Effects of rupatadine on Platelet activating factor (PAF)-induced human mast cell degranulation compared with desloratadine and levocetirizine (The MASPAF study)

R Muñoz-Cano, MD, PhD;[1,2] E Ainsua-Enrich, PhD;[3] I Torres-Atencio, PhD;[4] M Martin; PhD;[3] J Sánchez-Lopez, MD, PhD;[1,2] J Bartra, MD, PhD;[1,2] C Picado, MD, PhD[1,2,5]; J Mullol, MD,PhD;[2,5,6 *], A Valero, MD, PhD;[1,2,5 *]

(*), Both authors had similar senior responsibilities

Running title: Rupatadine inhibits PAF-induced mast cell degranulation

(1) Unitat d'Al·lèrgia. Servei de Pneumologia i Al·lèrgia Respiratòria. Hospital Clínic. Universitat de Barcelona. Barcelona, Catalonia, Spain.
(2) Immunoal·lèrgia Respiratòria Clínica i Experimental. Institut d'Investigacions Biomèdiques August Pi iSunyer (IDIBAPS). Barcelona, Catalonia, Spain.
(3) Biochemistry Unit, School of Medicine, Universitat de Barcelona. Barcelona, Catalonia, Spain.
(4) Pharmacology Department, School of Medicine, Universidad de Panamá. Panamá.
(5) Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES). Barcelona, Catalonia, Spain.
(6) Unitat de Rinologia i Clínica de l'Olfacte, Servei d'Oto-rino-laringologia, Hospital Clínic, Universitat de Barcelona. Barcelona, Catalonia, Spain.

Corresponding author:
Rosa Muñoz-Cano, MD, PhD
Unitat d'Al·lèrgia. Servei de Pneumologia i Al·lèrgia Respiratòria
Hospital Clínic, IDIBAPS
Villarroel 170
Barcelona 08036
Catalonia, Spain
Telf.+34932275540
FAX.+34932275455
Email: rmunoz@clinic.ub.es

Funding

This study has been supported by a Sociedad Española de Alergia e Immunología Clínica (SEAIC) grant, Ministerio de Economia y Competitividad-Carlos III Health Institute (FIS-PI09/1202), Spain and Grupo Uriach S.A., Barcelona, Spain. R.M-C. was the recipient of the "Rio Hortega" Fellowhip, Ministerio de Economia y Competitividad, Carlos III Health Institute, Spain.

Acknowledgments: We thank Maria Perez for technical support.

Conflict of interest: JM is or has been member of national and international scientific advisory Boards (consulting), received fees for lectures, and grants for research projects from ALK-Abelló, Boheringer-Ingelheim, Crucell, Esteve, FAES, GSK, Hartington Pharmaceuticals, Johnson & Johnson, MEDA Pharma, MSD, Novartis, Pierre Fabre, Sanofi-Aventis, Schering Plough, UCB, and Uriach Group. AV is or has been member of national scientific advisory Boards (consulting), received fees for lectures, and grants for research projects from Boheringer-Ingelheim, Esteve, FAES, GSK, Chiesi, MEDA Pharma, MSD, Novartis, Leti, Stallergenes, Sanofi-Aventis, Orion Pharma, Vifor, UCB, and Uriach Group. CP has been or is member of national and international scientific advisory boards (consulting), received fees lectures, and grants for research projects from Esteve, FAES, MSD, Novartis, Chiesi, Grupo Uriach, and GSK. JB is or has been member of national scientific advisory Boards (consulting), received fees for lectures from FAES, UCB, Grupo Uriach and Hall Allergy. RM-C, EA-E, IT-A, MM, JS declare no conflict of interest.

Meeting/Conferences: Part of this work has been presented as a poster at the Annual Meeting of the American Academy of Asthma, Allergy and Immunology (AAAAI) in New Orleans, February 2012.

Abstract

Rationale. Platelet activating factor (PAF) is a lipid mediator involved in the pathophysiology of several allergic diseases, such as the amplification of mast cell (MC) activation in anaphylaxis. Rupatadine is an antihistamine with demonstrated anti-PAF effect but its capacity to inhibit PAF-induced MC degranulation has not been fully evaluated.

Objective. To investigate the ability of rupatadine to inhibit PAF-induced MC degranulation compared with desloratadine and levocetirizine, to confirm dual rupatadine anti-H_1 and anti-PAF activities.

Methods. The human MC line LAD2 and primary MC (human lung tissue (hLMC)) were used. MC mediator release was evaluated by β-hexosaminidase and histamine release assay. Rupatadine (H_1 antagonist + PAF receptor antagonist), desloratadine and levocetirizine (H_1 antagonists) effects were compared in LAD2 and hLMC. Selective PAF receptor antagonists WEB2086, BN52021, and CV6209 were also tested. PAF receptor protein expression was evaluated in both LAD2 and hLMC.

Results. PAF receptor expression was confirmed in both LAD2 and hLMC. Among anti-PAF inhibitors, CV6209 and rupatadine inhibited PAF-induced MC degranulation in both LAD2 and hLMC. In LAD2, Rupatadine (5 and 10 μM) and levocetirizine (5 μM), but not desloratadine, inhibited PAF-induced β-hexosaminidase release. Rupatadine (1 -10 μM), levocetirizine (1-10 μM), and desloratadine (10 μM) inhibited PAF-induced histamine release. Rupatadine at 10 μM, but neither levocetirizine nor desloratadine, showed an inhibitory effect in hLMC degranulation.

Conclusions. This study shows that rupatadine and, to a lesser extent, levocetirizine, but not desloratadine, inhibit PAF-induced degranulation in both LAD2 and human lungMC. These findings support the dual anti-H_1 and anti-PAF effects of rupatadine in allergic disorders.

Key words: CV6209, desloratadine, LAD2, levocetirizine, mast cell, Platelet-activating Factor, rupatadine.

