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Involvement of histamine H₁ and H₂ receptor inverse agonists in receptor’s crossregulation

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Chemical compounds studied in this article

Amthamine dihydrobromide (PubChem CID: 16218912); Cetirizine dihydrochloride (PubChem CID: 55182); Chlorpheniramine maleate (PubChem CID: 5281068); Diphenhydramine hydrochloride (PubChem CID: 8980); Mepyramine maleate (PubChem CID: 5284451); Triprolidine hydrochloride (PubChem CID: 5702129); Cimetidine (PubChem CID: 2756); Ranitidine hydrochloride (PubChem CID: 3033332); Famotidine (PubChem CID: 5353622); 2,3-trifluormetilfenilhistamine dimaleate (PubChem CID: 71433958).

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Abstract

Histamine [2-(4-Imidazolyl)-ethylamine] modulates different biological processes, through histamine H$_1$ and H$_2$ receptors, and their respective blockers are widely used in treating allergic and gastric acid-related disorders. Histamine H$_1$ and H$_2$ receptor crossdesensitization and cointernalization induced by its agonists have been previously described. In this study, we show how this crosstalk determines the response to histamine H$_1$ and H$_2$ receptor inverse agonists and how histamine H$_1$ and H$_2$ receptor inverse agonists interfere with the other receptor’s response to agonists. By desensitization assays we demonstrate that histamine H$_1$ and H$_2$ receptor inverse agonists induce a crossregulation between both receptors. In this sense, the histamine H$_1$ receptor inverse agonists desensitize the cAMP response to amthamine, a histamine H$_2$ receptor agonist. In turn, histamine H$_2$ receptor inverse agonists interfere with histamine H$_1$ receptor signaling. We also determine that the crossdesensitization induced by histamine H$_1$ or H$_2$ receptor agonists alters the histamine inverse agonists receptor response: activation of histamine H$_1$ receptor affects cAMP response induced by histamine H$_2$ receptor inverse agonists, whereas histamine H$_2$ receptor agonist induces a negative regulation on the anti-inflammatory response of histamine H$_1$ receptor inverse agonists. Binding studies revealed that histamine H$_1$ and H$_2$ receptors cointernalize after stimulus with histamine receptor inverse agonists. In addition, the inhibition of the internalization process prevents receptor crossregulation. Our study provides new insights in the mechanisms of action of histamine H$_1$ and H$_2$ receptors that explain the effect of histamine H$_1$ and H$_2$ receptor inverse agonists and opens up new venues for novel therapeutic applications.
Graphical abstract:

Keywords
Histamine H₁ receptor inverse agonists; histamine H₂ receptor inverse agonists; GPCR crossregulation; histamine H₁ receptor; histamine H₂ receptor.

1. Introduction
Histamine [2-(4-Imidazolyl)-ethylamine] is an important mediator of many physiological and pathological processes including inflammation, gastric acid secretion, neuromodulation, regulation of immune function, cell proliferation and differentiation, among others. Histamine exerts its biological effects by binding to four different G protein-coupled receptor subtypes (H₁-H₄) (Panula et al., 2015).

Until now, the most clinically relevant uses of histamine receptor ligands are achieved through the interaction with histamine H₁ or H₂ receptors, which are widely expressed in many tissues. In this regard, histamine H₁ receptor antagonists/inverse agonists are used in the treatment of several allergic conditions, such as rhinoconjunctivitis,
urticaria, and atopic dermatitis, and are promptly available as prescription and/or as over-the-counter drugs (Church, 2016). On the other hand, histamine H2 receptor antagonists/inverse agonists have proved to be active agents for the treatment of duodenal and gastric ulcers, reflux, esophagitis and Zollinger–Ellison syndrome (Hershcovici and Fass, 2011; Sigterman et al., 2013). Additionally, since histaminergic ligands are low-cost drugs with no patent protection, there is a great interest to facilitate the repurposing of these drugs for other pathologies. Consequently, a deep understanding of their mechanisms of action is needed. The histamine H1 and H2 receptors are coexpressed in most human tissues and cell types, such as neurons, airway and vascular smooth muscle cells, endothelial cells, hepatocytes, epithelial cells, neutrophils, eosinophils, monocytes, dendritic cells, as well as T and B lymphocytes, among others (Jutel et al., 2009; Parsons and Ganellin, 2009). In most tissues, histamine H1 receptor couples to Gaq/11 leading to an increase in phosphoinositide metabolism, whereas histamine H2 receptor couples to Gs, triggering adenylyl cyclase (AC) activation and cyclic AMP (cAMP) accumulation (Panula et al., 2015). In these systems, the action of endogenous histamine may result from the balance and coordination of the signaling events activated by these, or even more, subtypes of histamine receptors. In this way, previous studies of our laboratory have described the existence of histamine H1 and H2 receptor crossregulation. In native and recombinant systems, both receptors desensitize when cells are exposed to a sustained stimulus with histamine H1 or H2 receptor agonists. Interestingly, this crossdesensitization does not depend on second messengers nor their downstream kinases, PKA or PKC, but on G protein-coupled receptor kinase 2 (GRK2) (Alonso et al., 2013). In addition, upon activation of histamine H1 or H2 receptor, both cointernalize in endosomes and form heteromers. Since these crossregulation mechanisms proved to be critical for the output
response to histaminergic stimulation, it would be expected that it also affects the response of the histamine H₁ and H₂ receptor inverse agonists used in the clinic. Nowadays, it is accepted that ligands which have been classically described as inverse agonists, due to their negative efficacy at modulating the G protein pathway, could also display some positive efficacy regarding receptor desensitization, internalization, or even signaling though another pathway (Kenakin, 2002; Pupo et al., 2016). In this sense, our laboratory has described that several histamine H₂ receptor inverse agonists behave as full agonists regarding histamine H₂ receptor desensitization and internalization, in spite of diminishing cAMP production (Alonso et al., 2014, 2015). In the present work, we hypothesized that histamine H₁ and H₂ receptor inverse agonists also induce the crossdesensitization between histamine H₁ and H₂ receptors, and that the crossregulation induced by histamine H₁ and H₂ receptor agonists affects the behavior of histamine H₁ and H₂ receptor inverse agonists. In order to address this issue, we analyzed the receptor’s crossregulation in U937 cells, which endogenously express histamine H₁ and H₂ receptors and in cotransfected HEK293 cells. We utilise clinically relevant ligands as mepyramine, chlorpheniramine, triprolidine and diphenhydramine (first-generation histamine H₁ receptor inverse agonists), cetirizine (second-generation histamine H₁ inverse agonist) and the widely used histamine H₂ receptor inverse agonists cimetidine, ranitidine and famotidine.

