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Pharmacological and SAR analysis of the LINS01 compounds at the human histamine H_1, H_2 and H_3 receptors

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Short Running Title: Pharmacology of LINS series at histamine receptors

Abstract

Histamine is a transmitter that activates the four receptors H_1R to H_4R. The H_3R is found in the nervous system as an auto and heteroreceptor, and controls the release of neurotransmitters, making it a potential drug target for neuropsychiatric conditions. We have previously reported that the 1-(2,3-dihydro-1-benzofuran-2-yl)methylpiperazines (LINS01 compounds) have selectivity for the H_3R over the H_4R. Here we describe their pharmacological properties at the human H_1R, H_2R in parallel

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with the H_3R, thus providing a full analysis of these compounds as histamine receptor ligands through reporter gene assays. Eight of the nine LINS01 compounds inhibited H_3R-induced histamine responses but no inhibition of H_2R-induced responses were seen. Three compounds were weakly able to inhibit H_1R-induced responses. No agonist responses were seen to any of the compounds at any receptor. SAR analysis shows that the *N*-methyl group improves H_3R affinity whilst the *N*-phenyl group is detrimental. The methoxy derivative, LINS01009, had the highest affinity.

Histamine is a biogenic amine that has important clinical and pathophysiological roles in allergy, gastric acid secretion and inflammatory diseases. Histamine effects occur by activation of histamine receptors of which there are four subtypes and all are G-protein coupled receptors (GPCRs), namely H_1R, H_2R, H_3R and H_4R.[1,2]

The H_1R is a G_q-coupled receptor and activation of this is important in allergy. There are many H_1R-antagonists in widespread clinical use for allergies such as hayfever (including oral agents e.g. certirizine, loratadine and chlorpheniramine, and topical preparations e.g. mepyramine). The H_2R is a G_s-coupled receptor. Activation is important in the production of gastric acid and H_2R-antagonists are widely used to reduce gastric acid production (e.g. ranitidine).[1] The H_3R is a G_i-coupled receptor and present in the CNS and on peripheral neurons. It is important for inhibiting the release of histamine (as an autoreceptor) but also negatively regulating the synthesis and release of many neurotransmitters. H_3R-antagonists have been investigated as potential treatments for several CNS disorders, including sleep disorders, schizophrenia, epilepsy, attention deficit and hyperactivity disorder and even obesity. There is also potential for H_3R-antagonists to have effects in neurodegenerative disorders such as Alzheimer's, Parkinson's disease and Huntington's chorea.[3-6] Recently, a H_3R antagonist (pitolisant) has been approval for the maintenance of wakefulness in patients with narcolepsy.[7,8] The H_4R is also a G_i-coupled receptor and is most widely expressed in bone marrow and on white blood cells and shown to be an important target to treat inflammation,[2] although to date no H_4R antagonist is available for clinical use. There is a considerable overlap between the selectivity of ligands for the H_3R and H_4R, with many H_3R ligands having high affinity for the H_4R, however some H_4R selective ligands have been identified (e.g. JNJ-7777120).[1,2] We have recently developed a series of compounds, originally designed based on the H_4R selective antagonist JNJ-7777120 chemophore, the 1-(2,3-dihydro-1-benzofuran-2-yl)methylpiperazines (LINS01) series (Figure 1).

Initial studies examining [^{3}H]-histamine binding to H_3R and H_4R membrane preparations of the first four compounds suggested micromolar affinities for these compounds, with higher affinity for the H_3R than H_4R.[9] A further study of other LINS01 compounds in HEK293 cells transiently expressing the H_3R or H_4R suggested the LINS01 compounds are antagonists or weak inverse agonists but once again that they had higher affinity for the H_3R than the H_4R.[10] Given the surprising finding that these LINS01 compounds had higher affinity for H_3R than H_4R, we decided to investigate the pharmacological profile of these ligands at the other histamine receptors, the H_1R and H_2R.

