

Discovery of a novel highly selective histamine H₄ receptor antagonist for the treatment of atopic dermatitis

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Discovery of a novel highly selective histamine H4
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ABSTRACT

The histamine H4 receptor (H4R), a member of G-protein coupled receptor family, has been considered as a potential therapeutic target for treating atopic dermatitis (AD). A large number of H4R antagonists have been disclosed, but no efficient agents controlling both pruritus and inflammation in AD have been developed yet. Here, we have discovered a novel class of orally available H4R antagonists showing strong anti-itching and anti-inflammation activity as well as excellent selectivity against off-targets. Pharmacophore-based virtual screening system constructed in house successfully identified initial hit compound **9**, and the subsequent homology model-guided optimization efficiently led us to discover pyrido[2,3-*e*]tetrazolo[1,5-*a*]pyrazine analogue **48** as a novel chemotype of potent and highly selective H4R antagonist. Importantly, orally administered compound **48** has a remarkable efficacy on anti-pruritus and anti-inflammation with favorable pharmacokinetic (PK) profile in several mouse models of AD. Thus, these data strongly suggest that our compound **48** is a promising clinical candidate for treatment of AD.

INTRODUCTION

Atopic dermatitis (AD) is a pruritic inflammatory skin disease, which is characterized by eczema, epidermal thickness, itching and predominant expression of the inflammatory Th2 cytokines including IL-4, IL-5 and IL-13.^{1, 2} Especially, mechanical skin injury caused by scratching aggravates the defect in skin barrier function and leads to the release of pro-inflammatory cytokines causing skin inflammation.³

The current first-line therapy for AD includes topical corticosteroids, topical calcineurin inhibitors, and adjuvant treatment of antihistamines (H1R antagonists) for pruritus. However, these agents cause dose-dependent side effects or have little efficacy on itching.^{4, 5} Recently, new biologics such as anti-IL-4R α antibody and anti-IL-31RA antibody are also being developed for AD as an injection.^{6, 7} However, orally available drug with sufficient efficacy for AD is still desired to be developed as widely-used one. Therefore, the large unmet medical need in the treatment of AD is the development of an orally available systemic agent to mitigate pruritus and inflammation in skin, simultaneously.⁸

Histamine is closely associated with pruritus and inflammation in AD.^{5, 9} Its receptors belong to a class of G-protein coupled receptors (GPCRs), and four subtypes have been identified so far (H1R, H2R, H3R, and H4R).^{10, 11} In particular, H4R, the most recently discovered subtype, is highly expressed in variety of organs and the several types of immune cells such as eosinophils, dendritic cells and Th2 cells that play important roles in allergic immune responses and diseases.^{12, 13} H4R knockout mice showed the attenuated skin inflammation in AD models,¹⁴⁻¹⁶ and its antagonists substantially reduced Th2 cytokine production, pruritus and skin

inflammation in several murine models mimicking human AD.^{15, 17, 18} These findings suggest that H4R is a promising therapeutic target for the treatment of AD.

Up to now, a number of H4R antagonists have been discovered through ligand-based or homology model-based virtual screening.¹⁹⁻³⁰ Among the representative H4R antagonists (Figure 1), compound **4**, **5** and **6** have advanced into Phase II clinical study. Compound **4** (Toreforant, JNJ-38518168)²² has entered in clinical trials for the treatment of asthma, rheumatoid arthritis, and psoriasis. It was known that compound **4** (toreforant) could not inhibit histamine-induced scratching in mice associated to AD.²² Although compound **5** (JNJ-39758979)²³ has showed a profound anti-pruritic effect in phase II clinical trial for moderate AD patients, the further development was discontinued due to drug-induced agranulocytosis caused by off-target effects.²³ Compound **6** (ZPL-3893787)²⁴ with very similar structure to **5** substantially reduced eczema area and severity index (EASI) score in Phase IIa proof-of-concept study, but its anti-pruritic efficacy was not statistically significant.³¹ Meanwhile, another aminopyrimidine-type H4R antagonists, compound **7** (A-940894)²⁶ and **8**²⁷ exhibited cross-activity on serotonin 5-HT₃ receptor (5-HT₃R) which shares very similar ligand recognition pattern with H4R.²⁷ Since 5-HT₃R is widely expressed in the central and peripheral nervous system and controls various physiological functions, its inhibition may cause CNS-related side effects.³²⁻³⁴ Thus, the development of oral H4R antagonist with high selectivity having dual effect of anti-pruritus and anti-inflammation in AD still remains a big challenge.

In this work, we developed a novel class of highly selective H4R antagonist. For a new scaffold hopping, a stratified virtual library with structural diversity was established from large sized commercially available database, and a pharmacophore model-based virtual screening was carried out. And then, the homology model-guided optimization of compounds followed to

improve the inhibitory activity for H4R and selectivity over off-targets, especially against 5-HT3R. The medicinal chemistry campaign resulted in the lead compound **48** presenting profound anti-pruritic and anti-inflammatory efficacies in pruritogen-induced acute itching models and oxazolone-induced mice AD model. This report describes the discovery and structure-activity relationship (SAR) of pyrido[2,3-*e*]tetrazolo[1,5-*a*]pyrazine derivatives as new H4R antagonists, and a structural rationale to achieve the selectivity against 5-HT3R based on computational models.

RESULTS AND DISCUSSION

Pharmacophore model-based virtual screening

In order to find a novel chemotype of H4R antagonists, we took advantage of a pharmacophore model-based virtual screening. Figure 2 shows a schematic representation of the virtual screening cascade. Initially, we constructed a structurally diverse representative library for a new scaffold hopping by conducting iterative clustering with Tanimoto similarity (similarity coefficient ≥ 0.9) in the ZINC12 database,³⁵ which is a free database of commercially-available compounds for virtual screening. The total size of in-house set for virtual screening (~500k molecules) is about 2% of the original database (~22 million molecules), but it still maintains structural and chemical diversity analyzed by principal component analysis of molecular fingerprints, HB donor, acceptors, rotatable bonds, MW, and clogP as compared to the original database. It allowed us to efficiently screen a huge size database without reducing the chance to find new chemotypes. In the next step, we generated a pool of pharmacophore models from known H4R antagonists depicted in Figure 1. The lowest energy conformation of each molecule was regarded as a bioactive conformation, and potential pharmacophore hypotheses were

carefully assigned based on our prior knowledge acquired by scaffold alignment and SAR of the published H4R antagonists. A total of 8 pharmacophore models with four features were created and used for virtual screening. Virtual hit structures with fit-score better than 2.7 were visually inspected, and 291 compounds with novel scaffolds were subjected to competitive radioligand binding assay for H4R. Among hit compounds with IC₅₀ value less than 10 μ M (data not shown), we have identified [1,2,4]triazolo[4,3-*a*]quinoxaline compound **9** (Table 1) which bears a novel tricyclic scaffold distinct from known H4R antagonists.

H4R homology model-guided optimization

Since H3R has the highest sequence homology with H4R among histamine receptors and many H3R antagonists also exhibit inhibitory activity for H4R, we included the competitive binding assay for H3R as well as H4R in our primary screening.³⁶ As an initial hit compound, **9** displayed good inhibitory activity for H4R and selectivity over H3R, 2.3 μ M and >100 μ M of IC₅₀, respectively (Table 1). To obtain the detailed insight for structure-guided rational design, we built a H4R homology model based on the recently published H1R crystal structure (3RZE.pdb).³⁷ The sequence identity and similarity between H4R and H1R is 24% and 47%, respectively. The homology model of H4R was constructed as similar as possible to the template, and was refined using MD simulation as detailed in experimental section.

The predicted docking pose of **9** in H4R homology model indicated a small space around 8-position of the [1,2,4]triazolo[4,3-*a*]quinoxaline scaffold (Figure 3a). Thus, 8-position of compound **9** was a good starting point for structural modification to improve activity. Targeting the small pocket at 8-position, we first introduced a subset of electron-withdrawing group or donating group (**10-12**). Surprisingly, **10** substituted with a chlorine atom displayed about 100-

fold enhanced inhibitory activity for H4R ($IC_{50} = 0.022 \mu M$) compared to **9**. Compound **11** with nitro group and **12** with methyl group also showed the slightly improved activity compared to **9**, likely due to filling effect of small pocket. The dramatic improvement of **10** in inhibitory activity compared to **11** and **12** strongly suggested the possibility of halogen bonding.

The predicted binding mode of **9** also suggests another important interaction, hydrogen bond between nitrogen in X position (Table 1) and amide of Gln347 (Figure 3a). To validate the hypothesis from the predicted binding mode in homology model, we synthesized and evaluated compound **13** lacking hydrogen bond acceptor capable of interacting with Gln347. As expected, the inhibitory activity of **13** was decreased extremely, and it may be due to the absence of hydrogen bonding source interacting with Gln347.

