# Analysis of histamine and modeling of ligand-receptor interactions in the histamine H-1 receptor for Magic Angle Spinning NMR studies

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# Analysis of histamine and modeling of ligand-receptor interactions in the histamine $H_1$ receptor for Magic Angle Spinning NMR studies

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**Abstract.** *Objective and Design:* Investigation of the principles of ligand-receptor interaction in histamine receptors can help to provide a solid foundation for structure-based drug design. Stable isotope labelling of the ligand 'Histamine' has been performed and 1D $^{13}C$ CP MAS and 2D Radio Frequency Dipolar Recoupling (RFDR) spectra for the ligand are presented. Hyperfine signals were well spread and did not suffer from any sizable line broadening. The production of $H_1$ receptor for Magic Angle Spinning NMR studies is currently in progress.
*Treatment:* An agonist binding domain is proposed using homology modeling, database searches and mutagenesis data for the $H_1$ receptor.
*Methods:* Homology modeling, Database searches for Expressed sequence Tag (ESTs), Magic Angle Spinning Nuclear Magnetic Resonance analysis of the ligand histamine.
*Results:* The three-dimensional receptor model and mutagenesis studies suggest that the amine of the agonist histamine may form an ion pair with the TM III Asp, whereas the imidazole ring of histamine may associate with TM V Asp and Thr.
*Conclusions:* Homology modeling studies confirms the absence of TM VIII in the $H_1$ receptor. According to the model the histamine in particular interacts with the transmembrane (TM) regions of the $H_1$ receptor structure, in particular TM helix III and V. This is in line with recent mutagenesis studies. Database search methods for ESTs have been used for electronic prediction of tissue distribution of $H_1$ receptor expression. The results indicate that the $H_1$ expression is highest in heart and skeletal muscle, which may be of importance for drug targeting.

**Key words:** Histamine $H_1$ receptors – G protein coupled receptors – MAS NMR – Ligand binding – ESTs

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## Introduction

The development of selective histamine receptor(s) agonists and antagonists has received considerable attention because of their potential use as pharmacological agents against a variety of human pathologies, including acid-peptic disorders, duodenal ulcers, asthma and allergies. Biotechnology using molecular biology, biophysics, and computational approaches provides an alternative approach for classical pharmacological screening to look at ligand-receptor interactions and receptor specificity, which should allow to design selective drugs based on detailed structural principles. Current therapies as described in a standard pharmacology text, for example Goodman & Gilman's 'The Pharmacological Basis of Therapeutics', are based on no more than 500 molecular drug targets. These traditional targets reveal what have been the most fruitful paths for therapeutic development in the past, and give a glimpse of where genomic sciences may yield drug-discovery success in the future. The cellular targets of most prescription drugs are proteins and a large proportion of these are plasma membrane bound. For the latter class, a drug does not need to enter the cell, but binds to the extracellular binding site of the protein and can control intracellular reactions from the outside. These molecular drug targets can be further categorized into biochemical classes such as receptors, enzymes, matrix adhesion factors and ion channels. In this report we will address specific approaches to study function, structure and relevance of a major pharmaceutical target family, namely G-Protein Coupled Receptors, exemplified in the histamine $H_1$ receptor.

### *G-Protein Coupled Receptors*

Biomembranes are based upon a bilayer matrix composed of a mixture of lipid and other amphiphilic molecules creating a thin but crucial separation between the inside and the out-

