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Chemical compounds studied in this article

Amthamine dihydrobromide (PubChem CID: 16218912); Cetirizine dihydrocloride (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:
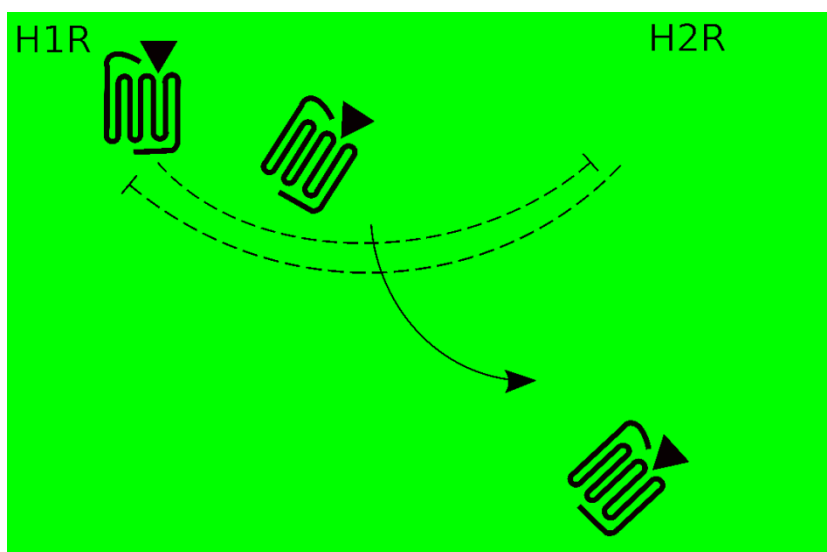
Keywords

Histamine H_1 receptor inverse agonists; histamine H_2 receptor inverse agonists; GPCR crossregulation; histamine H_1 receptor; histamine H_2 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 (H_1-H_4) (Panula et al., 2015).

Until now, the most clinically relevant uses of histamine receptor ligands are achieved through the interaction with histamine H_1 or H_2 receptors, which are widely expressed in many tissues. In this regard, histamine H_1 receptor antagonists/inverse agonists are used in the treatment of several allergic conditions, such as rhinoconjunctivitis,

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urticaria, and atopic dermatitis, and are promptly available as prescription and/or as over-the-counter drugs (Church, 2016). On the other hand, histamine H_2 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 H_1 and H_2 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 H_1 receptor couples to Gαq/11 leading to an increase in phosphoinositide metabolism, whereas histamine H_2 receptor couples to Gαs, 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 H_1 and H_2 receptor crossregulation. In native and recombinant systems, both receptors desensitize when cells are exposed to a sustained stimulus with histamine H_1 or H_2 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 H_1 or H_2 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 H_1 and H_2 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 H_2 receptor inverse agonists behave as full agonists regarding histamine H_2 receptor desensitization and internalization, in spite of diminishing cAMP production (Alonso et al., 2014, 2015). In the present work, we hypothesized that histamine H_1 and H_2 receptor inverse agonists also induce the crossdesensitization between histamine H_1 and H_2 receptors, and that the crossregulation induced by histamine H_1 and H_2 receptor agonists affects the behavior of histamine H_1 and H_2 receptor inverse agonists. In order to address this issue, we analyzed the receptor´s crossregulation in U937 cells, which endogenously express histamine H_1 and H_2 receptors and in cotransfected HEK293 cells. We utilise clinically relevant ligands as mepyramine, chlorpheniramine, triprolidine and diphenhydramine (first-generation histamine H_1 receptor inverse agonists), cetirizine (second-generation histamine H_1 inverse agonist) and the widely used histamine H_2 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 H_1 receptor agonist), bovine serum albumin (BSA), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides from *Escherichia Coli* (LPS), cimetidine, ranitidine

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hydrochloride, famotidine, cetirizine dihydrocloride, 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]cAMP, [^{3}H]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_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-$β_1$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_1 receptor (H_1R) and histamine H_2 receptor (H_2R) 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 [^{3}H]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-H$_1$R and pCEFLHA-H$_2$R. 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 (cyclooxigenase-2) forward 5′-

TTCAAATGAGATTGTGGGAAAATTGCT-3′ and reverse 5′-

AGATCATCTCTGCCTGAGTATCTT-3′, human IL-8 (interleukin-8) forward 5´-

CTGCGCCAACACAGAAATTA-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, 2×10^5 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 1×10^6 U937 cells/tube or 1×10^4 HEK293-H$_1$R, HEK293-H$_2$R or HEK293-H$_1$R-H$_2$R cells for 60 min at 4°C with increasing concentrations of [^{3}H]tiotidine or [^{3}H]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 μM amthamine stimuli decreased in a time- and concentration-dependent manner (Fig. 1A and B). Maximal desensitization was attained after 30 min and with 10 μM cetirizine. Besides, preexposure of U937 cells to mepyramine, chlorpheniramine, triprolidine, or diphenhydramine (10 μM; 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.

