# Discovery of Novel Human Histamine H4 Receptor Ligands by Large-Scale Structure-Based Virtual Screening

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A structure-based virtual screening (SBVS) was conducted on a ligand-supported homology model of the human histamine H4 receptor (hH4R). More than 8.7 million 3D structures derived from different vendor databases were investigated by docking to the hH4R binding site using FlexX. A total of 255 selected compounds were tested by radioligand binding assay and 16 of them possessed significant [$^3$H]histamine displacement. Several novel scaffolds were identified that can be used to develop selective H4 ligands in the future. As far as we know, this is the first SBVS reported on H4R, representing one of the largest virtual screens validated by the biological evaluation of the virtual hits.

## 1. Introduction

Histamine plays a crucial role as a major regulator of many cellular events. It serves as a mediator, for example, in hypersensitivity (allergic) responses, gastric acid secretion, neurotransmission, immunomodulation, cell differentiation, and embryonic development.[1] All these processes take place with the involvement of histamine receptors, of which four major types (H1R, H2R, H3R, and H4R[a]) have so far been identified.[2] H1 antagonists (antihistamines) are widely used in the treatment of allergy. Their therapeutic effect on allergic rhinitis and urticaria is well-known. On the other hand, in several allergic diseases, for example, bronchial asthma, H1 antagonists are not effective.[3] H2 antagonists are used in treating peptic ulcers, gastresophageal reflux disease, and gastrointestinal bleeding.[4,5] H3R was first cloned in 1999 by Lovenberg et al.[6] On the basis of its tissue distribution and the demonstrated pharmacological effects, H3 antagonists can have a therapeutic importance in cognitive disorders, dementia, and obesity.[7–9] H4R is the novel member of the histamine receptor family. It was cloned by several groups independently in 2000 and 2001.[10–14] In spite of the high sequence homology with H3R, the highly different expression pattern indicates different functions. H4R occurs in bone marrow, spleen, eosinophils, mast cells, basophils, CD8+ T cells, and dendritic cells[12,13,15–17] and, in contrast with H3R, it has virtually no expression in the CNS.[12,13] H4R mediates histamine-induced chemotaxis in mast cells and eosinophils, and this effect can be blocked by selective H4 antagonists.[15,18,19] H4R in common with H2R is involved in the secretion of interleukin 16 (IL-16) from CD8+ T cells.[20] It was also demonstrated that H4R antagonists cause a significant inhibition of polymorphonuclear cell influx into the peritoneum or pleural cavity in zymosan-induced neutrophilic inflammation models.[21,22] These biological functions and the expression pattern indicate a crucial role of H4R in allergy and inflammation, therefore, H4 antagonists can become the new antihistamines,[23] and H4 antagonists alone or in combination with H1 antagonists may be used against some allergic diseases effectively in the near future. H4R is therefore a promising target for drug design,[24] especially because a limited number of selective H4 ligands have only been discovered so far.

H4R is a member of class "A" in the G-protein-coupled receptor (GPCR) superfamily. Despite the fact that GPCRs are the targets for 60–70% of drugs in development today,[25] until quite recently, the only GPCR with an experimentally determined 3D structure was bovine rhodopsin.[26] However, during the preparation of this manuscript, the first crystal structures of a human GPCR were reported.[27,28] The bovine rhodopsin structure has been widely used as a template to generate relevant GPCR homology models for structure-based drug design. At the beginning, these GPCR models were exclusively used for binding site and ligand binding mode analyses.[29–32] The improvement of homology modeling, docking methods, scoring functions, and computational resources allowed successful structure-based virtual screenings (SBVS) on GPCRs.[33–36] In these studies, several novel GPCR ligands were reported indicating the general relevance of this technique in structure-based drug design. The protocol of SBVS can be built up in many ways. Two conditions, however, are essential for SBVS: a target structure and a ligand database. In our case, the target structure was a ligand-supported homology model of the human histamine H4 receptor (hH4R), which the model was previously demonstrated to be suitable for virtual screening by enrichment studies.[37] Virtual screenings typically start up with a database ranging from several hundred thousand to a million compounds. As a first step, this database is usually prefiltered by "drug-like" (or "lead-like") properties. These filters can be useful in eliminating compounds with undesirable physicochemical prop-

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[a] Abbreviations: GPCR, G-protein-coupled receptor; hH4R, human histamine H4 receptor; CNS, central nervous system; SBVS, structure-based virtual screening; vHTS, virtual high throughput screening; PC, personal computer; CPU, central processing unit; PBS, polybutylene succinate.

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erties. However, a reasonable number of drugs on the market do not fulfill the criteria of "rule-of-five", the most popular "drug-like" filter,[38] and therefore, a drug design project with such a prefiltering step would have never discovered them. As a next step, databases can be further reduced by using pharmacophore models, and docking is only applied for some thousand compounds in the last step of the workflow. One advantage of using pharmacophore models instead of docking is the reduction of the computational time. Generation of pharmacophore hypotheses, however, requires a reasonable set of ligands with measured biological activity. In the case of hH4R, the lack of such information prevents the application of ligand-based methods in virtual screening. It also has to be mentioned that these methods obviously miss ligands exploiting new parts of the binding site or a totally new cavity. Such limited number of chemotypes applied for pharmacophore generation increases the risk of missing actives. Docking requires reasonably more computational capacity, than pharmacophore searching. Then again, docking ligands into the same binding site is typically a parallelizable computational calculation, and therefore, it is possible to execute it on a cluster of PCs instead of a supercomputer. Screening for potential anticancer agents using distributed computing in the Screensaver project is a good example for this approach.[39] Clusters are effectively used in virtual high throughput screening (vHTS) and make it possible to dock even millions of compounds with high accuracy in a reasonable time frame.

In this study, we report a successful large-scale virtual screening on the homology model of hH4R. We compiled a compound database comprising more than 8.7 million entries that was screened against the hH4R homology model without any prefiltering using the docking program FlexX.[40] This protocol saved some valuable compounds from prefiltering, hereby minimizing the number of false negatives. Successful docking of all compounds yielded 255 compounds that were evaluated by radioligand binding assay, and 16 showed significant hH4R affinity. To the best of our knowledge, this is one of the largest validated SBVSs published so far.

## 2. Materials and Methods

**Materials.** Histamine dihydrochloride and thioperamide maleate were purchased from Sigma (St. Louis, MO). Imetit dihydrobromide, iodophenpropit dihydrobromide, dimaprit dihydrochloride, clobenpropit dihydrobromide, and (*R*)-(−)-α-methylhistamine dihydrobromide were purchased from Tocris Bioscience (Ellisville, MO). SK-N-MC cell line stably transfected with hH4R was previously decribed.[12] [$^3$H]histamine dihydrochloride (52.0 Ci/mmol) was purchased from Amersham (U.K.). Compound **1** (JNJ 7777120)[22] was provided by Johnson&Johnson Pharmaceutical Research and Development, LLC (San Diego, CA).

