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Clara C. Blad

A QUEST FOR CONNECTIONS

Ligands for the HCA_2, adenosine A_3 and GPR88 receptors

A quest for connections. Ligands for the HCA_2, adenosine A_3 and GPR88 receptors Clara Blad 2012

A quest for connections

ligands for the HCA_2, adenosine A_3 and GPR88 receptors

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ligands for the HCA_2, adenosine A_3 and GPR88 receptors

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Chapter 1

General introduction

G protein-coupled receptors in health and disease

G protein-coupled receptors (GPCRs), alternatively called 7-TM receptors (for 7 transmembrane domains), comprise a large family of eukaryotic membrane proteins. When a GPCR is activated, for example by a hormone or a neurotransmitter, it transfers the message to intracellular signaling cascades. The most well-known of these cascades involve G protein activation, but recently it has become clear that other possible signaling mechanisms exist. In the end, these GPCR signals mediate vital functions of the human body, such as perception of the world around us, communication between the brain and other parts of the body, energy storage or mobilization, movement and fertility.

Table 1. Examples of drugs acting on GPCRs. Adapted from [5].

GPCR	Drug	Indication
β_2 adrenergic	albuterol	asthma
angiotensin AT_1	losartan	hypertension
calcitonin	calcitonin	osteoporosis
dopamine D_2	haloperidol	schizophrenia
gonadotropin-releasing factor	goserelin	cancer
histamine H2	ranitidine	ulcer
serotonin 1D	sumatriptan	migraine
leukotriene	pranlukast	allergy, asthma
μ opioid	morphine	pain

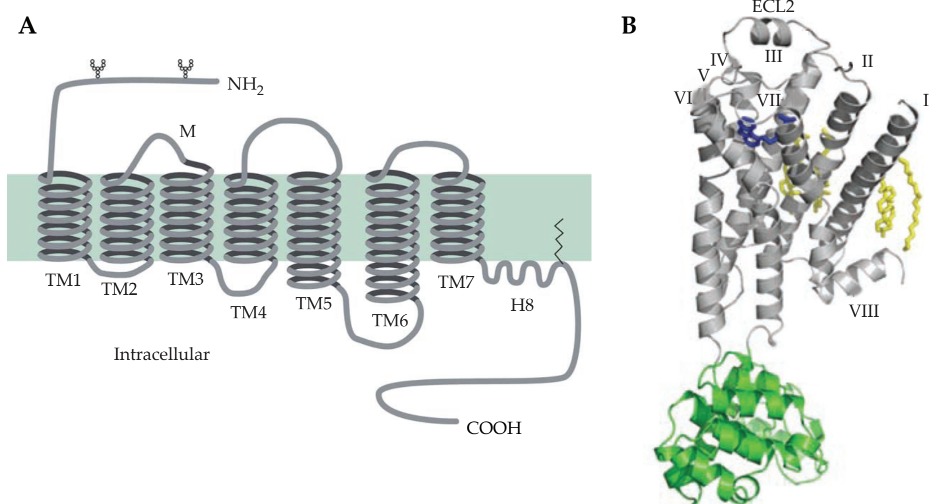

Figure 1. Common secondary structure of GPCRs. **A**: Schematic representation of a GPCR, indicating transmembrane domains 1-7 (TM1-7), the α-helical domain termed helix 8 (H8), a palmitoylation site next to it (zigzag line) and two potential glycosylation sites in the N-terminal tail (Y shapes). From [6]. **B**. High-resolution crystal structure of the β_2 adrenoceptor bound to carazolol (blue) together with the stabilizing T4 lysozyme protein (green) in place of the third intracellular loop. The helices that are shown next to each other in A form a barrel-like arrangement here, which is probably the form of the protein in vivo. The helices are indicated in roman numerals, including helix VIII in the C-terminal region. A small helix in extracellular loop 2 (ECL2), which may be typical for this receptor subtype, is also clearly visible. From [7].

A total of 799 verified human GPCRs are known, thus representing about 3% of all genes in our genome [1]. These receptors bind to a large variety of ligands including small organic compounds, lipids, peptides, proteins and even photons. Approximately 50% of the GPCR repertoire is dedicated to olfaction.

From a pharmacological point of view, GPCRs are interesting drug targets because they are implicated in many different pathophysiological processes. Furthermore, it is relatively straightforward to design molecules that interact with GPCRs and this type of drugs are successfully used in the clinic [2]. An estimated 30 to 40% of all available drugs target GPCRs, with annual sales of over 65 billion dollars [3]. Some examples are given in table 1. The variety of indications, for which these drugs are used, illustrates the omnipresence of GPCRs in the human body.

On a molecular level, all GPCRs have a common secondary structure. Their most striking features are the seven hydrophobic membrane-spanning alpha-helices (TM1-7) [4]. Extracellular and intracellular loops (EL and IL) connect the helices, and an extracellular N-terminal domain and intracellular C-terminus complete the protein (see figure 1A). The TM helices have been shown to form a barrel-like tertiary structure in the membrane, with TM1 and TM7 in close proximity (figure 1B).

GPCR classification

Several classifications have been proposed for the GPCR superfamily on the basis of primary structure, endogenous ligand specificity and species source. In a classical system by Kolakowski, the GPCRs are segregated into seven families or classes (A-F and O) [8]. A modern version of this system is used in the GPCRDB database www.gpcr.org [9], distinguishing the following classes: A: rhodopsin-like, B: secretin-like, C: metabotropic glutamate-like, D: pheromone receptors, E: cAMP receptors and F: frizzled/smoothened. After the human genome sequence became available, a slightly different system called GRAFS was proposed [10-12]. In this system, the classes are named Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin, plus a recent addition, Taste2. Thus, class B from the A-F system is divided in the Adhesion and Secretin classes, which reflects the inherent differences between these receptor clusters. Classes A (Rhodopsin), B (Secretin&Adhesion) and C (Glutamate) are most important in mammalian physiology. Of these, class A is by far the largest and most diverse. It contains 672 receptors, which includes 388 olfactory receptors [13]. The GPCRs that will be discussed in this thesis all belong to the class A, rhodopsin-like, GPCR family.

Properties of Rhodopsin-like GPCRs

Although the class A receptors are highly diverse in sequence and ligand binding properties, certain residues in the TM regions are conserved (see figure 2). Most striking are the microdomains D/ERY (TM3), CWxP (TM6) and NPxxY (TM7). Furthermore, two conserved cysteine residues are present in the extracellular domain, at the end of TM3 and in extracellular loop 2. They are thought to form a disulfide bridge in most class A GPCRs, which may be crucial to structural integrity and receptor function (see for example [14]).

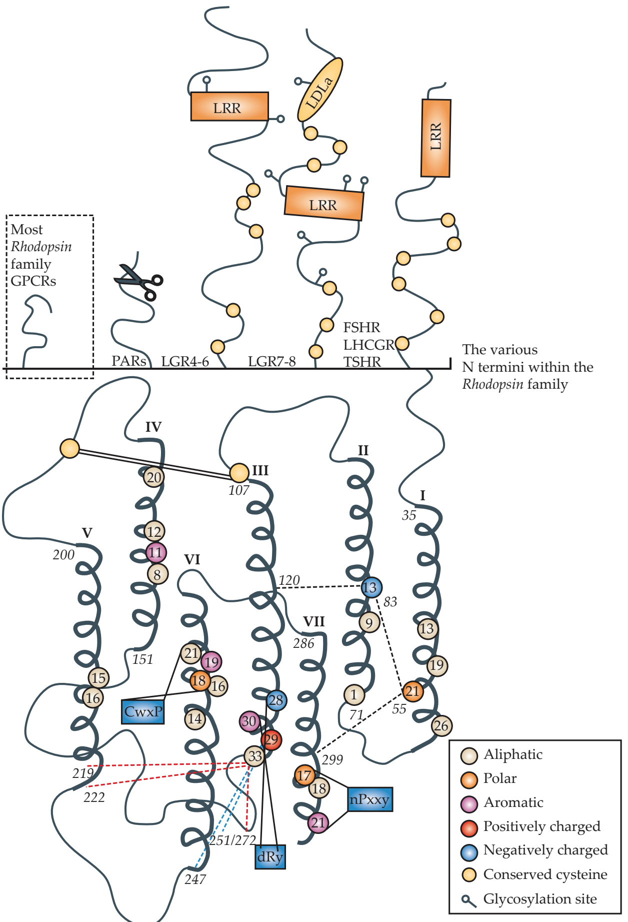

Figure 2. Conserved features and structural motifs within the Class A, rhodopsin-like, GPCRs. The lower part of the figure shows the conserved residues from eight diverse class A GPCRs. Conserved residues are shown as circles colour-coded for amino acid properties. In the blue boxes conserved class A motifs are indicated with uppercase letters for completely conserved positions, lowercase letters for well-conserved positions (>50%) and x for variable positions. Conserved cysteine residues are shown in yellow and the disulphide bridge between EL1 and EL2, which is conserved in most GPCRs, is indicated as two lines. Dashed black lines show hydrogen bonds within bovine rhodopsin whereas dashed blue lines (from DRY to 247 and 251) show the postulated ionic lock, which is thought to keep the receptor in the inactive state. Dashed red lines display Van der Waals interactions within the β_2-adrenoceptor model. In the upper part of the figure different N-termini of class A GPCRs are shown. The scissors indicate the cleavage site for the protease-activated receptors (PARs). From [13].

The position of the ligand binding site varies among class A receptors. Small ligands (such as biogenic amines, nucleosides, eicosanoids, lysophosphatidic acid and sphingosine-1-phosphate) bind in a so-called TM cavity, formed by the seven TM alpha helices ([15] and references therein). The ligands for the hydroxy-carboxylic acid receptors and the adenosine receptors, which are described in the present work, fall in this category. Larger ligands, such as peptides and glycoprotein hormones, interact with the N-terminus and/or the extracellular loops, and in some cases also with the outer portions of the transmembrane helices. Examples are oxytocin, vasopressin, opioids, thyroid-stimulating hormone (TSH) and follicle-stimulating hormone (FSH) [16].

High-resolution GPCR structures

Recently, some of the most important advances in the field have been made using X-ray crystallography. Structural analysis of GPCR molecules is very challenging and until recently, only the structure of rhodopsin was known [17-19]. In the last four years crystallization efforts finally paid off with the elucidation of high resolution crystal structures of squid rhodopsin, the β_2 adrenergic receptor (β_2AR), the β_1AR, the A_{2A} adenosine receptor (for a review see [20], see also figure 1), and most recently, the CXCR4 receptor [21], the dopamine D_3 receptor [22] and the histamine H_1 receptor [23]. These receptors were all stabilized in the inactive state, since this facilitates crystallization. For (rhod)opsin, the active apoprotein was also crystallized [24-25]. In 2011, active, transmitter-bound GPCRs have finally been crystallized, namely rhodopsin [26-27], the human β_2AR [28] and the adenosine A_{2A} receptor [29-30]. The newest structure of activated β_2AR is even complexed with a G_s protein [31]. This new (relative) wealth of structural information has increased our understanding of the structure of the specific GPCRs, the position of the ligand binding pockets and the orientation of the ligands in those pockets. The structures of the different receptors are remarkably similar, confirming that the secondary and tertiary structure of GPCRs is highly conserved, although there are, of course, subtle differences. For example, the binding pocket of the antagonist ZM241385 in the A_{2A} receptor was not as was anticipated on the basis of the β_2 adrenergic crystal structure with its antagonist carazolol. Therefore, care should be taken when homology models are used to predict the structure of a receptor that has not been crystallized yet. Comparison of active and inactive structures suggests that binding of an agonist results in conserved rearrangements near the binding site, which then propagate through the transmembrane domains to yield an active state. Binding of a G protein or substitute seems necessary to stabilize a fully active state [32].

Receptor activation leads to G protein signaling

GPCRs form an important connection between the outside and the inside of the mammalian cell. When an endogenous agonist like a hormone or a neurotransmitter binds to a GPCR, a conformational change occurs in the receptor protein, which commonly leads to the activation of a G protein (guanine nucleotide-binding protein) [33]. The G protein α subunit will release GDP from its GTPase catalytic site and bind GTP. This causes the G protein to dissociate from the GPCR, and divide into the α subunit and the $\beta\gamma$ complex. Both parts of the G protein can activate downstream effectors. The main Gα families are $G\alpha_s$, $G\alpha_{i/0}$ and $G\alpha_q$. $G\alpha_s$ stimulates adenylate cyclase, thus stimulating cAMP production, whereas $G\alpha_{i/0}$ has the opposite effect, inhibiting adenylyl cyclase. $G\alpha_q$ can stimulate phospholipase C, which causes an increase in cytoplasmic calcium concentrations. G$\beta\gamma$ dimers can activate phosphoinositide 3-kinases, which in turn activate other proteins, including MAP-kinases. Interestingly, these mitogen-activated protein kinases can also be activated by GPCRs via other, G protein-independent pathways [34]. When activated, they can influence gene expression. Other effects can also occur; the skin flushing side effect of drugs acting on the hydroxy-carboxylic acid receptor 2 (HCA_2) is possibly mediated by this pathway (see

chapter 2 and 3). The Gα subunit is deactivated when it converts GTP into GDP in its intrinsic GTPase domain. Gβγ dimers are deactivated when they bind a Gα subunit again.

The next step: internalization and arrestins

After a receptor has been activated, it is in many cases removed from the cell membrane. This internalization process is often (but not always) mediated by arrestins, which act as scaffold proteins that assemble a protein complex at the intracellular face of the receptor. Once inside the cell, the receptor protein can be broken down or recycled to the membrane after removal of the bound agonist [35-36]. Recruitment of arrestins to a receptor protein can also result in arrestin-mediated signaling, leading for example to the MAP-kinase activation mentioned above [37-38].

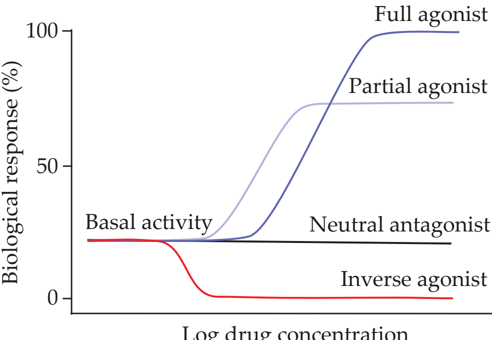

Figure 3. Dose-response curves of ligands with different efficacy. Many GPCRs also display some basal activity in absence of a ligand. From: [20].

Different ligands have different effects

Next to agonists, GPCR ligands exist that de-activate the receptor (inverse agonists), as well as ligands that block the binding site but do not change the receptor activation state (neutral antagonists) (figure 3). We can also distinguish full agonists, which fully activate the receptor, and partial agonists, which cannot cause full activation even at concentrations that fully occupy the receptor. This last type of ligands for the HCA_2 receptor is investigated in chapter 3. The existence of partial agonists suggests that GPCRs are not simply on-off switches. Rather, the receptor protein is now thought to exist in a range of conformational states. A bound ligand stabilizes a particular subset of states, and this determines which of all possible signaling pathways will be influenced, and also with which efficacy. In this more complex view of the receptor protein, a ligand is not simply an agonist, antagonist or inverse agonist, but can be, for example, an inverse agonist for the G_s pathway, and at the same time an agonist for the arrestin pathway [38-39]. In fact, this extreme example of 'biased signaling' has been demonstrated for the β_2-adrenoceptor, where propranolol functioned as an inverse agonist on the G_s protein and its downstream pathways, but as a partial agonist on (most likely arrestin-mediated) activation of extracellular signal-regulated kinase (ERK)1/2 [40]. Thus, one ligand binding to one receptor can have a whole spectrum of effects. A slightly different ligand may prompt the downstream cascades of

the same receptor in a different manner. These phenomena, termed ligand texture and ligand-directed signaling, have only recently been recognized [39]. Ligand bias on the HCA_2 receptor is discussed in chapters 2 and 3 of this thesis.

Manipulating downstream signaling

Understanding of the intracellular signaling cascades and how GPCRs stimulate them made it possible to bend these pathways to suit research purposes. For example, in immortalized cell lines expressing high levels of the chimeric G protein G_{qi5}, all GPCRs that normally interact with G_i proteins are forced to signal through the G_q pathway, leading to an easily detectable increase in intracellular calcium concentration [41-42]. G_{qi5} is identical to the G_q protein, except for five C-terminal residues which are exchanged for their G_i counterparts. These five amino acids determine the interaction with the receptor. Many variations of this strategy exist and are employed in the search for new GPCR ligands, often in a high-throughput screening setup where thousands of compounds can be tested per day. One example of such a screen is reported in chapter 7 of this work.

Allosteric modulators

A special class of GPCR ligands is formed by allosteric modulators. These compounds bind to the receptor at a site distinct from the so-called orthosteric binding site, where the endogenous ligand binds. Binding of the modulator can influence the conformation of the receptor and modify the affinity and/or efficacy of orthosteric ligands. Some allosteric modulators also have intrinsic efficacy themselves and can activate the receptor without binding of an orthosteric agonist. Allosteric modulators can be promising drug candidates since they may be more specific and have less side effects. Allosteric binding sites of synthetic ligands are, in principle, not evolutionarily conserved, which makes it less likely for an allosteric modulator to have affinity for related GPCRs [43]. Furthermore, an allosteric enhancer that makes the endogenous agonist more potent could boost the natural signal without constantly activating the receptor. This can be an important therapeutic advantage because it improves the timing and localization of receptor activation. Two allosteric GPCR modulators are currently on the market: the calcimimetic cinacalcet, which is a positive allosteric enhancer of Ca^{2+}-sensing receptors, and the anti-HIV drug maraviroc, an allosteric inhibitor of chemokine receptor CCR_5 [44]. In chapter 5 of this thesis a number of new allosteric modulators for the HCA_2 receptor are presented.

Orphan receptors and the quest for ligands

As mentioned above, a total of 799 human GPCRs have been identified, of which 369 are presumably non-sensory GPCRs (not involved in taste, vision or smell). Approximately 100 to 140 of these potential drug targets are still ‘orphans’, with no known endogenous ligand and in most cases no known function [1, 45].

Classic and reverse pharmacology

The first GPCRs that were purified and cloned in the 1980s, were receptors for known signaling molecules (adrenalin and noradrenalin) that had been studied for decades (for

a review see [46]). This approach, where receptors are identified to match the known signaling molecules, has later been termed the classic pharmacology approach (see figure 4, left scheme). After the first expression cloning of the β_2-adrenoceptor in 1986 [47], and the finding that this receptor probably shared the 7 transmembrane domain topology of rhodopsin, pioneers of GPCR research soon suspected that this feature was common to many receptors signaling through G proteins. The rapid homology cloning of many other GPCRs, including those for acetylcholine, serotonin and the neuropeptide substance K, confirmed their hypothesis [48-49]. Some of the receptors that were cloned did not have a known ligand, but in many cases they were readily paired to one of the many 'orphan' signaling molecules. However, in the 1990s the number of cloned receptors had vastly increased due to the advent of PCR techniques, and the number of known signaling molecules that were not yet coupled to a receptor was dwindling. This imbalance reversed the roles of transmitter and receptor in research: known orphan receptors were used to fish for novel signaling molecules, instead of the other way around. The term reverse pharmacology is used for this approach. In this thesis, it was applied on the orphan receptor GPR88 (chapter 7).

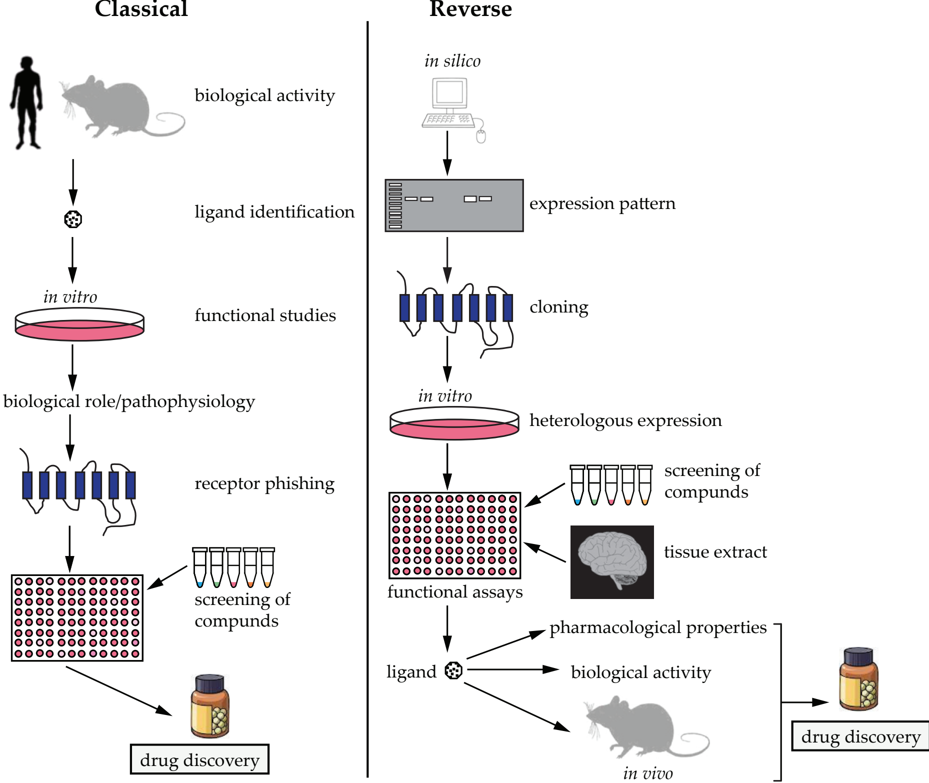

Figure 4. Flow charts of the classical pharmacology approach and the reverse pharmacology approach that arose in the 1990s, mostly due to advances in homology cloning. Adapted from [5, 50].

The differences between the classical approach and the reverse pharmacology approach are illustrated in figure 4. In reverse pharmacology, an orphan GPCR is first identified

by homology cloning using molecular biology techniques, or, since the sequence of the human genome is known, in silico. The GPCR of interest is then expressed in a heterologous cell system, followed by screening against a compound collection or purified tissue extracts for receptor activation. The use of tissue extracts increases the chances of finding the endogenous ligand. However, important technical difficulties are associated with the latter strategy, including low signal-to-noise ratio and failure to isolate the ligand from an active extract. Therefore, synthetic compound collections are often used instead. The first receptors that were deorphanized by reverse pharmacology were the 5-HT_{1A} serotonin receptor and the dopamine D_2 receptor [51-52]. In total, the application of the reverse pharmacology approach led to the 'deorphanization' of more than 150 GPCRs, coupling them to approximately 75 endogenous ligands. Many of these ligands were novel

Table 2. GPCRs listed as orphan receptors by IUPHAR with (putative) ligands identified from the literature.

Name	Pseudonyms	Ligand(s)	References
CCRL2	CRAM	CCL5 (RANTES), CCL19, chemerin	[53-56]
CMKRL1	ChemR23	chemerin, resolvin E1 (RvE1), SIV/HIV-1 coreceptor	[57-60]
CMKOR1	CXCR7, RDC1	CXCL12/SDF-1α	[61-62]
GPR1		chemerin; glucose/sucrose (S. cerevisiae), HIV/SIV coreceptor	[63-65]
GPR3		sphingosine 1-phosphate (S1P)	[66]
GPR4		protons, lysolipids (?)	[67-69]
GPR6		S1P (?)	[66, 70]
GPR12		tyrosol, S1P, sphingosylphosphorylcholine (SPC)	[66, 71-72]
GPR17		nucleotides, cysteinyl leukotriene (CysLT)	[73]
GPR18		N-arachidonoylglycine, Δ(9)-tetrahydrocannabinol	[70, 74-75]
GPR23	P2Y9, LPA4	lysophosphatidic acid (LPA)	[76]
GPR32		resolvin D1 (RvD1)	[77]
GPR34		lysophosphatidyl-L-serine	[78]
GPR35		kynurenic acid, 2-acyl lysophosphatidic acids	[79-80]
GPR37	PAEL	neuropeptide head activator	[81]
GPR39		Zn^{2+}	[82-83]
GPR55		lysophosphatidylinositol, cannabinoids	[84-85]
GPR63		S1P (?), dioleoylphosphatidic acid	[70, 86]
GPR65	TDAG8	protons, psychosine (?)	[87-88]
GPR68	OGR1	protons, sphingosylphosphorylcholine (?)	[68, 89-90]
GPR75		CCL5 (RANTES)	[91]
GPR84		medium-chain free fatty acids (FFA)	[92]
GPR92	GPR93, LPA5	LPA, farnesyl pyrophosphate, geranyl geranyl diphosphate	[93-96]
GPR119		oleoyl-lysophosphatidylcholine (OLPC), oleoylethanolamide (OEA), N-oleoyldopamine (OLDA)	[97-99]
GPR120		FFA	[100]
GPR132	G2A	protons, lysolipids (?), oxydized FFA (9-HODE, 11-HETE)	[101-103]
MAS1	Mas	Angiotensin 1-7, neuropeptide FF	[104-105]
MRGPRD		β-alanine	[106]
MrgprX1	MrgX1, SNSR4	BAM8-22, BAM22 (1-22) and related peptides	[107]
MrgprX2	MrgX2	corticostatin-14	[108]
OPN5	GPR99	photoreceptor (birds)	[109]
OXGR1	GPR99	α-ketoglutarate (2-oxoglutarate)	[110]
SUCNR1	GPR91	succinate	[110]

and unexpected signaling molecules, including calcium ions, trace amines, bile acids, kynurenic acid, protons, oleoylethanolamide, lysophosphatidylinositol, lysophosphatidic acid and a plethora of new neuropeptides.