Although **10** showed potent inhibitory activity against H4R and acceptable selectivity over H3R, it had moderate metabolic stability in human and mouse liver microsome and decreased FaSSIF solubility compared to **9** (Table 1). To investigate the effect of nitrogen inserted in the A ring on activity, metabolic stability and solubility, we synthesized **14** and **15** with nitrogen atom in the A ring of **9**. Intriguingly, compound **15** with nitrogen inserted at 6-position of the A ring had markedly improved metabolic stability in human and mouse liver microsome while maintaining inhibitory activity against H4R and selectivity over H3R. This effect of nitrogen insertion was also confirmed in the experiment with **10** and **16**. Nitrogen inserted compound **16** displayed a strong inhibitory activity against H4R and the better selectivity against H3R, metabolic stability, and FaSSIF solubility compared to **10**. These improvements in metabolic stability and solubility are presumably due to the increased hydrophilicity of **16** (experimental $\log D_{7.4} = 0.95$) compared to **10** (experimental $\log D_{7.4} = 2.12$).

The predicted binding mode of **16** was described in Figure 3b. The basic amine of *N*-methylpiperazine is expected to form ionic interaction with Asp94, which is essential for binding to H4R and conserved in other aminergic GPCR ligands.³⁸ In addition, the methyl group attached to piperazine occupies a small hydrophobic pocket composed of aromatic residues, Tyr72, Phe344, and Trp348 (Figure 3b). The nitrogen atom at X-position makes a strong hydrogen bonding with Gln347 as described previously. The pyrido[2,3-*e*][1,2,4]triazolo[4,3-*a*]pyrazine ring is shown to favorably form pi-pi interaction with Tyr95, Trp316, Tyr319, and Phe168 in extracellular loop 2. It also would form OH-pi interaction with Tyr319 and CH-pi interaction with Leu175. As shown in Figure 3b, the chlorine atom at 8-position is predicted to be located within the distance where chlorine atom could form the halogen bond with the carbonyl oxygen of Ser179. The predicted O-Cl distance is very close to the optimal distance 3.3Å for such interactions.^{39, 40} The inhibitory activities of **15-18** strongly support this interaction model. The IC₅₀ values of **15-18** were excellently correlated with the strength of the halogen bond by the magnitude of sigma-hole: H << Cl < Br < I. The significant decrease in H4R inhibitory activities of **19-21** may be attributed to loss of the halogen bond. Meanwhile, the introduction of small size substituents at the 7- and 9-positions (**22-26**) decreased inhibitory activity compared to compound **16**, which allowed us to confirm that there is limited space around those positions as expected in our binding model. As a result, the initial modification of the A ring part based on the homology model led to 8-chloropyrido[2,3-*e*][1,2,4]triazolo[4,3-*a*]pyrazine derivative **16** presenting good inhibitory activity against H4R, selectivity over H3R, and favorable metabolic stability and solubility.

Further optimization of amine group

A positively charged amine, an essential pharmacophoric feature of H4R ligands for the receptor binding, makes ionic interaction with Asp94 conserved in aminergic GPCRs. To investigate the effect of the basicity on the ionic interaction, we first replaced *N*-methylpiperazine (**16**, ACD pKa = 9.9 ± 0.4) with piperazine (**27**, ACD pKa = 8.9 ± 0.8), which resulted in slightly decreased inhibitory activity against H4R (Table 2). In addition to basicity, the methyl group itself is also predicted to contribute to inhibitory activity by optimal fitting to a small hydrophobic pocket composed of aromatic residues (Figure 3a-b). The size effect of methyl group was illustrated by the loss in inhibitory activity of **28** substituted with cyclopropyl causing steric clash with Tyr72. Additional methyl group in piperazine (**29-30**) and the homopiperazine derivatives (**31-32**) also resulted in decrease of inhibitory activity against H4R. It indicates that the binding site around positively charged amine would be spatially limited. The replacement of *N*-methylpiperazine with (*R*)-3-amino pyrrolidines as in compounds **33-35** resulted in a reduction in H4R inhibitory activity likely due to the repulsion between Phe344 and the puckered conformation of pyrrolidine ring. Interestingly, compound **37** with 3-methylamino-azetidine, whose three-dimensional coordinate of positively charged amine is very close to that of *N*-methylpiperazine, showed comparable inhibitory activity to that of **16**. However, addition or removal of a methyl group in 3-methylamino-azetidine caused a loss of inhibitory activity against H4R as shown in compound **36** and **38**. The lower activities of **39-40** against H4R relative to **16** are expected as a result of the small steric hindrance preventing the Asp94 from optimally interacting with the basic amine. Although **41** with the bicyclic piperazine group showed similar inhibition activity against H4R compared to **16** with the methylpiperazine group, it demonstrated rather lower selectivity against H3R. Considering both inhibitory activity for H4R and selectivity over H3R,

we concluded that the *N*-methylpiperazine (**16**) and *N*-methyl-3-amino-azetidine (**37**) are optimal amine substituents for pyrido[2,3-*e*][1,2,4]triazolo[4,3-*a*]pyrazine scaffold.

Improvement of selectivity based on 5-HT3R homology model

In order to explore the possibility of side-effects coupled with off-target binding, we carried out competitive binding assay of compound **16** against a broad panel of GPCR, ion channel, and transporters. Intriguingly, **16** showed very strong inhibitory activity against both 5-HT3R ($IC_{50} = 0.057 \mu M$) and H4R ($IC_{50} = 0.077 \mu M$) while it did not exhibit any significant inhibitory activity against other 55 membrane proteins (data not shown). This is consistent with the previous report for fragment library screening that illustrated remarkable similarities in ligand recognition between H4R and 5-HT3R.²⁷ To obtain structural clues for selectivity against 5-HT3R, we constructed a human 5-HT3R homology model based on X-ray crystallized mouse structure. Figure 4a (left panel) displays the predicted docking pose of **16** in 5-HT3R homology model. In the predicted binding mode, the pyrido[2,3-*e*][1,2,4]triazolo[4,3-*a*]pyrazine scaffold is expected to form pi-pi interaction with Trp85, CH-pi with Ile66 and strong hydrogen bond with Arg87. Moreover, the halogen bonding between the chlorine atom and side chain oxygen of Asp199 is also observed at a distance 3.5 Å. The triazole moiety is embedded between Val202 and Met223 via CH-pi or hydrophobic interaction. Compound **16** showed very similar interaction patterns in 5-HT3R with those in H4R. However, the basic amine of *N*-methylpiperazine is oriented for making cation-pi interactions with Trp178 and Tyr229 in 5-HT3R, while it forms ionic interaction with Asp94 in H4R. These interactions of basic amine in 5-HT3R are consistent with previous observation that a cation-pi interaction between the primary amine of serotonin and Trp178 (Trp183 in mouse 5-HT3R) critically contributes to ligand recognition of 5-HT3R.^{41, 42}

In aqueous media, non-covalent interactions between protein and ligand must compete with the desolvation penalty of ligand. The negative contribution on binding affinity of desolvation energy is further maximized when the ligand binds to the aromatic box such as Trp178, Tyr229 and Tyr148 in 5-HT3R. In the previous study, Salonen and co-workers⁴³ reported that the stepwise *N*-alkylation of ammonium ions making cation- π interactions at active site of Factor Xa dramatically improved binding affinity, which was related to desolvation cost of ligands.⁴³ These reports prompted us to calculate desolvation energies of basic amines including methylpiperazine and *N*-alkylated azetidines. As shown in Figure 4a (right panel), the desolvation energies, minus of solvation energies, are strongly correlated with % inhibition at 10 μ M against 5-HT3R. In addition, the negative contribution of hydrophilicity on the inhibitory activity against 5-HT3R is supported by calculated ACD logD_{7.4} values. Surprisingly, the *N*-methyl-3-amino-azetidine of **37** displayed significantly reduced inhibitory activity against 5-HT3R compared to **16** while maintaining potent inhibitory activity against H4R. The *N*-methyl-3-amino-azetidine of **37** has much higher desolvation energy than *N*-methylpiperazine of **16** and the high desolvation cost of **37** could not be overcome by the energy gain in cation- π interactions with 5-HT3R. On the contrary, *N*-methyl-3-amino-azetidine of **37** forms strong ionic interaction with Asp94 in H4R, and it would be sufficient to compensate for the desolvation penalty of the basic amine. All the molecular pairs in Table 3 indicate that the *N*-methyl-3-amino-azetidine group has better selectivity against 5-HT3 than *N*-methylpiperazine without exception.