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To confirm that histamine H_1 receptor inverse agonists desensitize histamine H_2 receptor response through histamine H_1 receptor, we performed the same assay using HEK293 cells which only express histamine H_2 receptor. As shown in Fig. 1E, no crossdesensitization occurs in the absence of histamine H_1 receptor. As expected, when both receptors are coexpressed in HEK293 cells, pretreatment with histamine H_1 receptor inverse agonists also desensitized the histamine H_2 receptor response to amthamine (Fig. 1F).

These findings show that inverse agonists acting selectively through histamine H_1 receptor induce the crossdesensitization of histamine H_2 receptor response, both in U937 native and HEK293 recombinant systems.

3.2. Activation of histamine H_2 receptor affects histamine H_1 receptor ligands anti-inflammatory response

Our next question was whether the pharmacological action of histamine H_1 receptor inverse agonists may be affected by the activation of histamine H_2 receptor. Histamine, acting through histamine H_1 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 proinflamamtory cytokine IL-6, through histamine H_1 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-H_1R-H_2R cells (Fig. 2A). Although it may seem that mepyramine reduced IL-6 promoter's activity, no significant differences were observed

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when compared to basal levels. Remarkably, cotreatment with the histamine H_2 receptor agonist amthamine, prevented the decrease of IL-6 promoter´s activity induced by all of the histamine H_1 receptor inverse agonists tested. When this assay was performed in HEK293 cells expressing only histamine H_1 receptor, response to histamine H_1 receptor inverse agonists was not modified by amthamine (Fig. 2B), indicating that amthamine achieves the interference by specifically binding to histamine H_2 receptor.

Next, we assessed if a histamine H_2 receptor agonist is able to desensitize histamine H_1 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 H_1 receptor inverse agonists, mepyramine, cetirizine, chlorpheniramine, triprolidine, and diphenhydramine inhibited COX-2 and IL-8 gene expression. Interestingly, cotreatment with amthamine, significantly reverted the anti-inflammatory effect of histamine H_1 receptor inverse agonists (Fig. 2C and D).

3.3. Activation of histamine H_1 receptor affects histamine H_2 receptor response to inverse agonists

Given the existence of a crossdesensitization between histamine H_1 and H_2 receptors induced by both agonists (Alonso et al., 2013) and that the stimulus with an histamine H_2 receptor agonist affects the response to histamine H_1 receptor inverse agonists, we evaluated whether histamine H_1 receptor agonists could affect histamine H_2 receptor response to inverse agonists in U937 cells. As expected, treatment with cimetidine, ranitidine and famotidine, classified as histamine H_2 receptor inverse agonists, caused a reduction in cAMP levels (Fig. 3A). However, pretreatment with an histamine H_1

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receptor agonist (10 µM; 30 min) annulled the effect of histamine H_2 receptor inverse agonists, indicating that histamine H_1 receptor activation interferes with histamine H_2 receptor response to inverse agonists. We verified that pretreatment with the histamine H_1 receptor agonist decreased histamine H_2 receptor response to inverse agonists in cotransfected HEK293 cells expressing both receptors (Fig. 3B). Conversely, the reduction in cAMP levels induced by histamine H_2 receptor inverse agonists was not modified in cells which only express histamine H_2 receptor (Fig. 3C), showing the specificity of the histamine H_1 receptor agonist effect.

3.4. Histamine H_2 receptor inverse agonists influence histamine H_1 receptor response

With the aim of establishing whether these ligands may interfere with histamine H_1 receptor response, we evaluated the effect of cimetidine, ranitidine, and famotidine on the histamine H_1 receptor agonist-induced IL-6 promoter activity. The concentration used for histamine H_1 receptor agonist corresponded to the maximum IL-6 promoter activity (data not shown). Treatment with all of the histamine H_2 receptor inverse agonists assayed reduced the luciferase signal induced by stimulation with an histamine H_1 receptor agonist in HEK293-H_1R-H_2R cells (Fig. 4A), while no interference was detected in HEK293-H_1R cells (Fig. 4B). This interference was also observed when cells were pretreated with the histamine H_2 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 H_1 receptor agonist induced gene expression of COX-2 and IL-8, and treatment with histamine H_2 receptor inverse agonists annulled the pro-inflammatory response (Fig. 4C and D).

