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FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra biochemických věd

CÍLENÉ MUTACE LIDSKÉHO HISTAMINOVÉHO H_4 RECEPTORU: POSTAVENÍ ARG-341 ZA VZÁJEMNÉHO PŮSOBENÍ H_4R AGONISTŮ CYANOGUANIDINOVÉHO TYPU

DIPLOMOVÁ PRÁCE

ve spolupráci s

UNIVERSITY OF REGENSBURG
FACULTY OF CHEMISTRY AND PHARMACY

Department of Pharmaceutical/Medicinal Chemistry II

Vedoucí diplomové práce:
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Regensburg, Hradec Králové 2010 Kateřina Ládová

CHARLES UNIVERSITY IN PRAGUE
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Department of Biochemical Sciences

SITE-DIRECTED MUTAGENESIS OF THE HUMAN HISTAMINE H_4 RECEPTOR: THE ROLE OF ARG-341 IN THE INTERACTION WITH CYANOGUANIDINE–TYPE H_4R AGONISTS

DIPLOMA THESIS

in cooperation with

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„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.“

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Hradec Králové, 2010

Kateřina Ládová

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ABSTRAKT

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Cílené mutace lidského histaminového H_4 receptoru: Postavení Arg-341 za vzájemného působení H_4R agonistů cyanoguanidinového typu

Lidský histaminový H_4 receptor (hH_4R) byl objeven v roce 2000. Předpokládá se, že H_4R zaujímá postavení v imunologických pochodech a může tak být potenciálním cílem vývoje nových léků v terapii zánětlivých onemocnění. Naklonování a exprese hH_4Ru odstartovaly hledání selektivních agonistů a antagonistů. Nedávno byl v sérii agonistů cyanoguanidinového typu identifikován vysoce účinný a receptorově selektivní H_4R agonista, UR-PI376, (2-cyano-1-[4-(1-*H*-imidazol-4-yl)butyl]-3-[(2-fenylthio)ethyl] guanidin), s jasnými preferencemi k hH_4Ru před H_4Rem myšovitých (mH_4R). Dle molekulárních modelových studií, UR-PI376 tvoří dvě vodíkové vazby mezi cyanoguanidinovým zbytkem a argininem 341 hH_4Ru, což vedlo k doměnce, že Arg-341 je důvodem, proč je UR-PI376 selektivnější k hH_4Ru než k lidskému histaminovému H_3 receptoru (hH_3R) a proč upřednostňuje hH_4R před mH_4Rem.
Abychom objasnili roli této aminokyseliny ve vzájemné interakci s cyanoguanidiny, vytvořili jsme tři mutanty: Arg-341 byl nahrazen serinem, glutamátem a alaninem pomocí tzv. „overlap-extension PCR“. Ve srovnání s hH_4Rem, u H_4Ru potkana a myši je Arg-341 nahrazen serinem, zatímco glutamát odpovídá psímu H_4Ru a hH_3Ru. H_4R mutanti byly exprimováni společně s $G\alpha_{i2}$ a $G\beta_1\gamma_2$ proteiny ve Sf9 hmyzích buňkách a charakterizováni prostřednictvím funkčních studií. Účinek agonistů histaminu a UR-PI376 a aktivita antagonisty thioperamidu byly měřeny metodou „steady-state GTPase activity assay“. Nicméně, žádné významné rozdíly v chování přirozeného hH_4Ru a jeho mutantů nebyly zaznamenány. Je tedy zřejmé, že receptorově a druhově specifický Arg-341 není zásadní pro vazbu UR-PI376 k hH_4Ru.

ABSTRACT

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Site-directed mutagenesis of the human histamine H_4 receptor: The role of Arg-341 in the interaction with cyanoguanidine-type H_4R agonists

The human histamine H_4 receptor (hH_4R) was discovered in 2000. The H_4R is supposed to be involved in immunological processes and is considered a potential drug target, e. g., for the treatment of inflammatory diseases. Cloning and expression of the hH_4R inspired to the search for selective agonists and antagonists. Recently, UR-PI376 (2-cyano-1-[4-(1*H*-imidazol-4-yl)butyl]-3-[(2-phenylthio)ethyl]guanidine) was identified within a series of cyanoguanidines as a highly potent and subtype-selective H_4R agonist with pronounced preference for the human over the murine H_4R (mH_4R). According to molecular modelling studies, the cyanoguanidine moiety of UR-PI376 forms charge-assisted hydrogen bonds with Arg-341 of the hH_4R, suggesting this amino acid brings about selectivity for the hH_4R over the hH_3R and is the reason for the preference of UR-PI376 for hH_4R over mH_4R as well.
To elucidate the role of this amino acid in the interaction with cyanoguanidines, three mutants were generated: Arg-341 was replaced by serine, glutamate or alanine, respectively, using overlap-extension PCR. Compared to the hH_4R, in the H_4Rs of rat and mouse, Arg-341 is replaced with Ser, whereas Glu is the corresponding amino acid in both, the canine H_4R and the human histamine H_3 receptor (hH_3R). The mutant H_4Rs were co-expressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ in Sf9 insect cells and characterized in functional studies. The potencies and efficacies of histamine and UR-PI376 (agonists) and the antagonistic activity of thioperamide were determined in steady-state GTPase activity assays. However, relevant differences between the wildtype and the mutant hH_4Rs were not observed. Obviously, Arg-341 can be eliminated to account for the H_4R subtype selectivity of UR-PI376 as well as for species selectivity.

CONTENTS

1 ABBREVIATIONS 1
2 INTRODUCTION 3
3 THEORETICAL PART 5
3.1 G-protein coupled receptors (GPCRs) 5
3.2 Histamine and its receptor subtypes 11
3.2.1 Histamine 11
3.2.2 Classical antihistamines, histamine H_1-receptor subtype 12
3.2.3 Histamine H_2-receptor subtype 14
3.2.4 Histamine H_3-receptor subtype 16
3.2.5 Histamine H_4-receptor subtype 18
3.3 Polymerase chain reaction (PCR) 23
3.4 Steady-state GTPase activity assay 25
4 AIM OF STUDY 28
5 EXPERIMENTAL PART 29
5.1 Materials and methods 29
5.1.1 Materials 29
5.1.2 Methods and conditions 34
5.2 Results 41
5.2.1 Site-directed mutagenesis 41
5.2.2 Protein determination – Lowry assay 42
5.2.3 Western blot analysis 43
5.2.4 Steady-state GTPase activity assay 46
5.3 Discussion 51
6 SUMMARY AND CONCLUSION 54
7 SUMMARY IN CZECH 55
8 REFERENCES 57

1 ABBREVIATIONS

Arg	arginine
ADP	adenosine diphoshate
Ala	alanine
ATP	adenosine triphosphate
Bmax	the maximal specific binding of a ligand
bp	base pair
BSA	bovine serum albumine
cAMP	cyclic 3', 5'-adenosine monophosphate
cDNA	complementary DNA
cH_xR	canine histamine H_x receptor (x = 1, 2, 3 or 4)
CNS	central nervous system
DAG	diacylglycerol
DAO	diamine oxidase
DMSO	dimethylsulfoxide
EC_{50}	molar concentration of the agonist causing 50 % of the maximal response
ECL	extracellular loop or enterochromaffin-like cells
EDTA	ethylendiaminetetraacetic acid
E_{max}	maximal response relative to histamine (1.00)
G-protein	guanidine nucleotide binding protein
GABA	γ-aminobutytic acid
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
gp	guinea pig
gpH_xR	guinea pig histamine H_x receptor (x = 1, 2, 3 or 4)
GDP	guanosine diphosphate
GERD	gastroesophageal reflux disease
Glu	glutamate
GTP	guanosine triphosphate
h	hour(s) or human
HDC	histidine decarboxylase
HNMT	histamine N-methyltransferase
HR	histamine receptor
hH_xR	human histamine H_x receptor (x = 1, 2, 3 or 4)

H_1R, H_2R, H_3R, H_4R	histamine receptor subtypes
ICL	intracellular loop
IP_3	inositol-1,4,5-trisphosphate
kb	kilo base pairs
MAPK	mitogen-activated protein kinase
mH_xR	murine histamine H_x receptor (x = 1, 2, 3 or 4)
min	minute(s)
P_i	inorganic phosphate
PCR	polymerase chain reaction
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC_β	phospholipase C_β
PPI	proton pump inhibitor
pEC_{50}	negative decadic logarithm of the molar concentration of the agonist causing 50 % of the maximal response
R	inactive state of a GPCR
R*	active state of a GPCR
RGS	regulator of G-protein signalling
rH_xR	rat histamine H_x receptor (x = 1, 2, 3 or 4)
RhoGEF	Ras homology guanine nucleotide exchange factor
rpm	revolutions per minute
s	second(s)
SEM	standard error of the mean
Ser	serine
Sf9	*Spodoptera frugiperda* insect cell line
T_m	melting temperature
TM	transmembrane domain
Tris	tris(hydroxymethyl)aminomethane
t.v.	total volume

2 INTRODUCTION

Histamine is an important biological mediator implicated in various physiological and pathophysiological processes in the body by way of four histamine receptor subtypes. All these receptors belong to the class A of the superfamily of G-protein coupled receptors (GPCR). Histamine H_1 receptor (H_1R) antagonists are widely used in allergic ailments whereas histamine H_2 receptor (H_2R) antagonists serve as antiulcer drugs. The third subtype of histamine receptor is mainly expressed in CNS. H_3R ligands have not been established in pharmacotherapy so far but they are under the clinical investigation, for instance, for the treatment of CNS disorders (e. g. schizophrenia) or obesity.

In contrast to the other histamine receptors, which were defined by pharmacological characterisation long before the cloning, the H_4 receptor (H_4R) was first cloned and reported as an orphan receptor and later on identified as a histamine receptor because of high sequence homology with the H_3R. Several research groups discovered the H_4R subtype independently in the years 2000 and 2001. It is known that the H_4R is expressed in cells of immune system suggesting a role in inflammation and immune response. Selective H_4R ligands, agonists and antagonists, are required for the investigation of that role of the H_4R. On the other hand, such compounds may be promising drugs, for instance, for the therapy of bronchial asthma, rheumatoid arthritis, pruritus and allergic rhinitis (see references in the text below).

The research group of Prof. Dr. A. Buschauer (University of Regensburg) identified the human H_3R and H_4R ligands among series of N^G-acylated imidazopropylguanidines. A considerable increase in the selectivity for H_4R over H_3R was achieved by structural modifications, particularly by replacement of the basic acylguanidine with a cyanoguanidine group. The compound UR-PI376 suggested the putative binding mode of cyanoguanidines with the hH_4R: UR-PI376 is in an energetically favourable conformation - in the given *Z* configuration - when the cyano group creates two additional charge-assisted hydrogen bonds with the guanidine moiety of Arg-341 residue in the hH_4R. As Arg-341 is characteristic of the hH_4R, this model can possibly explain the selectivity of UR-PI376 for the human over the murine H_4R and for the hH_4R over the hH_3R (Igel et al. 2009). To substantiate or falsify this working hypothesis, the relevance of Arg-341 had to be proven by exchange of Arg with other amino acids.

This thesis is divided into two parts – theoretical and experimental part. The first one is focused on characteristics of G-protein coupled receptor family, interactions of

receptors with ligands, the histamine receptor subtypes and, with respect to the intended modifications of the H_4R, principles of crucial methods used in site-directed mutagenesis (overlap-extension PCRs) and pharmacological investigation of ligands, i. e. functional studies in the steady-state GTPase activity assay. The experimental part describes the procedure and the evaluation of generated H_4R mutants, in which Arg-341 was replaced with serine (as in rat and mouse H_4R), glutamate (as in dog H_4R and hH_3R) and alanine, including expression of mutants in Sf9 insect cell system, immunoblotting and functional characterisation of the obtained mutants expressing membranes with agonists, histamine and UR-PI376, and inverse agonist thioperamide.

3 THEORETICAL PART

3.1 G-protein coupled receptors (GPCRs)

GPCRs represent a large protein family of transmembrane receptors, also named by some authors as a family of 7 transmembrane receptors, serpentine-like receptors or heptahelical receptors (Pierce et al. 2002, Kristiansen 2004). They take part in the communication between cell and outside world. The GPCR superfamily can be subgrouped in receptors for various endogenous ligands (about 400 “endo-GPCRs”) such as amines, peptides, glycoproteins, prostanoids, fatty acids, phospholipids, nucleosides, nucleotides, Ca^{2+} ions, and sensory receptors (about 400 “exo-GPCRs”) for diverse exogenous ligands such as odorants, bitter and sweet tastants, pheromones and photons of light. GPCRs include receptors for many hormones, paracrines, neurotransmitters or neuromodulators (Kristiansen 2004). As function and dysfunction of such receptors are associated with numerous human diseases, GPCRs represent the most important class of biological targets of marketed drugs and are considered promising targets for the development of new drugs as well.
Total number of identified GPCRs continually raises (Lagerström and Schiöth 2008) and comprises about 800 members, corresponds to about 2 % of the human genome (Jacoby et al. 2006, Lagerström and Schiöth 2008). Numerous different methods are used to identify, study and functionally characterise GPCRs including, for instance, cloning and expression, protein engineering, molecular modelling, site-directed mutagenesis, use of antibodies against of receptor epitopes, affinity labelling, chemical labelling, molecular pharmacology, determination ligand binding and functional activity of wildtype and mutant receptors, and so on (Kristiansen 2004).
Less than 150 genes are designated as orphans, proteins having similarity to receptors but whose endogenous ligands or function have not been conclusively identified yet (Wise et al. 2004). The effort to identified ligands for orphan GPCRs revealed, for instance, the existence the histamine H_4-receptor (Oda et al. 2000).

Members of the GPCR family share a common membrane topology: extracellular N-terminus, a cytoplasmatic C-terminus and seven α-helical transmembrane domains (TM1 - TM7) connected by three intracellular and extracellular loops (ICL 1 - 3 and ECL 1 - 3) (Figure 1, Ballesteros and Weinstein 1995, Kristiansen 2004).

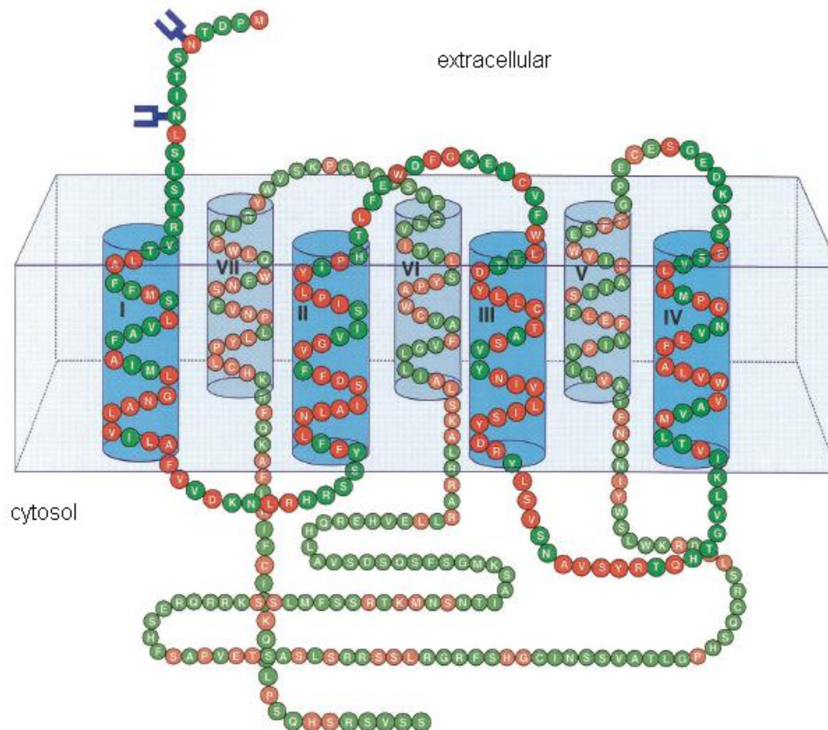

Figure 1. Snake plot of the human H_4-receptor (Nguyen et al. 2001). It represents seven transmembrane helices (TM I-VII), three extracellular loops and three intracellular loops with an extracellular N-terminal and an intracellular C-terminal peptide. *N*-linked glycosylation sites are indicated in blue. Amino acids in red represent residues shared with the histamine H_3 receptor.

The list of GPCRs has been maintained by the International Union of Pharmacology (IUPHAR, cf. http://www.iuphar-db.org/index.jsp) as well as by Kolakowski who created A - F classification system (Kolakowski 1994). GPCRs are grouped into 5 classes (GRAFS) based on homology and functional similarity. The 5 classes are named glutamate, rhodopsin (the largest group), adhesion, frizzled/taste2 and secretin receptor family (Fredriksson et al. 2003). We are interested in the largest family, which comprises hundreds of receptors including the histamine receptors. The rhodopsin family contains most of the GPCR drug targets, mainly amine and peptide receptors (Lagerström and Schiöth 2008).

