Journal of Receptors and Signal Transduction

ISSN: 1079-9893 (Print) 1532-4281 (Online) Journal homepage: http://www.tandfonline.com/loi/irst20

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To cite this article: Gomathi Nagarajan, Vairamani Mariappanadar, Muthu Tamizh, Ilango Kaliappan & Berla Thangam Elden (2016): Effect of H4R antagonist N-(2-aminoethyl)-5-chloro-1H-indol-2-carboxamides and 5-chloro-2-(piperazin-1-ylmethyl)-1H-benzimidazole on histamine and 4-methylhistamine-induced mast cell response, Journal of Receptors and Signal Transduction, DOI: 10.1080/10799893.2016.1247863

To link to this article: http://dx.doi.org/10.1080/10799893.2016.1247863

Published online: 03 Nov 2016.

JOURNAL OF RECEPTORS AND SIGNAL TRANSDUCTION, 2016
http://dx.doi.org/10.1080/10799893.2016.1247863

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RESEARCH ARTICLE

Effect of H4R antagonist *N*-(2-aminoethyl)-5-chloro-1*H*-indol-2-carboxamides and 5-chloro-2-(piperazin-1-ylmethyl)-1*H*-benzimidazole on histamine and 4-methylhistamine-induced mast cell response

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ABSTRACT

Context: The histamine plays a decisive role in acute and chronic inflammatory responses and is regulated through its four types of distinct receptors designated from H1 to H4. Recently histamine 4 receptor (H4R) antagonists have been reported to possess various pharmacological effects against various allergic diseases.

Objective: To investigate the inhibitory effect of *N*-(2-aminoethyl)-5-chloro-1*H*-indol-2-carboxamide (Compound A) and 5-chloro-2-(piperazin-1-ylmethyl)-1*H*-benzimidazole (Compound L) on H4R-mediated calcium mobilization, cytokine IL-13 production, ERK1/2, Akt and NF-κB activation in human mastocytoma cells-1 (HMC-1).

Materials and methods: Compounds A and L were synthesized chemically and their inhibitory effect on intracellular calcium release was analyzed by Fluo-4 calcium assay, cytokine measurement through ELISA and activation of signaling molecules by western blot.

Results: Pre-treatment with compounds A and L significantly reduced the H4R-mediated intracellular calcium release. Histamine and 4-methylhistamine (4-MH) induced Th2 cytokine IL-13 production in HMC-1 cells, was inhibited by compound A (77.61%, 74.25% at 1 μM concentration) and compound L (79.63%, 81.70% at 1 μM concentration). Furthermore, histamine induced the phosphorylation of ERK1/2, Akt and NF-κB was suppressed by compounds A and L at varying levels, ERK1/2 (88%, 86%), Akt (88%, 89%) and NF-κB (89%, 87%) in HMC-1 cells.

Discussion and conclusions: Taken together these data demonstrate that compound A and compound L may block H4R-mediated downstream signaling events.

ARTICLE HISTORY
Received 25 July 2016
Revised 30 August 2016
Accepted 8 September 2016
Published online 3 November 2016

KEYWORDS
H4R antagonist; mast cells; histamine; 4-methylhistamine; Compound A; Compound L

Introduction

Histamine has long been known to be the mediator that orchestrates inflammatory and allergic responses acting mainly through histamine receptors. During inflammation, histamine released from preformed stores in mast cells and basophils, acts on vascular smooth muscle cells and endothelial cells, leading to increased vasodilation and vascular permeability (1). Histamine contributes to the progression of allergic inflammatory responses by enhancing the secretion of proinflammatory cytokines like IL-1α, IL-1β, IL-6, as well as chemokines like RANTES and IL-8 both in several cell types and local tissues (2).

The pleiotropic effects of histamine are mediated by four subtypes of histamine receptors namely H1R, H2R, H3R and histamine 4 receptor (H4R) and belong to G-protein coupled receptor family (3). The most characteristic roles for H1R activation are smooth muscle contraction and increase in vascular permeability. Many of its functions contribute to allergic responses. The H2R functions as a key modulator for gastric acid secretion and H3R is predominantly expressed in the human central nervous system (4). Compared with H1R and H2R, the lately discovered H4R has more selective expression pattern and found mainly in the cells of hematopoietic origin, in particular mast cells, basophils, eosinophils and T-cells (3,5–8). H1R antagonists also referred as antihistamines have long been used to treat allergies, offering symptomatic relief in atopic nasal, conjunctival and skin disease. However, H1R antagonists are not optimally effective in asthma (9), where histamine is particularly involved in immune cell chemotaxis and pro-inflammatory responses. Recent reports indicate that, H4R is involved in the control of immune cell trafficking, pro-inflammatory mediator's release, increased expression of adhesion molecules and rearrangement of the actin cytoskeleton leading to immune cell migration from the bloodstream into the sites of inflammation (10,11). Recently, we also reported that in human mast cells H4R activation caused the production of various inflammatory mediators including, various cytokines, chemokines which are associated with allergic asthma and other inflammatory diseases (12).

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H4R plays a major role in immunomodulatory functions, therefore, it is now considered as a new drug target for the treatment of various inflammatory diseases including allergy and asthma (5). Experiments using the dual H3R/H4R antagonist thioperamide have shown the pathophysiological significance of H4R in inflammatory conditions, such as allergic asthma and other allergic disorders (6). Recent reports indicate that the H4R antagonist 1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine (JNJ7777120) was able to inhibit histamine induced chemotaxis and Ca^{2+} influx in mouse bone marrow derived mast cells, as well as tracheal mast cell migration from the connective tissue toward the epithelium (4,13,14). Furthermore, JNJ7777120 significantly blocked neutrophil infiltration in a mast cell-dependent mouse zymosan induced peritonitis model (4). In addition, only a few H4R antagonists were reported, and for instance A-940894, INCB38579 and VUF6007 were reported to have anti-inflammatory and anti-pruritic effects (4,15–18). Thus, only a limited number of selective H4R antagonists have been discovered so far.