Taking in consideration that histamine H_1 receptor activation mediates antiproliferative and apoptotic effects in U937 cells (Alonso et al., 2013), we analyzed the impact of histamine H_2 receptor inverse agonists in the antiproliferative response to an histamine H_1 receptor agonist. For this, U937 cells were incubated with an histamine H_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_1 receptor was inhibited by treatment with histamine H_2 receptor inverse agonists (Fig. 5A and B). FACS studies showed that treatment with the histamine H_1 receptor agonist induced an arrest in the cell cycle, evidenced by an increment in the SubG0 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_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_2 receptor inverse agonists tested, cimetidine, ranitidine, or famotidine (Fig. 5D).

Altogether, these results highlight the importance of the crossregulation between histamine H_1 and H_2 receptor in the final response to histaminergic ligands.

3.5. Cointernalization of histamine H_1 and H_2 receptors is responsible for the crossregulation induced by inverse agonists

In previous studies from our laboratory, we reported that histamine H_1 and H_2 receptors are cointernalized upon histamine H_1 or H_2 receptor agonist stimulation in U937, CHO and HEK293 cells (Alonso et al., 2013). In addition, treatment with histamine H_2 receptor inverse agonist leads to histamine H_2 receptor internalization (Alonso et al.,

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2014). In order to evaluate a putative reciprocal internalization of histamine H_1 and H_2 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 H_1 receptor binding sites in U937 cell membranes (58% respect to untreated cells), but also decreased the number of histamine H_2 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 H_1 receptor by 43% and 31%, respectively (Fig. 6B). With the intention of evaluating the histamine H_2 receptor-binding specificity of the ligands necessary to induce cointernalization, saturation binding assays with [^{3}H]mepyramine were performed in transfected HEK293-H_1R-H_2R and HEK293-H_1R cells exposed to ranitidine and famotidine. HEK293-H_1R-H_2R cells showed similar results to those obtained with U937 cells; where ranitidine and famotidine led to a 29% and 32% decrease in histamine H_1 receptor binding sites, respectively (Fig. 6C). On the other hand, ranitidine and famotidine failed to stimulate histamine H_1 receptor internalization in HEK293-H_1R cells, although cetirizine, the histamine H_1 receptor ligand, internalized histamine H_1 receptor by 45% (Fig. 6D).

These findings reveal that inverse agonists induce receptor cointernalization as part of the crosstalk between histamine H_1 and H_2 receptors.

In an attempt to determine if the cointernalization of histamine H_1 and H_2 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_1 and H_2 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_1 receptor inverse agonists was able to impair histamine H_2 receptor agonist response in the presence of dynasore (Fig. 7A), which efficiently inhibited histamine H_1 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_2 receptor crossdesensitization (Fig. 7B). Next, the effect of dynasore on the crossdesensitization induced by histamine H_2 receptor inverse agonists on histamine H_1 receptor response was evaluated in HEK293T-H_1R-H_2R cells. Figure 7C shows that neither, cimetidine, ranitidine nor famotidine were able to reduce IL-6 promoter´s activity induced by the histamine H_1 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_1 and H_2 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_1 or H_2 receptors, 2) histamine H_1 and H_2 receptor inverse agonists crossdesensitize the receptor´s response to their

agonist, and 3) the crointernalization of histamine H_1 and H_2 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 H_1 and H_2 receptors (Sriram and Insel, 2018). Many of the histamine H_1 and H_2 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 H_1 and H_2 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 H_1 or H_2 receptor agonists also influences the response to inverse agonists. In this way, the activation of whichever of

these receptors modifies the response of histamine H_1 or H_2 receptor inverse agonists in different systems. Thus, the anti-inflammatory effect of histamine H_1 receptor inverse agonists evaluated by the reduction in IL-8 and COX-2 gene expression in monocytic U937 cells was less pronounced when histamine H_2 receptor was activated by amthamine (Fig. 2C and D). On the other hand, cAMP response triggered by incubation with the histamine H_2 receptor inverse agonist was prevented when U937 cells were pretreated with the histamine H_1 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 H_1 and H_2 receptor inverse agonists have different efficacies in different tissues. Therefore, when considering targeting histamine H_1 or H_2 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 H_1 and H_2 receptors are coupled to different G proteins and that their crossinterference induced by histamine H_1 and H_2 receptor inverse agonists depends on the cointernalization mechanism. To date, histamine H_1 and H_2 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 H_1 receptor agonist, alone or in combination with specific histamine H_2 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_1R-H_2R 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 H_1 receptor inverse agonists can induce the internalization of their own receptor. In line with these findings, cetirizine showed to induce histamine H_1 receptor internalization both, in cells that endogenously express the receptor (U937) and in a recombinant system (HEK293T-H_1R and HEK293T-H_1R-H_2R) (Fig. 6). Moreover, regarding the crossregulation between histamine H_1 and H_2 receptor, here we describe a new efficacy for several histamine H_1 and H_2 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 H_1 and H_2 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 H_1 and H_2 receptors are ubiquitously expressed. In this sense, novel clinical applications for histamine H_1 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 H_2 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).