The LINS01 compounds (**1a-1i**) were synthesized as previously reported by our group (Figure 2, see supporting Information).[9-11] In case of the non-substituted phenols (to obtain compounds **1a-d**), the commercial 2-allylphenol was used as starting material. In case of R'-substituted molecules, the corresponding phenols **2e-h** were used as starting material, which were allylated with allyl bromide in conventional or ultrasonic conditions, with good to excellent yields. The yields in conventional or ultrasonic methods are comparable, however the ultrasonic methodology saved reaction time and thus was considered advantageous. The allyl-phenyl ethers **3e-h** were then thermally isomerized (>200 °C) to access the 2-allylphenols (**4a-h**) with good yield using a microwave-assisted Claisen rearrangement approach, using DMF as solvent. The dihydrobenzofurans (**5a-h**) were obtained through iodine-promoted cyclization from the corresponding *ortho*-allylphenols in an eco-friendly medium, using water or a mixture of ethanol-water as solvent. Finally, the iodinated heterocycle was used to alkylate the *N*-substituted piperazines in aprotic solvent (THF), with potassium carbonate as base, with moderate yields. A novel microwave-assisted methodology was employed to avoid the considerable excess of 1-phenylpiperazine to obtain compounds **1d** and **1i**, leading to moderate to good yields (50-70%). Although this method gave comparable yields to the conventional methodology, it also saved reaction time and required less 1-phenylpiperazine (1.1 eq.) indeed, then comprising the green chemistry principles.[12] The spectroscopic data for the final compounds (see Supporting Information) and intermediates are in accordance to the literature reports.[9-11] None of the LINS01 molecules were previously evaluated in H_1R and H_2R, and the compound **1i** was not evaluated in H_3R before. This is also the first report on the affinity data for these compounds through reporter gene assays.

Histamine stimulated a response that was 2.3 ± 0.1 fold over basal in the H_1-SRE-luciferase cells (pEC_{50} 6.78 ± 0.04; n=8, Figure 3a,b). This response was readily inhibited by mepyramine, a histamine H_1R antagonist, causing a parallel rightward shift of the histamine concentration response and yielding a pK_D value of 8.53 ± 0.05 (n=9). ICI162846 (an H_2R antagonist) and JNJ-7777120 (an H_4R antagonist) were

not able to antagonize the histamine response at concentrations of up to 10 µM. Clobenproprit (an H_3R/H_4R antagonist) caused a small rightward shift of the histamine response (pK_D 5.01 ± 0.11; n=9). Thus histamine responses are readily antagonized by mepyramine, but not ICI182846, clobenpropit or JNJ-7777120, confirming the presence of the H_1R in this cell line.[13]

Histamine also stimulated a response (pEC_{50} 7.50 ± 0.03; n=8) in the H_2-CRE-SPAP cells that was 3.8 ± 0.1 fold over basal (Figure 3c,d). This response was inhibited by the H_2R antagonist ICI162846 (pK_D 8.71 ± 0.04; n=9) whereas neither mepyramine nor clobenpropit nor JNJ-7777120 were able to cause a rightward shift of the histamine concentration response curve. This confirms the presence of the H_2R in this cell line.[13]

Histamine did not stimulate a response, nor inhibit the basal response, on its own in the CHO-H_3-CRE-SPAP cells, indicating the absence of a G_s-coupled stimulatory histamine receptor.[14] Histamine did however inhibit forskolin-induced CRE-SPAP production (pEC_{50} 6.66 ± 0.04; n=9) in a manner consistent with stimulation of a G_i-coupled histamine receptor (Figure 3e,f). This histamine G_i-induced agonist response was inhibited by clobenproprit (pK_D 9.18 ± 0.06; n=9). As expected, neither mepyramine nor ICI162846 were able to inhibit the histamine responses in this cell line. JNJ-7777120 cause a small rightward shift of the histamine-concentration response curve (pK_D 5.45 ± 0.07; n=4), very similar to that previously reported for the human H_3R (pK_D 5.29) and very different from that for the histamine H_4R (pK_D 8.40).[15] Thus the presence of the histamine H_3R was confirmed in this cell line.[14]