GPCRs are coupled to heterotrimeric guanine nucleotide binding proteins, called G-proteins, which are composed of a Gα-subunit and a Gβγ-complex (Figure 2, Gilman 1987, Offermanns 2003). The Gα-subunit, an intrinsic GTPase, obviously differs from other subunits and performances as an individual (Gilman 1987). The activity of Gα proteins is regulated according to a functional cycle by release of GDP and binding of GTP, interaction of the activated (GTP-loaded) form with effectors, hydrolysis of GTP to GDP and P_i, thereby restoring the inactive state. The Gα-subunit is bound to GDP in

basal state. Activation of GPCR, which can be agonist-free (constitutively active) or agonist occupied (stabilized by an agonist), leads to the conformational changes of the G-protein and results in dissociation of the Gα-subunit from GDP. This step appears to be the rate-limiting step of the G-protein cycle (Gilman 1987, Seifert et al. 2005). Then the ternary complex, typical for its high agonist affinity, is formed. The ternary complex consists of the agonist-bound receptor and nucleotide-free Gαβγ heterotrimer. Both agonist-free and agonist-bound GPCR advance the binding of the GTP to the Gα-subunit. Now G-protein is in the active state. The binding of GTP to the Gα-subunit induces a conformational change in the subunit and the dissociation of the heterotrimer into $G\alpha_{GTP}$ and Gβγ and in GPCR uncoupling from G-protein. Last item results in decrease in agonist affinity of the receptor, while the G-protein subunit, $G\alpha_{GTP}$ and Gβγ, increase or reduce the activity of effector systems. Way back to the basal state is mediated by the intrinsic GTPase activity of the Gα-subunit which cleaves GTP into GDP and $P_{i,}$ followed by the re-association of $G\alpha_{GDP}$ and the Gβγ complex, allowing the next G-protein cycle (Seifert et al. 2005). Besides GPCRs, the activity of G-proteins is also receptor independently modulated by a family of proteins named regulators of G-protein signalling (RGS) (Kimple 2009).

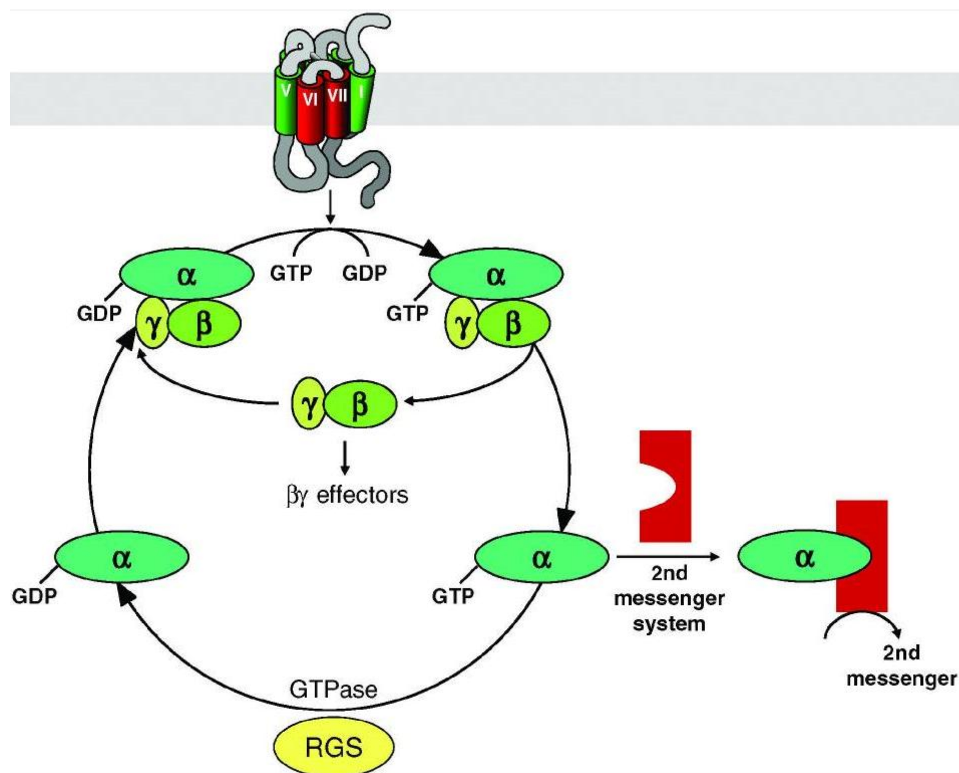

Figure 2. The G-protein cycle (Milligan and Kostenis 2006). See the text for explanation.

Gα-subunits are divided into four classes, named G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$ based on their structure and signalling pathway. Gα-subunits are not transmembrane polypeptides as GPCRs but based on their cDNA sequence would appear to be essentially soluble proteins (Kristiansen 2004, Milligan and Kostenis 2006). Widely expressed members of G_s-subfamily activate membrane-bound adenylyl cyclase that generates the second messenger 3´,5´-cyclic adenosine monophosphate (cAMP). In contrast, $G_{i/o}$-subfamily Gα-subunits are inhibitors of adenylyl cyclase (Kimple 2009, Milligan and Kostenis 2006). The second messenger cAMP exerts various effects on effector proteins, for instance, activation of the protein kinase A (PKA) or the mitogen-activated protein kinase (MAPK) pathway both modulating gene expression (Marinissen and Gutkind 2001). Phosphodiesterases inactivate cAMP and terminate the signal transduction. $G_{q/11}$ proteins are potent activators of phospholipase-$C_β$ ($PLC_β$) isoforms that cause a hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) and generate the second messengers inositol-1,4,5-trisphosphate (PI_3) and diacylglycerol (DAG). IP_3 binds to the IP_3 receptor (ligand-gated Ca^{2+}-ion channel), which is located in the membrane of the endoplasmatic reticulum and promotes the release of Ca^{2+}-ions from its intracellular store into the cytoplasm (Kimple 2009, Mokoshiba 2007). DAG activates the protein kinase C (PKC) and modulates the function of cellular proteins by phosphorylation. IP_3 is inactivated by dephosphorylation, whereas DAG is degraded by lipases or inactivated by phosphorylation (Berridge 1987, Payrastre et al. 2001). The $G_{12/13}$ subfamily plays a role in the activation Ras homology guanine nucleotide exchange factors (RhoGEFs) (Kimple 2009, Milligan and Kostenis 2006). The duration of GPCR signalling is controlled by the time when GTP remains bound to the Gα-subunit before its hydrolysis to GDP. RGS proteins are key modulators of GPCR signalling because they are able to accelerate the intrinsic GTP hydrolysis activity of Gα-subunits. (Kimple 2009) In addition, there are at least five different β- and twelve γ-subunits and it is known that the βγ complex plays a more important role than only being a binding partner for the Gα-subunit. Generally, they are considered to interact with effectors including a variety of ion channel subunits. Probably, the βγ-dimers cannot bind both an effector and the α-subunit concurrently. The Gα-subunits of individual G-proteins are substrates for ADP-ribosylation catalysed by bacterial toxins. Such toxins have been key tools in the discovery and classification of the G-proteins and are still used in many studies of G-protein function (Milligan and Kostenis 2006). Best characterized are the reactions carried out by toxins elaborated by *Vibrio cholerae* and *Bordetella pertussis* (Gilman 1987). There are also some diseases associated with G-protein activity. Cholera and a number of other bacterial exotoxins produce similar effects via the same mechanism, up-regulation of $Gα_i$-subunits is found in heart failure,

various cardiovascular phenotypes and aspects of metabolic syndrome are linked with polymorphic variants of the Gβ-subunit (Milligan and Kostenis 2006).

The influence of a ligand on a GPCR can be explained by the two-state model of GPCR activation (Figure 3A,B, Leff 1995). GPCR can occur in an active receptor state R* and an inactive receptor state R, which are in equilibrium. The ratio between R* and R describes the degree of constitutive activity of a GPCR. The inactive receptor state is able to isomerise to an active receptor state independently from agonist binding. Then the active receptor state can bind G-proteins and promote GDP/GTP exchange. Agonists stabilize the active conformation of the receptor. Full agonists bind and stabilize R*, which leads to a maximal biological response (efficiency). Inverse agonists particularly bind and stabilize R and reduce constitutive activity. Partial agonists and partial inverse agonists less stabilize the active and the inactive receptor state, respectively. Neutral ("silent") antagonists do not alter the equilibrium but only block the binding site of the natural ligand. Nevertheless, pure "neutral antagonists" are probably few in numbers. Many compounds earlier believed to be antagonists turned out to be inverse agonists (Kenakin 2003) depending on the used pharmacological assay and the degree of constitutive GPCR activity in the respective system. All in all, as demonstrated experimentally, the two-state model is not able to explain perfectly the real stage of GPCR activation. There can be many levels of constitutive activity and structurally different ligands versus their stabilization of relevant receptor state resulting in diverse biological responses (Seifert and Wenzel 2002).

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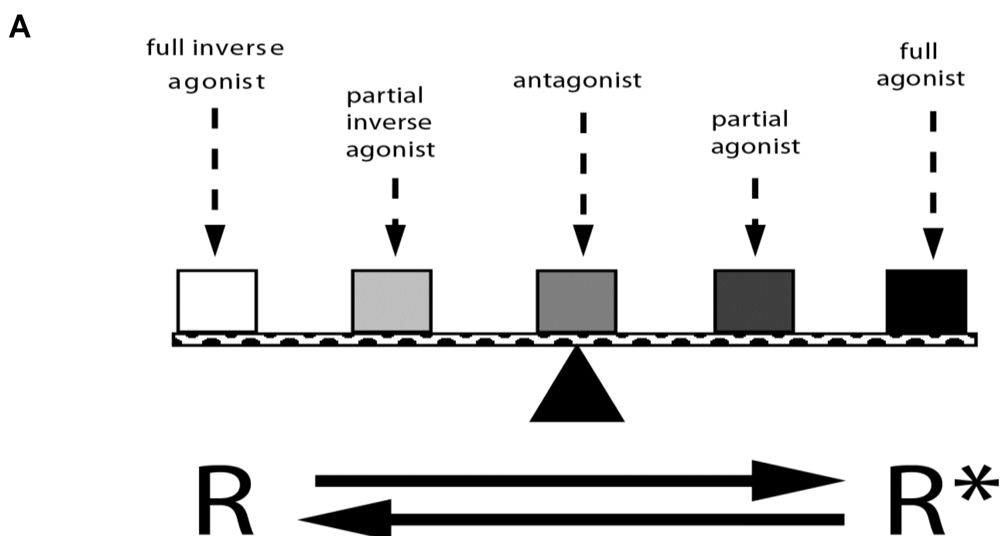

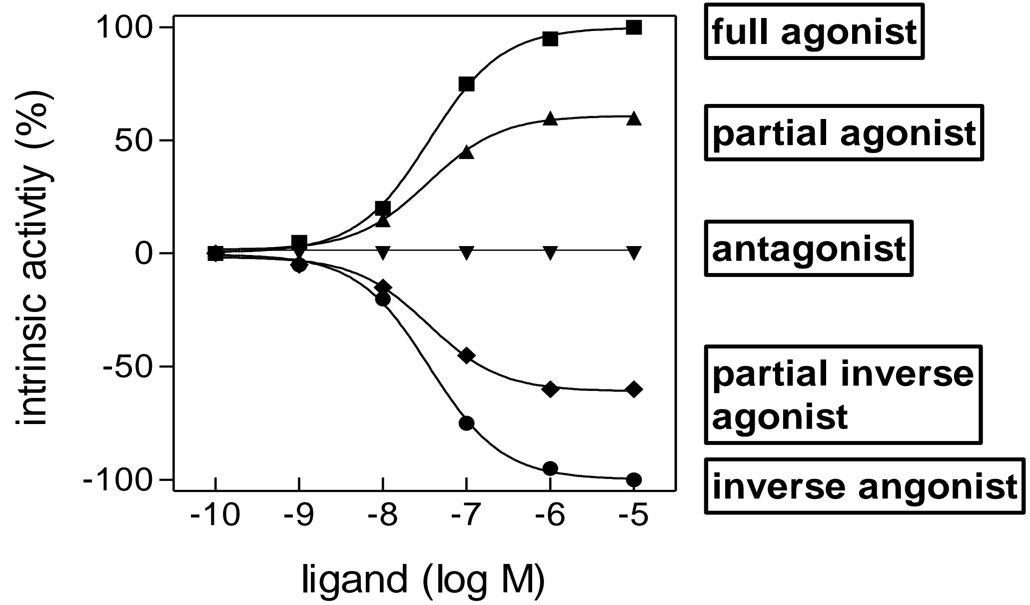

Figure 3A,B. The two-state model of GPCR activation (Seifert and Wenzel 2002). See text for explanation.

3.2 Histamine and its receptor subtypes

3.2.1 Histamine

Histamine is a biogenic amine and is known to be an important biological mediator. It has influences on different physiological and pathophysiological processes in the body and can cause a wide variety of pharmacological effects of varying intensity mediated by four histamine receptor subtypes (H_1R, H_2R, H_3R, H_4R). The biological responses range from CNS effects of histamine as a neurotransmitter and physiological stimulation of gastric acid secretion to pathophysiological reactions due to histamine release from mast cells or basophiles, e. g., from mild irritation and itching to anaphylactic shock and eventual death (Hough 2001, Igel et al. 2009, Mainitz and Novak 2007).

Figure 4. Structure of histamine.

Histamine, 2-(1*H*-imidazol-4-yl)ethanamine (Figure 4) was synthesized by Windaus and Vogt in 1907 (Windaus and Vogt 1907) and isolated from the mould ergot in 1910 by Sir Henry Dale and his colleagues at the Wellcome Laboratories from *Secale cornutum* (Barger and Dale 1910). This group and others carried out a series of experiments to explore its biological actions, but not until 1927 histamine was found by Best *et al.* to be a natural constituent of the body (Parsons and Ganellin 2006) and was identified as a mediator of anaphylactic reaction in 1932 (Mainitz and Novak, 2007). Nowadays this biogenic amine is known to produce a broad variety of effects on numerous organs, including contraction of smooth muscles (e. g. bronchi), vasodilatation, increased vascular permeability and mucus secretion, stimulation of gastric acid secretion and cardiac contractility, tachycardia, alterations of blood pressure, inhibition of autonomic transmitter release etc. (Mainitz and Novak 2007, Parsons and Ganellin 2006, Zhu et al. 2001). Histamine plays role in immunomodulation, haematopoiesis, wound healing, circadian rhythm and the regulation of histamine- and polyamine-induced cell proliferation and angiogenesis in tumor models and intestinal ischemia (Mainitz and Novak 2007). The regulation of sleep order, feeding and memory processes are connected with histamine localization in the brain, one of the last organs, where histamine was discovered. The existence of histaminergic neurones, located in tuberomammillary nucleus of the hypothalamus and

projected to all major areas of the brain, was confirmed in 1984 by Watanabe et al. (Parsons and Ganellin 2006, Watanabe et al. 1984).
Histamine is synthesized by the decarboxylation of the naturally occurring amino acid L–histidine by the enzyme histidine decarboxylase (HDC) and can be found in all human tissues with the largest concentration located in the lungs, the skin, connective tissues and at partially high concentrations in the gastrointestinal tract (Parsons and Ganellin 2006). It is stored in the vesicles of mast cells and basophilic leukocytes as an inactive form bond to heparin and a protein into a storage complex (Code and Mitchell 1957, Riley and West 1953). Additional sources are blood basophiles, blood platelets, enterochromaffin-like (ECL) cells in the stomach, endothelial cells and also in neurons (Mainitz and Novak 2007, Parsons and Ganellin 2006). The degradation of histamine is rapid and occurs by two main catabolic pathways via methylation by histamine N-methyltransferase (HNMT) and oxidative deamination by diamine oxidase (DAO), formerly called histaminase. The preferential metabolic pathway depends on localization of histamine. DAO is mainly specific for small intestine, colon ascendens, placenta and kidney, HNMT is widely expressed in liver and kidney, spleen, colon, prostate, ovary, spinal cord cells, bronchi and trachea, where HNMT is the key enzyme for histamine metabolism (Mainitz and Novak 2007, Ogasawara et al. 2006). The half-life of histamine is very short with less than 10 s in the rat and 20 – 30 s in the dog (Parsons and Ganellin 2006).