Resumen

Introducción. El factor de activación plaquetario (PAF) es un mediador lipídico que ha sido involucrado en la fisiopatología de diversas enfermedades alérgica, como la amplificación de la activación de los mastocitos (MC) en la anafilaxia. Rupatadina es un antihistamínico que ha demostrado también un efecto anti-PAF, pero no ha sido elucidada su capacidad para inhibir la degranulación mastocitaria inducida por PAF.
Objetivo. Evaluar la capacidad de rupatadina para inhibir la degranulación de los MC inducida por PAF en comparación con desloratadina y levocetirizina, con el objetivo de confirmar el efecto dual anti-H1 y anti-PAF de rupatadina.
Métodos. Para este estudio se utilizaron la línea celular de mastocitos humanos LAD2 y mastocitos primarios (mastocitos pulmonares (MP)). Los mediadores mastocitarios se midieron utilizando las pruebas de liberación de β-hexosaminidasa e histamina. Los efectos de rupatadina (antagonista H1 + antagonista del receptor del PAF), desloratadina y levocetirizina (antagonista H1) se compararon en LAD2 y MP. También se probaron los antagonistas selectivos del receptor del PAF WEB2086, BN52021, y CV6209. La expresión proteica del receptor del PAF fue evaluada tanto en LAD2 como en MP.
Resultados. La expresión del receptor del PAF fue confirmada en LAD2 y MP. De los inhibidores anti-PAF, CV6209 y rupatadina inhibieron la degranulación mastocitaria inducida por PAF, tanto en LAD2 como en MP. En LAD2, rupatadina (5 y 10 µM) y levocetirizina (5 µM), pero no desloratadina, inhibieron la liberación de β-hexosaminidasa inducida por PAF. Rupatadina (1 -10 µM), levocetirizina (1-10 µM), y desloratadina (10 µM) inhibieron la liberación de histamina inducida por PAF. Rupatadina a 10 µM, pero ni levocetirizina ni desloratadina, demostraron efecto inhibitorio alguno sobre la degranulación inducida en MP.
Conclusiones. Este estudio demuestra que rupatadina, y en menor medida levocetirizina, pero no desloratadina, es capaz de inhibir la degranulación inducida por PAF en LAD2 y mastocitos pulmonares. Estos hallazgos apoyan el efecto dual anti-H1 y anti-PAF de rupatadina para su uso en las enfermedades alérgicas. Palabras clave: CV6209, desloratadina, Factor de Activación de Plaquetas, LAD2, levocetirizina, mastocitos, rupatadina.

Introduction

Platelet-activating factor (PAF) is a lipid mediator released by many cells including mast cells (MC) and eosinophils[1] which are implicated in several allergic diseases such as asthma, allergic rhinitis, and anaphylaxis[2–4]. There is evidence that PAF can activate human lung mast cells (hLMC) but no skin MC, through PAF receptor and phospholipase C (PLC) γ1 and β2[5]. These findings provide a plausible mechanism whereby PAF mediates an amplification loop for MC activation in the generation of the allergic response.

The role of PAF in asthma has been well established, and several studies have demonstrated that it is associated with bronchoconstriction and bronchial hyperreactivity [6, 7]. Furthermore, PAF has been proposed to be the most potent inductor of vascular permeability among the mediators involved in nasal inflammation, with a principal role in rhinorrhea and nasal congestion [3, 8–11]. Finally, PAF levels and, more interestingly, PAF acetylhydrolase activity have been related to anaphylaxis severity[4, 12].

PAF receptor expression has been previously described in human MC. PAF receptor has also been found in hLMC, but not in skin MC[5]. No studies have been performed so far in the human MC line LAD2. PAF-induced mast cell degranulation is partially dependent on extracellular calcium as it has been demonstrated in human peripheral blood-derived mast cells[5] and in the human leukemia mast cell line HMC-1[13].

Despite the significant relevance of PAF in the pathophysiology,of allergic diseases the studies so far reported with PAF antagonist have failed to show any definitive and positive effect in bronchial symptoms[2, 14]. Moreover, there are no studies with selective PAF antagonists in human rhinitis or anaphylaxis. Lack of evidence of clinical efficacy of PAF antagonists probably accounts for their absence in the current

armamentarium of allergic airway disease and their use restricted to research purposes.

Rupatadine is a H_1 receptor antagonist indicated in the treatment of allergic rhinitis[15] and urticaria[16]. Rupatadine has the properties of an ideal second generation antihistamine including antiallergic/antiinflammatory effects such as cytokine secretion inhibition[8, 17]. In addition, rupatadine has shown a PAF receptor antagonism effect demonstrated in *in vitro* studies and in a wide series of *in vivo* pharmacological studies using animal models and humans[18]. More recently, the anti-PAF clinical effect of rupatadine has been demonstrated using an *in vivo* human nasal provocation test with PAF [9, 10]. However, the mechanism of action of rupatadine anti-PAF effect has not been fully evaluated in human MC[17, 19].

The objective of the MASPAF study was to investigate the rupatadine capability to inhibit PAF-induced MC degranulation. To confirm its anti-H1 and anti-PAF dual effect, rupatadine was compared with selective anti-PAF antagonists and second generation anti-H_1 (desloratadine and levocetirizine) with unknown PAF antagonist activity.

doi: 10.18176/jiaci.0117

Material and Methods

Reagents

PAF, desloratadine, WEB2086, and BN52021 were purchased from Sigma-Aldrich (Madrid, Spain) while CV6209 was from Enzo Life Sciences (Madrid, Spain). Rupatadine and levocetirizine were provided by Grupo Uriach S.A. (Barcelona, Spain).

Mast cell cultures

Mast cell lines. The LAD2 human mast cell line, provided by A. Kirshenbaum and D.D. Metcalfe (National Institutes of Health, Bethesda, MD, USA), was grown in StemPro-34 serum-free medium (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with StemPro-34 Nutrient and with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), and 100 ng/mL recombinant stem cell factor (Preprotech, Rocky Hill, NJ, USA)[10]. Cultures were kept at 37ºC in 5% CO_2 in a humidified incubator.