Though JNJ7777120 is considered to be the standard antagonist for H4R (16,19), the recent data show that JNJ7777120 activates β-arrestin in Gαi/o protein independent manner and exhibit partial agonist effect which indicates that it has dual activity and also it has a relatively short plasma half-life and limited bioavailability (4,5). Therefore, further investigations on the development of novel lead compounds to explore their therapeutic potential in treating allergic diseases are required. In our previous study, we have generated selective histamine receptor H4R antagonists using bioinformatics tools (20). Here in this study, we chemically synthesized (Scheme 1) and tested whether these compounds can inhibit H4R-mediated downstream signaling events in human mast cells (human mastocytoma cells-1 (HMC-1)).

Materials and methods

Materials

JNJ7777120, histamine, 5-chloroindol-2-carboxylic acid, 4-chloro-1,2-phenylene diamine and chloroacetic acid were purchased from Sigma–Aldrich (St. Louis, MO), Fluo-4 calcium assay kit from Invitrogen, IL-13 ELISA kit from R&D Systems (Minneapolis, MN). H4R siRNA, siRNA transfection medium, transfection reagent and 4-methylhistamine (4-MH) (H4R agonist), phospho-Akt antibody, phospho-ERK1/2 (Thr 177/Tyr160) and phospho-NF-κB antibody were purchased from Santa Cruz Biotechnology (Dallas, TX). Total ERK 1/2 antibody and total Akt antibody were purchased from Cell Signaling (Burlington, NC). Super Signal West Pico chemiluminescent substrate was purchased from Thermo Scientific and all the other basic chemicals were from Sigma (St. Louis, MO). IMDM, FBS, glutamine, penicillin and streptomycin were purchased from Gibco, Invitrogen, Carlsbad, CA.

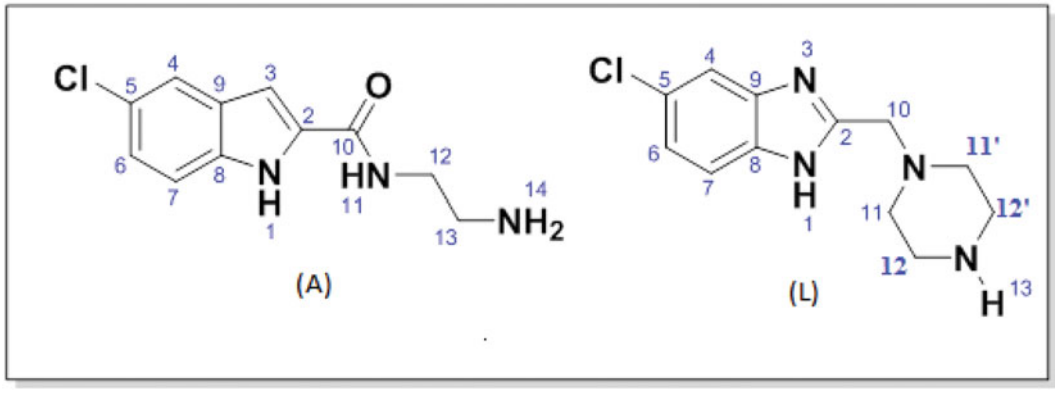
Methods

Synthesis of title compounds

Synthesis *of* N-*(2-aminoethyl)-5-chloro-1*H-*indol-2-carboxamide (compound A)* 5-Chloroindol-2-carboxylic acid (100 mg) dissolved in 10.0 ml of benzene was refluxed with $SOCl_2$ (1 ml) for 5 h at 100 °C. The excess of $SOCl_2$ was removed by distillation. The acid chloride was added to ethylenediamine (2.0 ml) and stirred overnight at room temperature. The precipitated solid was removed by centrifugation and the supernatant solvent was removed. The precipitate was dissolved in ethylacetate (10.0 ml), washed with water (2 × 5 ml) and the organic layer was evaporated to get a solid, after drying over anhydrous sodium sulfate. Yield: 40.0 mg. The purity of the compound was checked by thin layer chromatography (methanol 100%) and the structure of the compound was confirmed by NMR spectroscopy, δ values (assignment); 6.8, 7.2, 7.3, 7.6 (aromatic ring hydrogen); 3.5 (N–CH_2); 2.98 (CH_2–N). The molecular weight was confirmed by electrospray ionization mass spectrometry; m/z 238 $[M+1]^+$(Figure 1(A,B)) (21,22).

Synthesis *of 5-chloro-2-(piperazin-1-ylmethyl)-1*H-*benzimidazole (compound L)*

Step 1: Synthesis of N-carboxymethyl piperazine The solution of chloroacetic acid (1.98 g) and sodium hydroxide (0.8 g) in water is added drop wise to the stirred solution of piperazine (8.0 g) and sodium hydroxide (2.0 g) in water. After 12 h the separated solid was filtered and the filtrate was cooled in an



Scheme 1. Structure of Compound A and Compound L.