From the predicted binding mode and interaction pattern of **16** in H4R and 5-HT3R (Figure 4b), we hypothesized another modification point to improve selectivity against 5-HT3R. In the interaction modes in H4R and 5-HT3R, triazole of **16** (I part in Figure 4b) forms a π - π

interaction with Tyr95, Phe168 and Tyr319 in H4R while it makes hydrophobic interaction via CH- π interaction with Met223 and Val202 in 5-HT3R. To utilize this discrepancy in interactions with H4R and 5-HT3R for selectivity improvement, we modified triazole ring of **16** and summarized assay results in Table 3. Compound **43** and **46** showed decreased inhibitory activities against H4R relative to those of the parent **16** and **37** without improving selectivity over 5-HT3R. It was thought that the relative positions of three nitrogen atoms on triazole ring negatively affect to the displaced π - π interaction with Phe168 (Figure 3). However, the replacement of triazole with a tetrazole ring (**44** and **47**) clearly showed the improvement of selectivity over 5-HT3R without loss of inhibitory activity against H4R. The tetrazole ring is expected to weaken hydrophobic or CH- π interaction with Met223 and Val202 in 5-HT3R relative to triazole ring. Eventually, we derived compound **48** as a lead compound by introducing bromine instead of chlorine atom to reinforce halogen bond, which exhibits strong inhibitory activity against H4R and excellent selectivity over H3R as well as 5-HT3R.

Chemistry

Compounds **10-48** were synthesized through synthetic routes summarized in Schemes 1-6. Intermediates, 2,3-dichloropyrido[2,3-*b*]pyrazine analogues were obtained from corresponding pyridine-2,3-diamines and diethyl oxalate, and followed by chlorination (Scheme 1). Triazole ring formation progressed from condensation with corresponding hydrazinyl compounds and trimethyl orthoformate (or triethyl orthoformate). And then, amination was performed from respective amines with or without base (General procedure A in Scheme 2).²⁰ According to chemical reactivity of intermediates shown in Scheme 2, triazole ring formation and amination was conducted in reverse order (General procedure B). **13** was prepared as described in Scheme 3.²¹ **13b**, which was a mixture of regioisomer was used without further purification. Desired **13c**

was purified by column chromatography after the chlorination. The final step was performed through copper-catalyzed amination. **27-42** were prepared as shown in Scheme 4. **29**, **33**, **36**, and **39** were obtained by additionally reductive alkylation from **30**, **35**, **37**, and **40**, respectively. **43-48** were prepared by Scheme 5 and 6. Intermediate, **43f** was produced by bromination, palladium-catalyzed cyanation, followed by ring closure, and chlorination. Each final step progressed in the same way as Scheme 2. **44**, **45**, **47**, and **48** were obtained from cyclization using sodium azide in good yield (69-81%). It is recommended to be aware that intermediates **10b**, **12b**, **13d**, **14d**, and **43f** should be handled carefully during synthesis because their molecular structures are similar to known substances that induce chloracne.⁴⁴

Profile of compound **48**

The lead compound **48** displayed a potent inhibitory activity against human H4R with an IC₅₀ of 0.027 μ M while it did not show any noticeable binding affinity to other subtypes of histamine receptors, H1R, H2R, and H3R (Supporting Information, Table S1). In addition, the competitive binding assay against a wider panel of GPCR, ion channel, and transporters at the concentration of 10 μ M revealed that compound **48** is highly selective for H4R (Table S1). The inhibitory activity of **48** against mouse H4R (IC₅₀ = 0.29 μ M) was about 10 times weaker than that for human H4R. The pharmacological differences in species have been known to result from significant sequence variation between human and mouse H4R (68%).⁴⁵ In order to evaluate the functional behavior of compound **48** for human and mouse H4R, [³⁵S]GTP γ S binding assay was performed in parallel with H4R agonist histamine and H4R antagonist thioperamide (mouse) or JNJ-10191584⁴⁶ (human). Tested in antagonist mode, compound **48** showed a potent antagonism for both human and mouse H4R with IC₅₀ value of 0.028 μ M and 0.69 μ M, respectively, while

no activity for human and mouse H4R was observed in agonist mode (data not shown). The degrees of protein binding in human and mouse plasma are 73.5% and 65.2%, respectively.

The pharmacokinetic profiles of compound **48** and its precursors were assessed in ICR mice after oral administration (Table 4 and Figure 5). The plasma exposure of compound **16** at 50 mg/kg was dramatically increased compared to compound **10**, possibly due to improved mouse metabolic stability by inserting nitrogen atom in 6-position of the A ring (Table 1). Its plasma levels reached a $C_{\max}$ of 4.63 $\mu\text{g/mL}$ at 0.5 h (Table 4). At the same dose, compound **48** showed higher plasma exposure than **16** with a maximum plasma levels reaching a $C_{\max}$ of 7.69 $\mu\text{g/mL}$ at 0.5 h. There was no difference in pharmacokinetic properties between ICR and BALB/c mice (data not shown). The dose-dependent increase in systemic exposures (AUC) was also confirmed at doses of 2, 10 and 50 mg/kg.

***In vivo* efficacy of compound 48**

Because histamine is a major mediator in the pruritus of AD,⁴⁷⁻⁴⁹ anti-pruritic effect of compound **48** was evaluated using the histamine-induced pruritus model. Intradermal injection of histamine increased the incidence of scratching in mice, and the number of scratching was significantly decreased by oral administration of compound **48** in a dose dependent manner (Figure 6a). In the [³⁵S]GTP γ S functional assay, compound **48** showed the inhibitory activity against mouse H4R with an IC_{50} of 0.69 μM , and it exhibited about 50% reduction of number of scratching at 10 mg/kg in the histamine-induced pruritus model where the free concentration of compound **48** is 1.89 μM . Therefore, the plasma free concentration required for 50% reduction of scratching is about 3-fold higher than the *in vitro* IC_{50} value. Figure 6a clearly shows that the anti-pruritic efficacy of compound **48** is correlated well with its plasma free concentration and *in*

vitro activity. Compound **48** completely suppressed the histamine induced scratching behavior at 50 mg/kg. Anti-pruritic effect of compound **48** was also evaluated in the different acute pruritus mouse models mediated by substance P and compound 48/80 (C48/80) known as histamine-dependent triggers.⁴⁷⁻⁴⁹ Oral administration of compound **48** dramatically decreased scratching behavior at 50 mg/kg (Figure 6b-c) in both models as observed in histamine-induced model. These results suggest that our lead compound **48** efficiently suppresses itching induced by various pruritogens associated with AD.

We also evaluated anti-pruritic and anti-inflammatory efficacy of compound **48** in the oxazolone-induced murine AD model displaying structural barrier alterations and clinical signs highly similar to human AD.¹⁶ First, chronic AD was induced by repeated challenge with oxazolone in the ear skin of BALB/c mouse as described in experimental section. And then, compound **48** was orally administered twice a day for 3 weeks, and its anti-pruritic and anti-inflammatory efficacy were evaluated by counting the number of scratching and by measuring ear thickness, respectively. Challenge of oxazolone to the mice significantly increased scratching behavior and ear thickness, whereas treatment of compound **48** at 50 mg/kg significantly reduced scratching behavior and ear thickness (Figure 7a-b). In addition, the histological examination of challenged ear skin also revealed that compound **48** profoundly reduced epidermal thickness and hyperplasia shown in AD skin lesions (Figure 7c). In summary, our lead compound **48** presented dual anti-pruritic and anti-inflammatory effects in the *in vivo* pruritus and dermatitis murine models.

CONCLUSION

Here, we have detailed the lead identification and optimization process of a novel class of potent and highly selective H4R antagonist. Firstly, hit compound **9** with a novel tricyclic scaffold has been found from virtual screening using a set of knowledge-based pharmacophore models. The construction of representative subset library by iterative clustering allowed us to efficiently screen the large ZINC database without loss of structural diversity. The molecular modification was supported by homology model-based design, leading to the rapid identification of compound **16** with dramatically improved inhibitory activity for H4R and metabolic stability. However, compound **16** exhibited the selectivity issue for 5-HT3R as shown in many developed H4R antagonists due to the very similar ligand recognition pattern of 5-HT3 receptor with H4R. A structural clue to avoid this selectivity issue was obtained by comparison of interaction modes predicted in homology model of H4R and 5-HT3R, and the desolvation penalty of positively charged amine forming cation- π interaction as in 5-HT3R could be a good target point for selectivity improvement. To our best knowledge, this is the first report improving inhibitory activity and selectivity based on structural rationale derived from homology modelling of H4R and 5-HT3R. The final lead compound **48** has a structurally unique scaffold and was illustrated as a highly potent and selective H4R antagonist without noticeable activity on off-targets including other histamine receptors and 5-HT3R. In the *in vivo* test, **48** exhibited a good pharmacokinetic profile and remarkably reduced the number of scratching in acute-itching murine models induced by pruritogens such as histamine, substance P and C48/80. Furthermore, compound **48** showed the significant anti-pruritic and anti-inflammatory efficacy in oxazolone-induced murine model mimicking human AD. In conclusion, compound **48** is expected to be a promising candidate for the treatment of AD.