None of the LINS01 compound series stimulated any agonist responses in any of the cell lines (Figure 4). With the exception of **1a**, the LINS01 compounds were able to inhibit the H_3R histamine induced response to yield measureable pK_D values. The compound **1g** had the highest affinity (pK_D 7.18) followed by **1h** (pK_D 6.99; Figure 4, Table 1). Increasing concentrations of **1g** caused progressive rightward shifts of the histamine concentration response curve in keeping with competitive antagonism at the H_3R (Figure 4e). Three compounds did cause a small rightward shift of the histamine response in CHO-H_1-SRE-luciferase cells giving measureable pK_D values. Interestingly, **1a**, that did not have any measureable affinity for the H_3R, had a very weak affinity for the H_1R. None of the LINS01 ligands had any measureable affinity for the H_2R.

As the LINS01 molecules were originally designed to be similar to the H_4R selective antagonist JNJ-7777120,[9] it was expected that these molecules would also be H_4-selective. Indeed, two molecules (LINS01005 – **1d** and LINS01007 – **1e**) appear to have a dose-dependent anti-inflammatory effect in a murine mouse model of asthma that correlated with the H_4R affinity.[9,10] However, these studies have also shown that most of LINS01 molecules actually have higher affinity for the H_3R than the H_4R.[9,10] It was therefore important to know the pharmacological profile of these compounds for the human H_1R and H_2R.

The LINS01 compounds, together with mepyramine (selective H_1R antagonist), ICI 162864 (selective H_2R antagonist), clobenpropit (H_3R/H_4R antagonist) and JNJ-7777120 (H_4R antagonist) were studied using the functional read out of reporter gene assays in CHO cell lines stable expressing each of the human H_1R, H_2R and H_3R. For the G_q-coupled H_1R, an SRE-reporter was used, whereas for the G_s-coupled H_2R and G_i-coupled H_3R (both of which affect cAMP) a CRE-reporter was used.[13,14] Reporter gene assays have several advantages. They can be used to examine responses from receptors without the need for large extracellular or intracellular tags, which have the potential to alter the ligand-receptor binding or receptor-effector coupling respectively. The longer agonist incubation times (here, 5 h at 37 °C) means that the interaction between the ligand and the receptor is highly likely to reach equilibrium allowing the use of pharmacological analyses such as the rightward shift of agonist concentration responses (Gaddum equation)[13,14] to assess ligand affinity (often not possible with very short term second messenger responses such as calcium). Thus the efficacy of ligands (ability to stimulate a response) and the affinity of antagonist ligands can be assessed within one assay. Also unlike second messenger assays (e.g. calcium, cAMP), being downstream responses, reporter gene assays also read out from several different signaling cascades (e.g. cAMP and MAP kinase).[16]

None of the LINS01 compounds stimulated any agonist responses at any of the histamine receptors studied. Although each of the selective antagonists inhibited histamine responses with high affinity at their respective receptors, no inhibition of histamine responses were seen with the LINS01 compounds in the H_2R expressing cells. There is therefore no evidence for any ligand-receptor interaction for the LINS01 compounds with the H_2R. Three of the LINS compounds had a measureable affinity, albeit low, for the human H_1R. Of these, **1a** is most interesting in that this compound was the one compound from the LINS01 series that had no affinity for the H_3R. The compound **1a** therefore appears to have H_1R selectivity (although the H_1R affinity is very poor). All compounds with the exception of **1a** antagonized histamine responses yielding measurable pK_D values.

Looking into the SAR on the H_3R affinity, the importance of the *N*-alkyl group (R) is clear. The unsubstituted compound (**1a**) had no affinity for the H_3R although did for the H_1R. Substitution with either *N*-methyl (**1b**) or *N*-allyl (**1c**) groups led to improved H_3R affinity, suggesting there is an additional hydrophobic interaction of the R-group in the binding site. The *N*-phenyl group (**1d**) also decreased H_3R affinity, possibly due steric hindrance or by decreasing the basicity of the nitrogen. Thus the *N*-alkyl group is important for H_3R affinity, and possible lack of it important for H_1R affinity.