side of the cell, and between different compartments within each cell. Each biomembrane contains a variety of membrane-inserted and associated proteins, which determine to a large extent the properties of the membrane [1]. Important elements are the G-Protein Coupled Receptors (GPCRs) that employ heterotrimeric guanine-nucleotide binding proteins (G-proteins) to trigger a variety of intracellular signal transduction cascades [2–8]. The GPCR superfamily is probably the largest and most functionally differentiated gene family in our genome [9–10]. This class of proteins carries out a wide variety of functions. They are for instance, involved in controlling the traffic of metabolites across the membrane, and sending signals from events occurring outside the cell across the membrane into the cell so that proper action can be taken such as e.g. start or stop of cell division. They are triggered by an extracellular signal, that can be a protein, a peptide, a small organic molecule, an ion, or even mechanical or photon energy. This triggers conformational changes in their transmembrane helices propagating to the cytosolic side exposing binding sites for G proteins. G proteins mediate subsequent cellular responses. Dysfunction of GPCRs results in diseases as diverse as Alzheimer's, Parkinson's, diabetes, dwarfism, colour blindness, retina pigmentosa and asthma, and are also involved in depression, schizophrenia, sleeplessness, hypertension, impotence, anxiety, stress, renal failure, several cardiovascular disorders and inflammational processes. Consequently, GPCRs are of enormous importance for the pharmaceutical industry. They are the target for at least 50% of all existing prescription medication and of the top 200 best selling prescription drugs, more than 20% interact with GPCRs, providing worldwide sales of over $ 20 billion. Many well-known medical drugs such as beta blockers and anti-histamines, or drugs like opium and cannabis act on GPCRs. Numerous natural ligands, agonists and antagonists of GPCRs are used for therapeutic purposes. For instance, histamine $H_1$ antagonists act against allergic and anaphylactic reactions, hay fever, itching, and motion sickness. Other GPCR ligands have been shown to be analgesic, anti-inflammatory and anti-asthmatic agents.

Histamine receptors belong to the large opsin subclass or class A of the G-Protein Coupled Receptor family. They are currently subclassified into the four subtypes $H_1$, $H_2$, $H_3$ and $H_4$. These subtypes can be distinguished on the basis of their sensitivity to specific agonists and antagonists [11–14] and by their molecular weight, that ranges from 45–60 kDa. Histamine has one of the broadest spectra among signalling molecules in the human body, ranging from involvement in allergies to contributing to the regulation of circadian rhythm in the brain [15–16]. Most of its well-defined actions are mediated by the $H_1$, $H_2$ and $H_3$ receptor [17]. $H_4$ has only recently been identified (Table 1). The $H_1$ receptor is most prominent in smooth muscle effects, especially those caused by IgE-mediated responses. Bronchoconstriction and vasodilation, the latter by release of endothelium-derived relaxing factor (EDRF), are typical retracts, opening gaps in the permeability barrier and resulting in the formation of local edema. These effects are manifest in allergic reactions and in mastocytosis, a rare neoplasm of mast cells. $H_2$ receptor mediates gastric acid secretion by parietal cells in the stomach. It also has a cardiac stimulant effect. A third action is to induce negative feedback upon histamine release from mast cells. The $H_3$ receptor appears to be involved mainly in presynaptic modulation of histaminergic neurotransmission in the central nervous system. In the periphery, it appears to be a presynaptic heteroreceptor, which modulates the release of neurotransmitters other than those that stimulate it with modulatory effects on the release of other transmitters.

**Table. 1.** Histamine receptor subtypes showing their distribution, mechanism and Prototype Antagonist.

| Receptor Subtype | Distribution | Postreceptor Mechanism | Prototype Antagonist |
|---|---|---|---|
| $H_1$ | Smooth muscle | ↑ $IP_3$, DAG | Diphenhydramine |
| $H_2$ | Stomach, heart, mast cells | ↑ cAMP | Cimetidine |
| $H_3$ | Nerve endings, CNS | G protein-coupled | Impromidine |

There are several different GPCRs subfamilies, each with slightly different characteristics [8–10]. However, their general structure and function is remarkably conserved. In general, GPCRs constitute of a single seven-helical integral membrane protein encoded by a single gene. Activation of the receptor by binding of an agonist leads to a conformational change in receptor topology that propagates through the membrane domain and, in turn, binds and activates a G-protein at the intracellular side. So far little is known about 3D-structure and ligand-interaction patterns of the histamine receptors. Bioinformatics using DNA and protein sequence similarity search tools provides a fast and effective method to look at receptor selectivity, which may be used to design structural principles of selective drugs. X-ray crystallography is presently one of the most powerful methods to determine the molecular structures of crystalline materials. It reveals the 3D crystal structure of a protein at an atomic level that cannot yet be matched with any other physical method of structural analysis. However, producing suitable crystals of membrane proteins still is a major bottleneck, and so far the only GPCR that has yielded a crystal structure is the light receptor rhodopsin [18]. Meanwhile, solid state NMR spectroscopy has already yielded unprecedented detail in the study of the interactions between ligands and membrane proteins in a natural lipid environment [19]. We aim to exploit solid-state MAS NMR analyses and computational approaches for the elucidation of the structure of the ligand and its interaction with the $H_1$ receptor. For this purpose stable-isotope labelled histamine is required.