Primary human lung mast cells (hLMC). Healthy lung fragments confirmed by anatomic pathology examination and obtained during elective surgery were selected. hLMC were dispersed from chopped lung specimens by means of an enzymatic procedure and purified by using magnetic bead affinity selection with anti-CD117 antibody (MiltenyBiotec, Madrid, Spain) as described previously[20]. Mast cell purity, assessed by metachromatic staining, was greater than 98%. Cells were kept in culture in Dulbecco's Modified Eagle Medium (DMEM) (LifeTechnologies, Madrid, Spain) supplemented with FBS 10%, 100 ng/mL recombinant stem cell factor, IL-10 (1 ng/mL) and IL-6 (0.5 ng/mL) (Preprotech, Rocky Hill, NJ, USA) for up to 5 days.

Study Design

Most of the experiments were performed in LAD2 due to the limited availability of hLMC. Desloratadine and levocetirizine, antihistamines with no demonstrated anti-PAF effect, have been used in order to evaluate rupatadine differential anti-PAF

doi: 10.18176/jiaci.0117

effect. Conversely, CV6209, WEB2086 and BN52021 are PAF antagonists with demonstrated inhibitory effect [5,21,22].

Mast cell degranulation was evaluated using both β-hexosaminidase and histamine assays in LAD2 cells. First, time-course and dose-response assays were performed in order to determine the optimal dose and incubation time. PAF was tested at 1 μM for 15 and 30 minutes and at 10 μM for 5, 15 and 30 minutes. Second, mast cells were pre-incubated with antihistamines or anti-PAF for 30 minutes and then triggered with PAF 10 μM for 30 minutes (optimal dose and incubation time) for the degranulation assays. Rupatadine (1 μM, 5 μM, 10 μM), desloratadine (1 μM, 5 μM, 10 μM), levocetirizine (1 μM, 5 μM, 10 μM), CV6209 (0.2 μM and 2 μM), WEB2086 (1 μM, 10 μM and 100 μM) and BN52021 (1 μM, 10 μM and 100 μM) were tested. In order to evaluate the relationship between PAF-induced mast cell degranulation and calcium mobilization, several doses of PAF were tested (1μM, 10μM and 50 μM) in LAD-2 calcium assays.

Mast cell degranulation was evaluated in hLMC using histamine assays only. MC were pre-incubated with antihistamines or anti-PAF for 30 minutes and then triggered with PAF 10 μM for 30 minutes. Rupatadine, desloratadine and levocetirizine were tested at 10 μM because it was the only concentration inhibiting PAF-induced histamine release in LAD2. CV6209 was tested at 2 μM and was the only anti-PAF antagonists used for these experiments due to the lack of inhibitory effect showed by BN52021 and WEB2086 in LAD-2 (data not shown).

PAF receptor expression was evaluated in mast cells from both LAD2 and hLMC.

Study outcomes

***Trypan Blue Exclusion Test of Cell Viability*.** The dye exclusion test was used to determine the number of viable cells after PAF and antagonists (levocetirizine, rupatadine, desloratadine, CV6209, WEB2086, and BN52021) stimulation (all concentrations used in the experiments were tested). The technique is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not [23].

β-hexosaminidase release assay. B-Hexosaminidase was quantified in the supernatant by release of 4-p-nitrophenol from p-nitrophenil N-acetyl-beta-D-glucosaminide (1.3mg/ml) in a sodium citrate buffer (pH 4.5). Reaction was stopped by addition of sodium carbonate buffer 0.2 M (pH 10.7). 4-P-nitrophenol release was quantified by absorbance at 405nm. The percentage of β-hexosaminidase release was calculated as follows: β-Hexosaminidase release rate (%) = [(samples release minus spontaneous release) / (maximum release minus spontaneous release)] x 100.

Calcium mobilization. Calcium mobilization in LAD2 cells was followed by fluorimetric analysis of free calcium with Fluo-4-AM fluorescent dye (Molecular Probes, Invitrogen). A total of $0.2x10^6$ cells/point were loaded with 5 mM Fluo-4-AM for 30 min at 4ºC in the dark, washed twice with Tyrode's buffer, and resuspended. Fluorimetric measurements were done in a Modulus II Microplate Multimode Reader, Turner Bio- systems (Promega, San Luis Obispo, CA, USA), according to the manufacturer's instructions.

Enzyme-linked immunoabsorbent assay (ELISA) for histamine. Mast cell degranulation was also monitored by measuring the histamine released into the extracellular fluid. A commercial ELISA kit (Immunotech, Beckman Coulter Co., Germany) was used according to the manufacturer's instructions. The net percentage

of histamine release was calculated from the ratio of each sample, with spontaneous release subtracted to total histamine.

PAF receptor expression by Western Blot. Total mast cell lysates from $1x10^6$ cells for LAD2 and $0.5x10^6$ cells for hLMC were separated by SDS-PAGE (10%) and electrotransferred to polyvinylidenedifluoride membranes (Millipore, Bedford, MA, USA). Blot was probed with an antibody against PAF receptor (Vitro, Madrid, Spain). Blotted membranes were visualized with enhanced chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

Data are shown as a mean ± standard deviation (SD). Comparison between groups was performed using a non-parametric test (Mann-Whitney U test with Bonferroni-Holm correction). P value is adjusted depending on the number of comparisons (k). Therefore, a p value was considered statistically significant when was equal or below (0.05/k).

Results

PAF and antagonists toxicity

Only rupatadine and desloratadine 100 µM induced a moderate increase in mast cell mortality compared to diluent (DMSO 1%)(data not shown).

PAF receptor expression

PAF receptor expression (48 KDa band) was described in LAD2 for the first time and corroborated in hLMC[5] (Figure 1A).

PAF time-course and dose-response in LAD2 activation

PAF induced a dose- and time-dependent cell activation in LAD2. Although PAF induced a significant β-hexosaminidase release with both 1 and 10µM at both 15 and 30 minutes, we chose the concentration (10µM) and time (30 minutes) associated with the induced maximum mast cell release (33.5%), as the experimental positive control (Figure 1B).

PAF-induced calcium mobilization in LAD2

A PAF dose-dependent (10-50µM) response was observed. Differences between 50µM and 10µM were not statistically significant. PAF 1µM did not induce calcium influx nor did negative control (Figure 1C).

