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PULMONARY PHARMACOLOGY & THERAPEUTICS

Effect of Fenspiride, a Non-steroidal Antiinflammatory Agent, on Neurogenic Mucus Secretion in Ferret Trachea in Vitro

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SUMMARY: **Neural mechanisms contribute to control of mucus secretion in the airways. Fenspiride is a non-steroidal antiinflammatory agent which has a variety of actions, including inhibition of neurogenic bronchoconstriction. The effect of fenspiride on neurally-mediated mucus secretion was investigated in vitro in electrically-stimulated ferret trachea, using $^{35}SO_4$ as a mucus marker. Cholinergic secretory responses were isolated using adrenoceptor and tachykinin receptor antagonists. Tachykinin responses were isolated using cholinoceptor and adrenoceptor antagonists. Electrical stimulation increased cholinergic secretion by ~90% and tachykininergic secretion by ~40%. Fenspiride (1 μM–1 mM) tended to inhibit cholinergic secretion in a concentration-dependent manner, although only at 1 mM was inhibition (by 87%) significant. Inhibition by fenspiride of tachykininergic secretion was not concentration-dependent, and again significant inhibition (by 85%) was only at 1 mM. Inhibition was not due to loss of tissue viability, as assessed by restitution of secretory response after washout. Fenspiride also inhibited secretion induced by acetylcholine, but did not inhibit substance P-induced secretion. Histamine receptor antagonists increased basal secretion by 164%, whereas fenspiride did not affect basal secretion. We conclude that, in ferret trachea in vitro, fenspiride inhibits neurally-mediated mucus secretion, with antimuscarinic action the most plausible mechanism of action, but not necessarily the only mechanism.**

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KEY WORDS: **Cholinergic nerve, Fenspiride, Mucus, Mucus secretion, Neurogenic inflammation.**

INTRODUCTION

Mucus secretion in the respiratory tract is a protective response to inhaled irritants, and is under humoral and neuronal control. In mammalian airways, the predominant neural control is cholinergic, with a minor adrenergic component.[1] Capsaicin-sensitive 'sensory-efferent' nerves also contribute to control of secretion, although the extent of their contribution varies with species.[2] Suppression of airway neurogenic mucus secretion is of interest because neural mechanisms and mucus hypersecretion are implicated in the pathophysiology of certain severe respiratory conditions, most notably asthma[3] and chronic obstructive pulmonary disease (COPD).[4]

Fenspiride is a non-steroidal compound with a number of modes of action and end-organ effects. It has been shown to be antiinflammatory, anti-allergic and antioxidant. Its anti-secretory properties are of interest in the present study. Fenspiride inhibited SO_2-induced goblet cell hyperplasia in rats, with concomitant inhibition of increased hexose and fucose, indicative of mucus hypersecretion, in lavage fluid.[5] Fenspiride also exhibits neuronal inhibitory activity. For example, in guinea pigs, it reverses capsaicin-induced and citric acid-induced bronchoconstriction and cough,[6] and inhibits cholinergic and non-adrenergic, non-cholinergic (NANC) neural contraction of isolated bronchi.[7] Consequently, it would be of interest to investigate the effect of fenspiride on airway neurogenic mucus secretion.

In the present study, we investigated the regulatory effect of fenspiride on neurogenic mucus secretion in ferret trachea in vitro in Ussing-type chambers, using $^{35}SO_4$ as a mucus marker.[8,9] Ferret trachea was used because we have previously characterized the components of the secretory response to electrical stimulation: ~60% cholinergic and ~40% mediated via sensory-efferent nerves and tachykinin (substance P

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and neurokinin A) interaction with NK_1 receptors on the secretory cells.[10]