2. Materials and Methods

2.1. Materials

Cell culture medium, antibiotics, isobutylmethyl xanthine (IBMX), cAMP, prostaglandin E2 (PGE₂), 2,3-trifluormetilfenilhistamine dimaleate (histamine H₁ receptor agonist), bovine serum albumin (BSA), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides from Escherichia Coli (LPS), cimetidine, ranitidine
hydrochloride, famotidine, cetirizine dihydrochloride, chlorpheniramine maleate and
diphenhydramine hydrochloride were obtained from Sigma Chemical Company (St.
Louis, MO). Amthamine dihydrobromide, mepyramine maleate, triprolidine
hydrochloride, tiotidine and dynasore were acquired from Tocris Cookson Inc.
(Ballwin, MO). \[^{3}H\]\textit{cAMP}, \[^{3}H\]\textit{mepyramine and \[^{3}H\]}tiotidine, were purchased from
Perkin Elmer Life Sciences (Boston, MA). Fetal bovine serum (FBS) was purchased
from Natocor (Argentina). Other chemicals used were of analytical grade and obtained
from standard sources.

2.2. Cell culture and transfections

U937 cells, DC6 and AD3 derived clones, were cultured at 37°C in a humidified
atmosphere of 5% CO\textsubscript{2} in RPMI 1640 medium, and HEK293 cells were cultured in
Dulbecco´s modified Eagle´s medium (DMEM); all supplemented with 10% FBS and
50 μg/ml gentamicin. DC6 and AD3 cell lines were obtained by U937 stable
transfection with pcDNA3-HA-dynaminK44A or pcDNA3-β\textsubscript{1}arrestin (319–418)
respectively, and were previously characterized (Fernandez et al., 2008). U937 and
HEK293 cells were purchased from ATCC.

For transient transfection, HEK293 cells were grown to 80-90% confluency and the
cDNA constructs were transfected using K2 Transfection System. The transfection
protocol was optimized as recommended by the supplier (Biontex, Munich, Germany).
Usually, assays were performed 48 h after transfection. The human histamine H\textsubscript{1}
receptor (H\textsubscript{1}R) and histamine H\textsubscript{2} receptor (H\textsubscript{2}R) were subcloned previously in our
laboratory in pCEFL and pCEFLHA, respectively (Notcovich et al., 2010; Shayo et al.,
2001).

2.3. cAMP assay
For desensitization assays, cells were pretreated with agonists, inverse agonists, and/or inhibitors at the times shown in the corresponding figure legends. Cells were then washed and resuspended in fresh medium containing 1 mM IBMX, incubated for 3 min, and exposed to 10 μM amthamine, 10 μM cimetidine, 10 μM ranitidine, 10 μM famotidine, or 1 μM PGE2 for 10 min to determine whether the system was able to generate cAMP. The reaction was stopped by ethanol addition followed by centrifugation at 2000 x g for 5 min. The ethanol phase was then dried and the residue resuspended in 50 mM Tris-HCl pH 7.4, 0.1% BSA. cAMP content was determined by competition of [³H]cAMP for PKA, as previously described (Davio et al., 1995).

2.4. Reporter gene assay

HEK293 cells seeded on 24-well plates were cotransfected with the pIL6-luciferase reporter plasmid, pCEFL-H₁R and pCEFLHA-H₂R. In some experiments, cells were cotransfected with the plasmid constructs indicated in the corresponding figure legend or an empty vector to maintain the total amount of DNA. After 6 h, cells were seeded in 96-well plates, and after 24 h deprived from serum for another 16 h. Cells were then stimulated with the corresponding agents and luciferase activity was measured 6 h later with the Steady-Glo Luciferase Assay System according to the manufacturer's instructions (Promega Biosciences Inc. San Luis Obispo, CA, USA) using the GloMax 96 Microplate Luminometer (Promega Biosciences Inc. San Luis Obispo, CA, USA). Experimental reporter activity was normalized to control activity.

2.5. RT-PCR and quantitative real-time PCR

Total RNA was isolated from U937 cells using Quick-Zol reagent (Kalium Technologies) following the manufacturer's instructions. For the first-strand cDNA synthesis, 2 μg of total RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (AB) with random primers. Quantitative real-time PCR
(qPCR) was performed in triplicate using the resulting cDNA, the HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne) for product detection, and the following primers: COX-2 (cyclooxygenase-2) forward 5′-
TTCAATGAGATTGTGGGAAAATTGCT-3’ and reverse 5′-
AGATCATCTCTGCTGAGTATCTT-3’, human IL-8 (interleukin-8) forward 5′-
CTGCAGCAACACAGAAATTA-3’ and reverse 5′-ATTGCATCTGGCAACCCTAC-3’ and human β-Actin (β-Act) forward, 5′-GGACTTCGAGCAAGAGATGG-3′ and reverse 5′-AGCACTGTGTTGGCGTACAG-3′. The cDNA was amplified by 45 cycles of denaturing (30 s at 95°C), annealing (30 s at 60°C), and extension (30 s at 72°C) steps. The specificity of each primer set was monitored by analyzing the dissociation curve, and the relative mRNA quantification was performed using the comparative ΔΔCt method using β-Actin as the housekeeping gene.