Ligands recently proposed for class A orphans

In the receptor database of the International Union of Basic and Clinical Pharmacology (IUPHAR-db), 97 rhodopsin-like (class A) orphans are listed. However, a search of the recent literature yielded putative endogenous ligands for 33 of these receptors (see table 2). In some cases further studies may be needed, but we can assume that many of these receptors are now truly deorphanized. This shows that the deorphanization efforts still yield results. All but four of the new receptor-ligand pairs belong in 6 subgroups: proton/lysolipid (GPR4, GPR65, GPR68, G2A), chemokine (CCRL2, CMKRL1, CMKOR1, GPR1, GPR75), lipid mediators (GPR3, GPR6, GPR12, GPR23, GPR32, GPR34, GPR63, GPR92, GPR119), cannabinoid (GPR18, GPR55), Mas related (Mas, MRGPRD, MrgprX1, MrgprX2), and metabolic intermediates (GPR84, OXGR1, SUCNR1, GPR120). A number of these receptors seem to have more than one endogenous ligand. One could state that these receptors were not truly deorphanized when only one of the ligands was identified. Of course, it is impossible to prove that no additional ligands remain to be discovered for any given receptor. In chapter 6 of the current work, a new, possibly endogenous, ligand for the adenosine A_3 receptor is reported.

Deorphanized receptors and the regulation of food intake

Clearly, deorphanization of GPCRs has had a profound influence on our understanding of mammalian physiology. For example, particularly large advances have been made in our understanding of the regulation of food intake. Several novel neuropeptides have been discovered as ligands for orphan GPCRs, including leptin, ghrelin and orexin [for a review see 45]. Furthermore, several GPCRs have been shown to react to nutrients and metabolic intermediates. These receptors seem to function as sensors for food and metabolic status, which is an unexpected new role for GPCRs. Nutrient sensing receptors include the calcium sensing receptor, GPRC6A and the dimeric taste receptor complex T1R1/T1R3, which are promiscuous receptors for several L-α-amino acids and divalent cations, as well as the T1R2/T1R3 dimer, which responds to sugars and D-amino acids [111]. These receptors are expressed in taste tissue, the gastrointestinal tract, endocrine glands, adipose tissue, and/or kidney, where they regulate the release of hormones important for metabolism and the regulation of food intake. They can also influence gene expression, for example yielding an increase in the expression of nutrient transporters. Next to these class C receptors, several class A receptors are also involved in nutrient sensing. GPR92/93 expressed in the small intestine is activated by proteolytic degradation products and by lysophosphatidic acid [93-96]. Medium- and long-chain free fatty acids, from the hydrolysis of ingested fat and oil, activate the free fatty acid receptor 1 (FFA1) as well as GPR84 and GPR120 [92, 100, 112-114]. FFA1 is expressed in the islets of Langerhans in the pancreas and the gut, whereas GPR120 is expressed in the gut, in adipose tissue and in the lung. FFA1 activation stimulates the release of the hormone GLP-1 from the gut, a role which may be shared

by GPR120. Activated FFA1 also potentiates glucose-stimulated insulin release from the pancreas, and it might be involved in the toxic effect of chronic high circulating free fatty acid levels on pancreatic β cells [115]. GPR84 is expressed in immune cells and its role in physiology has not yet been elucidated. Short-chain free fatty acids, which are mainly fermentation products of carbohydrate fibers, are ligands of the FFA2 and FFA3 receptors [116-117]. FFA2 is primarily expressed in immune cells, and has been shown to play an important role in neutrophil recruitment during intestinal inflammation [118], and it is thought to be involved in leukocyte chemotaxis by sensing the presence of bacterial fermentation products. Additionally, it appears to be involved in energy homeostasis and appetite regulation [119], including the mediation of the anti-lipolytic effect of acetate and proprionate [120]. FFA3 is more widely expressed than FFA2, with the highest expression in adipose tissue, and more moderate levels in immune cells and tissues. It has been suggested that FFA3 mediates the effect of proprionate on leptin release [121]. Next to nutrients, intermediates in (energy) metabolism have also been identified as GPCR ligands, some of which were mentioned above (see table 2). More specifically, succinate and α-ketoglutarate (2-oxoglutarate), two intermediates from the citric acid cycle, are the endogenous ligands of GPR91 and GPR99, respectively, and the receptors are now named SUCNR1 and OXGR1 [110]. These receptors are predominantly expressed in the kidney. Succinate stimulates renin release via GPR91, which causes an increase in blood pressure. Intermediates of the β-oxidation process, which is upstream of the citric acid cycle, activate HCA_3 (GPR109B) [122]. The main ligand seems to be 3-hydroxy-octanoic acid. The same receptor has also been reported as a receptor for aromatic D-amino acids [123]. Two related receptors, HCA_2 (GPR109A) and HCA_1 (GPR81), are activated by the ketone body 3-hydroxybutyrate and by lactate, respectively [124-126]. Butyrate, a short-chain free fatty acid, may be an additional ligand for HCA_2 in the gut [127]. All three hydroxy-carboxylic acid receptors have an anti-lipolytic effect when activated. HCA_2 and HCA_3 are part of a negative feedback loop which keeps the release of fat stores in check under starvation conditions, whereas HCA_1 plays a role in the antilipolytic effect of insulin [review 128]. This family of hydroxy-carboxylic acid receptors is discussed in detail in chapter 2, and HCA_2 is the focus of chapters 3, 4 and 5.

Current challenges in deorphanization

After a peak in 2003 the deorphanization rate seems to be declining (see figure 5) [129]. This may be partly due to a shift in focus in the pharmaceutical industry. Many companies are currently faced with expiring patents of major blockbusters and increasing rules and regulations regarding safety, resulting in increased numbers of leads that fail to reach the market. The cost of compound screens for deorphanization is high and success not assured. Even if a ligand can be linked to an orphan receptor many years of additional R&D are needed to put a drug on the market. Less costly R&D programs could target the approximately 175 non-orphan GPCRs that are not currently targeted by drugs. Novel ligands, including allosteric modulators, or optimized versions of known ligands, can also be developed for the ~50 current GPCR drug targets. In the present climate pharmaceutical companies may more likely choose these R&D strategies.

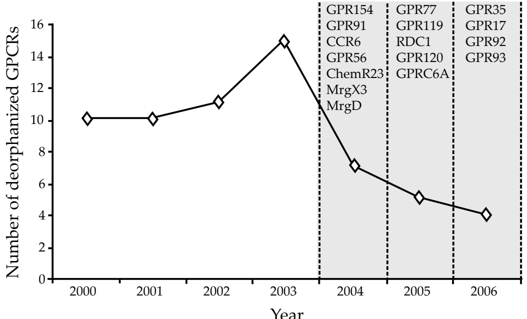

Figure 5. Declining rate of deorphanization since 2004. Adapted from [129].

Furthermore, a number of challenges complicate the identification of ligands for the remaining orphan GPCRs. Many of the remaining orphan GPCRs do not show high sequence homology to a cluster of GPCRs with similar ligands. When orphans are phylogenetically localized between two receptor subfamilies it may mean they bind ligands that share properties of both ligand families. Some other orphans, such as GPR88, do not show any significant homology to a known receptor subfamily. Purification of a known ligand type from tissue extracts is already a highly challenging task, and if the ligand properties are totally unknown another degree of complexity is added.

Why did traditional screening methods fail to bring to light the ligands for the remaining orphan GPCRs? It is possible that some of these orphans display non-traditional signaling, for example through G protein-independent signaling cascades. Traditional assays are

Table 3. Ligand-independent functions of orphan GPCRs. Adapted from [130].

GPCR	Orphan 7TM protein	Effect on function
$GABA_{B1}$	$GABA_{B2}$	Export to the cell surface and G protein coupling
DOR22a	DOR83b	Export and improvement of functionality
DOR43a	DOR83b	Export and improvement of functionality
T1R1	T1R3	Effect on receptor functionality and pharmacology
T1R2	T1R3	Effect on receptor functionality and pharmacology
MrgD	MrgE	Decrease of internalization; increase of ERK phosphorylation and intracellular [Ca^{2+}]
MT_1	GPR50	Loss of ligand binding and function
	ORF74	Constitutive activity responsible for oncogenic action of Herpesvirus 8
	UL33	Constitutive activity responsible for HCMV-related pathologies
	EBI2	Constitutive activity in Epstein-Barr virus-infected cells

DOR: *Drosophila* odorant receptors; EBI2: Epstein-Barr virus-induced receptor 2; ERK: extracellular signal-regulated kinase; HCMV: human cytomegalovirus; Mrg: Mas related gene; MT_1: melatonin receptor 1; Smo: Smoothened; T1R: taste receptor.

not equipped to identify this activation. The orphans may also need unknown interacting partners in order to function, for example another GPCR to form a functional heterodimer. In heterologous cell systems correct expression of functional GPCRs cannot always be obtained due to the absence of necessary protein partners.

Finally, some of the orphans may be 'real' orphans with no physiological ligand at all,

Table 4. Remaining class A orphan GPCRs from IUPHAR-db excluding the putatively liganded GPCRs shown in table 2. The receptors are classified according to a phylogenetic analysis by Joost and Methner [143]. The receptors at the bottom of the table were not included in this analysis.

Group	Orphan	Pseudonyms	Remarks
A03	GPR15	GPRF	
	GPR25		
A06	GPR22	GPRM	constitutive activity [144]
A08	MAS1L		
	MRGPRE		
	MRGPRF		
	MRGPRG	GPR169	
	MRGPRX3	SNSR1/2	
	MRGPRX4		
A09	GPR19	GPRJ	
	GPR50		
A10	LGR4	GPR48	
	LGR5	GPR49, GPR67	
	LGR6		
A11	GPR31	GPRV	
	GPR42		
	GPR82		
A12	GPR34	GPRy	
	GPR87		
A15	EBI2	GPR183	constitutive activity [140]
	P2RY5		
	P2RY8		
	P2RY10		
	GPR21	GPRL	
A18	GPR27	SREB1	
	GPR45		
	GPR52		
	GPR61		constitutive activity [145]
	GPR62		
	GPR78		constitutive activity [146]
	GPR85	SREB2	
	GPR88		
	GPR101		
	GPR173	SREB3	
	GPR20		constitutive activity [147]
	GPR26		constitutive activity [146]
	GPR33		pseudogene in most individuals [148]
	GPR37L1		
	GPR79		probable pseudogene [149]
	GPR83		
	GPR135		
	GPR139		
	GPR1		
	GPR142		
	GPR146		
	GPR148		
	GPR149		
	GPR150		
	GPR151	GalR4, GalRL	
	GPR152		
	GPR153		
	GPR160		
	GPR161		
	GPR162		
	GPR171		
	GPR174		
	GPR182		
	OPN3		
	TAAR2	GPR58	
	TAAR3		probable pseudogene in primates [150]
	TAAR4		probable pseudogene in primates [150]
	TAAR5		
	TAAR6		
	TAAR8		
	TAAR9		

that exert their function(s) through ligand-independent mechanisms. Some examples of orphan GPCRs with known ligand-independent activity are summarized in table 3 [130]. The top four 'orphan 7TM proteins' (indeed, they may not be receptors, nor G protein-coupled) in the table interact with the listed non-orphan GPCR to help it reach the cell surface (GABA, DOR) [131-133], change its functionality and/or signalling (DOR, T1R, Mrg) [131-132, 134-137], or inhibit its function (MT_1/GPR50) [138]. The bottom three orphans, all virus-encoded, do not need a ligand to function because they are active in the absence of a ligand [139-142]. Evolutionary analysis showing the presence or absence of evolutionary pressure on the protein as a whole, or on the predicted binding site, can be used to determine whether the protein is likely to be functional and whether it is likely to interact with a ligand in order to function.

Inventarisation of Class A orphan GPCRs

In table 4, all remaining class A (Rhodopsin-like) orphan receptors are shown, taken from the IUPHAR-db but not including the receptors listed in table 2. Some of the receptors display constitutive activity, which may indicate that these proteins do not need ligands to function. Others are likely to be pseudogenes in some or all individuals. Phylogenetic analysis showing sequence similarities between orphan and liganded receptors can give valuable clues where to start the quest for a ligand. In table 4 the orphan GPCRs are classified according to an analysis by Joost and Methner [143]. Another useful method to classify orphan receptors is using phylogenetic analysis of the residues predicted to line the ligand binding cavity, as was done by Surgand and colleagues [15]. This method yielded surprising results in the case of GPR88, which belongs to the Rhodopsin-like receptors on the basis of its full sequence, but was classified with the Glutamate-like receptors (class C) in this analysis.
Deorphanization of the 65 orphan GPCRs in table 4, and/or elucidation of their biological functions, could have a great impact on our understanding of mammalian physiology. Furthermore, keeping in mind that drugs on the market today target only ~50 GPCRs, understanding of these orphans may give rise to a whole range of new medicines.

Objectives and overview of this thesis

In the current work deorphanization and receptor-ligand pairing are a leading theme. I will describe studies on three GPCRs: the hydroxy-carboxylic acid receptor 2 (HCA_2), the orphan receptor GPR88 and the adenosine A_3 receptor (A_3R).
HCA_2 is a recently deorphanized GPCR that is of great interest as a drug target. In fact, one of its ligands, nicotinic acid, has been used as an anti-dyslipidemia drug for over 50 years. As described above, the endogenous ligand of HCA_2 is 3-hydroxybutyrate, which acts as a negative feedback signal to preserve fat tissue during times of starvation. The biological and pharmacological roles of the HCA_2 receptor and its two close family members HCA_1 and HCA_3 are reviewed in chapter 2. In my research, I explored the signaling cascades that are influenced upon HCA_2 activation by synthetic and endogenous agonists (chapter 3). In the same chapter I describe the in vivo effects of two partial agonists for HCA_2.

Furthermore, I investigated the binding of synthetic compounds to HCA_2 both in an orthosteric and in an allosteric manner (chapter 4 and 5).

As I have mentioned above, a receptor does not necessarily have only one unique ligand. For the adenosine A_3 receptor, there are indications that it binds an additional ligand next to its canonical agonist adenosine. This ligand could be involved in the resistance of muscle tissue to tumor metastases. I attempted to identify this elusive ligand, and identified the antiproliferative compound N^6-isopentenyl adenosine as an A_3R ligand that may or may not be endogenous (chapter 6).

GPR88 is an orphan GPCR expressed predominantly in two brain regions: the striatum and the central extended amygdala. GPR88 could be of major interest therapeutically, and I set out to identify a synthetic, if not endogenous, agonist for this receptor, screening a large number of compounds in a functional assay (chapter 7).

In this thesis, a recently discovered and deorphanized receptor is further investigated, expanding the ligand repertoire for two binding sites on the receptor; a new ligand is proposed for a GPCR long since paired to its ligand; and a ligand screen on an orphan GPCR is described, clearly identifying the challenges of such an operation. This research into the 'simple' interaction between small molecules and membrane proteins gives insights into human physiology and the mechanism of action of (future) drugs, and opens new horizons for pharmacotherapy.

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Chapter 2

Biological and pharmacological roles of hydroxy-carboxylic acid receptors

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CHAPTER 2

Abstract

The hydroxy-carboxylic acid (HCA) receptors HCA_1, HCA_2 and HCA_3 were previously known as GPR81, GPR109A and GPR109B, respectively, or as the nicotinic acid receptor family. They form a cluster of G protein-coupled receptors with high sequence homology. Recently, intermediates of energy metabolism, all hydroxyl-carboxylic acids, have been reported as endogenous ligands for each of these receptors. The HCA receptors are predominantly expressed on adipocytes and mediate the inhibition of lipolysis by coupling to G_i–type proteins. HCA_1 is activated by lactate, HCA_2 by the ketone body 3-hydroxy-butyrate and HCA_3 by hydroxylated β-oxidation intermediates, especially 3-hydroxy-ocatanoic acid. Both HCA_2 and HCA_3 are part of a negative feedback loop which keeps the release of fat stores in check under starvation conditions, whereas HCA_1 plays a role in the antilipolytic (fat conserving) effect of insulin.
HCA_2 was first discovered as the molecular target of the anti-dyslipidemic drug nicotinic acid (or niacin). Many synthetic agonists have since been designed for HCA_2 and HCA_3, but the development of a new, improved HCA-targeted drug has not been successful so far, despite a number of clinical studies. Recently, it has been shown that the major side-effect of nicotinic acid, skin flushing, is mediated by HCA_2 receptors on keratinocytes, as well as on Langerhans cells in the skin. In this chapter, we summarize the latest developments in the field of HCA receptor research, with emphasis on (patho)physiology, receptor pharmacology, major ligand classes and the therapeutic potential of HCA ligands.

List of non-standard abbreviations

5-HpETE, 5-hydroperoxy-eicosatetraenoic acid; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; BAC, bacterial artificial chromosome; CETP, cholesterol ester transfer protein; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; $CysLT_2$, cysteinyl-leukotriene 2; DP_1, prostaglandin D_2 receptor 1; EC_{50}, half-maximal effective concentration; EL, extracellular loop; ER, extended release; ERK, extracellular signal-related kinase; GTPγS, guanosine 5'-O-[gamma-thio]triphosphate; HCA, hydroxy-carboxylic acid; HDL, high density lipoprotein; IFN-γ, interferon-γ; IL, intracellular loop; LDL, low density lipoprotein; LTD_4, leukotriene D_4; MEF, monoethyl ester of fumaric acid; MMF, monomethyl ester of fumaric acid; PGD_2; prostaglandin D_2; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; PPARγ; peroxisome proliferator-activated receptor γ; PUMA-G, protein up-regulated in macrophages by INF-γ (murine HCA_2); TM, transmembrane domain; VLDL, very low density lipoprotein.

I. Introduction

In the past twenty years it has become clear that GPCR ligands include not only traditional hormones and neurotransmitters, but also ions and other endogenous molecules. In this chapter, we focus on a recently discovered GPCR family with affinity for several intermediates of energy metabolism. These metabolite ligands are all hydroxy-carboxylic acids, hence the novel nomenclature for this receptor family: hydroxy-carboxylic acid receptors [1]. HCA_1 (GPR81) is endogenously activated by lactate, HCA_2 (GPR109A) by 3-hydroxy-butyrate and HCA_3 (GPR109B) by 3-hydroxylated β-oxidation intermediates, especially 3-hydroxy-octanoic acid [2-5]. All three receptors are predominantly expressed in adipose tissue, where they couple to G_i proteins [1]. Activation of the receptors has an antilipolytic effect. Discovery of the endogenous HCA ligands has increased our understanding of the (patho)physiological roles of their receptors, and opens new avenues for research and drug discovery.

Of the HCA family, HCA_2 is most extensively studied since it is the target of the anti-dyslipidemic drug nicotinic acid (or niacin) [6-8]. Nicotinic acid has been used since the 1950's [9] and is still the most efficacious drug approved to raise HDL cholesterol plasma levels [10]. In HCA_2 knockout mice, the antilipolytic and triglyceride lowering effects of nicotinic acid are abolished [8]. Skin flushing, the major nicotinic acid side-effect, was also shown to be receptor dependent [11]. Separating this side-effect from the therapeutic effects of HCA_2 ligands is one of the major challenges in this field. In addition, it will be important to analyze the potential of HCA_1 and HCA_3 as therapeutic targets. This review will summarize current knowledge on the pharmacology and physiology of HCA receptors as well as the recent development of new synthetic ligands of this receptor class.

II. Identification and characterization of HCA receptors

A. Cloning of HCA receptors

The HCA_3 receptor (GPR109B) was first cloned from a human monocyte cDNA library and identified as an orphan G protein-coupled receptor, HM74 [12].

Lee et al. [13] discovered the HCA_1 receptor (GPR81) as another orphan GPCR by BLAST analysis. The cDNA of the HCA_1 receptor was then cloned from a bacterial artificial chromosome (BAC) clone carrying a region of human chromosome 12. The HCA_1 receptor cDNA showed high homology [11] to the HCA_3 receptor (GPR109B/HM74), and the genes encoding HCA_1 and HCA_3 receptors were localized in close proximity on the same BAC clone. The HCA_2 receptor (GPR109A) was originally identified in murine macrophages upon stimulation of cells with interferon-γ (INF-γ) and called "protein up-regulated in macrophages by INF-γ" (PUMA-G) [14]. In 2003, the human and rat HCA_2 receptors were cloned and shown to be highly homologous to the murine orthologue PUMA-G [6, 7].

B. Sequence alignment and phylogenetic tree

The HCA_2 and the HCA_3 receptor are highly homologous as they share 95% sequence identity on the protein level. In fact, HCA_3 differs from HCA_2 in only 16 amino acids of

which 12 are non-conservative changes which are clustered around extracellular loops 1 and 2. In addition, the HCA_3 receptor has an extended C-terminus of 24 amino acids. The HCA_1 receptor has almost 50% sequence homology with both HCA_2 and HCA_3. Most notably, HCA_1, HCA_2 and HCA_3 receptors share a conserved arginine residue in the third transmembrane helix which is supposed to be critically involved in ligand binding (see Section VI). While HCA_1 and HCA_2 receptors are present in the genome of numerous mammalian species including humans and rodents, the HCA_3 receptor is exclusively found in humans and higher primates like chimpanzee. The HCA_3 receptor obviously evolved through a relatively recent gene duplication, as indicated by its tandem location with HCA_2 on human chromosome 12 and its high level of sequence identity to HCA_2.
Several single nucleotide polymorphisms in the coding regions of genes encoding HCA_2 and HCA_3 receptors have been described [15]. The effects of these mutations on the physiological or pharmacological functions of the respective receptors are unknown.

C. Deorphanization of HCA receptors

1. HCA_1 receptor

In two recent studies it was shown that lactate was able to activate the HCA_1 receptor with half-maximal effective concentrations of 1.3 and 4.8 mM, respectively [5]. Lactate was a specific agonist of HCA_1 as it did not activate the closely related receptors HCA_2 and HCA_3. Various other hydroxy-carboxylic acids structurally related to lactate had a strongly reduced potency or were inactive towards HCA_1. For instance, 2- and 4-hydroxy-butyrate were weak agonists with an EC_{50} value of 8.5 and 15 mM, respectively, whereas 3-hydroxy-butyrate was completely inactive. Interestingly, the physiologically relevant stereoisomer (S)-lactate was much more potent and efficacious than (R)-lactate. Given the fact that plasma levels of (S)-lactate can reach concentrations sufficient to activate HCA_1, it is conceivable that lactate would be a physiologically relevant endogenous ligand of the HCA_1 receptor.

2. HCA_2 receptor

In 2005, the ketone body 3-hydroxy-butyrate was described as an endogenous ligand of HCA_2, the receptor of the anti-dyslipidemic drug nicotinic acid [3]. Racemic 3-hydroxy-butyrate activated human and mouse HCA_2 receptor with an EC_{50} value of 0.7 and 0.8 mM, respectively. 3-hydroxy-butyrate was a specific agonist of HCA_2 as it was inactive on the closely related receptor HCA_3. Other ketone bodies like acetoacetate or acetone had no activity on HCA_2. Short and medium chain fatty acids like butyrate, hexanoate and octanoate were also weak agonists on mouse and human HCA_2 with EC_{50} values ranging from 0.13 to 1.6 mM. While under physiological conditions plasma concentrations of short chain fatty acids would be too low to activate HCA_2, plasma levels of ketone bodies like 3-hydroxy-butyrate can increase during fasting and reach levels sufficient to activate the receptor.

3. HCA_3 receptor

Despite the high homology of the HCA_3 receptor to the nicotinic acid and ketone body

receptor HCA_2, HCA_3 is not activated by nicotinic acid or 3-hydroxy-butyrate. Recently, 2- and 3-hydroxylated medium-chain fatty acids have been identified as endogenous ligands of the orphan HCA_3 receptor [4]. 2- and 3-hydroxy-octanoate were specific agonists of HCA_3 with EC_{50} values of 4 and 8 μM, respectively. Under certain conditions, which go along with increased fatty acid β-oxidation rates, plasma concentrations of 3-hydroxy-octanoate reach levels sufficient to activate the HCA_3 receptor [4, 16, 17].

Moreover, aromatic D-amino acids like D-phenylalanine or D-tryptophan were shown to specifically activate the HCA_3 receptor [18]. Given the extreme rare occurrence of D-amino acids, it is unclear whether the ability of aromatic D-amino acids to activate HCA_3 is of physiological or pathophysiological significance.

D. Novel nomenclature of HCA receptors

Table 1. HCA receptor nomenclature. The structures of 2-OH-propanoate, 3-OH-butyrate and 3-OH-octanoate are shown.