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Likewise, histamine H_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_2 receptor antagonists/inverse agonists in heart failure pathologies rely, at least partially, on histamine H_1 and H_2 receptor crossdesensitization/cointernalization. Thus, histamine H_2 receptor antagonists/inverse agonists could also modulate the histamine H_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_2 receptor antagonists/inverse agonists in cardiac tissue.

This crosstalk may be responsible for the beneficial effects of histamine H_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_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_2 receptor led to upregulation on histamine H_2 receptor levels, sustained cointernalization of histamine H_1 receptor by ranitidine treatment may also lead to upregulation of histamine H_1

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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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Figure Legends:

Figure 1. Effect of histamine H_1 receptor inverse agonists on histamine H_2 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-H_2R (E) or HEK293-H_1R-H_2R cells (F) were exposed to 10 μM histamine H_1 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 H_2 receptor agonist on histamine H_1 receptor inverse agonists response. HEK293 cells cotransfected with IL-6-Luc, H_1R and H_2R (A) or IL-6-Luc and H_1R 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 H_2 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_1 receptor agonist on histamine H_2 receptor inverse

agonists response**. (A) U937, (B) HEK293-H_1R-H_2R, or (C) HEK293-H_2R cells were

exposed to 10 μM H_1 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_1 receptor agonist pretreatment. ns. not significant; #P < 0.05; ##P < 0.01;

###P < 0.001 respect to basal levels after histamine H_1 receptor agonist pretreatment.

**Figure 4. Effect of histamine H_2 receptor inverse agonists on histamine H_1 receptor

response.** (A-B) HEK293 cells cotransfected with IL-6-Luc, H_1R and H_2R (A) or IL-6-

Luc and H_1R coding constructs (B), were incubated for 10 min with 10 μM cimetidine

(CIM), ranitidine (RAN), famotidine (FAM) or amthamine (histamine H_2 receptor

agonist), and then treated with 10 μM histamine H_1 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

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to H_1 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 H_1 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 H_1

receptor agonist response, ***P < 0.001.

Figure 5. Effect of histamine H_2 receptor inverse agonists on the inhibition of cell

proliferation and apoptosis induced by histamine H_1 receptor agonist. U937 cells

were treated with 10 μM histamine H_1 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 H_1 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 H_1 and H_2 receptors induced by histamine

H_1 and H_2 receptor inverse agonists. (A) [^{3}H]Tiotidine or [^{3}H]mepyramine saturation

assays were performed in U937 cells: control (●) or treated for 90 min with 10 μM

cetirizine (CET) (■). (B) [^{3}H]Mepyramine saturation assays were performed in U937

cells: control (●) or treated for 90 min with 10 μM ranitidine (RAN) (□) or 10 μM

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famotidine (FAM) (○). (C-D) [^{3}H]Mepyramine saturation assays were performed in

HEK293-H$_1$R-H$_2$R (C) or HEK293-H$_1$R (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 H$_1$ and H$_2$ 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 H$_1$ 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 H$_2$ receptor agonist response without pretreatment. ns. not

significant. (C) HEK293 cells cotransfected with IL-6-Luc, H$_1$R and H$_2$R 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 H$_1$ receptor agonist for 6 h.

