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Computerized modeling techniques predict the 3D structure of H_4R: Facts and fiction

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1. ABSTRACT

The functional characterization of proteins presents a daily challenge for biochemical, medical and computational sciences, especially when the structures are undetermined empirically, as in the case of the Histamine H4 Receptor (H_4R). H_4R is a member of the GPCR superfamily that plays a vital role in immune and inflammatory responses. To date, the concept of GPCRs modeling is highlighted in textbooks and pharmaceutical pamphlets, and this group of proteins has been the subject of almost 3500 publications in the scientific literature. The dynamic nature of determining the GPCRs structure was elucidated through elegant and creative modeling methodologies, implemented by many groups around the world. H_4R which belongs to the GPCR family was cloned in 2000; understandably, its biological activity was reported only 65 times in pubmed. Here we attempt to cover the fundamental concepts of H_4R structure modeling and its implementation in drug discovery, especially those that have been experimentally tested and to highlight some ideas that are currently being discussed on the dynamic nature of H_4R and GPCRs computerized techniques for 3D structure modeling.

2. INTRODUCTION

Histamine is a physiological amine that regulates cellular functions and triggers the inflammatory response. Histamine interacts with receptors belonging to the G-protein coupled receptors (GPCRs) super–family. GPCRs are grouped into 6 classes (A-F) based on sequence homology and functional similarity (1, 2). The H_4 receptor (H_4R) belongs to class ''A'' of the GPCRs. To date, four histamine receptors activated by the same endogenous agonist, histamine are known (3) and one of them, H_4R is the most novel, cloned over a decade ago on the basis of its high sequence homology with the H_3 receptor (4-8) (Figure 1 and table 1). H_4R is mainly present in leukocytes and mast cells (5, 6). This expression pattern implies that it has a role in both immune and inflammatory responses. Indeed, a growing body of evidence indicates that H_4R is involved in chemotaxis, allergy, inflammation, autoimmune disorders and acts as a mediator release in various types of immune cells including mast cells, eosinophils, monocyte-derived dendritic cells and T cells. Moreover, H_4R is involved in the modulation of various interleukins (IL), such as IL-B4 and IL-16 (9, 10); suggesting that H_4R is a potential drug target for inflammatory diseases such as

Table 1. Sequence identities of human histaminegic receptors

	H_1R	**H_2R**	**H_3R**	**H_4R**
H_1R		19.6%	22.9%	21%
H_2R	35.6%		19.4%	19.9%
H_3R	32.5%	32.5%		**35.8%**
H_4R	29.4%	27.3%	**54.1%**	

The upper half of the matrix shows the sequence identity for the complete receptor sequence while the lower half shows the sequence identity of the trans-membrane domains.

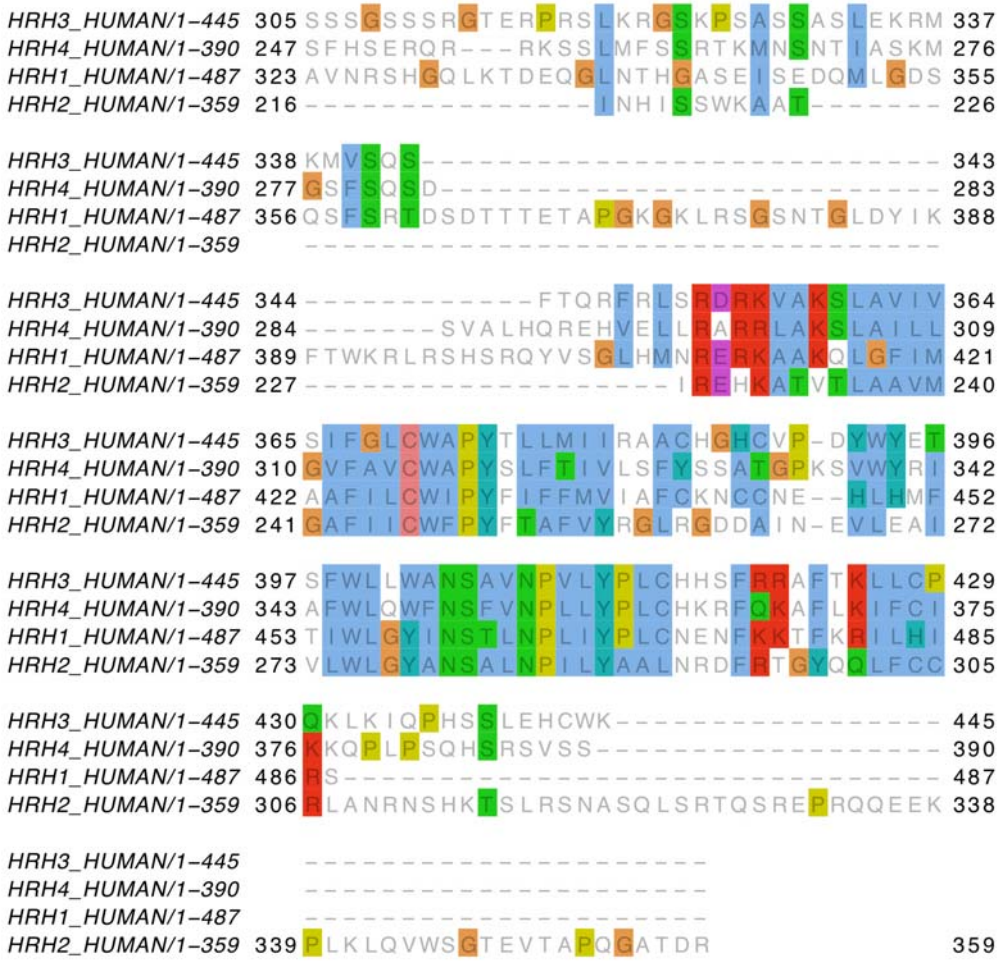


Figure 1. multiple sequence alignment of the four human histaminergic receptors (H_1-H_4). The trans-membrane domain of the four receptors is as following: Helices I to IV lie between 32-61 (30); 68-97 (30); 104-136 (33); 150-172 (23); 195-220 (26); 418-448 (31) and 458-478 (21) respectively. In parentheses we note the length of the helix.

allergy, bronchial asthma, rheumatoid arthritis rhinitis and pruritus (10, 11). The physiological activity of H_4R (and GPCRs) is beyond the scope of this article but useful information can be found elsewhere (12, 13).