3.2.2 Classical antihistamines, histamine H_1-receptor subtype

Because of the evidence that histamine plays a very important and active role in allergy and anaphylaxis research in this field was initially focused on compounds capable of antagonising the pathophysiological effects caused by histamine (Parsons and Ganellin 2006). The first histamine receptor antagonists (known as classical antihistamines but now called H_1-receptor antagonists) were already synthesized in the 30s of last century, i. e. over 20 years after the discovery and description of some of these effects of histamine (Hill et al 1997). In 1942 phenbenzamine was the first antihistamine used in patients. Subsequently, this compound was replaced by mepyramine (Figure 5), which is still in use against some topical skin disorders. Others antihistamines followed, such as diphenhydramine, chlorpheniramine and promethazine. Their use took place widely in the treatment of various allergic ailments like hay fever, allergic rhinitis and urticaria. We do not have to forget the use as hypnotics and as antiemetics, e. g., for the treatment of travel sickness, where side effects of antihistamines were employed (Parsons and Ganellin 2006). The hH_1R was cloned from the cattle in 1991 (Yamashita

et al. 1991) and from humans in 1993 (de Backer et al. 1993). It is a 56 kDa protein with 487 amino acids, coupling to the G_q-mediated signalling pathway, i. e. PIP_2 metabolism by phospholipase C, resulting in IP_3, DAG and an increase in calcium mobilization (Nakamura et al. 2000).

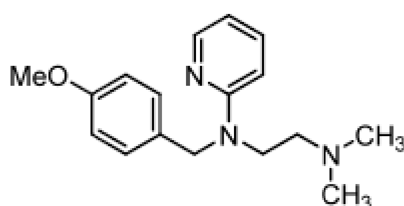

Figure 5. Mepyramine, classical antihistamine.

The H_1-receptor antagonists are divided into the first and the second generation (or "non-sedating") antagonists and they are used therapeutically. By contrast, H_1R agonists are only used as pharmacological tools to study the H_1R at the molecular level (Straßer et al. 2009). Antagonists reduce the effects of histamine on many tissues, notably vascular and extravascular smooth muscle, but it became apparent that some of the effects of histamine are refractory to inhibition by these drugs. For example, histamine stimulated gastric secretion was shown to be unresponsive to three different antihistamines (Hill et al. 1997). In 1966, Ash and Schild suggested the presence of two distinct histamine receptor subtypes based on the pharmacological effects of known histaminergic ligands (Leurs et al. 2009): "The specific antagonism of some actions of histamine by low concentrations of antihistamine drugs characterizes one type of receptor for which we suggest symbol H_1. Such receptors occur in guinea-pig ileum and bronchi. Several other actions of histamine, for example, stimulation of gastric acid secretion, inhibition of rat uterus and stimulation of isolated atria, cannot be specifically antagonized. These actions are likely to be mediate by other histamine receptors." (Ash and Schild 1966) Nowadays the favourite H_1R antagonists are second generation drugs such as cetirizine and fexofenadine (Figure 6) because of their reduced side effects such as sedation (Hill et al. 1997). The selective radioligand $[^3H]$mepyramine has been used to detect the distribution of H_1-receptors in body. H_1-receptors were found, for instance, in the brain, in smooth muscle from airways, in the gastrointestinal and the genitourinary system, the cardiovascular system, the adrenal medulla, endothelial cells and lymphocytes (Hill et al. 1977).

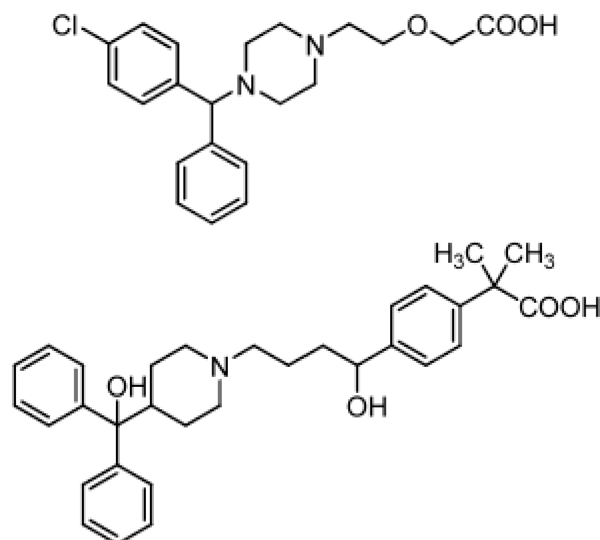

Figure 6. Structure of the "non-sedating" H_1R antagonists cetirizine and fexofenadine (down).

3.2.3 Histamine H_2-receptor subtype

The discovery of the first histamine H_2-receptor antagonists in the 1970s confirmed the existence of a second class of histamine receptors which can not be blocked by the "classical antihistamines", i. e. the H_1R antagonists (Parsons and Ganellin 2006). Between the years 1964 to 1972 were synthesized around 700 novel compounds including burimamide, the first antagonist used as a pharmacological tool for the definition of the H_2R (Black et al. 1972). Unfortunately this substance did not find a place in clinical field because of insufficient potency and lack of oral activity (Arrang et al. 1983). The development of the first clinically useful H_2R antagonist was continued with the thiourea metiamide, which was withdrawn due to the induction of agranulocytosis, and was finally successful with the cyanoguanidine cimetidine (Figure 7), the first H_2R antagonists launched onto the market mid of the 1970s. Cimetidine is a competitive antagonist of histamine in right atrium, uterus and parietal cells. The drug represents a success story in medicinal chemistry and a milestone in the treatment of gastric acid acid-related diseases such as gastric and duodenal ulcer as well as gastroesophageal reflux disease (GERD). After cimetidine, the more potent H_2R antagonists ranitidine (Figure 7), famotidine, nizatidine and roxatidine entered clinical use. Nowadays, the H_2R antagonists are widely replaced with blockers of the H^+/K^+-ATPase, the co-called proton pump inhibitors (PPIs).

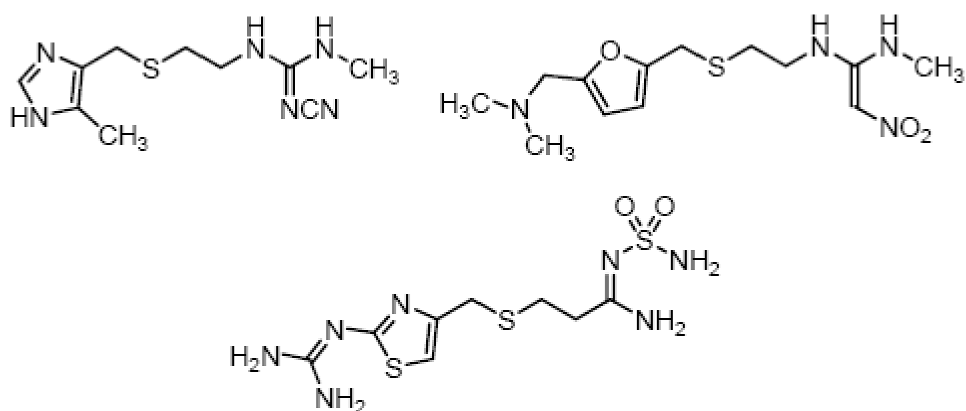

Figure 7. From the left, up, cimetidine, ranitidine and famotidine representing the group of H_2R antagonists.

Most H_2R antagonists are rather polar compounds, which do not enter the brain to a significant extent. By contrast, zolatidine, an experimental drug is a potent and selective brain-penetrating H_2R antagonist (Hill et al. 1997, Parsons and Ganellin 2006), which can be, in principle, used to investigate the role of H_2Rs in the CNS. H_2R agonists are not clinically used so far. 4-Methylhistamine (Figure 8, shown as 5-methylhistamine according to contemporary nomenclature, Black et al. 1972) was the first (weak) agonist described as H_2R–selective. However, recently this compound was reported to be much more potent and selective for the H_4R. Structurally different, highly potent H_2R agonists are compounds such as impromidine (Figure 8) and arpromidine (Buschauer 1989, Parsons and Ganellin 2006, Preuss et al. 2007c). Such imidazolylpropylguanidines achieve up to 400-fold higher H_2R agonistic potency than histamine and were developed as positive ionotropic vasodilators for the treatment of severe congestive heart failure, as agents inducing cell differentiation in acute myelogenous leukemia, and as anti-inflammatory drugs (Buschauer 1989, Dove et al. 2004, Mörsdorf et al. 1990, Preuss et al. 2007b).

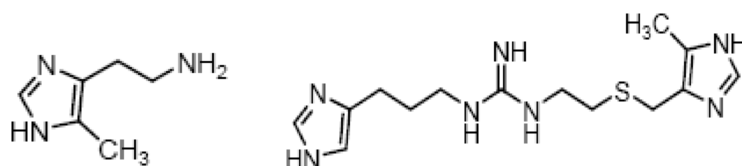

Figure 8. H_2R agonists (from the left): 5-methylhistamine and impromidine.

The human H_2R consists of 359 amino acids and is coupling to G_s-proteins, resulting in stimulation of adenylyl cyclase (Preuss et al. 2007b). The H_2R was first cloned from

canine gastric parietal cDNA by Gantz and colleagues (Gantz et al. 1991). The four H_2R species isoforms, human (hH_2R), canine (cH_2R), rat (rH_2R) and guinea pig (gpH_2R), are closely related to each other, sharing about 80 % identity of the amino acid sequences. H_2R mediated effects are, for instance, stimulation of gastric acid secretion by parietal cells (Black et al. 1972), increase in cardiac contractility and myeloid cell differentiation (Preuss et al. 2007b). The H_2R has also been identified in airway, uterine, vascular smooth muscle cells, neurons and finally, it can inhibit a variety of functions within the immune system. H_2-receptors on basophils and mast cells have been shown to block the release of histamine, inhibit antibody synthesis, T-cell proliferation, cell-mediated cytolysis and cytokine production. In the CNS, H_2Rs inhibit the long-lasting after-hyperpolarization and the accommodation of firing (Hill et al. 1997).

3.2.4 Histamine H_3-receptor subtype

The histamine H_3-receptor plays a role as a presynaptic autoreceptor, inhibiting synthesis and release of histamine from histaminergic neurones in the CNS (Arrang et al. 1983). Histaminergic neurones are located in the tuberomammillary nucleus of the hypothalamus and project to all major areas of the brain. In addition, the H_3R acts as a heteroreceptor on non-histaminergic neurones where it modulates the release of other neurotransmitters, such as acetylcholin, dopamine, GABA, 5-hydroxytryptamine, substance P and noradrenaline in the CNS and also in the periphery, for instance, in gastrointestinal tract, airways and cardiovascular system (Leurs et al. 2005, Parsons and Ganellin 2006, Sander et al. 2008). Studies on the effects of histamine and ligands of H_1- and H_2-receptors in the CNS revealed that burimamide, the H_2R antagonist, competitively inhibited the release of histamine in the brain, but other antagonists were less active, H_1R antagonists even ineffective (Parsons and Ganellin 2006). The existence of another histamine receptor subtype was suggested in 1983 (Arrang et al. 1983), further substantiated by pharmacological characterization in 1987 by Arrang and colleagues (Arrang et al. 1987) and proven by cloning in 1999 by Lovenberg and colleagues (Lovenberg et al. 1999). The H_3R contains an open reading frame of 445 amino acids and shows very low sequence similarity with the other two receptors, 22 % with H_1R and 20 % with H_2R, which explains why the H_3R gene was not cloned in the same time as previous receptors (Lovenberg et al. 1999). An additional member of histamine receptor family, the H_4-receptor, was soon discovered by similarity searching through genomic databases. The H_4R and the H_3R share about 60 % identity. This explains why many known H_3R ligands are active at the H_4R, too (Leurs et al. 2005).

Histamine acts as an agonist at all four receptors with lowest affinities for H_1R (pK_i=4.2) and H_2R (pK_i=4.3) and highest affinities at H_3R (pK_i=8.0) and H_4R (pK_i=7.8) (Lim et al. 2005). There is a large variety of H_3R isoforms. About 20 isoforms have been described, which vary in four regions of the receptor protein and show different pharmacological profiles. For instance, deletions in the third intracellular loop result in distinct agonist potencies, signalling properties and constitutive activity. It is also evident that H_3R isoforms are linked with genetic polymorphism. For example, at the position 280 can be found alanine or valine and this substitution is characteristic for patients with Shy-Drager syndrome, neurological orthostatic hypotension, which means neuronal degeneration and autonomic failure (Leurs et al. 2005). The H_3-receptor couples $G_{i/o}$ proteins, which is negatively coupled to adenylyl cyclase. Adenylyl cyclase stimulates the formation of cyclic AMP (cAMP), then activates protein kinase A and subsequently cAMP-responsive-element-binding protein to modulate the gene transcription. Furthermore, H_3R activation of $G_{i/o}$ proteins might result in the activation of other effector pathways, including mitogen-activated protein kinase (MAPK) and phospholipase A_2 to induce the release of arachidonic acid, the lowering of intracellular Ca^{2+} levels, and the inhibition of the Na^+/H^+ exchanger (Leurs et al. 2005).
(*R*)-α-Methylhistamine (Figure 9) was the first compound reported as a potent and selective H_3R agonist (*S* isomer is less potent), whereas thioperamide (Figure 10) was described as a selective and competitive antagonist, which is active in the nanomolar range and is able to cross the blood brain barrier (Arrang et al. 1987). The prodrug of (*R*)-α-methylhistamine, BP 2-94 (Figure 9), which showed improved oral bioavailabilty and pharmacokinetic properties compared to the parent compound, was the first H_3R agonist investigated in man. All H_3R agonists have a similar structure with histamine, containing a 4(5)-imidazolyl moiety. Agonists, such as imetit and immepip, show only limited selectivity for the H_3R over the related, later discovered H_4-receptor (Leurs e al. 2005).

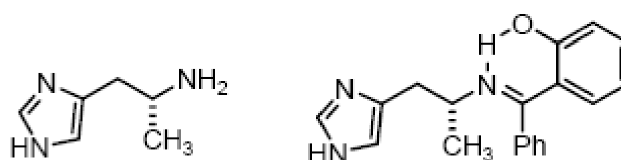

Figure 9. From the left side: H_3R agonist (*R*)-α-methylhistamine and the prodrug BP 2-94.

Thioperamide (Figure 10) is a high affinity antagonist for the investigations of H_3R but is not suitable for studies in patients because of potential liver toxicity. Moreover, thioperamide shows also high activity at the human H_4-receptor. H_3R antagonists are supposed to be of potential value in therapy of obesity, nasal decongestion and many

CNS disorders, for example memory and learning problems, Alzheimer´s disease, epilepsy, schizophrenia, sleep disturbance (Gemkow et al. 2009, Leurs et al. 2005, Parsons and Ganellin 2006). Many H_3R ligands (e. g., clobenpropit, proxyfan, ciproxifan, cipralisant) have been developed and several compounds entered clinical investigations, for instance, tiprolisant is currently in clinical trials for the treatment of schizophrenia (Gemkow et al. 2009).

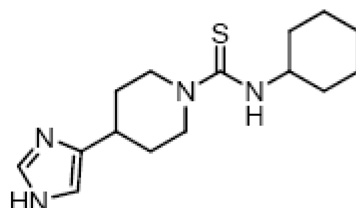

Figure 10. H_3R and H_4R antagonist/inverse agonist thioperamide.

3.2.5 Histamine H_4-receptor subtype

The investigation of histamine receptors by molecular biology brought first results in 1991 when the genes encoding for the bovine H_1- (Yamashita et al. 1991) and canine H_2- (Gantz et al. 1991) receptors were cloned (Parsons and Ganellin 2006). The molecular architecture of the third subtype was discovered few years later, in 1999 by the team of T. Lovenberg. They identified and cloned about 30 orphan GPCRs via expressed-sequence tags (ESTs) with the aim to discover new drug targets and to identify novel neurotransmitters and peptides. The GPCR97 cDNA, expressed abundantly in CNS, encodes the H_3R (Lovenberg et al. 1999). The cloning of the H_3R gene marked the entry of the novel histamine receptor subtype into the G-protein coupled receptor family (Leurs et al. 2009). This was already predicted by team of D. G. Raible in 1994, who characterized a novel histamine receptor on human eosinophils (Raible et al. 1994). Screening of human libraries and databases confirmed this idea in the years 2000 and 2001 and led several research groups independently to the cloning and identification of the fourth subtype of histamine receptors (Coge et al. 2001, Liu et al. 2001a, Morse et al. 2001, Nakamura et al. 2000, Nguyen et al. 2001, Oda et al. 2000, Zhu et al. 2001) which is closely related to the H_3R (Gbahou et al. 2006).