Receptor	Aliases	Naturally occurring ligands
HCA_1	GPR81, GPR104, TA-GPCR, LACR, FKSG80	2-OH-propanoate (lactate) $^-$O, O, OH
HCA_2	GPR109A, PUMA-G, HM74A, HM746, NIACR1	3-OH-butyrate $^-$O, O, OH
HCA_3	GPR109B, HM74, NIACR2	3-OH-octanoate $^-$O, O, OH 2-OH-octanoate, D-phenylalanine, D-tryptophan

In the past, various names were given to the receptors HCA_1 (GPR81), HCA_2 (GPR109A/HM74A/NIACR1) and HCA_3 (GPR109B/HM74/NIACR2) (see table 1). After the identification of HCA_2 (GPR109A) as the receptor of the anti-dyslipidemic drug nicotinic acid, HCA_1 (GPR81), HCA_2 (GPR109A) and HCA_3 (GPR109B) were often called the "nicotinic acid receptor family" or "niacin receptor family". This was, however, for two reasons misleading: firstly, nicotinic acid is unlikely to be the physiologically relevant ligand because its concentrations are too low to activate HCA_2 (GPR109A), and secondly, the two closely related receptors HCA_1 (GPR81) and HCA_3 (GPR109B) do not respond to nicotinic acid at reasonable concentrations and therefore are no nicotinic acid receptors. With the identification of hydroxy-carboxylic acids as the endogenous ligands of HCA_1, HCA_2 and HCA_3, the physiological and pathophysiological functions of these receptors could be clarified (see section IV). Based on sequence homology, ligand similarity and

their physiological role, HCA_1, HCA_2 and HCA_3 are now regarded as members of a novel subfamily of G protein-coupled receptors, the hydroxy-carboxylic acid (HCA) receptor family.

The orphan receptor GPR31 and the 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) receptor OXER1 are the receptors most closely related to HCA_1, HCA_2 and HCA_3 [19-21]. Interestingly, the arginine residue in transmembrane helix 3, which is conserved among HCA_1, HCA_2 and HCA_3 and which has been suggested to serve as a molecular anchor of the carboxylic group of HCA_1 receptor ligands, is also present in GPR31 and the 5-oxo-ETE receptor. OXER1 binds 5-oxo-ETE as well as, with lesser affinity, 5-hydroxy-eicosatetraenoic acid and 5-hydroperoxy-eicosatetraenoic acid (5-HpETE). Thus, OXER1, a receptor for polyunsaturated fatty acids with an oxo, hydroxy or hydroperoxy substitution in the 5-position may well be regarded as another member of the hydroxy-carboxylic acid receptor family.

III. Gene structure and tissue distribution

A. Gene structure

The genes encoding HCA_1, HCA_2 and HCA_3 receptors are tandemly located on human chromosome 12q24.31 and have likely evolved from gene duplication (see figure 1). In humans and other mammals which express HCA receptors the genes for HCA_1, HCA_2 and HCA_3 consist of each a single exon.

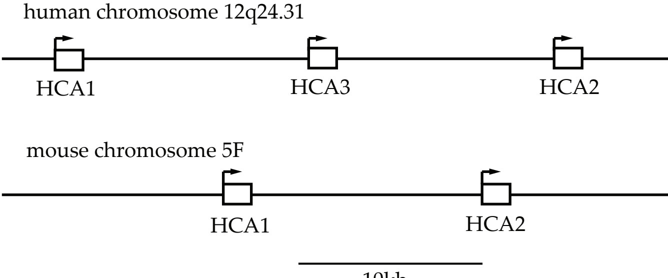

Figure 1. Gene structure of the HCA receptors. Schematic representation of the genomic organization of the genes encoding hydroxy carboxylic acid (HCA) receptors.

B. Tissue distribution

1. HCA_1 receptor

The HCA_1 receptor was originally reported to be expressed in human pituitary [13]. However, this has never been confirmed. Several studies have independently shown that HCA_1 is primarily expressed in white and brown adipose tissue of both humans and rodents [5, 7, 22-24]. Only minor amounts of mRNA of HCA_1 were detected in kidney, skeletal muscle or liver. In addition, expression of HCA_1 was increased during differentiation of 3T3-L1 preadipocytes [5, 22, 23]. In mouse and human adipocyte cell lines transcription of the HCA_1 gene was induced upon treatment with peroxisome proliferator-activated receptor γ (PPARγ) agonists.

2. HCA_2 receptor

Similar to the HCA_1 receptor, HCA_2 is highly expressed in human and murine white and brown adipose tissue [6-8]. Expression of HCA_2 was increased during differentiation of 3T3-L1 preadipocytes, as well as upon treatment with PPARγ agonists [23]. Moreover, the HCA_2 receptor is expressed in various immune cells including monocytes, neutrophils, macrophages, dendritic cells and epidermal Langerhans cells [14, 25-27]. In macrophages expression of HCA_2 was inducible by treatment with IFN-γ. A recent study demonstrated expression of the HCA_2 receptor in keratinocytes by utilizing advanced BAC-transgenic reporter mice for HCA_2 [28]. Similar to macrophages, expression of the HCA_2 receptor in keratinocytes and keratinocyte cell lines was induced by IFN-γ [29]. In addition, expression of GPR109A has also been reported in retinal pigment epithelium as well as in the intestinal epithelium [30, 31].

3. HCA_3 receptor

Expression of the HCA_3 receptor appears to be very similar to the expression pattern of the HCA_2 receptor and can be found to be highly expressed in white adipose tissue [6-8]. In addition, the HCA_3 receptor is expressed in various human immune cells including neutrophils, monocytes and macrophages [4, 12, 18, 32]. Evidence has also been provided for the expression of the HCA_3 receptor in epithelial cells of the colon [30].

IV. Physiological and pharmacological roles of HCA receptors

A. HCA_1 receptor

The HCA_1 receptor mediates the inhibitory regulation of adipocyte lipolysis by lactate [2, 5, 24]. Since plasma lactate levels are strongly increased under conditions of intensive physical exercise, it would be conceivable that lactate inhibits and thereby restricts the supply of fatty acids under anaerobic conditions. However, Ahmed et al. [24] studied wild-type and HCA_1 receptor-deficient mice which were trained to exercise at an intensity resulting in plasma lactate levels sufficient to activate the HCA_1 receptor and found that plasma concentrations of free fatty acids were not different between wild-type and HCA_1 receptor-deficient mice. Thus, there is so far no evidence for a role of lactate and its receptor in the regulation of lipolysis during intensive exercise.

Interestingly, the adipose tissue can convert more than 50% of the metabolized glucose to lactate, a process stimulated by insulin and glucose uptake [33]. Lactate is then released from adipocytes and taken up by the liver to serve as a substrate for gluconeogenesis and glycogen synthesis. Insulin-induced glucose uptake results in a several-fold increase in lactate levels in the adipose tissue [24, 34, 35]. In HCA_1 receptor-deficient mice as well as in HCA_1-deficient adipocytes, insulin-induced inhibition of lipolysis and insulin-induced decrease in adipocyte cAMP were strongly reduced [24]. This suggests that lactate acting through HCA_1 functions in an autocrine and paracrine fashion to mediate insulin-induced antilipolytic effects and thereby regulates lipolysis postprandially (figure 2A). When on a high fat diet, mice lacking HCA_1 showed a reduced weight gain (Ahmed et al., 2010). This

indicates that the lactate/HCA_1-mediated antilipolytic effects contribute to the increase in body weight under hypercaloric diet.

B. HCA_2 receptor

The ketone body 3-hydroxy-butyrate which activates the HCA_2 receptor with an EC_{50} of 0.7 mM has been described as the endogenous ligand of HCA_2 [3]. In fact, 3-hydroxy-butyrate plasma levels increase to 1-2 mM after an overnight fast and reach 6-8 mM during prolonged fasting [36]. It is very likely that the HCA_2 receptor activated by 3-hydroxy-butyrate at millimolar concentrations during starvation mediates a negative feedback regulation that controls the lipolytic rate [37] (figure 2B). This regulatory mechanism would help to avoid excessive triglyceride degradation and thereby save energy during food shortage.

The antidyslipidemic drug nicotinic acid activates HCA_2 receptors expressed on adipocytes resulting in a rapid decrease in the release of free fatty acids from fat cells. This in turn reduces the supply of free fatty acids to the liver, leading to a reduced synthesis of triglycerides and very low density lipoprotein (VLDL) as well as to a subsequent decrease of low density lipoprotein (LDL)-cholesterol levels [38]. It is less clear how nicotinic acid increases levels of high density lipoprotein (HDL)-cholesterol. It is possible that the decrease in triglyceride content of apolipoprotein B (ApoB) containing lipoproteins results in a decreased exchange of triglycerides for cholesteryl esters from HDL-particles mediated by the cholesterol ester transfer protein (CETP) eventually leading to increased HDL-cholesterol levels [39-41]. Consistent with this hypothesis, HDL-cholesterol elevation in response to nicotinic acid has been shown to depend on the presence of CETP [42, 43]. Whether the HCA_2 receptor mediates the increase in HDL-cholesterol levels in response to nicotinic acid is, however, currently not clear [44, 45]. It is also unknown whether the activation of HCA_2 receptors expressed by cells outside the adipose tissue plays a role during starvation. It is possible that elevated 3-hydroxy-butyrate levels during starvation activate HCA_2 receptors expressed on immune cells and thereby induce anti-inflammatory effects which could be advantageous under conditions of starvation.

Besides its antilipolytic effect, nicotinic acid has been shown to influence the function of the adipose tissue as an endocrine organ. Both in vitro and in vivo data indicate that nicotinic acid increases the release of adiponectin from adipocytes through HCA_2 [46, 47]. The anti-inflammatory and antidiabetic consequences of increased adiponectin plasma levels [48] may contribute to the beneficial effects of nicotinic acid.

Recently, studies in mice have shown that the anti-atherosclerotic effect of nicotinic acid is not only due to nicotinic acid-induced changes in lipid metabolism but also results from direct effects of nicotinic acid on HCA_2 expressed by immune cells. In contrast to atherosclerosis-prone LDL-receptor-deficient mice carrying the wild-type receptor, mice lacking HCA_2 or mice transplanted with HCA_2-deficient bone marrow showed strongly reduced anti-atherosclerotic effects in response to nicotinic acid. The nicotinic acid receptor HCA_2 is expressed by monocytes and macrophages including plaque macrophages, and nicotinic acid inhibits the recruitment of macrophages to atherosclerotic lesions in HCA_2-dependent manner. In addition, HCA_2 mediates a stimulatory effect of nicotinic

acid on the cholesterol efflux from macrophages. Thus, nicotinic acid appears to reduce the progression of atherosclerosis also through direct anti-inflammatory effects and stimulatory effects on the reverse cholesterol transport [49, in press].

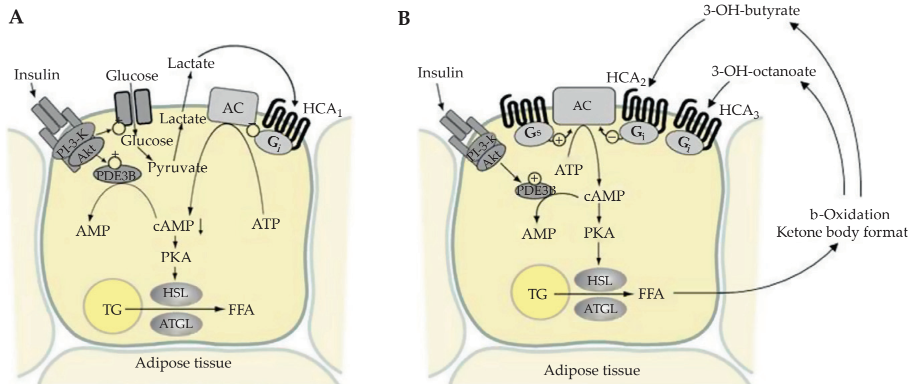

Figure 2. Biological roles of the HCA receptors. Functions of the recently deorphanized receptors HCA_1, HCA_2 and HCA_3. The lactate receptor HCA_1 mediates the acute anabolic effects of insulin on adipocytes and thereby helps to store energy after feeding (A). In contrast, HCA_2 and HCA_3 receptors are involved in the long-term regulation of lipolytic activity being receptors for the ketone body 3-hydroxy-butyrate (HCA_2) and the β-oxidation intermediate 3-hydroxy-octanoate (HCA_3). In situations of increased β-oxidation rates (e. g. during starvation) 3-hydroxy-butyrate and 3-hydroxy-octanoate plasma levels are increased and result in the inhibitory regulation of lipolysis via HCA_2 and HCA_3 receptors, respectively, in form of a negative feedback loop (B). Thereby HCA_2 and HCA_3 receptors help preserve energy stores during starvation. AC, adenylyl cyclase; TG, triglycerides; HSL, hormone-sensitive lipase; ATGL, adipocyte triglyceride lipase; FFA, free fatty acids; PKA, cAMP-regulated protein kinase.

Evidence has been provided that HCA_2 receptors expressed by epidermal Langerhans cells and keratinocytes mediate the major unwanted effects of nicotinic acid, the flush reaction. The symptoms of flushing consist of a cutaneous vasodilation as well as sensations of tingling and burning which impair patients' compliance [11, 25, 27, 50, 51]. Nicotinic acid or the antipsoriatic drug monomethyl fumarate induce a biphasic increase in dermal blood flow which is mediated by HCA_2 [28]. While the first phase is due to activation of HCA_2 on Langerhans cells, the second phase of the flush depends on HCA_2 expressed by keratinocytes. The Langerhans cell-mediated flushing involves cyclooxygenase-1 (COX-1), prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2). In contrast, keratinocyte-mediated flushing at later phases of the reaction involves cyclooxygenase-2 (COX-2) and PGE_2 [27, 28, 52, 53].

There is evidence indicating that the HCA_2 receptor expressed in intestinal epithelial cells responds to butyrate which is present in millimolar concentrations in the gut lumen, and that HCA_2 thereby functions as a tumor suppressor and anti-inflammatory receptor [30]. Furthermore, comparison of the HCA_2 potencies [3] and reported fecal concentrations of the short-and medium-chain fatty acids [54] suggests that pentanoate and hexanoate may also activate gut HCA_2.

C. HCA_3 receptor

Similar to HCA_2, the primary physiological role of the HCA_3 receptor appears to be the regulation of lipolysis. Plasma concentrations of the β-oxidation intermediate 3-hydroxy-

octanoate, which activates HCA_3 receptors at micromolar concentrations, are elevated under conditions of increased fatty acid oxidation such as starvation, diabetic ketoacidosis, various mitochondrial fatty acid β-oxidation disorders and under a ketogenic diet [4, 16, 17]. Under such conditions the HCA_3 receptor mediates a negative feedback loop to counterregulate pro-lipolytic stimuli in order to prevent excessive lipolysis which would result in the futile release and circulation of free fatty acids [1] (figure 2B). The fact that the HCA_3 receptor is only found in humans and chimpanzee suggests that a negative feedback loop of lipolysis mediated through 3-hydroxy-octanoate/HCA_3 has evolved in higher primates to economize the use of fatty acids during periods of starvation. Whether HCA_3 has potential physiological roles in immune cells or other organs is currently not known.

V. Receptor classification with pharmacological tools

Although nicotinic acid was introduced in man in the 1950s [9], structure-activity relationships for its target(s) were developed much later. Only in the 1980s Aktories and colleagues [55, 56] proposed the existence of a specific receptor for nicotinic acid and a related compound, acipimox. Progress being slow, members of the same laboratory explored a few more compounds related to nicotinic acid in a number of receptor assays, using membranes from rat adipocytes and rat spleen [57]. A few years later, the human HCA_2 receptor was cloned [7]. In that paper a number of nicotinic acid-like compounds were also tested. As most medicinal chemistry efforts have been directed towards the HCA_2 receptor we will discuss the synthetic ligands for this receptor first, followed by the more restricted information on the HCA_3 receptor. To our knowledge synthetic ligands for the HCA_1 receptor have only been reported in the patent literature, which is beyond the scope of this review. Last but not least, antagonists have not been disclosed for any of the HCA receptors, which is currently hampering a full pharmacological characterization of these receptors. A few years ago we published a review on the then available ligands, largely nicotinic acid-like compounds (Soudijn et al., 2007). The current review does not reiterate that but starts from there, and is organized according to chemical classes. We report representative structures of these classes in Figure 3.

A. Structure-activity relationships for the HCA_2 receptor

1. Nicotinic acid-like compounds

Lorenzen and colleagues [57] observed that nicotinic acid (1 in figure 3) displaces [^{3}H] nicotinic acid from and increases [^{35}S]GTPγS binding to rat epididymal adipocyte and spleen membranes with (sub)micromolar potency. The same two assays were used by Wise et al. [7] with similar results, now on the cloned human HCA_2 receptor. Apparently there are no huge species differences between rat and man for nicotinic acid itself. Two other marketed products, acifran (2 in figure 3) and acipimox (3 in figure 3), were also tested but showed lower potencies. Nicotinamide (4 in figure 3) was inactive, indicating that the carboxylic acid group is essential for activity. Gharbaoui et al. [58] evaluated other heterocyclic scaffolds, confirming that changing this moiety invariably led to compounds with lower potency than nicotinic acid, if at all.

1 (nicotinic acid) 2 (acifran) 3 (acipimox) 4 (nicotinamide)

5 (MK-0354) 6 (biphenyl anthranilic acid derivative)

7 (MK-6892) 8 (fumaric acid esters)
R^1 = H, R^2 = Me: MMF
R^1 = H, R^2 = Et: MEF

9 (pyridopyrimidinones) 10 (pyrazolopyrimidines)

11 (benzotriazoles) 12

Figure 3. Representative compounds from various chemical classes as HCA receptor agonists. (* denotes chiral center).

2. Pyrazoles

Pyrazole-3-carboxylic acid acted as a high-efficacy partial agonist in a rat tissue [^{35}S]GTPγS binding assay [57]. This finding was taken as the starting point for a synthetic program, both in academia and industry. Partial agonists may display tissue selectivity, thought to be potentially beneficial in the case of the HCA_2 receptor, as the side effect of flushing

might be separated from the desired action in dyslipidemia. Van Herk et al. [59] prepared two series of alkyl- and benzyl-substituted pyrazole-3-carboxylic acid derivatives; they were all partial agonists, and some compounds had micromolar affinity. This was particularly true for the butyl-substituted derivative. Gharbaoui et al. [58] and Skinner et al. [60] reported on a more extended series of pyrazoles largely corroborating the findings of Van Herk et al. [59]. In the latter publication it was shown that the carboxylic acid could not easily be replaced by a tetrazole bioisostere. Later, Semple and colleagues [61] reported on one particular exception, in which the carboxylic acid/tetrazole switch yielded a partial agonist. In mice this compound (MK-0354, 5 in figure 3) was as active as nicotinic acid in reducing the amount of plasma free fatty acids in vivo and had quite favorable pharmacokinetic properties, whilst not causing vasodilation in the mouse ear, a surrogate marker for flushing (see also section VIII). Imbriglio et al. [62] synthesized further MK-0354 analogues by introducing fluorinated phenyl substituents, the 2,3,5-trifluoro variant of which was 2-3 fold more potent than nicotinic acid in vitro. Similar derivatives, now with a carboxylic acid function were reported by Schmidt et al. [63] and Imbroglio et al. [64]. A further derivatization of MK-0354 with a cyclopropane extension was reported by Boatman et al. [65].

3. Acifran analogues

Acifran (5 in figure 3) was developed in the early 1980s as a lipid lowering agent. Only in 2006 Mahboubi and coworkers [66] synthesized and evaluated a small number of acifran analogs. The introduction of a para-fluoro substituent on the phenyl ring preserved activity in the in vivo animal model, while other modifications were not allowed. There was little selectivity with respect to the HCA_3 receptor. A further, more extensive, study was performed by Arena Pharmaceuticals [67]. Other substituents on the phenyl ring (e.g., 3-chloro) or replacement by thiophene yielded some compounds that showed slightly higher potency than acifran, but without a significant degree of selectivity towards the HCA_3 receptor. Some of the analogs were resolved into their individual stereoisomers, showing that invariably the (+)-isomer was the biologically active principle.

4. Anthranilic acid derivatives

High-throughput screening (HTS) campaigns at a number of companies, in particular Merck, led to the discovery of anthranilic acid derivatives as HCA_2 ligands, first reported by Shen et al. [68]. Such compounds [63] appear prone to have high plasma protein binding with a strong negative impact on the in vivo activity of the molecules, e.g. the biphenyl compound 6 in figure 3. Other anthranilic acid derivatives were reported by Shen et al. [69]. Partial hydrogenation of the anthranilic acid phenyl ring yielded compounds that retained activity on the HCA_2 receptor, elaborately explored by Raghavan et al. [70]. The authors concluded that the tetrahydro variants of anthranilic acid derivatives show improved oral bioavailability and better cytochrome P450 profiles. A recent publication [71] describes the discovery of (pre)clinical candidate MK-6892 (7 in figure 3). It was also found (Ding et al., 2010; Schmidt et al., 2010) that the cyclohexene ring system in such compounds can be further substituted.

5. Fumaric and other acids and their esters

A mixture of fumaric acid esters is on the market in Germany for the treatment of psoriasis. Interestingly, the monomethyl (MMF) and monoethyl (MEF) ester of fumaric acid (8 in figure 3), but not fumaric acid itself, have micromolar affinity for the HCA_2 receptor [29] (see also section VIII). A number of 'simple' acids rather than esters were tested by Ren et al. [72]. The two most potent compounds were trans-cinnamic acid and para-coumaric acid, although substantially less active than nicotinic acid. On the HCA_3 receptor trans-cinnamic acid was also active, while para-coumaric acid was not. Oral administration of trans-cinnamic acid to wild-type mice led to a significant reduction in plasma free fatty acid levels, whereas the compound was without effect in HCA_2 receptor KO animals. Further SAR on the HCA_2 receptor for this ligand class was recently reported by us. A rather restricted binding pocket on the receptor was delineated with trans-cinnamic acid itself being the largest planar ligand with appreciable affinity for the receptor [73 in press].

6. Pyridopyrimidinones

Peters et al. [74] reported on a very different scaffold from which HCA_2 receptor agonists were derived. The pyridopyrimidinones (e.g., 9 in figure 3) can be regarded as derivatives of nicotinamide, but that compound is inactive at HCA_2 receptors. Nevertheless submicromolar affinity and potency were observed in this series, although the compounds behaved poorly in pharmacokinetic studies.

7. Pyrazolopyrimidines as allosteric agonists

Shen and coworkers [75] described another series of agonists for the HCA_2 receptor with intriguing pharmacological activity, in particular 7 in figure 3. When tested alone it behaved as a partial agonist with 8-fold higher potency than nicotinic acid. Interestingly, the presence of 10 shifted the concentration-effect curve of nicotinic acid significantly to the left, suggestive of an allosteric mechanism of action. In a radioligand binding assay the pyrazolopyrimidine dose-dependently increased rather than displaced specific [^{3}H] nicotinic acid binding, yet another token of its nature as an allosteric enhancer.

8. Patent literature

Many companies have published patents on ligands for the HCA_2 receptor. As these publications are not peer-reviewed we refrain from discussing them here. However, the most remarkable developments in this area have been published in four recent reviews, to which we refer the interested reader [76-79].

B. Structure-activity relationships for the HCA_3 receptor

Despite the high (>95%) homology between HCA_2 and HCA_3 receptors, nicotinic acid is very selective for the HCA_2 receptor, whereas acifran is not (see e.g. [66, 72, 80]. The most extensive structure-activity study with acifran analogs [67] showed that an ethyl rather than a methyl substituent at the chiral center in acifran provided some selectivity for the HCA_3 receptor, whereas all other compounds were slightly selective for the HCA_2

receptor. Ren et al. identified ortho-coumaric acid as approx. 20-fold selective for the HCA_3 receptor, while the isomer para-coumaric acid was inactive at the HCA_3 receptor but not at the HCA_2 receptor (see paragraph A.5. in this section).
New HCA_3 receptor ligands were reported by Semple and coworkers [81]. In a screening campaign the authors discovered a benzotriazole compound (11 in figure 3) with nanomolar activity. Further exploration, e.g. by replacing the isopropyl group by 2-butyl, led to even more potent compounds, but without effect on the HCA_2 receptor.
Some 4-amino-3-nitrobenzoic acids, used as intermediates in the synthesis of the benzotriazoles, also displayed significant activity at HCA_3 receptors and selectivity over HCA_2 receptors [82]. Further substitution of the 4-amino group led to compounds with potencies in the higher nanomolar range. In the same publication the nitro-aryl moiety was substituted by a pyridine ring like in nicotinic acid, yielding HCA_3 receptor-selective full agonists.
Recently, the synthetic efforts were extended to the pyrazole carboxylic acids as a template for the HCA_3 receptor [83]. A similar substituted amino group as mentioned above was introduced to the pyrazole ring system. A typical representative (12 in figure 3) displayed high potency with an EC_{50} value of 3 nM, and over 1000-fold selectivity with respect to the HCA_2 receptor.
An intriguing conclusion from this research is that 'on average' ligands for the HCA_3 receptors have higher affinity than ever met for the HCA_2 receptor, despite intensive efforts on the latter receptor.