Here we present a 3D model for the $H_1$ receptor based upon homology modeling of the rhodopsin structure and discuss its potential for drug design. Further, in preparation for SSNMR studies of the $H_1$ receptor we have prepared uniformly labelled $^{13}C$, $^{15}N$-Histamine and characterized it by SSNMR.

## Materials and methods

### *Chemicals used*

U-$^{13}C_6$,$^{15}N_7$ Histidine.HCl.$H_2O$ with $^{13}C$ 98%; $^{15}N$ 96–99%, < 5% D-Isomer was obtained from Cambridge Isotope Laboratories Inc. L-Histidine decarboxylase type IIIS from Lactobacillus 30a was obtained from Sigma. Column material Dowex 50-X8, with mesh size of 200–400 was obtained from Applied Membranes, Inc.

### *Protein analysis*

Recombinant his-tagged human $H_1$ receptor produced in Sf9 cells and purified using immobilized metal affinity chromatography (Ratnala et al, in preparation). For population analysis, samples were taken up in PAGE sample buffer (2% sodium dodecyl sulphate (SDS), 0.04 M dithioerythrol (DTE) and 0.015% bromophenol blue in 0.5 M Tris pH 6.8). Samples were run on a 12% SDS-PAGE gel at 100 V for the 5% acrylamide stacking gel and 200 V for the running gel. Proteins were subsequently blotted onto a nitrocellulose membrane (1 h at 100 V) in ice-cold blot buffer (25 mM Tris, 0.2 M glycine in 20% methanol) using a Miniprotean system (BioRad, Melville, N.Y., USA).

### *Synthesis of uniformly labeled histamine*

Uniformly labelled $^{13}C$, $^{15}N$ Histamine was prepared from labelled histidine in good overall yield of 70% by means of an enzymatic conversion using L- Histidine decarboxylase type III-S from *Lactobacillus 30*. From 51 mg of U-$^{13}C_6$,$^{15}N_7$ Histidine.HCl.$H_2O$, 34 mg of very pure uniformly labelled U-$^{13}C_6$,$^{15}N_7$ Histamine.HCl.$H_2O$ was obtained. The reaction conditions were maintained at 37 °C, in presence of the enzyme histidine decarboxylase in 0.2 M ammonium acetate buffer at pH of 4.8 for 5 h to obtain a complete conversion. In the process of conversion, $CO_2$ was removed at regular intervals by evaporation. The raw reaction mixture was totally evaporated to form solid powder and purified by Chromatography the use of a column of 1 cm in diameter and 4 cm in height with ~ four gram of Dowex 50-X8, 200–400 mesh as column material and finally eluted with 4 M HCl. Recrystallization was performed from ethanol/diethyl ether. The uniformly labelled histamine crystals were obtained after 48 h incubation time at 2 °C and finally they were washed with diethyl ether dried and stored at room temp.

### *Computational and Bioinformatics approaches*

Gene expression levels for the $H_1$ receptor were estimated according to their publicly available ESTs and gene differential expressions were assessed using a previously described and validated statistical method. For this purpose we have used the 'UniGene' software program from The National Center for Biotechnology Information, National Library of Medicine, NIH, Bethesda, USA. This is an experimental system for automatically partitioning GenBank sequences into a non-redundant set of gene-oriented clusters. Each UniGene cluster contains sequences that represent a unique gene, as well as related information such as the tissue types in which the gene has been expressed and map location. The data is projected in the form of Electronic Northern. The UniGene dataset is mined for information about the number of unique clones per gene per tissue. Clones are assigned to particular tissues by applying data-mining heuristics to UniGene's library information file (H.lib.info) [20–22]. Consequently, the collection may be of use to the community as a resource for gene discovery. It can also be used by experimentalists to select reagents for gene mapping projects and large-scale expression analysis.

For the protein structure verification, graphics, and visual homology models of the human $H_1$ receptor, we have used the 'WHAT IF' software program from The Centre for Molecular and Biomolecular Informatics, University of Nijmegen, The Netherlands [23]. 'WHAT IF' is a versatile protein structure analysis program that can be used for mutant prediction, structure verification, molecular graphics, etc. Homology modeling was based upon the rhodopsin structure.