The histamine H_4R has about 40 % sequence homology with the H_3R (58 % in transmembrane domains) (Gbahou et al. 2006, Liu et al. 2001a, Morse et al. 2001, Nakamura et al. 2000, Nghuyen et al. 2001, Oda et al. 2000, Zhu et al. 2001). Homology with the other histamine receptors is lower, only about 19 %. That is why we had to wait for its discovery until the structure of the H_3R gene was found (Leurs et al. 2009, Oda et al. 2000). The size of the open reading frame of the H_4R cDNA is

1173 bp, consisting of 390 amino acids and one stop codon (Morse et al. 2001). It is presented in a single copy on chromosome 18q11.2. and is interrupted by two introns (7867 bp and more than 17 500 bp lenght). Introns divide the coding region into three parts, encoding amino acids number 1 - 65, 66 - 119 and 120 - 390 (Coge et al. 2001, Nakamura et al. 2000, Oda e al. 2000). The H_4R has an aspartate residue in the third transmembrane helix, which is supposed to interact with an amino group of the endogenous ligands (Shin et al. 2002). In addition, a glutamate in the fifth transmembrane helix presented in both H_3R and H_4R has been demonstrated to play an essential role in histamine binding as determined by site-directed mutagenesis analysis (Shin et al. 2002, Uveges et al. 2002). Meanwhile, two splice-isoforms have been identified, designated as H_4 (67) and H_4 (302) (van Rijn et al. 2008). There are deviations at three positions in the H_4R amino acid sequence in human population, which was also a reason for differences in the structure of the novel histamine receptor published by different laboratories: Val instead of Ala at position 138, Arg instead of His at position 206 and Arg instead of Gln at position 253 (Leurs et al. 2009, Morse et al. 2001, Zhu et al. 2000). The characterization of the human H_4R led to the cloning of animal cDNA of the H_4R, such as mouse, rat, guinea pig and pig, with only moderate homology (67 - 72 %) in protein sequence. The bovine and rabbit H_4R could not be cloned using the hH_4R cDNA in analysis because of their low homology with the human receptor; on the contrary, the monkey H_4R displays significantly higher homology: about 92 % of the human variant (Liu et al. 2001b, Oda et al. 2005). The H_4R species variants can differ in the response to numerous H_4R ligands, revealing differences in potencies and qualities of action and making ligand development very complicated. On the other hand, the H_4R shows similar tissue distribution in the different species variants (Lim et al. 2010). The H_4 receptor is the most distributed in bone marrow and peripheral blood, in smaller amount in spleen, thymus, small intestine, colon, heart and lung (Liu et al. 2001a, Morse et al. 2001, Nakamura et al. 2000, Nguyen et al. 2001, Oda et al. 2000, Zhu et al. 2001). However, there are still some conflicting reports about these localizations. The H_4R is preferentially expressed in various cells of the immune system, such as basophils, eosinophils, neutrophils, T-cells, dendritic cells, and mast cells (Leurs et al. 2009, Liu et al. 2001a, Morse et al. 2001, Oda et al. 2000, Parsons and Ganellin 2006, Zhu et al., 2001), suggesting an important role in inflammation and immune response (Gbahou et al. 2006). Therefore, H_4R antagonists are considered promising drugs for the treatment of bronchial asthma, allergic rhinitis, rheumatoid arthritis and pruritus (Lim et al. 2006). Furthermore, the H_4R can be found in the central nervous system (Connelly et al. 2009). The H_4R couples to pertussis toxin sensitive $G\alpha_{i/o}$ protein. Thereby, the H_4R activation inhibits the formation of cAMP,

which modulates protein kinase A and subsequently, cAMP-responsive-element-binding protein to modulate the gene transcription (Liu et al. 2001, Oda et al. 2000, Zhu et al. 2001). Moreover, stimulation of the H_4R leads to an increase in mitogen-activated protein kinase phosphorylation (Morse et al. 2001) as well as to calcium mobilization and thereby actin polymerisation, cell shape change, migration of mast cells, eosinophils and monocyte dendritic cells (Buckland et al. 2003, Hofstra et al. 2003). Pertussis toxin sensitive intracellular calcium increase in eosinophils induced by histamine, which can be blocked by the presumed selective H_3R antagonist thioperamide (but it is known, H_3R is not expressed in eosinophils as well as in the mast cells) as already reported by Raible et al. in 1994, suggested the existence of new histamine receptor subtype as mentioned above. At the beginning, due to lack of selective H_4R ligands, compounds known to interact more or less selectively with the other histamine receptor subtypes, were used to investigate the pharmacology and (patho)physiology of the H_4R. It was soon confirmed that most imidazole-containing H_3R ligands have affinity for the H_4R as well. For example, the H_3R antagonists, thioperamide and clobenprobit, showed antagonistic and agonistic activity, respectively (Morse et al. 2001, Nakamura et al. 2000, Oda et al. 2000, Zhu et al. 2001).

OUP-16 was the first agonist reported to show some selectivity for H_4R over H_3R (Figure 11, Hashimoto et al. 2003). Later on, 4-methylhistamine (Figure 8), which was originally used as an agonist to define the H_2-receptor, turned out to be at least 100-fold more potent at the H_4R (Lim et al. 2005). Clozapine (Figure 11), used in the treatment of schizophrenia, shows that an imidazole ring is not necessary for H_4R agonism. Most imidazole-containing H_3R ligands were found to have significant affinity for the H_4R. For example, 4-methylhistamine shows only 40-fold selectivity for H_3R as well as immepip. Small structural changes of the ligands can deeply influence the binding to other histamine receptor subtypes (Smits et al. 2009). Structural modifications of the non-imidazole H_2R agonist dimaprit led to the development of VUF8430 (Figure 11, Lim et al. 2009), which has been used as an H_4R agonist in vivo, despite of some affinity to other histamine receptor subtypes, in particular, at higher concentrations.

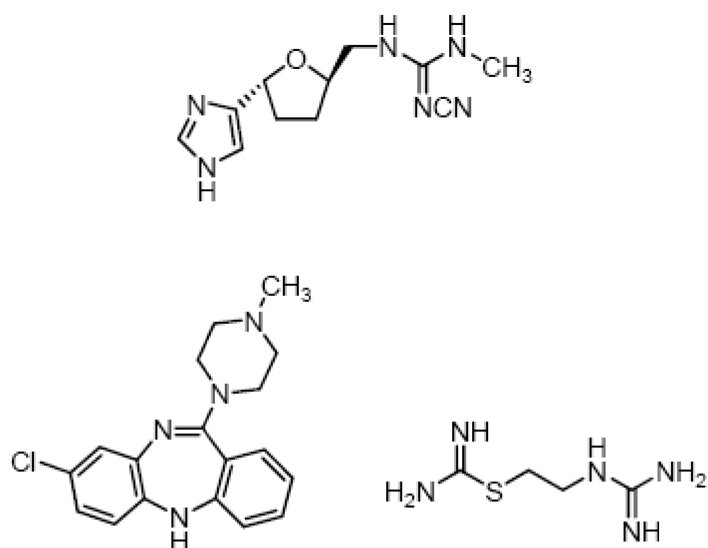

Figure 11. H_4R agonists (from the left, up): OUP-16, clozapine and VUF8430.

The first and potent H_4R antagonist (can also perform as a partial inverse agonist), the indole derivative JNJ7777120 (Figure 12), developed by Johnson and Johnson Pharmaceuticals, was demonstrated to be active on human, mouse and rat H_4Rs (Jablonowski et al. 2003).

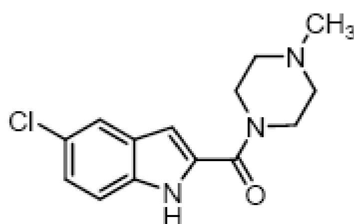

Figure 12. H_4R antagonist JNJ777120.

The investigation of numerous H_4R ligands, which were optimised for the human receptor, on H_4R of other species (e. g., in the mouse revealed) revealed considerable differences (up to 3 - 4 orders of magnitude) in potency. In addition to such discrepancies in potency, species dependent differences in HR subtype selectivities may contribute to misleading conclusions when such compounds are pharmacological studied in animals, for instance, in mice. This is one of the major problems with the currently known H_4R agonists.

Among a series of N^G-acylated imidazolylpropylguanidines, originally designated as histamine H_2R agonists, several ligands with a high affinity to the H_3R and H_4R were identified at the University of Regensburg. It was made an effort to increasing the

selectivity for the H_4R by chemical modification of the lead structures, among others, resulting in a series of cyanoguanidines. Within this group, 2-cyano-1-[4-(1*H*-imidazol-4-yl)butyl]-3-[(2-phenylthio)ethyl]guanidine, UR-PI376 (Figure 13) was identified as the most potent hH_4R agonist. This compound lacks agonistic activities at the hH_2R and hH_3R, has negligible activity at the hH_1R (Igel et al. 2009) and is one of the most potent and selective H_4R agonists known so far.

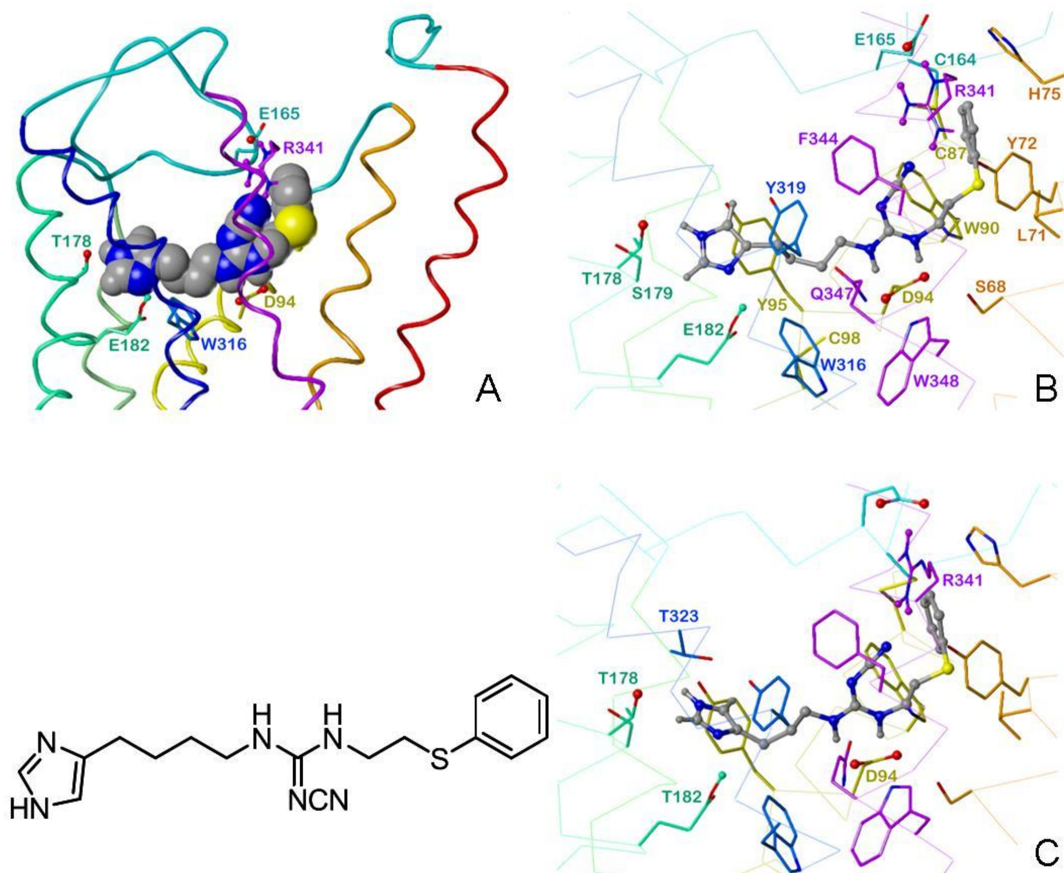

Figure 13. Structure of cyanoguanidine-type hH_4R agonist UR-PI376 and suggested model of the H_4R binding site (Igel et al 2009). A: Space fill representation of UR-PI376 within a tube model of the backbone. B, C: Detailed model of the binding modes of UR-PI376. For clarity of presentation, the labeling of amino acids in panel C is restricted to key and extra contacts.

A model of the H_4R binding site was suggested as shown in Figure 13 (for details Igel et al. 2009). The molecular modelling studies (Igel et al 2009) reveal the possibility, that cyanoguanidine moiety of UR-PI376 forms two charge-assisted hydrogen bonds with the carboxylate oxygens of Asp-94 and two additional charge-assisted hydrogen bonds with the guanidine moiety of Arg-341. It was suggested that the latter brings about selectivity for the hH_4R over the hH_3R and is the reason for the preference of UR-PI376 for hH_4R over mH_4R as well. Compared to the hH_4R, in the H_4Rs of rat and mouse,

Arg-341 is replaced with Ser, whereas Glu is the corresponding amino acid in both, the canine H_4R, and the human histamine H_3 receptor (hH_3R).

3.3 Polymerase chain reaction (PCR)

Nowadays is PCR the most important reaction of the molecular biology, the molecular pharmacology respectively. This technique allows amplification of DNA generating millions of copies of a particular DNA sequence. The heat-stable DNA polymerase, DNA as a template, two oligonucleotideprimers (sense and antisense), nucleotides and buffer containing e. g., magnesium and manganese, are all for one PCR. PCR amplification is performed according to repeated cycles (denaturation, annealing, elongation) of incubation at different temperatures. Within the denaturation the temperature climb up around 92 ˚C to separated strands of the template DNA. Then the temperature descends to 55 ˚C to allow annealing (hybridisation of the primers with complementary sequences flanking the DNA template), before elongation occurs again at elevated temperature (72 °C). This is the temperature optimum of the respective DNA-polymerase. Primers are extended in both directions and a new double-strand DNA is formed. Therefore this phase is called extension or elongation phase. The cycle is repeated depending on the amount of required product. The PCR is commonly carried out in a reaction volume of 10 – 200 µl in small reaction tubes (0.2 – 0.5 ml volumes) in a thermal cycler. The thermocycler is a microprocessor-controlled device heating and cooling the reaction mixture to achieve the temperatures required at each step of the reaction. Most thermocyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

Initial denaturation proceeds at 94 ˚C. Even higher temperatures can be used depending on the termostability of both the DNA-polymerase and the used DNA. For most templates 30 seconds are recommended. Some templates may require longer initial denaturation up to a few minutes because of their high complexity.

Denaturation is a very fast process. As well as the initial denaturation the temperature can start from 70 ˚C depend on the type of the enzyme. For example, the common *Taq*-DNA-polymerase is stable till 74 ˚C. Other DNA-polymerases (Phusion DNA-polymerase) are enzymatically active up to 98 ˚C. However, to minimize thermal deactivation and decomposition the denaturation time should be kept as short as possible. Five to ten seconds are usually enough for separation of strands.

Primer annealing critically depends on the sequence, reflected by the "melting temperature" (T_m). The T_m should be calculated with the nearest-neighbour method as results from primer T_m calculations can vary significantly depending on the method used. As a rule, primers longer than 20 nucleotides anneal at T_m +3 °C of the T_m short of primers. Melting temperatures give an orientation for the selection of the annealing temperature although experimental optimisation is usually required. The annealing time is recommended from 10 to 30 seconds.
Elongation is complete in usually 15 – 60 seconds per 1 kb of product. At too short elongation periods extension is insufficient, whereas too long exposure time may result in undesired products and thermal denaturation of the polymerase.
Final elongation is occasionally performed at a temperature of 70 – 74 °C for 5 to 15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. Final hold proceeds at 4 to 15 °C for an indefinite time, which may be employed for short-term storage of the reaction.

Template has to be the highest quality as possible because then the whole reaction works in the best way and we get high quality results. Theoretically, one DNA molecule is enough for PCR but in practice at least 1×10^5 DNA molecules are required to avoid errors. For image, 1×10^5 molecules correspond to approximately 0.5 pg plasmid-DNA, 300 ng human genomic DNA or cDNA of 1 µg entire RNA. There are different templates, which could be used for PCR: plasmid-DNA, cosmid-DNA, phage-DNA, DNA from last PCRs, genomic DNA, cDNA etc.
Efficiency of amplification determines how much new DNA is obtained. Ideally, in each cycle DNA molecules are doubled. During thirty cycles there are about one billion DNA molecules, after forty cycles one trillion molecules (1 µg 1 kb long DNA). The amplification factor is 1.6 to 1.7 per cycle.
Buffer solution provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.
Divalent cations, generally Mg^{2+}, are used. Excessive Mg^{2+} stabilizes DNA double strand and prevents complete denaturation of DNA. It can also stabilize spurious annealing of primer to incorrect template sites and decrease specificity. Conversely, inadequate Mg^{2+} may lead to lower product yield. The concentration of cations is chosen between 0.5 to 2.5 mM.
In addition, PCR can require other substances such as dimethylsulfoxide (DMSO) in case of a complex DNA-template with high contents of guanine and cytosine bases or supercoiled plasmids to relax for denaturation. Subsequently, after the addition DMSO, annealing temperature must be decrease. For bigger yield salts as KCl or NH_4SO_4 can

be use. The quality of nucleotides does not have substantial influence on PCR. Usually it is used the concentration 200 μM of each dNTP (10 mM dNTP mix) except dUTP-derivates and dITP.

PCR-primers complementary to the 3´-end of each of the sense and antisense DNA strand are normally 18 – 30 bp long and consist of 40 – 60 % guanidine and cytosine (G+C) with one, two, highly three G or C at the beginning for sufficient binding. Primers for "extra long" products should be 25 – 35 bp long. They would not have more than four same bases in a row (e.g. AAAA) to avoid frameshifts. The melting temperature of primers lies in the range 55 – 80 °C. It is necessary to prevent formation of secondary structures of primers, so called hair pins because it increases the probability of "wrong" hybridizations. This can be controlled by given computer programmes. Optimal concentration for 50 µl of total volume is 10 pM.

