

Histamine H1-receptor antagonists inhibit nuclear factor-kappaB and activator protein-1 activities via H1-receptor-dependent and -independent mechanisms

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Clinical and Experimental Allergy

Summary

Background Histamine H1-receptor antagonists are used to relieve the symptoms of an immediate allergic reaction. They have additional anti-inflammatory effects that could result from an inhibition of the transcription factors activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB). The implication of the H1-receptor in these effects is controversial. Diphenhydramine is a first-generation H1-receptor antagonist while mizolastine and desloratadine are second-generation compounds. Mizolastine is also an inhibitor of 5-lipoxygenase (5-LO), an enzyme that has been involved in NF-κB activation.

Objective We measured the ability of antihistamines to reverse histamine-induced smooth muscle contraction, an effect that involves the H1-receptor. We then investigated whether these drugs affect NF-κB and AP-1 activities in A549 lung epithelial cells, and whether this potential regulation involves H1-receptor and 5-LO.

Methods Muscle tone was measured on tracheal segments of guinea-pigs. The H1-receptor was overexpressed by transfection and detected by Western blotting and immunofluorescence microscopy. NF-κB and AP-1 activities were assessed by reporter gene assays in cells overexpressing or not overexpressing the H1-receptor. Production of regulated upon activation, normal T cell expressed and secreted (RANTES), a chemokine whose expression is induced through NF-κB, was measured using an immunoassay.

Results H1-receptor antagonists reversed histamine-induced contraction in a dose-dependent manner. Induction of AP-1 and NF-κB activities by histamine and the down-regulatory effect of antihistamines required overexpression of the H1-receptor. In contrast, when tumour necrosis factor-α and a phorbol ester were used to stimulate NF-κB and AP-1 activities, respectively, repression of these activities did not involve the H1-receptor. Indeed, repression was triggered only by a subset of H1-receptor antagonists and was not stronger after overexpression of the H1-receptor. Mizolastine and desloratadine dose-dependently decreased tumour necrosis factor-α-induced production of RANTES. Diphenhydramine, H2- and H3-receptor antagonists as well as selective inhibitors of 5-LO were ineffective in this assay.

Conclusion Repression of NF-κB and AP-1 activities by H1-receptor antagonists involves H1-receptor-dependent and -independent mechanisms but not 5-LO.

Keywords antagonists, histamine H1-receptor, 5-lipoxygenase, NF-κB, RANTES, transcription factor AP-1

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Background

The early-phase allergic reaction is potently inhibited by histamine H1-receptor antagonists, whether these are first generation like diphenhydramine or second generation

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like desloratadine and mizolastine [1–3]. Interestingly, desloratadine and mizolastine display additional anti-inflammatory activities *in vitro* and *in vivo*. In various target cells, these exert inhibitory effects on the expression of adhesion molecules [4, 5], and on the release of leukotrienes [6–8], inflammatory cytokines or chemokines [5, 7, 9–11]. In animal models, desloratadine inhibits bronchospasm [12], while mizolastine inhibits eosinophil migration [13] and neutrophil infiltration [14], and shows an overall anti-inflammatory effect in experimental colitis [14] and arachidonic acid-induced cutaneous reactions [15]. In these two latter studies, it was hypothesized that the anti-inflammatory properties of mizolastine involve its inhibitory effect on 5-lipoxygenase (5-LO) rather than H1-receptor antagonism. Indeed, 5-LO is the major enzyme involved in leukotriene synthesis from arachidonic acid [15], and it has also been shown to activate nuclear factor-kappaB (NF- κ B) [16]. In fact, the role of H1-receptor in mediating the additional anti-inflammatory effects of H1-receptor antagonists is not clear; while some authors postulate that these effects are independent of the H1-receptor [17–19], others relate them to H1-receptor blockade because histamine can activate the transcription factors NF- κ B and activator protein-1 (AP-1) through this receptor [20, 21]. NF- κ B and AP-1 regulate the expression of many pro-inflammatory mediators, including CCL5/regulated upon activation, normal T cell expressed and secreted (RANTES) [22], and are involved in the pathogenesis of chronic inflammatory diseases such as asthma and allergy [23, 24]. Allergic rhinitis is commonly treated with H1-receptor antagonists. This condition is often associated with asthma, for which it also constitutes a risk factor [25]. Hence, there is a growing interest in characterizing the additional anti-inflammatory properties of H1-receptor antagonists in lower airway cells that are involved in the pathophysiology of asthma. Alveolar inflammation is present in asthma [26, 27]. Alveolar epithelial type II cells as well as the A549 cell line, which has retained many features of this cell type, are able to synthesize inflammatory mediators that attract and activate neutrophils, basophils, eosinophils, monocytes and T lymphocytes [28].

In the present work, we measured the potency of diphenhydramine, desloratadine and mizolastine to decrease histamine-induced muscle contraction, an effect known to be mediated through the H1-receptor. The effects of these H1-receptor antagonists were then analysed in A549 cells. We provide evidence that mizolastine and desloratadine down-modulate AP-1 and/or NF- κ B activities through H1-receptor-dependent and -independent pathways.