EXPERIMENTAL SECTION

Chemistry

Commercially available reagents and solvents were used without further purification. Hit compound **9** was purchased from Synnovator. Thin layer chromatography (TLC) analyses were carried out on Merck Kieselgel 60F₂₅₄ plates or NH plates of Fuji Silysia, Ltd.. ¹H and ¹³C NMR spectra were verified by a Bruker DRX 300 (300 MHz), a Bruker Avance III 400 (400 MHz) using tetramethylsilane and residual solvents as internal standards. Chemical shifts (δ) are expressed as parts per million (ppm) and coupling constants (*J*) as herz (Hz). Resonance patterns are reported with the notation s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), td (triplet of doublets), br (broad peak), brs (broad singlet) and m (multiplet). Some carbon peaks of piperazine ring are frequently merged with DMSO peak or overlapped each other and not observed. Analytical reverse-phase ultra-high-performance liquid chromatography mass (UPLC-MS) was carried out on Ascentis Express C18 column (50 $\times$ 2.1 mm, 2.7 μ m) using Waters Acquity UPLC and PDA instruments and high-performance liquid chromatography mass (HPLC-MS) was carried out on ACE 5C18 column (50 $\times$ 4.6 mm, 5.0 μ m) using Waters 2767 LC/MS system. Normal phase and reverse-phase flash column chromatography were performed with flash cartridge with Yamazen medium pressure liquid chromatography (MPLC) system, FR-260 and Biotage SNAP cartridges equipped with Isora One, respectively. The yields reported were not optimized. The purities of all compounds are >95% at two wavelengths (225 and 254 nm) and are determined on Xbridge column (150 $\times$ 4.5 mm, 5 μ m) equipped with HPLC, Shimadzu LC-2010A system. Analytical conditions are as follows (Eluent A: CH₃CN - 1% to 100%, 0.1% TFA containing, gradient; Eluent B: H₂O - 99% to 0%, 0.1% TFA containing,

gradient; analytical time: 20 min; oven temperature, 25 °C; flow rate, 1.0 mL/min). High-resolution mass (HRMS) was measured with JEOL, JMS-700 spectrometer. Synthetic procedures and analytic data for compound **9-48** were described in Supporting Information.

Pharmacophore Model

The structures of seven known H4R antagonists except **4** (Figure 1) were built using the Schrödinger suite implemented in Maestro molecular modeling software.⁵⁰ After ionized at pH = 7.0 ± 2.0 using Epik module, the structures were minimized using the Polak-Ribiere-conjugate-gradient method with default parameters in MMFFs force field. The conformational searches were done using Systematic Pseudo-Monte Carlo (SPMC) method by applying the generalized Born/solvent accessible surface (GB/SA) continuum solvation model. The lowest energy conformation for each H4R antagonist was validated by analyzing distribution for torsional angles of similar structures stored in the CSD (Cambridge Structure Database).⁵¹ Based on prior knowledge of scaffold alignment and SAR analysis of analogues from original literature (data not shown), a pool of potential common feature pharmacophore hypotheses for each lowest energy conformation was created using Discovery Studio,⁵² assuming that the biologically active conformations are fairly close to the global minimum energy conformations. Five functional features were considered in assigning pharmacophore variants: hydrogen bond acceptor, hydrogen bond donor, hydrophobic group, aromatic ring, and positively charged group.

Library Preparation and Virtual Screening

A representative subset of all purchasable ZINC database was created using pipeline pilot.⁵³ All purchasable molecules of the ZINC12 database³⁵ containing ~22 million were sorted by ascending molecular weight. Tanimoto similarity index of the first ranked molecule and the rest

of molecules was calculated, and the molecules with similarity higher than a specified cutoff, 0.9 were classified into the same cluster. It was iteratively conducted to all remaining compounds of the database. Finally, the most diverse of 1,126 clusters were created with the least redundancy. As an in-house virtual screening set, total of 493,834 molecules were selected in proportion to the size of each cluster. Conformational database for virtual screening was generated in Discovery studio with default parameters (maximum 250 conformers and fast search method). Virtual screening for a pool of pharmacophore models was conducted with fast search method.

Homology Modeling and Molecular Docking

The H1R X-ray structure (pdb code: 3RZE) was selected as a template structure for H4R homology model and was obtained from the RCSB (Research Collaboratory for Structural Bioinformatics) Protein Data Bank (<http://www.rcsb.org/>).⁵⁴ The T4-lysozyme inserted to assist crystallization was removed and was not modeled. The amino acid sequence of human H4R (accession number: Q9H3N8) was retrieved in UniProtKB sequence database (<http://www.uniprot.org/>).⁵⁵ The target and template sequences were aligned using ClustalW method, followed by manual adjustment to avoid gaps in transmembrane. Three-dimensional structure of human H4R was generated with adjusted alignment result and default parameters in Prime of Schrödinger suite.⁵⁰ The loops were refined using the refine loops tool with the extended high-loop refinement procedure. Five models were generated and were scored by probability density function (PDF) total energy. The model with the lowest energy value, which indicates the highest similarity with the template, was selected as a final model. The lowest energy conformation of compound **3** was aligned to the 3D structure of doxepin, a ligand bound to H1R in X-ray structure. The initial structure of H4R–compound **3** complex was minimized using CHARMM implemented in Discovery studio with all backbone atoms fixed. Through the

subsequent molecular dynamics simulation performed with restraints on protein backbone atoms in the NPT ensemble for 10ns, we derived the final homology model of human H4R complexed with compound **3** which was used to generate pharmacophore finding hit compound **9**. The centroid coordinates of compound **3** within refined homology model were used for generating the receptor grid box to define ligand binding site for molecular docking. The hit compound **9** was docked into grid box using standard precision mode of Glide program in Schrödinger suite.⁵⁰ The OPLS_2005 force field and all default parameters were applied during docking calculation. The docking pose with the lowest Glide score was minimized again with CHARMM program and used for analogue docking. The human 5-HT3R homology model (accession number: P46098-1) of extracellular N-terminal domain that comprises the orthosteric ligand-binding site was built based on the mouse 5-HT3R X-ray structure (pdb code: 4PIR) in the same way as described in H4R homology model.

PAINS

All of the biologically tested compounds (Table 1-3) were checked for the presence of PAINS substructures using PAINS-Remover online web server.⁵⁶ None of the compounds were classified as a pan-assay interference compound.

Calculation of Solvation Energy

The conformation search of the protonated amine structure was performed utilizing MMFFs force field. Conformations within 2 kcal/mol of the observed lowest conformation were further optimized with MP2/6-31G** basis set using the density functional theory (DFT) program Jaguar in Schrödinger package.⁵⁰ For each amine structure with the lowest single point energy,

electrostatic solvation energy in water was computed using the standard Poisson-Boltzmann continuum solvation model in Jaguar program.

Histamine Receptor Radioligand Binding Assay

Competitive radioligand binding assays for H3R and H4R were performed using the commercially available membrane preparation of CHO-K1 cells that express human or mouse H4R and human H3R (Perkin Elmer, ES-393-M400UA for human H4R, ES-392-M400UA for human H3R, and multispan, Cm1030 for mouse H4R). [³H]Histamine (Perkin Elmer, NET732) was used for H4R binding assay and [³H]N α -methylhistamine (Perkin Elmer, NET1027) was for H3R binding assay. All binding assays were performed in 50 mM Tris-HCl buffer (pH 7.4 at room temperature) in the presence or absence of competing ligands. The mixtures of cell membrane lysate, radioligand and competing ligands were incubated in a total assay volume of 200 μ L in 96-well plates for 30 min at 27 °C and then harvested on 96-well glass fiber plates (Millipore, MSFBN6B50) that were pretreated with 0.5% PEI, followed by washing for three times using ice-cold 50 mM Tris-HCl buffer (pH=7.4 at 4 °C). The radioactivity retained on the filters was measured by liquid scintillation counting in a Microbeta counter (Perkin-Elmer Life and Analytical Sciences, Inc., USA).

Off-Target Selectivity Assay of Compound 48

Binding affinities to various proteins including receptors, ion channels and transporters were assessed based on competitive radioligand binding technique. These assays were performed by Eurofins Cerep, in study number 100040480 (Celle l'Evescault, France). The experiment was accepted in accordance with Eurofins validation Standard Operating Procedure.

GTP γ ³⁵S Functional Assay of Compound 48

The functional activity on recombinant mouse and human H4R was evaluated using GTP γ ³⁵S binding assays. The agonistic and antagonistic activities of compound 48 were assessed using reference agonist (R- α -methyl histamine for both of human and mouse H4R) and reference antagonists (JNJ10191584 for human and thioperamide for mouse H4R). The assays were performed by Euroscreen FAST, Ogeda SA (Belgium).

Intrinsic Clearance (CL_{int}) Determination in Microsomes

The metabolic stability of each compound was evaluated in pooled liver microsomes of CD-1 mice and humans. Test compounds (5 μ M) were incubated with mice (Corning, 452701) and human (Corning, 452117) liver microsomes activated with 1 mM NADPH, and loss of compound was followed at 0, 10, 20, and 30 min. The percentages of remaining compounds were determined by UPLC-UV. Intrinsic clearance (CL_{int}) based on microsomal protein concentration was calculated from the elimination rate constant assuming first order kinetics.