### *Biophysical approaches*

For SSNMR analysis of the ligand, four milligram of pure uniformly labeled histamine powder was loaded into a 4 mm zirconium oxide rotor and sealed with a Kel-F Cap. CP/MAS spectra were recorded with a Bruker AV-400 spectrometer operating at a $^{13}C$ frequency of 50 MHz, and equipped with a 4 mm MAS probe. The 1-D spectrum as well as 2-D RFDR spectra were recorded using a MAS frequency of 8 kHz. The $^{15}N$ CP/MAS spectra were recorded with a Bruker DSX-750 spectrometer operating at $^{15}N$ frequency of 40 MHz. The 1-D spectrum was recorded using a MAS frequency of 12 kHz in a cramps rotor in a 4 mm MAS probe. The chemical shifts were referenced using external glycine (34.3 ppm for $^{15}N$ and 176.04 ppm for $^{13}C$ respectively).

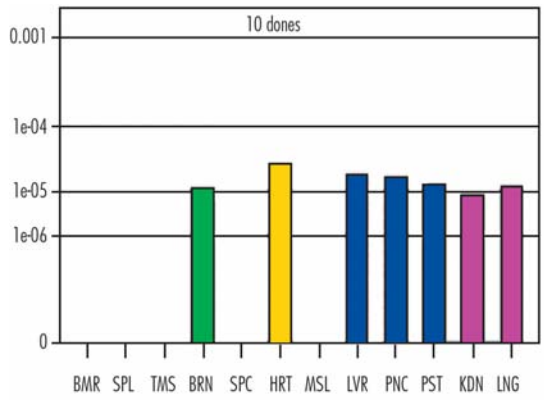
## Results

### *Modeling of tissue distribution of $H_1$ receptor expression*

We acquired an integrated molecular analysis of genomes and their expression with the help of UniGene clustering. This program helps to accelerate gene discovery through the use of arrayed cDNA libraries, and to aid in the accumulation of sequence, map, and expression information for all genes. The UniGene clustering process takes EST sequence information, as well as those of the known genes, and automatically partitions it into a non-redundant set of gene-oriented clusters. In theory, each UniGene cluster contains sequences that represent one unique gene. Thus a verified representative clone in a cluster can be used to determine the expression level of that particular gene. The electronic expression results were calculated by dividing the number of clones per gene by the number of clones per tissue, and are presented on a root scale designed to be visually parallel with the experimental tissue vector presentations. Figure 1 shows an electronic northern for a set of normal human tissues.

### *Homology modeling of the $H_1$ receptor*

The three-dimensional structures of bovine rhodopsin (a GPCR) and bacterio-rhodopsin (an archaebacterial proton pump) have been determined. In spite of considerable diversity at the sequence level, both structures reveal an overall seven transmembrane helix architecture. The precise packing arrangements of the transmembrane helices differ considerably, however, indicating that within the same scaffold large structural versatility is allowed, probably a quite different function. While a common structural framework of seven transmembrane helices has been proposed for the GPCR superfamily, there is no significant sequence similarity between the GPCR subfamilies: the rhodopsin-like, secretin-like, cAMP, and metabotropic receptors thus probably bear their own unique 'seven transmembrane' signatures. Unexpectedly the crystal structure of rhodopsin shows the presence of an eight amphipatic helix on the intracellular surface [18].




| Tissue | | Clones per gene | Total clones |
|---|---|---|---|
| BMR | Bone marrow | 0 | 26,809 |
| SPL | Spleen | 0 | 13,489 |
| TMS | Thymus | 0 | 3,451 |
| BRN | Brain | 3 | 274,393 |
| SPC | Spinal cord | 0 | 506 |
| HRT | Heart | 1 | 35,078 |
| MSL | Skeletal muscle | 0 | 23,264 |
| LVR | Liver | 1 | 55,430 |
| PNC | Pancreas | 1 | 58,927 |
| PST | Prostate | 1 | 81,135 |
| KDN | Kidney | 1 | 121,315 |
| LNG | Lung | 2 | 167,397 |

**Fig. 1.** Expression of human $H_1$ receptor in normal human tissues based on quantifying ESTs from various tissues in Unigene clusters from a sample size of 155 Homo sapiens.