MATERIALS AND METHODS

Tracheal preparation for measurement of secretion

Male ferrets (Froxfield Farms, Froxfield, Hampshire, UK) weighing 1–2 kg were used throughout, and were housed 'free range' in a single room with food and tap water freely available. Procedures were approved by the Home Office (UK), and have been described in detail previously.[10,11] The ferrets were terminally anaesthetized with pentobarbitone sodium (Sagatal; 60 mg/kg), dissected along their ventral midline and bled via an incision in the left ventricle. Tracheae were excised and placed into warmed, aerated Krebs–Henseleit solution (bubbled with 95% O_2, 5% CO_2 at 37°C, pH 7.4) of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_2$25.5 and glucose 5.05. After removing loose connective tissue and fat, the tracheae were cut longitudinally along the posterior membrane, pinned out flat and cut transversely into four equal segments. Each segment was mounted and clamped between two perspex half-chambers (Ussing-type), which separated the tracheal segment into a 'luminal' side (i.e. mucus-producing) and a 'submucosal' side. Each half-chamber was independently superfused with warmed (37°C), aerated (95% O_2, 5% CO_2) Krebs–Henseleit solution which was circulated by gas lift pumps. The cross-sectional area exposed to the Krebs was 1.12 cm^2 for each segment. For stimulation of nerves, two pairs of pins piercing the trachea bilaterally and connecting the two halves of the chamber, were fed to an electrical field stimulator (model S88; Grass Instruments, Quincy, USA). Tissue was stimulated at 10 Hz (submaximal), 0.5 ms and 50 V for 5 min (see below).

Radiolabelling of mucus

At time 0 h, $Na_2{}^{35}SO_4$ (100 μCi; Amersham International plc, Bucks. UK) was added to the submucosal side of the Ussing chamber and left in contact with the submucosa for the duration of the experiment (all experimental drugs were added to the luminal half-chamber). For the first 2 h after $^{35}SO_4$ addition, the luminal side of the chamber was drained of liquid and replaced with fresh Krebs–Henseleit every 30 min. After 2 h, luminal collections (containing secretions) were made every 15 min. Luminal fluid was collected into tubes containing 5 g of guanidine hydrochloride (6 M final concentration) to dissolve the mucus, then assayed for $^{35}SO_4$-labelled mucus macromolecules as described below.

Measurement of $^{35}SO_4$ output

The collected luminal samples from the secretory experiments were transferred from the guanidine tubes to dialysis bags (Visking size 18/32″ to retain molecules of $\sim$12 kDa or greater: Medicell International Ltd., London, UK) and exhaustively dialysed against distilled water ($\sim$7 l) containing unlabelled Na_2SO_4 in excess to displace free $^{35}SO_4$ (i.e. $^{35}SO_4$ not bound to mucins). After six changes of water, all detectable unbound $^{35}SO_4$ had been removed from the samples. Throughout dialysis, sodium azide (0.01% w/v) was present in the water to inhibit bacterial growth. After 48 h dialysis, individual samples were weighed and 1 ml duplicates of each were taken. Duplicates were mixed with 2 ml scintillant (Ultima Gold XR; Canberra Packard Ltd., Pangbourne, UK) and radioemission determined in a liquid scintillation spectrometer (model 1900CA, Canberra Packard Ltd.). Total radioactivity per collection was determined by multiplying the 1-ml-aliquot counts (in disintegrations per minute, dpm) by the weight of the samples, on the assumption that 1 ml weighed 1 g.

Protocols

The effect of fenspiride was determined on $^{35}SO_4$ output (i.e. mucus secretion) induced by electrical stimulation or autonomic agonists. All drugs (fenspiride, autonomic agonists and receptor antagonists) were added to the luminal half chamber (see below for timings). The tissue was stimulated (10 Hz, 50 V, 0.5 ms) for the first 5 min of the 15 min incubation period. Collections were made from the luminal side every 15 min to determine both basal and stimulated secretion rate. Fenspiride was dissolved in distilled water, and an equivalent volume of water was used as control.

To isolate cholinergic neural secretion, the tissue was pretreated with propranolol, phentolamine (10 μM each) and the tachykinin NK_1 receptor antagonist CP 99 994 (3 μM)[12] to remove respectively α-adrenergic, β-adrenergic and tachykininergic neural influences. When investigating tachykininergic neural secretion, cholinergic and adrenergic neural influences were removed using atropine, propranolol and phentolamine (10 μM each). Autonomic antagonists were added 1 h prior to stimulation, and were present throughout the stimulation period. Fenspiride (1 μM-10 mM) was added 30 min before stimulation. Treatment groups were: 1) sham stimulation, 2) cholinergic stimulation with water control, 3) tachykininergic stimulation with water control, 4) cholinergic stimulation with fenspiride (at each concentration), and 5) tachykininergic stimulation with fenspiride. Different tissue segments were used for each concentration of fenspiride.