2. H_4R AGONISTS AND ANTAGONISTS

Based on experiments using animal models, H_4R antagonists show reasonable therapeutic potential for treatment of allergy, inflammation, asthma and colitis (14-17). Although they have not been tested for efficacy in autoimmune disorders, there is an emerging role for histamine and mast cells in autoimmune processes (18). These studies and others, indicate that H_4R may play an essential role in autoimmune diseases and suggest that antagonists for the H_4 receptor may open new venues for their treatment.

Unlike H_4 receptor antagonists, H_4 receptor agonists seem to be less useful therapeutically. Nevertheless, they are valuable in the exploration of the functionalities of H_4R (19-21). A cohort of H_4R agonists have been reported (22), however only a limited number of selective H_4R agonists (23) have been identified so far: 4(5)-methylhistamine (20), VUF 8430 (17) and OUP-16 (21) (Figure 2).

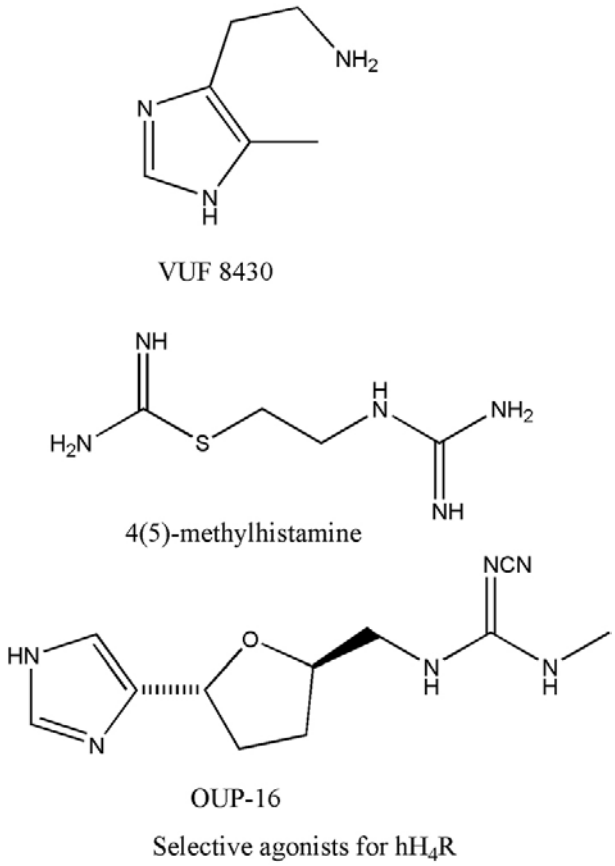


Figure 2. Structural formulas of the selective H_4R agonists.

3. GPCRs AND THEIR STRUCTURE MODELING TECHNIQUES

G-protein coupled receptors (GPCRs) are the largest integral membrane protein family in the human genome. They have a typical structural topology consisting of seven transmembrane helices (7TMH) connected by intracellular and extracellular loops, with an extracellular N-terminal and an intracellular C-terminal (24) (Figure 3). GPCRs derive their name from their ability to recruit and regulate the activity of intracellular heterotrimeric G-proteins. GPCRs are also known as seven-transmembrane domain receptors, 7TM receptors, heptahelical receptors, serpentine receptor, and G protein-linked receptors (GPLR). Their main role is to transduce a signal across the cell membrane. Such signals emerge from interactions of GPCRs with extracellular agents, known as "ligands" or "agonists". These ligands are highly diverse entities (e.g., ions, biogenic amines, nucleosides, lipids, peptides, proteins, and even light). Ligand binding is followed by a conformational change that results in a decreased affinity of GPCR to G-proteins. Thus, the binding of such agonists and GPCRs results in signal transduction that induces a cascade of intracellular responses (12, 13, 25).

GPCRs are major contributors to information flow into cells and, as such, are involved in a wide range of physiological processes and diseases, including but not limited to those affecting the cardiovascular, nervous, endocrine, and immune systems. The cardinal involvement of GPCRs leads to their designation as drug targets in a multitude of therapeutic areas, to the effect that GPCRs are considered to be the largest group of drug targets to date. It has been estimated that GPCRs comprise ~45% of the currently marketed drug targets (26-29), and 60-70% of the drugs in development nowadays (30).

GPCRs can recognize structurally diverse ligands ranging from photons to ions, amino acids, small organic molecules, lipids, peptides, or proteins. The location of the ligand binding pocket is known for many of these receptors (31, 32). For instance, small organic molecules are known to bind within the transmembrane domain (TMD) (Figure 4), while peptides and proteins interact with the N-terminus and/or the extracellular loop regions. Despite these differences in ligands and ligand-binding sites, the majority of GPCRs can interact and activate one or more of the 16 known α-subunits of heterotrimeric G-proteins. Furthermore, compelling recent evidence suggests that some GPCRs can also activate G protein-independent signaling pathways (33, 34).

The number of known GPCRs is in the thousands, and many more are being discovered as a result of recent advances in genomics and proteomics. Structures

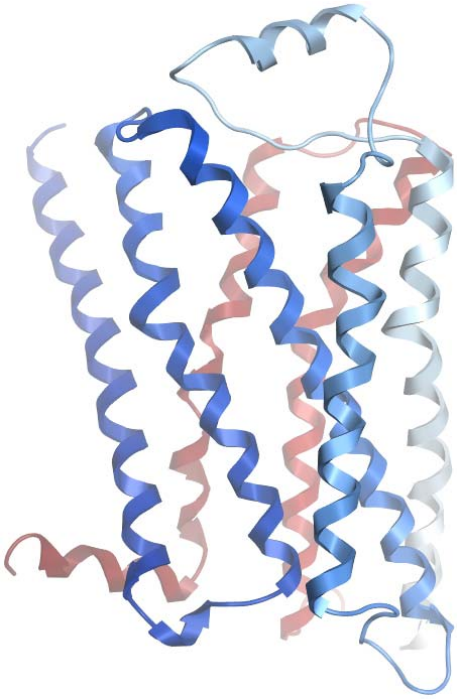


Figure 3. Cartoon representation of Rhodopsin-like family GPCRs, seen from aside in a direction parallel to the membrane surface. The general fold is composed of N-terminus, 3 extracellular loops, 3 intracellular loops, C-terminus and 7 trans-membrane helices, colored in blue to orange from N-terminus and C-terminus. Drawings were done by using the software MOE 2009.10 (http://www.chemcomp.com/software-moe2009.htm/).