Luciferase activity was determined as detailed in Materials and Methods. Data represent
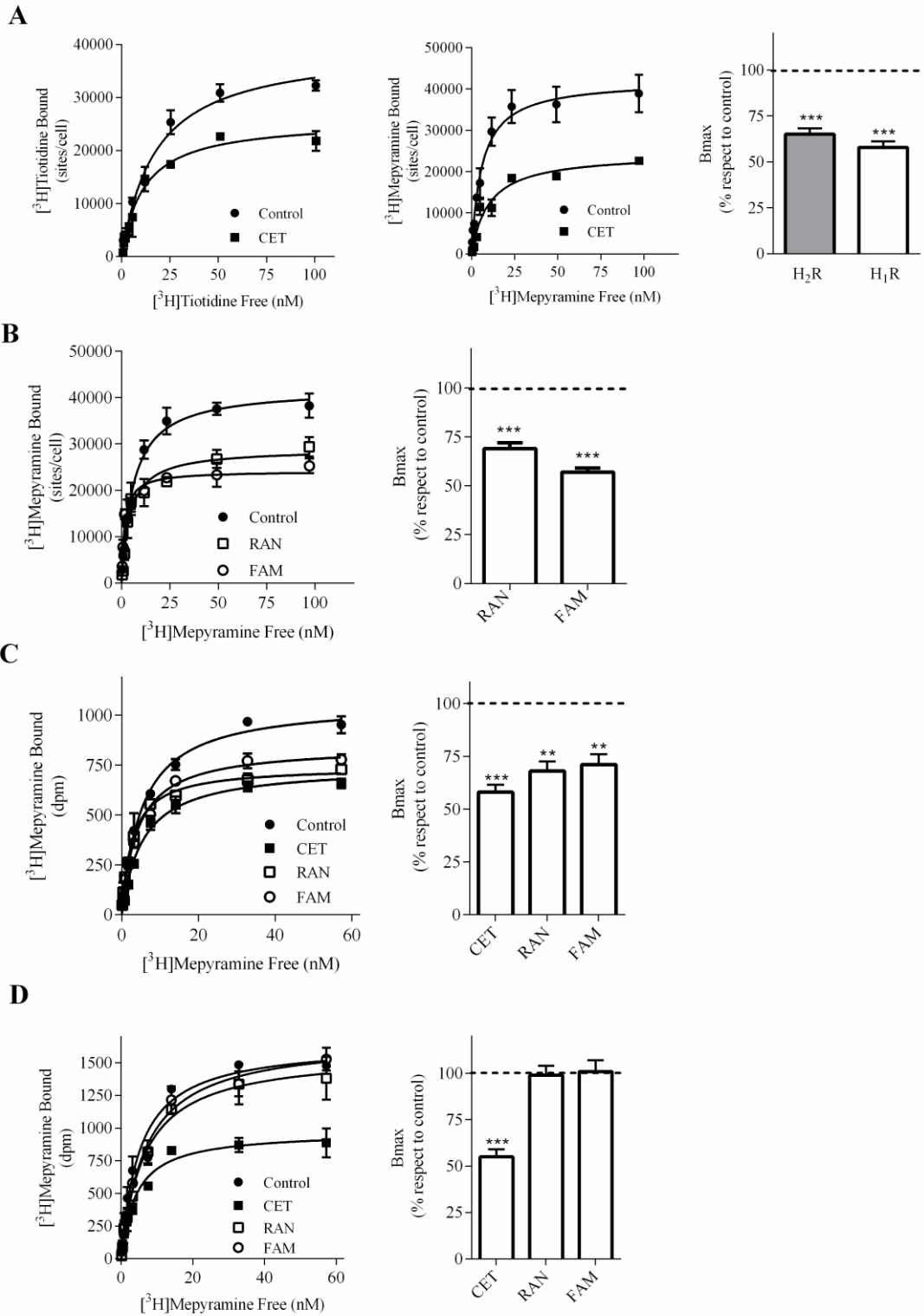
mean ± S.E.M. (n = 3). ns. not significant; #P < 0.05; ##P < 0.01; ###P < 0.001 respect to

histamine H$_1$ receptor agonist response.