Several H_3R ligands reported in the literature present the *N*-arylpiperazine moiety such as DL-80[17] which present medium to low affinity to H_3R, but with certain selectivity over H_4R. However these compounds present a longer linker than the LINS01 compounds, and thus the position of the most basic nitrogen changes accordingly. Docking studies[18,19] suggest that a salt bridge between a basic nitrogen and Glu206 is a key interaction to the antagonist activity. Figure 5 shows the proposed interactions of several antagonists with H_3R, and the hypothetical interaction of **1g**. The alignment of UCL2190, DL-80 and **1g** (Figure 5) suggests that the nitrogens supposed to interact with Glu206 and the aromatic rings of these molecules can occupy the same positions in space, supporting this hypothesis.

We have previously shown that only compounds **1e** and **1f** showed any measureable affinity for the H_4R, suggesting that the R' group may play the role in the H_4R affinity. Compound **1e** also presented similar pK_i for both receptors (~6.0).[10] The high homology between these two receptors explains the non-selectivity of several ligands from literature. It is known that H_3R and H_4R share two highly conserved amino acids in the binding site, an aspartate (Asp114 and Asp94, respectively) and an glutamate (Glu206 and Glu182, respectively), which interact with the primary amine and imidazole, respectively, of histamine. This explains the higher affinity of histamine for these receptors than for H_1R and H_2R.[20] The interaction with Glu206 generally determines the affinity of H_3R ligands and possibly the low affinity of these ligands to H_1R and H_2R, since Glu206 is not present in these receptors. By observing the present results, we believe that the more basic nitrogen of LINS01 compounds interact with Glu206 by analogy.

The results also suggest that some volume in the R' region is tolerated by H_3R. As can be noted in Table 1, compounds **1e**-**1h** present a substituent in R' and the binding affinities kept similar. The presence of the bulky *t*-butyl group in **1h** maintained the binding affinity value ~7.0, corroborating this hypothesis. However, the more polar methoxy group led to better affinity (pK_D 7.18), suggesting that polar atoms can improve the potency without increasing lipophilicity indeed. Molecules with excessive lipophilicity can lead to pharmacokinetic issues in the development stage, and thus should be avoided at initial stages.[21]

Ligand efficiency metric analysis (see Supporting Information) is a suitable approach to evaluate the contribution of each atom of a given molecule to the binding affinity.[21,22] The concept of ligand efficiency (LE) arouse from the need to measure whether a potency of a ligand derives from an adequate fit with the target or simply by doing many nonspecific interactions with it.[23] Literature considers that a good LE value to a lead-like molecule is at least >0.3, while market drugs present an average LE value >0.45.[21,22] All LINS01 compounds presented LE >0.3 regarding H_3R affinity, and thus can be considered promising compounds for further development. Compounds **1b**, **1f** and **1g** must be highlighted due to their high LE values (>0.52), and with exception of the *N*-phenyl derivatives **1d** and **1i**, the LINS01 molecules present LE values comparable to drugs that are doing well in the market. The fit quality (FQ) is a size-independent measurement of LE, i.e. it allows direct comparison of the values independently from the molecular size.[24] Again, the LINS01 compounds present very good values for FQ and are considered interesting lead compounds.

Derivations of LE concept considering the lipophilicity also appeared, such as the lipophilicity-corrected ligand efficiency (LELP) and the lipophilic ligand efficiency (LLE), in order to ponder the LE with the lipophilicity (mainly measured by clogP, see Supporting Information).[25,26] Excessive hydrophobicity should be avoided during drug discovery process because it limits the water solubility (which directly impacts on pharmacokinetics) and favors nonspecific interactions with biological proteins. The optimum LELP values are considered in the range from -10 to +10, while LLE values should be between 5 and 7.[21] Once again, the *N*-phenyl derivatives **1d** and **1i** showed LELP values outside the desired values, indicating that the phenyl group led to excessive lipophilicity. This lipophilicity can be verified by the high clogP values for these molecules. Considering that the lipophilicity usually increases during the development,[25] these molecules are not promising for further modifications, or these modifications should consider more hydrophilic groups. The LLE value of **1g** is within the optimal value, indicating that the insertion of methoxy group in R' led to improved affinity and contributed to reduce the lipophilicity of the molecule at the same time, leading to a clogP value similar to that from the non-substituted molecule **1b**.

