Life Sciences 197 (2018) 67–72

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

In vitro pharmacology of ambroxol: Potential serotonergic sites of action

J.D. Hull[a], R.A. Lyon[b,*]

[a] Procter & Gamble, Greater London Innovation Centre, Egham, Surrey TW20 9NW, UK
[b] Procter & Gamble, Mason Business Center, Mason, OH 45040, USA

ARTICLE INFO

Keywords:
Ambroxol
Radioligand binding
Serotonin
Receptor
5-HT3
5-HT transporter
SERT

ABSTRACT

Aims: Ambroxol is a muco-active agent with multiple, clinically relevant effects in the airway. Despite its widespread use and well documented clinical efficacy, there are few data on its mechanism of action and receptor pharmacology beyond sodium channel blockade and inhibition of guanylate cyclase. Accordingly, in vitro studies were conducted to determine its overall receptor pharmacology and possible sites of action.

Materials and methods: In vitro radioligand binding/enzyme inhibition studies were conducted at 62 receptors, ion channels and enzymes using standard techniques. Additional in vitro studies were conducted to establish the potency of ambroxol at selected sites.

Key findings: These studies indicate that ambroxol has affinity for the 5-HT_3 serotonin receptor, as well as affinity for the 5-HT serotonin transporter (SERT), with IC_{50} values of 17,600 nM and 19,500 nM respectively. In vitro functional studies in isolated guinea pig colon indicate that ambroxol is a 5-HT_3 serotonin receptor antagonist with an IC_{50} value of 36,000 nM.

Significance: Together, these studies indicate that ambroxol may exert its beneficial properties via antagonism of the 5-HT_3 serotonin receptor and/or inhibition of serotonin uptake (5-HT transport: SERT), in addition to its reported effects at the sodium channel and guanylate cyclase.

1. Introduction

Ambroxol (*trans*-4-(2-amino-3,5-dibrombenzylamino)-cyclohexanol), an active metabolite of bromhexine (2,4-dibromo-6-{[cyclohexyl(methyl)amino]methyl}aniline), is a widely used muco-active agent [18,22]. It is used to facilitate the removal of mucus from the airways and decrease mucus tenacity associated with a variety of respiratory diseases including episodes of acute upper respiratory tract infection [18]. Ambroxol is reported as having multiple clinically relevant actions including reduction of mucus viscosity, stimulation of airway surfactant secretion [11], anti-inflammation [1] and analgesia [9,10],

Although the clinical actions and efficacy of ambroxol are well known and described, the molecular mechanisms responsible for these activities are not well understood. Previous investigations have identified sodium and calcium channel blockade as possible mechanisms of ambroxol [31–33]. In particular, these studies indicate that ambroxol has a preferential effect on the sodium 1.8 (Na_v 1.8) channel [32] with lower affinity for the Na_v 1.2 channel, with in vitro potencies (IC_{50} values) of 34,000 nM and 110,000 nM respectively. Ambroxol is reported to have much lower potency (i.e., IC_{50} value of 140,000 nM) in inhibiting calcium channels in vitro [33], with ambroxol showing no selectivity for calcium channel subtypes (i.e., equipotent at L and N subtypes). In addition, there is one report that ambroxol inhibits the activation of guanylate cyclase with an IC_{50} of 3900 nM [24].

Given these data, and the lack of any other reported pharmacology, we investigated the potential interaction of ambroxol at 62 receptors, ion channels and enzymes to provide additional mechanistic understanding.

2. Materials and methods

Ambroxol HCl was obtained from Sigma Aldrich. Animals used for tissue collection in the preparation of in vitro assays received appropriate oversight by an Institutional Animal Care and Use Committee (IACUC) or equivalent animal welfare oversight review board (ORB) in compliance with relevant animal welfare regulations.