To determine viability at the end of the experiment, tissues (for vehicle control and fenspiride at 1 mM) were drained and replenished with Krebs–Henseleit every 15 min for an extra hour (to wash out drug treatment). At 4 h, ACh (10 μM) was added to the baths to stimulate secretion.

To investigate whether any neuro-inhibition was via post-junctional activity, fenspiride (100 μM) was added 30 min prior to either substance P (1 μM) or ACh (10 μM), the latter given at the same time point as electrical stimulation. Treatment groups were: 1) water vehicle + water vehicle, 2) water + substance P, 3) fenspiride + substance P, 4) water + ACh, 5) fenspiride + ACh.

To determine whether any inhibition of secretion was due to an anti-histamine action by fenspiride, the effect of antihistamines on cholinergic neural mucus secretion was investigated. The histamine receptor antagonists pyrilamine and cimetidine (both 100 μM) were added in combination instead of fenspiride prior to electrical stimulation.

For all protocols, only one tracheal segment was used for each experimental procedure (e.g. cholinergic stimulation in the presence of one concentration of fenspiride). Because each trachea yielded four segments it was possible, for the most part, to investigate baseline, stimulated and drug responses for a single trachea (e.g. sham stimulation, cholinergic stimulation, cholinergic stimulation in the presence of fenspiride at one concentration, and cholinergic stimulation in the presence of a second concentration of fenspiride). The order of experimental treatments was preplanned and was rotated daily around the four chambers to account for any differences between chambers or in response between tracheal segments.

Data analysis

In Results, data are expressed as the arithmetic mean and one standard error of the mean (SEM), with *n*-values the number of animals. Because baseline dpm varied between tracheal segments, responses obtained from individual segments were converted to percentage changes in radiolabel output for the differences between response to drug or electrical stimulation and the preceding collection. The concentration of fenspiride giving 50% inhibition of the stimulated secretory response (IC_{50}) was calculated by non-linear regression using Graphpad Prism software (version 2.0: Microsoft, San Diego, USA). Significance of changes in secretion pre- and post-drug or electrical stimulation in the same group were assessed using the Wilcoxon sign-rank sum test. The significance of differences between groups was assessed using the Mann–Whitney U-test. For all tests, the null hypothesis of no difference between control and treatment groups was rejected at $P<0.05$ (two-tail).

Drugs and chemicals

The following drugs and chemicals were used (supplier in parenthesis, the chemicals whose suppliers are not specified in parentheses were purchased from Sigma Chemical Co., Poole, UK): acetylcholine chloride; atropine sulphate (Phoenix Pharmaceuticals Ltd., Pharma Hameln, G.m.b.H., Germany); cimetidine; dimethylsulphoxide (DMSO); pentobarbitone sodium B.P. (Sagatal; RMB Animal Health Ltd., Dagenham, UK); phentolamine mesylate (Ciba Laboratories, Horsham, UK); propranolol hydrochloride (Imperial Chemical Industries Ltd., Macclesfield, UK); pyrilamine; substance P (SP); $Na_2{}^{35}SO_4$, (Amersham). Fenspiride was supplied by Institute Recherche Internationale Servier, France. CP 99,994 was a kind gift from Pfizer Inc., Groton, Connecticut, USA.

RESULTS

Baseline radioactivity in the studies was of the order of 600 dpm. There were no significant differences between treatment groups.

Effect of fenspiride on cholinergic neural mucus secretion, and viability

In the presence of adrenergic and tachykinin receptor antagonists, a 5 min electrical stimulation of ferret tracheal segments in vitro increased cholinergic $^{35}SO_4$ output 10-fold above sham stimulation (Fig. 1A). Fenspiride (1 μM–1 mM) had no significant effect on basal secretion but tended to inhibit cholinergic secretion in a concentration-dependent manner, although only at 1 mM was inhibition (by 87%) significant (Fig. 1A).

At the end of the experiment, ACh (10 μM) was added to the system to test tissue viability after fenspiride (1 mM). Tissue response to ACh in vehicle-treated tracheal segments was 65 $\pm$ 12% increase in secretion above baseline, and in fenspiride-treated segments was 74 $\pm$ 4% (not significantly different).

Effect of fenspiride on tachykininergic neural mucus secretion

Electrical stimulation of tracheal segments in the presence of atropine, propanolol and phentolamine increased $^{35}SO_4$ output seven-fold above sham stimulation (Fig. 1B). Fenspiride (10 μM–1 mM) had no effect on basal secretion. Similar to fenspiride inhibition of cholinergic-induced secretion (above), fenspiride only significantly inhibited (by 85%) tachykininergic secretion at 1 mM (Fig. 1B). However, there was no tendency to a concentration-related inhibitory effect.