Solubility Assay

FaSSIF (pH=6.5) and SGF (pH=1.2) solutions of test compounds were incubated at 37 °C for 0.5 and 2 h, respectively. Samples were collected using a 0.45 μ m syringe filter and the concentration was measured by UPLC-UV.

Animals

All experiments were conducted in accordance with the National Research Council's Guidelines (IACUC, Korea) for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Experiments Committee of Sungkyunkwan University.

PK Study in ICR Mice

Pharmacokinetic studies were performed on ICR mice (n=3 for each group). After oral administration of test compounds, blood was sampled at 0.5, 1, 2, 4, 7, and 24 h post dose. Plasma concentrations were quantitated by LC-MS/MS. Pharmacokinetic parameters were calculated from non-compartmental analysis using Phoenix WinNonlin (Pharsight, Palo Alto, CA).

Pruritogen-induced Acute Pruritus in Mouse and Quantification of Scratching Behavior

Pruritic responses to histamine, substance P and compound 48/80 (C48/80) were examined in female ICR mice (n=8-10 per group). For the induction of scratching, the hair on the back skin was clipped 24 h before intradermal injection of pruritogens. Mice were received an oral dose of compound **48**, dissolved in a vehicle of 20% hydroxylpropyl- β -cyclodextrin, 30 min before intradermal injection of histamine (300 nmol), substance P (100 nmol) and C48/80 (100 μ g), respectively. Histamine, substance P and C48/80 (Sigma Chemical Co.) were dissolved in saline. Immediately after intradermal injection of histamine, the mice were returned to an acrylic cage (approximately 30 $\times$ 30 $\times$ 30 cm, access production) and scratching responses were video-recorded from above and was quantified by counting the number of bouts of scratching for 20 min immediately after the intradermal injection. A bout of scratching was defined as 3 or more rapid back and forth motions of the hindpaw directed to the site of sensitization

Oxazolone-induced AD Model

The hair on the abdominal skin of each female BALB/c mice (n=8 per group) was shaved with a clipper 1 day before experiments. Mice were then sensitized on day 0 by the topical single application of 50 μ L of 1% oxazolone (Sigma Chemical Co.) in acetone/corn oil (4:1) on the shaved abdominal skin. On day 7, 25 μ L of 0.2% oxazolone and acetone/corn oil vehicle was applied repeatedly to the right and left ear, respectively, 3 times per week. Compound **48** was orally administered 30 min before the challenge, twice a day for 3 weeks. Scratching behavior was quantified by counting the number of bouts of scratching for one hour after application of oxazolone to the ear on day 25, as described above. On days 27, the ear thickness was measured with the dial thickness gauge (IDC1012, Mitutoyo) and the ears were removed for analysis. Change in ear thickness (Δ ear thickness) was expressed by thickness of the right (oxazolone-challenged ear) – thickness of the left ear (acetone/corn oil-challenged ear). For histological analysis of challenged ear skin, ear tissue was fixed with 4% paraformaldehyde, embedded in paraffin and sectioned at 3 μ m. Sections were stained with hematoxylin-eosin.

Statistical Analysis

All data are represented as the mean $\pm$ S.E.M. Student's t-test was used to compare two groups. Dunnett's test or Kruskal-Wallis test in GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA) was used for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

ASSOCIATED CONTENT

Supporting Information

Selectivity data of compound **48** against off-targets; synthetic procedures and analytical data for compound **9-48** (PDF)

Molecular formula strings and some data (CSV)

Predicted binding mode of compound **16** in H4R homology model (PDB)

Predicted binding mode of compound **16** in 5-HT3R homology model (PDB)

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The manuscript was written through contribution of all authors. All authors have approved to the final version of manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AD, Atopic dermatitis; DIPEA, *N,N*-diisopropylethylamine; EtOH, ethanol; H4R, histamine H4
receptors; 5-HT3R, Human ligand-gated ion channel serotonin 5-HT3 receptors; MeOH,
methanol; SAR, Structure-activity relationships; TEA, triethylamine.

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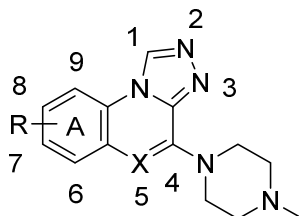
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Table 1. Structure-guided SAR of compound **9**

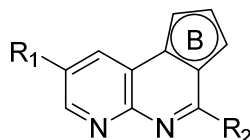
Compd	A ring	R	X	Inhibitory activity (IC ₅₀ , μM)		Metabolic stability (CL _{int} , μL/min/mg)		Solubility (μg/mL)	
				H4R	H3R	Mouse	Human	SGF	FaSSSIF
1 (JNJ-7777120)				0.029	>100	128	6.50	1414	378
9	C	H	N	2.3	>100	73.1	11.5	>2000	1187
10	C	8-Cl	N	0.022	4.4	29.1	5.83	>2000	227
11	C	8-NO ₂	N	0.17	41	10.4	2.63	1900	80.2
12	C	8-Me	N	0.57	15	23.3	7.51	>2000	1943
13	C	8-Cl	C	6.2	>100	8.43	0.86	>2000	>2000
14	9-N	H	N	3.0	>100	50.2	8.21	1923	>2000
15	6-N	H	N	3.0	>100	5.36	0.61	>2000	>2000
16	6-N	8-Cl	N	0.077	90	1.98	0.60	>2000	1244
17	6-N	8-Br	N	0.067	71	3.13	1.64	1929	679
18	6-N	8-I	N	0.034	>100	3.74	2.81	1249	126
19	6-N	8-Me	N	0.51	>100	2.91	5.17	1997	1905
20	6-N	8-CF ₃	N	0.28	43	2.05	1.31	>2000	1231
21	6-N	8-CN	N	0.24	>100	2.80	3.49	1742	417
22	6-N	7,8-diCl	N	0.18	>100	5.28	2.55	1948	163
23	6-N	7-OEt, 8-Cl	N	0.52	>100	3.56	3.28	ND	
24	6-N	7-OCH ₂ CF ₃ , 8-Cl	N	0.24	>100	4.94	5.72	ND	
25	6-N	7-CN, 8-Cl	N	0.27	36	0.69	1.63	ND	
26	6-N	8-Br, 9-Me	N	0.30	24	7.62	3.84	1802	583

Table 2. Effects of varying the amine-substitutions of compound **16** on inhibitory activity and selectivity

Clc1cc2nc3c(ncn3R)n2

Compd	R	Inhibitory activity (IC ₅₀ , μM)		Compd	R	Inhibitory activity (IC ₅₀ , μM)	
		H4R	H3R			H4R	H3R
16		0.077	90	35		6.3	>100
27		0.13	>100	36		1.2	>100
28		>10	>100	37		0.065	>100
29		0.38	>100	38		5.2	>100
30		0.76	>100	39		0.43	>100
31		2.9	11	40		0.49	>100
32		>10	>100	41		0.052	24
33		4.8	>100	42		>10	>100
34		4.1	>100				

Table 3. Effects of varying around the B-ring of compound **16** on inhibitory activity and selectivity



Compd	R ₁	B ring	R ₂	Inhibitory activity ^a			Compd	R ₁	B ring	R ₂	Inhibitory activity ^a		
				H4R	H3R	5-HT3R					H4R	H3R	5-HT3R
				(IC ₅₀ , μM)	(% Inh.)						(IC ₅₀ , μM)	(% Inh.)	
16	Cl			0.077	90	100	37	Cl			0.065	>100	50
43	Cl			0.17	75	96	46	Cl			1.1	>100	32
44	Cl			0.062	73	85	47	Cl			0.053	>100	18
45	Br			0.028	49	89	48	Br			0.027	>100	14

^aIC₅₀ values in μM for H4R and H3R. Percent inhibition values at 10 μM for 5-HT3R.

Table 4. Mean pharmacokinetic parameters of compound **10**, **16**, and **48** after oral administration to female ICR mice (n=3)

Compd	Dose (mg/kg)	C _{max} (µg/mL)	T _{max} (h)	AUC _{all} (µg·h/mL)
10	50	0.676	0.67	1.68
16	50	4.63	0.50	23.6
48	2	0.180	0.50	0.251
	10	1.82	0.50	5.40
	50	7.69	0.50	62.6

Figure Legends

Figure 1. Structures of representative H4R antagonists

Figure 2. Overview of pharmacophore-based virtual screening for H4R antagonists. A pool of pharmacophore models based on known H4R antagonists was subjected to screen structurally diverse in-house library stratified from commercially available ZINC database. In the pharmacophore models (right panel), five functional features are represented by distinct color codes: hydrogen bond acceptor (green), hydrogen bond donor (magenta), hydrophobic group (cyan), aromatic ring (orange), and positive charge group (red).