2.6. Cell proliferation

U937 cells were seeded at 1x10^5 cells/ml in 24-wells plates, incubated for 24 h, 48 h or 72 h with different combinations of compounds as indicated in the figure legend and then counted in a Neubauer chamber.

2.7. Cell cycle analysis

U937 cells growing in exponential phase were treated as indicated in the figure legend for 72 h. Then, cells were centrifuged at 1000 x g for 5 min, resuspended in one volume of phosphate buffered saline (PBS) and fixed and permeabilized by vigorous addition of nine volumes of ice-cold 70% (v/v) ethanol and stored at -20°C for a minimum of 24 h, prior to analysis. Cells at a density of approximately 1x10^6 were resuspended in 0.1 ml staining solution [20 μg/ml propidium iodide (PI) and 100 μg/ml RNase A in PBS, pH 7.4] and incubated in the dark at room temperature for 30 min. The percentage of cells in the sub-G0/G1, G0/G1, S, and G2/M cell cycle phases was determined by a FACS
Scan Flow Cytometer (Becton-Dickinson, Laguna Hills, CA). Data from at least three independent experiments were analyzed using FlowJo V10 program.

2.8. Determination of phosphatidylserine exposure at the cell surface by annexin V binding assay

U937 cells growing in exponential phase were treated with the corresponding compounds as indicated in the figure legend or 2% (v/v) dimethylsulfoxide (DMSO; positive control) for 72 h. Following cold PBS washing, 2x10^5 cells were incubated with fluorescein isothiocyanate (FITC)-labeled annexin V and PI according to the manufacturer's instructions (Invitrogen) and analyzed using a FACS Scan Flow Cytometer (Becton-Dickinson). The different cell subpopulations were identified according to the annexin V/PI staining pattern, as follows: cells labeled with annexin V only were considered to be at an early apoptotic stage, cells labeled with annexin V and PI were considered to be at a late apoptotic stage, and cells labeled with PI only were considered necrotic.

2.9. Radioligand binding assay

Triplicate assays were performed in 50 mM Tris-HCl, pH 7.4. Saturation studies were performed by incubating 1x10^6 U937 cells/tube or 1x10^4 HEK293-H1R, HEK293-H2R or HEK293-H1R-H2R cells for 60 min at 4°C with increasing concentrations of [3H]tiotidine or [3H]mepyramine, in the absence or presence of 10 μM unlabeled tiotidine or mepyramine, respectively. Specific binding was calculated by subtraction of nonspecific binding from total binding. For HEK293 cells, the incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4. For U937 cells, rapid filtration under reduced pressure onto Whatman GF/B glass-fiber filters, followed by three washes with 3 ml ice-cold buffer was performed. Experiments on intact cells were carried out at 4°C to avoid ligand internalization.
2.10. Statistical analysis

Statistical analysis (from assays performed by triplicate from at least three independent experiments), binding data, sigmoidal dose-response, and desensitization fittings were performed with GraphPad Prism 6.00 for Windows, GraphPad Software (San Diego, CA). Statistical analysis was performed using one- or two-way ANOVA, followed by Dunnett’s or Bonferroni’s multiple comparisons post test. Cell proliferation and cell cycle statistical analysis were carried out by one-way ANOVA followed by Tukey’s multiple comparison post test. Values of $P<0.05$ were considered to indicate statistically significant differences.

3. Results

3.1. Histamine H$_1$ receptor inverse agonists affect histamine H$_2$ receptor response

In order to evaluate the potential crossregulation between histamine H$_1$ receptor inverse agonists and histamine H$_2$ receptor response, we first analyzed the effect of cetirizine pretreatment on histamine H$_2$ receptor response to amthamine (a potent and selective histamine H$_2$ receptor agonist). In U937 cells pretreated with cetirizine, cAMP production following 10 $\mu$M amthamine stimuli decreased in a time- and concentration-dependent manner (Fig. 1A and B). Maximal desensitization was attained after 30 min and with 10 $\mu$M cetirizine. Besides, preexposure of U937 cells to mepyramine, chlorpheniramine, tripolidine, or diphenhydramine (10 $\mu$M; 30 min) induced a significant desensitization of histamine H$_2$ receptor in response to amthamine, whereas the response to PGE2 remained unaffected (Fig. 1C and D). The concentration used for amthamine and PGE2 corresponded to the maximum cAMP response in U937 cells (data not shown). These results suggest the specificity of the histamine H$_2$ receptor desensitization.
To confirm that histamine H₁ receptor inverse agonists desensitize histamine H₂ receptor response through histamine H₁ receptor, we performed the same assay using HEK293 cells which only express histamine H₂ receptor. As shown in Fig. 1E, no crossdesensitization occurs in the absence of histamine H₁ receptor. As expected, when both receptors are coexpressed in HEK293 cells, pretreatment with histamine H₁ receptor inverse agonists also desensitized the histamine H₂ receptor response to amthamine (Fig. 1F).

These findings show that inverse agonists acting selectively through histamine H₁ receptor induce the crossdesensitization of histamine H₂ receptor response, both in U937 native and HEK293 recombinant systems.

3.2. Activation of histamine H₂ receptor affects histamine H₁ receptor ligands anti-inflammatory response

Our next question was whether the pharmacological action of histamine H₁ receptor inverse agonists may be affected by the activation of histamine H₂ receptor. Histamine, acting through histamine H₁ receptor, plays a key role in the induction of early-phase allergic symptoms and inflammatory actions. Its activation upregulates the production and release of cytokines, chemokines and adhesion molecules by inflammatory cells, epithelium and endothelium (Asako et al., 1994; Kimura et al., 2004; Kordulewska et al., 2017; Matsubara et al., 2005; Yamauchi et al., 2007). It has also been reported that histamine promotes the induction of the proinflammatory cytokine IL-6, through histamine H₁ receptor in several systems (Cadman et al., 1994; Delneste et al., 1994; Kohda et al., 2002). In concordance, we observed that cetirizine, chlorpheniramine, triprolidine, or diphenhydramine significantly reduced the constitutive IL-6 promoter activity in transfected HEK293-H₁R-H₂R cells (Fig. 2A). Although it may seem that mepyramine reduced IL-6 promoter’s activity, no significant differences were observed
when compared to basal levels. Remarkably, cotreatment with the histamine H₂ receptor agonist amthamine, prevented the decrease of IL-6 promoter’s activity induced by all of the histamine H₁ receptor inverse agonists tested. When this assay was performed in HEK293 cells expressing only histamine H₁ receptor, response to histamine H₁ receptor inverse agonists was not modified by amthamine (Fig. 2B), indicating that amthamine achieves the interference by specifically binding to histamine H₂ receptor.