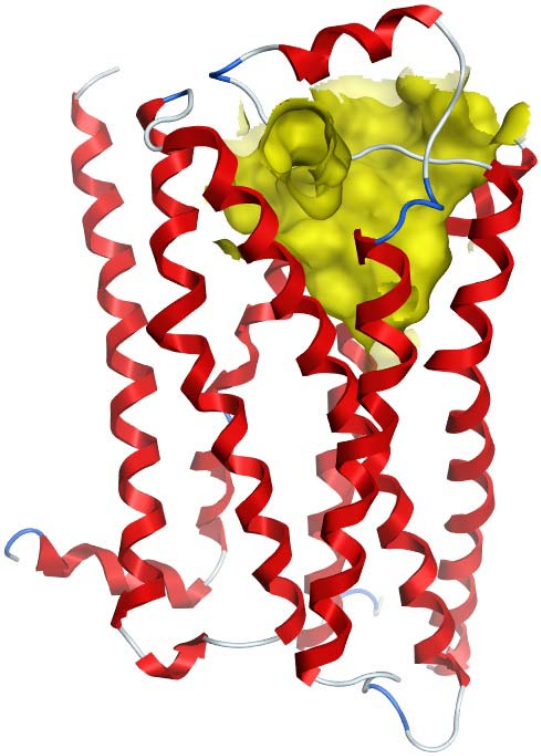


Figure 4. The binding pocket region of histaminergic receptors colored in yellow. The pocket is formulated by residues from helices III-VII in upper part as well as second and maybe third extra-cellular loops.

of these drug targets should be elucidated in order to employ them by methods of "Structure Based Drug Design" (SBDD) (35). The structural aspects of GPCRs are however a source of constant debate during the last two decades. Difficulties in understanding GPCR functioning mechanisms are also related to the lack of high-resolution structural information on more than 99% of these proteins. Computational modeling of GPCRs has, hence, to face the difficulties due to the lack of high-resolution information even on the ground state.

From the molecular point of view, all GPCRs share a common molecular architecture, being composed of a single polypeptide chain folded into a bundle of seven α-helical TMs connected by three extracellular and three intracellular loops. The N-terminus is located in the extracellular space, while the C-terminus is in the cytosol (Figure 3 shows general GPCR topology). Given that structure-based drug discovery is an efficient method to rationally design novel drugs and improve the properties of old drugs, the scientific community has been striving for a long time to shed light onto the elusive structure-function relationships of GPCRs employing a variety of direct biophysical and indirect biochemical methods (36). Direct experimental study of GPCR structures is currently too complicated because of their native membrane environment (37), resulting in limitations in the purification and crystallization processes. Up-to-date, only five GPCRs: Bovine rhodopsin, squid rhodopsin, beta2 adrenergic receptor (beta2aR), beta1 adrenergic receptor (beta1aR) and A2A adenosine receptor have been structurally solved by high-resolution crystallography and their 3D structures are elucidated (38-44). The prospects for elucidating the structures of other GPCR are not very high, and await a major breakthrough (45, 46). Structural information on GPCRs could be attained by techniques of electron crystallography, electron paramagnetic resonance, UV absorbance and fluorescence spectroscopy, nuclear magnetic resonance (NMR) spectroscopy (47-49) as well as computer modeling. The predicted structures could also be validated by some other experimental techniques such as substituted cysteine accessibility method (SCAM) (50, 51) and site directed mutagenesis (52, 53).

3.1. *De novo* and *ab initio* models

Two alternative computational approaches were used in order to build molecular models of GPCRs without using specific homologous template structures: *de novo* (knowledge-based approaches) and *ab initio* (first-principles approaches). *De nov*o protein design and *ab initio* structure prediction are two problems at the forefront of research in the fields of structural biology and chemistry. The goal of an *ab initio* technique is to characterize the 3D structure of a protein using only the amino acid sequence as input. Based on the independent folding concept introduced by Anfinsen (54), *ab initio* modeling attempts to simulate the physical forces that drive protein folding, using energy functions such as molecular mechanics force fields as well as statistical functions.

De novo protein design involves the production of novel protein sequences that adopt a desired fold. The experimentally resolved structures of the five GPCRs are considered to be the prototypes of the main family of GPCRs, referred to as type A. The rhodopsin crystal structure has often been used as a template in the homology modeling of the transmembrane region of several GPCR subtypes. This paradigm is based on experimental evidence that part of the template conformation is similar to other GPCRs (29, 38, 47, 49, 55).

Based on the cryoelectron microscopy density maps, a cohort of helices models using hydrophobicity properties of receptors and geometric parameters were built (56). Similar methods were developed to predict structures of GPCRs, as well as ligand binding sites and relative binding affinities (57). Although the GPCR conformational space is small compared to water-soluble proteins, it is still very complicated especially due to Loops of variable lengths, non-conserved non-canonical elements and unexpected structural diversity (40, 42), when compared to the rhodopsin crystal structure. This is further complicated by the presence of cavities that can accommodate water molecules and/or different ligands. A further source of complexity is the possible influence of interacting GPCR subunits or other proteins of the signaling cascade. Thus, although *de novo* and *ab initio* methods may suggest reasonable TM arrangements of GPCRs, the accuracy of their predictions is limited by the experimental information available. As a result, homology modeling is considered a more reliable technique whenever applicable (58). Moreover, "*de novo/ab initio*" predictions for GPCR sequences shorter than 10 residues have been quite reliable when using reasonable physical-chemical force fields incorporating explicit water molecules or implicit solvation models rather than a vacuum environment. These however, increase the computational complexity of modeling, and are thus, not applicable to larger proteins. This would suggest that physical-based GPCR modeling should be abandoned in favor of a non-physical method, such as homology modeling. However, due to insertions and deletions, the loop regions in GPCRs exhibit variable length and low sequence identity (below 30%) (59). Hence, it is not reasonable to initiate homology modeling based on the loops of known GPCR structures. De *novo/ab initio* computational approaches using either coarse-grained backbone dihedral sampling (60) or Monte Carlo (MC) simulations in a temperature annealing protocol combined with a scaled collective variables (SCV) technique (61) have been shown to accurately predict loop regions of GPCRs with decreasing performance at increasing loop lengths. Prediction of longer and interacting motifs is still a very challenging task (62). The reasons for the failure of current "*de novo/ab initio*" approaches in predicting long peptides stem from the difficulty in carrying out sufficiently complete searches of their conformational spaces, as well as insufficiently accurate force fields (63).