VI. Mutagenesis and receptor modeling studies

A. HCA_1 receptor

Prior to the deorphanization of HCA_1, Ge and coworkers [22] constructed a chimeric cysteinyl-leukotriene 2 ($CysLT_2$) receptor where the intracellular domains (all ILs and the C-terminus) were replaced by the HCA_1 sequence. The endogenous $CysLT_2$ ligand, leukotriene D_4 (LTD_4), activated the receptor and this resulted in stimulation of G_i. Wild-type $CysLT_2$ predominantly couples to G_q, so these findings suggested that HCA_1 is a G_i coupled receptor. A first clue regarding the biological function of HCA_1 was obtained when LTD_4 was shown to inhibit lipolysis in primary mouse adipocytes expressing the chimera. After lactate was identified as the endogenous HCA_1 ligand, a homology model was constructed of the binding pocket [5]. Four conserved residues predicted to interact with lactate were mutated to alanine in separate mutant receptors: Arg99Ala (TM3), Tyr233Ala (TM6), Arg240Ala (TM6) and Thr267Ala (TM7). Stimulation of $[^{35}S]GTP\gamma S$ binding by lactate was absent in all four mutants, suggesting that these residues are all needed for receptor activation by lactate.

B. HCA_2 and HCA_3 receptors

The HCA_2 and HCA_3 receptors have different ligand repertoires and SAR, although there is some cross-selectivity (see section V). For example, nicotinic acid binds almost exclusively to HCA_2, whereas acifran [84] stimulates both HCA_2 (EC_{50} = 1.9 μM) and HCA_3 (EC_{50} = 90 μM). This is surprising since these receptors have > 95 % amino acid sequence

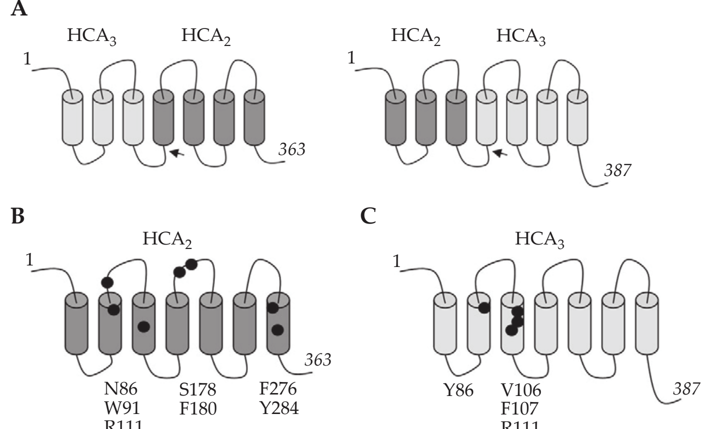

Figure 4. HCA_2/HCA_3 chimeras and residues involved in ligand binding. **A.** Schematic representation of HCA_2/HCA_3 chimeras discussed in Tunaru et al. [85] and Ahmed et al. [4]. The arrow indicates the juncture point. **B.** Schematic representation of HCA_2 indicating the positions of residues important for ligand binding (black dots). **C.** Schematic representation of HCA_3 indicating the position of residues important for ligand binding (black dots).

identity. To investigate which amino acids are involved in ligand selectivity, Tunaru and coworkers [85] constructed HCA_2/HCA_3 chimeras and introduced point mutations in both native receptors. For the chimeras, the receptors were cut at the interface of IL2 and TM4, and then the fragments were joined together at this point (see figure 4A). On the chimera with the HCA_2 receptor C-terminally, acifran had HCA_2-like potency (EC_{50} = 2 μM). At the same time, this receptor was insensitive to nicotinic acid. It was also HCA_3-like in its response to 2-oxo-octanoic acid, which is a close analogue of the endogenous HCA_3 ligand [4]. The HCA_3 N-terminal part seemed indeed responsible for the affinity of 2-oxo-octanoic acid, since the 'reverse' chimera with the HCA_2 sequence N-terminally did not respond to this compound. Using the site-directed mutagenesis approach, a HCA_2 mutant containing HCA_3 residues on three positions was constructed. The mutations Asn86Tyr, Trp91Ser (interface of TM2 and IL1) and Ser178Ile (EL2) prevented HCA_2 from responding to nicotinic acid and lowered the potency of acifran to HCA_3-like levels (see figure 4B). As a next step, the positively charged arginine residues in HCA_2 were investigated as likely counterparts for the negatively charged carboxylate function in nicotinic acid and acifran. All four arginine residues in the sequence were mutated to alanine, and only the Arg111Ala (TM3) mutant was rendered insensitive to nicotinic acid. The HCA_3 single mutant Arg111Ala, which was later constructed, also showed a deficient binding of the ligand 2-hydroxy-octanoic acid, which is another analogue of the endogenous HCA_3 ligand [4] (see figure 4C). This suggests that the acidic moiety of the ligands is anchored at the same position in both HCA_2 and HCA_3. Interestingly, the HCA Arg111 corresponds to the conserved Asp residue important for agonist binding in the biogenic amine receptors. In HCA_2, three more residues seem implicated in nicotinic acid binding: Phe180 (EL2), Phe276 and Tyr284 (both TM7) [85]. In a later paper, the roles of HCA_2 residues Asn86, Met103 and Leu107 were investigated further [4]. Mutations into corresponding HCA_3 residues (respectively Tyr, Val and Phe) conferred a full HCA_3-like response to 2-hydroxy-octanoic acid. Conversely, the highly similar mutant Asn86Tyr,

Trp91Ser, Met103Val showed no response to this ligand. In the paper describing the HCA_2/HCA_3 chimeras, a receptor homology model for HCA_2 was also presented [85]. The model was based on the rhodopsin crystal structure [86], although the binding mode of the large ligand retinal did not give many clues on how to dock the small HCA receptor ligands. It was proposed that Trp91, Phe276 and Tyr284 interact with the pyridine ring of nicotinic acid, whereas a hydrogen bond may be present between Ser178 and the nitrogen. An independently constructed HCA_2 homology model, also based on the rhodopsin structure, was used to dock an anthranilic acid derivative [87]. The proposed binding pocket was lined by residues mainly from TM3, TM5, TM6 and EL2, which could bind the ligand in an extended conformation. Three residues important for nicotinic acid binding, Arg111 (TM3), Ser178 (EL2) and Phe276 (TM7), were also implicated here. Recently the repertoire of available GPCR crystal structures has grown considerably [88-91]. These new templates have not been exploited yet for HCA receptor homology modeling, but may yield better models in the near future.

VII. Signal transduction and receptor desensitization

A. G protein coupling

It was previously shown that nicotinic acid-induced effects are sensitive to treatment with pertussis toxin which specifically inactivates α-subunits of G_i-type G proteins [56]. During the last decade numerous studies have shown that HCA_1, HCA_2 and HCA_3 receptor-mediated effects are sensitive to pertussis toxin [2, 4-8, 18, 22]. Thus, HCA_1, HCA_2 and HCA_3 receptors couple to G_i/G_o -type G proteins.

B. Downstream signaling pathways

Agonists of HCA_1, HCA_2 and HCA_3 receptors have been shown to inhibit adenylyl cyclase activity and thereby to decrease cAMP levels in various cells after heterologous expression of the receptors as well as in primary adipocytes [4-8, 24, 92]. Since cAMP is the major intracellular regulator of lipolysis by stimulating cAMP-dependent kinase to activate lipolytic enzymes, a decrease in cAMP results in an antilipolytic effect in adipocytes [93].

Activation of G_i-type G proteins in immune cells results in stimulation of phospholipase C β-isoforms most likely through the release of βγ-subunits of G proteins [94], and activation of HCA_2 and HCA_3 receptors in neutrophils, macrophages or other immune cells has been shown to result in increases in free intracellular Ca^{2+} concentrations [4, 11, 18, 26, 95]. An increase in the intracellular Ca^{2+} concentration induced by nicotinic acid via HCA_2 receptors may lead to the activation of Ca^{2+}-sensitive phospholipase A_2 (PLA_2) and subsequent formation of prostanoids [11, 27, 95]. PLA_2 can also be activated by phosphorylation through extracellular signal-regulated kinase (ERK) which can be activated via HCA receptors as well [4, 5, 8, 92, 96].

C. Receptor desensitization

Some of the effects mediated by the HCA_2 receptor are subject to desensitization [97]. For instance, nicotinic acid-induced flushing and nicotinic acid-induced increases in intracellular Ca^{2+} concentrations via the HCA_2 receptor desensitize within minutes [26, 27]. Whether

these desensitization phenomena are due to effects on the receptor itself or at the level of downstream signaling processes is unclear. There is some evidence that heterologously expressed HCA_1 and HCA_2 receptors internalize in response to full agonists [5, 92, 98]. In contrast, a partial agonist, which did not induce ERK phosphorylation, did not induce receptor internalization [92]. Ligand-dependent internalization of HCA_2 appears to involve G protein-coupled receptor kinase 2 (GRK2) and arrestin 3 [98].

D. Receptor oligomerization

It is generally accepted that some G protein-coupled receptors can form dimers or oligomers. In a recent study it was shown by bioluminescence resonance energy transfer that HCA_2 and HCA_3 receptor constructs can interact when heterologously expressed in human embryonic kidney cells [80]. However, the existence of homo-/heterodimers of HCA_2 and HCA_3 in native tissues and the implications of oligomerization for receptor function remain unknown.

VIII. Therapeutic potential of HCA receptor ligands

Although all members of the HCA receptor family are potentially interesting drug targets, only HCA_2 is currently exploited as such. Therefore, this section will focus on this receptor alone.

A. Nicotinic acid alone

The use of high-dose nicotinic acid in the clinic has a long history starting in the 1950's [9]. A number of clinical studies demonstrated the usefulness of this compound as a lipid-modulating drug, although patient comfort and compliance are compromised by the common skin flushing side-effect [for reviews see: 10, 99; see also section IV]. Nowadays anti-flushing formulations of nicotinic acid are usually chosen over the crystalline form of the drug.

B. Nicotinic acid with anti-flushing strategies

Flushing is a widely occurring side effect of high-dose nicotinic acid. It seems to decrease with continued nicotinic acid treatment, but still up to one in five patients stops the treatment due to this side effect [100]. Administration of nicotinic acid with a meal seems to attenuate the flushing, probably due to a slower absorption rate. Acetylsalicylic acid, which is an inhibitor of prostaglandin synthesis, is also effective against flushing [101]. The more elegant versions of these strategies are extended-release nicotinic acid [102] and a combined formulation of nicotinic acid with laropiprant [52]. Modern extended-release (ER) nicotinic acid has been proven efficacious and has overcome the initial hepatotoxicity problems by decreasing the half-life compared to earlier sustained-release preparations [103]. In all of the clinical trials discussed below, ER nicotinic acid was used instead of an immediate-release formulation. Laropiprant is a prostaglandin D_2 receptor 1 (DP_1) antagonist. Like aspirin, it can reduce the cutaneous vasodilatation which is at the core of the flushing syndrome (see sections IV and VII). Nicotinic acid with laropiprant and

nicotinic acid alone (4 weeks 1 g/day followed by 20 weeks 2 g/day) were shown to have comparable lipid-modifying effects and safety profiles except for flushing, which was less severe with laropiprant [104]. In a separate study, severity of flushing in the first 16 weeks of nicotinic acid treatment was evaluated in patients with dyslipidemia [105]. Despite higher nicotinic acid doses, patients receiving the combination treatment experienced significantly less flushing. Furthermore, a lower number of patients from the combination group discontinued the trial due to flushing (7.4% vs 12.4%). The data from these and all other trials evaluating nicotinic acid/laropiprant have recently been compiled by McKenney and coworkers [106]. A fixed-dose formulation (1000 mg nicotinic acid/20 mg laropriprant) is on the market in Europe since 2008, but the FDA has requested further studies before introduction in the USA.

C. Nicotinic acid in combination with other lipid-altering drugs

HMG-CoA reductase inhibitors ('statins') are the first choice for lowering cholesterol plasma levels. Therefore, it was a logical step to investigate if combination of statins with nicotinic acid had any added benefit. An early example of such a trial showed that combination treatment with simvastatin and nicotinic acid for 3 years significantly improved the lipid profile in patients with coronary heart disease and low HDL cholesterol [107]. Furthermore, the frequency of a first cardiovascular event was only 3% in the treatment group versus 24% in the placebo group. Overall, the therapeutic efficacy of the combination was much better than simvastatin alone [see for example: 108].

An important surrogate endpoint in the evaluation of lipid-modifying therapy is the effect on the size of atherosclerotic lesions, usually measured by carotid intima-media thickness (CIMT) [99]. Statin monotherapy does not greatly influence atherosclerotic plaque formation, whereas statin/nicotinic acid combination treatment has been shown to reduce lesion development in several clinical trials. In the ARBITER 2 study, patients on statins received supplementary nicotinic acid or placebo therapy during one year [109]. CIMT was significantly increased in the placebo group, whereas it was unchanged in the group receiving nicotinic acid treatment, although the difference between the groups did not reach significance. In the more recent ARBITER6-HALTS study the effects of ER nicotinic acid or ezetimibe as add-on therapy with statin treatment were assessed [110]. Ezetimibe inhibits cholesterol absorption from the gut. After 14 months of treatment, a significant reduction in mean and maximal CIMT was observed in the nicotinic acid/statin group; HDL cholesterol was significantly increased, whereas it was decreased in the ezetimibe/statin group. Although the majority of the patients completed the study, it was halted prematurely because of an unexpected increase in atherosclerosis in the ezetimibe/statin group [see also: 111, 112]. In another very recent clinical study, participants received ezetimibe/statin combination treatment with or without additional nicotinic acid [113]. This triple therapy significantly improved lipid levels including HDL, LDL and apolipoprotein AI, and was well tolerated overall. Small and transient side-effects on fasting glucose were seen with nicotinic acid use.

The nicotinic acid/statin combination therapy is currently being evaluated further in two large trials. The endpoints are progression of cardiovascular disease, incidence of

major cardiovascular events and cardiovascular disease-associated mortality. The AIM-HIGH study compares ER nicotinic acid/simvastatin with simvastatin monotherapy and enrolled 3300 patients suffering from cardiovascular disease, low HDL cholesterol and high triglyceride levels (see at http://clinicaltrials.gov/ct2/show/NCT00120289). The HPS2-THRIVE compares ER nicotinic acid/simvastatin/laropiprant with simvastatin alone in patients with coronary heart disease (N>20,000) (see at http://clinicaltrials.gov/ct2/show/NCT00461630). The results of these trials are expected between 2011 and 2013. If a significant benefit of nicotinic acid is shown, a much more widespread use of the drug can be expected.

D. Fumaric acid derivatives

Fumaric acid esters, which have now been identified as HCA_2 agonists [29], have been used for the treatment of psoriasis as early as the 1950's [114]. Although their mechanism of action is still poorly understood, good and prolonged clinical efficacy and an acceptable safety profile have been reported for the oral administration of a mixture of monoethyl- and dimethylfumarate [115, 116]. For a recent retrospective analysis of almost 1000 patients see Reich et al. [117]. Dimethylfumarate (DMF) is quickly metabolized in vivo to monomethylfumarate (MMF), which has a higher potency at HCA_2 [29; see also section V]. DMF was also evaluated in a phase II trial including 240 patients for the treatment of relapsing-remitting multiple sclerosis [118]. Administration of 3 times 240 mg/day reduced the mean number of new brain lesions emerging between the 12th and 24th week of treatment by 70%, compared to placebo. It has not been conclusively demonstrated that activation of HCA_2 is at the basis of the therapeutic effects of fumaric acid esters, but the receptor is a plausible mediator as it is definitely expressed in immune cells. Nicotinic acid itself has also been suggested for the treatment of multiple sclerosis [119]. If HCA_2 agonists can indeed act as anti-inflammatory or immunosuppressive drugs, the treatment of several auto-immune diseases could be improved with these relatively safe and inexpensive compounds.

E. Clinical candidates

An HCA_2 agonists, MK-0354, was selected on the basis of low or negligible ERK1/2 activation in vitro and greatly reduced flushing in animal studies [61]. In a phase II study, 2.5 g of MK-0354 lowered free fatty acid levels in dyslipidemic patients consistently, comparable to 1 g of ER nicotinic acid. Little flushing was observed with MK-0354; however, no clinically meaningful lipid modification occurred either [120]. The recently completed trial with another HCA_2 agonist, MK-1903 (structure not disclosed), seems to have yielded similar results (see at http://www.clinicaltrial.gov/ct2/show/NCT00847197). Merck announced that the HDL cholesterol elevation was not large enough to meet the objectives for efficacy, and no safety problems were mentioned (see at http://drugdiscovery.pharmaceutical-business-review.com/news/merck_to_discontinue_development_of_mk1903_091224/). It seems that clinical efficacy of HCA_2 agonists cannot (yet) be accurately predicted at the preclinical stage. A better understanding of the mechanism of action of nicotinic acid could decrease the risk of attrition at a late stage of drug development.

IX. Conclusion

The first steps towards understanding the physiological relevance of the HCA receptor family have only recently been taken. These receptors seem to have evolved to recognize hydroxylated intermediates of energy metabolism with a relatively low affinity, in order to regulate lipolysis. HCA_1 seems to contribute to insulin-induced anti-lipolysis, and also to the weight gain induced by a hypercaloric diet. HCA_2 is important for the conservation of adipose tissue under starvation conditions, next to its pharmacological role as the high-affinity nicotinic acid receptor. HCA_3 seems to be part of the same negative feedback loop to limit lipolysis during starvation. HCA_2 and HCA_3 may have additional roles in the immune system, but further studies are needed in that area.

Despite rather spectacular effects on lipid levels and also on atherosclerosis progression, and the introduction of reduced-flushing formulations, the use of the antidyslipidemic drug nicotinic acid is still stunted by its side-effects. Other HCA_2 ligands, including partial agonists and biased agonists, may be better tolerated. However, despite extensive efforts of the pharmaceutical industry, no new HCA_2 ligands have been successful as clinical candidates so far. More research is needed to enable the identification of genuinely promising molecules. Possibly the most important enigma is how nicotinic acid induces HDL elevation. Recent animal experiments suggest that nicotinic acid reduces the progression of atherosclerosis also via lipid-independent anti-inflammatory effects and by increasing cholesterol efflux from plaque macrophages. Lipid-independent beneficial effects of nicotinic acid, in particular anti-inflammatory effects, deserve further analysis in the future. HCA_1 also has potential as a drug target for antilipolysis, and it is very unlikely that agonists for this receptor cause flushing because no skin expression has been detected. HCA_1 antagonists may reduce weight gain, but no antagonists are known to date for any of the HCA receptors. Thus, studies in animals have revealed unexpected physiological and pharmacological roles of HCA receptors. Much more work on the generation of new agonistic and antagonistic ligands of HCA receptors and their analysis in in vitro and in vivo models is required to explore all options to harness HCA receptors as targets to prevent and treat a variety of diseases like dyslipidemia, adipositas, cardiovascular diseases or chronic inflammatory and immune diseases.

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Chapter 3

Effects of pyrazole partial agonists on HCA_2-mediated flushing and VLDL-triglyceride levels in mice

This chapter is based on: Li Z, Blad CC, van der Sluis RJ, de Vries H, Van Berkel TJC, IJzerman AP, Hoekstra M, Br J Pharmacol **2012**; 167(4): 818-25

Abstract

Background and purpose: Niacin can effectively treat dyslipidemic disorders. However, its clinical use is limited due to the cutaneous flushing mediated by the nicotinic acid receptor HCA_2. In the current study, we evaluated two partial agonists for HCA_2, LUF6281 and LUF6283, with respect to their anti-dyslipidemic potential and cutaneous flushing effect.

Experimental approach: In vitro potency and efficacy studies with niacin and the two HCA_2 partial agonists were performed using HEK293T cells stably expressing human HCA_2. Normolipidemic C57BL/6 mice received either niacin or HCA_2 partial agonists (400 mg/kg/day) once a day for 4 weeks for evaluation of their effects in vivo.

Key results: Radioligand competitive binding assay showed K_i values for LUF6281 and LUF6283 of 3 μM and 0.55 μM. [^{35}S]-GTPγS binding determined niacin > LUF6283 > LUF6281 as rank order of their potency. All three compounds similarly reduced plasma VLDL-triglyceride concentrations, while LUF6281 and LUF6283 - in contrast to niacin - did not also exhibit the unwanted flushing side effect in C57BL/6 mice. Niacin lowered the adipose tissue expression of lipolytic genes HSL and ATGL by 50% ($P<0.05$). LUF6281 and LUF6283 unexpectedly did not. In contrast, the decrease in the VLDL-triglyceride concentration upon LUF6281 and 6283 treatment was associated with a parallel >40% decrease ($P<0.05$) in the expression level of APOB within the liver.

Conclusions and Implications: The current study identifies LUF6281 and LUF6283, two HCA_2 partial agonists of the pyrazole class, as promising drug candidates to achieve optimal lipid-lowering while successfully avoiding the unwanted flushing side effect.

Introduction

Niacin, also known as nicotinic acid, is the most effective agent currently available to treat dyslipidemic disorders [1]. It lowers plasma levels of pro-atherogenic lipids, including chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and triglycerides (TG) in normolipidemic as well as hypercholesterolemic subjects [2]. Several clinical trials have shown that nicotinic acid reduces cardiovascular disease and myocardial infarction incidence, providing a solid rationale for the use of niacin in the treatment of atherosclerosis [3-4]. The G protein-coupled receptor GPR109A, also known as PUMA-G in mouse and HM74A in humans, has been identified as a high-affinity receptor for niacin [5-6]. We now know that the endogenous ligand for GPR109A is 3-hydroxybutyrate, and this receptor has recently been renamed as hydroxy-carboxylic acid receptor 2 (HCA_2) [7]. Despite its established cardiovascular benefits, the clinical use of niacin has been limited due to the cutaneous flushing, a well-recognized adverse skin effect from niacin therapy. Flushing has been cited as the major reason for the discontinuation of this therapy [8]. The nicotinic acid receptor HCA_2 expressed in the skin is a critical mediator of niacin-induced flushing [9]. Niacin stimulates HCA_2 in epidermal Langerhans cells and keratinocytes, causing the cells to produce vasodilatory prostaglandin D2 (PGD_2) and prostaglandin E2 (PGE_2), which leads to cutaneous vasodilation [10-13].

For the past decade, the pharmacology of HCA_2 has been studied and full or partial agonists for HCA_2 have been developed in an attempt to achieve the beneficial effects of niacin while avoiding the unwanted flushing side effect [14]. Based on the structure-activity relationship of niacin-related molecules, several potent agonists for HCA_2 have been identified, including acipimox, acifran, 3-pyridine-acetic acid, 5-methylnicotinic acid, pyridazine-4-carboxylic acid, and pyrazine-2-carboxylic acid [15-16]. However, the challenge remains that HCA_2 partial agonists failed to mimic the beneficial effects of niacin on LDL-cholesterol, triglycerides or HDL-cholesterol despite the absence of flushing events in clinical studies [17]. Further understanding of the medicinal chemistry of HCA_2 is needed to pharmacologically dissociate the anti-lipolytic and vasodilatory effects of niacin by acting on HCA_2.

In the current study, we assessed the properties of two HCA_2 partial agonists, LUF6281 and LUF6283, of the pyrazole class, which were developed in our laboratory [18]. We first characterized these two compounds in vitro, using a radioligand binding assay, [^{35}S]-GTPγS assay and ERK phosphorylation assay. The ERK phosphorylation assay was included because it has been suggested that ERK1/2 phosphorylation downstream from HCA_2 correlates positively with skin flushing [19]. Subsequently, we determined the cutaneous flushing effect and the lipid-lowering potential of these two partial agonists in normolipidemic C57BL/6 mice which represent a good mouse model to study the VLDL-triglyceride lowering effect of niacin [20].

Methods

Materials

[^{3}H]-nicotinic acid (60 Ci/mmol) was obtained from BioTrend (Köln, Germany). [^{35}S]-

GTPγS (1250 Ci/mmol) was obtained from Perkin Elmer (Waltham, MA).

Cell culture and membrane preparation

Human embryonic kidney (HEK) 293T cells stably expressing human HCA_2 were cultured in DMEM supplemented with 10% newborn bovine serum, 0.4 mg/mL G418, 50 IU/mL penicillin and 50 μg/mL streptomycin. The cells were harvested by scraping in cold PBS, centrifuged at 1000 x g for 10 minutes and resuspended in cold 50 mM Tris-HCl buffer, pH 7.4. Then a DIAX 900 electrical homogenizer (Heidolph, Schwabach, Germany) was used for 15 seconds to obtain cell lysis. The suspension was centrifuged at 225000 x g for 20 minutes at 4 °C and the supernatant was discarded. The pellet was resuspended in Tris-HCl, and the homogenization and centrifugation steps were repeated. The membranes were resuspended in cold assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4) and the protein content was determined using BCA assay (Thermo Scientific, Waltham, USA). During membrane preparation the suspension was kept on ice at all times. Membrane aliquots were stored at -80°C until the day of use.