Next, we assessed if a histamine H₂ receptor agonist is able to desensitize histamine H₁ receptor anti-inflammatory response in a cellular system of pathophysiological relevance. For this purpose, we differentiated U937 cells into macrophage-like cells with PMA and activated them with LPS. In order to evaluate the inflammatory response, IL-8 cytokine and COX-2 enzyme transcript levels were measured using quantitative real-time polymerase chain reaction. Histamine H₁ receptor inverse agonists, mepyramine, cetirizine, chlorpheniramine, tripolidine, and diphenhydramine inhibited COX-2 and IL-8 gene expression. Interestingly, cotreatment with amthamine, significantly reverted the anti-inflammatory effect of histamine H₁ receptor inverse agonists (Fig. 2C and D).

3.3. Activation of histamine H₁ receptor affects histamine H₂ receptor response to inverse agonists

Given the existence of a crossdesensitization between histamine H₁ and H₂ receptors induced by both agonists (Alonso et al., 2013) and that the stimulus with an histamine H₂ receptor agonist affects the response to histamine H₁ receptor inverse agonists, we evaluated whether histamine H₁ receptor agonists could affect histamine H₂ receptor response to inverse agonists in U937 cells. As expected, treatment with cimetidine, ranitidine and famotidine, classified as histamine H₂ receptor inverse agonists, caused a reduction in cAMP levels (Fig. 3A). However, pretreatment with an histamine H₁
receptor agonist (10 µM; 30 min) annulled the effect of histamine H₂ receptor inverse agonists, indicating that histamine H₁ receptor activation interferes with histamine H₂ receptor response to inverse agonists. We verified that pretreatment with the histamine H₁ receptor agonist decreased histamine H₂ receptor response to inverse agonists in cotransfected HEK293 cells expressing both receptors (Fig. 3B). Conversely, the reduction in cAMP levels induced by histamine H₂ receptor inverse agonists was not modified in cells which only express histamine H₂ receptor (Fig. 3C), showing the specificity of the histamine H₁ receptor agonist effect.

3.4. Histamine H₂ receptor inverse agonists influence histamine H₁ receptor response

With the aim of establishing whether these ligands may interfere with histamine H₁ receptor response, we evaluated the effect of cimetidine, ranitidine, and famotidine on the histamine H₁ receptor agonist-induced IL-6 promoter activity. The concentration used for histamine H₁ receptor agonist corresponded to the maximum IL-6 promoter activity (data not shown). Treatment with all of the histamine H₂ receptor inverse agonists assayed reduced the luciferase signal induced by stimulation with an histamine H₁ receptor agonist in HEK293-H₁R-H₂R cells (Fig. 4A), while no interference was detected in HEK293-H₁R cells (Fig. 4B). This interference was also observed when cells were pretreated with the histamine H₂ receptor agonist amthamine (Fig. 4A), in a similar way to that previously described for inositol 1,4,5-trisphosphate response (Alonso et al., 2013).

Consistently, in U937 cells differentiated with PMA, stimulation with an histamine H₁ receptor agonist induced gene expression of COX-2 and IL-8, and treatment with histamine H₂ receptor inverse agonists annulled the pro-inflammatory response (Fig. 4C and D).
Taking in consideration that histamine H\textsubscript{1} receptor activation mediates antiproliferative and apoptotic effects in U937 cells (Alonso et al., 2013), we analyzed the impact of histamine H\textsubscript{2} receptor inverse agonists in the antiproliferative response to an histamine H\textsubscript{1} receptor agonist. For this, U937 cells were incubated with an histamine H\textsubscript{1} receptor agonist alone or in combination with cimetidine, ranitidine, or famotidine for 3 days and cell number, cell cycle stage and apoptosis were evaluated. The antiproliferative effect mediated by histamine H\textsubscript{1} receptor was inhibited by treatment with histamine H\textsubscript{2} receptor inverse agonists (Fig. 5A and B). FACS studies showed that treatment with the histamine H\textsubscript{1} receptor agonist induced an arrest in the cell cycle, evidenced by an increment in the SubG\textsubscript{0} and a reduction in the S population compared to the control cells. Cotreatment with cimetidine, ranitidine or famotidine prevented this arrest, which was in accordance with the proliferative response observed (Fig. 5C). We also evaluated early and late apoptosis by PI and annexin V binding assays. Following U937 cells’ exposure to the histamine H\textsubscript{1} receptor agonist, a reduction in viable cells with a concomitant increase in early and late apoptotic subpopulations was detected. This induction of apoptosis was blocked in the presence of all three histamine H\textsubscript{2} receptor inverse agonists tested, cimetidine, ranitidine, or famotidine (Fig. 5D).

Altogether, these results highlight the importance of the crossregulation between histamine H\textsubscript{1} and H\textsubscript{2} receptor in the final response to histaminergic ligands.