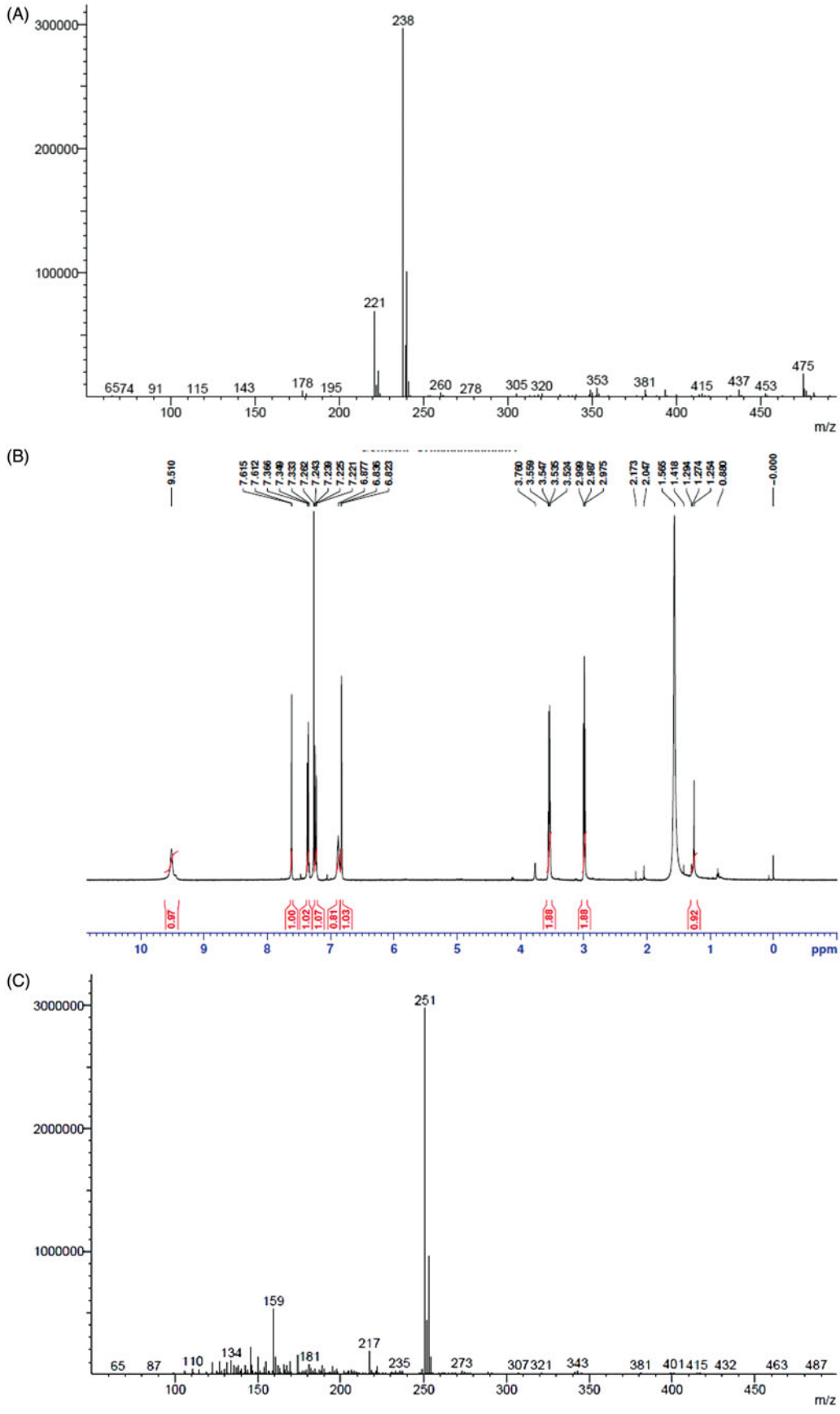


Figure 1. Characterization of compound A and compound L. (A) The electrospray ionization mass spectrometry of compound A with peak 238. (B) The NMR spectrum for compound A. (C) The electrospray ionization mass spectrometry of compound L with peak 251. (D) The NMR spectrum for compound L.

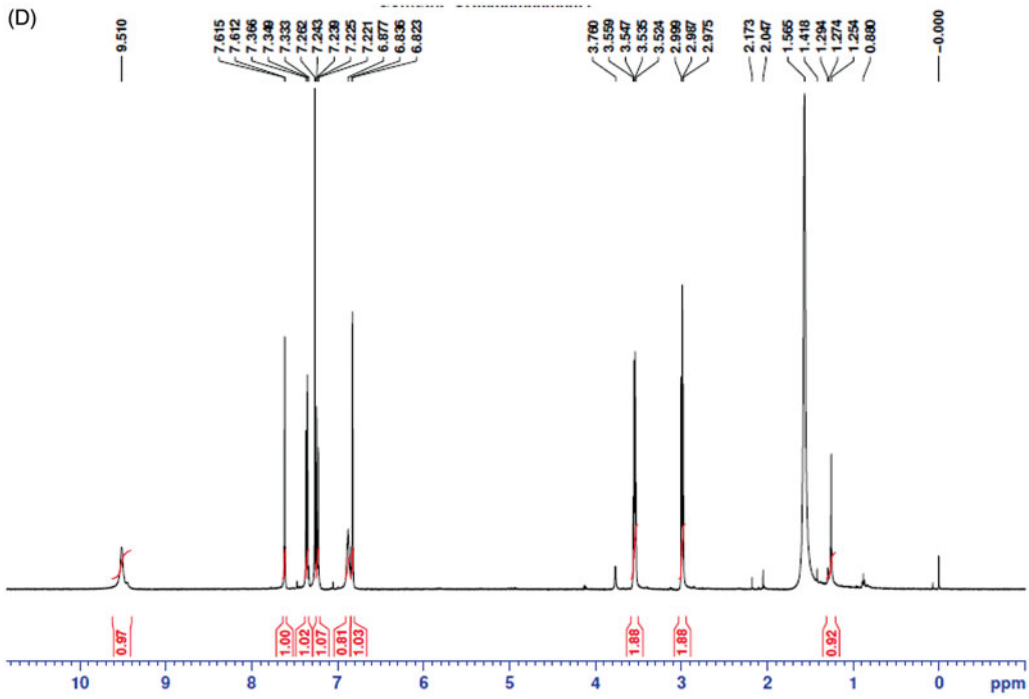


Figure 1. Continued.

ice bath. The solution was made alkaline with 10% sodium hydroxide solution and extracted with ethylacetate (20 ml) to remove excess of piperazine. Further, it was acidified and extracted with ethylacetate (20 ml) to remove excess of chloroacetic acid. The filtrate was then treated with excess of methanol. The precipitate formed was centrifuged and the supernatant was decanted. The precipitate was dried and a white solid was obtained. Yield: 8.0 g, the molecular weight of the synthesized compound was confirmed by electrospray ionization mass spectrometry; m/z: 145 $[M+1]^+$.