3.2 Homology (comparative) modeling

Due to the lack of experimental 3D-structures of other GPCRs, one could hope to gain some information from approximations based on molecular models. While "*de novo/ab initio*" modeling is not practical yet for any protein (64-66), "homology"/"comparative" modeling are

quite established methods (65, 67) and are expected to be especially successful for modeling the GPCR superfamily A since it is considered to have the general features of five potential templates (68). Indeed, many GPCR structures have been modeled recently, based on the template of bovine rhodopsin/beta2-adrenergic receptor/ A2A adenosine receptor, by using its backbone coordinates and adding the appropriate side chains for each sequence (69-73). Such homology modeling of GPCRs has been aided mainly by experimental information from point mutations and other experimental data sources (74-76).

In homology modeling, the modeled protein retains some of the features of the selected template. The length of helices in the modeled TMD remains similar to those of the template receptor while the loops are generally not included in the template construction, except in those rare cases where loop lengths and compositions are similar to those of the template. Other approaches for constructing models of GPCRs suggest that GPCRs could differ in their structure from rhodopsin or other known receptors even though their general features are similar (77, 78). There are few indications to justify such deviations from the template structure, when constructing models for other GPCRs. A review by Baker and Sali (79) has shown that a homology model for a protein at medium size or larger with a sequence identity of less than 30% to the template crystal structure is unreliable. The averaged sequence identity of TMHs of hGPCRs to bovine rhodopsin/squid rhodopsin/beta2-adrenergic receptor/beta1-adrenergic receptor/A2A adenosine receptor rests outside the regimen boundaries of traditional homology modeling (29). Others in the modeling community think that while this "rule" is correct for globular proteins, it is doubtful whether this "rule" could be extended to membrane proteins. Moreover, this rule does not specify how identity should be distributed along a sequence. As much as the GPCRs superfamily is united by an overall structural topology and an ability to recruit and regulate the activity of G proteins, sequence identity between superfamily members, despite the conserved transmembrane cores, is too low. Significant sequence conservation is found, however, within several subfamilies of GPCRs. The subfamily of rhodopsin-like GPCRs is by far the largest (more than 85% of GPCRs) and is characterized by the presence of some 35 (out of ~190) highly conserved residue positions in the TMD, that may be crucial for folding and/or involved in binding and/or in activation (80).

By sequence analysis of the TMD of 302 GPCRs, Palczewski and his colleagues (81) came to the conclusion that it is reasonable to speculate that the overall fold of these receptors is highly conserved". One of the implications of this study is that it is reasonable to use the overall structures of the available reference receptors to model the TMD of other GPCRs using homology modelling.

Another obstacle in the modelling of GPCRs is the conformational change that may coincide with activation. In contrast to inactive state of GPCRs, no crystal structures of active states are available to date. Given the absence of experimental structural information, several investigators have applied computational strategies to predict activated models of GPCRs (60, 82-87). Although the rhodopsin activated models generated to date appear to satisfy most of the experimental data known for GPCRs, novel predictions deriving from their analyses still await experimental validation. Moreover, it remains to be determined whether or not all GPCRs share the same activated forms.

A recently published study by Rayan (29) examined to what extent are the structures of the five G-protein coupled receptors useful as templates for constructing models of other GPCRs. A quantitative measure of conservation in GPCRs family was helpful to decide upon the exact parts of the receptors that could be used as templates for such comparative modeling, and those that should be optimized. The study assigned the parts of the structure of the reference receptor that may be used as a template, and suggested the construction of the remaining parts by other methods that allow deviations from the crystal structure of the template.

4. 3D STRUCTURE MODELS OF H_4 RECEPTOR

H_4 receptor was modeled by several groups based on bovine rhodopsin and human beta2-adrenergic receptor. Table 2 summarizes the published H_4R models.

4.1. Rhodopsin-based homology modeling era 2000-2007

During the last decade, studies were done to define the histamine-binding site of H_4R. Shin *et al* (2002) (88) performed molecular modeling and site-directed mutagenesis to predict identity and functional importance of amino acids residing in the histamine binding pocket. The results indicated that Asp94 in transmembrane region 3 (TM3) and Glu182 in TM5 are critically involved in histamine binding. Asp94 was found to most likely serve as a counter-anion to the cationic amino group of histamine, whereas Glu182 interacts with the N^{τ} nitrogen atom of the histamine imidazole ring via an ion pair. In contrast, Thr178 and Ser179 in TM5 were found not to be significantly involved in either histamine binding or receptor activation. These results resembled those for the analogous residues in the H1 histamine receptor but contrasted with findings regarding the H2 histamine receptor. These results also demonstrated that Asn147 in TM4 and Ser320 in TM6 could play a role in receptor activation, but were not involved in histamine binding. Taken together, this data indicated that although histamine seemed to bind to the H_4R in a fashion similar to that predicted for the other histamine receptor subtypes, there are also important differences that can probably be exploited for the discovery of novel H_4-selective compounds. The molecular model of the human H_4 receptor was constructed from the structure of rhodopsin using the Look program (Molecular Application Group, Palo Alto, CA) which uses the SEGMED program (89). A model of histamine was docked to the H_4 receptor homology-based model at a site corresponding to its expected binding pocket in the H_1 and H_2 receptors, based on prior mutagenesis data. The resulting complex model was further refined by molecular mechanics minimization.