3.5. Cointernalization of histamine H\textsubscript{1} and H\textsubscript{2} receptors is responsible for the crossregulation induced by inverse agonists

In previous studies from our laboratory, we reported that histamine H\textsubscript{1} and H\textsubscript{2} receptors are cointernalized upon histamine H\textsubscript{1} or H\textsubscript{2} receptor agonist stimulation in U937, CHO and HEK293 cells (Alonso et al., 2013). In addition, treatment with histamine H\textsubscript{2} receptor inverse agonist leads to histamine H\textsubscript{2} receptor internalization (Alonso et al.,
In order to evaluate a putative reciprocal internalization of histamine H1 and H2 receptors induced by inverse agonists of both receptors, we measured the number of cell surface \(^{3}H\)tiotidine or \(^{3}H\)mepyramine binding sites after incubating U937 or transfected HEK293 cells with the ligands. Exposure to cetirizine (10 µM; 90 min) not only reduced the number of histamine H1 receptor binding sites in U937 cell membranes (58% respect to untreated cells), but also decreased the number of histamine H2 receptor membrane sites (65% respect to untreated cells) (Fig. 6A). In the same way, treatment with ranitidine and famotidine (10 µM; 90 min) internalized histamine H1 receptor by 43% and 31%, respectively (Fig. 6B). With the intention of evaluating the histamine H2 receptor-binding specificity of the ligands necessary to induce cointernalization, saturation binding assays with \(^{3}H\)mepyramine were performed in transfected HEK293-H1R-H2R and HEK293-H1R cells exposed to ranitidine and famotidine. HEK293-H1R-H2R cells showed similar results to those obtained with U937 cells; where ranitidine and famotidine led to a 29% and 32% decrease in histamine H1 receptor binding sites, respectively (Fig. 6C). On the other hand, ranitidine and famotidine failed to stimulate histamine H1 receptor internalization in HEK293-H1R cells, although cetirizine, the histamine H1 receptor ligand, internalized histamine H1 receptor by 45% (Fig. 6D).

These findings reveal that inverse agonists induce receptor cointernalization as part of the crosstalk between histamine H1 and H2 receptors.

In an attempt to determine if the cointernalization of histamine H1 and H2 receptors induced by inverse agonists is responsible for the crossregulation process or if an additional mechanism triggers it, we blocked the internalization of the receptors and evaluated their crossregulation. Given that arrestin and dynamin proved to be essential for the internalization of several GPCRs including histamine receptors (Fernandez et al.,
2008; Wolfe and Trejo, 2007), we pharmacologically or genetically inhibited arrestin and dynamin and assessed the effect on histamine H<sub>1</sub> and H<sub>2</sub> receptor crossregulation. First, we evaluated the response to amthamine after cetirizine, chlorpheniramine and triprolidine pretreatment in the presence of dynasore, a dynamin inhibitor, in U937 cells. None of the histamine H<sub>1</sub> receptor inverse agonists was able to impair histamine H<sub>2</sub> receptor agonist response in the presence of dynasore (Fig. 7A), which efficiently inhibited histamine H<sub>1</sub> receptor internalization (supplementary Fig. 1). Similar results were observed in U937 derived clones DC6 and AD3, obtained by stable transfection with a dynamin or arrestin dominant negative mutant respectively; cetirizine, chlorpheniramine, and triprolidine did not induce histamine H<sub>2</sub> receptor crossdesensitization (Fig. 7B). Next, the effect of dynasore on the crossdesensitization induced by histamine H<sub>2</sub> receptor inverse agonists on histamine H<sub>1</sub> receptor response was evaluated in HEK293T-H<sub>1</sub>R-H<sub>2</sub>R cells. Figure 7C shows that neither, cimetidine, ranitidine nor famotidine were able to reduce IL-6 promoter’s activity induced by the histamine H<sub>1</sub> receptor agonist when internalization was blocked.

Altogether, these results indicate that the inhibition of the internalization process prevents the receptor’s crossdesensitization, and thus the cointernalization induced by inverse agonists is responsible for the crossregulation between histamine H<sub>1</sub> and H<sub>2</sub> receptors.

4. Discussion

The major findings of the present study are that: 1) the response to inverse agonists may be crossregulated by the activation of either histamine H<sub>1</sub> or H<sub>2</sub> receptors, 2) histamine H<sub>1</sub> and H<sub>2</sub> receptor inverse agonists crossdesensitize the receptor’s response to their
agonist, and 3) the cointernalization of histamine H₁ and H₂ receptors is responsible for the crossdesensitization induced by inverse agonists.

One hundred and thirty-four GPCRs are targets for drugs approved in the United States and European Union and it is estimated that approximately 35% of approved drugs target GPCRs. Among the receptors with the highest number of approved drugs are the histamine H₁ and H₂ receptors (Sriram and Insel, 2018). Many of the histamine H₁ and H₂ receptor antagonists, now reclassified as inverse agonists, have been successfully used in the clinic, achieving the desired effects, although side effects do happen. The concept of inverse agonism arose from experimental observations showing that certain drugs were able to reduce the activity of receptor systems that were active even in the absence of agonists. These ligands bind preferentially and stabilize the inactive conformation of the receptors. However, whether inverse agonism is essential or important for these drugs to exert their medicinal actions has not been clarified yet.

From the moment these drugs were implemented to date, several concepts have changed, including GPCR crossregulation and pluridimensional efficacy of GPCR ligands, which allow researchers to continue elucidating new aspects of these drugs´ mechanisms of action.