Group efficiency (GE) is also a metric value to evaluate the contribution of an inserted group to the potency.[27] LINS01 molecules present a common dihydrobenzofuranyl-methylpiperazine core with structural modifications in the basic nitrogen (R) and in the aromatic ring (R'). As can be noted in Tables 1 and 2, modifications in the R-group lead to important variations in the binding affinities. The presence of *N*-methyl group led to important improvement in the affinity to H_3R (GE = 2.29), indicating that this group should be maintained to good H_3R affinity. The *N*-

allyl group also increased the affinity, but as it present 3 heavy atoms its efficiency was lower than *N*-methyl (GE = 0.85). In counterpart the *N*-phenyl group of **1d** presented low GE value. The higher the GE value, the higher the contribution of the group to increase the potency. For example, the addition of a group with one heavy atom (HA = 1) and a GE value of 0.52 in a molecule with molecular weight <300 means an increase of 2.3-fold in potency.[21,27] This means that the *N*-methyl group contributes very importantly to the binding affinity, while the *N*-phenyl group neither and thus its insertion in the molecule do not justify. The contribution of the R' group in the potency, as indicated by GE values, is less prominent. In the case of chlorine atom in **1e**, negative GE value was observed, stating the negative contribution of this atom to the affinity. The best contribution was given by the methoxy group of **1g** (GE = 0.35).

In summary, the dihydrobenzofuranyl-methylpiperazine (LINS01) molecules are selective H_3R antagonists with no intrinsic activity at either the human histamine H_1R, H_2R or H_3R and no, or extremely poor, affinity for the H_1R or H_2R. Although the best ligands (**1g** and **1h**) have greater than 100-fold H_3R selectivity (over H_1R or H_2R), their affinities for the human H_3R (66 nM and 100 nM) clearly show potential scope for further improvement with different chemical modifications. As future directions, this chemical scaffold should be used to design novel derivatives in order to increase the affinity to H_3R taking advantage on the selectivity profile of such compounds.

Conflicts of interest

The authors declare none.

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Figure Captions

Figure 1. Structure of LINS01 compounds.

Figure 2. Reagents and conditions. **a.** Phenol, allyl bromide (2 eq.), K_2CO_3 (2 eq.), acetone, 60 ºC,))) 4h or conventional 12 h; **b.** 200 ºC, MW (300 W max., 300 psi max.), DMF, 1 h; **c.** I_2 (1.1 eq.), water, 4-8 h; **d.** Piperazines (NH, *N*-Me, *N*-allyl, *N*-Ph, 2-4 eq.), K_2CO_3 (1 eq.), THF, 80 ºC, 24 h *or* 1-phenylpiperazine (1.1 eq.), MW (300 psi max, 300 W max), 120 ºC, 1.5 h.

Figure 3. Reporter gene production in response to in the absence and presence of different antagonist ligands in CHO-H1-SRE-luciferase cells (**a** and **b**), CHO-H2-CRE-SPAP cells (**c** and **d**) and CHO-H3-CRE-SPAP cells (**e** and **f**). Bars represent basal CRE-SPAP production, that in response to 10 µM histamine, 10 µM PDBU, 3 µM forskolin, or antagonist alone. Data points are mean ± SEM of triplicate determinations. These single experiments are representative of 4 (**a**), 8 (**b**), 4 (**c**), 8 (**d**), 4 (**e**) and 8 (**f**) separate experiments.

Figure 4. Reporter gene production in response in response to different LINS01 ligands CHO-H1-SRE-luciferase cells (**a** and **b**), CHO-H2-CRE-SPAP cells (**c** and **d**) and CHO-H3-CRE-SPAP cells (**e** and **f**). Bars represent basal CRE-SPAP production, that in response to 10 µM histamine, 10 µM PDBU or 3 µM forskolin alone. Data points are mean ± SEM of triplicate determinations. These single experiments are representative of 4 separate experiments in each case.