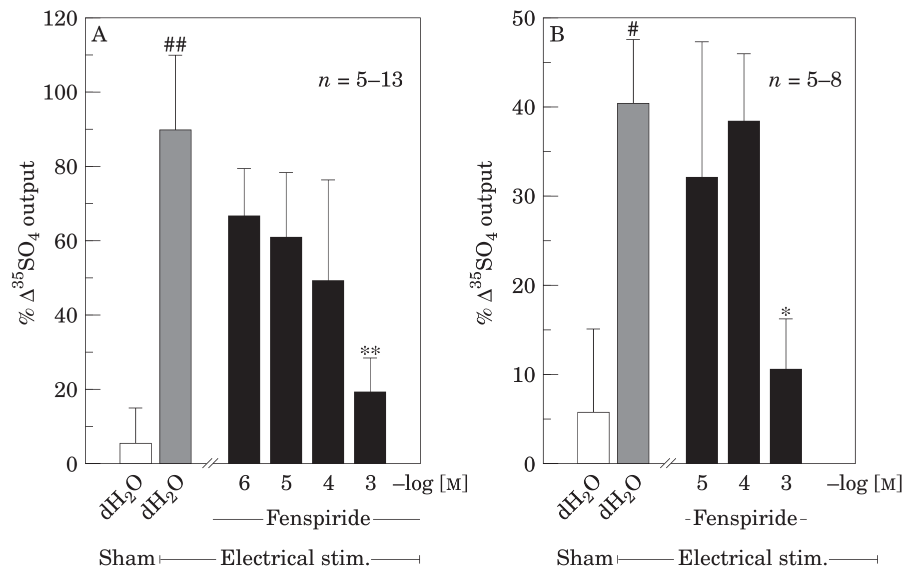

Fig. 1 Effect of fenspiride on neurogenic mucus secretion in ferret trachea in vitro. Tracheal segments were stimulated electrically at 10 Hz, 50 V, 0.5 ms for 5 min. A) Effect on cholinergic stimulation: propranolol, phentolamine (10 μM each) and the tachykinin NK_1 receptor antagonist CP 99,994 (3 μM) were used to exclude adrenergic and tachykininergic influences. B) Effect on tachykininergic stimulation: atropine, propranolol and phentolamine (10 μM each) were used to exclude cholinergic and adrenergic influences. Data are mean percent change in output of macromolecules labelled in situ with $^{35}SO_4$ (a marker for mucus) for 5–13 animals per group; vertical bars are one SEM. dH_2O, distilled water vehicle. $^{\#}P$<0.05, $^{\#\#}P$<0.01 vs. sham-stimulated vehicle control groups; $^{*}P$<0.05, $^{**}P$<0.01 vs. stimulated vehicle control groups. Sham (□); Vehicle stimulated (■); Fenspiride stimulated (■).

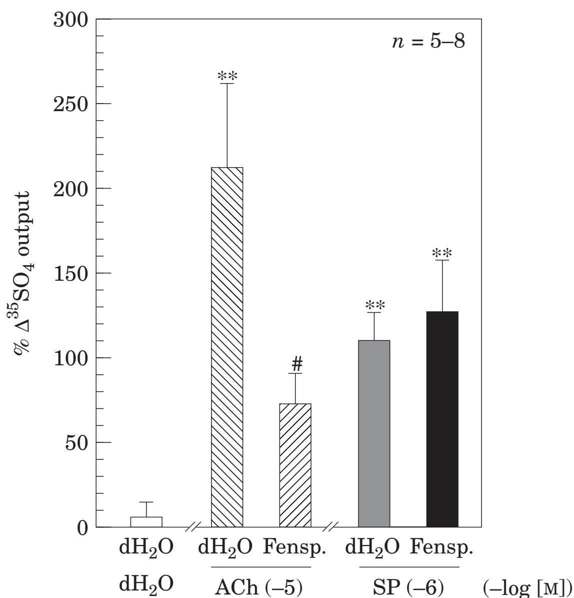

Fig. 2 Effect of fenspiride (0.1 mM) on substance P (SP)- and acetylcholine (ACh)-induced mucus secretion in ferret trachea in vitro. Data are mean percent change in output of macromolecules labelled in situ with $^{35}SO_4$ (a marker for mucus) for 5–8 animals per group; vertical bars are one SEM. dH_2O, distilled water vehicle. $^{\#}P$ <0.05 vs. ACh-stimulated vehicle control group; $^{**}P$<0.01 vs. vehicle-vehicle control group. Sham (□); dH_2O + ACh (10 μM) (▧); Fenspiride (100 μM) + ACh (▨); dH_2O + SP (1 μM) (■); Fenspiridine (■).