Materials and methods

Reagents

12-O-tetradecanoyl-phorbol-13-acetate (TPA), transferin-polylysine, poly-L-lysine, spermine, histamine, the

first-generation H1-receptor antagonist diphenhydramine, the histamine H2-receptor antagonist cimetidine, the histamine H3-receptor antagonist thioperamide and the selective inhibitors of 5-LO AA-861 and BW A4C were purchased from Sigma (St Louis, MO, USA). Human recombinant TNF- α was purchased from BD Pharmingen (San Diego, CA, USA). Luciferin and dithiothreitol were obtained, respectively, from Euromedex (Souffelweyheim, France) and Promega (Madison, WI, USA). Coenzyme A was purchased from Roche (Mannheim, Germany). Mizolastine was obtained from Sanofi-Aventis (Tours, France). Desloratadine was kindly provided by Urquima SA (Barcelona, Spain). Solutions of histamine, antihistamines and inhibitors of 5-LO were initially prepared at 10^{-2} M following the manufacturer's recommendations. Further dilutions were made up in cell culture medium or in Chenoweth-Koelle solution. At these dilutions, no toxic effects of the drugs or the solvents were observed on A549 cells as assessed by trypan blue exclusion.

Measurement of muscle tone

Adult male Dunkin Hartley guinea-pigs (Charles River Laboratories, L'Arbresle, France), weighing 350–400 g and fasted 18 h before the experiment, were used. Guinea-pigs were killed by a blow to the head. The tracheae were excised, cleaned of adhering adipose and connective tissue. The contractility of tracheal segments (four tracheal rings in all cases) was measured by adopting the method described previously [29]. Tissues were suspended in organ baths containing a Chenoweth-Koelle buffer. The solution was maintained at 37 °C and gassed continuously with a mixture of 95% O₂, 5% CO₂. The tracheal rings were pre-contracted to a stable maximum by incubation with histamine (10 μ M) for 20–25 min. Antihistamines were then added and changes in tension were measured isometrically with a myograph transducer connected to a physiograph (Narco Bio-system, Houston, TX, USA). In all the experiments, indomethacin, a cyclooxygenase inhibitor, was added at 1.4 μ M to the buffer in order to prevent spontaneous tone development due to the prostanooids released. These experiments were carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health.

Cell culture

A549 human lung epithelial cells and HeLa human epithelioid cervix cells were maintained in Ham's F12/Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mM glutamine. The day

before transfection and/or stimulation, cells were seeded in medium containing 5% charcoal/dextran-treated fetal calf serum.

Reporter plasmids

The reporter construct – 517/+63 Coll Luc contains the luciferase gene driven by part of the collagenase promoter with its single AP-1 site (a gift from Peter Herrlich, Institute of Genetics, Karlsruhe, Germany). The 3xIgk Cona Luc plasmid contains three tandem repeats of the NF- κ B response element from the immunoglobulin κ chain linked to the conalbumin minimal promoter and the luciferase gene (a gift from Alain Israël, Institut Pasteur, Paris, France). The pJ7-*LacZ* plasmid contains the SV40 early promoter linked to the β -galactosidase gene (a gift from Malcolm Parker, Imperial Cancer Research Fund, London, UK).

Regulated upon activation, normal T cell expressed and secreted immunoassay

One hundred thousand cells were stimulated by 10 ng/mL of TNF- α or 10^{-5} M of histamine in the absence or in the presence of increasing doses of antihistamines or 5-LO inhibitors. The concentration of RANTES in the supernatant was determined 24 h later using an ELISA kit (R&D Systems, Minneapolis, MN, USA).

Cloning of H1-receptor cDNA and construction of a myc-tagged H1-receptor

The H1-receptor complete coding sequence was cloned from HeLa cells by RT-PCR. Five micrograms of RNA were subjected to a RT reaction at 25 °C for 10 min and then at 42 °C for 50 min. The reaction mixture contained 0.5 mM dATP, dGTP, dCTP, dTTP (Eurogentec, Seraing, Belgium), 20 U RNasin ribonuclease inhibitor, 5 μ M hexamers, 10 mM dithiothreitol (Promega) and 200 U of Moloney Murine Leukaemia Virus Reverse Transcriptase in 25 μ L supplied buffer (Invitrogen, Cergy Pontoise, France). The RT was denatured by heating to 70 °C for 15 min. Amplification was performed with 10 μ L of a 1:10 dilution of RT reaction, 1 U *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and the following primers: 5'-TGCTCTAGAC CATGAGCCTCCCAATTCT-3' and 5'-AACTGCAGTTAG GAGCGAATATGCAGAATTCT-3'.

PCR conditions were 45 s at 94 °C for denaturation, 30 cycles of 45 s at 94 °C, 45 s at 53 °C and 2 min 30 s at 72 °C. Cycles were followed by a final extension step at 72 °C for 10 min. The PCR product was digested by *Xba*I, phosphorylated in 5' with polynucleotide kinase and inserted between the *Xba*I and *Sma*I sites of pCI (Promega) to obtain the expression vector pCIH1R. The H1-receptor cDNA cloned from HeLa cells has a sequence that is

identical to that described in the National Center for Biotechnology Information database (accession number AB041380) as checked using a FASTA package of sequence comparison program [30]. Two oligonucleotides were designed to introduce a c-myc epitope (EQKLISEEDL) at the N-terminus of the H1-receptor. This receptor has no conventional signal peptide and, therefore, the myc tag is conserved after post-translational processing. Briefly, the two oligonucleotides, 5'-CTAGACCATGGAGCAGAAGCT GATCTCTGAGGAGGACCTGAGCCTCCCAATTCTCTCTGC CTCTTAGAAGACAAGATGTGTGAGGGCAACAAGACCAC TATGGCCAGCCCCAG-3' and 5'-CTGGGGGCTGGCCA TAGTGGTCTTGTGTCCTCACACATCTGTCTTCTAAGGGC AGGAGGAATTGGGGAGGCTCAGGTCTCTCTCAGAGATCA GCTTCTGCTCCATGGT-3', were phosphorylated in 5' with polynucleotide kinase and annealed. This linker was then substituted into the *Xba*I-*Pvu*II fragment of pCIH1R to generate the expression vector pCImycH1R.