**Homology Modeling.** The development of hH4R homology models suitable for vHTS was reported elsewhere.[37] Here we only summarize the most important steps of homology modeling and evaluation. Homology models of hH4R were developed based on the crystal structure of bovine rhodopsin. Histamine has two major anchoring points at the hH4R binding site. The crucial role of Asp94 (3.32) and Glu182 (5.46) was demonstrated by site-directed mutagenesis.[41] After docking histamine to the binding site, we rotated the carboxylate group of Asp94 (3.32) around the Cα−C$\beta$ axis to accommodate histamine more favorably. Docking into this model (indicated as "Model C" in ref 37) resulted in improved binding modes of histamine having simultaneous interactions with Asp94 (3.32) and Glu182 (5.46). Enrichment tests revealed that this model is able to select known H4 ligands from random decoys. Highest enrichment factors were around 40–50, as calculated at the top 0.5% of the database. Other models with better performance reported in that study were not available when our vHTS project was initiated.

**Database Preparation.** A significant part of the docking molecules was collected from the ZINC database.[42] These compounds are commercially available, except the NCI compounds, which are accessible for academic researchers only. Compounds from the ZINC database are available in multiple tautomeric forms and protonation states. All of these were added to our database. We have updated the compound collections by the vendors where it was possible. If a vendor's compound collection has changed significantly since the last ZINC update, we used the new database from the vendor's Web site directly instead of the ZINC version. ZINC compounds are in a "ready-to-dock" format. Others were prepared for docking in two steps. First, the "washing" module of MOE (version 2005.06; Chemical Computing Group Inc., Montreal, Canada) was applied to generate the physiologically relevant protonation state of the compounds. Second, 3D coordinates were generated by Concord 4.0 in Sybyl7.0 (Tripos Associates, St. Louis, MO). These compounds were only considered in one tautomeric or protonation state during docking. In summary, our database comprises 8743666 3D structures of small molecules (5066235 unique compounds) stored in mol2 format by vendors.

**Virtual Screening.** Docking calculations were performed by FlexX version 2.0.1. FlexX was running with formal charges assignment, other parameters were set as default. The active site was defined as 5 Å radius around residues Asp94 (3.32), Glu182 (5.46), and Asn350 (7.45). High throughput docking was exclusively done using the "ClusterGrid" production grid system developed by the National Information Infrastructure Institute (NIIF) at Hungary. "ClusterGrid" is basically built up of semidedicated worker nodes of PC laboratories located at Hungarian Universities. Semidedication basically means that the laboratories are used in normal operation during the day and are reloaded to grid mode in the evening. The PC laboratories are interconnected with a high speed national backbone network. Hundreds of CPUs were used simultaneously for the calculations. The FlexX license for 1000 CPUs was kindly provided by BioSolveIT GmbH. FlexX was deployed on-demand to worker nodes. A total of 300 compounds were submitted for FlexX as a single job. The executables, input data, and results were stored on a distributed grid storage during the calculations. This script always checked whether the job was completely and successfully finished. If not, the remaining compounds were resubmitted. Scoring of the successfully docked ligand poses was performed by the CScore module in Sybyl7.0 on a minicluster with 4 CPUs.

**Radioligand Binding Assay.** SK-N-MC cells transfected with human H4 receptor were collected in PBS centrifuged at 3000 g for 10 min, at 4 °C. Pellets were collected and homogenized in 50 mM Tris pH 7.5 containing 5 mM EDTA. After centrifugation at 40000 g (25 min, at 4 °C) pellets were rehomogenized in 50 mM Tris pH 7.5 containing 5 mM EDTA, aliquoted, and stored at −70 °C until use. For the H4 competition binding studies, cell membranes were incubated in plastic tubes with 10 nM [$^3$H]histamine in 50 mM Tris pH 7.5 containing 5 mM EDTA, with or without test compounds, for 60 min at 25 °C. Nonspecific binding was defined using 100 $\mu$M unlabeled histamine. After incubation, the membranes were filtered through GF/C filters pretreated with 0.5% polyethylenimine using an M48 Brandel cell harvester. Filters were placed into scintillation cocktail (OPTIPHASE "HISAFE" 3) and quantified by a Tri-Carb 2900TR liquid scintillation analyzer. Data were analyzed using GraphPad Prism (Graph-Pad Software, San Diego, CA). $K_i$ values were calculated according to Cheng and Prusoff (1973). Inhibition, $K_i$, and $K_d$ values (average ± S.D.) were determined from three independent experiments unless otherwise indicated.

**Clustering of the In Vitro Hits.** Compounds with significant H4 affinity were clustered by different methods (ring systems, branch fragments, and pharmacophores) with ChemTK Lite version 4.0.2.[43] Final clustering was based on the combination of the results of ChemTK with manual adjustments.

**Table 1.** 3D Structures Docked Successfully to the hH4R Binding Site by FlexX

| source | vendor | URL | No of successfully docked 3D structures | No of 3D structures in the top 2000 | No of 3D structures in the top 45000 | No of compounds ordered | No of actives |
|---|---|---|---|---|---|---|---|
| ZINC | ACB-Eurochem[a] | | 107705 | 1 | 47 | 0 | 0 |
| ZINC | Ambinter | www.ambinter.com | 641100 | 125 | 2856 | 1 | 0 |
| ZINC | Asinex | www.asinex.com | 341431 | 146 | 1745 | 38 | 0 |
| ZINC | ChemBridge | www.chembridge.com | 476345 | 147 | 2800 | 29 | 2 |
| ZINC | Comgenex | www.comgenex.com | 35066 | 1 | 95 | 3 | 0 |
| ZINC | Enamine | www.enamine.net | 557158 | 160 | 2590 | 17 | 0 |
| ZINC updated | InterBioScreen | www.ibscreen.com | 589900 | 115 | 2088 | 23 | 1 |
| ZINC | Life Chemicals | www.lifechemicals.com | 57505 | 6 | 183 | 0 | 0 |
| ZINC | InterChim | www.interchim.com | 380502 | 57 | 1417 | 0 | 0 |
| ZINC | Maybridge | www.maybridge.com | 63481 | 33 | 499 | 5 | 0 |
| ZINC | Nanosyn | www.nanosyn.com | 81914 | 46 | 805 | 1 | 0 |
| ZINC | National Cancer Institute (NCI) | http://dtp.nci.nih.gov | 249747 | 204 | 2079 | 58 | 5 |
| ZINC updated | Otava | www.otavachemicals.com | 450768 | 42 | 1402 | 0 | 0 |
| ZINC | Peakdale Molecular | www.peakdale.com | 11584 | 13 | 110 | 0 | 0 |
| ZINC | Ryan Scientific | www.ryansci.com | 75555 | 30 | 510 | 2 | 1 |
| ZINC | Sigma Aldrich | www.sigmaaldrich.com | 51380 | 15 | 276 | 0 | 0 |
| ZINC | Specs | www.specs.net | 361408 | 84 | 1625 | 12 | 0 |
| ZINC | TimTec | www.timtec.net | 157451 | 43 | 992 | 0 | 0 |
| Vendor | AnalytiCon Discovery | www.ac-discovery.com | 8460 | 0 | 4 | 0 | 0 |
| Vendor | Apollo | www.apolloscientific.co.uk | 6983 | 0 | 5 | 0 | 0 |
| Vendor | Aurora Fine Chemicals | www.auroratinechemicals.com | 30854 | 3 | 98 | 0 | 0 |
| Vendor | Biotech | www.biotech-us.com | 119823 | 76 | 1257 | 8 | 1 |
| Vendor | ChemDiv | www.chemdiv.com | 540450 | 82 | 1932 | 20 | 3 |
| Vendor | ChemTI | www.chemti.com | 1176915 | 196 | 10058 | 0 | 0 |
| Vendor | CiVentiChem | www.cvchem.com | 256455 | 16 | 524 | 0 | 0 |
| Vendor | EMC Microcollections | www.microcollections.de | 23805 | 1 | 11 | 0 | 0 |
| Vendor | Florida | http://ufark12.chem.ufl.edu/ | 28614 | 5 | 100 | 0 | 0 |
| Vendor | Key Organics | www.keyorganics.ltd.uk | 46799 | 0 | 93 | 0 | 0 |
| Vendor | LaboTest | www.labotest.com | 156288 | 45 | 1222 | 0 | 0 |
| Vendor | MDPI | www.mdpi.org | 9660 | 2 | 27 | 1 | 0 |
| Vendor | Meta Database (KEGG, Akos sets) | http://ligand.info | 470782 | 106 | 2603 | 0 | 0 |
| Vendor | Micro Source | www.msdiscovery.com | 1688 | 0 | 4 | 0 | 0 |
| Vendor | Moscow MedChemLabs | www.mosmedchemlabs.com | 176606 | 20 | 683 | 0 | 0 |
| Vendor | Oakwood Products | www.oakwoodchemical.com | 11383 | 0 | 10 | 0 | 0 |
| Vendor | Pharmeks | www.pharmeks.com | 123940 | 21 | 382 | 18 | 1 |
| Vendor | Princeton BioMolecular Research | www.princetonbio.com | 575780 | 111 | 2497 | 2 | 0 |
| Vendor | Spectrum Info | www.spectrum.kiev.ua | 8132 | 0 | 4 | 0 | 0 |
| Vendor | Toslab | www.toslab.com | 14694 | 1 | 73 | 0 | 0 |
| Vendor | Tripos (Leadquest) | www.leadquest.com | 44452 | 1 | 35 | 1 | 0 |
| Vendor | Vitas-M Laboratory | www.vitasmlab.com | 221103 | 46 | 1259 | 6 | 0 |
| Vendor | Chemical Block[b] | www.chemblock.com | 0 | 0 | 0 | 10 | 2 |
| | Σ | | 8743666 | | | 255 | 16 |