We have built a model for the $H_1$ receptor based on the rhodopsin structure using the 'WHAT IF' program. The available receptor sequences show seven hydrophobic alpha-helical domains, commonly interpreted as seven transmembrane helices. These hydrophobic alpha helices pack into a barrel like conformation that embeds itself in the cell membrane (Fig. 2). The presence of an $\alpha$-helix, the eighth helix, H8, in rhodopsin has been a striking finding from the crystal structure. Helix eight, being perpendicular to the TM helical bundle, is oriented toward the outside of the bundle and continues until the palmitoylation anchoring sites. In the case of $H_1$ receptor we have not noticed any eighth helix as per the model predicted by the 'WHAT IF' program. On the other hand we could observe a close correspondence between the modeling of positions of residues important for ligand binding and mutagenesis studies [24]. Mutagenesis studies [25–26] it was predicted that the Asp in TM III and Asp and Thr V are involved in ligand binding. In the model the TM helix III is indeed positioned close to TM helices V and VI. The amine of the histamine may form an ion pair with the TM III Asp, while the imidazole ring may associate with TM V Asp and Thr. Despite these encouraging results, it was not possible to fit the molecular structure from crystalline Histamine in the putative binding site of the $H_1$ receptor model. Hence modeling data alone is insufficient for high-resolution structural drug design, and spectroscopic data from e.g. SSNMR studies will be needed in order to provide on the spatial and electronic structure of histamine in the agonist-binding pocket of its receptor target.

*Characterization of histamine by solid-state NMR*

The 1-D $^{13}C$ CP/MAS spectra of uniformly labeled histamine showed two distinct peaks in the range of 20–40 ppm, where aliphatic carbons resonate, and three distinct peaks in the range of 110–140 ppm, typical for aromatic carbons (Fig. 3a). This allowed a preliminary assignment. 1D $^{15}N$ CP/MAS spectra show three resonances, with a distinct peak at 50 ppm, characteristic for aliphatic amino groups, and two distinct peaks in the range of 170–180 ppm, in the region for aromatic nitrogen (Fig. 3c). A 2-D RFDR analysis (Fig. 3b) allowed a complete $^{13}C$ assignment using cross peaks to identify correlation networks.

*Protein expression*

The histamine results are encouraging and show that a full analysis of ligand protein interactions using established NMR methods [19] is well within reach, once a sufficient amount of purified receptor can be made available. For large

**Fig. 2.** Generated 3D structure of a human $H_1$ receptor based on the WHAT IF program illustrating barrel structure. Colors residues on a gradient from red (negative hydrophobicity) to blue, passing through white at a hydrophobicity of zero. The hydrophobicity scale used in the figure is the Kyte-Doolittle scale [28].