Transient transfection of A549 cells

Cells were transfected with DNA complexed to replication-deficient adenovirus, transferrin-polylysine and polylysine as described previously [31]. For NF- κ B activity assays, 100 000 cells were transfected with 60 ng 3xIgk Cona Luc, 500 ng of either pCIH1R or pCI and 25 ng of pJ7-*LacZ*. For AP-1 activity assays, 100 000 cells were transfected with 60 ng of – 517/+63 Coll Luc, 100 ng of either pCIH1R or pCI and 25 ng of pJ7-*LacZ*. pJ7-*LacZ* was used for normalization of transfection efficiency. For immunofluorescence microscopy, 100 000 cells were transfected with 600 ng of either pCIH1R, pCImycH1R or pCI. For Western blot analysis, one million cells were transfected with 4 μ g of either pCImycH1R or pCI.

Reporter gene assays

The day after transfection, cells were pre-treated for 1 h with antihistamines at various concentrations and further incubated for 4 h after adding either 10 ng/mL of TNF- α , 10 ng/mL of TPA or histamine at 10^{-5} M. Luciferase and β -galactosidase assays were performed as described previously [31]. Luciferase activity was divided by β -galactosidase activity to normalize values for variations in transfection efficiency. Measurement of β -galactosidase activity in transfected cells indicated that antihistamines did not affect the expression of reporter genes non-specifically.

Western blot analysis

To analyse H1-receptor expression, cells were lysed in buffer containing 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40 and a protease inhibitor cocktail (Roche). After 10 min on ice, cell

extracts were subjected to one cycle of freeze-thaw, and cleared by centrifugation at $20\,000 \times g$ for 10 min at 4°C . Forty micrograms of total proteins were subjected to SDS-PAGE on 12% gels and blotted onto nitrocellulose. Blots were probed with anti-H1-receptor antibodies at $1\ \mu\text{g}/\text{mL}$ (Chemicon International, Temecula, CA, USA) anti- β -actin antibodies diluted 1 : 5000 (Sigma). After incubation with peroxidase-conjugated secondary antibodies, immunoblots were developed in 0.2 mM coumaric acid, 1.25 mM luminol and 0.009% hydrogen peroxide mixture and exposed to a cooled charge-coupled device camera (Kodak, Rochester, New York).

Immunofluorescence microscopy

Cells were seeded into an eight-well glass slide (Nalge Nunc International, Naperville, IL, USA). Twenty-four hours post-transfection, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. After treatment with NH_4Cl 50 mM and blocking in PBS containing 3% bovine serum albumin and 0.1% (v/v) Tween 20, cells were incubated for 1 h at room temperature with $10\ \mu\text{g}/\text{mL}$ of a monoclonal anti-c-myc antibody (gift of Bernard Mouillac, INSERM U469, Montpellier, France). A 1 : 50 dilution of normal goat serum was then added for 15 min. Cells were then incubated for 30 min at room temperature with $5\ \mu\text{g}/\text{mL}$ of FITC-conjugated goat anti-mouse IgG (Immunotech, Marseille, France). After removal of the chambers, slides were mounted in Citifluor (Citifluor, London, UK) and observed with a fluorescence microscope (Olympus, Tokyo, Japan). When using the anti-H1-receptor antibody, which is directed against the third intracellular loop of the H1-receptor, cells were permeabilized as follows: after treatment with NH_4Cl , cells were incubated 4 min at 0°C with 0.5% (v/v) Triton X-100 in PBS. The anti-H1-receptor antibody was used at a concentration of $5\ \mu\text{g}/\text{mL}$ and the FITC-conjugated goat anti-rabbit (Nordic Immunological Laboratories, Tilburg, the Netherlands) at a dilution of 1 : 100. Controls were performed with primary antibody alone, secondary antibody alone and isotypic control plus secondary antibody.

Statistical analysis

The results obtained with A549 cells are expressed as the mean $\pm$ SEM of at least three independent experiments performed in duplicate, and those obtained with tracheal segments are expressed as the mean $\pm$ SEM of six independent experiments. Data were analysed using the Instat software (GraphPad Software, San Francisco, CA, USA). Dose responses were analysed by ANOVA while the Welch *t*-test was used for comparison between two treatment groups. Statistical significance was set up at $P < 0.05$.

Results

Histamine H1-receptor antagonists reverse the contractile effect of histamine

Diphenhydramine, desloratadine and mizolastine were analysed using an assay that typically measures an H1-receptor-mediated effect. Their ability to inhibit histamine-induced smooth muscle contraction was assessed using tracheal segments isolated from guinea-pigs (Fig. 1). All three compounds reversed histamine-induced contraction in a dose-dependent manner. A maximal inhibition of 100% was observed at the highest tested dose. The concentrations of diphenhydramine, mizolastine and desloratadine producing half-maximal inhibitory effect (EC_{50}) were found to be 0.27, 0.85 and $5.9\ \mu\text{M}$, respectively.