Figure 3. Predicted binding mode of compound **9** and **16** in H4R homology model constructed from H1R X-ray structure (pdb code: 3RZE). (a) The carbon atoms of compound **9** and the key residues of H4R are shown in yellow and gray, respectively. The hydrogen bond and ion interaction are depicted by red dashed lines. (b) The binding mode of compound **16** in opposite-side view of (a). The distance and angles of halogen-bonding interaction is depicted by magenta line. All distances are in angstroms.

Figure 4. Predicted binding mode of compound **16** in 5-HT3R homology model based on mouse 5-HT3R X-ray structure (pdb code: 4PIR) and interaction diagram in 5-HT3R and H4R. (a) Left panel; compound **16** is shown as yellow carbon color, while residues are shown as gray carbon of stick representation. Right panel; the 5-HT3R selectivity profiles of amine substituents are clearly correlated with the calculated solvation energy values. ^aInhibitory activities against human H4R, IC₅₀ in μ M. ^bInhibitory activities against human 5-HT3R, % inhibition at 10 μ M. ^cSolvation free energies calculated using Jaguar program in kcal/mol. ^dPredicted ACD logD_{7.4} values. (b) Interaction diagram of compound **48** in 5-HT3R and H4R.

Figure 5. Plasma concentration of compound **10**, **16**, and **48** after oral administration to female ICR mice at 50 mg/kg (n=3). Data expressed as mean $\pm$ SD

Figure 6. Effect of compound **48** on scratching behavior induced by pruritogens. (a) The number of scratching induced by histamine with treatment of indicated dose of compound **48**. The grey dots indicate the free concentrations in plasma at T_{max}. (b,c) The effect of compound **48** on substance P and C48/80-induced scratching. Each column represents the mean $\pm$ S.E.M. ($n = 8-10$). $**p < 0.01$, $***p < 0.001$ vs S (Saline) and $^{+++}p < 0.001$ vs V (Vehicle).

Figure 7. Effect of compound **48** on the scratching behavior and the total ear and epidermal thickness in oxazolone induced AD model. The compound was orally administered twice a day for 3 weeks. (a) The number of scratching behavior of mice in each group 1h after oxazolone challenge on day 25. (b) Change of total ear thickness in sensitized mice challenged with oxazolone. (c) Epidermal thickness in the ear skin challenged with oxazolone. Each column represents the mean $\pm$ S.E.M. ($n = 8$). $**p < 0.01$, $***p < 0.001$ vs control and $^{+++}p < 0.001$ vs vehicle.