Figure 5. Proposed interactions of H_3R ligands with Glu206 (blue dashed lines). (**A**) UCL2190; (**B**) DL-80; (**C**) LINS01009 (**1g**); (**D**) Alignment of UCL2190 (yellow), DL-80 (purple) and LINS01009 (grey) considering the aromatic ring and basic nitrogen involved in the interaction with Glu206.

Tables

Table 1. pK_D values for the human H_1R, H_2R and H_3R.

Compounds	H_1R pK_D (n)	H_2R pK_D (n)	H_3R pK_D (n)
1a	5.27 ± 0.10 (4)	<5.00 (4)	<5.00 (4)
1b	5.66 ± 0.10 (4)	<5.00 (4)	6.67 ± 0.07 (5)
1c	<5.00 (4)	<5.00 (4)	6.87 ± 0.07 (5)
1d	<5.00 (4)	<5.00 (4)	5.61 ± 0.13 (4)
1e	<5.00 (8)	<5.00 (8)	6.31 ± 0.06 (8)
1f	<5.00 (4)	<5.00 (4)	6.80 ± 0.10 (6)
1g	<5.00 (10)	<5.00 (10)	7.18 ± 0.04 (15)
1h	<5.00 (8)	<5.00 (8)	6.99 ± 0.02 (8)
1i	5.87 ± 0.04 (8)	<5.00 (8)	6.29 ± 0.03 (8)
mepyramine	8.53 ± 0.05 (9)	<5.00 (8)	<5.00 (9)
ICI162846	<5.00 (9)	8.71 ± 0.04 (9)	<5.00 (9)
clobenpropit	5.01 ± 0.11 (9)	<5.00 (8)	9.18 ± 0.06 (9)
JNJ-7777120	<5.00 (4)	<5.00 (4)	5.45 ± 0.07 (4)

pK_D values for ligands as determined in reporter assays from the rightward parallel shift of a histamine concentration response curve. Values are mean ± SEM of n separate determinations.

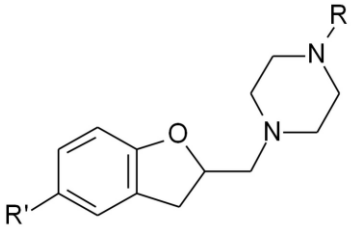
Table 2. Ligand metric analyses of the LINS01 compounds considering the H_3R affinity.

Compounds	clogP	LE	LELP	LLE	FQ	GE
1a	1.30	>0.43	<3.04	>3.70	>0.56	-
1b	1.90	0.54	3.53	4.77	0.74	>2.29[a]
1c	2.54	0.50	5.13	4.33	0.74	>0.85[a]
1d	3.59	0.35	10.28	2.02	0.59	>0.14[a]
1e	2.73	0.48	5.68	3.58	0.69	-0.49[b]
1f	2.32	0.52	4.48	4.48	0.74	0.18[b]
1g	1.93	0.52	3.73	5.25	0.78	0.35[b]
1h	3.58	0.46	7.85	3.41	0.74	0.11[b]
1i	4.02	0.37	10.73	2.27	0.65	0.93[c]
clobenpropit	2.67	0.62	4.25	6.51	0.98	-

[a]Calculated for the R-group based on **1a**.

[b]Calculated for the R'-group based on **1b**.

[c]Calculated for R'-group based on **1f**.



1a: R = H; R' = H (LINS01001)
1b: R = Me; R' = H (LINS01003)
1c: R = Allyl; R' = H (LINS01004)
1d: R = Ph; R' = H (LINS01005)
1e: R = Me; R' = Cl (LINS01007)
1f: R = Me; R' = Me (LINS01008)
1g: R = Me; R' = MeO (LINS01009)
1h: R = Me; R' = *t*Bu (LINS01010)
1i: R = Ph; R' = Me (LINS01012)