The polymerase, the typical enzyme for PCR is *Taq*-DNA-polymerase from a termophilic bacterium, *Thermus aquaticus*. It has an optimum of activity (5´,3´-DNA-polymerase and 5´,3´-exonuclease activity) around 74 °C at pH = 8 (Mülhardt 2006).

3.4 Steady-state GTPase activity assay

Steady-state GTPase activity assay is a very efficient method for functional characterisation of GPCR agonists, antagonists and inverse agonists. The Sf9 expression cell system has been shown to be a highly sensitive model system for analysis of constitutive activity of GPCRs. Steady-state GTPase activity assay monitors the outcome of receptor activation at the G-protein level, i. e. the result of GDP/GTP exchange and GTP hydrolysis, and enables comparison of various receptors with each other independently of the expression level. In the GTPase assay is possible to use various G_i/G_o-coupled GPCRs expressed in recombinant mammalian and insect cells. In case of insect cells, it is necessary to co-express GPCRs with G_i/G_o-proteins because insect cells do not express G-proteins capable of coupling to mammalian GPCRs. GTPase assays with G_s-coupled GPCRs were demonstrated to be more efficient with GPCR-$G\alpha_s$ fusion proteins than with co-expressed proteins. G_q-coupled GPCRs are provided with RGS proteins.

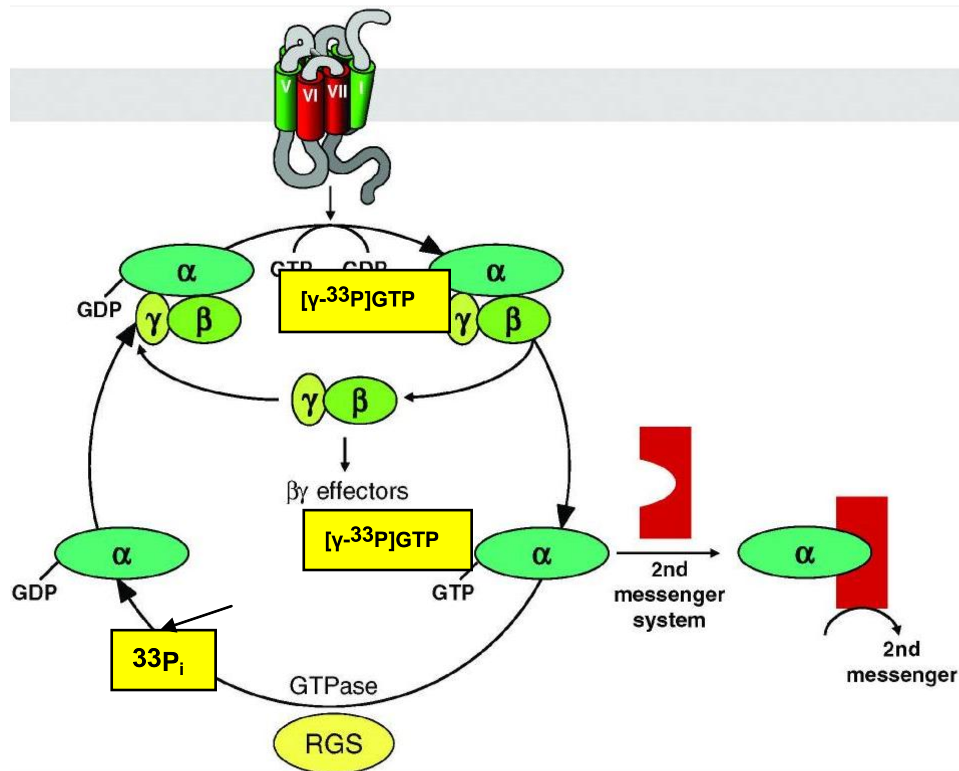

Figure 14. The G-protein cycle (Milligan and Kostenis 2006). The active state of the receptor interacts with the G-protein. Subsequently, GDP is released, radioactively labeled GTP ([γ-^{33}P]GTP) associates with the Gα-subunit, induces conformational changes and induces the dissociation of Gα from Gβγ subunits and uncoupling from the GPCR. The intrinsic GTPase activity of the Gα-subunit mediates the cleavage of GTP into GDP and $^{33}P_i$. GDP-loaded Gα re-associates with Gβγ to allow the next G-protein cycle. The degradation of [γ-^{33}P]GTP is determined by liquid scintillation counting of $^{33}P_i$.

A procedure for the steady-state GTPase assay is described in detail in chapter *Material and methods*. Assays are performed (at least) in triplicate, and it is preferred to include as many ligands as possible in one run with respect to comparison of pharmacological data. Unlabeled GTP (1 mM) is added in some tubes to evaluate non-enzymatic degradation of radioactively labeled GTP. Results are analyzed by nonlinear regression in the given computer program. GTPase activity, expressed as pmoles of P_i released per mg of membrane protein per min, is calculated in following way:

$$pmol\ mg^{-1}\ min^{-1} = \frac{(cpm\ total - cpm\ blank)\ x\ pmol\ GTP/tube}{cpm\ total\ added\ x\ min\ incubation\ x\ mg\ protein}\ x\ \frac{1000}{600}$$

Explanation:

cpm total	radioactivity of $^{33}P_i$ from binding tubes, except from tubes containing unlabeled 1 mM GTP
cpm GTP	radioactivity of $^{33}P_i$ from binding tubes containing unlabeled 1 mM GTP
pmol GTP/tube	concentration of unlabeled GTP
cpm total added	the radioactivity of [γ-^{33}P]GTP added to each tube
mg protein	absolute amount of membrane protein added per tube
min incubation	reaction mixture incubated with [γ-^{33}P]GTP, usually for 20 min
1000	100 µl of reaction mixture + 900 µl of charcoal = 1000 µl
600	600 µl of supernatant added to Scinti-Vital coctail

(Seifert and Wieland 2005).

4 AIM OF STUDY

Arg-341 of the human H_4R was recently suggested to play a crucial role in H_4R species- and receptor subtype-selectivity of cyanoguanidine-type H_4R agonists such as UR-PI376. The aim of this diploma thesis was to substantiate or falsify this working hypothesis by:

1. Site-directed mutagenesis of the human histamine H_4R: the exchange Arg-341 with alanine, serine (as in rat and mouse H_4R) or glutamate (as in dog H_4R and hH_3R) and expression of mutants in Sf9 insect cells

2. Functional of wildtype and mutant H_4Rs by investigation of histamine, UR-PI376 and thioperamide in the steady-state GTPase activity assay

5 EXPERIMENTAL PART

5.1 Materials and methods

5.1.1 Materials

5.1.1.1 Overlap – extension PCR (polymerase chain reaction)

5x Phusion® HF Reaction Buffer from Finnzymes (contains $MgCl_2$); dNTP-Mix from Fermentas; DMSO; Phusion DNA Polymerase from Finnzymes; PCR–termocycler Biometra®; pcDNA 3.1 plasmid with the hH_4R sequence from the UMR cDNA Resource Center at the University of Missouri-Rolla (Rolla, MO) used as a template for the generation of pVL1392-SF-hH_4R-His6; DNA primers were synthesized by MWG Biotech (Ebersberg, Germany)

Primers with given mutations and a new *Pst*I site were constructed:

hH_4R → hH_4R-R341**A***

Primer 1A/2-forward:

24 bp, GC-content = 45.8 %, T_m = 55.2 ºC

5´- GAT TAT TCA TAC CGT CCC ACC ATC -3´

Primer 1A-reverse:

26 bp, GC-content = 38.5 %, T_m = 55.1 ºC (44 bp, GC-content = 40.9 %, T_m = 64.5 ºC)

5´- **CAG** CCA AAA TGC AAT ***AGC*** ATA CCA AAC TGA TTT AGG ACC TGT TG -3´

Primer 1B-forward:

25 bp, GC-content = 40.0 %, Tm = 54.7 ºC (43 bp, GC-content = 41.9 %, T_m = 65.3 ºC)

5´- ***GCT*** ATT GCA TTT TGG **CTG** CAG TGG TTC AAT TCC TTT GTC AAT C -3´

Primer 1B/2-reverse:

22 bp, GC-content = 50.0 %, T_m = 55.4 ºC

5´- GTA ACA ACG GTT GGG TCT AGT G -3´

$hH_4R \rightarrow hH_4R$-R341**S**

Primer 1A/2-forward:

24 bp, GC-content = 45.8 %, T_m = 55.2 ºC

5´- GAT TAT TCA TAC CGT CCC ACC ATC -3´

Primer 1A-reverse:

26 bp, GC-content = 38.5 %, T_m = 55.1 ºC (44 bp, GC-content = 38.6 %, T_m = 63.2 ºC)

5´- **CAG** CCA AAA TGC AAT ***ACT*** ATA CCA AAC TGA TTT AGG ACC TGT TG -3´

Primer 1B-forward:

25 bp, GC-content = 40.0 %, Tm = 54.7 ºC (43 bp, GC-content = 39.5 %, T_m = 64.9 ºC)

5´- ***AGT*** ATT GCA TTT TGG **CTG** CAG TGG TTC AAT TCC TTT GTC AAT C -3´

Primer 1B/2-reverse:

22 bp, GC-content = 50.0 %, T_m = 55.4 ºC

5´- GTA ACA ACG GTT GGG TCT AGT G -3´

$hH_4R \rightarrow hH_4R$-R341**E**

Primer 1A/2-forward:

24 bp, GC-content = 45.8 %, T_m = 55.2 ºC

5´- GAT TAT TCA TAC CGT CCC ACC ATC -3´

Primer 1A-reverse:

26 bp, GC-content = 38.5 %, T_m = 55.1 ºC (44 bp, GC-content = 38.6 %, T_m = 63.8 ºC)

5´- **CAG** CCA AAA TGC AAT ***TTC*** ATA CCA AAC TGA TTT AGG ACC TGT TG -3´

Primer 1B-forward:

25 bp, GC-content = 40.0 %, Tm = 54.7 ºC (43 bp, GC-content = 39.5 %, T_m = 64.6 ºC)

5´- ***GAA*** ATT GCA TTT TGG **CTG** CAG TGG TTC AAT TCC TTT GTC AAT C -3´

Primer 1B/2-reverse:

22 bp, GC-content = 50.0 %, T_m = 55.4 ºC

5´- GTA ACA ACG GTT GGG TCT AGT G -3´

*Given mutations, where arginine-341 (R341) was replaced with alanine (A), serine (S) and glutamate (E), are demonstrated with red italics in the yellow field; black letters in the yellow field demonstrate a nucleotide recognised with enzyme *PstI*.

5.1.1.2 Electrophoresis

Agarose from Lonza; Ethidium Bromide; TAE-Buffer (40 mM Tris-Acetate, 1 mM EDTA); DNA Ladder and 6x Loading Dye from Fermentas; Biometra Bio Doc Analyze 2.0 digital

5.1.1.3 DNA purification

QIAquick Gel extraction Kit from QIAGEN: QIAquick Spin Columns, Buffer QG, Buffer PE (concentrate), Buffer EB (10 mM Tris/HCl, pH = 8.5), Collection tubes (2 ml), Loading Dye; Ethanol (96 - 100 %); Microcentrifuge; Isopropanol (100 %); VWR Digital Heatblock

5.1.1.4 Restriction enzymes

BSA 10x from New England BioLabs; 10x Buffers NEB 1-4 from New England BioLabs; Enzymes *Sac*I*,* XbaI*, Xho*I*, PstI* (20000 U/ml) from New England BioLabs; Minicentrifuge; VWR Digital Heatblock

5.1.1.5 Ligation and transformation

CIP-vector: pVL1392 (c = 22 ng/µl); 10x Ligation Buffer and T_4 DNA Ligase from New England BioLabs; LB medium pH = 7.5 (Tryptone, Yeast extract, NaCl); Agar-plates (Tryptone, Yeast extract, NaCl, Agar); Ampicillin (100 mg/ml); *Escherichia coli* (TOP 10, stored at -80 °C); SOC medium pH = 7.0 (Tryptone, Yeast extract, NaCl, KCl, Glucose); Minicentrifuge; VWR Digital Heatblock

5.1.1.6 Mini/Maxiprep

QIAprep Spin Miniprep Kit: Buffer P1 (50 mM Tris/HCl, pH = 8.0; 10 mM EDTA in 100 µg/ml Rnase A), Buffer P2 (200 mM NaOH; 1 % SDS (w/v)), Buffer N3, Buffer PB, Buffer PE (concentrate), Buffer EB (10 mM Tris/HCl, pH = 8.5), Ethanol (96 - 100 %), Microcentrifuge, QIAprep spin column, 1.5 ml microcentrifuge tubes

QIAprep Spin Maxiprep Kit: Buffer P1 (50 mM Tris/HCl, pH = 8.0; 10 mM EDTA in 100 µg/ml Rnase A), Buffer P2 (200 mM NaOH; 1 % SDS (w/v)), Buffer P3 (1 M potassium

acetate, pH = 5.0), Buffer QBT (750 mM NaCl; 50 mM MOPS, pH = 7.0; 15 % Isopropanol (v/v); 0.15 % Triton® X-100 (v/v)), QIAgen-tip, Tip holder, Buffer QC (1 M NaCl; 50 mM MOPS, pH = 7.0; 15 % Isopropanol (v/v)), Buffer QF (1.25 M NaCl; 50 mM Tris/HCl, pH = 8.5; 15 % Isopropanol (v/v)), tubes or vessels with suitable capacity, refrigerated centrifuge capable of 20000 x g

5.1.1.7 Protein expression and membrane preparation

BaculoGold™ Transfection Kit (BD Biosciences, San Diego, CA): pVL1392 Baculovirus Transfer Vector, Transfection Buffer A, Transfection Buffer B, *Spodoptera frugiperda* (Sf9) insect cells; Baculovirus encoding $G\alpha_{i2}$ from Department of Pharmacology, University of Southwestern Medical Center, Dallas, TX; Baculovirus encoding $G\beta_1\gamma_2$ from Department of Pharmacology and Toxicology, University Ulm, Germany
Membrane preparation: PBS-Buffer (100 mM NaCl; 80 mM Na_2HPO_4; 20 mM Na H_2PO_4), Lysis-Buffer (10 mM Tris/HCl pH = 7.4; 1 mM EDTA; 10 µg/ml Benzamidine; 10 µg/ml Leupeptin; 200 µM PMSF), 10x Binding-Buffer (Tris base 454 g/5l; $MgCl_2$ x H_20 127.5 g/5l; EDTA 18.8 g/5l, pH = 7.4)

5.1.1.8 Protein determination – Lowry assay

Reagents for the assay from Bio Rad (Bio Rad, Hercules, CA), Biophotometer (Lowry measurement); Bovine Serum Albumine (BSA) 2 mg/ml; 10x Binding-Buffer (Tris base 454 g/5l; $MgCl_2$ x H_20 127.5 g/5l; EDTA 18.8 g/5l, pH = 7.4)

5.1.1.9 Western blot analysis

Gel-electrophoresis equipment from Bio Rad (Bio Rad, Hercules, CA); Anti-Flag M1 antibody (1:1000) from Sigma, $G\alpha_{i1/2}$ (1:1000) and Gβ common (1:1000) antibodies from Calbiochem; Antimouse (1:2000), Antirabbit (1:10000) antibodies from Sigma
Gel (12 %): Buffer A (Tris; 10 % SDS, pH = 8.0), Buffer B (Tris; 10 % SDS; pH = 6.8), Acrylamide, 50 % Glycerol, TEMED, 10 % APS; Running-Buffer (Tris; Glycin; SDS; pH = 8.3); Blotting-Buffer (Tris; Glycin; Methanol); TBS-Buffer (NaCl; 1 M Tris/HCl pH = 7.6); TWEEN; Low-fat milk in TBS-Buffer; Ponceau S dye; Film wrap and imaging densitometer from Bio Rad

5.1.1.10 Steady-state GTPase activity assay

Ligands (10 µl in 100 µl t. v.): Histamine 1 mM (dissolved in Millipore water) diluted in a row 1:10 to 1 nM; Thioperamide 1 mM (dissolved in Millipore water) diluted in a row 1:10 to 1 nM; UR-PI376 1 mM (dissolved in 20 % DMSO) diluted in a row 1:10 in 20 % DMSO to 1 nM
Rea-Mix (30 µl in 100 µl t. v.): 1M Tris/HCl pH = 7.4, 10 mM EDTA pH = 7.4, 500 mM $MgCl_2$, 1 M NaCL, 10 mM AppNHp, 10 µM GTP, 10 mM ATP, Creatine phosphate (CP) from Sigma, Creatine kinase (CK) from Sigma; 6.7 % BSA
20 mM Tris/HCl pH = 7.4 (20 µl in 100 µl t. v.); Membranes (15 µg protein/20 µl); 10 mM Tris/HCl pH = 7.4; [γ-^{33}P]GTP prepared in Department of pharmacology and toxicology, University Regensburg, Germany using GDP and [^{33}P]phosphoric acid, (15,15 mCi/ml, obtained from Perkin Elmer) using an enzymatic labelling procedure; Active coal (NaH_2PO_4/HCl pH = 2.0; Active coal from Sigma); VWR Digital Heatblock; Centrifuge 13000 rpm; Scinti-Vials; Radioactive samples were counted in Perkin Elmer Tricarb 2800TR liquid scintillation analyzer; GraphPadPrism Software from GraphPad, USA for data analysis

5.1.2 Methods and conditions

5.1.2.1 Overlap - extension PCR

The point mutations in the sequence of the hH_4R were generated by sequential overlap-extension PCR with pVL1392-SF-hH_4R-His6 as the template. PCR 1A was used to amplify a DNA fragment consisting of the cleavable signal peptid from influenza hemagglutinin (S), the Flag epitope (F) recognized by the M1 monoclonal antibody, and the N-terminal part of the hH_4R. The sense primer (primer 1A/2-forward) annealed on pVL1392 prior to the 5´-end of SF. The antisense primer (primer 1A-reverse) encoded the sequence according to the given mutation and a new *Pst*I site. In PCR 1B, the DNA sequence for the C-terminal part of the hH_4R and a hexahisitdine tag was amplified using pVL1392-SF-hH_4R-His6 as the template. The hexahistidine tag allows future purification and provides additional protection against proteolysis. The sense primer (primer 1B-forward) encoded the sequence according to the given mutation and a new *Pst*I site. The antisense primer (primer 1B/2-reverse) annealed on pVL1392 vector after the stop codon of the hH_4R. In PCR 2, the products of PCR 1A and PCR 1B annealed in the region of new created point mutations and the new *Pst*I site. The sense primer of PCR 1A and the antisense primer of PCR 1B were used.