33

A

B

C

D

A

B

C
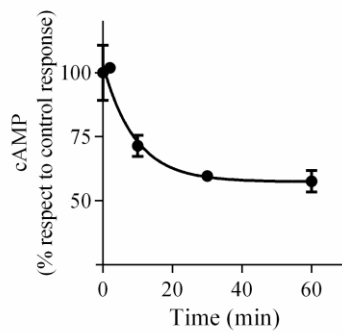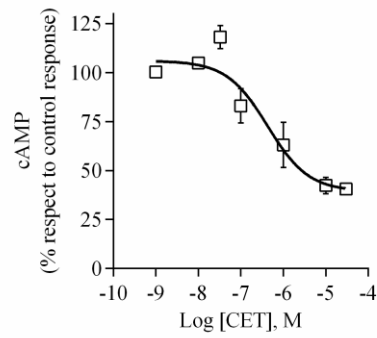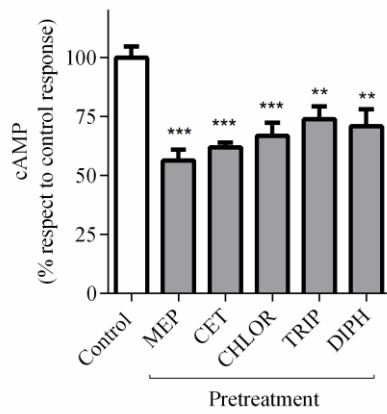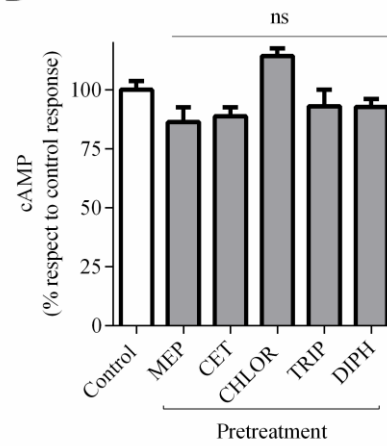