Effect of PAF antagonists on PAF-induced mast cell degranulation

In LAD2, CV6209 inhibited PAF-induced β-hexosaminidase release at 0.2 µM (45% $p<0.01$) and 2 µM (56% $p<0.001$) (Figure 2A) and histamine release at 2 µM (30% $p<0.01$) (Figure 2B). WEB2086 and BN52021 did not inhibit PAF-induced MC release neither in LAD2 nor in hLMC (data not shown).

In hLMC, CV6209 at 2 µM inhibited PAF-induced histamine release (24% $p<0.0\text{~~0~~}1$) (Figure 2C).

Effect of rupatadine, levocetirizine, and desloratadine on PAF-induced mast cell degranulation

β-hexosaminidase release in LAD2. Only Rupatadine 10 µM (48%) significantly ($p<0.01$) inhibited PAF-induced β-hexosaminidase release. No statistical differences were found with Rupatadine 5 µM (30%), 25 µM (34%) and 100 µM (no inhibition) and levocetirizine from 1 to 100 µM (Figure 3A and 3B, respectively). Desloratadine did not show any inhibitory effect at the tested concentrations (Figure 3C).

Histamine release in LAD2. Rupatadine 5 µM (29%) and 10µM (30%) and levocetirizine 5 µM (25%), 10 µM (27%) and 25 µM (25%) significantly ($p<0.01$) inhibited PAF-induced histamine release (Figure 4A and 4B). Desloratadine did not significantly inhibited histamine release at any of the concentrations tested (Figure 4C).

Histamine release in hLMC. Rupatadine 10 µM (12%) significantly ($p<0.01$) inhibited PAF-induced histamine release but this effect was not observed with either desloratadine (2%) or levocetirizine (4%) (Figure 5).

DISCUSSION

LAD2 is one of the three human well established MC lines and usually serves as replacements of mature tissue MC[24]. PAF receptor expression has been demonstrated in several cell types, including platelets, eosinophils and hLMC [25–27], but never in LAD2. In our study, we have demonstrated for the first time the existence of PAF receptor in LAD2. We also corroborated that the PAF receptor is present in hLMC[5].

In the MASPAF study we have demonstrated that rupatadine and, to lesser extent, levocetirizine inhibited PAF-induced human MC degranulation while suggesting that this effect could be mediated through PAF receptor. Desloratadine, an antihistamine without anti-PAF effect, failed to show any consistent inhibitory effect.

In addition to mast cell degranulation, this study has also confirmed the ability of PAF to mobilize intracellular calcium as previously demonstrated by other authors [5, 13]. This evidence and the inhibitory effect demonstrated by PAF specific antagonists such as CV6209 suggest that PAF effect in our model may be mediated by PAF receptor activation.

Recently, Kajiwara et al. have demonstrated the activation of human MC through PAF receptor[5], reaching a 20-30% of total histamine release in hLMC at a concentration as low as 1 nM[5]. The concentration used in our study was higher (10 µM) than the one used in the Kajiwara study [5], probably due to differences in the cell types used (LAD2 and hLMC versus MC derived from peripheral blood-derived progenitors) and the high inter-individual sensitivity in response to PAF stimulation observed in hLMC. Alevizos et al. [19] published a study using LAD2 stimulated with PAF that shows similarities and discrepancies with our study. Although histamine release was induced with a PAF concentration as low as 0.01µM, β-hexosaminidase release could not be induced at that dose. In our study, PAF 10µM induced both histamine and β-

hexosaminidase release without any impact on cell viability. Moreover, we could not demonstrated calcium mobilization with PAF concentrations below 10μM, suggesting that lower concentrations of PAF would not activate the PAF receptor in LAD2.

In our study, CV6209 inhibited PAF-induced histamine and β-hexosaminidase release in both LAD2 and hLMC. However, PAF antagonists BN52021 and WEB2086 did not shown any inhibitory effect (data not shown). Although several studies have compared rupatadine with WEB2086 and BN52021, demonstrating a greater effect of these PAF antagonists in PAF-induced mortality in mice or platelet aggregation in rabbits and human samples[18], their effect in mast cells has never been evaluated. On the other hand, it has been demonstrated that CV6209 inhibits PAF-induced histamine release in human peripheral blood-derived mast cells, but it has never been compared to rupatadine[5]. Our results showed that rupatadine inhibitory effect was comparable to CV6209 in LAD2, although CV6209 effect was greater compared to rupatadine in hLMC.

In the present study, we have demonstrated that rupatadine consistently inhibited PAF-induced degranulation, blocking both histamine and β-hexosaminidase release, in both LAD2 and hLMC.

In one of the few studies investigating the role of rupatadine in MC, Alevizos et al. compared the effect of rupatadine and diphenhydramine in PAF-induced histamine and cytokine release in LAD2[19]. The discrepancies in rupatadine effect observed in Alevizos and our study could be related to differences in the concentrations used for PAF. We used a significantly higher dose of PAF (10 μM) as a degranulation trigger, and found that rupatadine is capable of inhibiting PAF-induced degranulation in a range that goes from 1 μM to 10 μM. Surprisingly, even though using a concentration of PAF 10x lower, Alvezios could show that only rupatadine at 25 μM had a significant inhibitory effect. Furthermore, both H1 receptor antagonists diphenhydramine in Alvezios study and desloratadine in ours, did not show any inhibitory effect in

doi: 10.18176/jiaci.0117

histamine or cytokine production induced by PAF, suggesting a lack of anti-PAF effect[19]. Other previous studies performed with rupatadine in human MC, specifically in cord blood MC, have shown that rupatadine 25μM inhibited IgE-mediated TNF-α and cytokine (IL-6, IL-8, IL-10, and IL-13) release[17]. In HMC-1 and LAD2, rupatadine 10μM to 50μM inhibited cytokine release induced by substance P without any impact on cell viability at any concentration[17].