PCR_1A for hH_4R → hH_4R-R341**A** (Product length: 1211 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1A/2-forward (10 µM), 2.5 µl primer 1A-reverse (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; DNA pVL1392-SF-hH_4R-His6 (0.1 µg/µl) was added in amount of 0.5 µl with 1.5 µl DMSO, 0.5 µl without DMSO, 1 µl with 1.5 µl DMSO and 1 µl without DMSO; refilled with Millipore water. Initial denaturation: 98 °C 30 s; 30 cycles of denaturation: 98 °C 8 s, annealing: 58 °C 20 s and elongation 72 °C 18 s; final elongation: 72 °C 5 min; final hold: 4 °C.

PCR_1A for hH_4R → hH_4R-R341**S** (Product length: 1211 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1A/2-forward (10 µM), 2.5 µl primer 1A-reverse (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; DNA pVL1392-SF-hH_4R-His6 (0.1 µg/µl) was added in amount of 0.5 µl with 1.5 µl DMSO, 0.5 µl without DMSO, 1 µl with 1.5 µl

DMSO and 1 µl without DMSO; refilled with Millipore water. Initial denaturation: 98 °C 30 s; 30 cycles of denaturation: 98 °C 8 s, annealing: 58 °C 20 s and elongation 72 °C 18 s; final elongation: 72 °C 5 min; final hold: 4 °C.

PCR_1A for hH_4R → hH_4R-R341**E** (Product length: 1211 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1A/2-forward (10 µM), 2.5 µl primer 1A-reverse (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; DNA pVL1392-SF-hH_4R-His6 (0.1 µg/µl) was added in amount of 0.5 µl with 1.5 µl DMSO, 0.5 µl without DMSO, 1 µl with 1.5 µl DMSO and 1 µl without DMSO; refilled with Millipore water. Initial denaturation: 98 °C 30 s; 30 cycles of denaturation: 98 °C 8 s, annealing: 58 °C 20 s and elongation 72 °C 18 s; final elongation: 72 °C 5 min; final hold: 4 °C.

PCR_1B for hH_4R → hH_4R-R341**A** (Product length: 458 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1B/2-reverse (10 µM), 2.5 µl primer 1B-forward (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; DNA pVL1392-SF-hH_4R-His6 (0.1 µg/µl) was added in amount of 0.5 µl with 1.5 µl DMSO, 0.5 µl without DMSO, 1 µl with 1.5 µl DMSO and 1 µl without DMSO; refilled with Millipore water. Initial denaturation: 98 °C 30 s; 30 cycles of denaturation: 98 °C 8 s, annealing: 57.7 °C 20 s and elongation 72 °C 7 s; final elongation: 72 °C 5 min; final hold: 4 °C.

PCR_1B for hH_4R → hH_4R-R341**S** (Product length: 458 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1B/2-reverse (10 µM), 2.5 µl primer 1B-forward (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; DNA pVL1392-SF-hH_4R-His6 (0.1 µg/µl) was added in amount of 0.5 µl with 1.5 µl DMSO, 0.5 µl without DMSO, 1 µl with 1.5 µl DMSO and 1 µl without DMSO; refilled with Millipore water. Initial denaturation: 98 °C 30 s; 30 cycles of denaturation: 98 °C 8 s, annealing: 57.7 °C 20 s and elongation 72 °C 7 s; final elongation: 72 °C 5 min; final hold: 4 °C.

PCR_1B for hH_4R → hH_4R-R341**E** (Product length: 458 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1B/2-reverse (10 µM), 2.5 µl primer 1B-forward (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; DNA pVL1392-SF-hH_4R-His6 (0.1 µg/µl) was added in amount of 0.5 µl with 1.5 µl DMSO, 0.5 µl without DMSO, 1 µl with 1.5 µl DMSO and 1 µl without DMSO; refilled with Millipore water. Initial denaturation: 98 °C 30 s; 30 cycles of denaturation: 98 °C 8 s, annealing: 57.7 °C 20 s and elongation 72 °C 7 s; final elongation: 72 °C 5 min; final hold: 4 °C.

PCR_2 for hH_4R → hH_4R-R341**A** (Product length: 1652 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1A/2-forward (10 µM), 2.5 µl primer 1B/2-reverse (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; product from PCR_1A and product from PCR_1B were added in amount of 2 µl with 1.5 µl DMSO, 2 µl without DMSO 5 µl with 1.5 µl DMSO, 5 µl without DMSO; refilled with Millipore water. Initial denaturation: 98 °C 30 s; 28 cycles of denaturation: 98 °C 8 s, annealing: 59 °C 20 s and elongation 72 °C 20 s; final elongation: 72 °C 5 min; final hold: 4 °C.

PCR_2 for hH_4R → hH_4R-R341**S** (Product length: 1652 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1A/2-forward (10 µM), 2.5 µl primer 1B/2-reverse (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; product from PCR_1A and product from PCR_1B were added in amount of 2 µl with 1.5 µl DMSO, 2 µl without DMSO 5 µl with 1.5 µl DMSO, 5 µl without DMSO; refilled with Millipore water. Initial denaturation: 98 °C 30 s; 28 cycles of denaturation: 98 °C 8 s, annealing: 59 °C 20 s and elongation 72 °C 20 s; final elongation: 72 °C 5 min; final hold: 4 °C.

PCR_2 for hH_4R → hH_4R-R341**E** (Product length: 1652 bp)

The reaction ran in 50 µl t. v.; 10 µl 5x Phusion HF-Buffer, 1 µl dNTP-Mix (10 mM), 2.5 µl primer 1A/2-forward (10 µM), 2.5 µl primer 1B/2-reverse (10 µM), 0.5 µl DNA-Polymerase in each 0.5 ml PCR-tube; product from PCR_1A and product from PCR_1B were added in amount of 2 µl with 1.5 µl DMSO, 2 µl without DMSO 5 µl with 1.5 µl DMSO, 5 µl without DMSO; refilled with Millipore water. Initial denaturation:

98 °C 30 s; 28 cycles of denaturation: 98 °C 8 s, annealing: 59 °C 20 s and elongation 72 °C 20 s; final elongation: 72 °C 5 min; final hold: 4 °C.

5.1.2.2 Electrophoresis

Control electrophoresis:
10 µl of 1 kb DNA Ladder
18 µl of mixture consisting of 3 µl PCR product, 3 µl Loading Dye and 12 µl water
150 V, 40 min

Electrophoresis before DNA purification:
15 µl of 1 kb DNA Ladder
56 µl of mixture consisting of 9 µl Loading Dye and 47 µl PCR product
150 V, 40 min

5.1.2.3 Restriction enzymes

Control digestion: 20 µl t. v., incubation for 1 h

Digestion for following use: digest enzymes *Sac*I and *Xba*I (2 µl each) were used before ligation to cleave PCR_2 products in 50 µl t. v., incubation for 2 h

5.1.2.4 Ligation and transformation

Ligation was provided with relevant carrier – vector, which was cut in the same sides like DNA-fragments (inserts). The vector pVL1392 (9600 bp) was dephosphorylated (CIP vector) by calf intestine alkaline phosphatase to prevent interaction between its two compatible ends. Phosphate was removed from 5´-end of a vector. The ligation ran in 20 µl t.v.: 50 ng pVL1392, 2 µl 10x Ligation Buffer and insert:

$$mass_{insert}\ [ng] = 5 \times mass_{vector}\ [ng] \times length_{insert}\ [bp] / length_{vector}\ [bp]$$

The mixture was firstly warmed up to 45 °C for 5 min and secondly incubated with 1 µl of T_4 DNA-ligase over night at 4 °C. Next day the ligation enzyme was deactivated by 65 °C for 10 min. The ligation product was transformed into *E.coli* to amplify the plasmid: 4 µl of product after ligation in 100 µl cell-suspension and 900 µl SOC medium inoculated onto agar plates in the presence of selective antibiotic and incubated over

night at 37 °C. Bacterial cultures were grown from a single colony picked from a freshly streaked selective agar plates. A single colony was then inoculated in 3 ml Luria-Bertani (LB) medium containing the appropriate selective antibiotic and incubated over night at 37 °C by vigorous shaking. More than 16 h of incubation was not recommended because of possible cell lysis and thereby lower plasmid yield. The next day the bacterial cells were harvested by centrifugation at >13000 rpm for 3 min and pellets were treated in miniprep/maxiprep kit.

5.1.2.5 Miniprep/Maxiprep

Miniprep and maxiprep kits are based on alkaline lysis of bacterial cells followed by adsorption of DNA onto the silica membrane in the presence of high salt. The relevant buffers ensure that only DNA is adsorbed, whereas RNA, cellular proteins and metabolites do not remain on the membrane and are found in the flow-through. Endonucleases were efficiently removed by a wash step with Buffer PB, salts were removed by washing with Buffer PE and DNA was eluted from column with Buffer EB. The method was followed according to QIA Spin miniprep/maxiprep kit instructions.

5.1.2.6 Sequencing

Sequencing was provided by Entelechon, the synthetic genes company, Regensburg, Germany.

5.1.2.7 Protein expression and membrane preparation

Sf9 cells were cultured in 250 ml Erlenmayer flasks at 28 °C and 150 rpm. Cells were maintained at a density of 0.5 – 6.0 x 10^6 cells per ml and were suspended in fresh medium at each passage (three times weekly) and for each infection. The BaculoGold™ transfection Kit was used according to the manufacture´s instructions to generate recombinant baculoviruses encoding mutants hH_4R-R341A, hH_4R-R341S or hH_4R-R341E. After transfection of Sf9 cells (4 x 10^6 cells per 3 ml) with pVL1392 plasmids encoding for mutants and incubation for 7 days (→P1), virus stocks were generated by two amplifications. In the first amplification cells were seeded at 2 x 10^6 cells per ml (t.v. 50 ml) and infected with 1 ml of the supernatant of the initial culture. Cells were cultured for 7 days. The supernatant was harvested at 3000 rpm for 15 min and stored at 4 °C (→P2). Five millilitres of P2 supernatant were cultured with 3 x 10^6 cells per ml (t.v. 100 ml) for 48 h and the supernatant was harvested as previously

(→P3). For membrane preparation, cells were seeded at 3 x 10^6 cells per ml in 50 ml of cell suspension, infected with 1:100 dilutions of P3 amplification baculovirus stocks and cultured for 48 h (hH_4R and mutants were always expressed together with G-protein subunits $G\alpha_{i2}$ and $G\beta_1\gamma_2$). Sf9 cells were harvested at 1000 rpm for 10 min and membrane preparation (all steps at 4 °C) followed. Cells were washed in PBS-Buffer and centrifuged at 1000 rpm for 10 min, lysed in Lysis-Buffer and centrifuged at 500 rpm for 5 min. The supernatant was removed and centrifuged at 18000 rpm for 20 min, resuspended in Lysis-Buffer and re-centrifuged. Membranes were homogenized in Binding-Buffer and stored at -80 °C.

5.1.2.8 Protein determination – Lowry assay

Protein determination was provided with Lowry assay: BSA 2 mg/ml was diluted (in Millipore water) in a row: 2000 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml. Reagents were prepared according to manual instructions. In 1.5 ml tubes were added following compounds (Table 1), mixed and after 15 min incubation at room temperature protein concentration was determined using BSA-calibration curve. Absorbance was measured at 750 nm.

Table 1. Scheme of preparation for protein determination

	Sample*	Standard	Blank
Sample	20 µl	---	---
Standard	---	20 µl	---
Binding-Buffer	---	---	20 µl
Reagent A´	100 µl	100 µl	100 µl
Reagent B	800 µl	800 µl	800 µl

* Columns: the hH_4R or mutants as a sample, BSA in given concentrations as a standard and Binding-Buffer as a blank. In lines there are components required for measurement.

5.1.2.9 Western blot analysis

The protein expression level was investigated by Western blot analysis. For this assay membranes were diluted with 10 mM Tris/HCl pH = 8.0 to the concentration 2 µg/µl and after homogenization with 2x Lämli-Buffer to the concentration 1 µg/µl. Samples were loaded into wells in the gel. The first lane was reserved for a standard, a commercially available mixture of proteins having defined molecular weights and forming visible, coloured bands. The last lane was filled with 2x Lämli-Buffer that ensures the right run in the gel. Membrane proteins were separated on SDS polyacrylamid gel containing 12 % (w/v) total acrylamide, at constant voltage (120 V) for 150 min and after that transferred onto nitrocellulose (0.45 µm) membrane and ran at constant current (250 mA) for 120 min. The uniformity of protein transferred from gel to the membrane was checked by staining the membrane Ponceau S dye. Membranes were incubated with anti-Flag M1, $G\alpha_{i1/2}$ or Gβ common antibodies over night at 4 °C. The next day imunnoreactive bands were visualized by enhanced chemoluminescence using goat antimouse IgG or donkey antirabbit IgG coupled to peroxidase. Immunoblots were scanned with imaging densitometer.

5.1.2.10 Steady-state GTPase activity assay

Membranes were thawed, sedimented at 13000 rpm for 10 min at 4 °C and resuspended in 10 mM Tris/HCl pH = 7.4. Reaction mixtures contained 20 µl of suspended membranes, 10 µl ligands at various concentrations (each concentration performed in triplicate), 20 µl 20 mM Tris/HCl pH = 7.4 and 30 µl Rea-Mix. Enzyme activities were corrected for spontaneous degradation of [γ-^{33}P]GTP via unlabeled GTP 1 mM which was added in last three tubes instead of ligands. Reaction mixtures (80 µl) were incubated for 2 min at 25 °C before addition of 20 µl of [γ-^{33}P]GTP (1.7 - 2.0 µCi/µl). Reaction ran 20 min at 25 °C and was terminated by the addition of 900 µl of active coal to absorb nucleotides. Mixtures with active coal were centrifuged for 7 min at 4 °C at 13000 rpm. Six hundreds microlitres of the supernatant fluid were removed and $^{33}P_i$ was determined by liquid scintillation counting. The experimental conditions chosen ensured that no more than 10 % of total amount of [γ-^{33}P]GTP added was converted to $^{33}P_i$. Sigmoid concentration-response curves of agonists and inverse agonists were analyzed by nonlinear regression using the built-in function in the GpaphPadPrism program.

GTPase assay have been performed for each mutant in three repetitions.

5.2 Results

5.2.1 Site-directed mutagenesis

The given mutations were prepared through the sequential overlap-extension PCRs according to suggestions based on molecular modelling studies (Igel et al. 2009). We obtained three point mutants of the hH_4R:

$hH_4R \rightarrow hH_4R$-R341**A**

Arginine-341 in the hH_4R was replaced by the conserved amino acid alanine according to the suggestion how this exchange may by involved in the interaction of the hH_4R with cyanoguanidine-type agonists.

$hH_4R \rightarrow hH_4R$-R341**S**

Arginine-341 in the hH_4R was replaced by serine. In the mouse and rat H_4R serine is placed instead of arginine at the position 341 and therefore the mutant was the interest of study the species selectivity of cyanoguanidine-type agonists.

$hH_4R \rightarrow hH_4R$-R341**E**

Arginine-341 in the hH_4R was replaced by glutamate. Glutamate is present in the dog H_4R and in the hH_3R and therefore the mutant was the interest of study the species selectivity and histamine receptor subtype selectivity of cyanoguanidine-type agonists.