Effect of fenspiride on substance P- and ACh-induced secretion

Exogenous substance P (1 μM) increased $^{35}SO_4$ output 14-fold above vehicle control, a response unaltered by fenspiride (100 μM) (Fig. 2). Exogenous ACh (10 μM) increased secretion 24-fold above vehicle controls. Fenspiride, at the same concentration (100 μM) which failed to inhibit substance P-induced secretion, inhibited ACh-induced secretion by 68% (Fig. 2).

Effect of antihistamines on cholinergic neural mucus secretion

The histamine receptor antagonists pyrilamine and cimetidine in combination (100 μM each) increased basal $^{35}SO_4$ output by 164% (i.e. prior to electrical stimulation) (Fig. 3). In this series of experiments, in the absence of the antihistamines, cholinergic neural stimulation increased $^{35}SO_4$ output by 57% (Fig. 3). Prior incubation with the antihistamines significantly inhibited the cholinergic neural secretion by 90%.

DISCUSSION

In the present study in ferret trachea in vitro, fenspiride inhibited neurally-mediated $^{35}SO_4$ output. The $^{35}SO_4$ output is consistent with secretion of mucus glycoconjugates by submucosal glands, because there are few goblet cells but numerous submucosal glands in ferret trachea.[11,13] By autoradiography, there is selective uptake of $^{35}SO_4$ by ferret tracheal submucosal glands, rather than epithelium.[8] Stimulation of ferret trachea in vitro increased radioactive counts in the incubation medium, with concomitant loss of autoradiographic grains from the glands.[8] In cats, $^{35}SO_4$ labelled tracheal washings have a molecular weight

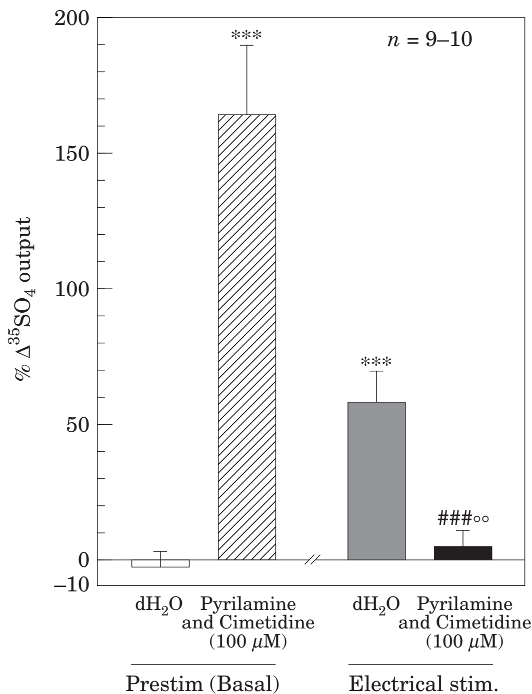

Fig. 3 Effect of antihistamines (pyrilamine and cimetidine in combination) on basal and cholinergic neural mucus secretion in ferret trachea in vitro. Propranolol, phentolamine (10 μM each) and the tachykinin NK_1 receptor antagonist CP 99,994 (3 μM) were used to exclude adrenergic and tachykininergic influences at stimulation parameters of 10 Hz, 50 V, 0.5 ms for 5 min. Data are mean percent change in output of macromolecules labelled in situ with $^{35}SO_4$ (a marker for mucus) for 9–10 animals per group; vertical bars are one SEM. dH_2O, distilled water vehicle; Prestim, effect of drug on basal mucus secretion prior to electrical stimulation. ****P*<0.01 vs. Prestim vehicle control group; $^{###}$*P*<0.001 vs. Prestim antihistamine group; $^{\circ\circ}$*P*<0.001 vs. stimulated vehicle control. dH_2O Prestim (□); dH_2O Stim (▨); Pyr. + Cim. Prestim (■); Pyr. + Cim. Stim. (▧).

and buoyant density characteristic of a mucin molecule.[9] Thus, the output of $^{35}SO_4$ observed herein is indicative of tracheal submucosal gland mucus secretion.