2.1. Radioligand binding and enzyme assays

The following radioligand binding and enzyme assays were conducted by NovaScreen Biosciences Corporation, a Perkin Elmer company (Hanover, MD) using established, validated assays and standard automated techniques. For the initial screen, ambroxol was solubilized in buffer prior to each assay and tested at a single concentration

* Corresponding author.
E-mail address: lyon.ra@pg.com (R.A. Lyon).

https://doi.org/10.1016/j.lfs.2018.02.002
Received 10 October 2017; Received in revised form 19 January 2018; Accepted 2 February 2018
Available online 03 February 2018
0024-3205/

(10 μM) in duplicate. Inhibition of 50% or greater at the 10 μM concentration of ambroxol was considered a positive finding to proceed to additional testing. Each assay included a positive control compound with known affinity for the site tested. Positive assays were followed up with complete radioligand binding competition studies. These studies included 8–9 concentrations of ambroxol in duplicate over 4–5 orders of magnitude, along with positive control assays (full competition curves), to determine an IC_{50} (concentration of ambroxol producing a 50% inhibition of binding/activity).

The initial screening data are reported as the mean of duplicate samples. Competition study data were analyzed using a proprietary program (NovaScreen Biosciences Corporation) to determine the IC_{50} and Hill coefficient (n_H). The affinity constant (Ki) was calculated according to the Cheng-Prusoff equation [5]: $K_i = IC_{50}/1 + [D]/K_D$ where IC_{50} is the concentration of ambroxol inhibiting 50% of the signal, K_D is the affinity constant of the radiolabelled drug and D is the concentration of radiolabelled drug used in the assay.

The following assay conditions and controls were employed in this study (target, tissue or cell source, radioligand, drug used to determine non-specific binding or substrate (for enzyme assays) and positive control (Table 1).

The 5-HT_3 receptor radioligand binding assay (competition study) was also performed by Nova Screen Biosciences Corporation using mouse N1E-115 cells. Cell membrane homogenates were incubated with 0.35 nM ^{3}H-GR-65630 for 60 min at 25 °C in the absence and presence of test compound (8–9 concentrations, duplicate assays) in a 20 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. Non-specific binding was determined with 1.0 μM MDL-72222. Samples were vacuum filtered, and counted in scintillation cocktail. Data are expressed as the % inhibition of the control specific binding. IC_{50} and Ki values were determined as described above. For additional details see Lumis and Kilpatrick [17], Hoyer and Neijit [14] and Tyers [28].

The 5-HT transporter (SERT) radioligand binding assay was performed by Cerep (Poiters, France) using the human 5-HT transporter expressed in CHO cells. Cell membrane homogenates were incubated for 60 min at 22 °C with 2.0 nM ^{3}H-imipramine in the absence and presence of test compound (7–9 concentrations, duplicate assays) in a buffer containing 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl and 0.1% BSA. Non-specific binding was determined using 10 μM imipramine. Samples were vacuum filtered and counted in scintillation cocktail. Data are expressed as the % inhibition of the control specific binding. IC_{50} and Ki values were determined as described above. For additional details, see www.cerep.com and Tatsumi [27].

The 5-HT_3 in vitro functional assay (to determine agonist or antagonist activity) was also conducted by Cerep (Poiters, France) using isolated guinea pig colon. Segments of guinea pig distal colon were suspended in 20 ml organ baths containing an oxygenated (95% oxygen, 5% carbon dioxide) and pre-warmed (37 °C) physiological salt solution containing NaCl (118.0 mM), KCl (4.7 mM), $MGSO_4$ (0.6 mM), $CaCl_2$ (1.3 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (25.0 mM) and glucose (11.0 mM), pH 7.4. pyrilamine (1 μM), methysergide (1 μM) and GR-113808 (0.1 μM) were included in the assay to block histamine H_1, 5-HT_2 and 5-HT_4 receptors respectively. The tissues were connected to force transducers for isometric tension recordings. Tissues were stretched to a resting tension of 1 g, then allowed to equilibrate for 60 min during which time they were washed and readjusted for tension [2]. The assays were carried out using semi-automated isolated organ systems (8 organ baths) with multi-channel data acquisition.

2.1.1. Evaluation of agonist activity

Tissues were first exposed to a submaximal concentration of serotonin (10 μM) to verify responsiveness and obtain a control contractile response. Tissues were then exposed to increasing concentrations of ambroxol (1 μM–100 μM) at 40 min intervals. If a contractile response was found, this response was tested versus the competitive antagonist MDL-72222 to confirm the involvement of 5-HT_3 receptors in the response.