The present study has also shown that desloratadine has no inhibitory effect and levocetirizine inhibited PAF-induced degranulation of LAD2 at some concentrations but not in hLMC. However, Weller et al. demonstrated that desloratadine 1μM was able to inhibit histamine release in isolated human skin MC[28]. Queralt M et al. reported a similar effect of loratadine and rupatadine in canine mast cells and HMC-1, after stimulation with the calcium ionophore-A23187, Con A and anti-IgE. However, in our study desloratadine, a metabolite of loratadine, has shown a no inhibitory effect when stimulating with PAF in LAD2 and hLMC [29]. The different mast cell phenotype observed in human skin and human lung[30, 31] as well as the different trigger used (anti-IgE, calcium ionophore, Con A and substance P)[28] could account for this discrepancy[32]. Although, desloratadine, a second generation antihistamine, has demonstrated to have some antiallergic effects[33], and to be up to 10 times more potent than loratadine in antagonizing histamine-induced increases in nasal microvascular permeability[34], in the light of our findings it lacks anti-PAF effect.

As far as we know, no studies with levocetirizine in MC have been ever published. As a second generation antihistamine, some anti-allergic effects have also been reported[35] but none of them related to PAF. Although to a lesser extent than rupatadine, levocetirizine inhibited PAF-mediated LAD2 degranulation (mainly histamine release at several concentrations) a finding that suggests it also has a potential anti-PAF activity in LAD2 but not in primary hLMC.

doi: 10.18176/jiaci.0117

It is of interest to note that the concentration of antihistamine necessary to decrease mast cell activation *in vitro* conditions in our study may be higher than *in vivo*. Single cell models are just partially representative of what is happening in a whole organism (a human being) and the role of metabolic pathways present in the *in vivo* model is neglected *in vitro* (cell-based) study. Furthermore, cell culture environment does not exactly reproduce the *in vivo* conditions. Therefore, our study must be considered as an *in vitro* model to investigate the drug effect on mast cell activation/mediator release but the concentrations used are not useful as a reference for the doses to be used for patient's treatment (an *in vivo* search of dose is always mandatory).

Finally, the strongest inhibitory effect of rupatadine in PAF-induced mast cell degranulation from both LAD2 and hLMC clearly correlates with its clinical effect in allergic rhinitis patients[10], where rupatadine but not levocetirizine showed an inhibitory effect of total nasal symptoms score.

In conclusion, we have demonstrated that rupatadine and, to a lesser extent, levocetirizine have anti-PAF activity in human MC. The dual anti-H1 and anti-PAF activity of rupatadine may provide to rupatadine a greater anti-inflammatory / anti-allergic activity, already reported in previous studies[10].

References

1. Spina D, Coyle AJ, Page CP. The role of platelet activating factor in allergic inflammation. *Pulm. Pharmacol.* 1989; 2: 13–19.

2. Kuitert LM, Angus RM, Barnes NC, Barnes PJ, Bone MF, Chung KF, Fairfax AJ, Higenbotham TW, O'Connor BJ, Piotrowska B, Rozniecki J, Uden S, Walters EH, Willard CJ. Effect of a Novel Potent Platelet-activating Factor Antagonist Modipafant, in Clinical Asthma. *Am. J. Respir. Crit. Care Med.* 1995; 151: 1331–1335.

3. Mullol J, Bousquet J, Bachert C, Canonica WG, Gimenez-Arnau A, Kowalski ML, Martí-Guadaño E, Maurer M, Picado C, Scadding G, Van Cauwenberge P. Rupatadine in allergic rhinitis and chronic urticaria. Allergy 2008. p. 5–28.

4. Vadas P, Gold M, Perelman B, Liss GM, Lack G, Blyth T, Simons FER, Simons KJ, Cass D, Yeung J. Platelet-activating factor, PAF acetylhydrolase, and severe anaphylaxis. *N Engl J Med* 2008/01/04 ed. 2008; 358: 28–35.

5. Kajiwara N, Sasaki T, Bradding P, Cruse G, Sagara H, Ohmori K, Saito H, Ra C, Okayama Y. Activation of human mast cells through the platelet-activating factor receptor. *J Allergy Clin Immunol* 2010; 125: 1137–1145 e6.

6. Kuitert L, Barnes N. PAF and asthma--time for an appraisal? *Clin Exp Allergy* 1995; 12: 1159–1162.

7. Chan-Yeung M, Lam S, Chan H, Tse KS, Salari H. The release of platelet-activating factor into plasma during allergen-induced bronchoconstriction. *J. Allergy Clin. Immunol.* 1991/03/01 ed. 1991; 87: 667–673.

8. Mullol J, Bousquet J, Bachert C, Canonica GW, Giménez-Arnau A, Kowalski ML et al. Rupatadine in the management of allergic disorders: 2014 update. *Allergy* 2015; 70: 1–24.

9. Muñoz-Cano R, Valero A, Roca-Ferrer J, Bartra J, Sanchez-Lopez J, Mullol J, Picado C. Platelet-activating factor nasal challenge induces nasal congestion and reduces nasal volume in both healthy volunteers and allergic rhinitis patients. *Am. J. Rhinol. Allergy* 2013; 27: 48–52.

10. Muñoz-Cano R, Valero A, Izquierdo I, Sanchez-Lopez J, Domenech A, Bartra J MJ. Evaluation of nasal symptoms induced by platelet activating factor, after nasal challenge in both healthy and allergic rhinitis subjects pretreated with rupatadine, levocetirizine or placebo in a cross-over design. *Allergy Asthma Clin Immunol* 2013; 9: 43.

11. Alfaro V. Role of histamine and platelet-activating factor in allergic rhinitis. *J. Physiol. Biochem.* 2004/10/02 ed. 2004; 60: 101–111.

12. Gill P, Jindal NL, Jagdis A VP. Platelets in the immune response: Revisiting platelet-activating factor in anaphylaxis. *J Allergy Clin Immunol* 2015; 135: 1424–1432.

13. Nilsson G, Metcalfe DD, Taub DD. Demonstration that platelet-activating factor is capable of activating mast cells and inducing a chemotactic response. *Immunology* 2000/02/26 ed. 2000; 99: 314–319.