Herein, fenspiride inhibited cholinergic neural secretion with a tendency to concentration-dependency. However, inhibition was only maximal and significant at 1 mM fenspiride. Similarly, fenspiride significantly inhibited tachykininergic neural secretion only at 1 mM, although without any trend to concentration-dependency. The inhibitory effects were not due to killing of the tissue by the high concentration (1 mM) of fenspiride because, following washout, ACh caused marked secretion which was not different from control. The pattern of inhibition of neurogenic mucus secretion observed herein is the converse of those reported for fenspiride-inhibition of neurogenic bronchial smooth muscle contraction in guinea pigs in vitro.[7] In the latter study, fenspiride concentration-dependently inhibited tachykininergic neural contraction, while cholinergic contraction was only affected at high concentrations of fenspiride. The reasons for this discrepancy are unclear, but there are a number of possibilities. Firstly, innervation of the secretory system might not mimic the bronchomotor system For example, in guinea-pig, ACh is a potent spasmogen but comparatively weak secretagogue. Secondly, ferret and guinea-pig airways have different primary sources of mucus.[13,14] Guinea-pig mucus is predominantly goblet cell-derived, with little submucosal gland input; the opposite is true for ferret airways. This difference may be reflected in the basic pharmacology of the secretory system: ACh is a potent secretagogue in ferret trachea,[15] whereas in guinea-pig it is much less potent.[16]

The present data on the effects of fenspiride on secretion induced by substance P (i.e. not inhibited) and ACh (i.e. inhibited) compliment the neurogenic results: that fenspiride tends to inhibit the cholinergic rather than the tachykinin receptor-mediated response. The ACh data also indicate that the inhibitory effect of fenspiride is post-junctional rather than pre-junctional (i.e. that fenspiride is a cholinoceptor antagonist in this system). A post-junctional effect of fenspiride is also indicated for inhibition of cholinergic neural bronchial contraction,[7] albeit at high concentrations.

A mode of action of fenspiride beyond being an anticholinergic is not established in the present study, particularly as the drug has a variety of actions and interacts with a number of receptors. For example, fenspiride has high affinity at α_1-adrenoceptors and histamine H_1 receptors.[17] An α_1-adrenoceptor interaction can be excluded in the present study because the α-adrenoceptor antagonist phentolamine was used throughout. It is also unlikely that fenspiride is acting as a histamine antagonist because the antihistamines used herein (pyrilamine and cimetidine) induced marked mucus output. In contrast, fenspiride did not alter baseline secretion. Induction of mucus secretion by histamine H_1 and H_2 antagonists has been reported in the airways, including human bronchi,[18,19] and gut.[20] Other possible antisecretory mechanisms of action for fenspiride include inhibition of TNF-α production[21] and phosphodiesterase (PDE) type IV inhibition.[22] Firstly, there is no precedent for production of cytokines with acute electrical stimulation, and induction of mucus secretion by TNF-α begins at 4 h,[23] which is beyond the 15 min incubation period used herein. Secondly, PDE type IV inhibitors increase secretion of mucus,[24] which is in contrast to the inhibition by fenspiride observed herein.

Finally, our present data have clinical implications. Fenspiride is usually administered orally at a daily dose of 80 mg. At this dose, fenspiride achieves a maximal plasma concentration of 206 ng/ml.[25] This is equivalent to a drug concentration of 0.7 mM, which is similar to the effective concentration of 1 mM observed in the present study. A number of severe

conditions of the respiratory tract have hypersecretion of mucus as a characteristic feature, including asthma[3] and COPD.[4] Fenspiride shows benefit in treatment of chronic sinusitis[26] and reduces cough and expectoration associated with chronic bronchitis.[27] It would be of interest to extend these observation to more formal studies in respiratory diseases with mucus hypersecretion.

In conclusion, in ferret trachea in vitro, the non-steroidal antiinflammatory agent fenspiride inhibits neurally-mediated mucus secretion, with anti-muscarinic action the most plausible mechanism of action, but not necessarily the only mechanism.

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