Expression of H1-receptor in A549 cells

To analyse the expression of the H1-receptor in A549 cells, Western blot experiments were performed. These revealed the presence of a protein of the expected size for the H1-receptor (55 kDa; Fig. 2a). Expression of the H1-receptor was confirmed at the RNA level, H1-receptor transcripts being detected by RT-PCR and Northern blotting experiments (data not shown). To further investigate the role of H1-receptor in mediating the effect of mizolastine, this receptor was overexpressed by transiently transfecting A549 cells with the vector pCIH1R. Because only 20% of the cells were transfected, Western blot analyses with an anti-H1-receptor antibody showed only a slight increase in H1-receptor overall expression after

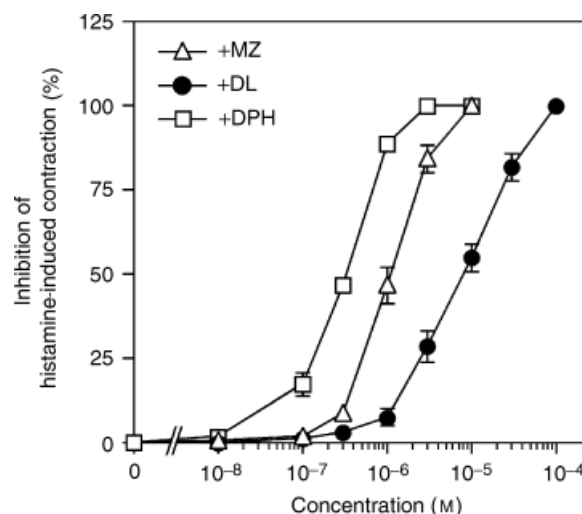


Fig. 1. Inhibitory effects of diphenhydramine, desloratadine and mizolastine on muscle contraction induced by histamine. Tracheal segments were isolated from guinea-pigs and pre-contracted with $10\ \mu\text{M}$ of histamine. After 20–25 min, antihistamines were added and concentration-response curves were determined, taking the intensity of the initial contraction as 100%. DL, desloratadine; DPH, diphenhydramine; MZ, mizolastine.

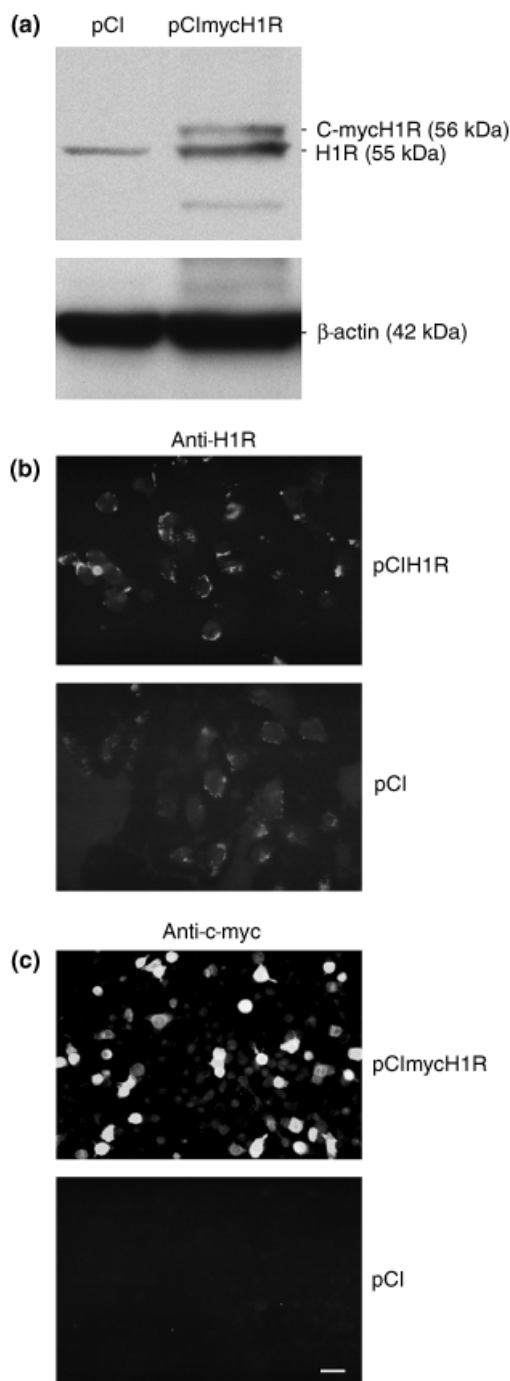


Fig. 2. Expression of endogenous and exogenous H1-receptor in A549 cells. (a) Cells were transfected either with the c-myc-tagged H1-receptor expression vector (pCImycH1R) or the empty vector (pCI). After 24 h, cell extracts were analysed by Western blotting. The blot was probed with an anti-H1-receptor antibody and an anti- β -actin antibody. (b) Cells were transfected either with the H1-receptor expression vector (pCIH1R) or pCI. Cells were then permeabilized and analysed by indirect immunofluorescence microscopy using an anti-H1-receptor antibody. (c) Cells were transfected either with the c-myc-tagged H1-receptor expression vector (pCImycH1R) or pCI. Cells were analysed by indirect immunofluorescence microscopy using an anti-c-myc antibody. The scale represents 50 μ m.