Table 2. summary of the distinct models of H_4 receptor reported in literature

Model (M)	Species	Ligands	Ref. Structure	Docking
M1 (88)	Bovine	AN	Rhodopsin	Homology-based molecular modeling- SEGMED
M2 (90)	Bovine	AN	Rhodopsin	Homology modeling followed by FlexX docking
M3 (94)	Bovine	AG- AN	Rhodopsin	Homology Modelling followed by Energy minimization and MD (docking with fixed main chain and non-fixed side chains)
M4 (96)	Bovine	AG-AN	Rhodopsin	Protein backbone constrained MD (NAMD 2.6 package)
M5 (97)	Bovine	AG- AN	Rhodopsin	Sequence alignment followed by docking with fixed backbone as well as without constraints (FlexX, FlexX-Pharm).
M6 (100)	Human	AG- AN	β2-adrenergic G protein-coupled receptor	Sequence alignment, homology modeling, and energy minimization
M7 (101)	Human	AG- AN	β2-adrenergic G protein-coupled receptor	- Inactive model: manual followed by MD using energy minimization - Active model: distance-restrained MD (GROMACS)
M8 (23)	Human	AG	β2- adrenergic receptor	Homology Modelling followed by Energ minimization and MD
M9 (103)	Bovine	AN	Rhodopsin	Automated pseudoreceptor construction algorithm and MD
M10 (104)	Human	AG- AN	β2- adrenergic receptor	Structure prediction without using homology – MembStruk, docking with HierDock/MscDock comparing energy to known ligand binding energies
M11 (84)	Bovine	AG	Opsin in complex with a C-terminal peptide derived from the G-alpha subunit of transducin and the inactive model (23)	Homology Modeling of the active model in interaction with $G\alpha_{i2}$-CTs with the software suite Sybyl

Abbreviations: AG, Agonist; AN, Antagonist.

Later in 2008, Kiss and coworkers (90) developed homology models of human histamine H_4 receptor (hH_4R) by utilizing crystal structure of bovine rhodopsin as a template. Histamine has two major anchoring points at the hH_4R binding site, Asp94 and Glu182. Following histamine docking to the binding site, the carboxylate group of Asp94 was rotated around the C_α-C_{beta} axis to accommodate histamine more favorably. Docking resulted in improved binding modes of histamine having simultaneous interactions with Asp94 and Glu182. This study was conducted by utilizing structure-based virtual screening (SBVS) of a ligand-supported homology model of the hH_4R (91-93). More than 8.7 million 3D structures derived from different vendor databases were investigated by docking known chemical compounds to the hH_4R binding site using the FlexX program. A total of 255 selected compounds were tested by radio-ligand binding assay and 16 of them show significant [3H]histamine displacement. Several novel scaffolds were identified for further development of selective H_4 ligands. Enrichment tests revealed that this model is able to select highly efficient known H_4 ligands from random decoys. An enrichment factor of 40–50 was attained through analysis of the top 0.5% of the ranked database. This enrichment factor indicates that the homology model in this case could be used for virtual screening and antagonist discovery.

As it was previously stated, Asp94 and Glu182 were shown to participate in the GPCR ligand binding. It was postulated in the rhodopsin-based homology model that Asp94 interacts in its anionic state, whereas Glu182 interacts in its neutral form. Jongejan and colleagues tested those two options by applying point mutations Asp94Asn and Glu182Gln (94). The Asp94Asn mutation abolished all detectable binding affinities for all ligands. The Glu182Gln mutant causes a 1000-fold decrease in histamine-like ligands without causing an affinity change in other ligands. The proposed model for agonist binding as well as ab initio calculations for histamine and the recently described selective, nonimidazole agonist VUF 8430 (17) (Figure 2) can explain the observed differences in binding to the H4R mutants. These studies provide a molecular understanding for the action of a variety of H4 receptor-ligands. The model of the transmembrane domains of the H_4R plus helix 8 that expands parallel to the membrane was constructed by homology modeling using the crystal structure of bovine rhodopsin (PDB code 1L9H) as a template. The residues which are considered to be mostly conserved in the Rhodopsin-like family of GPCRs were aligned in both sequences. The derived model of the wild type H_4R was employed to build the molecular models for the mutated receptors by changing the atoms implicated in the amino acid substitutions by interactive computer graphics. Using the proposed binding mode of histamine in the H_1R as a starting point, initial docking poses were obtained. This served as the starting point for physical-based molecular mechanics simulations. Energy minimization was performed while fixing all receptor atoms, except for the side chain atoms of the residues in direct contact with the ligand. Short (10-20 ps) MD simulations were performed *in vacuo* to overcome energetic barriers and explore conformational space. The validity of the proposed model of recognition of histamine and VUF 8430 was subsequently confirmed with *ab initio* geometry optimizations, using quantum mechanical simulation at the 6-31G(d,p) level of theory (95). Jongejan *et al* (94) found that the glutamic acid residue Glu182 is the source of the increased affinity of histamine observed for both the H_3R and H_4R using mutational analysis. In the resultant H_4R model, TM3 is repositioned relative to the core architecture of rhodospin due to the presence of two unique glycine residues in TM2 in the rhodopsin structure. These result in a slight bend of TM3, at position 99, toward TM5. Asp94, the major site of interaction for ligands containing a

protonated moiety, becomes therefore situated in even closer proximity to Glu182 in TM5.

To differentiate between the active and inactive forms of the receptor, molecular dynamics (MD) simulations were performed on H_4R homology-based models. Jojart (96) carried out MD simulations in an explicit membrane (POPC/TIP3P) and water molecule environment on the homology model of the hH4R. The MD simulations were conducted on the receptor alone, in complex with its endogenous activator histamine and with the selective hH_4R antagonist JNJ7777120. These models were built by using the crystal structure of Bovine rhodopsin as a template, which was the only GPCR with an experimentally determined 3D structure (84). The complex structures were obtained after docking experiments and subsequent optimization. During the simulation of the histamine-hH_4R complex, considerable changes occurred in the hH_4R structure as well as in the interaction pattern of histamine at the binding site. These changes were in agreement with experimental data published on GPCR activation. In particular, the intracellular side of TM6 moved significantly away from TM3 and TM7. As previously reported, histamine's ethylamine showed interaction with Glu182 and its imidazole hydrogen bonded to Asp94 alternating to Glu162. Moreover, histamine formed an H-bond with Asn147 serving as a donor, a residue previously proved to be important in hH_4R activation as its mutation to non H-bond donor residues lowers hH4R activation. The MD simulations of the native hH_4R and the JNJ7777120-hH_4R complex suggest that these models represent an inactive conformation of hH4R. MD simulation in the presence of JNJ7777120 resulted in the movement of the intracellular side of TM6 in the direction of TM3, opposite of its outward movement characteristic to activation (96). This modeling strategy provided an ensemble of 3D structures for both active and inactive receptors. As the authors suggested, this ensemble is potentially useful for structure based drug design (96).