Step 2: Synthesis of compound L 4-Chloro-1,2-phenylene diamine (1.0 g) was taken in 50 ml of xylene and reflexed with *N-carboxymethyl piperazine* (0.8 g) for 6 h. Xylene was decanted and the residue was dissolved in ethyl acetate and the compound was purified by column chromatography over silica gel. Yield: 70 mg. The purity of the compound was checked by thin layer chromatography (methanol:ethylacetate 1:1) and the structure of the compound was confirmed by NMR spectroscopy, δ values (assignment): 7.3, 7.5, 7.6 (aromatic ring hydrogens); 3.9 ($C–CH_2–N$); 3.2 ($N–CH_2$); 2.8 ($CH_2–NH$). The molecular weight of the synthesized compound was confirmed by electrospray ionization mass spectrometry; m/z 251 $[M+1]^+$(Figure 1(C,D)).

Culture of HMC-1 cell line

HMC-1 cells were kindly provided by Dr. Joseph H Butterfield, Mayo Clinic (Rochester, MN), and cultured in IMDM supplemented with 10% FBS, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 ng/ml).

Cytotoxicity assay

Cell toxicity of H4R antagonists compound A and L was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on HMC-1 cells. Briefly, the cells were seeded on to 96-well plates at a density of 0.1×10^5 cells/well in IMDM medium supplemented with 1% BSA. Different concentrations in the range 1 nM–100 μM of compounds A and L were added to the cells and were incubated for 24 h. MTT reagent was added and incubated for 4 h. The farmazone crystals formed were dissolved in DMSO and the absorbance was taken at 570 nm using ELISA reader (Biotek Synergy HT multimode reader).

Small interfering RNA (siRNA) mediated gene silencing of histamine H4R in HMC-1 cells

The HMC-1 cells (50,000 cells/well) were plated in six-well plates. For histamine H4R gene silencing, a human siRNA targeting H4 receptor (Santa Cruz Biotechnology, Dallas, TX) was employed. The siRNA transfection was performed according to the manufacturer's instruction. In brief, cells were incubated with transfection mixture containing 100 nmol/L of siRNA for 5–6 h and then incubated with 1 ml of serum-containing media further for 24 h.

Measurement of intracellular calcium levels

Intracellular calcium levels were measured in HMC-1 cells using Fluo-4 calcium assay kit and performed according to the manufacturer's instructions. In brief, HMC-1 cells

and siRNA mediated H4R transfected cells were washed and suspended in assay buffer and plated in black flat-bottom 96-well plates. Mast cells (2×10^5) were pre-treated with JNJ7777120 (10 μM), compound A and compound L (10 μM, 1 μM) for 10 min, labeled with Fluo-4 dye and incubated for 1 h at 37 °C. The plates were then read at excitation 494 nm and emission 516 nm. The basal reading for 100 s was taken, then the cells were stimulated with histamine (10 μM) or 4-MH (10 μM) and change in fluorescence was measured for a total of 600 s. (Biotek Synergy HT multimode reader).

Estimation of IL-13 by ELISA

The HMC-1 cells (1×10^6 cells/ml per well in a basal medium) were washed and preincubated with JNJ7777120 (10 μM), compound A, compound L (10 μM, 30 min) and then stimulated with 4-MH or histamine (10 μM). Supernatants were collected and stored at −80 °C until further use. Concentration of IL-13 in supernatant was estimated using sandwich ELISA kit according to the manufacturer's instructions. The reactions were measured at 450 nm (Biotek Synergy HT multimode reader).

Western blotting

The HMC-1 cells (1×10^6 cells/ml) were lysed in radioimmunoprecipitation assay (RIPA) buffer. The proteins concentration was quantified by Bradford method and was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The blots were probed with rabbit anti-human H4R antibody, anti-phospho-ERK1/2, anti-phospho-Akt antibody and anti-phospho-NF-κB p65 antibody. Respective blots were stripped and reprobed with β-actin, antibodies followed by anti-mouse-IgG-HRP antibodies (Santa Cruz). The bands were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo scientific) and the image was captured using multi-imaging system (Cell Biosciences). Images from multiple gels (at least three determinations) were quantitated using the fluorchem Q software.

Statistical analysis

The difference in estimated parameters between the groups was analyzed using one-way ANOVA with Bonferroni's test. Data are expressed as mean ± SD. All the parameters were analyzed at 99% confidence intervals and p values of $<.01$ were considered to be statistically significant except for dendrogram analysis where the parameters were analyzed at 95% confidence intervals and p values of; $<.05$ were considered to be statistically significant. Statistical analysis of the data was performed using GraphPad Prism version 6.00, version 7.00, San Diego, CA.

Results

Cytotoxic effect of compound A and compound L on HMC-1 cells

The compounds A and L were checked for its cytotoxic effect on HMC-1 cells at different concentrations from 1 nM to 100 μM. The compounds were not found to be toxic to the cells at different concentration used (Figure 2).