It has been described that GPCRs functionally interact with other pathways in several ways. In particular, histamine H₁ and H₂ receptor agonists interfere with the agonist´s response of the other GPCR. This crossregulation has already been described in recombinant and naïve systems involving GRK2-dependent crossdesensitization and cointernalization/ heterodimerization of both receptors, and has proven to be part of histamine´s fine regulation to achieve a final response (Alonso et al., 2013). Now, we show that the crossdesensitization induced by histamine H₁ or H₂ receptor agonists also influences the response to inverse agonists. In this way, the activation of whichever of
these receptors modifies the response of histamine H₁ or H₂ receptor inverse agonists in
different systems. Thus, the anti-inflammatory effect of histamine H₁ receptor inverse
agonists evaluated by the reduction in IL-8 and COX-2 gene expression in monocytic
U937 cells was less pronounced when histamine H₂ receptor was activated by
amthamine (Fig. 2C and D). On the other hand, cAMP response triggered by incubation
with the histamine H₂ receptor inverse agonist was prevented when U937 cells were
pretreated with the histamine H₁ receptor agonist (Fig. 3A). These results evidence how
the efficacy of these inverse agonists is affected by the presence and activation of the
other subtype of histaminergic receptor. This crosstalk may explain why histamine H₁
and H₂ receptor inverse agonists have different efficacies in different tissues. Therefore,
when considering targeting histamine H₁ or H₂ receptors, it would be necessary to take
into account not only the selectivity, affinity and residence time of ligands, but also the
expression levels of both receptors and their crossregulation in order to accomplish the
desired effect.

It is important to note that histamine H₁ and H₂ receptors are coupled to different G
proteins and that their crossinterference induced by histamine H₁ and H₂ receptor
inverse agonists depends on the cointernalization mechanism. To date, histamine H₁ and
H₂ receptor inverse agonist have shown to interfere with the response of other GPCRs
that share the same signaling pathway. In fact, treatment with these drugs stabilizes a
conformation of the receptor that, although it is inactive, it may couple and recruit G-
protein making it less available for other unrelated receptors that signal through the
same pathway (Monczor et al., 2003; Tubio et al., 2010).

Our results show that interference may occur among receptors that do not share the
same signaling pathway and determine cell’s fate. Thus, the exposure to histamine H₁
receptor agonist, alone or in combination with specific histamine H₂ receptor inverse
agonists, ultimately determines whether U937 cells arrest their cell cycle and engage with apoptotic processes or proliferate instead (Fig. 5).

It is important to mention that the IC$_{50}$ of cetirizine needed to achieve histamine H$_2$ receptor desensitization was 0.43 µM or 170 ng/ml (Fig. 1B), becoming clinically relevant since pharmacokinetic studies after oral administration of the clinically used dose (10 mg/day) reported a maximal plasma concentration of 311 ng/ml. Among all the tested histamine H$_1$ receptor inverse agonist, cetirizine showed the highest efficacy leaving a residual activity of histamine H$_2$ receptor between 40% and 60% for HEK293-H$_1$R-H$_2$R and U937 cells, respectively (Fig.1C and F). This interference in histamine H$_2$ receptor response was similar to that achieved by histamine H1 receptor agonist (Alonso et al., 2013), denoting a control of the histamine response through histamine H$_2$ receptor by histamine H$_1$ receptor.

Several GPCRs regulate their functions through cointernalization, which explains the signaling crossdesensitization reported in somatostatin 2A/opioid receptors and adenosine A2A/dopamine D2 receptors (Hillion et al., 2002; Pfeiffer et al., 2002), and can even trigger new intracellular signaling pathways. In this sense, Smith et al. described that the cointernalization of the protease-activated receptor-4/purinergic receptor P2Y12 heterodimer is necessary for β-arrestin recruitment to endosomes and Akt signaling (Smith et al., 2017). Regarding histamine H$_1$ and H$_2$ receptors, cointernalization and heterodimerization have been described upon histamine H$_1$ or H$_2$ receptor agonist stimulus, although receptor cointernalization is not the only mechanism of desensitization (Alonso et al., 2013). In reference to histamine H$_2$ receptor inverse agonists, cimetidine, ranitidine, and famotidine have shown to elicit histamine H$_2$ receptor internalization as part of their pluripotential efficacy, in an arrestin and dynamin dependent manner (Alonso et al., 2014). This report provides the first evidence
that histamine H₁ receptor inverse agonists can induce the internalization of their own receptor. In line with these findings, cetirizine showed to induce histamine H₁ receptor internalization both, in cells that endogenously express the receptor (U937) and in a recombinant system (HEK293T-H₁R and HEK293T-H₁R-H₂R) (Fig. 6). Moreover, regarding the crossregulation between histamine H₁ and H₂ receptor, here we describe a new efficacy for several histamine H₁ and H₂ receptor inverse agonists, as they induce the cointernalization of both receptors, interfering in the signaling cascade of receptors that have never been challenged to their own ligands. Further investigations are necessary in order to unravel what happens after internalization, whether the receptors heterodimerize in the endosomes and may, eventually, trigger some type of intracellular signaling. Our findings open an interesting field of study related to histamine H₁ and H₂ receptor inverse agonists.

The repurposing of well-characterized and well-tolerated drugs in order to treat illnesses for which they were not originally intended has emerged as an attractive alternative to a long and costly process of drug development. Repositioning antihistaminergic ligands seems a promising idea given that histamine exerts a variety of actions throughout the body and that histamine H₁ and H₂ receptors are ubiquitously expressed. In this sense, novel clinical applications for histamine H₁ receptor antagonists/inverse agonists are currently being studied for the management of different pathological situations, such as inflammatory-related conditions (in combination with glucocorticoids) (Zappia et al., 2015), analgesia (Stein et al., 2016) or neurodegenerative and sleep disorders (Kim and Song, 2017; Krystal, 2015). In the same way, evidence for anti-cancer effects of the histamine H₂ receptor inverse agonist cimetidine has been reported in various types of neoplasias, including glioblastoma, cholangiocarcinoma, malignant melanoma, renal cell carcinoma, colorectal and gastric cancer (Dana et al., 2017; Pantziarka et al., 2014).
Likewise, histamine H\textsubscript{2} receptor antagonist/inverse agonist use was recently associated with a lower risk for incident heart failure and better preserved stroke volume, left ventricular end-diastolic volume, and mass/volume ratio over time in community dwelling adults (Leary et al., 2016). It would be interesting to determine whether beneficial effects of histamine H\textsubscript{2} receptor antagonists/inverse agonists in heart failure pathologies rely, at least partially, on histamine H\textsubscript{1} and H\textsubscript{2} receptor crossdesensitization/cointernalization. Thus, histamine H\textsubscript{2} receptor antagonists/inverse agonists could also modulate the histamine H\textsubscript{1} receptor-mediated pro-inflammatory response to endogenous histamine, aiding the resolution of the cardiac disease. We believe that these newly described pharmacological behaviors may encourage and clarify the mechanisms of histamine H\textsubscript{2} receptor antagonists/inverse agonists in cardiac tissue.