[a] We could not contact "ACB-Eurochem", and their compounds were deleted from ZINC early after we finished the virtual screening. [b] The compound library of "Chemical Block" was not screened against the hH4R, although some compounds among the virtual hits of other companies were purchasable from this vendor.

## 3. Results

**Virtual Screening.** In summary, 8743666 structures, representing 5066235 unique compounds, were docked by FlexX successfully into the hH4R binding site (Table 1).

The successfully docked and scored ligands were ranked by the best scoring function combination found previously by enrichment tests,[37] that is, the docked poses for a compound were ranked by ChemScore, and the best poses for all the compounds were ranked by the own score of FlexX. After that, we applied a two-step selection procedure to find the most promising compounds for in vitro testing.

First, the docking solutions for the best 2000 structures were inspected visually considering the following criteria: (i) the ligand has to be positioned entirely into the binding site, and (ii) protonation state and the tautomeric form of the ligand has to be acceptable. A compound was only considered for in vitro testing if both of these criteria have been met. In addition, interaction(s) with Asp94 (3.32) or Glu182 (5.46) was taking into account as a positive feature. Consequently, 128 compounds were selected for in vitro screening. From among these, 66 (referred to "top_2000" below) were on stock at the vendors and arrived at our laboratory.

Second, we inspected the top 45000 ligands. Because it was not possible to analyze the binding mode one by one, we were searching for compounds with at least one protonated amine function and another potential H-bond donor group. We have found 461 molecules meeting the above criteria. For these compounds bit packed MACCS Structural Keys fingerprint was generated by MOE, and a diverse subset of 229 compounds were selected with maximum Tanimoto index of 0.7. A total of 166 ("top_45000") of these were available at the vendors. In the case of 23 additional compounds, it was not possible to order them from the vendor anymore, however, close analogs possessing a very similar core structure were available. Therefore, 23 analog compounds ("top_45000_analog") were also ordered for in vitro testing.

**In Vitro Screening.** First we examined the binding of [$^3$H]histamine to membranes from SK-N-MC cells expressing

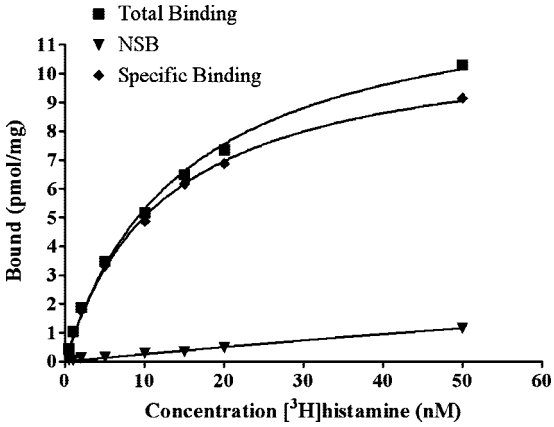



**Figure 1.** [$^3$H]Histamine binding to SK-N-MC cells stably transfected with hH4R. Cells were incubated with increasing concentrations of [$^3$H]histamine. Nonspecific binding (NSB) was determined in the presence of 100 $\mu$M histamine. $K_d$ value was determined from three independent experiments. Data from one representative measurement are shown.

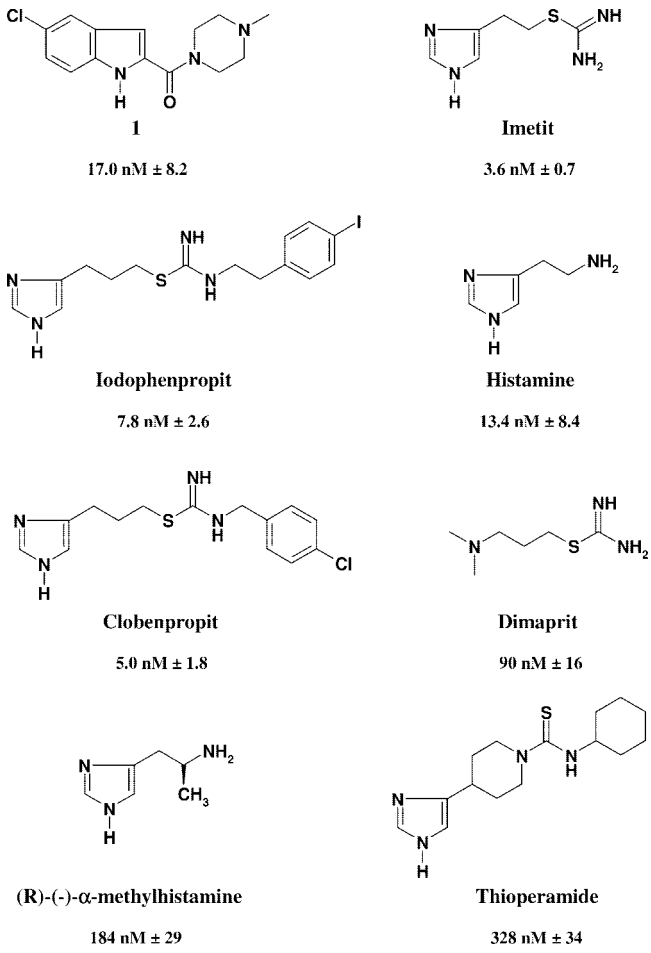



**Figure 2.** [$^3$H]Histamine binding to SK-N-MC cells stably transfected with hH4R. Membranes were incubated with 10 nM [$^3$H]histamine. $K_i$ (nM) values were determined from three independent experiments.

hH4R. Scatchard analysis revealed that [$^3$H]histamine binds with high affinity to hH4R with a $K_d$ of 12.8 ± 3.5 nM (Figure 1).