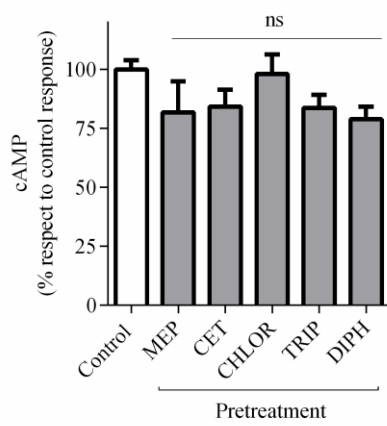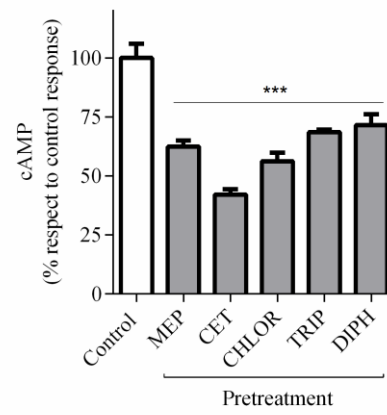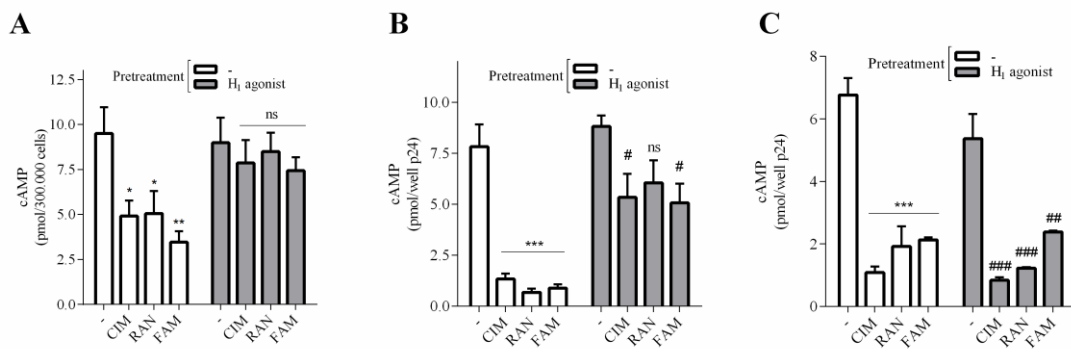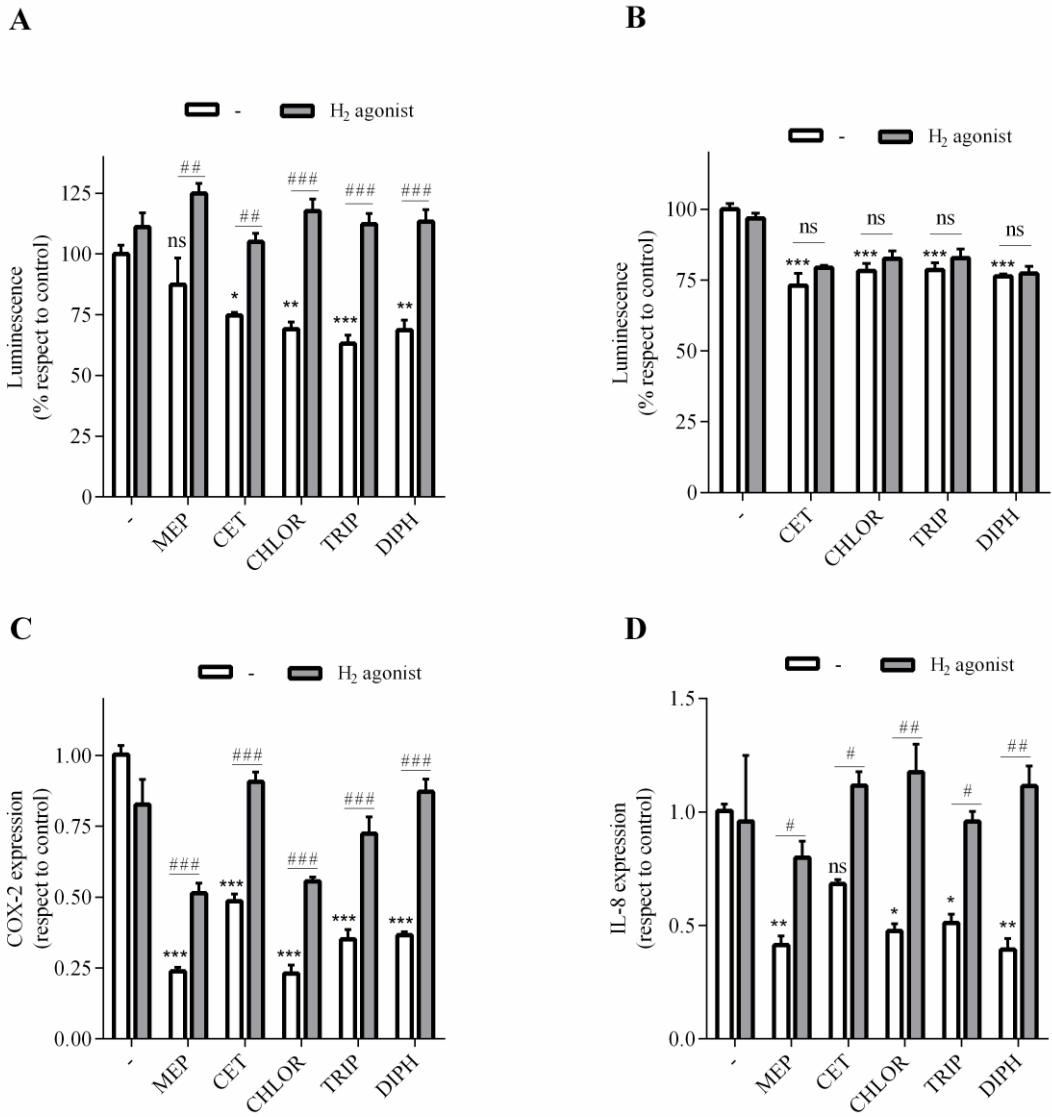