[^{3}H]-nicotinic acid displacement assay

Membranes of our stable HEK293T-HCA_2 cell line (50 μg protein per tube) were incubated for 1 hour at 25°C with 20 nM [^{3}H]-nicotinic acid and with increasing concentrations of the test compounds in assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4). The total assay volume was 100 μL. To assess the total binding, a control without test compound was included. The non-specific binding was determined in the presence of 10 μM unlabeled nicotinic acid. Final DMSO concentration in all samples was ≤0.25%. The incubation was terminated by filtering over GF/B filters using a 24-sample harvester (Brandel, Gaithersburg, USA). The filters were washed 3 times with 2 mL cold buffer (50 mM Tris HCl, pH 7.4). Filters were transferred to counting vials and counted in a Perkin Elmer LSA Tri-Carb 2900TR counter after 2 hours of extraction in 3.5 mL Emulsifier Safe liquid scintillation cocktail (Perkin Elmer, Waltham, USA).

[^{35}S]-GTPS binding assay

This assay was performed in 96-well format in 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.4 at 25°C with 1 mM DTT, 0.5% BSA and 50 μg/mL saponin freshly added. HEK-HCA_2 membranes (5 μg protein per well in 25 μL) were pre-incubated with 25 μL of 40 μM GDP and 25 μL of increasing concentrations of the test compounds, for 30 minutes at room temperature. Then, 25 μL [^{35}S]-GTPγS was added (final concentration 0.3 nM) and the mixture was incubated for 90 minutes at 25°C with constant shaking. The incubation was terminated by filtration over GF/B filterplates on a FilterMate harvester (PerkinElmer). The filters were dried and 25 μL Microscint 20 (PerkinElmer) was added to each filter. After ≥3 hours of extraction the bound radioactivity was determined in a Wallac Microbeta Trilux 1450 counter (PerkinElmer, MA, USA).

ERK1/2 phosphorylation assay

The assay was performed using the AlphaScreen SureFire Phospho-ERK1/2 kit

(PerkinElmer, MA, USA), following the kit protocol. Briefly, a 96-well cell culture plate was coated with poly-D-lysine and HEK cells stably expressing human HCA_2 were seeded at 50,000 cells/well in 200 μL DMEM supplemented with 10% newborn bovine serum, 0.4 mg/mL G418, 50 IU/mL penicillin and 50 μg/mL streptomycin. After overnight incubation the cells were serum starved for 4 h in the same medium lacking the serum, and then the medium was replaced by 90 μL prewarmed PBS and incubated for an additional 30 minutes. Increasing concentrations of the test compounds were diluted in prewarmed PBS and 10 μL was added per well for stimulation. After 5 minutes the stimulation solution was removed from the plates, the wells were washed once in ice-cold PBS and 100 μL lysis buffer was added per well. After 15 minutes of incubation and shaking at room temperature, the lysates were mixed by pipetting and 4 μL was transferred to a 384-well OptiPlate (PerkinElmer, MA, USA). The reaction mix was prepared according to the kit protocol (60 μL reaction buffer and 10 μL activation buffer with 1 μL of the donor and acceptor beads each) and 7 μL mix was added to each proxyplate well. After 2 h the plate was read on an EnVision multilabel plate reader (PerkinElmer, MA, USA).

Animals

Twelve week old female C57BL/6 mice were fed a regular cholesterol-free chow diet containing 4.3% (w/w) fat (RM3, Special Diet Services, Witham, UK). Mice received niacin, or HCA_2 partial agonists LUF6281 and LUF6283 (400 mg/kg/day in 50% (v/v) DMSO in PBS) once a day for 4 weeks via oral gavage. After euthanization, mice were bled via orbital exsanguination and perfused in situ through the left cardiac ventricle with ice-cold PBS (pH 7.4) for 20 minutes. Liver and fat were dissected and snap-frozen in liquid nitrogen. Animal care and procedures were performed in accordance with the national guidelines for animal experimentation. All protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Measurement of skin flushing

Cutaneous flushing in C57BL/6 mice was assessed by monitoring the change of the skin temperature at the mouse paw location. Temperature measurements were recorded using a non contact infrared thermometer (Pro Exotics PE-1 Infrared Temp Gun, Littleton, USA). The probe was held at a distance of 1 to 2 mm from the metacarpal pad of the mouse paw, and temperature readings were taken from a circular area approximately 3 mm in diameter. Animals were habituated to handling and to the infrared probe before use. Skin temperature was initially recorded from the abdominal area, tail, ear, and paw, after which it was determined that mouse paw skin temperature yielded the most reliable and consistent results. During the experiment, the animals were dosed with niacin or partial agonists LUF6281 and LUF6283 (400 mg/kg/day) via oral gavage (10:00-11:00 AM), and the paw temperature was measured every 10 minutes for a period of 60 minutes in total. Three readings from the center area of mouse paw were recorded routinely for each time point. Baseline paw temperature was recorded right before animals were dosed. All the administration was performed in conscious mice to avoid the interference of the anesthetics on skin temperature.

Plasma lipid analysis

The distribution of cholesterol over different lipoproteins in plasma was determined by fast protein liquid chromatography (FPLC) through a Superose 6 column (3.2 x 30 mm; Smart-System, Pharmacia, Uppsala, Sweden). Cholesterol content of the lipoprotein fractions was measured using the enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany).

Quantitative real-time PCR

Total RNA was isolated using acid guanidinium thiocyanate (GTC)-phenol-chloroform extraction. Briefly, 500 μL of GTC solution (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine) was added to each sample, followed by acid phenol:chloroform extraction. RNA in the aqueous phase was precipitated with isopropanol. The quantity and purity of isolated RNA were examined using an ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE, USA). One microgram of RNA from each sample was converted into cDNA by reverse transcription with RevertAid™ M-MuLV Reverse Transcriptase (Promega, Madison, WI, USA). Negative controls without addition of reverse transcriptase were prepared for each sample. Quantitative real-time PCR was carried out using ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. 36B4, beta-actin, and GAPDH were used as internal housekeeping genes. The gene-specific primer sequences used are listed in Table 1. Amplification curves were analyzed using 7500 Fast System SDS software V1.4 (Applied Biosystems, Foster City, CA, USA).

Table 1. Primers used for quantitative real-time PCR analysis

Gene	Forward primer	Reverse Primer
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
beta-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
GAPDH	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
ATGL	TGCCCTCAGGACAGCTCC	TTGAACTGGATGCTGGTGTTG
HSL	CTGACAATAAAGGACTTGAGCAACTC	AGGCCGCAGAAAAAAGTTGAC
APOB	ATGTCATAATTGCCATAGATAGTGCCA	TCGCGTATGTCTCAAGTTGAGAG
MTP	AGCTTTGTCACCGCTGTGC	TCCTGCTATGGTTTGTTGGAAGT

Statistical analysis

Analysis of the in vitro studies was performed using Prism 5.0 software (GraphPad Software Inc., La Jolla, USA). Nonlinear regression was used to determine IC_{50} values from competition binding curves. The Cheng-Prusoff equation was then applied to calculate K_i values [21]. [^{35}S]-GTPγS and pERK curves were analysed by nonlinear regression to obtain EC_{50} values. For the in vivo studies, data were analyzed by T-test or one way ANOVA with Student-Newman-Keuls post test (Instat GraphPad software, San Diego, USA) were appropriate. Statistical significance was defined as $p<0.05$. Data are expressed as means+SEM.

Results

NIACIN **LUF6281** **LUF6283**

Figure 1. Chemical structures of niacin, LUF6281, and LUF6283.

The chemical structures of niacin and the HCA_2 partial agonists LUF6281 and LUF6283 are shown in Figure 1. The affinity of the compounds for HCA_2 was determined by a competitive binding assay using radiolabeled nicotinic acid and a HEK293T cell line stably expressing the human HCA_2 nicotinic acid receptor (Figure 2). The K_i values determined for LUF6281 and LUF6283 were 3.1 μM and 0.55 μM, respectively (Table 2).

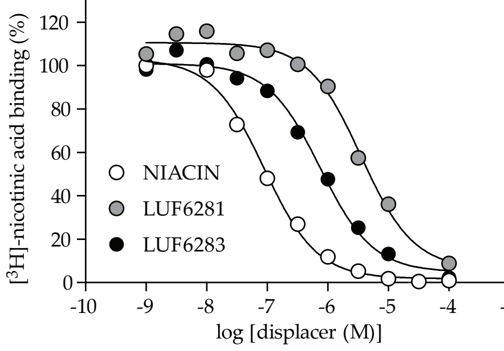

Figure 2. Competitive radioligand binding assay using 20 nM [3H]-nicotinic acid revealing the relative affinities of niacin, LUF6281 and LUF6283. The assay was performed on HEK293T-HCA_2 membranes (50 μg/tube). A representative experiment is shown (of N=3).

Table 2. In vitro biochemical characterization of the HCA_2 agonists niacin, LUF6281, and LUF6283. Values are means±SEM (N≥3).

	K_i (μM)	EC_{50}-[35S]-GTPγS (μM)	EC_{50}-pERK1/2 (μM)	EC_{50} ratio GTPγS/pERK1/2
Niacin	0.04 ± 0.02	0.41 ± 0.11	0.02 ± 0.004	21
LUF6281	3.1 ± 0.5	8.60 ± 1.00	1.37 ± 0.31	6
LUF6283	0.55 ± 0.01	3.10 ± 0.13	0.32 ± 0.06	10

As functional readout the potencies and intrinsic efficacies of niacin, LUF6281, and LUF6283 were measured by their ability to stimulate [35S]-GTPγS binding. The results show that LUF6281 and LUF6283 are partial agonists compared to niacin (100%), with intrinsic efficacies of 55±4% and 76±3%, respectively (N=3). The rank order of their potency was niacin > LUF6283 > LUF6281, with EC_{50} values of 0.41, 3.1 and 8.6 μM, respectively (Figure 3; Table 2).

The second functional assay monitored ERK1/2 phosphorylation upon HCA_2 activation by the different compounds. All compounds appeared to be high efficacy full agonists (Figure 4; Table 2). The EC_{50} values obtained here were 20 nM for niacin, 1.4 μM for

LUF6281 and 0.32 μM for LUF6283. Thus, all compounds seemed to be more potent in the pERK1/2 assay than in the [^{35}S]-GTPγS assay. Importantly, this difference was much more pronounced for niacin (21-fold) than for LUF6283 (10-fold) and LUF6281 (6-fold) (Table 2). To examine the vasodilatory effects of these compounds in vivo, we used normolipidemic C57BL/6 mice to assess the cutaneous flushing. Normal paw skin temperature of C57BL/6 mice is approximately 26.4°C (n=30). Flushing was measured as absolute increase in mouse paw skin temperature. As anticipated, niacin treatment induced a strong increase in skin temperature (+3°C; P<0.001). However, neither of the partial agonists displayed a significant temperature rise. At 20 minutes after compound administration, LUF6281 and LUF6283 induced a temperature increase of maximally 0.6°C (n = 10 per group), which was significantly lower than the temperature rise observed in the niacin group (P<0.001; Figure 5) and not different from the temperature rise induced by mouse handling alone. Both of the HCA_2 partial agonists thus avoided the unwanted flushing side effect seen upon niacin exposure in mice.

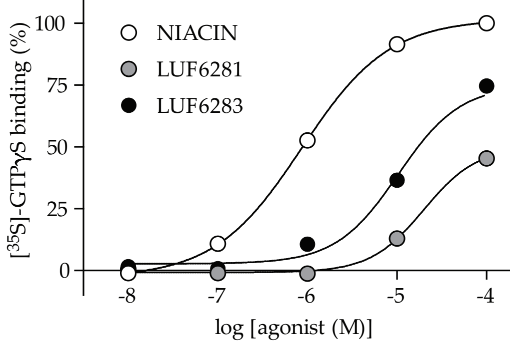

Figure 3. Concentration-response curves of niacin, LUF6281 and LUF6283 in a [^{35}S]-GTPγS binding assay, showing the relative potencies and intrinsic efficacies. Niacin is a full agonist, whereas LUF6281 and LUF6283 are partial agonists in this assay. The assay was performed on HEK293T-HCA_2 membranes (5 μg/tube). A representative experiment is shown (of N=3).

To evaluate the lipid lowering potential of the LUF compounds, we tested their effect on plasma lipid levels. Although treatment with niacin or the LUF compounds for 4 weeks did not alter the plasma total cholesterol or triglyceride concentrations (data not shown), separation of plasma lipoproteins by FPLC in combination with analysis of the lipid content across the FPLC fractions showed that both niacin and the two partial agonists

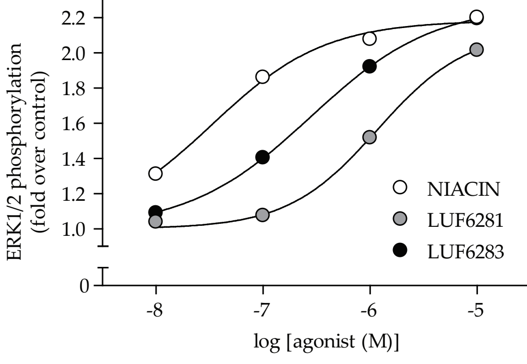

Figure 4. Concentration-response curves of niacin, LUF6281 and LUF6283 in an ERK1/2 phosphorylation assay, showing the relative potencies and intrinsic efficacies. All ligands are full agonists in this assay. The assay was performed on attached HEK293T-HCA_2 cells. A representative experiment is shown (of N=3-5).

greatly reduced plasma VLDL-triglyceride concentrations in C57BL/6 mice (Figure 6).

It is well established that niacin lowers plasma triglycerides through its anti-lipolytic action in adipocytes. In accordance, in white adipose tissue of niacin-treated mice we detected a marked decrease in the relative mRNA expression level of key lipolytic enzymes adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL; Figure 7). Strikingly, no effect of the partial agonists on adipose tissue ATGL/HSL expression was noted. In contrast, in liver, LUF6281 and LUF6283 significantly reduced (-50%; $P<0.05$) the expression of apolipoprotein B (APOB), the essential protein moiety of triglyceride-rich VLDL/LDL particles, while niacin did not (Figure 8). None of the treatments significantly changed the expression level of microsomal triacylglycerol transfer protein (MTP) in livers of C57BL/6 mice, suggesting that the loading of APOB with lipids was not affected. Combined, these findings suggest that the partial agonists lower plasma VLDL-triglycerides levels by interfering with hepatic VLDL production rather than inhibiting adipocyte lipolysis.

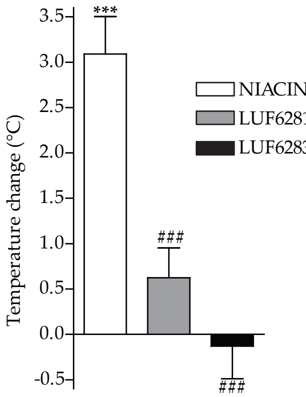

Figure 5. Mouse flushing after administration of niacin, LUF6281, and LUF6283. The cutaneous vasodilation was determined by change in paw skin temperature in C57BL/6 mice. Mice received niacin, LUF6281, or LUF6283 (400 mg/kg/day) via oral gavage. Data are expressed as the change of skin temperature at 20 min after compound administration (n=10 per group). ***$P<0.001$ vs untreated mice; ### $P<0.001$ vs niacin-treated mice.

Discussion and conclusions

Although niacin can effectively lower lipid levels through inhibition of adipocyte lipolysis, its clinical use has been restricted by its off target cutaneous flushing effect that is mediated by HCA_2 located in skin Langerhans cells and keratinocytes [12, 22]. To overcome this problem, a variety of niacin derivates have been developed in the past that act as partial or biased agonists for HCA_2 [18-19, 23]. To date, the most promising lead compound has been the pyrazole MK-0354 which was shown to stimulate the G protein pathway associated with anti-lipolysis in adipocytes without inducing parallel ERK1/2 phosphorylation, an in vitro measure for flushing [23]. Also in pre-clinical mouse studies MK-0354 showed promising results as no vasodilatation (flushing) was observed while the anti-lipolytic activity was retained. Unfortunately, in clinical trials this compound eventually failed since it did not provide the beneficial effect of niacin on plasma lipid levels [17]. Although the clinical failure of MK-0354 argues against a high potential for this class of compounds in general, our current studies indicate that there is still hope for pyrazoles. More specifically, here we present data on the potential of two novel partial HCA_2 agonists of the pyrazole class to lower plasma VLDL-triglyceride levels without

causing the off target flushing response.

Our in vitro studies suggest that niacin, LUF6281, and LUF6283 may all have a certain bias, since these compounds all show a higher potency for ERK1/2 phosphorylation than for G protein activation. Furthermore, LUF6281 and LUF6283 were both partial agonists in the [^{35}S]-GTPγS assay but high efficacy full agonists in the ERK1/2 phosphorylation assay. The fold difference in potency was dependent on the compound; niacin was 21-fold more potent for ERK phosphorylation, while LUF6283 and LUF6281 were respectively 10-fold and only 6-fold more potent. The high potency of niacin for activation of the MAP kinase pathway may explain why this compound causes flushing so effectively. Previous findings of Walters et al. have suggested an important role for an additional pathway, i.e. beta-arrestin dependent signaling, in ERK phosphorylation and the flushing response

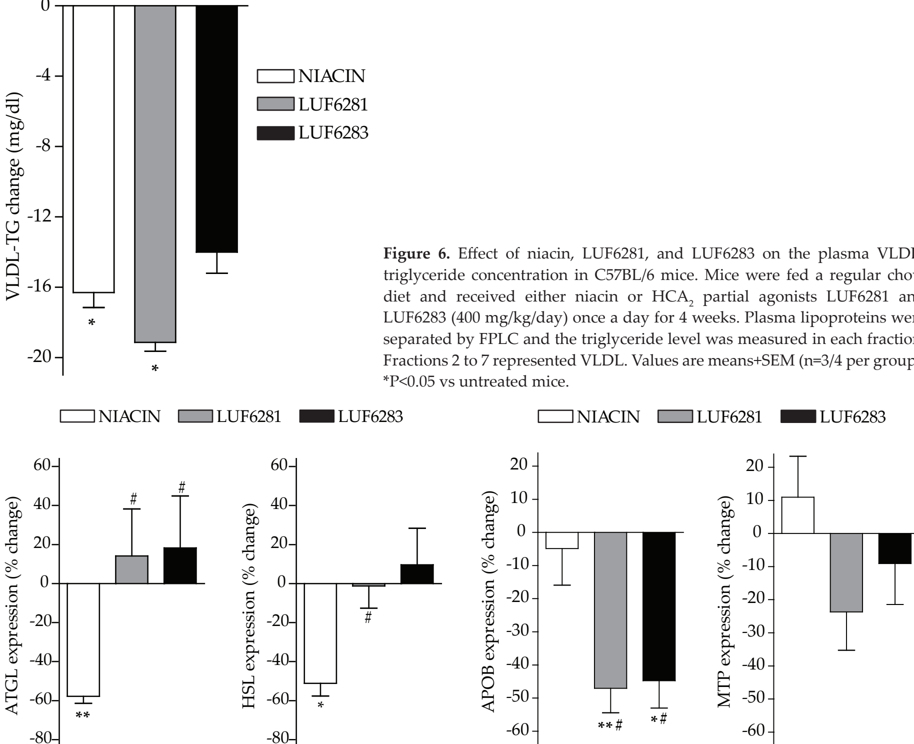

Figure 6. Effect of niacin, LUF6281, and LUF6283 on the plasma VLDL-triglyceride concentration in C57BL/6 mice. Mice were fed a regular chow diet and received either niacin or HCA_2 partial agonists LUF6281 and LUF6283 (400 mg/kg/day) once a day for 4 weeks. Plasma lipoproteins were separated by FPLC and the triglyceride level was measured in each fraction. Fractions 2 to 7 represented VLDL. Values are means+SEM (n=3/4 per group). *P<0.05 vs untreated mice.

Figure 7. Effect of niacin, LUF6281, and LUF6283 on relative gene expression levels of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) in white adipose tissue of C57BL/6 mice. Data are presented as percent change relative to the untreated group. Values are means+SEM (n=5/6 per group). *P<0.05 ** P<0.01 vs untreated mice; # P<0.05 vs niacin-treated mice.

Figure 8. Effect of niacin, LUF6281, and LUF6283 on hepatic relative gene expression levels of apolipoprotein B (APOB) and microsomal triacylglycerol transfer protein (MTP) in C57BL/6 mice. Data are presented as percent change relative to the untreated group. Values are means+SEM (n=5/6 per group). *P<0.05 ** P<0.01 vs untreated mice; # P<0.05 vs niacin-treated mice.

associated with niacin treatment [24]. Since we do not possess data on the effect of our compounds on beta-arrestin membrane recruitment, we cannot draw a firm conclusion whether our compounds are biased partial agonists. However, since all three compounds

in the current study are more potent in the ERK-phosphorylation assay (lower EC_{50} values), we do not anticipate that biased agonism is the reason for their negligible flushing effect. Although, unlike MK-0354, our pyrazole compounds are still active in the ERK1/2 assay, we hypothesized that their relatively low ratio of GTPγS over ERK1/2 might still attenuate the flushing response. Indeed, our in vivo findings in C57BL/6 mice confirm that the pyrazoles do not provoke a similar flushing response as niacin.

Both pyrazoles however induced a similar level of VLDL-triglyceride lowering as observed with niacin treatment, suggesting that they do also exhibit the anti-lipolytic activity of niacin. Strikingly, niacin treatment decreased the relative expression level of the lipolytic genes ATGL and HSL in adipose tissue, while the two LUF compounds did not. From these combined findings it seems that the novel partial agonists – in contrast to niacin – actually do not execute their VLDL-triglyceride lowering action by modulating adipocyte lipolysis. Our further gene expression analysis suggests that the compounds rather lower plasma triglyceride levels by inhibiting VLDL production, since they decrease the hepatic relative expression level of APOB. The assembly and secretion pathway of VLDL in the liver involves the transfer of lipid by MTP to APOB during translation and then the fusion of APOB-containing precursor particles with triglyceride droplets to generate mature VLDL particles [25-26]. APOB is thus essential for proper assembly and secretion of APOB-containing lipoproteins [27] and several novel lipid lowering therapies are therefore based on decreasing APOB transcription and mRNA stability by antisense oligonucleotides [28-29]. As our new HCA_2 partial agonists target another metabolic pathway, i.e. hepatic VLDL production, as compared to both niacin and MK-0354 (inhibition of adipocyte lipolysis), we anticipate that they may still also effectively achieve lipid lowering in the human situation. However, their efficacy in the clinical setting remains to be validated.

In conclusion, the current study identifies the two HCA_2 partial agonists LUF6281 and LUF6283 as promising drug candidates to achieve the beneficial lipid lowering effect of niacin without producing the unwanted flushing side effect.

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Chapter 4

Structure-activity relationships of trans-substituted-propenoic acid derivatives on the nicotinic acid receptor HCA_2 (GPR109A)

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Supplementary information can be found online with this publication.

Abstract

Nicotinic acid (niacin) has been used for decades as an antidyslipidemic drug in man. Its main target is the Hydroxy-Carboxylic Acid receptor HCA_2 (GPR109A), a G protein-coupled receptor. Other acids and esters such as methyl fumarate also interact with the receptor, which constituted the basis for the current study. We synthesized a novel series of substituted propenoic acids, such as fumaric acid esters, fumaric acid amides and cinnamic acid derivatives, and determined their affinities for the HCA_2 receptor. We observed a rather restricted binding pocket on the receptor with trans-cinnamic acid being the largest planar ligand in our series with appreciable affinity for the receptor. Molecular modeling and analysis of the structure-activity relationships in the series suggest a planar trans-propenoic acid pharmacophore with a maximum length of 8 Å and out-of-plane orientation of the larger substituents.

Since the 1950's nicotinic acid (niacin) has been used as an antidyslipidemic drug in man. Even today nicotinic acid is the most efficacious drug to raise the levels of HDL, the "good" cholesterol [1]. In 2003 different groups identified that the lipid-lowering actions of nicotinic acid are mediated by the G protein-coupled receptor HCA_2. HCA_2 is also known as GPR109A, HM74A, NIACR1 or, in mice, as PUMA-G. It is a member of a G protein-coupled receptor subfamily involved in metabolism, with HCA_3 (GPR109B) and HCA_1 (GPR81) as closely related members [2-5]. The HCA_2 receptor is primarily expressed in adipocytes, spleen tissue, retinal pigment epithelium [6], intestinal epithelium [7] and various immune cells such as monocytes and macrophages [8]. Unfortunately, the HCA_2 expression in a type of epidermal macrophages known as Langerhans cells is the cause of flushing of the skin, a harmless but unpleasant side effect which undermines treatment compliance [3, 9].