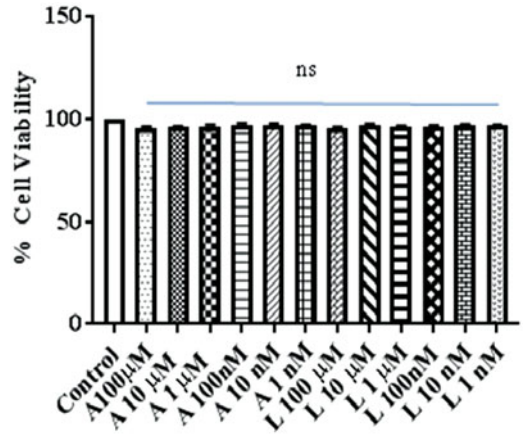


Figure 2. Effect of compounds A and L on cytotoxicity in HMC-1 cells. The data show the % cell viability of HMC-1 cells after the treatment of cells with different concentrations (100 μM–1 nM) of compounds A and L. Values are expressed as mean ± SD of three experiments done in triplicates. ns was considered as statistically non-significant.

Effect of compounds A and L on histamine and 4-MH induced calcium mobilization

A rise in intracellular calcium ion concentration triggers cellular activation, which in turn leads to the release of inflammatory mediators from mast cells. Histamine a natural agonist for H4R and 4-MH, a synthetic agonist which has high affinity and binds preferentially to H4R were used as agonist in the study. We examined the effect of compounds A and L on histamine and 4-MH induced intracellular calcium release in HMC-1 cells. We found that histamine and 4-MH (10 μM) induce the intracellular calcium release, whereas JNJ7777120 (10 μM), compounds A and L (10 μM, 1 μM) inhibited the intracellular calcium release in HMC-1 cells. Interestingly, compound L was found to be more effective than compound A at 10 μM concentrations in inhibiting the intracellular calcium release in response to histamine and 4-MH (Figure 3(A,B)). The H4R siRNA gene silencing decreased the 4-MH-induced intracellular calcium release. In addition, no significant effect was observed on JNJ7777120, compounds A and L pre-treatment on H4R-mediated intracellular calcium release. These data indicate that compounds A and L can inhibit the H4R activation in HMC-1 cells.

Effect of compounds A and L on H4R mediated IL-13 release in HMC-1 cells

Since IL-13 is thought to be a critical mediator of allergic asthma, we examined the effect of compounds A and L on H4R mediated IL-13 release by using histamine and 4-MH in HMC-1 cells. As shown in Figure 4, histamine (10 μM) was able to induce IL-13 secretion up to 1576 pg/ml. Pre-treatment with the compounds A and L at 1 μM concentration decreased the histamine (10 μM) induced IL-13 cytokine release up to 352.84 pg/ml (77.61%) and 321.03 pg/ml (79.63%). 4-MH (10 μM) induced IL-13 (1494 pg/ml) was