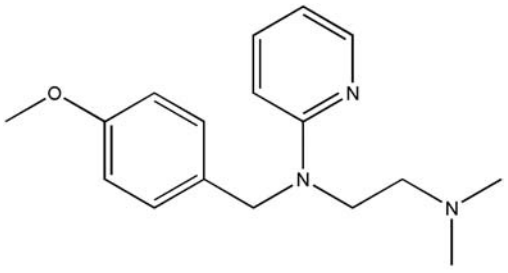
Subsequent studies were also conducted to check for differences in binding mode between agonist and antagonist H_4R ligands, based on the crystal structure of bovine rhodopsin and distinct known H_4 receptor ligands (97) [histamine, OUP-16 (the first reported H4 agonist with a considerable selectivity over H_3R (98)) and JNJ7777120 (the first reported selective H4 antagonist (16))]. Fishing experiments were performed by Kiss *et al* (97) to figure out whether these hH_4R models can pick up "actives from haystack". The impact of receptor conformation and the effect of different sets of random decoys, docking methods (FlexX, FlexX-Pharm) and scoring functions (FlexX-Score, D-Score, PMF-Score, G-Score, ChemScore) were investigated. It was found that two agonists (histamine and OUP-16) (Figure 2) form complementary interactions with Asp94, Glu182 and Thr323, whereas JNJ7777120 interacts with Asp94 and Glu182 only. These results suggest a role of Thr323 in ligand binding and presumably also in receptor activation. The models that were optimized in the presence of an agonist (histamine) and an antagonist (JNJ7777120), were compared in more detail. A conclusion was drawn that the type of the ligand which is utilized in modeling and model refinement can significantly influence the efficacy in virtual screening. Six initial hH_4R models were built by the MODELLER program. In accordance with the sequence alignment, the model contained a disulfide-bond between residues Cys87 (at the C-terminus of TM4 and Cys164 (in the second extra-cellular loop). Several tests have been performed to check the quality of the more suitable hH_4R model, by assessing the Ramachandran plots and packing quality. The overall quality of the model and the template were quite similar. As a final test, HARMONY (99) was utilized to evaluate whether the residues of the model adopt a structural environment that frequently occurs in protein structures. HARMONY indicated that the model and the template possess quite the same overall quality (35).

4.2. The human beta$_2$-adrenergic receptor era: 2007-recently

Studies were further extended to detect variations in binding mode in response to species variations after human beta$_2$-adrenergic receptor (h-beta$_2$AR) was finally crystallized. Using the natural variation in histamine H_4 receptor protein sequence, Lim and his colleagues (100) tried to identify amino acids involved in the binding of H_4 receptor agonists. After identification of a domain between the top of TM4 and the top of TM5 as being responsible for the differences in agonist affinity between human and mouse H_4 receptors, detailed site-directed mutagenesis studies were performed. These studies identified Phe169 in the second extracellular loop as the single amino acid responsible for the differences in agonist affinity between the human and mouse H_4Rs. Phe169 is part of a Phe-Phe motif, that existes in the beta$_2$AR, which was structurally determined by crystallographic methods (42). These results point to an important role of the second extracellular loop in the agonist binding to the H_4 receptor and provide a molecular explanation for the species difference between human and mouse H_4 receptors.

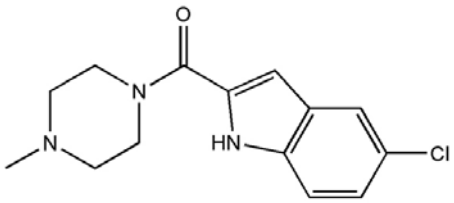
The human H_4R was modeled based on the crystal structure of beta$_2$-adrenergic receptor (Protein Data Bank code 2RH1), which lacks the N-terminal tail and contains a T_4 ligase structure in the third intracellular loop (IL) (42). The latter was removed in the model template. A large part of IL3 of the H_4R was removed to fit the length of the IL3 of the template. Alignment constraints were applied to avoid gaps in TM domains between Thr146 and Gly128, Leu167, and Pro149 of beta$_2$-adrenergic receptor and H_4R, respectively. In extracellular loop 2 (EL2), constraints were put between Cys191 and Cys164, Phe193, and Phe168 as well as Tyr174 and Ser156 of the beta$_2$AR and H_4R respectively. This alignment was used to run homology modeling and resulted in models with a preserved disulfide bridge.

Another study by Deml *et al* (101) was aimed to explore the value of dual H_1R / H_4R antagonists as anti-allergy drugs and to address the question of whether H_1R ligands bind to hH_4R based on the crystal structure of h-beta$_2$AR. In an acute murine asthma model, the H_1R antagonist mepyramine (Figure 5) and the H_4R antagonist JNJ7777120 (Figure 6) exhibited synergistic inhibitory

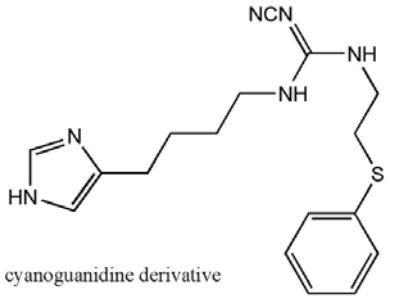


Mepyramine

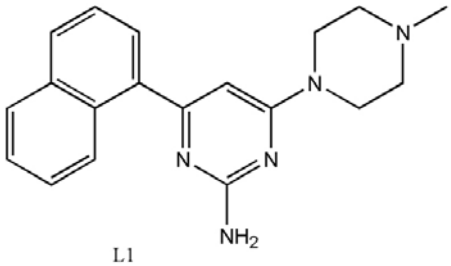
Figure 5.H_1R antagonist.