Subsequent competition binding experiments were performed with 10 nM [$^3$H]histamine. Eight known H4R ligands were evaluated for binding to the hH4R, to compare their $K_i$ values with the ones reported in the literature (Figure 2).

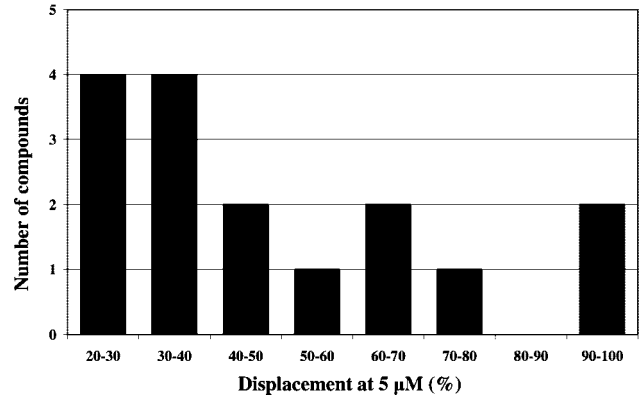



**Figure 3.** Distribution of the activities of the in vitro hits.

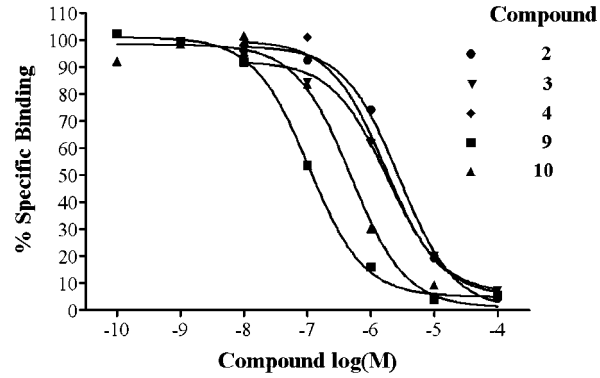



**Figure 4.** [$^3$H]Histamine binding to SK-N-MC cells stably transfected with hH4R. Membranes were incubated with 10 nM [$^3$H]histamine. $K_i$ values were determined from two (compounds **2**, **3**, and **4**) or three (compounds **9** and **10**) independent experiments: compound **2**, 1.48 $\mu$M ± 0.11; compound **3**, 1.24 $\mu$M ± 0.33; compound **4**, 897 nM ± 77; compound **9**, 85 nM ± 34; compound **10**, 219 nM ± 44. Data from one representative measurement are shown.

Consistent with the saturation experiment using [$^3$H]histamine, the $K_i$ value for unlabeled histamine was 13.4 ± 8.4 nM. All the $K_i$ values for other known H4 ligands were in good agreement with the data reported in the literature.[11–14] Then, we have tested the most promising compounds derived from the vHTS in a concentration of 5 $\mu$M by radioligand binding assay. Altogether, 255 compounds (66, "top_2000"; 166, "top_45000"; and 23, "top_45000_analog") were evaluated in vitro. A total of 16 out of the 255 compounds showed a significant (>20%) displacement of [$^3$H]histamine, which means an overall hit rate of 6.3% (Figure 3).

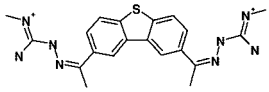
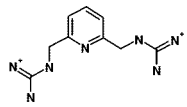
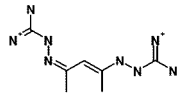
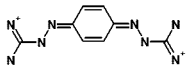
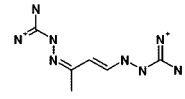
Three compounds were derived from "top_2000", 12 from "top_45000", and 1 from "top_45000_analog". Compounds **2**, **3**, **4**, **9**, and **10** showed a displacement above 60%, therefore, we have determined their $K_i$ values (Figure 4).

## 4. Discussion

To the best of our knowledge, no SBVS study has been published in the field of H4R so far. A structure-based approach can be utilized in the presence of a high quality model of the hH4R. In our case, the previously developed and published hH4R homology model was shown to be suitable for virtual screening by enrichment studies.[37] Therefore, we set up a large-scale SBVS, where we aimed to screen the database with more than 8.7 million entries by docking to the hH4R binding site.

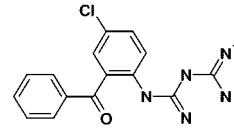
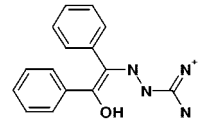
**Database Preparation.** As a practical aspect in the preparation of the database, we preferred compounds that were commercially or academically available.

**Table 2.** Group I of the In Vitro Hits[a]

| Structure | Compound | Displacement | Rank (by score) |
|---|---|---|---|
|  | 2 | 72.0% ± 1.4 | 480. |
|  | 3 | 67.8% ± 1.7 | 12134. |
|  | 4 | 64.3% ± 6.8 | 3309. |
|  | 5 | 44% ± 12 | 20589. |
|  | 6 | 33.9% ± 2.5 | 2885. |

[a] Displacement values were determined from three independent experiments at an inhibitor concentration of 5 $\mu$M in the presence of 10 nM [$^3$H]histamine.

**Table 3.** Group II of the In Vitro Hits[a]

| Structure | Compound | Displacement | Rank (by score) |
|---|---|---|---|
|  | 7 | 37.1% ± 1.1 | 2453. |
|  | 8 | 34.7% ± 6.5 | 229. |

[a] Displacement values were determined from three independent experiments at an inhibitor concentration of 5 $\mu$M in the presence of 10 nM [$^3$H]histamine.

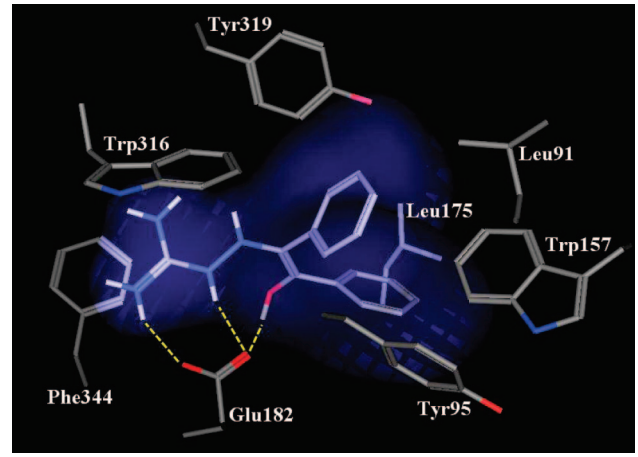



**Figure 5.** Binding mode of compound **8** from group II of the in vitro hits at the hH4R. Ionic interaction and H-bonds with Glu182 (5.46) are marked with dashed yellow lines.