All these mutants were successfully coexpressed with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ using Sf9 insect cell expression system and further detected by immunoblotting. The hH_4R or its three mutants were investigated in steady-state GTPase activity assay.

5.2.2 Protein determination – Lowry assay

Table 2. The protein concentration in membranes expressing the hH_4R or its mutants using Lowry method of protein determination

	Concentration [µg/ml]
hH_4R	1141
hH_4R-R341**A**	1139
hH_4R-R341**S**	1329
hH_4R-R341**E**	1183

5.2.3 Western blot analysis

Expression of the hH_4R, hH_4R-R341A, hH_4R-R341S or hH_4R-R341E was investigated by immunoblotting with M1 anti-Flag, $G\alpha_{i1/2}$ and Gβ common antibodies. In immunoblotting with M1 anti-Flag antibody we obtained bands of the hH_4R and receptor mutants located at ~45 kDa (Figure 15). Immunoblotting using $G\alpha_{i1/2}$ and Gβ common antiobodies also confirmed the expression of $G\alpha_{i2}$ and $G\beta_1\gamma_2$ proteins (Figures 16, 17).

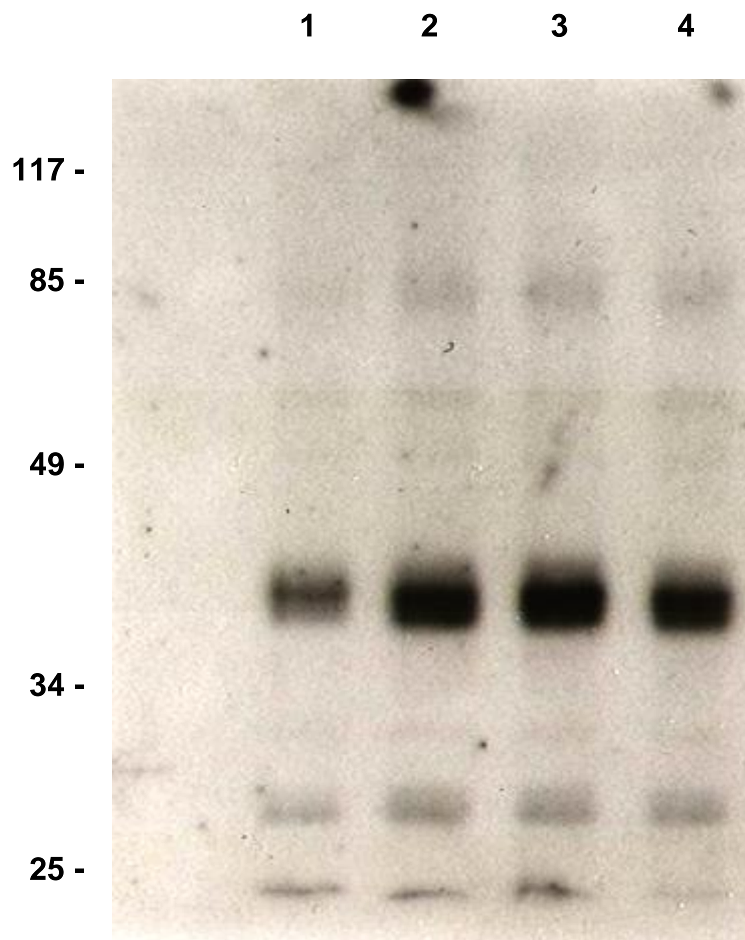

Figure 15. Western blot analysis of membranes expressing the hH_4R or its mutants with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ using M1 anti-Flag antibody. (1) hH_4R, (2) hH_4R-R341A, (3) hH_4R-R341E, (4) hH_4R-R341S. Numbers on the left side of the immunoblot indicate molecular masses of marker proteins in kDa.

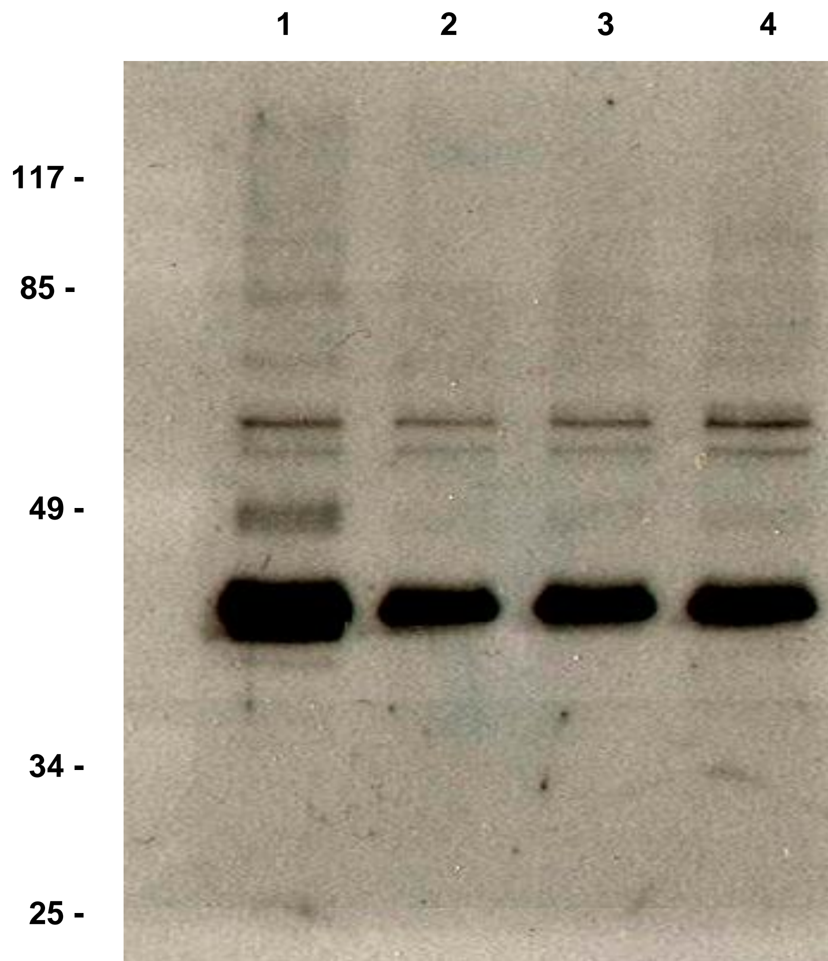

Figure 16. Western blot analysis of membranes expressing the hH_4R or its mutants with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ using $G\alpha_{i1/2}$ antibody. (1) hH_4R, (2) hH_4R-R341A, (3) hH_4R-R341S, (4) hH_4R-R341E. Numbers on the left side of the immunoblot indicate molecular masses of marker proteins in kDa.

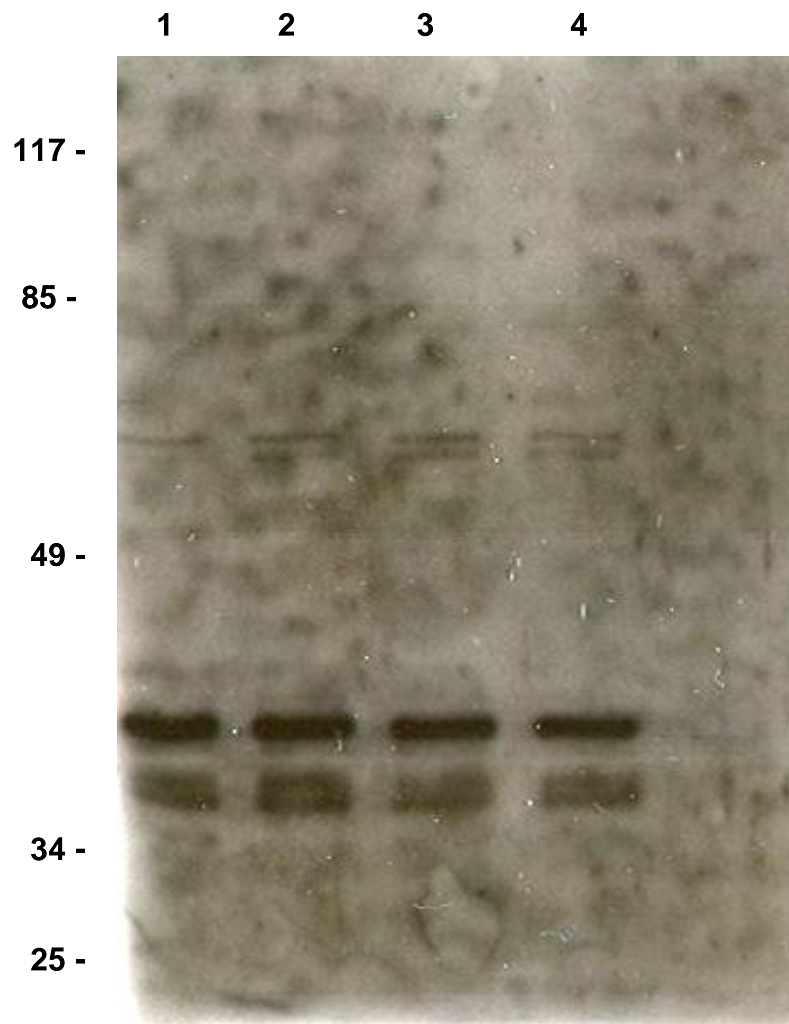

Figure 17. Western blot analysis of membranes expressing the hH_4R or its mutants with $G\alpha_{i2}$ and $G\beta_1\gamma_2$ using Gβ common antibody. (1) hH_4R, (2) hH_4R-R341A, (3) hH_4R-R341S, (4) hH_4R-R341E. Numbers on the left side of the immunoblot indicate molecular masses of marker proteins in kDa.

5.2.4 Steady-state GTPase activity assay

The potencies and efficacies of three ligands (histamine, UR-PI376 and thioperamide) at the wildtype and mutant H_4Rs were determined in steady-state GTPase activity assay. The efficacies of tested ligands were referred to the efficacy of the full agonist histamine (HIS). Efficacies and potencies of the selected ligands at the hH_4R can be compared with efficacies and potencies of the same ligands at three mutants. Comparing the hH_4R with hH_4R-R341A, hH_4R-R341S or hH_4R-R341E did not show differences in the GTPase activity by tested ligands (Table 3). Figures 18, 19 and 20 show representative concentration-response curves of HIS, UR-PI376 and THIO for each mutant. Efficacies and potencies of tested ligands at the hH_4R were kindly provided by Irena Brunskole, Department of Pharmaceutical/Medicinal Chemistry II, University Regensburg (unpublished data).

Table 3. Potencies (pEC_{50} ± SEM) and efficacies (E_{max} ± SEM) of tested ligands at the hH_4R or its point mutants in the GTPase activity assay

	Histamine*	
	pEC_{50}	**E_{max}**
HH_4R	7.60 ± 0.12	1.00
hH_4R-R341**A**	7.45 ± 0.19	1.00
hH_4R-R341**S**	7.52 ± 0.16	1.00
hH_4R-R341**E**	8.02 ± 0.48	1.00

	UR-PI376	
	pEC_{50}	**E_{max}**
HH_4R	7.33 ± 0.10	0.98 ± 0.04
hH_4R-R341**A**	7.20 ± 0.34	1.40 ± 0.20
hH_4R-R341**S**	7.53 ± 0.04	1.25 ± 0.20
hH_4R-R341**E**	7.43 ± 0.32	1.09 ± 0.13

Table 3. (continued)

	Thioperamide	
	pEC$_{50}$	**E$_{max}$**
hH$_4$R	6.87 ± 0.14	-0.87 ± 0.08
hH$_4$R-R341**A**	7.27 ± 0.09	-1.11 ± 0.08
hH$_4$R-R341**S**	7.07 ± 0.11	-1.08 ± 0.21
hH$_4$R-R341**E**	7.15 ± 0.21	-1.79 ± 0.01

* In steady-state GTPase activity assay the agonists, histamine and UR-PI376, and inverse agonist, thioperamide were investigated as described under *Methods and Conditions.* Reaction mixtures contained membranes (15 µl protein/tube) and ligands ranging from 1 nM to 10 µM. All data are presented as mean of three independent experiments in triplicate ± SEM. Agonist potencies were given as pEC$_{50}$ values (negative decadic logarithm of the molar concentration of the agonist causing 50 % of the maximal response). Maximal responses were expressed as E_{max} values. The E_{max} value of histamine was set to 1.00, *E*max values of other compounds were referred to this value.

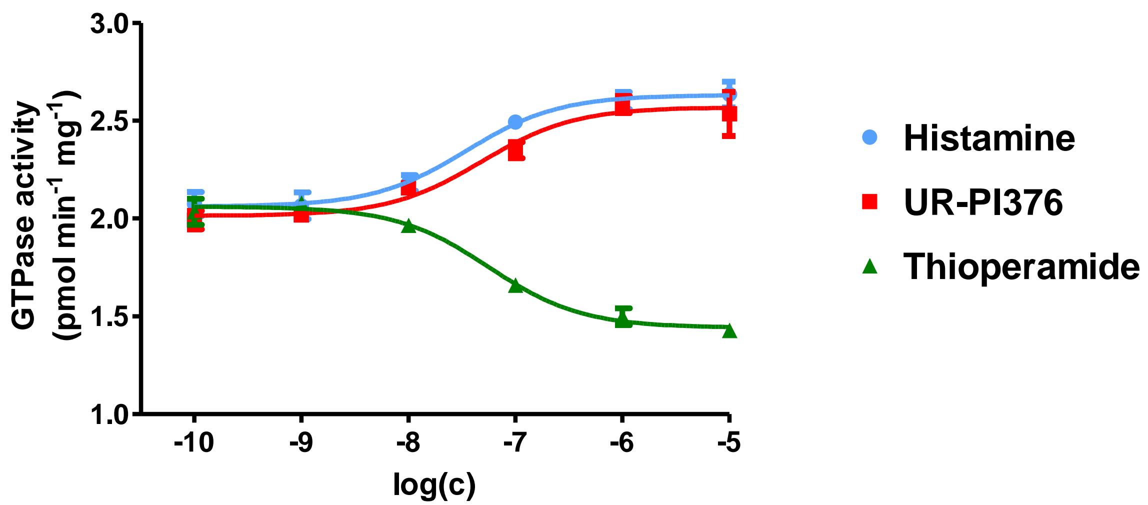

Figure 18. Concentration-response curves for hH_4R agonists, histamine (blue curve) and UR-PI376 (red curve), and inverse agonist, thioperamide (green curve) at hH_4R-R341A in steady-state GTPase activity assay.

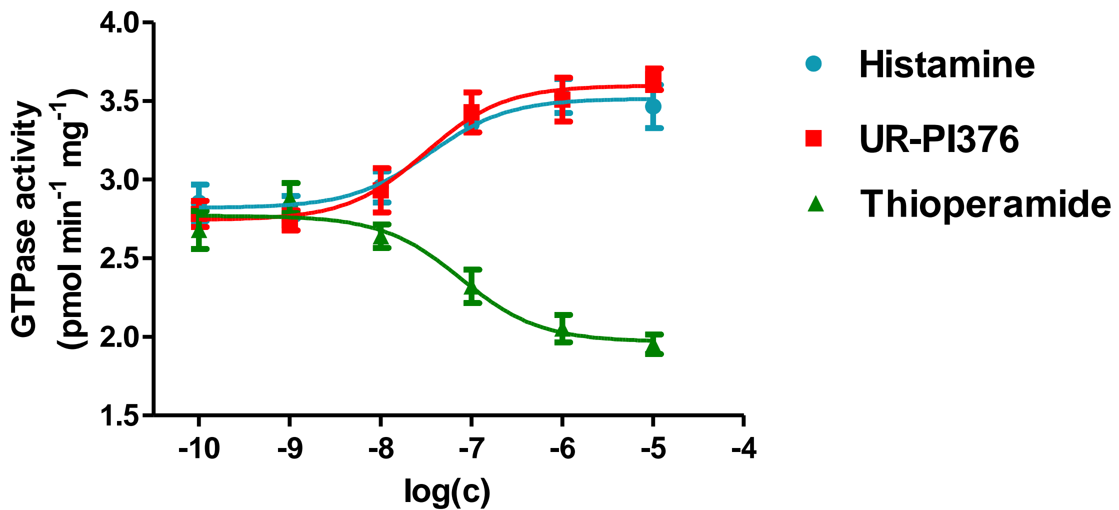

Figure 19. Concentration-response curves for hH_4R agonists, histamine (blue curve) and UR-PI376 (red curve), and inverse agonist, thioperamide (green curve) at hH_4R-R341S in steady-state GTPase activity assay.