14. Spence DP, Johnston SL, Calverley PM, Dhillon P, Higgins C, Ramhamadany E, Turner S, Winning A, Winter J, Holgate ST. The effect of the orally active

platelet-activating factor antagonist WEB 2086 in the treatment of asthma. *Am J Respir Crit Care Med* 1994; 149: 1142–1148.

15. Martínez-Cócera C, De Molina M, Martí-Guadaño E, Pola J, Conde J, Borja J, Pérez I, Arnaiz E, Izquierdo I. Rupatadine 10 mg and cetirizine 10 mg seasonal allergic rhinitis: A randomised, double-blind parallel study. *J. Investig. Allergol. Clin. Immunol.* 2005; 15: 22–29.

16. Dubertret L, Zalupca L, Cristodoulo T, Benea V, Medina I, Fantin S, Lahfa M, Pérez I, Izquierdo I, Arnaiz E. Once-daily rupatadine improves the symptoms of chronic idiopathic urticaria: a randomised, double-blind, placebo-controlled study. *Eur. J. Dermatol.* 2007; 17: 223–228.

17. Vasiadi M, Kalogeromitros D, Kempuraj D, Clemons A, Zhang B, Chliva C, Makris M, Wolfberg A, House M, Theoharides TC. Rupatadine inhibits proinflammatory mediator secretion from human mast cells triggered by different stimuli. *Int. Arch. Allergy Immunol.* 2009; 151: 38–45.

18. Merlos M, Giral M, Balsa D, Ferrando R, Queralt M, Puigdemont A, Garcia-Rafanell J, Forn J, García-Rafanell J, Forn J. Rupatadine, a new potent, orally active dual antagonist of histamine and platelet-activating factor (PAF). *J. Pharmacol. Exp. Ther.* 1997/01/01 ed. 1997; 280: 114–121.

19. Alevizos M, Karagkouni A, Vasiadi M, Sismanopoulos N, Makris M, Kalogeromitros D, Theoharides TC. Rupatadine inhibits inflammatory mediator release from human laboratory of allergic diseases 2 cultured mast cells stimulated by platelet-activating factor. *Ann. Allergy, Asthma Immunol.* 2013; 111: 542–547.

20. Kulka M, Metcalfe DD. Isolation of tissue mast cells. *Curr. Protoc. Immunol.* 2010/09/04 ed. 2010; Chapter 7: Chapter 7:Unit 7.25.

21. Dupre DJ, Le Gouill C, Rola-Pleszczynski M, Stankova J. Inverse agonist activity of selected ligands of platelet-activating factor receptor. *J. Pharmacol. Exp. Ther.* 2001/09/19 ed. 2001; 299: 358–365.

22. Northover AM, Northover BJ. Inhibition of NO-synthase and degranulation of rat omental mast cells in vitro. *Mediators Inflamm.* 1996; 5: 257–261.

23. Strober W. Trypan blue exclusion test of cell viability. *Curr. Protoc. Immunol.* 2008/04/25 ed. 2001; Appendix 3: Appendix 3B.

24. Guhl S, Babina M, Neou A, Zuberbier T, Artuc M. Mast cell lines HMC-1 and LAD2 in comparison with mature human skin mast cells--drastically reduced levels of tryptase and chymase in mast cell lines. *Exp. Dermatol.* 2010; 19: 845–847.

25. Ishii S, Nagase T, Shimizu T. Platelet-activating factor receptor. *Prostaglandins Other Lipid Mediat.* 2002/11/16 ed. 2002; 68-69: 599–609.

26. Kato M, Kita H, Tachibana A, Hayashi Y, Tsuchida Y, Kimura H. Dual signaling and effector pathways mediate human eosinophil activation by platelet-activating factor. *Int. Arch. Allergy Immunol.* 2004. p. 37–43.

27. Shirasaki H, Seki N, Kikuchi M, Kanaizumi E, Watanabe K, Konno N, Himi T. Expression and localization of platelet-activating factor receptor in human nasal mucosa. *Ann Allergy Asthma Immunol* 2005/09/03 ed. 2005; 95: 190–196.

28. Weller K, Maurer M. Desloratadine inhibits human skin mast cell activation and histamine release. *J Invest Dermatol* 2009/06/12 ed. 2009; 11: 2723–2726.

29. Queralt M, Brazis P, Merlos M, de Mora F, Puigdemont A. In vitro inhibitory effect of rupatadine on histamine and TNF-alpha release from dispersed canine skin mast cells and the human mast cell line HMC-1. *Inflamm. Res.* 2000; 49: 355–360.

30. Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proc. Natl. Acad. Sci. U. S. A.* 1986; 83: 4464–4468.

31. Lowman MA, Rees PH, Benyon RC, Church MK. Human mast cell heterogeneity: histamine release from mast cells dispersed from skin, lung, adenoids, tonsils, and colon in response to IgE-dependent and nonimmunologic stimuli. *J. Allergy Clin. Immunol.* 1988; 81: 590–597.

32. He SH, He YS, Xie H. Activation of human colon mast cells through proteinase activated receptor-2. *World J. Gastroenterol.* 2004; 10: 327–331.

33. Agrawal DK. Anti-inflammatory properties of desloratadine. Clin. Exp. allergy 2004. p. 1342–1348.

34. Kreutner W, Hey JA, Anthes J, Barnett A, Young S, Tozzi S. Preclinical pharmacology of desloratadine, a selective and nonsedating histamine H1 receptor antagonist. 1st communication: receptor selectivity, antihistaminic activity, and antiallergenic effects. *Arzneimittelforschung.* 2000; 50: 345–352.

35. Walsh GM. The anti-inflammatory effects of levocetirizine--are they clinically relevant or just an interesting additional effect? *Allergy Asthma. Clin. Immunol.* 2009; 5: 14.