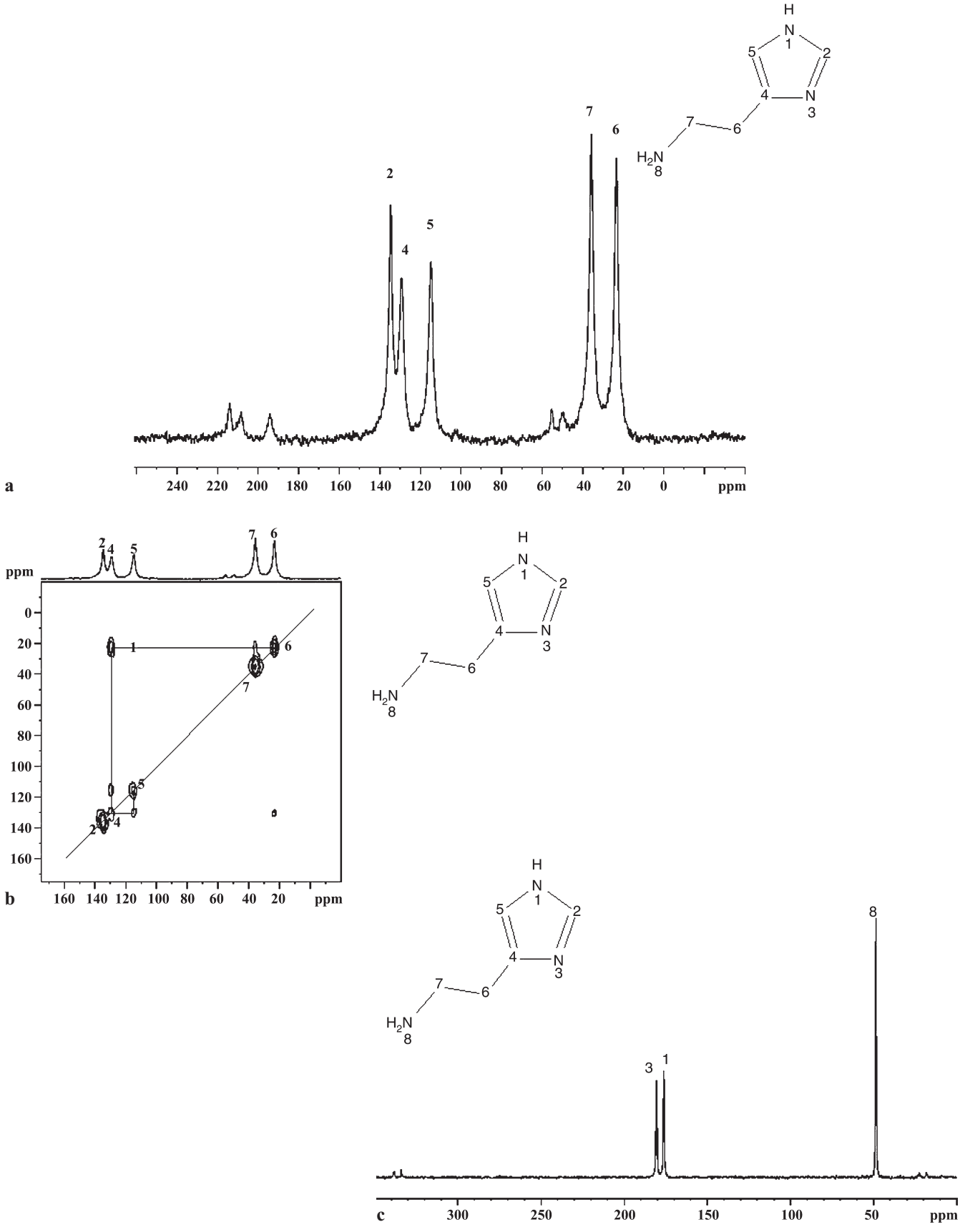



**Fig. 3.** (a) CP/MAS $^{13}C$ NMR spectrum of U-$^{13}C$,$^{15}N$ -Labelled Histamine with a spinning frequency of 8 kHz. (b) 2-D RFDR/MAS $^{13}C$ NMR spectrum of $^{13}C$, $^{15}N$ -Labelled Histamine. (c) CP/MAS $^{15}N$ NMR spectrum of $^{13}C$, $^{15}N$ -Labelled Histamine with a spinning frequency of 12 kHz.

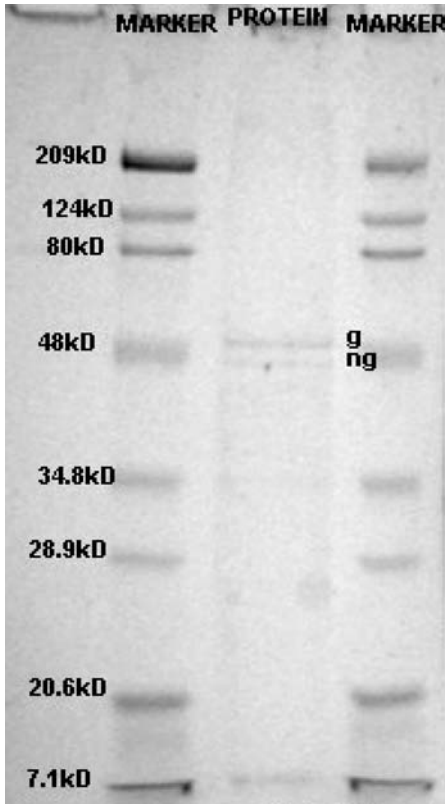



**Fig. 4.** 12% SDS-PAGE analysis of purified $H_1$ receptor. Proteins were identified by silver staining. MARKER is the molecular weight standard. Positions of glycosylated (g) and non-glycosylated (ng) receptor are marked on the right. MW of $H_1$ receptor is ~ 50 kDa.

scale expression and purification of functional Human $H_1$ receptor we are using recombinant baculovirus in combination with IMAC. SDS-PAGE analysis of purified receptor is shown in Fig. 4.