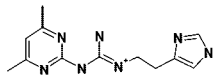
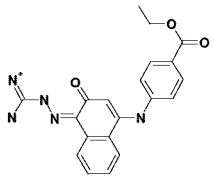
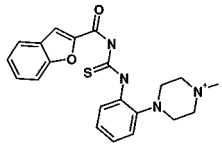
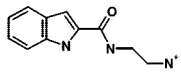
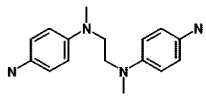
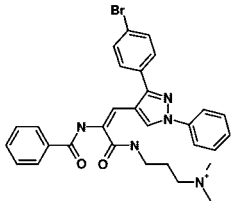
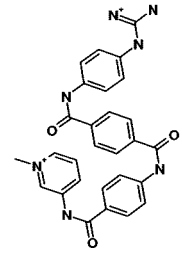
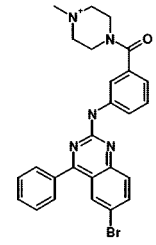
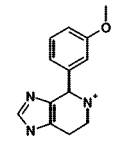
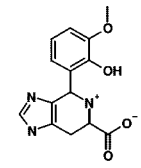
It is difficult to draw the line between "drug-like" and "nondrug-like" compounds, which is further supported by the numerous definitions of "drug-likeness" in the literature.[38,44,45] Also, several well-known, existing drugs are of "nondrug-like" properties. Moreover, Fialkowski et al. showed that molecular weight distribution of drugs is virtually identical to that of all chemicals stored in the Beilstein database.[46] This would argue with the concept suggesting molecular weight distributions of drug-like and random compounds differ significantly.[44] Another important aspect to consider is that "drug-likeness" can be improved during the "hit-to-lead" process to meet the required standards. Therefore, we did not apply any "drug-like" prefiltering of our database.

**Virtual Screening.** During docking, scoring, and evaluation of the results of our large-scale virtual screening, we have made some valuable observations. One of these is that a significant degree of redundancy can be established between the molecular databases of different vendors. Our database comprises 8743666 3D structures of small molecules. Considering one protonation and tautomeric state, the number of unique compounds in our database is 5066235. The observed redundancy is in line with the finding reported by Baurin et al.[47] These authors analyzed a chemical library of 2.7 million commercially available compounds and found that only 1.6 million of these were unique structures.

In analyzing the binding mode of the best molecules, we found that some of the ligands were positioned partially outside the protein. This was because our model lacks the membrane environment and also due to the fact that the hH4R binding site is relatively small. It also has to be mentioned that the hH4R homology model is based on the inactive crystal structure of bovine rhodopsin, where the second extracellular loop is considerably folded down to the transmembrane part. This could result in the shrinkage of the binding site of homology models. Therefore, one of the criteria used to select molecules for in vitro testing was that the ligand has to be positioned entirely in the binding site.

We also found compounds that were not protonated correctly during the MOE "washing" process. A typical error was the protonation of both nitrogens on a piperazine ring. Although this method is less than accurate, in the case of a database with millions of compounds, it was the only reliable alternative. It should be noted that, without "washing" the compounds with MOE, all tertiary amines would have been represented in their neutral form, showing thus no interaction with Asp94 (3.32) or Glu182 (5.46), resulting in several false negatives. ZINC compounds were considered in several different protonation states and tautomeric forms during the virtual screening. We found several compounds represented in a very unlikely protonation form, for example, with protonated pyridines, that are dominant only under pH = 5. Accordingly, as a second criterion by selecting molecules for in vitro screening, we have checked whether the protonation state of the ligand is reasonable. In our firm opinion, proper handling of protonation and tautomerism remains a cardinal question of virtual screening, and mistreatment of this aspect causes numerous false (mainly negative) results.

**Table 4.** Singletons of the In Vitro Hits[a]

| Structure | Compound | Displacement | Rank (by score) |
|---|---|---|---|
|  | 9 | 95.8% ± 4.3 | 35454. |
|  | 10 | 92.6% ± 0.6 | 34297. |
|  | 11 | 50.3% ± 4.7 | 894. |
|  | 12 | 46.9% ± 4.1 | 4446. |
|  | 13 | 34.3% ± 7.3 | 16721. |
|  | 14 | 29.4% ± 0.7 | 3470. |
|  | 15 | 26.0% ± 4.2 | 18074. |
|  | 16 | 23.0% ± 5.7 | 42960. |
| ANALOG score: -11.463  ORIGINAL score: -24.724  | 17 | 20.9% ± 1.0 | 21442. |

[a] Displacement values were determined from three independent experiments at an inhibitor concentration of 5 $\mu$M in the presence of 10 nM [$^3$H]histamine.

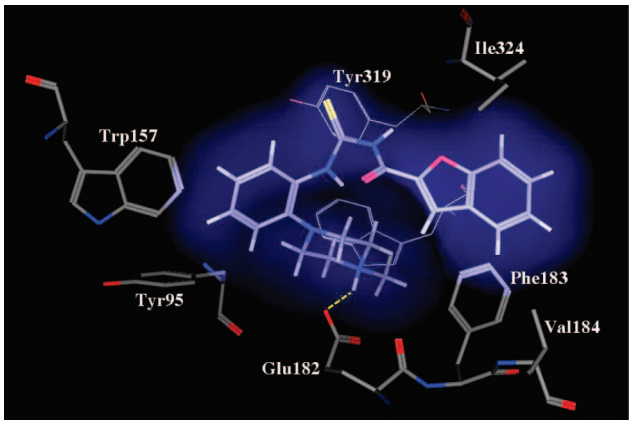



**Figure 6.** Binding mode of compound **11** at the hH4R. Ionic interaction with Glu182 (5.46) is marked with dashed yellow line.

After completing the screening phase, we used a two-step selection method to find promising molecules for in vitro screening. During the first selection, the structure of the best scoring compounds as well as their binding mode was analyzed carefully. This selection embraced a relatively small number of compounds (2000). The number of compounds analyzed in the second selection step (45000) was solely defined according to the enrichment results in our previous study,[37] where the highest enrichment factors were achieved by testing the top 0.5% of the database.

**In Vitro Screening.** The total of 255 selected compounds was investigated in a hH4R binding assay. We have found several novel scaffolds that can be used to develop new H4 ligands. This is in agreement with the extensive comparative virtual screening study of Evers et al.[48] They concluded that ligand-based approaches outperform structure-based techniques on amine-binding GPCRs, however, SBVS is a useful tool in GPCR drug design to identify novel ligands, especially when only limited ligand information is available. Also, effectiveness of SBVS on homology models highly depends on the quality of the protein model.