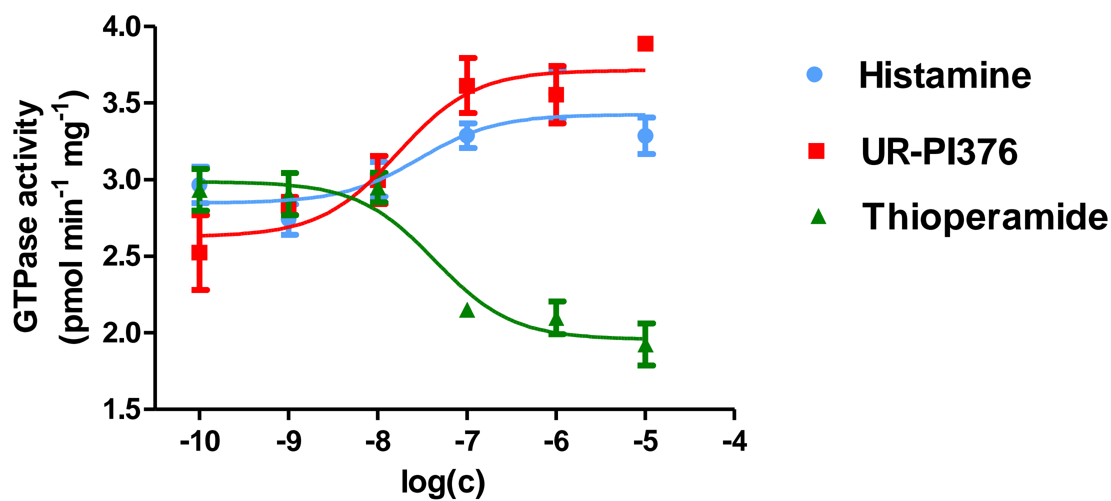

Figure 20. Concentration-response curves for hH_4R agonists, histamine (blue curve) and UR-PI376 (red curve), and inverse agonist, thioperamide (green curve) at hH_4R-R341E in steady-state GTPase activity assay.

5.3 Discussion

High affinity ligands for the human H_4R often show pronounced discrepancies in terms of potency (up to several orders of magnitude), selectivity (in particular H_4R versus the closely related H_3R) and even the quality of action (agonism vs. antagonism), when studied in a different species. This is one of the major problems with the currently known H_4R agonists. Therefore, in view of the relatively wide divergence in amino acid sequence among the various H_4R species orthologs (Figure 21), conclusions drawn from results of experiments in animals, for instance, performed to validate the target in a disease model in the mouse, may be misleading. However, the investigation of "natural mutants", i. e. the species variants, of a receptor may be very efficient to identify crucial amino acids for ligand binding, receptor activation and receptor subtype selectivity and species selectivity (Lim et al. 2010). Usually, such studies include modifications of the receptor proteins, e. g., preparation of chimeric GPCRs and site-directed mutagenesis, to verify the respective hypothesis.

Hm	100						
Mk	93	100					
Pg	70	70	100				
Dg	71	71	71	100			
Gp	62	64	61	61	100		
Rt	68	68	66	65	61	100	
Ms	67	66	65	66	62	85	100
	Hm	Mk	Pg	Dg	Gp	Rt	Ms

Figure 21. Percent homology of amino acid sequences of the histamine H_4-receptor among different species. Hm: human, Mk: monkey (*M. fascicularis*), Pg: pig, Dg: dog, Gp: guinea pig, Rt: rat; and Ms: mouse (Lim et al. 2010).

Although the homology of the human and mouse H_4R is only in the range of 67 % (Figure 22), investigations revealed that single amino acids play a major role in different agonist binding. Phenylalanine 169 in the second extracellular loop of the hH_4R was identified as a key amino acid, responsible for different agonist affinity between the human and mouse H_4Rs (Lim et al. 2008).

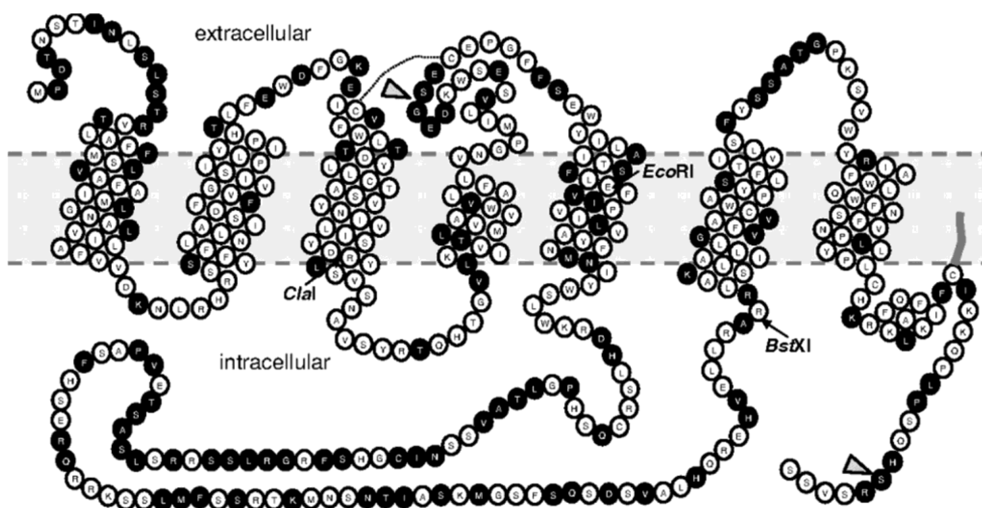

Figure 22. Snake plot of the human H_4R protein (Lim et al. 2008). The residues of the human H_4R identical with those of the mouse H_4R are demonstrated as open circles; filled circles indicate differences between two species homologues.

It may be assumed that imidazolylbutylcyanoguandines, bearing a space-filling substituent as in UR-PI376 (2-cyano-1-[4-(1*H*-imidazol-4-yl)butyl]-3-[(2-phenylthio)ethyl]guanidine), may adopt a similar orientation as histamine. The potent H_4R agonist UR-PI376 is devoid of agonistic activities at the other histamine receptors including the hH_3R (Igel et al. 2009) and is therefore one of the most potent and selective H_4R agonists known so far. In addition, UR-PI376 is up to 100 times less potent at the murine H_4R (P. Igel, personal communication). This phenomenon stimulated speculations about a potential role of Arg-341 in both, receptor subtype selectivity and species selectivity and gave rise to molecular modelling studies, suggesting the binding mode shown in Figure 24.

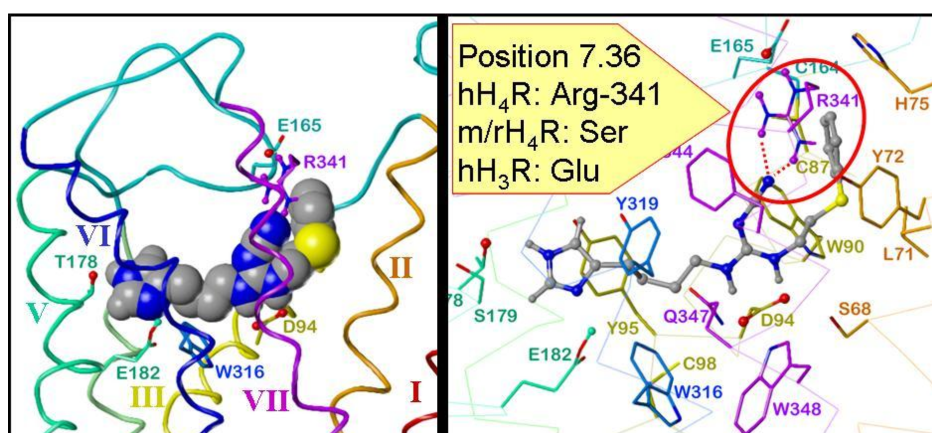

Figure 24. Suggested binding mode of UR-PI376 in the wildtype hH_4R, possible role of Arg-341 in position 7.36 of the H_4R and (highlighted in arrow) corresponding amino acid in mouse/rat H_4R and human H_3R, respectively (modified from Igel et al. 2009; graphic provided by Prof. Dr. A. Buschauer). The cyanoguanidine moiety of UR-PI376 is suggested to form forms two charge-assisted hydrogen

bonds with the carboxylate oxygens of Asp-94 and two additional charge-assisted hydrogen bonds with the guanidine group of Arg-341.

In the present diploma work the Arg-341 residue present in the hH_4R was replaced by site-directed mutagenesis with Ser, which is found in that position in rat and mouse H_4Rs, with Glu, which is the corresponding amino acid in the cH_4R and the hH_3R, and with Ala. The receptors were expressed in Sf9 cells and functionally investigated in GTPase activity assays. The expression of the hH_4R was detected with M1 anti-Flag antibody (Figure 15). For nonfused hH_4R two bands have already been reported. A relatively weak band exhibited the molecular mass ~46 kDa and a very intense band was located at ~43 kDa. The bands at 30 - 35 and 26 kDa were described as probably atypically migrating H_4R species (Schneider et al. 2008). $G\alpha_{i2}$ protein was detected with $G\alpha_{i1/2}$ antibody (Figure 16). We also detected $G\beta_1\gamma_2$ protein with Gβ common antibody (Figure 17).
UR-PI376 is a potent agonist (pEC_{50} = 7.47) with almost full intrinsic activity (E_{max} = 0.93) at the hH_4R in the steady-state GTPase activity assay in Sf9 insect cell membranes expressing the hH_4R-RGS19 fusion protein plus $G\alpha_{i2}$ plus $G\beta_1\gamma_2$ (Igel et al. 2009). By contrast, the GTPase assays performed with membrane preparations of H_4R mutants expressing Sf9 cells revealed no relevant difference compared to the data obtained with wildtype hH_4R. This was true in view of the potencies and intrinsic activities of the agonists, histamine and UR-PI376, as well as for the antagonist/inverse agonist thioperamide, although, according to the suggested model, Arg-341 was expected to play a different role in the interactions of these ligands with the H_4R. Therefore, the results of the investigations performed in the diploma work do not support the hypothesis that charge-assisted hydrogen bonds between the cyanoguanidine group and Arg-341 (see Figure 13) are involved in the differential binding of this class of H_4R agonists. Very recently, molecular determinants of agonist and antagonist binding to H_4R species variants were suggested by Lim et al. (Lim et al. 2010) on the basis of pharmacological studies using chimeric receptors and site-directed mutagenesis. Although cyanoguanidine-type agonists were not included in this study, future investigations should take into account residues in positions 4.57 and 5.39 as well as Phe-169 in the second extracellular loop as suggested previously (Lim et al. 2008).

6 SUMMARY AND CONCLUSION

Pharmacological data of cyanoguanidine-type H_4R agonists and molecular modelling studies of the hH_4R suggested Arg-341 to be a key amino acid in terms of potency, receptors selectivity and species selectivity. Based on this model, the hH_4R was subjected to site-directed mutagenesis and the mutant receptors were pharmacologically characterized with the aim to test the working hypothesis. Arg-341 was replaced by serine (as in rat and mouse hH_4R), glutamate (as in dog hH_4R and human H_3R) and alanine. The synthesized mutant receptors were expressed using Sf9 insect cell system and recombinant proteins were determined in immunoblot analysis and steady-state GTPase activity assay. The potent and selective cyanoguanidine-type hH_4R agonist, UR-PI376, as well as histamine and the H_4R inverse agonist thioperamide were investigated at mutant versus wildtype H_4Rs.
Overlap-extension PCR was used to obtain the point mutations of the hH_4R. PCR products were then restricted with digestion enzymes and ligated with the new vector suitable for transformation in *E. coli*. Sequencing after Maxi preparation confirmed the correctness of the whole newly created plasmids. Plasmids pVL1392 were transfected into Sf9 insect cells and membranes were prepared for further detection. Protein determination using Lowry assay and Western blot analysis confirmed that expression in Sf9 insect cells was successfully done. The molecular masses of the hH_4R and receptor mutants were ~45 kDa.
In steady-state GTPase activity assay, efficacies and potencies of histamine, UR-PI376 and thioperamide did not differ between the hH_4R and its point mutants. These data can conclude that Arg-341 is neither the key amino acid in terms of histamine receptor subtype selectivity of UR-PI376 and related compounds nor a molecular determinant of ligand binding to H_4R species variants. It is obvious that further work should focus on other amino acids than Arg-341 to explain the differences in potency and selectivity of cyanoguanidine-type H_4R agonists. Suggestions, based on very recently published data collected from the investigation of a structurally distinct set of compounds on chimeric and point mutant receptors of a large variety of H_4R species variants should be considered. The identification of the molecular determinants of species-selective H_4R binding are very important with respect to the design of new drugs avoiding the problems with the currently known H_4R agonists in terms of target validation in animals.

7 SUMMARY IN CZECH

Histamin je důležitý biologický mediátor, který ovlivňuje nejrůznější fyziologické a patofyziologické pochody v těle prostřednictvím čtyř histaminových receptorů. Všechny tyto receptorové podtypy patří do třídy A nadrodiny receptorů spřažených s G-proteinem (GPCR). Klasická antihistaminika (dnes H_1 antagonisté, H_1R) našla uplatnění jako antialergika, zatímco antagonisté H_2-receptoru (H_2R) jsou účinní v terapii peptidického vředu a gastroesofageální refluxní choroby (GERD). Třetí podtyp histaminových receptorů je exprimován v převážné míře v centrální nervové soustavě (CNS) a jeho využití potenciálně spadá do oblasti léčby onemocnění CNS a obezity. Zatím však není dostatek informací o ligandech H_3-receptoru (H_3R), které by našly uplatnění v klinické praxi (Parsons and Ganellin 2006). V letech 2000 a 2001 byl objeven nezávisle několika vědeckými skupinami čtvrtý podtyp histaminový receptorů (H_4R), a to na základě vysoké homologie se třetím subtypem (Coge et al. 2001, Liu et al. 2001a, Morse et al. 2001, Nakamura et al. 2000, Nguyen et al. 2001, Oda et al. 2000, Zhu et al. 2001). H_4R je exprimován v buňkách imunitního systému (Leurs et al. 2009, Liu et al. 2001a, Morse et al. 2001, Oda et al. 2000, Parsons and Ganellin 2006, Zhu et al., 2001), což předpovídá jeho úlohu v léčbě zánětu a nemocí imunitního systému, např. astma bronchiale, revmatoidní artritidy, dny či alergické rýmy (Gbahou et al. 2006, Lim et al. 2006).
Tým Prof. Dr. A. Buschauera na Univerzitě Regensburg identifikoval lidské histaminové H_3R (hH_3R) a H_4R (hH_4R) komponenty v sérii N^G-acylovaných imidazopropylguanidinů, původně vyvinutých jako H_2R agonisté. Pomocí strukturálních modifikací s cílem zjistit selektivitu ke čtvrtému subtypu byl získán agonista cyanoguanidinového typu, UR-PI376, 2-cyano-1-[4-(1-*H*-imidazol-4-yl)butyl]-3-[(2-fenylthio)ethyl]guanidin. UR-PI376 je prototypem agonistů cyanoguanidinového typu v komplexu s hH_4R, kdy se tvoří vodíkové vazby mezi cyano skupinou a guanidinovým zbytkem aminokyseliny Arg-341. Na místě Arg-341 u H_4R myši a potkana se vyskytuje serin, u psa a u hH_3R se nachází glutamát (Igel et al. 2009). Naskytla se tedy otázka, jakou roli hraje Arg-341 v interakci s H_4R cyanoguanidinovým typem agonistů.

Pomocí polymerázové řetězové reakce (PCR) jsem vytvořili tři mutanty hH_4Ru, kde jsme Arg-341 nahradili serinem (Ser), glutamátem (Glu) a jako konzervativní aminokyselina byl zvolen alanin (Ala). PCR produkty jsme dále nastřihli restrikčními endonukleázami a ligovali s vektorem vhodným pro transformaci do buněk *E. Coli*. Sekvenování potvrdilo správnost nukleotidových sekvencí nově vzniklého plazmidu. Mutanty hH_4Ru jsme co-exprimovali s $G\alpha_{i2}$ and $G\beta_1\gamma_2$ proteiny prostřednictvím Sf9 hmyzích imunitních buněk a po membránové preparaci následovala měření proteinové koncentrace Lowryho metodou a zjištění relativní

molekulové hmotnosti mutantů (Western blot). Relativní molekulová hmotnost všech exprimovaných mutantů se pohybovala okolo ~ 45 kDa.

Vzájemné interakce hH_4R ligandů s mutanty hH_4R jsme testovali metodou „steady-state GTPase aktivity assay“. Do reakce jsme použili plného agonistu (histamin), agonistu cyanoguanidinového typu (UR-PI376) a inverzního agonistu (thioperamid). Účinnost, vyjádřená jako záporný logaritmus molární koncentrace agonisty, který způsobí 50 % maximální odpovědi (pEC_{50}), a jako maximální odpověď relativní k histaminu (E_{max}) byly srovnávány s účinností nezmutovaného hH_4R. Získaná data bohužel nepotvrdila, že by Arg-341 byl klíčovou aminokyselinou pro receptorovou a druhovou specifitu ligandy UR-PI376. Je zřejmé, že budoucí práce budou zaměřeny na jiné aminokyseliny, které by vysvětlily rozdíly v účinnosti a selektivitě H_4R agonistů cyanoguanidinového typu.

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