 226:129-37.
 16. Gibbs BF, Plath KES, Wolff HH, Grabbe J. Regulation of mediator secretion in human basophils by p38 mitogen-activated protein kinase: phosphorylation is sensitive to the effects of phosphatidylinositol 3-Kinase inhibitors and calcium mobilization. *J Leukocyte Biol* 2002; 72:391-400.
 17. Bechgaard E, Nielsen A. Bioavailability of bromhexine tablets and preliminary pharmacokinetics in humans. *Biopharm Drug Dispos* 1982; 3:337-44.
 18. van Riemsdijk MM, van der Klauw MM, Stricker BH, Herings RM. Skin reactions attributed to the use of bromhexine. *Ned Tijdschr Geneesk* 1994; 138: 2446-8.
 19. Beeh KM, Beier J, Esperester A, Paul LD. Anti-inflammatory properties of ambroxol. *Eur J Med Res* 2008; 13:557-62.
 20. Berni Canani M, De Luca L, Vuilleumier P, Berni Canani R. Study of the activity of ambroxol in children with aspecific bronchial hyperreactivity. *Pediatr Med Chir* 1987; 9:327-31.
 21. Melillo G, Cocco G. Ambroxol decreases bronchial hyperreactivity. *Eur J Respir Dis* 1986; 69:316-20.
 22. Fegiz G. Prevention by ambroxol of bronchopulmonary complications after upper abdominal surgery: double-blind Italian multicenter clinical study versus placebo. *Lung* 1991; 169:69-76.
 23. Vytiska-Binstorfer E, Salzer H, Simbruner G. Ambroxol in comparison with betamethasone for the stimulation of antepartal lung maturity. A clinical double-blind study. *Zentralbl Gynakol* 1986; 108:220-9.
 24. Mezzetti M, Colombo L, Marini GM, Crusi V, Pierfederici P, Mussini E. A pharmacokinetic study on pulmonary tropism of ambroxol in patients under thoracic surgery. *J Em Surg Intensive Care* 1990; 13: 179-85.
 25. Aerts NE, Dombrecht EJ, Bridts CH, Hagendorens MM, de Clerck LS, Stevens WJ, Ebo DG. Simultaneous flow cytometric detection of basophil activation marker CD63 and intracellular phosphorylated p38 mitogen-activated protein kinase in birch pollen allergy. *Cytometry B Clin Cytom* 2008; 76B:8-17.
 26. Hasegawa I, Niisato N, Iwasaki Y, Marunaka Y. Ambroxol-induced modification of ion transport in human airway Calu-3 epithelia. *Biochem Biophys Res Commun* 2006; 343:475-82.
 27. Weiser T, Wilson N. Inhibition of tetrodotoxin (TTX)-resistant and TTX-sensitive neuronal Na(+) channels by the secretolytic ambroxol. *Mol Pharmacol* 2002; 62:433-8.
 28. Felix K, Pairet M, Zimmermann R. The antioxidative activity of the mucoregulatory agents: Ambroxol, bromhexine and N-acetyl-L-cysteine. A pulse

- radiolysis study. Life Sci 1996; 59:1141-7.
29. Gillissen A, Scharling B, Jaworska M, Bartling A, Rasche K, Schultze-Werninghaus G. Oxidant scavenger function of ambroxol *in vitro*: a comparison with N-acetylcysteine AC. Res Exp Med (Berl) 1997; 196:389-98.