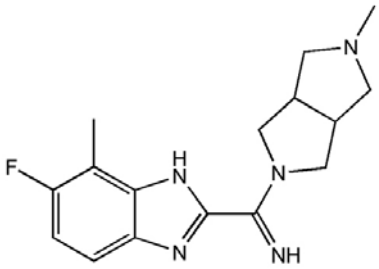
JNJ-7777120



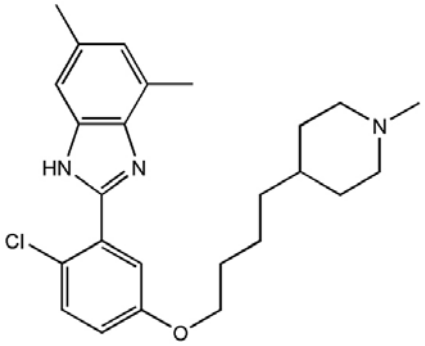
cyanoguanidine derivative



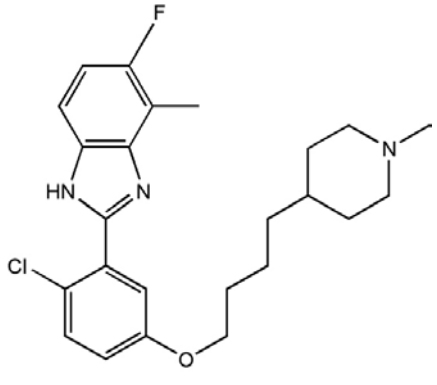
L1



L2



L3



L4

Figure 6. Structural formulas of some selective H_4R antagonists.

effects on eosinophil accumulation in the bronchoalveolar lavage fluid. As assessed in competition binding experiments 18 H_1R antagonists and 22 H_1R agonists showed lower affinity to hH_4R than to hH_1R. Most compounds were neutral antagonists or inverse agonists. Twelve phenylhistamine-type hH_1R partial agonists were also hH_4R partial agonists. Four histaprodifen-type hH_1R partial agonists were hH_4R inverse agonists. Dimeric histaprodifen was a more efficacious hH_4R inverse agonist than the reference compound thioperamide. Suprahistaprodifen was the only histaprodifen acting as hH_4R partial agonist. Suprahistaprodifen was docked into the binding pocket of inactive and active hH_4R models in two different orientations, predominantly stabilizing the active state of hH_4R.

Collectively, the synergistic effects of H_1R and H_4R antagonists in an acute asthma model and the overlapping interaction of structurally diverse H_1R ligands with hH_1R and hH_4R indicate that the development of dual H_1R/H_4R antagonists is a worthwhile and technically feasible goal for the treatment of type-I allergic reactions. For generation of an inactive hH_4R model, the sequence of hH_4R was aligned to h-beta$_2$AR. Based on this alignment, the homology model of the inactive hH_4R was generated using the crystal structure of the h-beta$_2$AR (Protein Data Bank code 2rh1). Loops with different length compared with the h-beta$_2$AR were modeled using the Loop Search module of SYBYL (Tripos, St. Louis, MO). Because of the lack of sufficient experimental data concerning the structure of the third intracellular loop and parts of the C terminus, both were included only partially in the modeling studies. This approximation should not have much influence to the modeling of the binding-mode of the ligand. Thereafter, the minimized receptor was manually placed in a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine membrane bilayer (104 molecules), and suprahistaprodifen was positioned manually into the proposed binding-pocket in two different orientations. To refine the hH_4R homology model, representing the inactive state, distance-restrained molecular dynamic simulations using the constraints of the inactive conformation were also performed, with explicit simulation of water molecules (84). The active model of the hH_4R was generated with a distance-restrained MD simulation, based on the constraints for the active conformation. In addition, distance restraints for the hydrogen-bonds of the transmembrane helices were applied. All simulations were carried out as described for H_1R by Strasser and colleagues (102) where Dimeric histaprodifen was docked into the binding pocket of gpH_1R. Hydrogen bonds and electrostatic interactions were then detected between dimeric histaprodifen and Asp116, Ser120, Lys187, and Glu190. To assess the influence of the Tyr72Asn mutation in hH4R on interaction with suprahistaprodifen, the corresponding mutants were generated for the inactive and active hH_4R. Subsequently, suprahistaprodifen was docked in both orientations and MD simulations were performed as described for the wild-type hH_4R by Niv and colleagues (84).

To explore the putative binding site of the cyanoguanidines, Igel and coworkers (23) developed in 2009 a homology model of the hH_4R based on the crystal structures of the h-beta$_2$AR. One compound in the cyanoguanidines family (see cyanoguanidine derivative in Figure 6) was manually docked in an energetically favorable conformation, while taking into consideration results from *in vitro* mutagenesis and modeling approaches. The binding site of the compound mentioned above, consisting of 20 amino acids with side chains not more than 3 Angstrom distant from the ligand, is located between TM2 and TM7 and the imidazole moiety was docked. In this binding mode, Glu182 is assumed to be protonated and serves as hydrogen bond donor for the π nitrogen. The τ nitrogen forms another H bond with the side chain oxygen of Thr178. However, a similar bi-dentate interaction is possible with the couple Ser179 /Glu182 if the imidazole ring is assumed to be coplanar with the butyl chain. Both the Thr178 and the Ser179 - Alanine mutations lead to an only 3 to 4-fold reduction of histamine affinity and potency. Thus, no definitive conclusion about the presence and the partner of a second hydrogen bond can be drawn. In this mode, the cyanoguanidine moiety is stacked with the phenyl ring of Phe344 and forms two charge-assisted hydrogen bonds with the carboxylate oxygens of Asp94 (distances 2.0-2.1 Angstrom), an amino acid proven to be essential for histamine binding by *in vitro* mutagenesis. This arrangement allows the arylthioalkyl substituent of the compound, like the isopropyl group of carazolol in the crystal structure of the beta2-adrenoceptor, to point outward. The cyano-group in the Z configuration forms two additional charge-assisted hydrogen bonds with the guanidine moiety of Arg341 which is also involved in a salt bridge with Glu165 (in the second extra-cellular loop). This arginine is species-specific (rat and mouse H4R: serine) and replaced by a glutamate in the hH_3R. Thus, interactions with Arg341 are suggested to contribute to the hH_4R subtype and species selectivity of the cyanoguanidines. The binding mode is more likely, since a nearly perpendicular conformation of the imidazolyl ring with respect to an alkyl chain is energetically favorable and present in the crystal structure of histamine monohydrobromide, too.