Due to the discovery of the HCA_2 receptor, industrial and academic groups have now started or intensified synthetic research lines to improve on the poor safety and pharmacokinetic properties of nicotinic acid. The majority of promising novel agonists, such as derivatives of acifran, anthranilic acids, anthranilic acid bioisosteres, xanthines, barbituric acid and pyrazole-3-carboxylic acids, was published and/or patented by GSK, Merck, Arena, Schering-Plough, Roche, Incyte, and our group [10]. Recently, 'simple' acids such as trans-cinnamic acid and 4-hydroxy-cinnamic acid have been described as modestly active HCA_2 receptor agonists with potencies in the higher micromolar range. Cinnamic acid derivatives had been described before as anti-inflammatory compounds and as suppressors of elevated blood lipid levels in atherosclerosis [11-13].

Some other simple acid derivatives, i.e. methyl fumarate and ethyl fumarate, were also reported as potent agonists for the HCA_2 receptor [14]. These fumarates have long been known as anti-psoriasis compounds [15]. They are micromolar affinity agonists for the HCA_2 receptor, but have not been extensively explored in a synthetic structure-activity approach. Therefore, we decided to investigate the medicinal chemistry of such fumaric and cinnamic acid derivatives in more detail. These constrained propenoic acid derivatives appeared also useful in a pharmacophore analysis, which we also performed.

The fumaric acid esters 2 and 3 (Table 1) were commercially available. Compounds 4-24 (Table 1 and Supplementary information) were synthesized according to two methods; A) starting from fumaric acid (1), the appropriate alcohol and EDC dissolved in DMF.[16] B) starting from a mixture of fumaric acid (1) and the suitable alcohol dissolved in DMF under microwave conditions [17]. Method A resulted in a mixture of both trans (4, 9-11, 13) and cis isomers of the desired esters, even if the reaction was carried out at 0 °C. Due to the difficult separation of the two isomers this method was eventually not preferred. According to method B, described by Averyanov [17], an equimolar mixture of 1 and the appropriate alcohol in DMF was heated in a sealed tube in the microwave at 180 °C. This method resulted solely in the desired trans substituted fumarates (5-8, 12, 14-24). With both methods also a substantial amount of the disubstituted fumarates was formed.

The propenoic acids with aromatic rings (49, 52-54, 57 and 58) that were not commercially available were prepared in a 32-74% yield (Table 2 and Supplementary information), catalyzed by piperidine via the Knoevenagel condensation of the commercially available

aromatic aldehydes and malonic acid (47) [18].

All compounds listed in Tables 1 and 2 were tested at 10 μM in radioligand binding assays for displacement of [^{3}H]-nicotinic acid (20 nM) from the human HCA_2 receptor stably expressed in HEK293 cells. Homologous displacement with unlabeled nicotinic acid yielded a K_i value of 64 nM for nicotinic acid (data not shown). Both the methyl and ethyl fumarates (2 and 3) also displayed submicromolar affinities for the HCA_2 receptor (Table 1), comparable to data reported by Tang [14]. In comparison with the reference agonist nicotinic acid, only a 3- (methyl derivative) or 7-fold (ethyl derivative) lower affinity was obtained. The unsubstituted fumaric acid (1) did not display any appreciable affinity towards the receptor, suggesting that the intact ester is crucial for receptor activity. In a series of aliphatic fumarate esters, increasing size did not substantially affect the receptor affinity. The propyl, butyl and pentyl substituents (4, 6 and 7) showed affinities between 0.7 and 1.0 μM, which is in the same range as the ethyl derivative. The larger hexyl substituent (8) resulted in a slightly poorer K_i value of 2.5 μM. Branched aliphatic compounds were also less tolerated e.g. derivatives 5 and 9.. A phenyl substituent (10) was not well tolerated either, but introduction of a spacer between the fumarate moiety and the aromatic system resulted in a gain of affinity. The methylene spacer, as in 11 (K_i = 3.5 μM), appeared to be optimal since α-methylbenzyl (12), phenylethyl (13) and phenylpropyl (14) substituents

Table 1. Affinities of substituted fumaric acid esters **1-24** in radioligand binding assays of the human HCA_2 receptor.

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Compound	R	K_i (μM) or % disp.[a]
1	H	10%
2	Me	0.18 ± 0.03
3	Et	0.41 ± 0.02
4	Pr	1.0 ± 0.1
5	iPr	4.2 ± 0.9
6	Bu	0.76 ± 0.19
7	Pe	0.70 ± 0.05
8	Hex	2.5 ± 0.03
9	cHex	17%
10	Phenyl	10%
11	Benzyl	3.5 ± 0.2
12	Benzyl-α-methyl rac.	5.7 ± 0.1
13	Phenyl ethyl	10 ± 1
14	Phenyl propyl	26%
15	2-Br Benzyl	0%
16	2-OMe Benzyl	5%
17	3-Br Benzyl	9.8 ± 0.6
18	3-Cl Benzyl	8.9 ± 1.5
19	3-F Benzyl	2.4 ± 0.6
20	3-OMe Benzyl	4%
21	4-Br Benzyl	21%
22	4-Cl Benzyl	14%
23	4-Me Benzyl	30%
24	4-OMe Benzyl	7%

[a] K_i ± SEM (n = 3), % Displacement at 10 μM (average of n = 2, with less than 10% difference between the two values). K_i values were determined in full displacement studies on membranes from HEK293T cells stably expressing HCA_2 (GPR109A), using [^{3}H]-nicotinic acid as the radioligand. Single point displacement assays were carried out using 10 μM of the test compound and 20 nM [^{3}H]-nicotinic acid

resulted in K_i values of 5.7 μM and 10 μM, and 26% of radioligand displacement at 10 μM, respectively. Subsequently, various additional substitutions of the benzylic ring system were explored. The binding pocket of the HCA_2 receptor was not able to accommodate the ortho substituted compounds 15 and 16 at a concentration of 10 μM. Meta substitution, on the other hand, was better tolerated (17-20). The 3-bromo- and 3-chloro-benzyl derivatives (17 and 18) showed a slight decrease in affinity and the smaller 3-fluoro-benzyl compound (19) a slight increase in affinity with respect to the unsubstituted benzyl fumarate. On the contrary the 3-methoxy-benzyl derivative 20 showed no affinity for the receptor. Furthermore, introduction of para substituents such as halogen, methyl or methoxy (21-24) resulted in a reduced affinity.

Table 2. Affinities of *trans*-substituted-propenoic acids **25**-**58** in radioligand binding assays on the human HCA_2 receptor.

Compound	R	R^1	K_i (μM) or % disp.[a]
25	Me	H	6%
26	Et	H	19%
27	Me	Me	0%
28	Phenyl	H	4.9 ± 1.8
29	2-OH Phenyl	H	4%
30	2-Me Phenyl	H	0%
31	3-OH Phenyl	H	0%
32	3-Me Phenyl	H	0%
33	3-Cl Phenyl	H	6%
34	3-NO_2 Phenyl	H	0%
35	4-OH Phenyl	H	14 ± 2
36	4-Me Phenyl	H	2%
37	4-Cl Phenyl	H	17%
38	4-OMe Phenyl	H	0%
39	4-NH_2 Phenyl	H	2%
40	4-$N(CH_3)_2$ Phenyl	H	0%
41	3,4-di-OH Phenyl	H	4%
42	3-OMe, 4-OH Phenyl	H	0%
43	3,4-OCH_2O- Phenyl	H	7%
44	Phenyl	Me	1%
45	Phenyl	Phenyl	0%
46	Phenyl	NHCOMe	0%
48	Pyridin-3-yl	H	3%
49	Pyrrol-2-yl	H	7%
50	Furan-3-yl	H	14 ± 2.5
51	Furan-2-yl	H	8.1 ± 0.8
52	5-Br-furan-2-yl	H	9%
53	5-Me-furan-2-yl	H	7%
54	5-Et-furan-2-yl	H	14%
55	5-(4-Cl-Ph)-furan-2-yl	H	0%
56	Thiophen-2-yl	H	5.5 ± 0.3
57	3-Br-thiophen-2-yl	H	6%
58	4-Br-thiophen-2-yl	H	6%

[a] See footnote Table 1

Since the ester moiety in methyl and ethyl fumarate can be hydrolysed in vivo[19], we investigated the non-hydrolysable amide linker as an alternative. However, these trans amide isosteres of compounds 2, 3, 10 and 11 were not able to bind to the receptor at 10 μM (data not shown).

To further explore the SAR, a number of cis anologs of the active trans fumaric acid derivatives were synthesized and tested, namely the maleic acid esters and maleic acid amides. Nevertheless none of these cis compounds interacted with the receptor (data not shown).

Next, a series of trans substituted propenoic acids (25-46, 48-58) were tested for their

affinities (Table 2). The small aliphatic compounds 25-27 were without effect, while the phenyl derivative 28 (cinnamic acid) showed micromolar affinity. In our assay, compound 28 showed a higher affinity (K_i = 4.9 μM) compared to the K_i value of 36 μM reported by Ren and colleagues.[20] To explore this lead, commercially available aromatic substituted trans propenoic acids were tested for their affinities (29-43). Only the 4-hydroxy derivative 35 was able to bind with an affinity of 14 μM. Related substituents such as 4-methoxy (38) and 4-amino (39) decreased the affinity dramatically. In general, except for the 4-hydroxy, aromatic substitution is not tolerated on the ortho, meta or para position. Also substituents at the β-position of cinnamic acid (44-46) resulted in a dramatic decrease in binding. Replacement of the phenyl moiety in cinnamic acid by aromatic isosteres such as 3-pyridinyl (48) and 2-pyrrole (49) resulted in a significant loss of affinity. On the contrary, 2-furanyl (51) and 2-thiophenyl (56) were accommodated like the phenyl compound. 3-Furanyl substitution (50) resulted in a 2 fold decrease compared to the 2-furanyl derivative 51. As in the cinnamic acid series, the 5-substituted 2-furanyl derivatives (52-55) and both the 3-bromo and 4-bromo-substituted 2-thiophenyl derivatives (57, 58) were devoid of affinity for the receptor.

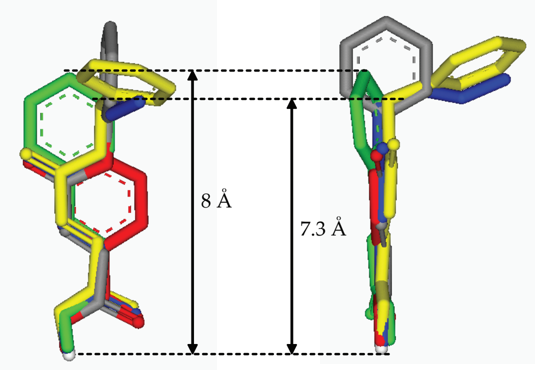

Figure 1. Aligned pharmacophore model (left- nicotinic acid in plane, right – nicotinic acid 90 degrees rotated) constructed of the active compounds nicotinic acid (red), butyl fumarate **6** (blue), benzyl fumarate **11** (yellow) and cinnamic acid **28** (green) and the inactive compound phenyl fumarate **10** (grey).

To visualize the SAR, a pharmacophore model was generated by manually superimposing the minimized structures of: nicotinic acid, cinnamic acid 28, fumaric acid esters 6 and 11, and the inactive phenylfumaric acid ester 10 (Figure 1). The alignment of the two sp^2 carbons of the propenoic fragment, which all the compounds have in common, resulted in a planar and constrained pharmacophore. The carbonyl oxygen of the ester function of compounds 6, 10 and 11 and the nitrogen of nicotinic acid overlay smoothly as a hydrogen acceptor region. This might explain the improved binding characteristics of the fumaric acid esters compared to cinnamic acid and also why the trans configuration is superior over the cis substituted propenoic acids. Molecular modeling and analysis of the structure-activity relationships in the series suggest a planar trans-propenoic acid pharmacophore with a maximum length of 8 Å, because this is the size of the largest planar ligand (28) in our series with appreciable affinity for the receptor. Larger compounds need an out-of-

plane orientation as in the case of the fumaric acid ester series (2-24).
Molecular modeling studies of the Merck Research group based on anthranilic acid derivatives confirmed the importance of the planar orientation of the carboxylic acid function and the nearby α,β sp^2 carbon atoms [21][22]. Full saturation of the phenyl ring in anthranilic acid resulted in inactive compounds. If the double bond in the α,β position was maintained, as in tetrahydro-anthranilic acids, the planar orientation and also the affinity was regained however [22].
In conclusion, methyl fumarate, ethyl fumarate and cinnamic acid have been published as agonists for the HCA_2 receptor [14, 20]. Our synthetic program confirmed the affinity of these compounds for the HCA_2 receptor and further explored the structure-activity relationships for a series of derivatives. Molecular modeling studies and the analysis of the structure-activity relationships in the series suggest a planar trans-propenoic acid pharmacophore with a maximum length of 8 Å and out-of-plane orientation of the larger substituents.

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Chapter 5

Novel 3,6,7-substituted pyrazolopyrimidines as positive allosteric modulators for the hydroxy-carboxylic acid receptor 2 (GPR109A)

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Supporting information: Tables S1-S3, figures S1 and S2, additional notes, and biological methods are included at the end of this chapter, and on http://pubs.acs.org supporting information on the synthesis and characterization of chemical compounds is also available.

Abstract

A number of pyrazolopyrimidines were synthesized and tested for their positive allosteric modulation of the HCA_2 receptor (GPR109A). Compound 24, an efficacious and potent agonist and allosteric enhancer of nicotinic acid's action, was the basis for most other compounds. Interestingly, some of the compounds were found to increase the efficacy of the endogenous ligand 3-hydroxybutyrate and enhance its potency almost 10-fold. This suggests that the pyrazolopyrimidines may have therapeutic value when given alone.

Abbreviations

NA, nicotinic acid; 3-OHB, 3-hydroxybutyrate; $[^{35}S]$-GTPγS, ^{35}S-labeled guanosine 5'-O-[gamma-thio]triphosphate; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle Medium; DTT, dithiothreitol; GDP, guanosine 5'-diphosphate; GPCR, G protein-coupled receptor; HCA, hydroxy-carboxylic acid receptor; HEK, human embryonic kidney cells; PAM, positive allosteric modulator (allosteric enhancer).

Introduction

Hydroxy-Carboxylic Acid Receptor 2 (HCA_2) [1] or GPR109A is a G protein-coupled receptor (GPCR) that was first identified in 2001 [2]. Two years later its involvement in the anti-lipolytic action of nicotinic acid (NA) was reported [3-5] and 3-hydroxybutyrate was identified as its endogenous ligand in 2005 [6] (for a review [1, 7]). A broad range of synthetic ligands (agonists only) for this receptor has been developed [7-9]. An intriguing class among those are substituted pyrazolopyrimidines, as recently reported by Shen and colleagues [10]. These compounds act as agonists, some with potencies comparable to nicotinic acid, but were also suggested to bind allosterically to HCA_2. One compound in particular (25 in the present study) was shown to behave as a positive allosteric modulator (PAM) as well, by significantly enhancing the potency of nicotinic acid at HCA_2 (~100-fold at 1 μM). We decided to further investigate the pyrazolopyrimidines by synthesizing a new series of derivatives and evaluating their activity on HCA_2 in several [^{3}H]-nicotinic acid and [^{35}S]-GTPγS binding assays.

Chemical synthesis

3 R^2 = Me
4 R^2 = OEt
5 R^2 = Et
6 R^2 = Pr

1 R = 4-*i*PrPh
2 R = H

	R^1	R^2
7	4-*i*PrPh	Me
8	4-*i*PrPh	OH
9	4-*i*PrPh	Cl
10	4-*i*PrPh	H
11	4-*i*PrPh	Et
12	4-*i*PrPh	Pr
13	H	Me
14	Br	Me

	R^1	R^2
15	4-*i*PrPh	Me
16	4-*i*PrPh	H
17	4-*i*PrPh	Et
18	4-*i*PrPh	Pr
19	Br	Me

20-30 R^1 = 4-*i*PrPh, Table 1
31 R^1 = Br

32-43 Table 1

[a]Reagents and conditions: (a) EtOH, reflux, 3 h; (b) $POCl_3$, N,N-dimethylaniline, reflux, 3 h; (c) NaOAc, 5% Pd/C, H_2 2.5 bar, RT, 1 h; (d) NBS, DCM, 0 °C, 1.5 h, RT, 16 h; (e) LiOH, H_2O/MeOH/THF, RT, 16 h; (f) R^3-NH_2, EDC*HCl, DCM, RT, 4 h; (g) R^1-$B(OH)_2$, 31, $Pd(Ph_3P)_4$, Na_2CO_3, toluene/H_2O, MW 150 °C, 2 h.

Scheme 1. Synthetic route to the substituted pyrazolo[1,5-a]pyrimidine-carboxamides **20-43**[a]

The synthetic route used to obtain the pyrazolo[1,5-a]pyrimidine-6-carboxamides **20**-**30** and **32**-**43** is depicted in Scheme 1. The 1*H*-pyrazol-5-amine **1** [10] or the commercially available compound **2** were ring closed with the respective ethyl 2-(ethoxymethylene)-3-oxoate [11] (**3**, **5** or **6**) or the available diester **4** in EtOH at reflux temperature, resulting in the ethyl-pyrazolo[1,5-a]pyrimidine-6-carboxylates (**7**, **8** and **11**-**13**) [10, 12-13] in good yields. The 7-hydroxypyrazolopyrimidine **8** was converted into the 7-chloro analogue (**9**) in the presence of $POCl_3$ [13] followed by palladium-catalyzed reductive dechlorination [13]

Table 1. Different measures for HCA_2 receptor activation in [^{35}S]-GTPγS binding assays

Nr.	R¹	R²	R³	[^{35}S]- GTPγS binding: %E_{max} cpd. alone (SEM)[a]	EC$_{50}$ shift NA[b]	%E_{max} NA (SEM)[c]
20	4-*i*Pr	Me		3 (1)	0.60	129 (9)
21	4-*i*Pr	Me		23 (1)	0.32	135 (5)
22[d]	4-*i*Pr	Me		22 (8)	0.36	134 (7)
23	4-*i*Pr	Me		1 (2)	1.07	128 (4)
24[d]	4-*i*Pr	Me		71 (17)	0.04	109 (6)
25[d]	4-*i*Pr	Me	(R)	5 (2)	0.53	115 (9)
26[d]	4-*i*Pr	Me	(R)	22 (0.4)	0.22	100 (1)
27	4-*i*Pr	Me		41 (6)	0.15	139 (10)
28	4-*i*Pr	H		10 (3)	0.62	106 (7)
29	4-*i*Pr	Et		69 (7)	0.08	151 (6)
30	4-*i*Pr	Pr		58 (20)	0.17	151 (9)
32	4-Me	Me		52 (4)	0.13	113 (7)
33	3-Me	Me		17 (2)	0.25	119 (5)
34	2-Me	Me		3 (1)	0.58	91 (6)
35	4-Et	Me		64 (9)	0.13	153 (15)
36	4-*t*Bu	Me		80 (5)	0.09	141 (4)
37	4-OMe	Me		20 (3)	0.24	129 (3)
38	4-O*i*Pr	Me		50 (3)	0.09	139 (2)
39	4-Ph	Me		41 (4)	0.16	139 (6)
40	4-Cl	Me		42 (9)	0.16	141 (14)
41	3-Cl	Me		16 (6)	0.37	126 (13)
42	3,4 diCl	Me		31 (5)	0.19	139 (12)
43	4-CF_3	Me		47 (6)	0.13	147 (8)

a) Percentage of [^{35}S]-GTPγS binding in the presence of 10 μM of the test compound alone (100% is the E_{max} at 100 μM nicotinic acid); b) the shift in EC_{50} of nicotinic acid (NA) in the presence of 10 μM of the test compound (1 is without test compound and values less than unity indicate increased affinity of nicotinic acid, e.g. 0.1 means a 10-fold shift); c) the E_{max} reached at 100 μM nicotinic acid (NA) in the presence of 10 μM of the test compound (100% is without test compound); d) also reported by Shen et al [10] (**26**, racemate only). Values are means (± SEM) of 3 independent experiments performed in duplicate.

to give the pyrazolo[1,5-a]pyrimidine **10**. The 3-position of the pyrazolo[1,5-a]pyrimidine compound **13** [14] was functionalized using NBS [15] to the versatile 3-bromo analogue **14**. Subsequently, hydrolysis of the ethyl esters **7**, **10**-**12** and **14** with LiOH resulted smoothly in the corresponding acids (**15**-**19**). The final pyrazolo[1,5-a]pyrimidine carboxamides **20**-**30** and the 3-bromo building block **31** were synthesized from the corresponding acids (**15**-**19**) and a range of amines by use of coupling reagent EDC*HCl. Finally a Suzuki reaction with building block 31 and various arylboronic acids, under microwave conditions, yielded the 3-aryl compounds 32-43 [16].

Biological evaluation and discussion

To evaluate the compounds we performed [^{3}H]-nicotinic acid binding assays (see Supporting Information, Table S1) and [^{35}S]-GTPγS binding assays (Table 1) on HEK-HCA_2 membranes. In Table 1 the following results are reported: a) the percentage of [^{35}S]-GTPγS binding in the presence of 10 μM of the test compound alone; b) the shift in EC_{50} value of nicotinic acid in the presence of 10 μM of the test compound; c) the E_{max} reached at 100 μM nicotinic acid in the presence of 10 μM of the test compound (100% is without test compound).

Structure-activity relationships

Compounds **20**-**23** show that linker lengths of 2 or 3 carbons between the carboxamide and the phenyl group are preferred for agonism and potentiation of nicotinic acid's effects. However, linker length does not appear to influence the E_{max} of nicotinic acid, which was approximately 130% for all four compounds. Introduction of an ether function in the linker (**24**) resulted in a higher activity in the [^{35}S]-GTPγS binding assay. So, the compound acted as an agonist in its own right, while the EC_{50} value of nicotinic acid was increased approximately 25-fold without a change in its E_{max} value. This compound also considerably slowed the dissociation of [^{3}H]-nicotinic acid from the receptor and increased the equilibrium specific binding of the radioligand to an extent that was not surpassed by any of the other test compounds (Supporting Information, Table S1). Introduction of a methyl substituent on the linker (**25**) was not beneficial in our hands, as opposed to the findings of Shen et al [10]. Removal of the phenyl group yielded **26**, which enhanced nicotinic acid's activity somewhat better. Replacing the ether of **24** by a secondary amine to obtain aniline **27** rendered the compound more active than **25** in all respects.

Next, a small series was synthesized with varying substituents on the 7-position (R^2, Table 1). Absence of the methyl substituent (**28**) severely reduced the activity compared to **24**. The ethyl and propyl substituted compounds (**29** and **30**) behaved highly similar to each other, and also to the parent derivative **24** except for the enhancement of nicotinic acid's E_{max} value by **29** and **30** but not **24**. Finally, a series of derivatives of **24** with varying substituents on the 3-phenyl ring was then tested (R^1, Table 1, compounds **32**-**43**). A methyl substituent on the para position (**32**) resulted in more agonism and potentiation of nicotinic acid compared to methyl substituents on the *meta* (**33**) or *ortho* (**34**) positions. Compounds with ethyl (**35**) and *tert*-butyl (**36**) substituents had activities highly similar to the isopropyl-substituted compound **24**, except that **35** and **36** significantly increased

the E_{max} value of nicotinic acid, which was not affected by **24**. Compounds substituted with methoxy (**37**) or isopropoxy (**38**) were also highly active. These compounds, like methyl derivatives **32** and **33**, seemed to have a more modest agonistic activity compared to their activity as enhancers of nicotinic acid potency. A phenyl derivative (**39**) retained activity in the [^{35}S]-GTPγS binding assay. *Meta*-substitution (**41**) and, to a lesser extent, *para*-substitution (**40**) with chlorine deminished activity. The 3,4-dichloro-substituted compound (**42**) behaved very similarly to the 4-chloro derivative (**40**). A trifluoromethyl group (**43**) conferred good enhancement of nicotinic acid potency and efficacy, paired with a moderate agonistic activity. To investigate any correlation between the agonistic and modulating effects of the pyrazolopyrimidines, the [^{35}S]-GTPγS binding activation and the potency shift of nicotinic acid, both at 10 μM, were plotted against each other (Supporting Information Figure S2), yielding a nonlinear correlation. The plot suggests that the ability of the pyrazolopyrimidine agonists to stabilize active receptor conformations contributes to, or even determines, the modulator strength of the compounds.