Scheme 1. Synthesis of Intermediate^a

Scheme 2. General procedure A and B^a

Scheme 3. Synthesis of compound **13**^a

Scheme 4. Synthesis of amine derivatives^a

Scheme 5. Synthesis of compound **43** and **46**^a

Scheme 6. Synthesis of compound **44**, **45**, **47**, **48**^a

Figure 1

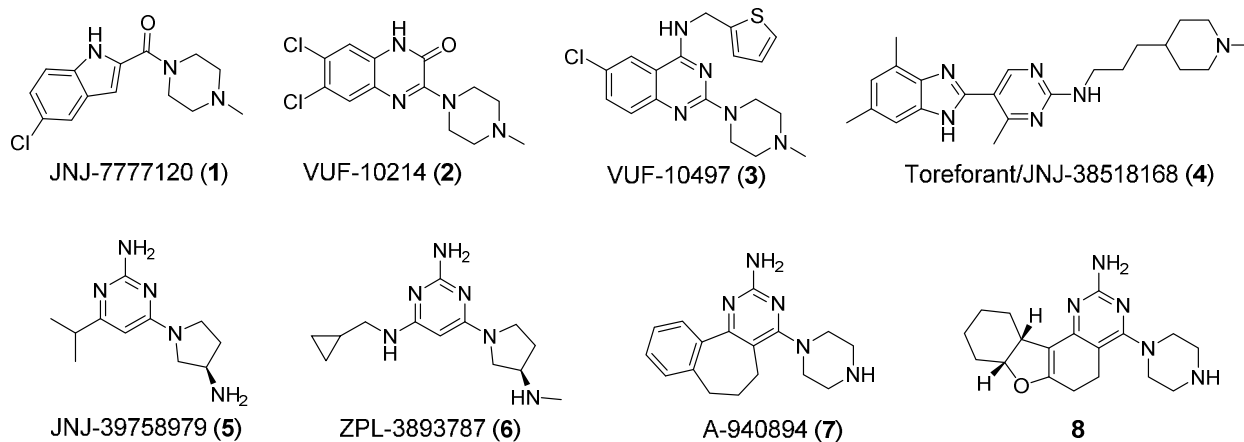


Figure 2

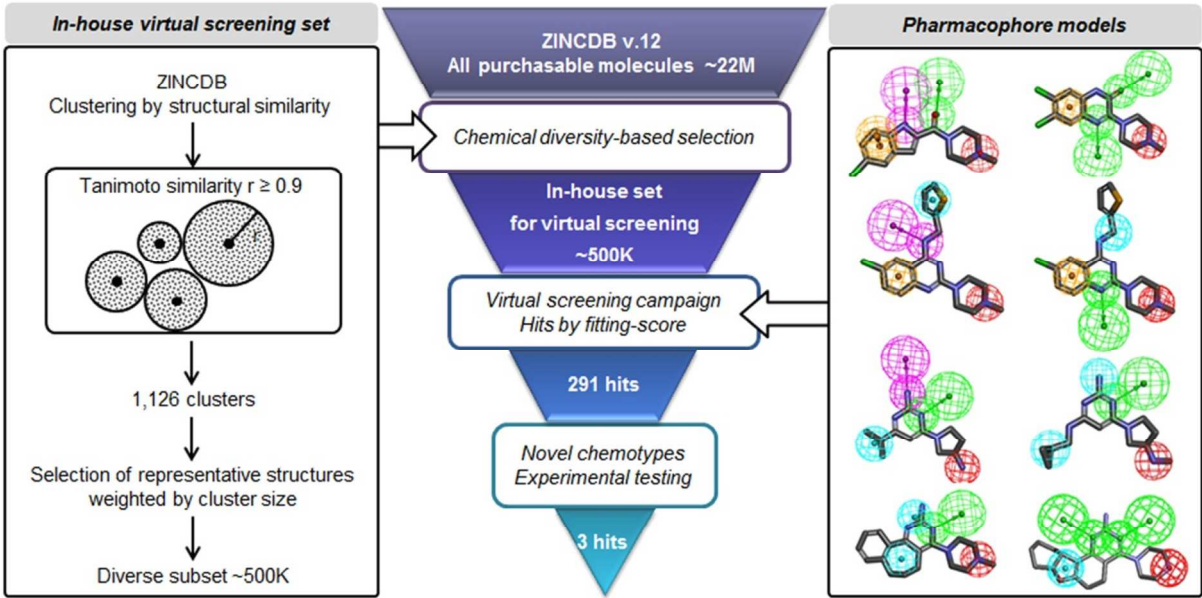


Figure 3

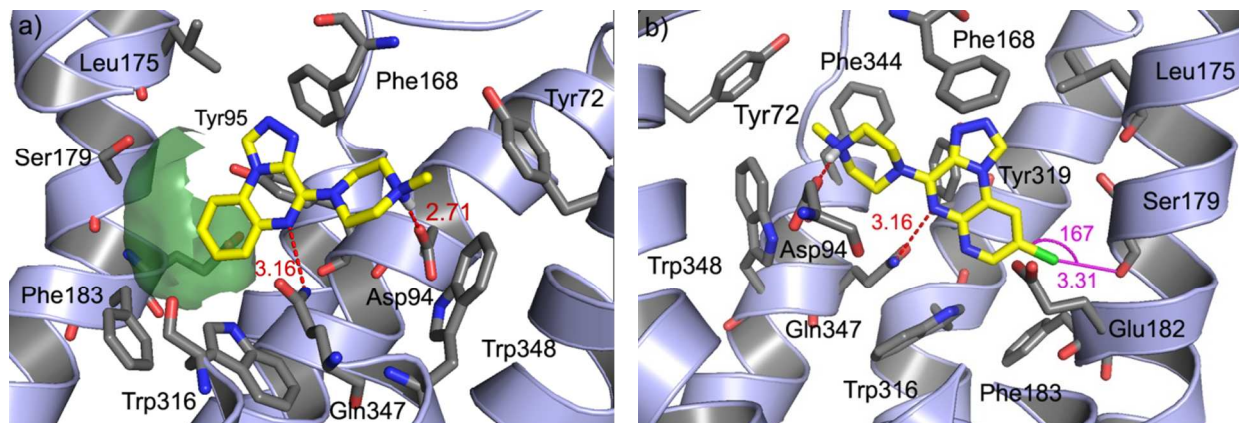


Figure 4

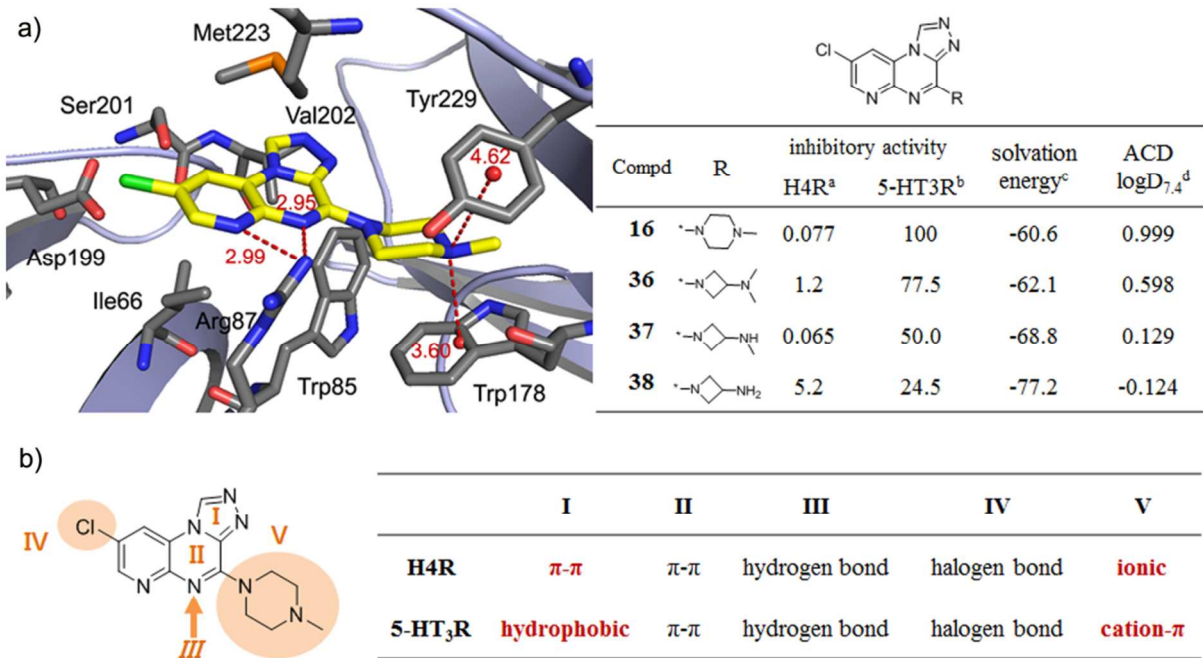


Figure 5

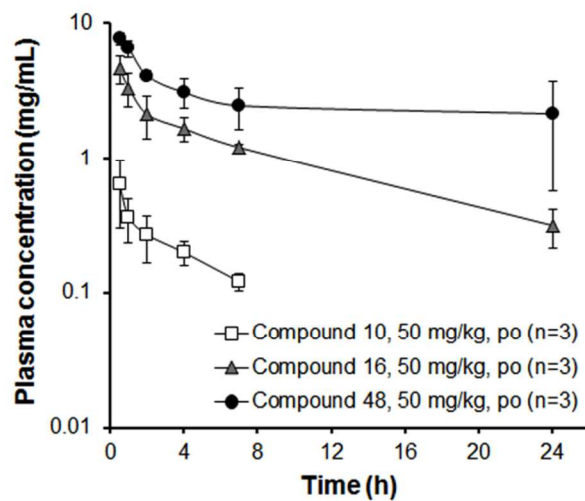


Figure 6

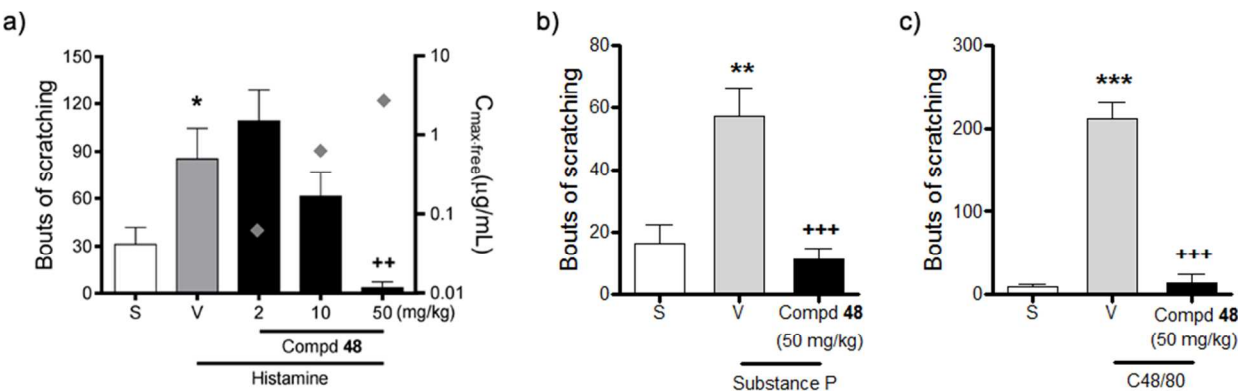
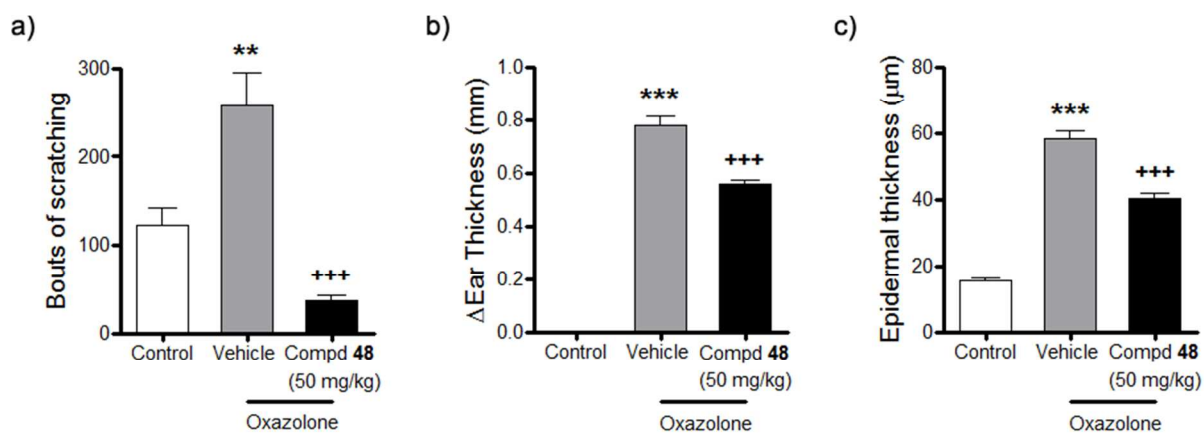
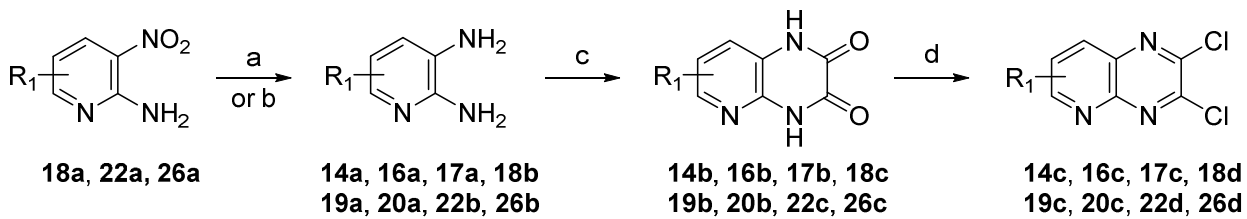


Figure 7

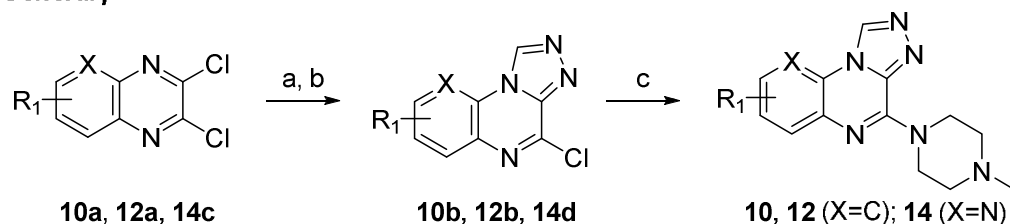
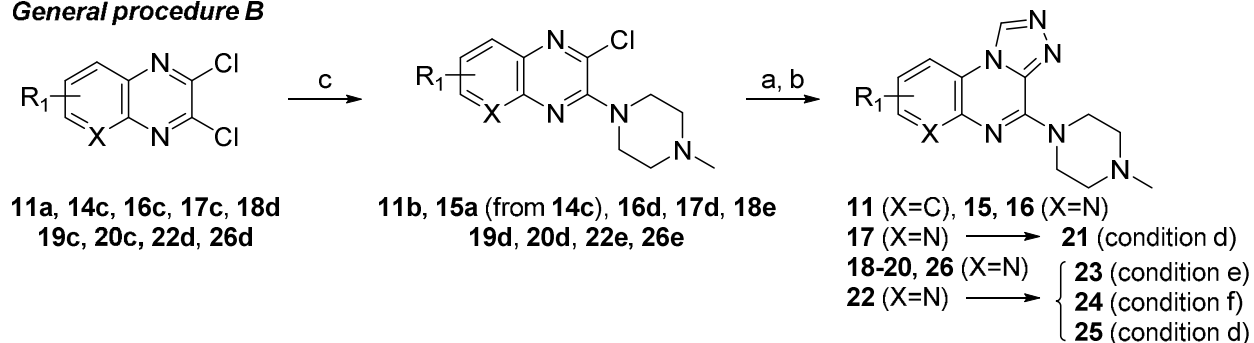