4.3. Ligand-based prediction, a new era

New approaches were developed to transfer ligand information into a homology-based receptor model. One study done by Tanrikulu *et al* (103) presented a computer-assisted method for the generation of pseudo-receptor model for the putative ligand binding site based on a three-dimensional alignment of known histamine H_4 receptor ligands. Following alignment, hydrogen bond donors/acceptors were projected outwards in the appropriate binding distances and geometries to provide pseudo-receptor atoms. Each pseudo-atom was then weighted according to the number of atoms that generated it in each ligand as well as the number of ligands that created it and a correlation vector was obtained. The resulting model was used for virtual screening of a large collection of commercially available compounds with two bioactive chemotypes retrieved.

The pseudo-receptor model was also used to find the putative ligand binding pocket within the transmembrane domain of the receptor together with a

homology model based on the beta2-adrenergic receptor template. The homology model was simulated using molecular dynamics, with an explicitly simulated environment of water and lipids. For each frame of a molecular dynamics simulation of a homology-based H_4 receptor model, potential ligand binding pockets were automatically extracted and their compatibility with the pseudoreceptor used as a selection criterion. The best-matching pocket fits perfectly with existing mutation data and previously published hypotheses suggesting Glu182 as the preferred binding partner of a positively charged moiety of H_4 receptor ligands. This new pseudoreceptor approach has demonstrated its suitability for both structure-based prioritization of protein receptor models, and ligand-based virtual screening with the aim to perform scaffold hopping. An automated pseudo-receptor construction algorithm (PRPS, pseudoreceptor point similarity) was developed and used to transfer ligand information into a homology-based receptor model for the H_4R antagonist binding pocket.

The MembStruk method to predict the 3D structure of several GPCRs including the H_4R without utilizing homology modeling techniques was developed recently (104). Predicted structures were validated by using the HierDock procedure (105) or MSCDock (106, 107) in order to predict their binding sites, binding configuration, and binding energies to known high affinity ligands (agonists and antagonists). Prediction did not depend on experimental data but rather compared to it. The predicted structure for the ligand-GPCR complex was then used to predict which mutations would dramatically decrease or increase binding. HierDock/MSCDock was thus applied to successfully predict the binding site structure and binding energy of some ligands including those which bind to H_4R.

To explore possible structure–function relationships of the hH_4R as a receptor species with high constitutive activity, molecular modeling of an active hH_4R state in complex with $G\alpha_{i2}$-CTs was performed (108). This study was based on the model of the putative active state of the hH_4R, the inactive state model (23) and the recent crystal structure of opsin in complex with a C-terminal fragment of transducin [Protein Data Bank, 3DQB] (109). The alignment of the active hH_4R model with the inactive h-beta$_2$AR structure shows that the main difference consists of an outward tilt of TM6, resulting in a distance of approximately 6.5Angstrom at the intracellular end (position of Arg297) (108). The bottom of TM5 and TM7 deviate by approximately 2 and 2.5Angstrom, respectively. The segments TM2–TM4 were found to be rather well aligned (rms fit of the backbone atoms approximately 1.2Angstrom). At the intracellular end of TM5 (N terminus of CL3), two residues of the hH_4R, Asp205 and His206, are nearly unique among all biogenic amine GPCRs. Asp205 forms a salt bridge with Arg299, which may be regarded as an ionic lock stabilizing the active receptor state. In summary, 14 contacts may be formed under participation of hH_4R residues from TM2, TM3, CL2, CL3, TM6, and the C-terminal helix 8. Interactions of the $G\alpha_{i2}$ C terminus with the receptor seem to enforce the proper fold of the last four $G\alpha_{i2}$ residues. In the hH_4R–$G\alpha_{i2}$-CT model described by Schneider (108).

5. CONCUDING REMARKS

In silico methodologies for modeling hH_4R, together with emerging experimental data, lead various research groups to construct good quality models for the receptor. Validating the models via docking experiments gives promising results but still requires improvement. Most models have a sufficient level of accuracy to allow an effective discrimination between binders and non-binders. However, those models were not tested on the capability to rank the affinity of a series of analogues in order to be exploited for lead-optimization purposes. We believe that flexible docking via utilizing more than one three-dimensional model of the receptor at once, could improve the docking results. Such hypothesis is under evaluation in our laboratory.

One of the underlying difficulties in integrating the various models produced emanates from the lack of an organized depository for modeled proteins. This requires laboratories interested in comparative studies to individually collect the models. We suggest the collection of all hH_4R models for a comparative study geared at enabling the optimization of binding prediction. Using existing docking suits, agonists and antagonists of known binding affinities will be docked to the various models. The hH_4R models will be ranked according to the correlation between the predicted and experimental binding affinities of docked ligands.

We should notify that while revising the final version of this manuscript, a crystal structure for CXCR4 Chemokine GPCR was released (Wu B, Chien EY, Mol CD *et al:* Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. Science.2010; 330(6007): 1066-71). No model for H4R based on this new template is published yet.

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Abbreviations: H_4R: Histamine H4 Receptor; GPCRs: G-protein coupled receptors; GPLR:G protein-linked receptors; SBDD: Structure Based Drug Design; beta1aR: beta1 adrenergic receptor; NMR: nuclear magnetic resonance; SCAM: substituted cysteine accessibility method; SBVS: structure-based virtual screening; MD: molecular dynamics; IL: intracellular loop; EL: extracellular loop.

Key Words: H_4R - H_4 receptor, homology modeling, 3D-structure prediction, G-protein coupled receptors (GPCRs), Drug Discovery, Review

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