The NCI database contains almost 250000 compounds, which represents the 2.9% of our screened database. A significantly higher rate of NCI molecules were found among the virtual hits:

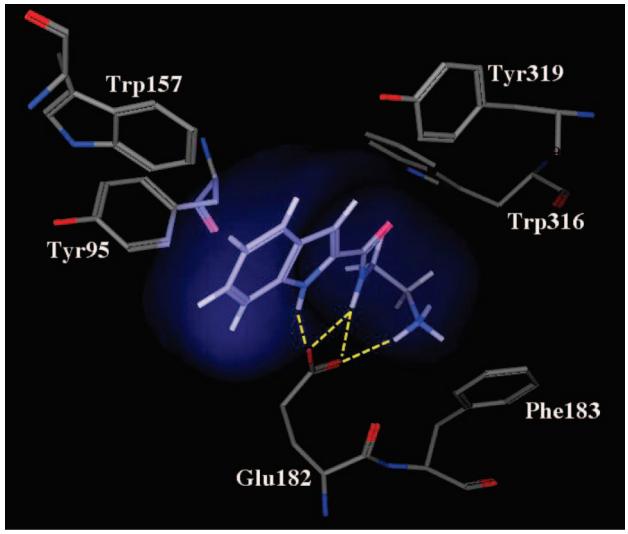



**Figure 7.** Binding mode of compound **12** at the hH4R. Ionic interaction and H-bonds with Glu182 (5.46) are marked with dashed yellow lines.

10.2% of the best 2000 and 4.6% of the best 45000 were NCI compounds. Even the in vitro hit rate of NCI compounds (8.6%, 5 in vitro hits/58 tested) was higher than the average (6.3%). In total, 5 out of the 16 in vitro hits were derived from the NCI database (31.3%). In our opinion, the NCI database is reasonably diverse, and it contains several unique structures. Therefore, we highly recommend the use of the NCI database for virtual screening.

Out of the 16 in vitro hits, 7 compose 2 groups and the remaining 9 are singletons. Group I of the in vitro hits has five members (Table 2).

All of these contain two cationic guanidinium functions linked by a lipophilic chain or ring system. Even a considerably bulky lipophilic system is well tolerated between the two positive centers (compound **2**). This part of the compounds has several substitutional possibilities to exploit. It is worth mentioning that compounds **4** and **6** only differ in a methyl group, however, this methyl group has a significant effect on the affinity. Analyzing the predicted binding mode of these compounds, however, we have not found any reasonable difference in their orientation at the binding site (see figure in Supporting Information). They both create two ionic interactions with Asp94 (3.32) and Glu182 (5.46), and the central part is positioned in a lipophilic cavity composed by residues Tyr95 (3.33), Trp157 (EC2 loop), Leu175 (5.39), Trp316 (6.48), and Tyr319 (6.51). Therefore, the increase of the lipophilic character at this part of the molecules can significantly influence their binding affinity. Members of this group created at least one ionic interaction with either Asp94 (3.32) or Glu182 (5.46).

The members of group II showed a similar displacement of histamine (Table 3).

They contain one cationic guanidinium group and a lipophilic aromatic system. According to the docking results they both form an ionic interaction with Glu182 (5.46), and their aromatic rings were found in the lipophilic surroundings of Leu91 (3.29), Tyr95 (3.33), Trp157 (EC2 loop), Leu175 (5.39), Trp316 (6.48), Tyr319 (6.51), and Phe344 (7.40). The hydroxyl group of compound **8** created an additional H-bond with Glu182 (5.46) (Figure 5).

With regard to the singletons, the two most potent in vitro hits (compounds **9** and **10**) showed a histamine displacement above 90% (Table 4).

Compound **11** has a protonated piperazine moiety that interacts with the side chain of Glu182 (5.46). Other parts of this compound create lipophilic interactions with several amino acids of hH4R, for example, Tyr95 (3.33), Trp157 (EC2 loop), Phe183 (5.47), Val184 (5.48), Trp316 (6.48), Tyr319 (6.51), and Ile324 (6.56) (Figure 6).

Compound **12** is a close relative of the first reported selective H4 antagonist, compound **1**.[49] It lacks the 5-chloro substituent on the indole-ring and contains a simple ethylamine side chain instead of the 4-methyl-piperazin moiety. H4R affinity of compound **1** analogs was previously published in a detailed study.[50] In this study, the p$K_i$ value of some analogs with a flexible side chain instead of a piperazine ring was also determined. The affinity of these more flexible compounds was more than 2 orders of magnitude lower than that of compound **1**. This is in line with our results, that is, compound **12** showed a moderate displacement of histamine in our radioligand binding assay. During the database preparation, we did not realize that a close relative of compound **1** was part of the database. On the other hand, our model ranked this molecule correctly into the top 0.5%, as a potential H4 active compound. Compound **12** formed one ionic interaction and two additional H-bonds with Glu182 (5.46) at the hH4R binding site. Lipophilic interactions were also formed with Tyr95 (3.33), Trp157 (EC2 loop), Trp316 (6.48), and Tyr319 (6.51) (Figure 7).

Compound **17**, which is an analog of the original virtual hit, was identified as a moderately active H4 ligand (Table 4). We decided to order analog compounds, because the original ones were no longer available from the vendors, but the basic structure of these hits seemed promising for the development of novel H4 agonists or antagonists. After hit identification, we docked compound **17** into the hH4R binding site and compared its binding mode and score with that of the original compound. We found significant differences: the COOH- and OH- groups of the original compound formed interactions with Thr178 (5.42) and Glu182 (5.46), respectively. On the other hand, these interactions were missing in the case of the analog. Furthermore, the scores of the best pose for the original and the analog compounds were –24.724 and –11.463, respectively. According to the analysis of binding modes and scores, we suggest that the original compound may exhibit a higher affinity than compound **17**.

Summarizing the binding mode analysis of the in vitro hit structures, every hit compound created at least one interaction with Asp94 (3.32) or Glu182 (5.46). This reinforces the hypothesis that these residues play crucial roles in ligand binding to hH4R.

The average hit rate of our hH4R in vitro screening was 6.3%. The hit rates, concerning "top_2000", "top_45000", and "top_45000_analog" were 4.6, 7.2, and 4.3%, respectively. It is interesting to note that "top_45000" selection yielded the highest hit rate. Moreover, the two most potent compounds were also found by this selection method. The first selection was based on the visual inspection of binding modes for the 2000 top-scoring hits, while the latter required no human intervention just filtering the top 0.5% of the ranked database, as suggested by preceding enrichment studies. These results underline the impact of hit selection approaches in virtual screening. "Top_45000_analog" selection resulted in the smallest hit rate as expected. Although structural cores were identical to those of the original hits altered substitution patterns are probably responsible for differential binding characteristics. Consequently, we have identified only one hit by testing analogs of the unavailable virtual hits. Docking calculations with this hit

revealed an altered binding mode relative to the original hit that might explain its moderate affinity to hH4R.

## 5. Conclusions

Structure-based virtual screening (SBVS) is becoming a general tool for computer-aided drug design. In recent years, several successful SBVS were published even on GPCR homology models. To the best of our knowledge, in this study, we report one of the largest validated SBVSs ever published. Moreover, this is the first structure-based drug design study reported on the human histamine H4 receptor (hH4R). After screening more than 8.7 million compounds virtually, we have identified several novel structures with a significant H4 affinity. These can serve as starting points in the development of selective H4 ligands in future.