Figures

Figure 1. (A) Expression of PAF receptor by western Blot in mast cells from both LAD2 (lane 1) and human lung (n=3) (lane 2-4). Effect of PAF in mast cell (LAD2) degranulation. PAF time-course and dose response on (B) β-hexosaminidase release and (C) and calcium mobilization. Results are represented as mean ± SD of 3 independent experiments. ***, p<0.001; ****, p<0.0001 compared to controls (no PAF). β-hex (%):β-Hexosaminidase release rate. RFU: relative fluorescence units.

Figure 1

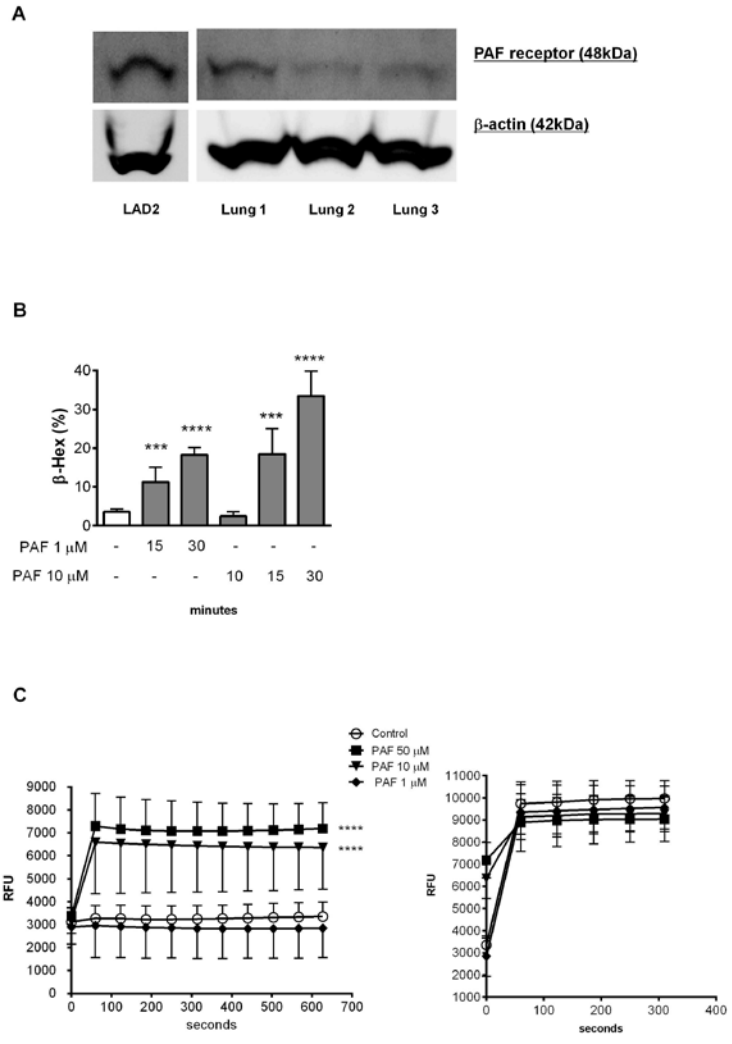

Figure 2. Effect of the specific anti-PAF receptor antagonist (CV6209) on PAF-induced mediator release from mast cell. Inhibitory effect on (A) β-hexosaminidase and (B) histamine release from LAD2 cell line, and (C) on histamine release from in primary lung mast cells. Results are represented as mean ± SD of 3 independent experiments. ****, p<0.0001 and ***, p<0.001 compared to controls (no PAF); †††, p<0.001 and ††, p<0.01 compared to PAF. β-hex (%):β-Hexosaminidase release rate. Histamine (%): histamine release rate.

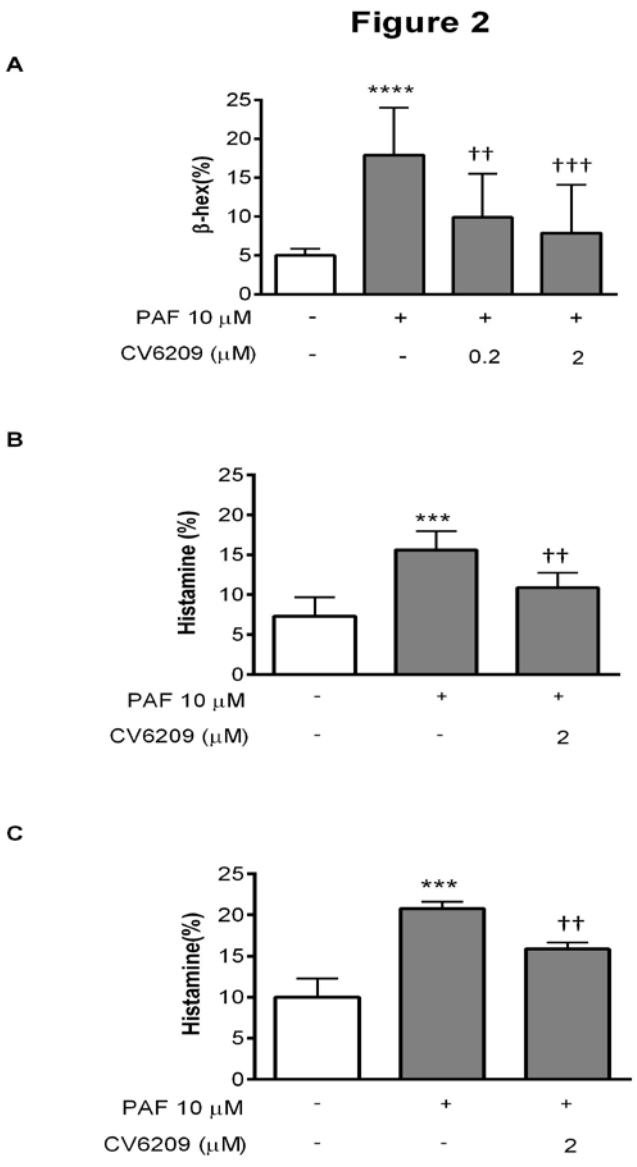

Figure 3. Effect of second generation antihistamines on PAF-induced β-hexosaminidase release in LAD2 cell line. (A) Rupatadine and, (B) to lesser extent levocetirizine, but (C) not desloratadine inhibit β-hexosaminidase release. Results are represented as mean ± SD of 3 independent experiments..***, p<0.001 and **, p<0.01 compared to controls; ††, p<0.01 compared to PAF. β-hex (%):β-Hexosaminidase release rate.