Modulation of nicotinic acid-mediated receptor activation

We next examined the effects at 1, 3 and 10 μM of five selected enhancers on the concentration-effect curves for nicotinic acid in [^{35}S]-GTPγS binding assays. Figure 1 shows the results of a representative experiment, and the average values obtained from 3 independent experiments are discussed below. Compound 24 reached 71±17% receptor

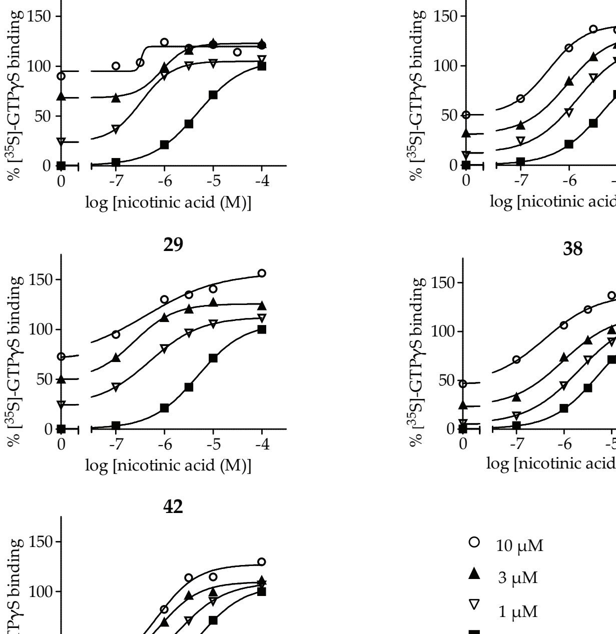

Figure 1. Dose-response curves of nicotinic acid in the presence of 0, 1, 3 and 10 μM pyrazolopyrimidines. The data are from [^{35}S]-GTPγS binding assays performed on HEK-HCA_2 membranes. Representative graphs from one experiment performed in duplicate.

activation at 10 μM without any nicotinic acid present, and nicotinic acid further increased activation to 109±6%, with a 24-fold increased potency compared to control.
At concentrations of 3 μM and 1 μM, 24 increased [^{35}S]-GTPγS binding to 51±14% and 30±5% respectively, and caused shifts in the EC_{50} value of nicotinic acid of approximately 5-fold for both concentrations. Compound 27 caused modest increases in both the potency (2-fold at 1 μM, 5-fold at 3 μM and 7-fold at 10 μM) and the E_{max} values (120±3% at 1 μM, 129±3% at 3 μM and 139±10% at 10 μM) of nicotinic acid, which seemed to follow the increase in [^{35}S]-GTPγS binding due to agonist activity alone (13±2% at 1 μM, 31±2% at 3 μM and 41±6% at 10 μM). Compound 29 was highly efficacious in all aspects: an agonist in its own right (28±3, 53±5 and 69±7% receptor activation at 1, 3 and 10 μM, respectively) and a positive allosteric modulator of nicotinic acid's EC_{50} (5, 9 and 13-fold shifts) and E_{max} (122±7, 129±6 and 151±6% of control) values. Both 38 and 42 were in every respect less potent and efficacious than 29. As an agonist, 38 was more potent and efficacious than 42, causing more receptor activation at 1 μM (15±6 vs 5±3%), 3 μM (27±2 vs 16±3%) and 10 μM (50±3 vs 31±5%). The modulation of the EC_{50} value of nicotinic acid was similar at 1 μM (both 2-fold) and 3 μM (5-fold vs 4-fold) but at 10 μM 38 was the more active compound again (11-fold vs 5-fold shift). Both compounds increased the E_{max} of nicotinic acid similarly (139±12; 139±6% at 10 μM). However, compound 42 showed a relatively low potency compared to 38 since no effect was seen at 1 μM (compared to 111±4% for 38) and only a small effect at 3 μM (114±3% for 42 compared to 123±5% for 38). For EC_{50} values and E_{max} values of 24, 29, 30, 32, 35, 36 and 38, tested again in the absence of nicotinic acid, see Table S2 in Supporting Information. Some further observations are discussed on page S7 of Supporting Information.

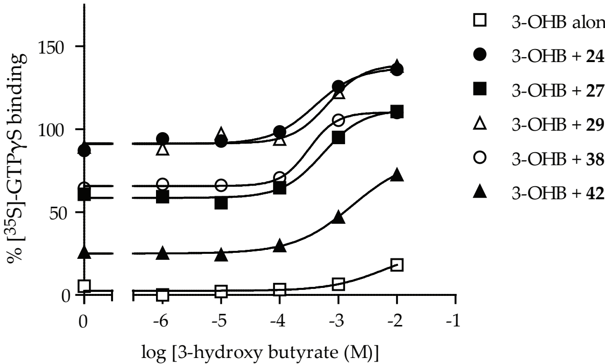

Figure 2. Concentration-response curves of 3-hydroxybutyrate in the presence and absence of 10 μM pyrazolopyrimidine in the presence and absence of 10 μM pyrazolopyrimidine. The data are from [^{35}S]-GTPγS binding assays performed on HEK-HCA_2 membranes. Representative graphs from one experiment performed in duplicate (see also Table S3 in SI).

Positive allosteric modulation of 3-hydroxybutyrate potency and efficacy

Allosteric modulators that enhance the potency of nicotinic acid on the HCA_2 receptor are of interest clinically since they could greatly reduce the daily dose of nicotinic acid when used in combination therapy. Clearly, the pyrazolopyrimidines fit this picture, but many of them can also activate HCA_2 on their own. In vivo, the activity of the endogenous

ligand 3-hydroxybutyrate (3-OHB) may also be enhanced by the pyrazolopyrimidines, but this cannot be assumed a priori since allosteric enhancement is probe dependent [17]. Therefore, 3-OHB dose-response curves were recorded in the presence of five compounds, and four of these increased the potency of 3-hydroxybutyrate approximately 8-fold (Figure 2 and Supporting Information Table S3). The modulators caused an increase in the intrinsic efficacy of the endogenous ligand as well. It should be noted that in a [^{35}S]-GTPγS binding assay 3-OHB was previously reported as a high-efficacy partial agonist for HCA_2 [18], whereas it behaved as a low-efficacy partial agonist with approx. 30% intrinsic efficacy in our hands. This may have to do with lower levels of receptor expression in our preparation, as we explicitly selected clones for physiological rather than high expression levels.

Future perspectives and conclusion

With the expanded ligand repertoire reported in this study other functional assays with the pyrazolopyrimidines can be performed, including ERK 1/2 phosphorylation assays. Activation of ERK 1/2 induced by HCA_2 agonists in vitro has been suggested to be predictive of the skin flushing side effect in vivo [19]. Furthermore, cooperativity with probes (orthosteric ligands) that are not structurally related to nicotinic acid should be examined. In the future, mutagenesis studies will hopefully shed light on the binding mode of the allosteric modulators and on how these ligands trigger the changes in receptor activation. In conclusion, we presented several pyrazolopyrimidine derivatives that do not displace [^{3}H]-nicotinic acid from HCA_2, but are capable of activating this receptor, which indicates an allosteric binding mode. Next to their agonistic effects these compounds potentiate the action of nicotinic acid and the endogenous ligand 3-hydroxybutyrate. Therapeutically, such positive allosteric modulators may represent an interesting alternative in the search for HCA_2 receptor ligands.

Experimental section

Chemistry

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 400 (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz) spectrometer. Chemical shifts (δ) are reported in ppm relatively to Me_4Si. Purity was confirmed ≥95% by HPLC performed on a Gilson 306 system (detection at 254 nm) equipped with an analytical C18 column in combination with a gradient of mixture A: 1 MeCN/9 H_2O, B: 9 MeCN/1 H_2O. High resolution mass spectra were recorded on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) with an electro spray ion source in positive mode resolution R = 60000 at m/z 400 (mass range m/z = 150-2000). Reactions were routinely monitored on TLC using Merck silica gel F_{254} plates. Microwave reactions were performed in an Emrys Optimizer (Biotage AB). Yields were not optimized. The final products were purified by column chromatography and/or by recrystallization.

General procedure for the synthesis of pyrazolo[1,5-a]pyrimidine carboxamides (20-31)

To a solution of the appropriate pyrazolo[1,5-a]pyrimidine-6-carboxylic acid (15-19, see Supporting Information) (1.0 eq.) and the substituted amine (1.2 eq) in DCM (20 mL per mmol) was added EDC*HCl (1.2 eq.) at room temperature. After 4 h the reaction mixture was concentrated and purified by column chromatography.

3-(4-Isopropylphenyl)7-methyl-N-(2-phenoxyethyl)pyrazolo[1,5-a] pyrimidine-6-carboxamide (24).

Started from acid 15 (0.33 mmol) and 2-phenoxy-ethylamine to give 90 mg (64%) as a yellow solid. ^{1}H NMR ($CDCl_3$): δ 8.48 (s, 1H), 8.14 (s, 1H), 7.88 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 11.2 Hz, 2H), 7.25 (d, J = 8.0 Hz, 2H), 6.95 (t, J = 7.6 Hz, 1H), 6.87 (d, J = 8.0 Hz, 2H), 6.76 (t, J = 5.6 Hz, 1H), 4.22 (t, J = 5.2 Hz, 2H), 3.82 (q, J = 5.2 Hz, 2H), 2.94 (m, 1H), 2.89 (s, 3H), 1.28 (d, J = 6.8 Hz, 6H). HRMS calcd. for $[C_{25}H_{26}N_4O_2+H]^+$ 415.21285, found 415.21274.

General procedure for the preparation of 3-(aryl)-7-methyl-N-(2-phenoxyethyl) pyrazolo[1,5-a]-pyrimidine-6-carboxamides via Suzuki-coupling (32-43)

According to a modified procedure of Berger [16]. A mixture of 31 (1.0 eq.), the substituted-phenylboronic acid (2.0 eq.), tetrakis(triphenyl-phosphine)palladium(0) (0.03 eq.) and sodium carbonate (3.0 eq.) in toluene (3.0 mL) and H_2O (0.5 mL) was heated in the microwave for 2 h at 150 °C. Water was added and the organics were extracted with DCM. The organic layer was dried, concentrated and purified by column (1% MeOH/DCM). Final products were obtained by recrystallization from MeOH.

*3-(4-Chlorophenyl)-7-methyl-N-(2-phenoxyethyl)pyrazolo[1,5-a] pyrimidine-6-carboxamide (**40**)*

Started from 31 (0.33 mmol), and (4-chlorophenyl)boronic acid (0.66 mmol), yield 82 mg (62%). ^{1}H NMR ($CDCl_3$): δ 8.62 (s, 1H), 8.50 (s, 1H), 7.89 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.31 (t, J = 8.0 Hz, 2H), 7.00 (t, J = 7.6 Hz, 1H), 6.92 (d, J = 8.4 Hz, 2H), 6.42 (s, 1H, NH), 4.21 (t, J = 5.2 Hz, 2H), 3.94 (q, J = 5.6 Hz, 2H), 3.03 (s, 3H). HRMS calcd. for $[C_{22}H_{19}ClN_4O_2+H]^+$ 407.12693, found 407.12679.

Biology

[^{35}S]-GTPγS binding assay

This assay was performed in 96-well format in 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.4 at 25 °C with 1 mM DTT, 0.5% BSA and 50 μg/mL saponin freshly added. HEK-HCA_2 membranes (5 μg protein per well in 25 μL) were pre-incubated with 25 μL of 40 μM GDP, in absence or presence of test compound, and 25 μL increasing concentrations of the orthosteric ligand, for 30 min at room temperature. Then, 25 μL [^{35}S]-GTPγS was added (final concentration 0.3 nM) and the mixture was incubated 90 min at 25 °C with constant shaking. The incubation was terminated by filtration over GF/B filterplates on a FilterMate harvester (PerkinElmer). The filters were dried and 25 μL Microscint 20 (PerkinElmer) was added to each filter. After ≥3 h extraction the bound

radioactivity was determined in a Wallac microbeta Trilux 1450 counter.

Data analysis

Analysis of the results was performed using Prism 5.0 software (GraphPad Software Inc., La Jolla, USA). Nonlinear regression was used to determine IC_{50} values from competition binding curves. The Cheng-Prusoff equation [20] was then applied to calculate K_i values. [^{35}S]-GTPγS curves were analysed by nonlinear regression to obtain EC_{50} values.

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Supporting Information

Table S1. Effects on [^{3}H]-nicotinic acid binding of a series of 3-(aryl)-7-methyl-*N*-(substituted)pyrazolo[1,5-a] pyrimidine-6-carboxamides (measured at 10 μM).

Cpd.	R^1	R^2	R^3	% [^{3}H]-nicotinic acid binding (±SEM) in equilibrium[a]	% [^{3}H]-nicotinic acid binding (±SEM) after 30 min dissociation[b]
20	4-*i*Pr	Me		96 (7.8)	91 (5.9)
21	4-*i*Pr	Me		108 (10.5)	114 (10.9)
22	4-*i*Pr	Me		111 (5.0)	99 (7.7)
23	4-*i*Pr	Me		101 (6.8)	112 (9.6)
24	4-*i*Pr	Me		149 (6.1)	133 (2.3)
25	4-*i*Pr	Me	(R)	109 (5.9)	92 (6.2)
26	4-*i*Pr	Me	(R)	69 (4.0)	118 (15.7)
27	4-*i*Pr	Me		107 (7.6)	115 (8.1)
28	4-*i*Pr	H		102 (3.4)	108 (18.7)
29	4-*i*Pr	Et		143 (3.2)	152 (1.4)
30	4-*i*Pr	Pr		148 (9.0)	136 (3.8)
32	4-Me	Me		114 (12.1)	130 (2.4)
33	3-Me	Me		117 (9.0)	145 (11.3)
34	2-Me	Me		111 (4.2)	113 (9.1)
35	4-Et	Me		147 (4.4)	148 (23.4)
36	4-*t*Bu	Me		142 (12.0)	147 (11.5)
37	4-OMe	Me		126 (6.4)	144 (14.3)
38	4-O*i*Pr	Me		143 (6.7)	162 (8.2)
39	4-Ph	Me		106 (3.5)	100 (2.0)
40	4-Cl	Me		108 (0.6)	96 (2.6)
41	3-Cl	Me		101 (4.4)	102 (5.3)
42	3,4 diCl	Me		96 (5.3)	101 (2.6)
43	4-CF_3	Me		119 (4.5)	103 (1.7)

a) the percentage of [^{3}H]-nicotinic acid specifically bound to HEK293-HCA_2 membranes at equilibrium in the presence of 10 μM of the test compound (100% is without test compound).
b) the percentage of [^{3}H]-nicotinic acid still bound to HEK293-HCA_2 membranes after 30 minutes of dissociation at 15 °C induced by 10 μM of nicotinic acid in the presence of 10 μM of the test compound (100% is without test compound - values above 100% indicate a slower dissociation rate in the presence of the test compound). Values are means (± SEM) of 3 independent experiments performed in duplicate.

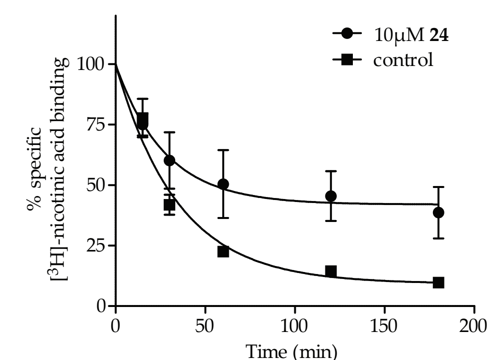
Modulation of [^{3}H]-nicotinic acid binding

In Figure S1-A the effect of 10 μM 24 on the full dissociation curve of the radioligand is shown. The data indicate that in the presence of 24 the dissociation rate is unchanged ($t_{1/2}$ = 30 min for both curves) but that a plateau is reached at 40% specific binding in the presence of the modulator. The control curve reached a plateau at 10%.

For the compounds that markedly increased the binding of [^{3}H]-nicotinic acid in equilibrium assays (Table S1) we attempted to determine an EC_{50} of this effect. However, at 10 μM none of the curves had reached a plateau, and the solubility of the compounds did not permit an increase above this concentration. The curves for three selected compounds, 24, 29 and 38, are shown in Figure S1-B.

Correlation between potentiation of nicotinic acid and allosteric agonism

A



B

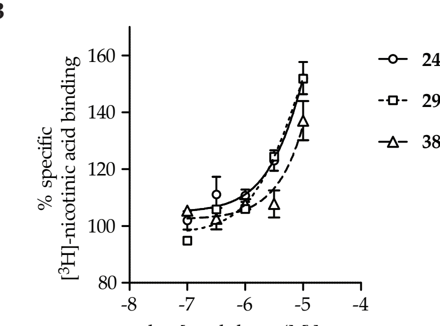


Figure S1. A. Dissociation curves with and without 10 μM **24** at 15 °C. After 3 hours association of 20 nM [^{3}H]-nicotinic acid to HEK-HCA_2 membranes, dissociation was started by addition of 10 μM nicotinic acid. The data points are means ± SEM for 3 independent experiments performed in duplicate. **B.** Binding curves (equilibrium) of compounds **24**, **29** and **38**. The assays were performed using 20 nM [^{3}H]-nicotinic acid and 50 μg HEK-HCA_2 membranes per assay point, and incubation was 1h at 25 °C. The data points are means ± SEM of 3 independent experiments performed in duplicate.

To investigate any correlation between the agonistic and modulating effects of the pyrazolopyrimidines, the [^{35}S]-GTPγS binding activation and the potency shift of nicotinic acid, both at 10 μM, were plotted against each other (Figure S2), yielding a nonlinear correlation. The plot suggests that the ability of the pyrazolopyrimidine agonists to stabilize active receptor conformations contributes to, or even determines, the modulator strength of the compounds.

The compounds at the bottom right of the curve are the most potent modulators and also the most active agonists. More to the left are compounds that are not potent or efficacious agonists, but still fairly active as modulators. However, it appears that a 3-fold or greater shift in nicotinic acid potency is always coupled to some agonist activity in this series. Closest to the origin of the graph is a cluster of compounds, 41, 33, 37 and 26. Replacing the 4-isopropyl by a 3-methyl (33), 3-chloro (41) or 4-methoxy (37) appears to decrease the agonist action significantly while preserving appreciable modulator strength. Removal of the phenyl from the 6-position substituent (R^3) followed by methylation (26) seems to have a similar effect.

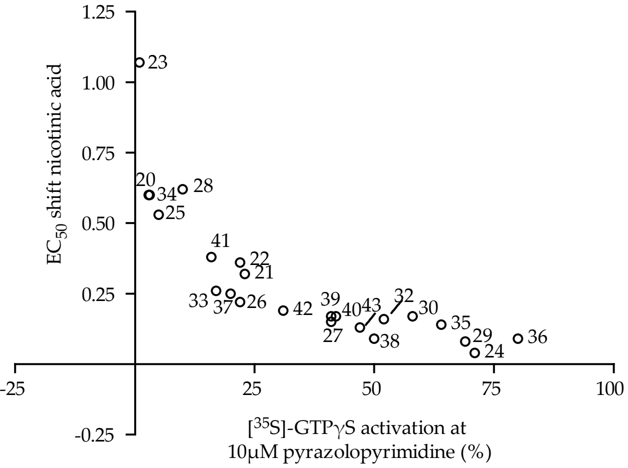


Figure S2. Correlation between potency enhancement of nicotinic acid and agonistic activity of the pyrazolopyrimidines on HCA_2

Modulation of the intrinsic efficacy of nicotinic acid

As described above, many of the active pyrazolopyrimidines are allosteric agonists for HCA_2. In the concentration-effect curves of nicotinic acid with the modulator, this was observed as an increase in the basal receptor activation level. At the same time, many of the same compounds also increased the maximal [^{35}S]-GTPγS binding. In most cases, nicotinic acid still caused an increase in [^{35}S]-GTPγS binding of 100%, identical to the range of the curve in absence of a modulator and typical of a full agonist. However, there were a few cases where the allosteric modulator changed the intrinsic efficacy of nicotinic acid. Thus, in the presence of compounds 24, 32, 36, and, to a lesser extent, 26 and 29, nicotinic acid appeared to be a partial agonist (Table 1). For 36 and 29 this may be due to their high agonist activity and the apparent maximum in E_{max} at approximately 150%, but for the other derivatives this postulated E_{max} ceiling was not reached. For example, in the presence of 10 μM 24, the basal receptor activation was on average 71% and the E_{max} was 109%, which means nicotinic acid only caused a 38% increase in [^{35}S]-GTPγS binding (see Table 1 and Figure 2). At 3 μM 24, nicotinic acid still behaved as a partial agonist with the concentration-effect curve ranging from 51% to 107%, on average.

The lowest concentration of 1 μM 24 had a negligible effect on the intrinsic efficacy of

nicotinic acid: here, the basal [^{35}S]-GTPγS binding was 30% and the maximum was 120%. Compounds 20 and 23 had the opposite effect and made nicotinic acid behave as a superagonist. The effect was highly similar for the two allosteric ligands: in the presence of 10 μM modulator, the basal [^{35}S]-GTPγS binding was unchanged, whereas the E_{max} was increased to approximately 130% (see Table 1 and 2). Thus, the modulators increased the intrinsic efficacy of nicotinic acid by 30%. These enhancers were not tested at lower concentrations.

Pyrazolopyrimidines as allosteric agonists

Further characterization of the most active allosteric agonists in concentration-effect curves (in absence of nicotinic acid) revealed that 24, 35 and 36 have a similar potency to nicotinic acid on HCA_2 (Table S2). The other compounds all seem to have a 2- to 3-fold lower potency, although the difference is not significant. All pyrazolopyrimidines are partial agonists; the most efficacious derivative, 24, has an intrinsic efficacy close to 90%. None of the pyrazolopyrimidines increased [^{35}S]-GTPγS binding in non-transfected HEK cells (data not shown).

Table S2. Agonism of the pyrazolopyrimidines in [^{35}S]-GTPγS assays on HEK-HCA_2 membranes. The values are means (SEM) of 3 independent experiments performed in duplicate.

	EC_{50} (μM) (SEM)	**% EC_{max} (SEM)**
nicotinic acid	4.7 (0.5)	100
24	3.0 (0.6)	87 (8)
29	9.0 (6.8)	75 (3)
30	9.2 (6.7)	70 (2)
32	9.7 (6.5)	65 (6)
35	3.8 (1.3)	72 (5)
36	3.0 (0.6)	70 (9)
38	9.9 (6.4)	64 (6)

Allosteric enhancement of the endogenous ligand 3-hydroxybutyrate

Table S3. Potency and intrinsic efficacy (basal activation by modulator minus E_{max}) of 3-hydroxybutyrate (3-OHB) in the presence and absence of 10 μM pyrazolopyrimidine. The data is from [^{35}S]-GTPγS binding assays performed on HEK-HCA_2 membranes. See also Figure 2. The values are means (SEM) of 3 independent experiments performed in duplicate.

	EC_{50} (μM) (SEM)	**% efficacy (SEM)**
3-OHB alone	3.82 (2.04)	29 (3)
3-OHB+**24**	0.54 (0.14)	39 (6)
3-OHB+**27**	0.62 (0.05)	46 (4)
3-OHB+**29**	0.51 (0.15)	36 (6)
3-OHB+**38**	0.55 (0.16)	51 (8)
3-OHB+**42**	2.58 (1.40)	69 (10)

Experimental Section – Pharmacology

[^{3}H]-Nicotinic acid (60 Ci/mmol) was purchased from BioTrend (Koeln, Germany). [^{35}S]-GTPγS (1250 Ci/mmol) was purchased from Perkin Elmer (Waltham, MA).

Cell culture and membrane preparation

Human embryonic kidney (HEK) 293T cells stably expressing human HCA_2 were cultured in DMEM supplemented with 10% newborn bovine serum, 0.4 mg/mL G418, 50 IU/mL penicillin and 50 μg/mL streptomycin. The cells were harvested by scraping in cold PBS, centrifuged at 1000g for 10 min and resuspended in cold 50 mM Tris-HCl buffer, pH 7.4. Then a DIAX 900 electrical homogenizer (Heidolph, Schwabach, Germany) was used for 15 sec to obtain cell lysis. The suspension was centrifuged at 100 000g for 20 min at 4 °C and the supernatant was discarded. The pellet was resuspended in Tris-HCl, and the homogenization and centrifugation steps were repeated. The membranes were resuspended in cold assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4) and the protein content was determined using a BCA assay (Thermo Scientific, Waltham, USA). During membrane preparation the suspension was kept on ice at all times. Membrane aliquots were stored at -80 °C until the day of the assay.

[^{3}H]-nicotinic acid binding assays

Competition assays

Membranes of the HEK293T-HCA_2 cell line (50 μg protein per tube) were incubated 1 hour at 25 °C with 20 nM [^{3}H]-nicotinic acid and increasing concentrations of the test compound in assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4). The total assay volume was 100 μL. To assess the total binding a control without test compound was included. The non-specific binding was determined in the presence of 10 μM unlabeled nicotinic acid. Final DMSO concentration in all samples was ≤ 0.25%. The incubation was terminated by filtering over GF/B filters using a 24-sample harvester (Brandel, Gaithersburg, USA). The filters were washed 3 times with 2 mL cold buffer (50 mM Tris HCl, pH 7.4). Filters were transferred to counting vials and counted in a Perkin Elmer LSA Tri-Carb 2900TR counter after 2 h extraction in 3.5 ml Emulsifier Safe liquid scintillation cocktail (Perkin Elmer, Waltham, USA).