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**Note Added after ASAP Publication.** This manuscript was released ASAP on May 7, 2008, with errors in Figure 3 and Tables 2–4. The correct version was posted on May 21, 2008.

**Supporting Information Available:** Binding mode of compounds **4** and **6** and LC-MS data for the in vitro hits. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Nissinen, M. J.; Karlstedt, K.; Castren, E.; Panula, P. Expression of histidine decarboxylase and cellular histamine-like immunoreactivity in rat embryogenesis. *J. Histochem. Cytochem.* **1995**, *43*, 1241–1252.
(2) Hough, L. B. Genomics meets histamine receptors: New subtypes, new receptors. *Mol. Pharmacol.* **2001**, *59*, 415–419.
(3) Zhang, M.; Thurmond, R. L.; Dunford, P. J. The histamine H4 receptor: A novel modulator of inflammatory and immune disorders. *Pharmacol. Ther.* **2007**, *113*, 594–606.
(4) Carballo, F. Efficiency of potent gastric acid inhibition. *Drugs* **2005**, *65*, 105–111.
(5) Pettit, M. Treatment of gastroesophageal reflux disease. *Pharm. World Sci.* **2005**, *27*, 432–435.
(6) Lovenberg, T. W.; Roland, B. L.; Wilson, S. J.; Jiang, X.; Pyati, J.; Huvar, A.; Jackson, M. R.; Erlander, M. G. Cloning and functional expression of the human histamine H3 receptor. *Mol. Pharmacol.* **1999**, *55*, 1101–1107.
(7) Leurs, R.; Blandina, P.; Tedford, C.; Timmerman, H. Therapeutic potential of histamine H3 receptor agonists and antagonists. *Trends Pharmacol. Sci.* **1998**, *19*, 177–183.
(8) Takahashi, K.; Suwa, H.; Ishikawa, T.; Kotani, H. Targeted disruption of H3 receptors results in changes in brain histamine tone leading to an obese phenotype. *J. Clin. Invest.* **2002**, *110*, 1791–1799.
(9) Hancock, A. A. The challenge of drug discovery of a GPCR target: Analysis of preclinical pharmacology of histamine H3 antagonists/inverse agonists. *Biochem. Pharmacol. Clin.* **2006**, *71*, 1103–1113.
(10) Nguyen, T.; Shapiro, D. A.; George, S. R.; Setola, V.; Lee, D. K.; Cheng, R.; Rauser, L.; Lee, S. P.; Lynch, K. R.; Roth, B. L.; O'Dowd, B. F. Discovery of a novel member of the histamine receptor family. *Mol. Pharmacol.* **2001**, *59*, 427–433.
(11) Oda, T.; Morikawa, N.; Saito, Y.; Masuho, Y.; Matsumoto, S. Molecular cloning and characterization of a novel type of histamine receptor preferentially expressed in leukocytes. *J. Biol. Chem.* **2000**, *275*, 36781–36786.
(12) Liu, C.; Ma, X.; Jiang, X.; Wilson, S. J.; Hofstra, C. L.; Blevitt, J.; Pyati, J.; Li, X.; Chai, X.; Carruthers, N.; Lovenberg, T. W. Cloning and pharmacological characterization of a fourth histamine receptor (H(4)) expressed in bone marrow. *Mol. Pharmacol.* **2001**, *59*, 420–426.
(13) Morse, K. L.; Behan, J.; Laz, T. M.; West, R. E. Jr.; Greenfeder, S. A.; Anthes, J. C.; Umland, S.; Wan, Y.; Hipkin, R. W.; Gonsiorek, W.; Shin, N.; Gustafson, E. L.; Qiao, X.; Wang, S.; Hedrick, J. A.; Greene, J.; Bayne, M.; Monsma, F. J., Jr. Cloning and characterization of a novel human histamine receptor. *J. Pharmacol. Exp. Ther.* **2001**, *296*, 1058–1066.
(14) Zhu, Y.; Michalovich, D.; Wu, H.; Tan, K. B.; Dytko, G. M.; Mannan, I. J.; Boyce, R.; Alston, J.; Tierney, L. A.; Li, X.; Herrity, N. C.; Vawter, L.; Sarau, H. M.; Ames, R. S.; Davenport, C. M.; Hieble, J. P.; Wilson, S.; Bergsma, D. J.; Fitzgerald, L. R. Cloning, expression, and pharmacological characterization of a novel human histamine receptor. *Mol. Pharmacol.* **2001**, *59*, 434–441.
(15) Hofstra, C. L.; Desai, P. J.; Thurmond, R. L.; Fung-Leung, W. P. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. *J. Pharmacol. Exp. Ther.* **2003**, *305*, 1212–1221.
(16) Lippert, U.; Artuc, M.; Grutzkau, A.; Babina, M.; Guhl, S.; Haase, I.; Blaschke, V.; Zachmann, K.; Knosalla, M.; Middel, P.; Kruger-Krasagakis, S.; Henz, B. M. Human skin mast cells express H2 and H4, but not H3 receptors. *J. Invest. Dermatol.* **2004**, *123*, 116–123.
(17) Voehringer, D.; Shinkai, K.; Locksley, R. M. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* **2004**, *20*, 267–277.
(18) Buckland, K. F.; Williams, T. J.; Conroy, D. M. Histamine induces cytoskeletal changes in human eosinophils via the H(4) receptor. *Br. J. Pharmacol.* **2003**, *140*, 1117–1127.
(19) Ling, P.; Ngo, K.; Nguyen, S.; Thurmond, R. L.; Edwards, J. P.; Karlsson, L.; Fung-Leung, W. P. Histamine H4 receptor mediates eosinophil chemotaxis with cell shape change and adhesion molecule upregulation. *Br. J. Pharmacol.* **2004**, *142*, 161–171.
(20) Gantner, F.; Sakai, K.; Tusche, M. W.; Cruikshank, W. W.; Center, D. M.; Bacon, K. B. Histamine h(4) and h(2) receptors control histamine-induced interleukin-16 release from human CD8(+) T cells. *J. Pharmacol. Exp. Ther.* **2002**, *303*, 300–307.
(21) Takeshita, K.; Sakai, K.; Bacon, K. B.; Gantner, F. Critical role of histamine H4 receptor in leukotriene B4 production and mast cell-dependent neutrophil recruitment induced by zymosan in vivo. *J. Pharmacol. Exp. Ther.* **2003**, *307*, 1072–1078.
(22) Thurmond, R. L.; Desai, P. J.; Dunford, P. J.; Fung-Leung, W. P.; Hofstra, C. L.; Jiang, W.; Nguyen, S.; Riley, J. P.; Sun, S.; Williams, K. N.; Edwards, J. P.; Karlsson, L. A potent and selective histamine H4 receptor antagonist with anti-inflammatory properties. *J. Pharmacol. Exp. Ther.* **2004**, *309*, 404–413.
(23) Fung-Leung, W. P.; Thurmond, R. L.; Ling, P.; Karlsson, L. Histamine H4 receptor antagonists: The new antihistamines. *Curr. Opin. Invest. Drugs* **2004**, *5*, 1174–1183.
(24) de Esch, I. J.; Thurmond, R. L.; Jongejan, A.; Leurs, R. The histamine H4 receptor as a new therapeutic target for inflammation. *Trends Pharmacol. Sci.* **2005**, *26*, 462–469.
(25) Lundstrom, K. Structural genomics of GPCRs. *Trends Biotechnol.* **2005**, *23*, 103–108.
(26) Palczewski, K. K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Le Trong, I.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **2000**, *289*, 739–745.
(27) Rasmussen, S. G. F.; Choi, H. J.; Rosenbaum, D. M.; Kobilka, T. S.; Thian, F. S.; Edwards, P. C.; Burghammer, M.; Ratnala, V. R.; Sanishvili, R.; Fischetti, R. F.; Schertler, G. F.; Weis, W. I.; Kobilka, B. K. Crystal structure of the human $\beta$2 adrenergic G-protein-coupled receptor. *Nature* **2007**, *450*, 383–387.
(28) Cherezov, V.; Rosenbaum, D. M.; Hanson, M. A.; Rasmussen, S. G. F.; Thian, F. S.; Kobilka, T. S.; Choi, H. J.; Kuhn, P.; Weis, W. I.; Kobilka, B. K.; Stevens, R. C. High-resolution crystal structure of an engineered human $\beta$2-adrenergic G protein coupled receptor. *Science* **2007**, *318*, 1258−1265.
(29) Furse, K. E.; Lybrand, T. P. Three-dimensional models for $\beta$-adrenergic receptor complexes with agonists and antagonists. *J. Med. Chem.* **2003**, *46*, 4450–4462.
(30) Costanzi, S.; Mamedova, L.; Gao, Z. G.; Jacobson, K. A. Architecture of P2Y nucleotide receptors: Structural comparison based on sequence analysis, mutagenesis, and homology modeling. *J. Med. Chem.* **2004**, *47*, 5393–5404.
(31) Salo, O. M.; Lahtela-Kakkonen, M.; Gynther, J.; Jarvinen, T.; Poso, A. Development of a 3D model for the human cannabinoid CB1 receptor. *J. Med. Chem.* **2004**, *47*, 3048–3057.
(32) ter Laak, A. M.; Timmerman, H.; Leurs, R.; Nederkoorn, P. H.; Smit, M. J.; Donne-Op den Kelder, G. M. Modelling and mutation studies on the histamine H1-receptor agonist binding site reveal different binding modes for H1-agonists: Asp116 (TM3) has a constitutive role in receptor stimulation. *J. Comput.-Aided Mol. Des* **1995**, *9*, 319–330.
(33) Clark, D. E.; Higgs, C.; Wren, S. P.; Dyke, H. J.; Wong, M.; Norman, D.; Lockey, P. M.; Roach, A. G. A virtual screening approach to finding novel and potent antagonists at the melanin-concentrating hormone 1 receptor. *J. Med. Chem.* **2004**, *47*, 3962–3971.