Scheme 1



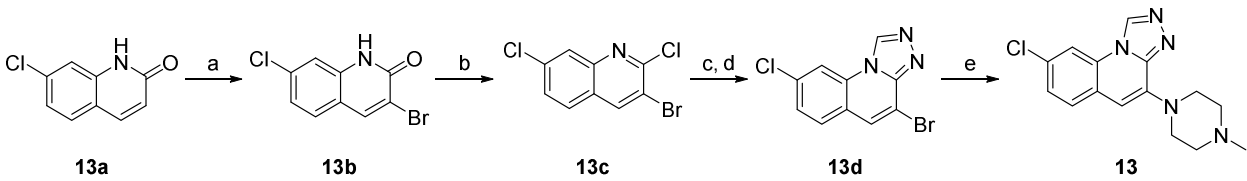
^aReagents and conditions: a) Fe (10 equiv), conc HCl, H₂O/EtOH, 100 °C; b) SnCl₂ (4.2 equiv), conc HCl, 80~100 °C; c) diethyl oxalate, reflux; d) POCl₃, reflux

Scheme 2

General procedure A**General procedure B**

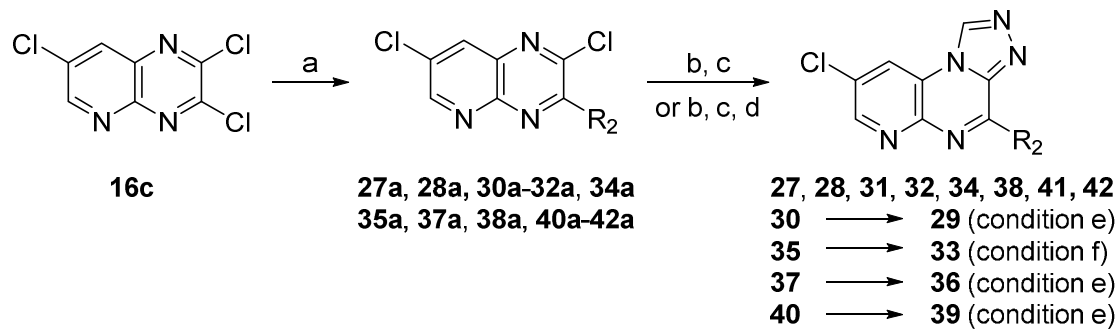
^aReagents and conditions: a) $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ (2.0~5.0 equiv), EtOH, rt~50 °C; b) $\text{HC}(\text{OMe})_3$, reflux; c) *N*-methyl piperazine (1.0~2.0 equiv), TEA (2.0~10 equiv), DCM or *N*-methyl piperazine (2.0~10 equiv), DMF; d) $\text{Zn}(\text{CN})_2$ (1.0 equiv), $\text{Pd}(\text{PPh}_3)_4$ (10 mol%), DMF, microwave (60 W, 90 °C); e) EtONa (1.2 equiv), EtOH, microwave (60 W, 90 °C); f) NaH (20 equiv), $\text{CF}_3\text{CH}_2\text{OH}$, microwave (60 W, 90 to 120 °C)

Scheme 3



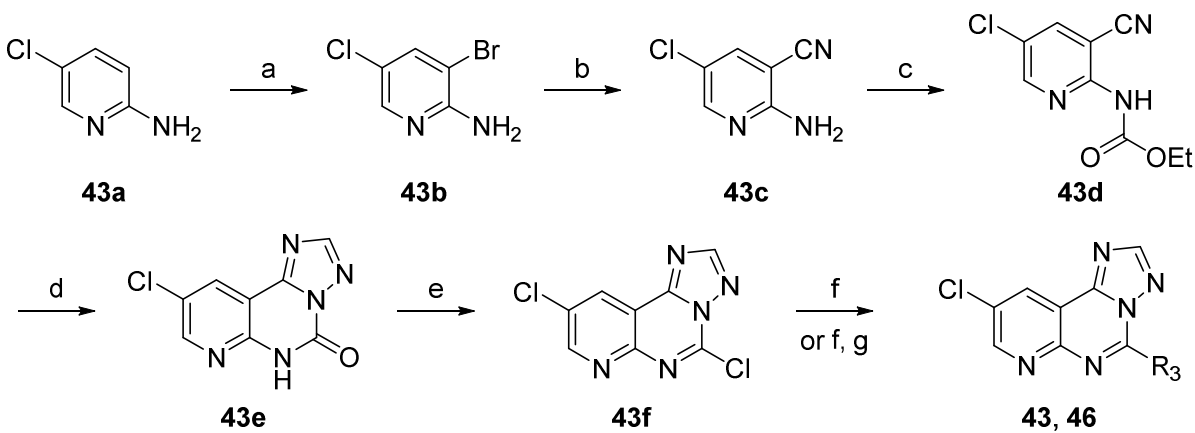
^aReagents and conditions: a) Br_2 (1.5 equiv), DMF, 60 °C; b) POCl_3 , reflux; c) $\text{NH}_2\text{NH}_2\text{H}_2\text{O}$ (2.5 equiv), EtOH, 60 °C; d) $\text{HC}(\text{OEt})_3$, 130 °C; e) *N*-methyl piperazine (2.1 equiv), CuI (2.1 equiv), L-proline (1.0 equiv), K_3PO_4 (2.1 equiv), DMSO, 120 °C

Scheme 4



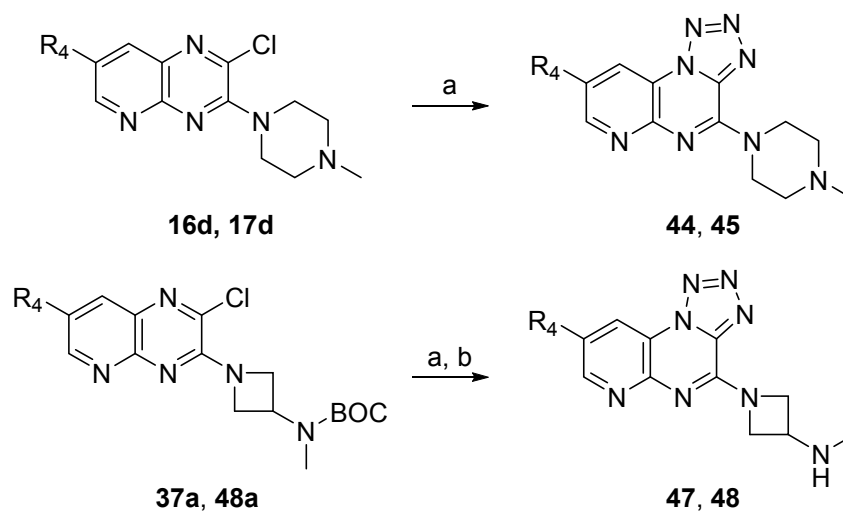
^aReagents and conditions: a) amines (1.0~1.5 equiv), TEA (2.0~10 equiv), DCM; b) $\text{NH}_2\text{NH}_2\text{H}_2\text{O}$ (2.0~5.0 equiv), EtOH, 0~50 °C; c) $\text{HC}(\text{OMe})_3$, 70~100 °C or $\text{HC}(\text{OEt})_3$, 70~90 °C; d) TFA/DCM, 0 °C~rt; e) 37% HCOH (1.5~6.0 equiv), NaBH_4 (3.0~6.0 equiv), MeOH, rt; f) HCOONa (4.2 equiv), $\text{HCOOH}/\text{HCONH}_2$, 100 °C

Scheme 5



^aReagents and conditions: a) Br₂ (1.0 equiv), CHCl₃, rt; b) Zn(CN)₂ (1.5 equiv), Pd(PPh₃)₄ (10 mol%), NMP, 120 °C; c) ethyl chloroformate, NaHCO₃ (3.0 equiv), 2-butanone, reflux; d) formic hydrazide (1.0 equiv), diphenyl ether, 180 °C; e) POCl₃, DIPEA (2.0 equiv), reflux; f) amines (2.0 equiv), DMF or amines (1.5 equiv), TEA (3.0 equiv), DMF; g) TFA/DCM, rt

Scheme 6



^aReagents and conditions: a) NaN_3 (9.0 equiv), EtOH, 70 °C; b) TFA/DCM, rt.

TABLE OF CONTENTS GRAPHIC (TOC)