This crosstalk may be responsible for the beneficial effects of histamine H\textsubscript{2} receptor inverse agonists on heart pathologies and may also explain unwanted effects of these drugs on other tissues. In this way, Allen et al. reported an anaphylactoid reaction following cessation of high-dose ranitidine in a 19-year-old female with mast cell activation syndrome, hypermobile Ehlers-Danlos syndrome and postural tachycardia syndrome (Allen et al., 2018). The authors suggest that patients who take ranitidine, after withdrawal, can suffer an exacerbated effect of histamine caused by upregulation of histamine H\textsubscript{2} receptor and raised histamine levels due to histidine decarboxylase induction, which is in concordance with previous in vitro studies (Alonso et al., 2015; Monczor and Fernandez, 2016; Smit et al., 1996). Considering our present results, it is feasible that in the same way that sustained internalization of histamine H\textsubscript{2} receptor led to upregulation on histamine H\textsubscript{2} receptor levels, sustained cointernalization of histamine H\textsubscript{1} receptor by ranitidine treatment may also lead to upregulation of histamine H\textsubscript{1}.
receptor, which may explain the observed anaphylactoid reaction after cessation of ranitidine treatment.

In conclusion, our findings support the notion that the crosstalk between histamine H\(_1\) and H\(_2\) receptor signaling is not restrictive to agonist ligands and, as a result, may have profound consequences regarding treatment with histamine H\(_1\) and H\(_2\) receptor antagonists/inverse agonists. Receptor agonists crossregulate receptor inverse agonists response and receptor inverse agonists crossregulate histamine response. Considering the large number of cell types in different tissues that express histamine H\(_1\) and H\(_2\) receptors, the clinically widespread use of antagonists/inverse agonists acting through both receptors in the treatment of several human diseases, and the advantage of drug repositioning, the accurate characterization of ligands’ mechanisms of action should allow us to reinterpret side effects of drugs and/or to ascribe new uses.

**Disclosure statement**

The authors have no conflict of interest.

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References


Jutel, M., Akdis, M., Akdis, C.A., 2009. Histamine, histamine receptors and their role in

https://doi.org/10.1111/j.1365-2222.2009.03374.x

Kenakin, T., 2002. DRUG EFFICACY AT G PROTEIN–COUPLED RECEPTORS.
https://doi.org/10.1146/annurev.pharmtox.42.091401.113012


https://doi.org/10.1016/j.imbio.2016.11.004


Panula, P., Chazot, P.L., Cowart, M., Gutzmer, R., Leurs, R., Liu, W.L.S., Stark, H.,


Smith, T.H., Li, J.G., Dores, M.R., Trejo, J., 2017. Protease-activated receptor-4 and

https://doi.org/10.1074/jbc.M117.782359


https://doi.org/10.1124/mol.117.111062


https://doi.org/10.1213/ANE.0000000000001326


https://doi.org/10.1074/jbc.M109.099689


https://doi.org/10.2332/allergolint.O-06-446

Figure Legends:

**Figure 1. Effect of histamine H₁ receptor inverse agonists on histamine H₂ receptor response.** (A) U937 cells were incubated with 10 µM cetirizine at different time points and washed with PBS. cAMP response to 10 µM amthamine was determined as detailed in Materials and Methods. (B) U937 cells were incubated with different concentrations of cetirizine (CET) for 30 min, washed with PBS, and cAMP response to 10 µM amthamine was determined. (C-D) U937 cells were exposed to 10 µM mepyramine (MEP), chlorpheniramine (CHLOR), triprolidine (TRIP), or diphenhydramine (DIPH) for 30 min, washed with PBS, and cAMP response to 10 µM amthamine (C) or 10 µM PGE2 (D) was determined. (E-F) HEK293-H₂R (E) or HEK293-H₁R-H₂R cells (F) were exposed to 10 µM histamine H₁ receptor inverse agonists for 30 min, washed with PBS, and cAMP response to 10 µM amthamine was determined. 100% corresponds to amthamine response without pretreatment (control). Data represent mean ± S.E.M. (n = 3). ns. not significant; **P < 0.01; ***P < 0.001 respect to control.

**Figure 2. Effect of histamine H₂ receptor agonist on histamine H₁ receptor inverse agonists response.** HEK293 cells cotransfected with IL-6-Luc, H₁R and H₂R (A) or IL-6-Luc and H₁R coding constructs (B), were incubated for 10 min with 10 µM amthamine (grey bars) or not (white bars), and then treated with 10 µM mepyramine (MEP), cetirizine (CET), chlorpheniramine (CHLOR), triprolidine (TRIP), or diphenhydramine (DIPH) for 6 h. Luciferase activity was determined as detailed in Materials and Methods. 100% correspond to basal activity without histamine H₂ receptor agonist treatment (control). Data represent mean ± S.E.M. (n = 3). ns. not
significant, *P < 0.05; **P < 0.01; ***P < 0.001 respect to control. # P < 0.01; ## P < 0.001. (C-D) U937 cells incubated for 24 h with 100 nM PMA were exposed for 10 min with 10 µM amthamine (grey bars) or not (white bars), treated with 10 µM mepyramine (MEP), cetirizine (CET), chlorpheniramine (CHLOR), triprolidine (TRIP), or diphenhydramine (DIPH) for 18 h and stimulated with 1 µg/ml LPS for 4 h. COX-2 (C) and IL-8 (D) mRNA levels were quantified by qPCR as detailed in Materials and Methods. Data represent mean ± S.E.M. (n = 3). ns. not significant, *P < 0.05; **P < 0.01; ***P < 0.001 respect to control. # P < 0.05; ## P < 0.01; ### P < 0.001.