## Discussion

The UniGene program offers an alternative, bioinformatic approach to profile gene expression. It suggests a tissue distribution of $H_1$ receptor expression, the electronic results generated by Unigene were verified using RT-PCR, cDNA-library screening, exon trapping and Northern blot analysis. Both Northern analysis and RT-PCR were facilitated by the expression profile deduced from dbEST. RT-PCR was most powerful to link computer-predicted exons and to verify intron/exons borders (Den Dunnen JT et al. unpublished data). These types of alternative screening techniques will also help in drug targeting in the future generation of drugs. Consequently, the collection of these databases may be of use to the community as a resource for gene discovery and will aid in drug targeting studies.

The first crystal structure of a GPCR (rhodopsin) revealed an eighth amphipathic helix running parallel to the membrane surface, that appears to cap or terminate the basic seven-helical receptor bundle. This eighth helix in rhodopsin participates in rhodopsin-G protein interaction and possibly in essential rhodopsin-phospholipid interactions [27]. Based on 'snake-like plots for mutant data of $H_1$ receptors' from GPCR database obtained from (www.gpcr.org) and the three-dimensional GPCR models of $H_1$ receptor using the 'WHAT IF' program, we could not find any evidence for such an eighth helix in the $H_1$ receptor. But we did observe a very long third intracellular loop in our $H_1$ receptor model, which certainly will be involved in binding the heterotrimeric G-protein. Very little experimental data is available on ligand structure and ligand-receptor interaction in the binding site of the histamine receptors. Residues in TM5 of the $H_1$ receptors have been indicated to be required for histamine binding according to site-directed mutagenesis studies and was it was also proposed that Asn207 in TM5 of the $H_1$ receptor is involved in hydrogen binding with the $ImN^r$ nitrogen atom of histamine. This data could be verified based on our model system of the $H_1$ receptor, the mutagenesis data comply in reference to the proper positions of the TM helices but could not comply with a binding site that can fit the crystal coordinates of histamine in the putative binding pocket.

High-resolution three-dimensional information on structure and ligand binding of a GPCR is crucial for the understanding of its function and for the structure-based design of drugs. Presently, in silico modelling clearly cannot yet provide such information, and we have to rely on X-ray crystallography and NMR spectroscopy. While X-ray crystallography can provide full structures at atomic resolution, it cannot easily provide dynamic information and in addition requires a quite unnatural condition in the form of well-ordered crystals. NMR spectroscopy has as main advantages that the receptor can be maintained in a natural lipid environment and that physical, chemical and dynamic information can be obtained. However, fluid-state NMR is not an option for solving GPCR structures since the mixed micelles of detergent solubilized GPCRs are too large and solubilization may also affect the structure. On the other hand, solid state magic-angle spinning (MAS) NMR spectroscopy has already demonstrated its value in the study of the interactions between ligands and receptors in natural lipid environments [19]. Electronic and chemical analysis of the structure of the ligand in situ is best performed by means of ultra high field (750 MHz) MAS NMR using chemical shift data, correlation spectroscopy and distance measurements. Comparison with the free ligand yields 'ligation shifts' that can be interpreted in terms of electronic and chemical structure of the ligand in the binding site, and of interaction with protein residues. Distance measurements will yield constraints for the ligand structure. The chemical shifts of the free in solid state ligand already have been measured. The chemical shifts in the solid ligand are more comparable with the crystal structure than the free ligand in solution, since the purity of the histamine powder we have used for the characterization of histamine by solid-state NMR is in a microcrystalline state and there is a negligible chance that the histamine occurs in the ionic state. Contrastingly, free ligand in solution state probably will exist in different ionic states. So, it was a worthwhile choice for us to characterise histamine by solid-state NMR rather than solution-state NMR. Eventually, such SSNMR data will be combined with molecular modelling to present a detailed structural model for the histamine binding region in the seven-helical domain.

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