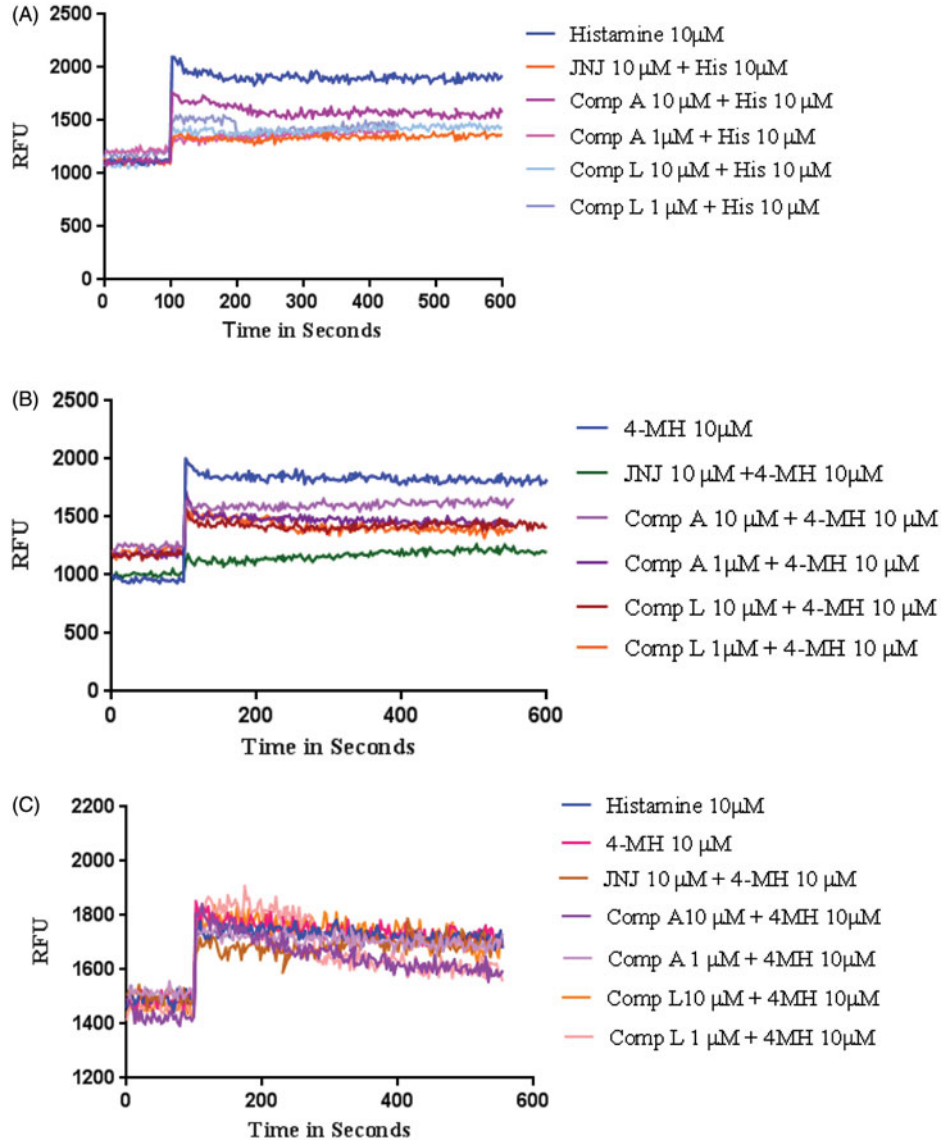


Figure 3. Effect of compounds A and L on histamine and 4-MH induced intracellular calcium release in HMC-1 cells. Data showing the inhibitory effect of compounds A and L (pretreated for 10 min) on intracellular calcium release at the concentration of 10 μM and 1 μM against (A) histamine (10 μM) and (B) 4-MH (10 μM) with H4R standard antagonist JNJ7777120 (10 μM), (C) Effect of compounds A and L on 4-MH induced intracellular calcium release in H4R siRNA transfected cells. Data shown are representative of three similar experiments.

inhibited by compounds A and L to the level of 384.65 pg/ml (74.25%) and 273.31 pg/ml (81.70%), respectively.

Effect of compounds A and L on ERK 1/2, Akt and NF-κB signalling molecules in HMC-1 cells

Furthermore, the effect of compounds A and L on signaling molecules such as NF-κB, ERk1/2 and Akt in histamine-induced mast cell activation was checked. We found that histamine (10 μM) induced phosphorylation of ERK1/2, Akt and NF-κB p65 in HMC-1 cells. Compound A (10 μM, 1 μM, 100 nM) was able to inhibit histamine-induced ERK1/2, Akt and NF-κB phosphorylation at (88%), (88%) and (89%), respectively. Compound L (10 μM, 1 μM, 100 nM) was also able to inhibit phosphorylation of ERK1/2, Akt and NF-κB at (86%), (89%), (87%) in HMC-1 cells (Figure 5).

Discussion

In the present study, we chemically synthesized the H4R antagonist, compound A and compound L that were generated using bioinformatics structure-based virtual screening strategies (20) and its inhibitory effect on H4R-mediated mast cell functions were studied using HMC-1 cells. Our compounds A and L were able to inhibit the effect of histamine and 4-MH induced calcium mobilization and Th2 cytokine IL-13 release. We have also shown that the compounds were able to inhibit the activation of ERK1/2, Akt and NF-κB when stimulated with histamine in HMC-1 cells.

The discovery of H4R led to diligent efforts on the development of antagonists targeting H4R based on its involvement in inflammatory and immunomodulatory functions (23–30). So far several antagonists such as JNJ7777120, A-940894,