transfection with pCIH1R (data not shown). However, overexpression of the H1-receptor was clearly demonstrated after transfection with pCImycH1R, an expression vector for a myc-tagged H1-receptor that can be discriminated by size from the endogenous receptor. Indeed, in pCImycH1R-transfected cells, the anti-H1-receptor antibody detected a band of 56 kDa, corresponding to the myc-tagged H1-receptor, in addition to the endogenous protein of 55 kDa (Fig. 2a). Expression of the H1-receptor in pCIH1R, pCImycH1R or mock-transfected cells was further analysed by immunofluorescence microscopy. As expected, the anti-H1-receptor antibody stained permeabilized cells just beneath the plasma membrane and the intensity of fluorescence was higher in pCIH1R-transfected cells than in mock-transfected cells that harbour only the endogenous H1-receptor (Fig. 2b). With the antibody directed against the extracellular c-myc tag, an intense cell surface staining was observed on pCImycH1R-transfected cells but not on mock-transfected cells (Fig. 2c). Altogether, these data indicate that the H1-receptor was overexpressed as a transmembrane protein after cell transfection.

Inhibition of nuclear factor-kappa B activity by histamine H1-receptor antagonists

We first examined whether overexpressing H1-receptor affects the regulation of NF- κ B activity by TNF- α and histamine using a reporter gene assay. Exogenous H1-receptor had no significant effect on basal or TNF- α -induced NF- κ B activity (Fig. 3a). Of note, histamine increased NF- κ B activity only after overexpression of the H1-receptor (Fig. 3a). Therefore, the effect of antihistamines on histamine-induced NF- κ B activity was analysed in this context (Fig. 3b). Data show a dose-dependent repression of this activity by mizolastine (EC_{50} = 5 nM) and desloratadine (EC_{50} = 13 nM), while histamine H2-receptor antagonist cimetidine and histamine H3-receptor antagonist thioperamide were ineffective. Diphenhydramine produced a significant inhibition of NF- κ B activity only at 10 μ M, which is also approximately its EC_{50} , assuming that its maximal effect would have been similar to that obtained with desloratadine or mizolastine.

These antihistamines were next evaluated for their effect on TNF- α -induced NF- κ B activity (Fig. 3c). In the absence of exogenous H1-receptor, mizolastine and desloratadine at 10 μ M inhibited this activity by 43% and 21%, respectively. Lower concentrations of mizolastine and desloratadine were inactive in this assay (data not shown). Overexpression of the H1-receptor did not significantly modify their inhibitory effect (Fig. 3c). None of the other antihistamines, including diphenhydramine, antagonized TNF- α -induced NF- κ B activity whether the H1-receptor was overexpressed or not (Fig. 3c).