(34) Evers, A.; Klebe, G. Successful virtual screening for a submicromolar antagonist of the neurokinin-1 receptor based on a ligand-supported homology model. *J. Med. Chem.* **2004**, *47*, 5381–5392.
(35) Evers, A.; Klabunde, T. Structure-based drug discovery using GPCR homology modeling: successful virtual screening for antagonists of the alpha1A adrenergic receptor. *J. Med. Chem.* **2005**, *48*, 1088–1097.
(36) Varady, J.; Wu, X.; Fang, X.; Min, J.; Hu, Z.; Levant, B.; Wang, S. Molecular modeling of the three-dimensional structure of dopamine 3 (D3) subtype receptor: discovery of novel and potent D3 ligands through a hybrid pharmacophore- and structure-based database searching approach. *J. Med. Chem.* **2003**, *46*, 4377–4392.
(37) Kiss, R.; Noszál, B.; Rácz, Á.; Falus, A.; Erõs, D.; Keserû, G. M. Binding mode analysis and enrichment studies on homology models of the human histamine H4 receptor. *Eur. J. Med. Chem.* **2008**, in press.
(38) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23*, 3–25.
(39) Richards, W. G. Virtual screening using grid computing: the screensaver project. *Nat. Rev. Drug Discovery* **2002**, *1*, 551–555.
(40) Rarey, M.; Kramer, B.; Lengauer, T.; Klebe, G. A fast flexible docking method using an incremental construction algorithm. *J. Mol. Biol.* **1996**, *261*, 470–489.
(41) Shin, N.; Coates, E.; Murgolo, N. J.; Morse, K. L.; Bayne, M.; Strader, C. D.; Monsma, F. J., Jr. Molecular modeling and site-specific mutagenesis of the histamine-binding site of the histamine H4 receptor. *Mol. Pharmacol.* **2002**, *62*, 38–47.
(42) Irwin, J. J.; Shoichet, B. K. ZINC—A free database of commercially available compounds for virtual screening. *J. Chem. Inf. Model.* **2005**, *45*, 177–182.
(43) Miller, D. W. A chemical class-based approach to predictive model generation. *J. Chem. Inf. Comput. Sci.* **2003**, *43*, 568–578.
(44) Oprea, T. I. Property distribution of drug-related chemical databases. *J. Comput.-Aided Mol. Des.* **2000**, *14*, 251–264.
(45) Kelder, J.; Grootenhuis, P. D.; Bayada, D. M.; Delbressine, L. P.; Ploemen, J. P. Polar molecular surface as a dominating determinant for oral absorption and brain penetration of drugs. *Pharm. Res.* **1999**, *16*, 1514–1519.
(46) Fialkowski, M.; Bishop, K. J. M.; Chubukov, V. A.; Campbell, C. J.; Grzybowski, B. A. Architecture and evolution of organic chemistry. *Angew. Chem., Int. Ed.* **2005**, *44*, 7263–7269.
(47) Baurin, N.; Baker, R.; Richardson, C.; Chen, I.; Foloppe, N.; Potter, A.; Jordan, A.; Roughley, S.; Parratt, M.; Greaney, P.; Morley, D.; Hubbard, R. E. Drug-like annotation and duplicate analysis of a 23-supplier chemical database totaling 2.7 million compounds. *J. Chem. Inf. Comput. Sci.* **2004**, *44*, 643–651.
(48) Evers, A.; Hessler, G.; Matter, H.; Klabunde, T. Virtual screening of biogenic amine-binding G-protein coupled receptors: comparative evaluation of protein- and ligand-based virtual screening protocols. *J. Med. Chem.* **2005**, *48*, 5448–5465.
(49) Jablonowski, J. A.; Grice, C. A.; Chai, W.; Dvorak, C. A.; Venable, J. D.; Kwok, A. K.; Ly, K. S.; Wei, J.; Baker, S. M.; Desai, P. J.; Jiang, W.; Wilson, S. J.; Thurmond, R. L.; Karlsson, L.; Edwards, J. P.; Lovenberg, T. W.; Carruthers, N. I. The first potent and selective non-imidazole human histamine $H_4$ receptor antagonists. *J. Med. Chem.* **2003**, *46*, 3957–3960.
(50) Terzioglu, N.; van Rijn, R. M.; Bakker, R. A.; de Esch, I. J.; Leurs, R. Synthesis and structure–activity relationships of indole and benzimidazole piperazines as histamine H4 receptor antagonists. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5251–5256.

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