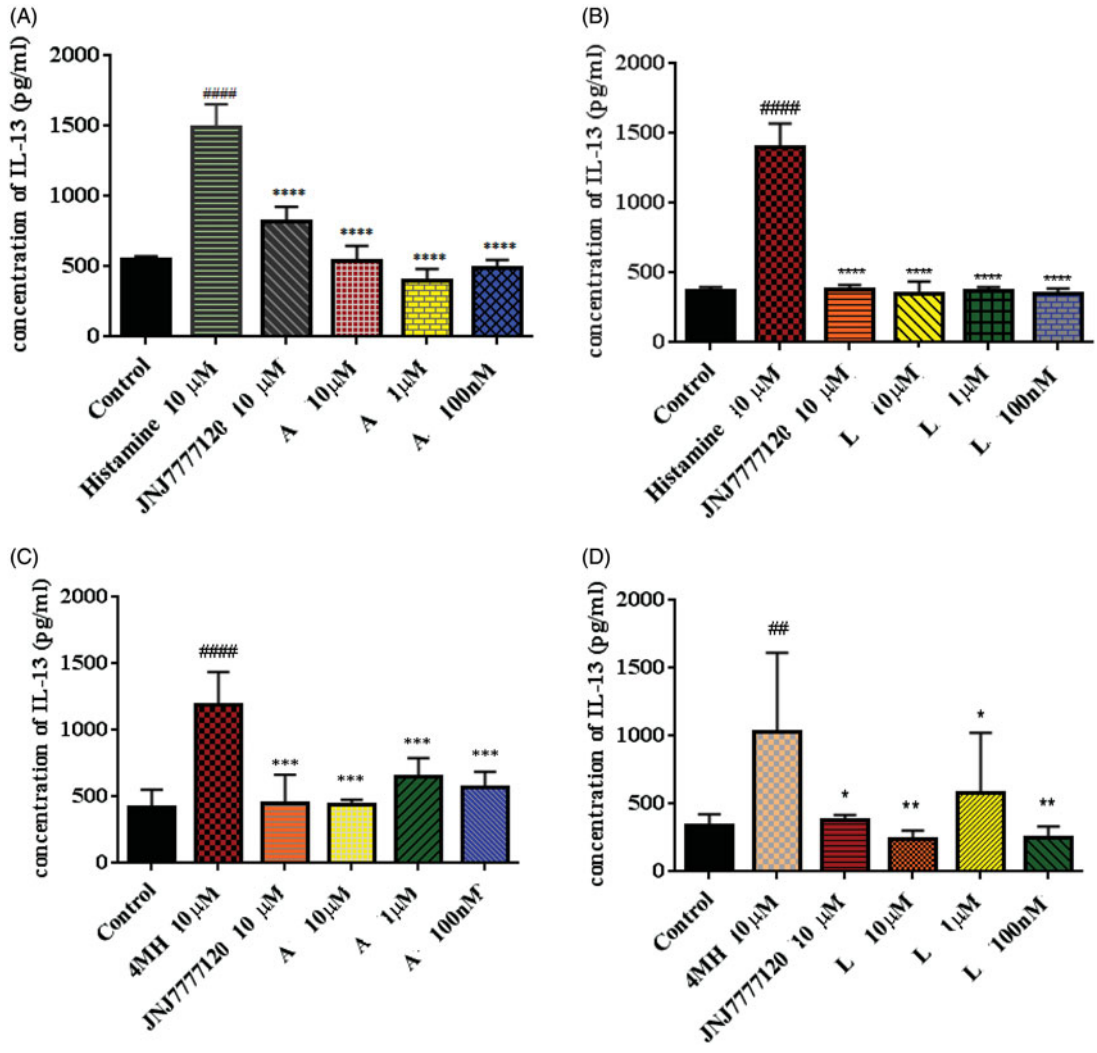


Figure 4. Effect of compounds A and L on histamine and 4-MH induced IL-13 release in HMC-1 cells. The figure shows the level of IL-13 production after 24 h stimulation with histamine and 4-MH (10 μM) and inhibitory effect of JNJ7777120 (10 μM), compounds A and L (pre-treated for 10 min at the concentration of 10 μM, 1 μM and 100 nM) against histamine (10 μM) (A, B) and 4-MH (10 μM) (C, D) and JNJ7777120 (10 μM). Values are expressed as mean ± SD of three experiments done in triplicates. ##$p < .001$ significantly different from control. $^{**}p < .001$ and $^{***}p < .0001$ significantly different from histamine or 4-MH.

A-943931, A-987306, INCB38579, VUF6007, VUF11489, were tested for their effect against H4R and has shown distinct effect on different cell types and various animal models of allergy and inflammation (4,15–18,23,32–49,55). PF-3893787 (NCT00856687), UR-63325 (NCT01260753), KD1157 (54), JNJ39758979 (NCT01679951, NCT00941707, NCT01862224, NCT01823016 and NCT02295865) are in various stage of clinical trials. JNJ39758979 has shown preclinical and safety in volunteers (50,51).

Among the antagonists, an indolecarboxamide-containing JNJ7777120 was considered as the first standard selective H4R antagonist (54). JNJ7777120 is used in various studies by different groups. Previous studies have reported that H3R antagonist such as thioperamide, clobenpropite and H3R agonist such as imetit and R-α-methylhistamine bind to the H4R with different affinity from that of the H3R and further it has shown to inhibit or activate the H4R (55).

Recent reports suggest that mast cells express H1R, H2R and H4R but not H3R except for brain mast cells. The Gαq/11 coupled H1R and Gα/io coupled H4R play a major role in allergic inflammation. The Gαs-coupled H2R is associated with gastric acid secretion. H3R is involved in neurotransmission and is not expressed in HMC-1 cells. The stimulation of H4R reduces forskolin-induced cAMP formation activation of MAPK and enhanced Ca^{++} mobilization and chemotaxis, without affecting degranulation, which enables the selective recruitment of effector cells and enhancement of allergic responses (53).

Accumulated evidence suggests that selective H4R antagonists have shown to inhibit histamine-induced chemotaxis and calcium responses in eosinophil and bone marrow-derived mast cells (8,15,17). Histamine-mediated calcium release was inhibited by A-940894 dose dependently in mouse bone marrow-derived mast cells (15). The previous report by Shin et al. (18) has shown that INCB38579 a small molecule antagonist inhibited H4R-mediated calcium mobilization in HEK-293 cells stably co-expressing the human, mouse or rat histamine H4R. JNJ7777120 and thioperamide were also shown to inhibit the calcium response in bone marrow-derived mast cells when stimulated with histamine (4,30). In the present study, compounds A, L and JNJ7777120 have shown to inhibit intracellular calcium release in HMC-1