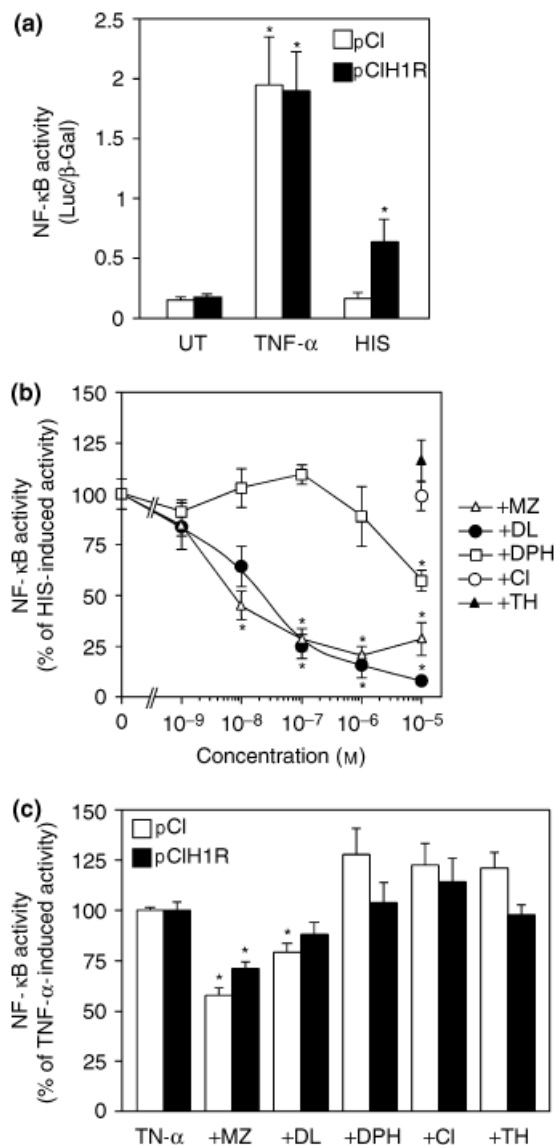


Fig. 3. Inhibition of nuclear factor-kappa B (NF-κB) activity by histamine H1-receptor antagonists. (a) Cells were transfected with an NF-κB luciferase reporter gene construct, a constitutive β-galactosidase reporter gene construct and either an H1-receptor expression vector (pCIH1R) or the corresponding empty vector (pCI). Cells were left untreated (UT) or stimulated for 4 h with TNF-α at 10 ng/mL or histamine at 10⁻⁵ M (HIS), and then lysed to determine the ratio of luciferase activity (Luc) over β-galactosidase activity (β-Gal). **P* < 0.05 vs. corresponding untreated, by the Welch *t*-test. (b) Cells were transfected with an NF-κB-dependent reporter gene construct, a constitutive β-galactosidase reporter gene construct and pCIH1R. These were then pre-treated for 1 h with antihistamines at various concentrations as indicated and stimulated for 4 h with histamine at 10⁻⁵ M (HIS). Histamine-induced NF-κB activity was given the nominal value of 100%. **P* < 0.01 vs. histamine alone, by ANOVA with the Bonferroni post-test. (c) Cells were transfected as in part (a). These were then pre-treated for 1 h with antihistamines at 10⁻⁵ M and stimulated for 4 h with TNF-α at 10 ng/mL. TNF-α-induced NF-κB activity was given the nominal value of 100%. **P* < 0.01 vs. TNF-α alone, by the Welch *t*-test. CI, cimetidine; DL, desloratadine; DPH, diphenhydramine; MZ, mizolastine; TH, thioperamide.

Inhibition of activator protein-1 activity by histamine H1-receptor antagonists

To investigate the effect of antihistamines on AP-1 activity and the implication of the H1-receptor, A549 cells were co-transfected with an AP-1 reporter gene construct and the expression vector pCIH1R or the corresponding empty vector pCI. These were then stimulated either by the phorbol ester TPA or histamine. We have shown previously that TNF-α stimulates AP-1 activity in A549 cells only weakly [32], while TPA is known to be a strong inducer. The exogenous H1-receptor affected neither basal nor TPA-induced AP-1 activity (Fig. 4a). In contrast, histamine increased AP-1 activity only after overexpression of the H1-receptor (Fig. 4a). Therefore, the effect of antihistamines on histamine-induced AP-1 activity was analysed in this context (Fig. 4b). Mizolastine and desloratadine dose-dependently repressed this activity with an EC₅₀ of 70 and 300 nM, respectively, while cimetidine and thioperamide had no effect. Diphenhydramine had a significant inhibitory effect only at the highest tested concentration of 10 μM.

These antihistamines were then evaluated for their effect on TPA-induced AP-1 activity in the presence or in the absence of exogenous H1-receptor (Fig. 4c). Mizolastine at 10 μM inhibited this activity by around 25% in pCI-transfected cells and its effect was not significantly modified when the H1-receptor was overexpressed (Fig. 4c). Lower concentrations of mizolastine were inactive in this assay (data not shown). None of the other antihistamines, including desloratadine and diphenhydramine, antagonized TPA-induced AP-1 activity whether the H1-receptor was overexpressed or not (Fig. 4c).

Mizolastine and desloratadine inhibit tumour necrosis factor-α-induced regulated upon activation, normal T cell expressed and secreted production in A549 cells

In A549 cells, TNF-α-induced RANTES expression is mediated through NF-κB [33]. In these cells, RANTES production was strongly induced by TNF-α but barely by histamine (Fig. 5a). We investigated the ability of antihistamines to inhibit TNF-α-induced RANTES production. Both mizolastine and desloratadine reduced RANTES release in a dose-dependent manner (EC₅₀ = 2 × 10⁻⁷ M and 3 × 10⁻⁶ M, respectively). An inhibition of 40% and 27% was obtained with 10⁻⁵ M of mizolastine and desloratadine, respectively (Fig. 5b). The inhibitory effect was specifically associated with the second-generation H1-receptor antagonists because a first-generation H1-receptor antagonist (diphenhydramine), a histamine H2-receptor antagonist (cimetidine) and a histamine H3-receptor antagonist (thioperamide) were unable to reduce TNF-α-induced RANTES release (Figs 5b and c). Selective inhibitors of 5-LO (AA-861 and BW A4C) also had no significant effect in this assay (Fig. 5c).