**Figure 3. Effect of histamine H<sub>1</sub> receptor agonist on histamine H<sub>2</sub> receptor inverse agonists response.** (A) U937, (B) HEK293-H<sub>1</sub>R-H<sub>2</sub>R, or (C) HEK293-H<sub>2</sub>R cells were exposed to 10 µM H<sub>1</sub> agonist for 30 min (grey bars) or not (white bars), washed with PBS, and cAMP response to 10 µM cimetidine (CIM), ranitidine (RAN) or famotidine (FAM) was determined as indicated in Materials and Methods. Data represent mean ± S.E.M. (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001 respect to basal levels without histamine H<sub>1</sub> receptor agonist pretreatment. ns. not significant; #P < 0.05; ##P < 0.01; ###P < 0.001 respect to basal levels after histamine H<sub>1</sub> receptor agonist pretreatment.

**Figure 4. Effect of histamine H<sub>2</sub> receptor inverse agonists on histamine H<sub>1</sub> receptor response.** (A-B) HEK293 cells cotransfected with IL-6-Luc, H<sub>1</sub>R and H<sub>2</sub>R (A) or IL-6-Luc and H<sub>1</sub>R coding constructs (B), were incubated for 10 min with 10 µM cimetidine (CIM), ranitidine (RAN), famotidine (FAM) or amthamine (histamine H<sub>2</sub> receptor agonist), and then treated with 10 µM histamine H<sub>1</sub> receptor agonist (grey bars) or not (white bars) for 6 h. Luciferase activity was determined as detailed in Materials and Methods. Data represent mean ± S.E.M. (n = 3). ns. not significant; ###P < 0.001 respect
to H₁ agonist response. **P < 0.001. (C-D) U937 cells incubated for 24 h with 100 nM PMA were exposed for 10 min with 10 µM cimetidine (CIM), ranitidine (RAN) or famotidine (FAM), and then treated with 10 µM histamine H₁ receptor agonist for 18 h. COX-2 and IL-8 mRNA levels were quantified by qPCR as detailed in Materials and Methods. Data represent mean ± S.E.M. (n = 3). ###P < 0.001 respect to histamine H₁ receptor agonist response, ***P < 0.001.

Figure 5. Effect of histamine H₂ receptor inverse agonists on the inhibition of cell proliferation and apoptosis induced by histamine H₁ receptor agonist. U937 cells were treated with 10 µM histamine H₁ receptor agonist, alone or in combination with 10 µM cimetidine (CIM), ranitidine (RAN) or famotidine (FAM) for 24, 48 and 72 h (A) or 72 h (B–D). After treatment, cell proliferation (A and B), cell cycle stage (C) and phosphatidylserine exposure at the cell surface by annexin V binding (D) were evaluated as detailed in Materials and Methods. (A) Data were calculated as the means ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. (B) Data represent mean ± S.E.M. (n = 3). ###P < 0.001 respect to control cells. ***P < 0.001 respect to histamine H₁ receptor agonist response. (C) Data represent mean ± S.E.M. (n = 3). **P < 0.01 respect to control cells. (D). Data represent mean ± S.E.M. (n = 3). ***P < 0.001 respect to control cells.

Figure 6. Cointernalization of histamine H₁ and H₂ receptors induced by histamine H₁ and H₂ receptor inverse agonists. (A) [³H]Tiotidine or [³H]mepyramine saturation assays were performed in U937 cells: control (●) or treated for 90 min with 10 µM cetirizine (CET) (■). (B) [³H]Mepyramine saturation assays were performed in U937 cells: control (●) or treated for 90 min with 10 µM ranitidine (RAN) (□) or 10 µM
famotidine (FAM) (○). (C-D) [³H]Mepyramine saturation assays were performed in HEK293-H₁R-H₂R (C) or HEK293-H₁R (D) cells: control (●) or treated for 90 min with 10 µM cetirizine (CET) (■), 10 µM ranitidine (RAN) (□) or 10 µM famotidine (FAM) (○). Data were calculated as the mean ± S.D. of assay triplicates. Similar results were obtained in at least three independent experiments. Right (A-D): Data represent the percentage Bmax value fitted by nonlinear regression of the saturation assay, calculated as the means ± S.E.M. (n = 3). 100% corresponds to untreated cells (control). ***P < 0.001; **P < 0.01 respect to control cells.

Figure 7. Receptor internalization is involved in the crossdesensitization induced by histamine H₁ and H₂ receptor inverse agonists. (A) U937 cells pretreated for 30 min with 80 µM dynasore (grey bars) or not (white bars), were exposed for 30 min to 10 µM cetirizine (CET), chlorpheniramine (CHLOR), or triprolidine (TRIP) washed with PBS, and cAMP response to 10 µM amthamine was determined. (B) U937, AD3 and DC6 cells were exposed for 30 min to different 10 µM histamine H₁ receptor inverse agonists as indicated in the figure, washed with PBS, and cAMP response to 10 µM amthamine was determined. Data represent mean ± S.E.M. (n = 3). ***P < 0.001 respect to histamine H₂ receptor agonist response without pretreatment. ns. not significant. (C) HEK293 cells cotransfected with IL-6-Luc, H₁R and H₂R coding constructs, were pretreated for 30 min with 80 µM dynasore (Dyn) (grey bars) or not (white bars), exposed for 10 min to 10 µM cimetidine (CIM), ranitidine (RAN) or famotidine (FAM), and then treated with 10 µM histamine H₁ receptor agonist for 6 h. Luciferase activity was determined as detailed in Materials and Methods. Data represent mean ± S.E.M. (n = 3). ns. not significant; #P < 0.05; ##P < 0.01; ###P < 0.001 respect to histamine H₁ receptor agonist response.
Author Agreement

All authors have seen and approved the final version of the manuscript being submitted.