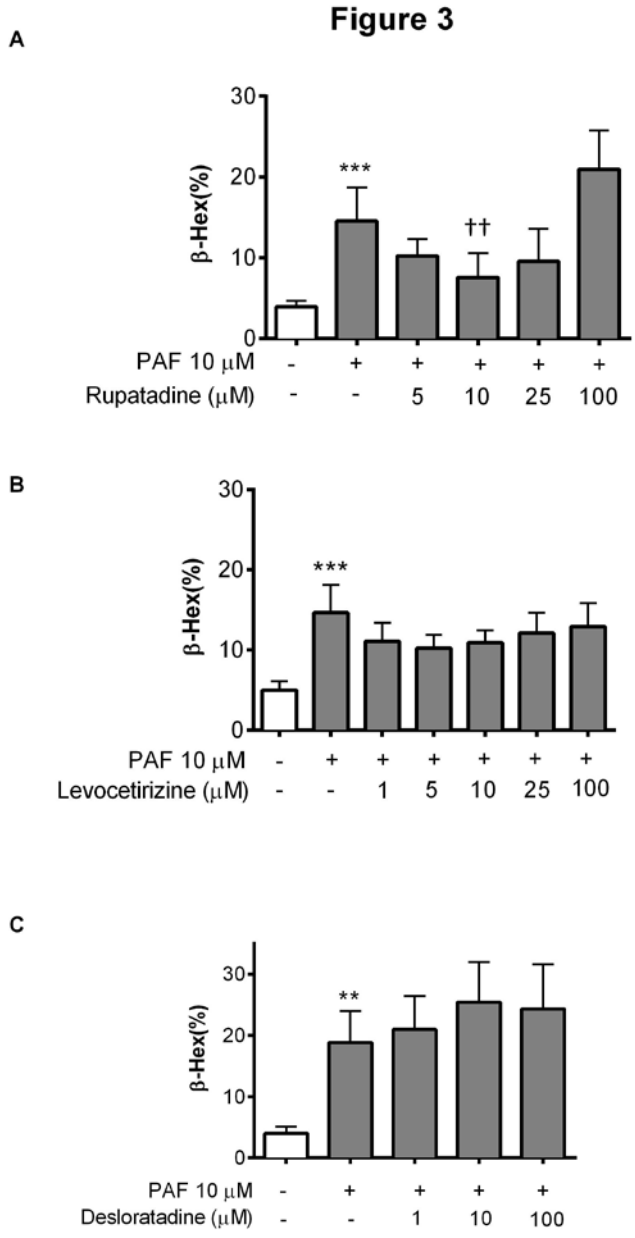

Figure 4. Effect of second generation antihistamines on PAF-induced histamine release in LAD2 cell line. (A) Rupatadine and (B) levocetirizine, and (C) to lesser extent desloratadine inhibit histamine release. Results are represented as mean ± SD of 3 independent experiments. ***, p<0.001 compared to controls. ††, p<0.01 and †, p<0.05 compared to PAF. Histamine(%):histamine release rate.

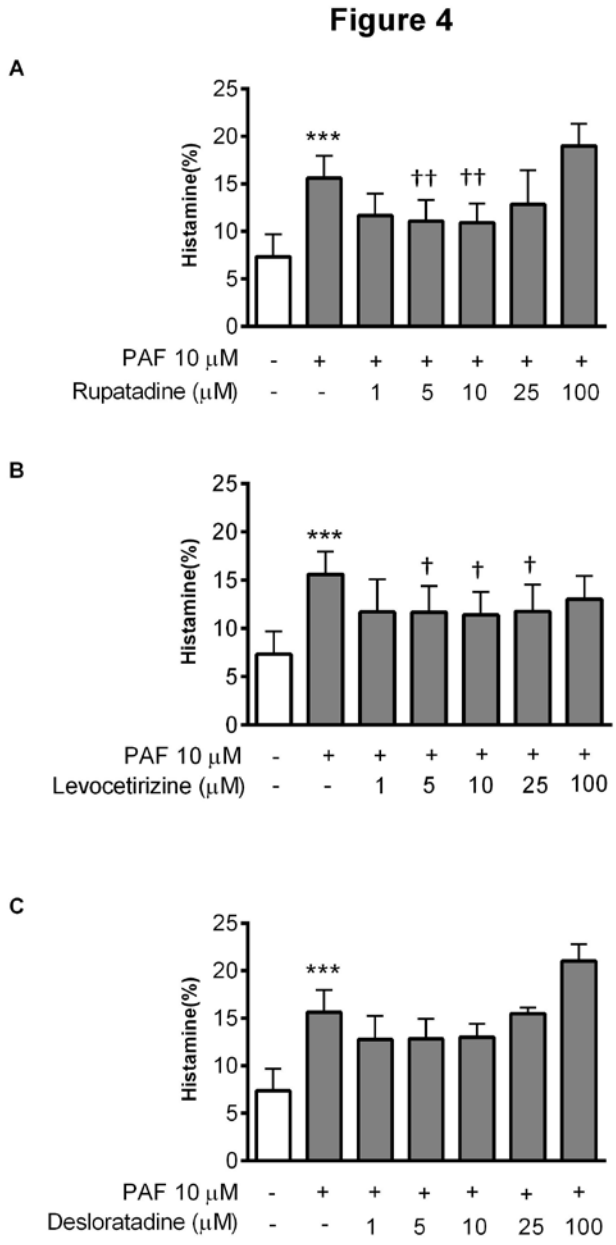

Figure 5. Effect of second generation antihistamines on PAF-induced histamine release in primary human lung mast cells. Rupatadine but not levocetirizine nor desloratadine inhibits histamine release. Results are represented as mean ± SD of 3 independent experiments. ***, p<0.001 compared to controls. ††, p<0.01 compared to PAF. Histamine(%):histamine release rate.

Figure 5