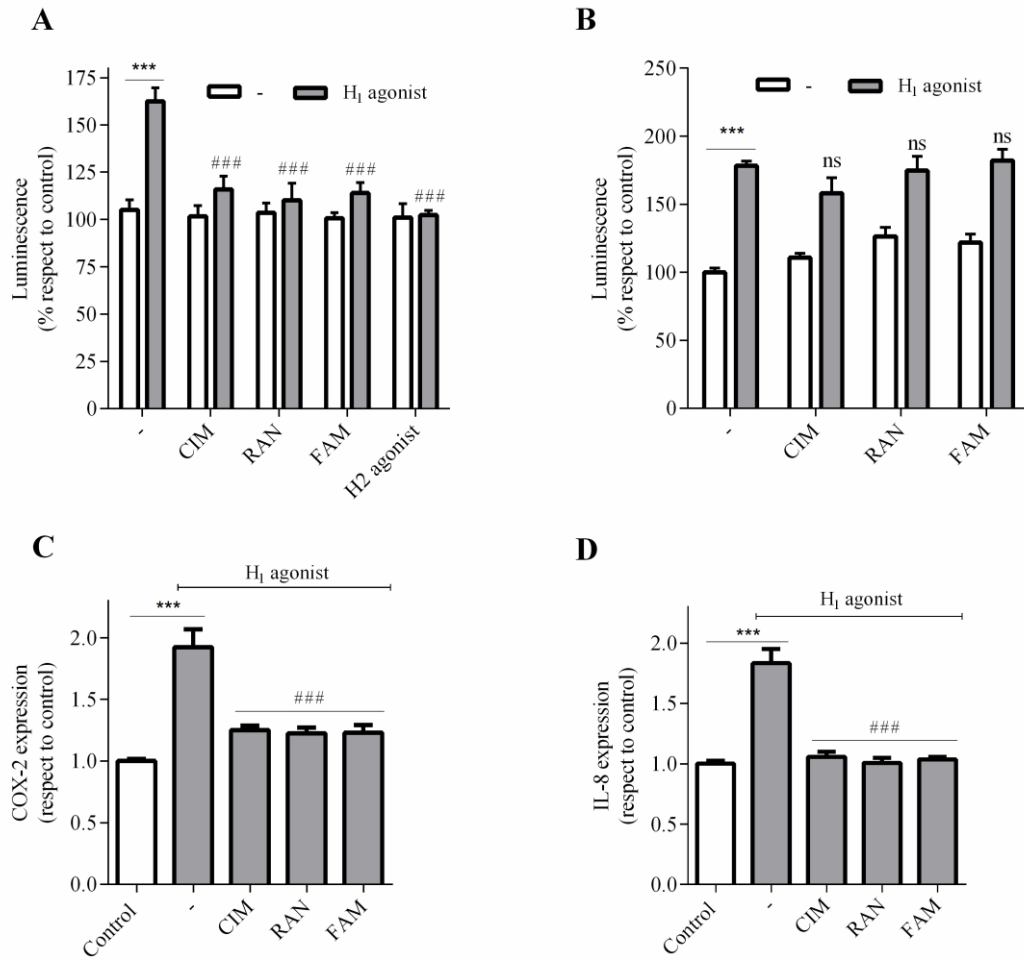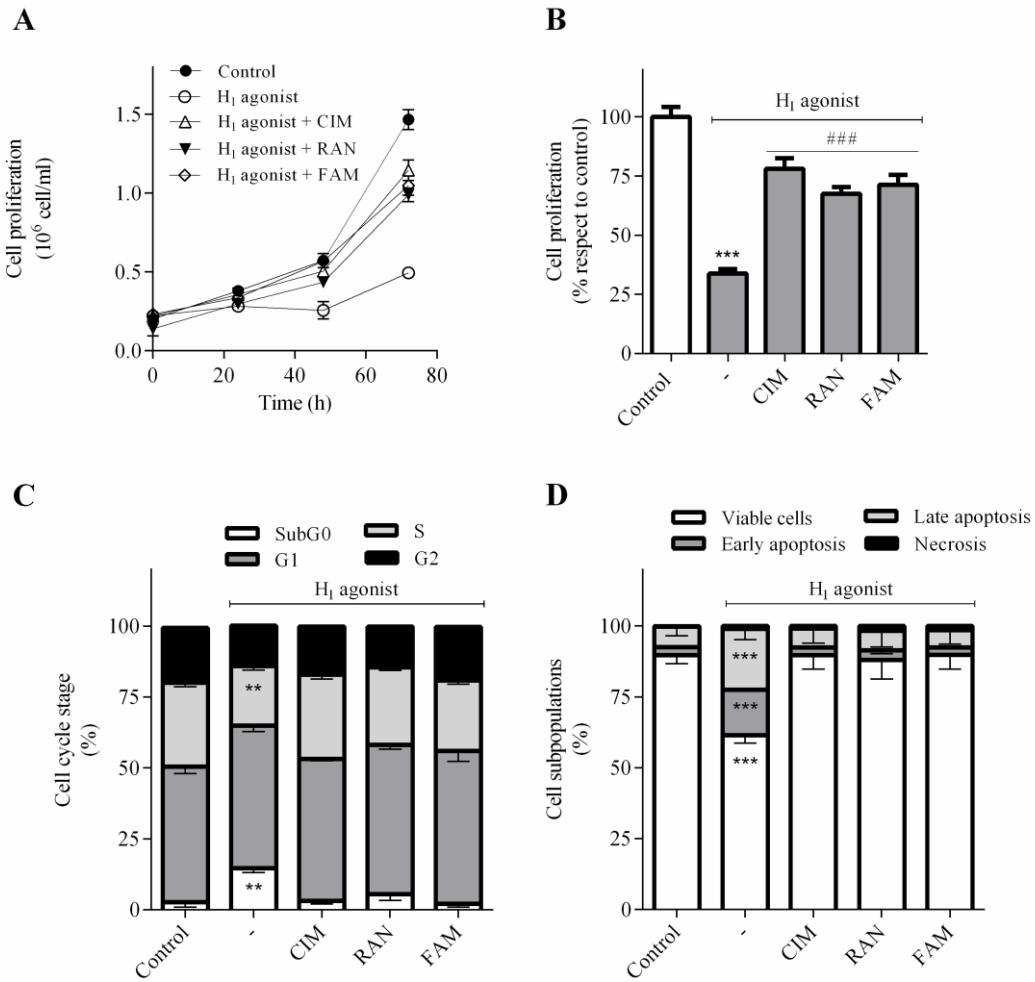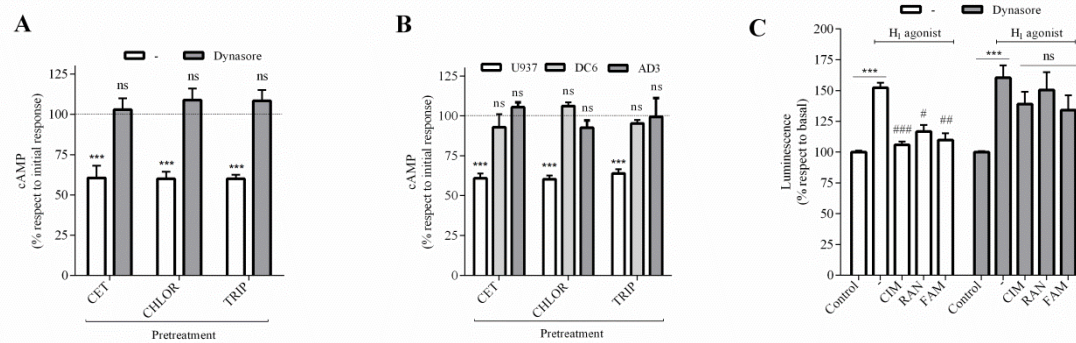
Author Agreement

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