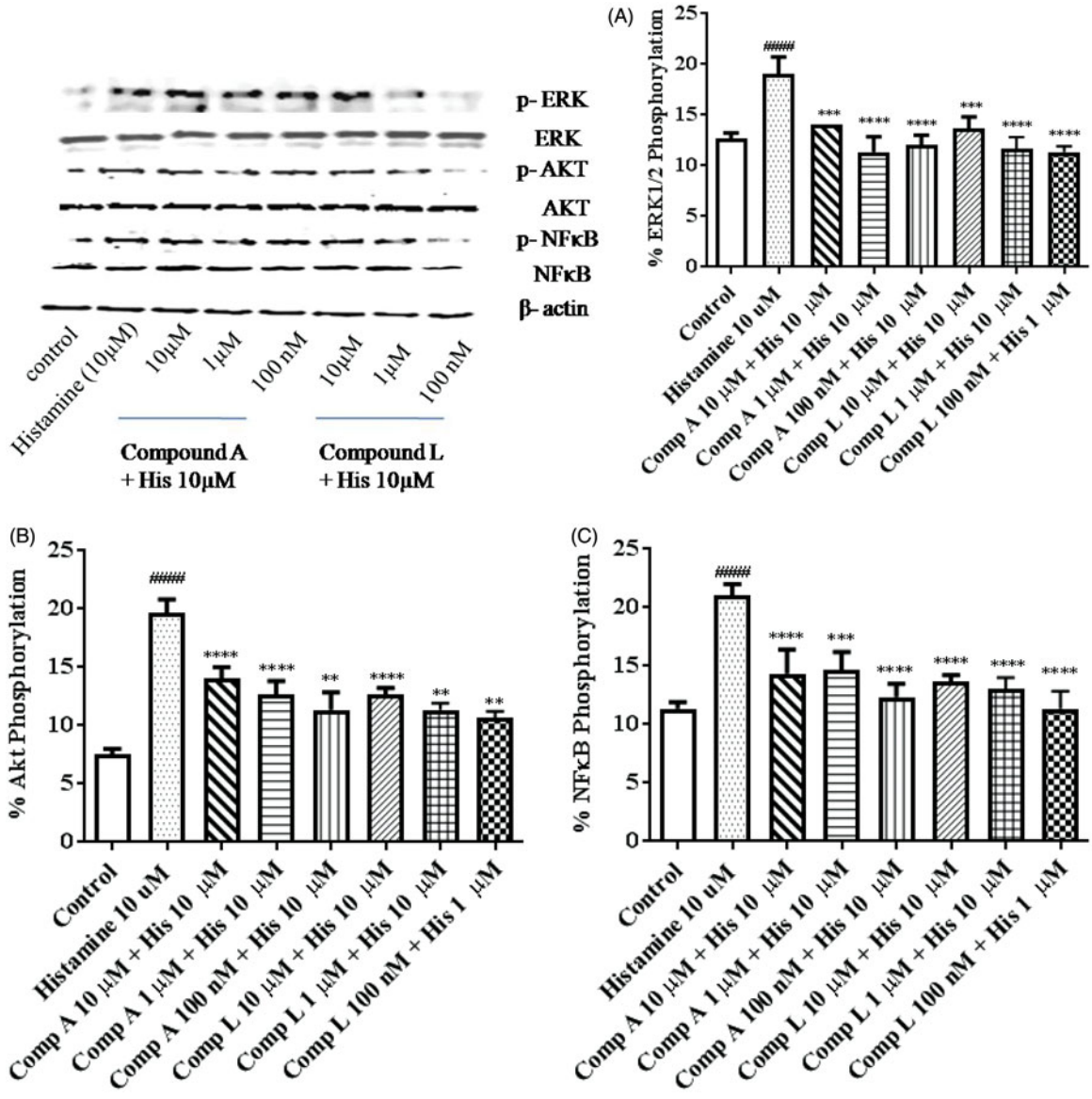


Figure 5. Effect of compounds A and L on histamine-induced downstream signaling in HMC-1 cells. HMC-1 cells (1×10^6) were pretreated with compounds A and L for 10 min and stimulated with histamine (10 μM). Cell lysates were separated on SDS-PAGE and blots were probed with anti-phospho-ERK1/2, anti-phospho-Akt and anti-phospho-NFκB antibody. Respective blots were stripped and reprobed with total or β-actin antibody followed by anti-mouse IgG-HRP antibodies. Immunoreactive bands were visualized by SuperSignal West Pico chemiluminescent substrate. Blots shown here is representative of three similar experiments. Bar graph represents summary of ERK1/2, Akt and NFκB phosphorylation. Bands were quantified by densitometry and expressed as percentage. Values are analyzed using one-way ANOVA with Bonferroni's test. Data were expressed as mean ± SD of three experiments. #$p < .05$ was considered significantly different from control. *$p < .05$ was considered significantly different from histamine.

cells. Since H4R expression is silenced by H4R siRNA, there is very less or no binding site available for the compounds. Therefore, there is no significant effect observed with compounds A and L on 4-MH induced intracellular calcium release in H4R siRNA transfected HMC-1 cells. Whereas, compounds A and L showed inhibition in H4R-mediated intracellular calcium release in HMC-1 cells. This shows that compounds A and L may be specific to H4R and doesn't show any effect through other receptors (H1, H2 and H3) at the used concentrations.

Selective antagonism or gene knockout of H4R has been demonstrated to reduce allergic airway inflammation in a mouse model (30,52). The H4R antagonist JNJ7777120 and VUF6007 have shown anti-inflammatory effect in rat model (31). Furthermore, it has shown that H4R antagonist JNJ7777120 has anti-puritic and anti-inflammatory function in a mouse model (18). H4R antagonist treatment has shown significantly to reduce the level of IL-13 in bronco alveolar lavage fluid and tissue of mice model of allergic asthma (9). Therefore, in the present study we compared the potencies of compound A and compound L in the inhibition of IL-13 release. Compound A and compound L were found to inhibit IL-13 release to basal level against 4-MH and histamine.

NF-κB is a central transcription factor which plays a major role in regulating the immune response to allergy, inflammation and infection. Previously it was thought that H1R activates NF-κB in cos-7 cells, later it was found that H4R was also involved in the activation of NF-κB (56,57). Furthermore, previous reports have shown that histamine-induced phosphorylation of ERK, MEK and Akt in mast cells, whereas

JNJ7777120 inhibited histamine induced NF-κB, Akt and ERK phosphorylation in mast cells and has also shown to inhibit the phosphorylation of NF-κB in a rat model of inflamed knee tissue (58–60). Similarly, in the present study our compounds A and L were also able to inhibit histamine induced ERK1/2, Akt and NF-κB phosphorylation in HMC-1 cells.

Taken together, our data demonstrate that compound A and compound L were shown to inhibit the H4R mediated calcium mobilization, IL-13 cytokine release and able to inhibit the downstream signaling molecules such as ERK1/2, Akt and NF-κB.

Conclusions

Therefore, the present study suggests that compounds A and L can inhibit H4R-mediated allergic and inflammatory reaction and can also provide new therapeutic agents for various allergic and inflammatory diseases. Further, the effect of these compounds on allergic asthma mice model is under progress.

Acknowledgements

We are grateful to Dr. Joseph H. Butterfield (Mayo Clinic, Rochester, MN) for HMC-1 cells. This work was supported by SRM University, Chennai, Tamil Nadu, India.

Disclosure statement

The authors declare no conflict of interest.

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