2.1.2. Evaluation of antagonist activity

Tissues were exposed to 10 μM 5-HT to obtain a control contractile response, followed by responses every 40 min. Increasing concentrations of ambroxol (1.0 μM–100 μM) were added 30 min prior to 5-HT and the antagonist effect was assessed. MDL-72222 was also tested in this fashion as a control. Results were expressed as a percent of the control (5-HT) response. The IC_{50} was determined by linear regression of the concentration-response curve.

Radioligand binding and functional data were plotted using GraphPad Prism software (version 4 for Windows) using non-linear regression (one site fit analysis). Goodness of fit was determined using r^2.

3. Results

Ambroxol was initially tested at a single concentration of 10 μM in 62 assays, including receptor, ion channel, transporter and enzyme assays as listed in the Materials and methods. The results of the initial screening at 10 μM are shown in Table 2. Of the 62 receptors, transporters, channels and enzymes tested, a 10 μM concentration (duplicate samples) of ambroxol produced ~50% inhibition (or more) at two of these sites including the serotonin 5-HT_3 receptor (65% inhibition) and the 5-HT transporter (SERT) (47% inhibition). Ambroxol also produced 40% inhibition at the sodium site 2, 14% inhibition at the L-type calcium channel and 2% inhibition at the N-type calcium channel. (No further evaluations were performed at the latter two sites as they did not approach the 50% pre-determined criteria for activity.)

Ambroxol was then tested in radioligand binding competition studies ($n = 2$) at the 5-HT_3 receptor and the 5-HT transport site (SERT) using standard methods. Each compound was tested at 7–9 concentrations over 3–4 orders of magnitude. Ambroxol demonstrated an IC_{50} value of 17,600 nM for the 5-HT_3 receptor as determined using ^{3}H-GR-65630 in mouse N1E-115 cells (Table 3). The control compound MDL-7222 had an IC_{50} of 6.0 nM (Ki of 2.9 nM) (Table 3) which is typical for this assay. The competition curves for ambroxol and MDL-72222 at the 5-HT_3 receptor are shown in Fig. 1. The Hill coefficient (n_H) for ambroxol was 1.0, indicating a single site interaction. Ambroxol demonstrated an IC_{50} value of 19,500 nM for the 5-HT transport site (SERT) as determined using ^{3}H-imipramine in CHO cells expressing the human recombinant SERT (Table 2). The control compound imipramine had an IC_{50} value of 2.6 nM (Table 2) which is typical for this assay. The competition curves for ambroxol and imipramine at the 5-HT transporter are shown in Fig. 2. Again, the Hill coefficient (n_H) for ambroxol was 1.0 in this assay, indicating inhibition at a single site.

Following radioligand binding competition studies, ambroxol was then tested for its ability to stimulate 5-HT_3 receptors (direct agonist activity) or inhibit the 5-HT_3 response to serotonin (antagonist activity) in isolated guinea pig colon. Ambroxol did not demonstrate any agonist activity in this assay at concentrations up to 100 μM (data not shown). On the contrary, ambroxol demonstrated a dose-related competitive antagonism of the contractile response to serotonin. The IC_{50} for ambroxol was determined to be 36,000 nM (mean of 2 assays); the IC_{50} for MDL-72222 was determined to be 3000 nM which is typical for this assay. These data are shown in Fig. 3 along with control data for the 5-HT_3 antagonist MDL-72222. No functional study was conducted for the 5-HT transporter (SERT) as previous studies have shown a good correlation of affinity between radioligand binding data and in vitro functional data for the 5-HT transport site [21,27].

The above data indicate two possible serotonergic mechanisms of action for ambroxol: antagonism of 5-HT_3 receptors and inhibition of 5-HT re-uptake (SERT). The affinity/potency of ambroxol for these targets are in approximately the same order of magnitude as previously reported data for sodium channels and inhibition of nitroprisside stimulation of guanylate cyclase. Current data and previously reported data