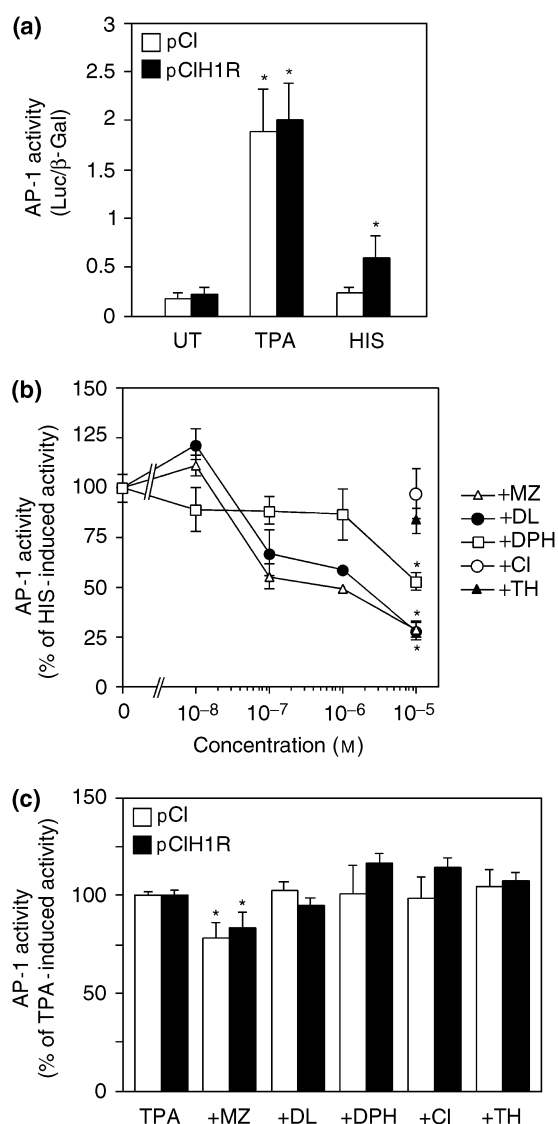


Fig. 4. Inhibition of activator protein-1 (AP-1) activity by histamine H1-receptor antagonists. (a) Cells were transfected with an AP-1 luciferase reporter gene construct, a constitutive β -galactosidase reporter gene construct and an H1-receptor expression vector (pCIH1R) or corresponding empty vector (pCI). Cells were left untreated (UT) or stimulated for 4 h with TPA at 10 ng/mL or histamine at 10^{-5} M (HIS), and then lysed to determine the ratio of luciferase activity (Luc) over β -galactosidase activity (β -Gal). **P* < 0.05 vs. corresponding untreated, by the Welch *t*-test. (b) Cells were transfected with an AP-1 luciferase reporter gene construct, a constitutive β -galactosidase reporter gene construct and pCIH1R. These were then pre-treated for 1 h with antihistamines at various concentrations as indicated and stimulated for 4 h with histamine at 10^{-5} M (HIS). Histamine-induced AP-1 activity was given the nominal value of 100%. **P* < 0.05 vs. histamine alone, by ANOVA with the Bonferroni post-test. (c) Cells were transfected as in part (a). These were then pre-treated for 1 h with antihistamines at 10^{-5} M and stimulated for 4 h with TPA at 10 ng/mL. TPA-induced AP-1 activity was given the nominal value of 100%. **P* < 0.05 vs. corresponding TPA alone, by the Welch *t*-test. CI, cimetidine; DL, desloratadine; DPH, diphenhydramine; MZ, mizolastine; TH, thioperamide.

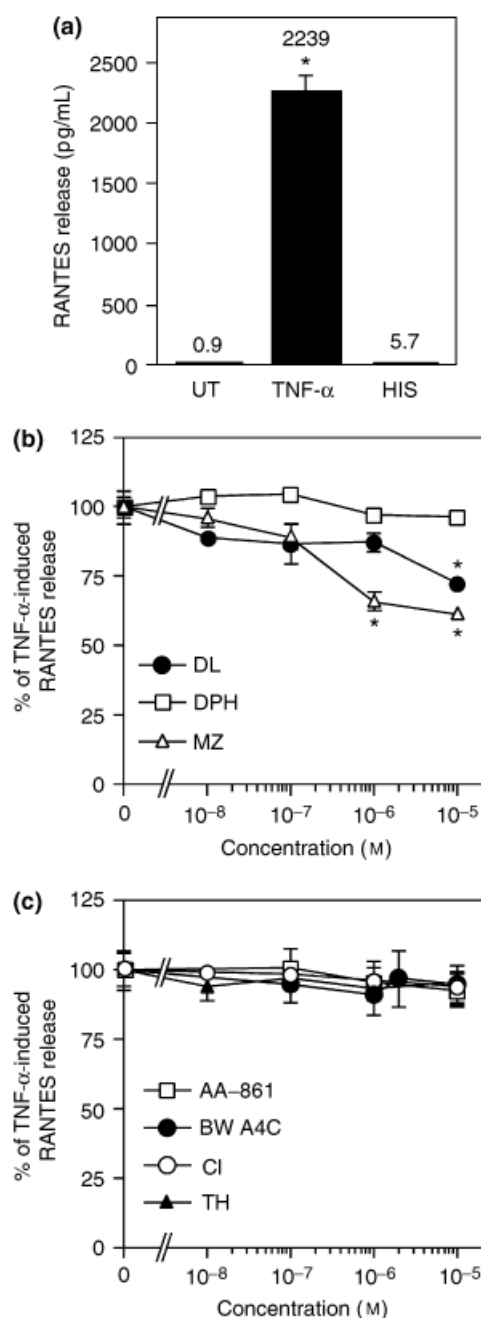


Fig. 5. Inhibition of regulated upon activation, normal T cell expressed and secreted (RANTES) release by mizolastine and desloratadine. (a) Cells were left untreated (UT) or stimulated with TNF- α at 10 ng/mL or histamine at 10^{-5} M (HIS). The concentration of RANTES in supernatant was determined by ELISA. **P* < 0.001 vs. untreated, by the Welch *t*-test. (b) Cells were stimulated for 24 h with TNF- α at 10 ng/mL in the presence of increasing concentrations of either mizolastine (MZ), desloratadine (DL) or diphenhydramine (DPH). The concentration of RANTES in the supernatant was determined. **P* < 0.01 vs. TNF- α alone, by ANOVA with the Bonferroni post-test. (c) Cells were stimulated for 24 h with TNF- α at 10 ng/mL in the presence of increasing concentrations of either histamine H2-receptor antagonist cimetidine (CI), histamine H3-receptor antagonist thioperamide (TH) or the 5-L0 inhibitors AA-861 or BW A4C. The concentration of RANTES in the supernatant was determined.

Discussion

Recently, several studies highlighted additional anti-inflammatory effects of second-generation H1-receptor antagonists. Indeed, these drugs not only antagonize an immediate allergic reaction but also affect the chemotaxis and activation of inflammatory cells during the late-phase reaction (cf. 'Background' for details and references). A number of investigators consider that the additional anti-inflammatory properties of H1-receptor antagonists are unrelated to H1-receptor antagonism [14, 15, 17–19]. However, other studies are in favour of an H1-receptor-mediated mechanism. Indeed, histamine was shown to activate NF- κ B [20, 34, 35] and AP-1 [21, 36] through the H1-receptor. Furthermore, H1-receptor antagonists can behave as inverse agonists [37], inhibiting H1-receptor constitutive activation of NF- κ B [34]. The implication of the H1-receptor in the additional effects of H1-receptor antagonists thus remains controversial.

In the present report, we demonstrate that the second-generation H1-receptor antagonists mizolastine and desloratadine inhibit NF- κ B activity via two distinct pathways: one involving the H1-receptor, and the other being independent from this receptor. We show that mizolastine also represses AP-1 activity via these two mechanisms.

Evidence for an H1-receptor-mediated mechanism was provided when histamine was used as an inducer of AP-1 and NF- κ B activities. Firstly, overexpression of the H1-receptor led to a significant induction of AP-1 and NF- κ B activities by histamine. Secondly, these activities were inhibited by H1-receptor antagonists, whether these were first- (diphenhydramine) or second-generation (mizolastine and desloratadine) molecules, but neither by the histamine H2-receptor antagonist cimetidine nor by the histamine H3-receptor antagonist thioperamide. Of note, there is no strict correlation between the EC₅₀ of desloratadine, diphenhydramine or mizolastine in the various assays and the reported affinity for the H1-receptor (1, 2.5 and 22 nM, respectively), which is measured as the capacity to displace tritiated pyrilamine from this receptor [38]. This apparent discrepancy has been reported by others [39] and reflects differences in the experimental conditions between functional assays and binding assays. Also, antihistamines were added before histamine in the reporter gene assays while these were added after histamine in the smooth muscle relaxation assay, providing an explanation as to why a given compound displayed such different EC₅₀ values in the two types of assays. The increased potency of second-generation antagonists seen when these were added before histamine is most likely due to their slow dissociation rate from the histamine H1-receptor [38], while, inversely, the low efficiency of diphenhydramine in the same experimental setting is probably due to its rapid dissociation rate.

Evidence for an H1-receptor-independent mechanism was obtained by using TNF- α and TPA as inducers of NF- κ B and AP-1 activities, respectively. Firstly, these stimuli do not signal via the H1-receptor. Secondly, in this context, NF- κ B and AP-1 activities were not repressed by a subset of H1-receptor antagonists. Involvement of the H1-receptor was further ruled out by showing that the inducing effect of TNF- α and TPA, and the inhibitory effect of mizolastine and desloratadine were not significantly modified after overexpression of this receptor. Previous studies with second-generation H1-receptor antagonists have reported that they can diminish DNA binding of NF- κ B [17–19] or AP-1 [40], but as opposed to the present work, involvement of the H1-receptor was not investigated.

RANTES is a chemokine involved in allergic inflammation and asthma. It attracts eosinophils, monocytes, mast cells and lymphocytes, activates basophils and induces histamine synthesis and release from these cells [41, 42]. Therefore, the capacity of mizolastine and desloratadine to inhibit RANTES production is likely to be relevant for allergy and asthma. We have demonstrated previously that RANTES expression is mediated exclusively through NF- κ B in A549 cells [33]. It has been suggested that mizolastine could repress NF- κ B through direct inhibition of 5-LO [15, 16]. According to this hypothesis, 5-LO inhibitors used at selective concentrations [43, 44] should also affect RANTES release. However, this was not observed in the present study. Of note, we found that A549 cells, whether untreated or stimulated with TNF- α , express a very low level of 5-LO because detection of the corresponding transcript required nested PCR (data not shown). Thus, it seems unlikely that mizolastine down-regulates NF- κ B activity through inhibition of 5-LO in A549 cells, possibly because the amount of 5-LO is too low in these cells. Nevertheless, this regulatory pathway cannot be excluded in other cell types in which 5-LO is present at higher levels.

Although the peak plasma concentrations of desloratadine and mizolastine after previously usual dosage are 10^{-8} and 10^{-6} M, respectively [3, 45], their concentration in the lung might be much higher. Indeed, weak bases, including H1-receptor antagonists, have been shown to accumulate in the lung where their concentration can be over 100-fold higher than in the plasma [46–48]. In A549 lung epithelial cells, RANTES release and the activity of transcription factors were inhibited in a histamine H1-receptor-independent manner by mizolastine and desloratadine at 10^{-6} or 10^{-5} M, which are concentrations likely to be reached in the lung. Moreover, the same concentrations of H1-receptor antagonists were required to repress the expression of inflammatory mediators in other studies [18, 19]. These observations suggest that treatment with mizolastine or desloratadine might be beneficial for asthmatic patients. In this regard, second-

generation H1-receptor antagonists have been shown to prevent the development of asthma in atopic children [49], and to have some efficacy as a single agent on mild asthma [50–53]. The usefulness of these drugs in the treatment of asthma requires to be assessed further, particularly by determining whether these affect inflammatory parameters in the lungs of asthmatic patients.

Competing interests

C. R. has received reimbursement of congress fees from Sanofi-Aventis. The rest of the authors have declared that they have no conflict of interest.

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