Dissociation assays

HEK-HCA_2 membranes (50 μg protein per tube) were incubated 3 h at 15 °C with 20 nM [^{3}H]-nicotinic acid in 50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4, in a total volume of 100 μL. Then, 5 μL nicotinic acid (final concentration 10 μM), in absence or presence of test compound (final concentration 10 μM) was added. In the single point screen dissociation was terminated after 30 min at 15 °C, and for full curves dissociation was measured at different time points. Non-specific binding was determined in the presence of 10 μM nicotinic acid, added prior to the membranes. Otherwise the assay was performed as the competition assay.

Chapter 6

Putative role of the adenosine A_3 receptor in the anti-proliferative action of N^6-(2-isopentenyl)adenosine

This chapter is based on: Blad CC, von Frijtag Drabbe Künzel JK, de Vries H, Mulder-Krieger T, Bar-Yehuda S, Fishman P, IJzerman AP. Purinergic Signal **2011**; 7(4): 453-62.

Abstract

We tested a panel of naturally occurring nucleosides for their affinity towards adenosine receptors. Both N^6-(2-isopentenyl)adenosine (IPA) and racemic zeatin riboside were shown to be selective human adenosine A_3 receptor (hA_3R) ligands with affinities in the high nanomolar range (K_i values of 159 and 649 nM, respectively). These values were comparable to the observed K_i value of adenosine on hA_3R, which was 847 nM in the same radioligand binding assay. IPA also bound with micromolar affinity to the rat A_3R. In a functional assay in CHO cells transfected with hA_3R, IPA and zeatin riboside inhibited forskolin-induced cAMP formation at micromolar potencies. The effect of IPA could be blocked by the A_3R antagonist VUF5574.

Both IPA and reference A_3R agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-N-methylcarboxamide (Cl-IB-MECA) have known antitumor effects. We demonstrated strong and highly similar antiproliferative effects of IPA and Cl-IB-MECA on human and rat tumor cell lines LNCaP and N1S1. Importantly, the antiproliferative effect of low concentrations of IPA on LNCaP cells could be fully blocked by the selective A_3R antagonist MRS1523. At higher concentrations, IPA appeared to inhibit cell growth by an A_3R-independent mechanism, as was previously reported for other A_3R agonists. We used HPLC to investigate the presence of endogenous IPA in rat muscle tissue, but we could not detect the compound.

In conclusion, the antiproliferative effects of the naturally occurring nucleoside IPA are at least in part mediated by the A_3R.

Introduction

Four adenosine receptors have been cloned and pharmacologically characterized: A_1, A_{2A}, A_{2B} and A_3. Of these subtypes, the A_3R has been identified last [1]. The hA_3R has a wide distribution in the body. High expression is observed in liver, lungs and immune cells, whereas the brain, testes, placenta and heart display moderate expression levels [2-3]. Furthermore, a very high expression level is observed in tumor cell lines and cancer tissues, making the A_3R an interesting target for the treatment of cancer (for a review, see [4]). A recent study even showed that the 3-fold A_3R upregulation in human colorectal cancer was reflected, via an unknown mechanism, in peripheral blood cells [5]. This could make the A_3R a promising biomarker for this and possibly other types of cancer. We and others have previously shown that A_3R agonists have antiproliferative effects on tumor cells in vitro and in vivo, characterized by induction of G_0/G_1 cell cycle arrest and apoptosis [4]. It has been shown that, in most cases, a deregulation of the NF-κB and Wnt signaling pathways is at the basis of these effects [4]. However, other mechanisms may be more important in specific cell types. For example, in A375 human melanoma cells, A_3R stimulation activated phosphatidylinositol 3-kinase (PI3K) which induced Akt phosphorylation, finally resulting in reduced levels of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) [6]. Another recent study suggested that A_3R upregulation may precede asbestos-induced malignant mesothelioma formation [7]. The A_3R agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-N-methylcarboxamide (Cl-IB-MECA) prevented TNF-α-mediated cell survival after asbestos exposure in vitro by acting on the de-regulated Akt/NF-κB pathways, showing that A_3R is a possible target for cancer prevention as well as treatment. Interestingly, A_3R agonists do not inhibit the growth of normal cells [7-9]. Moreover, these ligands can act as cytoprotective agents, for example by preventing the myelotoxic effects of chemotherapy [10]. In view of the promising preclinical data, the safety profile of the A_3R agonist IB-MECA has been evaluated in phase I clinical studies [11]. This agonist was tolerated very well. Related A_3R agonist Cl-IB-MECA is currently in phase I/II clinical trials for hepatocellular carcinoma ([12] and trial ID: NCT00790218).

On the basis of these studies and observations we hypothesized that endogenous A_3R agonists might also contribute to the body's natural defense mechanism against tumors. In particular, such compounds may be excreted by striated muscle cells, protecting the tissue from metastases [13]. We have shown that extracts from muscle cells inhibited tumor proliferation while protecting bone marrow cells in vivo. These effects were dependent on A_3R activation. However, they could not be attributed to adenosine, as the effect was not sensitive to ADA and could not be reproduced by administration of adenosine alone [13]. Thus, we set out to identify other endogenous agonists for the A_3R.

In the current work we report the affinity of a panel of naturally occurring nucleosides for the human adenosine receptors, while focusing on the hA_3R. Then, we assessed the potency of the two higher affinity compounds, the nucleosides IPA and racemic zeatin riboside, in cAMP generation assays. The effect of IPA and the reference A_3R agonist Cl-IB-MECA on tumor cell line proliferation was also determined. We provide evidence that the antiproliferative effects of IPA are indeed linked to the A_3R as they can be partially

blocked by a selective antagonist for that receptor. We also present a HPLC method to investigate the IPA content of muscle tissue. However, endogenous IPA could not be detected in our analysis.

Materials & Methods

Materials

[^{3}H]-DPCPX and [^{125}I]-AB-MECA were purchased from Amersham Biosciences (Roosendaal, the Netherlands). [^{3}H]-ZM241385 and [^{3}H]-MRS1754 were obtained from Tocris Cookson, Ltd. (Bristol, UK). All nucleosides listed in table 1 were supplied by Sigma-Aldrich (Zwijndrecht, the Netherlands). Ammonium acetate and EDTA for analysis of muscle tissue were obtained from Fluka (Zwijndrecht, the Netherlands), whereas methanol for the HPLC analysis was from Biosolve (Valkenswaard, the Netherlands).
Chinese hamster ovary (CHO) cells expressing the human adenosine A_1 receptor were kindly provided by Dr. A. Townsend-Nicholson (University College of London, UK). Human embryonic kidney (HEK) 293 cells stably expressing the human adenosine A_{2A} receptor were a gift from Dr. E. Wang (Biogen/IDEC, San Diego, CA, USA). CHO cells expressing the human adenosine A_{2B} receptor were donated by Dr. S. Rees (GSK, Stevenage, UK) and both CHO and HEK293 cells expressing the hA_3R were kindly provided by Dr. K.-N. Klotz (University of Wuerzburg, Germany). RBL-2H3 cells were a kind gift of Dr. Frank Redegeld (Utrecht University, the Netherlands). LNCaP (human prostate carcinoma) and N1S1 (rat hepatocellular carcinoma) cells were purchased from the American Type Culture Collection (Manassas, Virginia, USA).
Fresh Wistar rat cadavers from an untreated control group of another study were kindly provided by the animal facility of the Leiden/Amsterdam Center for Drug Research (Leiden, the Netherlands).

Radioligand binding studies

Cell culture and membrane preparation

CHO cells expressing the human A_1 receptor were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 μg/ml), penicillin (50 IU/ml) and G418 (0.2 mg/ml) at 37 °C and 5% CO_2. HEK 293 cells stably expressing either the human A_{2A} adenosine receptor or the hA_3R were grown in DMEM containing 10% newborn calf serum, streptomycin (50 μg/ml), penicillin (50 units/ml) and G418 (0.5 mg/ml) at 37 °C and 7% CO_2. Membranes were prepared as previously described [14]. ADA was added in the final preparations at 0.8 IU/ml, except for the membranes containing the A_3R, where no ADA was included.

Human adenosine A_1 receptor

Affinity for the human A_1 receptor was determined on membranes from CHO cells expressing the human receptors, using [^{3}H]-DPCPX as the radioligand. Membranes containing 10 μg of protein were incubated in a total volume of 200 μl of 50 mM Tris/HCl (pH 7.4) and [^{3}H]-DPCPX (final concentration 1.6 nM) for 1 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of 10 μM CPA. The incubation

was terminated by filtration over Whatman GF/B filters under reduced pressure with a Brandell harvester. Filters were washed three times with ice-cold buffer and placed in scintillation vials. Packard Emulsifier Safe (3.5 ml) was added, and after 2 h radioactivity was counted in a Perkin-Elmer Tri-Carb 2900 β-scintillation counter.

Human adenosine A_{2A} receptor

Affinity for the human A_{2A} receptor was determined on membranes from HEK293 cells stably expressing this receptor, using [^{3}H]-ZM241385 as the radioligand. Membranes containing 30 μg of protein were incubated in a total volume of 200 μl of 50 mM Tris/HCl (pH 7.4) and [^{3}H]-ZM241385 (final concentration 1.7 nM) for 2 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of 10 μM CGS21680. Filtration and counting were performed as described for the A_1 receptor.

Human adenosine A_{2B} receptor

At the human A_{2B} receptor radioligand displacement was determined on membranes from CHO cells stably transfected with this receptor, using [^{3}H]-MRS1754 as the radioligand. Membranes containing 20 μg of protein were incubated in a total volume of 100 μl of 50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 0.01 w/v % CHAPS (pH 8.26 at 5 °C), and [^{3}H]-MRS1754 (final concentration 1.2 nM) for 1 h at 25 °C in a shaking water bath. Nonspecific binding was determined in the presence of 1 mM NECA. Filtration and counting were performed as described for the A_1 receptor.

Human A_3R

The affinity at the hA_3R was measured on membranes from HEK293 cells stably expressing this receptor, using [^{125}I]-AB-MECA as the radioligand. Membranes containing 35 μg of protein were incubated in a total volume of 100 μl of 50 mM Tris/ HCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.01% w/v CHAPS (pH 8.26 at 5 °C), and [^{125}I]-AB-MECA (final concentration 0.10 nM) for 1 h at 37 °C in a shaking water bath. Nonspecific binding was determined in the presence of 100 μM R-PIA. The incubation was terminated by filtration over Whatman GF/B filters under reduced pressure with a Brandell harvester. Filters were washed three times with ice-cold buffer and placed in counting tubes. Radioactivity was counted in a Perkin-Elmer Wallac 1470 Wizard gamma-counter.

Rat A_3R

The affinity at the rat A_3R was measured on membranes from RBL-2H3 (rat basophilic leukemia) cells endogenously expressing this receptor, using [^{125}I]-AB-MECA as the radioligand. Membranes of these cells were prepared as described previously [15]. Membranes containing 60 μg of protein were incubated in a total volume of 100 μl of 50 mM Tris/ HCl, 10 mM $MgCl_2$ (pH 7.7 at 22 °C), and [^{125}I]-AB-MECA (final concentration 0.20 nM) for 1 h at 37 °C in a shaking water bath. Nonspecific binding was determined in the presence of 100 μM R-PIA. Filtration and counting were performed as described for the hA_3R.

Second messenger studies: cAMP production in cells expressing the hA_3R

CHO cells expressing the hA_3R were grown overnight as a monolayer in 24 well tissue culture plates (400 µl/well; 2 x 10^5 cells/well) in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% newborn calf serum, streptomycin (50 µg/ml), penicillin (50 IU/ml) and G418 (0.2 mg/ml) at 37 °C and 5% CO_2. To determine the potencies of IPA, adenosine and zeatin riboside, cAMP generation was performed in DMEM/N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) buffer (0.60 g HEPES/50 ml DMEM pH 7.4). Each well was washed twice with HEPES/ DMEM buffer (250 µl), after which the PDE inhibitors rolipram (50 µM) and cilostamide (50 µM) were added to each well. This mixture was incubated for 30 min at 37 °C followed by the introduction of either the compound of interest (10 µM), reference compound Cl-IB-MECA (10 µM) or DMEM/HEPES. After a further 10 min of incubation, forskolin was added (10 µM). After a subsequent 15 min, incubation was stopped by aspirating the assay medium and by adding 200 µl of ice-cold 0.1 M HCl. The amount of cAMP was determined by competition with [^{3}H]-cAMP for protein kinase A (PKA). Briefly, the sample, approximately 1.8 nM [^{3}H]-cAMP, and 100 µl of PKA solution were incubated on ice for at least 2.5 h. The incubations were stopped by rapid dilution with 2 ml of ice-cold Tris/ HCl buffer (50 mM, pH 7.4), and bound radioactive material was then recovered by filtration through Whatman GF/C filters. Filters were additionally rinsed with 2 x 2 ml of Tris/HCl buffer, Packard Emulsifier Safe (3.5 ml) was added, and after 2 h radioactivity was counted in a Perkin-Elmer Tri-Carb 2900 β-scintillation counter.
In the assays evaluating the effects of ADA and VUF5574, intracellular cAMP levels were measured using a LANCE cAMP 384 kit (PerkinElmer, The Netherlands) as described previously [16]. To each well, 5 µL of the agonist Cl-IB-MECA, adenosine or IPA (10 µM final concentration) in stimulation buffer (PBS with 5 mM Hepes, pH 7.4 supplemented with 0.1% BSA, rolipram (50 µM) and cilostamide (50 µM)) was added in the absence (control) or presence of ADA (0.8 IU/mL) or VUF5574 (1 µM). Then 4.5 µL hA_3-CHO cell suspension in stimulation buffer was seeded into a 384-well plate (approximately 5000 cells/ well), which was followed by incubation for 15 min at room temperature. Subsequently, 2.5 µL forskolin (1 µM) was added and the mixture was incubated for 30 min at room temperature. Then, detection mix (6 µL) and cAMP antibody solution (6 µL) were added and incubated for 3h. Intracellular cAMP levels were measured using a TR-FRET assay on a Victor spectrometer (PerkinElmer, The Netherlands) according to instructions of the supplier. All data reflect the average of at least three independent experiments performed in duplicate.

Proliferation assays on tumor cell lines

Effect of IPA on the proliferation of tumor cell lines

Cell proliferation was studied in both N1S1, a rat hepatocellular carcinoma cell line, and LNCaP, a human prostate carcinoma cell line. The cells were grown in RPMI 1640 with penicillin (100 units/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and 10% fetal

bovine serum (FBS). The cells were maintained in T-75 flasks at 37 °C in a 5% CO_2 incubator and transferred to freshly prepared medium twice weekly. For the in vitro studies with the LNCaP cell line serum-starved cells were used. In these experiments FBS was omitted from the culture medium for 18 h and the experiment was carried out on monolayers of cells in RPMI medium supplemented with 1% FBS. For the N1S1 cells no serum starvation was used and the assay was carried out in the growth medium of the cells.
The cells (1.5 x 10^4/ml) were incubated in 96-well microtiter plates in the presence of 25 μM adenosine or various concentrations of Cl-IB-MECA and IPA (0.01, 0.1, 1, and 10 μM). A 24 h [^{3}H]-thymidine incorporation assay was used to evaluate cell growth, except for the experiments evaluating the effect of EHNA (10 μM), which lasted 48 hours. For the last 18 h of incubation, each well was pulsed with 1 μCi [^{3}H]-thymidine. Cells were harvested and the [^{3}H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ, USA).

Effect of IPA on the proliferation of LNCaP cells in the presence of the A_3R antagonist MRS1523
The effect of IPA on the proliferation of LNCaP cells in the presence of the A_3R antagonist MRS1523 was also examined. The cells (1.5 x 10^4/ml) were incubated in 96-well microtiter plates with IPA (0.01, 0.1, 1 and 10 μM) in the absence or presence of MRS1523 (0.01, 0.1 and 1 μM). Cell growth was evaluated in a 24-hour [^{3}H]-thymidine incorporation assay as described above.

Data analysis
All radiologand displacement curves and cAMP concentration-effect curves were analyzed with GraphPad Prism software (version 5.0). Statistical analysis of the results of the cAMP production assays was done using an unpaired Student's t-test.

HPLC analysis of the presence of IPA in rat muscle tissue
The presence of IPA in rat muscle tissue was investigated by HPLC analysis. Fresh rat muscle tissue (hind leg vastus lateralis and semimembranosus) was used. About 5 gram of tissue was weighed exactly and cut into small pieces of about 5x5x5 mm. The material was spiked with IPA and/or internal standard cyclopentyladenosine in methanol (2.5 μg). Then, 30 ml of 5 mM ammonium acetate (pH 5.2), 50 mM EDTA was added and the material was treated with a Diax 900 homogenizer (Heidolph, Schwabach, Germany) equipped with a 1.5 cm probe, for 1.5 min at speed 1. The homogenate was subjected to 4 freeze-thaw cycles in liquid nitrogen and a 60 °C water bath. The sample was centrifuged at 6000 g at 4 °C for 30 min and the supernatant was additionally centrifuged at 200000 g at 4 °C for 45 min. After the centrifugation steps, the clear supernatant was filtered over Miracloth to remove fat particles and transferred to an activated C18 solid phase extraction column (Grace, Deerfield, USA). After passing the sample, the column was washed with 25 ml 5 mM ammonium acetate and the material of interest was eluted with 5 ml methanol. The methanol was evaporated in a vacuum centrifuge and the sample was reconstituted in 0.5 ml 5 mM ammonium acetate after which HPLC analysis was performed.

The system consisted of a gradient solvent delivery system (Gilson, Den Haag, the Netherlands), equipped with a Gilson 115 UV detector, monitoring at 270 nm. The column was a Discovery® RP 18 (Supelco, Zwijndrecht, the Netherlands), 125 x 4.6 mm, packed with 5 μM particles. As eluent a gradient between solvent A (10% methanol in 5 mM ammonium acetate) and solvent B (90% methanol in 5 mM ammonium acetate) was applied with a flow of 0.6 ml/min. The profile of the gradient was as follows; 0 min, 0% B; 10 min, 0% B; 15 min, 37.5% B; 35 min, 37.5% B; 40 min, 100% B; 50 min, 100% B; 51 min, 0% B and 60 min, 0% B. For analysis, 100 μl of sample in 5 mM ammonium acetate pH 5.2 was injected by an autoinjector. Data were recorded and processed using ADchrom software (Leiden University, the Netherlands).

Results

Radioligand binding studies on human adenosine receptors

The affinities of the naturally occurring modified nucleosides 1-methyl-adenosine, 2’-O-methyl-adenosine, IPA, trans-zeatin riboside and racemic zeatin riboside were determined in radioligand binding studies on all four subtypes of human adenosine receptors (table 1). The affinity of adenosine for the A_1, A_{2A} and A_{2B} receptors could not be assessed in this experimental set-up due to the essential presence of ADA in the assays. Both IPA and the racemic mixture of cis- and trans-isomers of zeatin riboside showed affinities in the higher nanomolar range (K_i values of 159 nM and 643 nM, respectively) for the hA_3R, in the same range as the affinity of adenosine itself. The affinities of both zeatin riboside and IPA at the other adenosine receptor subtypes were negligible, given the lack of displacement at a test concentration of 1 μM.

Table 1. Chemical structures of naturally occurring modified nucleosides and their effects in radioligand binding studies at human adenosine A_1, A_{2A}, A_{2B} and A_3 receptors.

	Name	R¹	R²	% displacement			K_i or % displ.
				hA_1	hA_{2A}	hA_{2B}	hA_3
1a	adenosine	-H	-OH	ND	ND	ND	847±60 nM
1b	N^6-(2-isopentenyl)-adenosine (IPA)	$-CH_2CHC(CH_3)_2$	-OH	14	0	0	159±19 nM
1c	trans-zeatin riboside	$CH_2CHC(CH_3)CH_2OH$-	-OH	2	0	6	31%
1d	racemic zeatin riboside	$-CH_2CHC(CH_3)CH_2OH$	-OH	17	0	0	643±27 nM
1e	2’-O-methyladenosine	-H	-OH	0	0	8	0%
2	1-methyl-adenosine	-	$-OCH_3$	2	0	4	34%

% displacement at 1 μM (N=2) or K_i value (N=3) is shown; data are means (±S.E.M.) of N experiments performed in duplicate.

Affinity of IPA for the rat A_3R

The affinity of IPA for the rat A_3R was also determined in radioligand binding studies (figure 1). A K_i value of 4.69 ± 0.20 μM was found for the rat orthologue, so the affinity of IPA for the rat A_3R was lower than for the hA_3R.

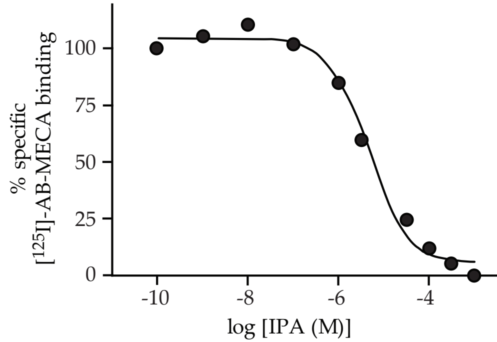


Figure 1. Competition binding experiment on RBL-2H3 cell membranes endogenously expressing the rat A_3R. IPA displaced [^{125}I]-AB-MECA from the rat A_3R with a K_i value of 4.69 ± 0.20 μM (N=3). Data from one representative experiment is shown. Incubation was 1 h at 37 °C in 50 mM Tris/ HCl, 10 mM $MgCl_2$ (pH 7.7)

Effect of IPA on the cAMP production in cells expressing the hA_3R

The potency of adenosine, IPA and racemic zeatin riboside to modulate cAMP production was determined in intact CHO cells stably expressing the hA_3R (table 2). Adenosine and IPA inhibited forskolin-stimulated cAMP accumulation with similar potencies in the low micromolar range and virtually identical intrinsic activities.

Table 2. Potency of naturally occurring nucleosides on the human adenosine A_3 receptor, as inhibitors of forskolin-stimulated cAMP production in CHO cells expressing the hA_3R.

	EC_{50} (μM)
adenosine	2.9 ± 1.1
N^6-(2-isopentenyl)-adenosine (IPA)	2.0 ± 0.7
racemic zeatin riboside	5.8 ± 1.8

EC_{50} value (N=4, except zeatin riboside N=3) is shown; data are means (±S.E.M.) of N experiments performed in quadruplicate.

In a second assay the effects of ADA and the selective hA_3R antagonist VUF5574 were investigated (figure 2). The selective A_3R agonist Cl-IB-MECA was included for comparison next to adenosine and IPA. As expected, adenosine and IPA inhibited cAMP production to a similar extent when added at 10 μM, whereas Cl-IB-MECA had a more potent effect. Inclusion of ADA abolished the effect of adenosine, whereas IPA and Cl-IB-MECA were insensitive to the enzyme. Modulation of the second messenger pathway by each of the agonists was largely and significantly inhibited by the antagonist VUF5574.

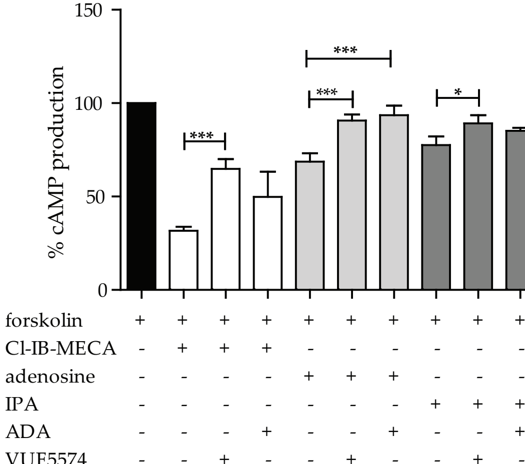


Figure 2. Effect of ADA and A_3R antagonist VUF5574 in cAMP accumulation assays. In CHO cells stably expressed with hA_3R, forskolin-stimulated cAMP production was inhibited by adenosine, IPA and Cl-IB-MECA, all at 10 μM. The effects of the concomitant presence of either the enzyme ADA (0.8 IU/ml) or the antagonist VUF5574 (10 μM) were also assessed. The cells were incubated for 15 min at room temperature with the ligand(s) of interest ±ADA. Then forskolin was added to the reaction mixture for a further 30 min. Data are means (± S.E.M.) of four experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t-test. * $P<0.05$; *** $P<0.001$

Effect of IPA on the proliferation of tumor cell lines

In view of the reported anti-tumor effects of agonists for the A_3R we investigated the effect of IPA on the proliferation of human prostate carcinoma cells (LNCaP) and rat hepatocellular carcinoma cells (N1S1). In both tumor cell lines IPA inhibited the incorporation of [^{3}H]-thymidine in a similar fashion as the reference A_3R agonist Cl-IB-MECA (figures 3 and 4). At 10 μM, IPA even had a significantly greater effect than Cl-IB-MECA on both cell lines ($P<0.01$). An approximate EC_{50} value of 1 μM for IPA was established in the LNCaP cell line, whereas the potency on the N1S1 cell line seemed somewhat lower. Adenosine, at a concentration of 25 μM, had only a modest effect on N1S1 proliferation and no effect on the LNCaP cell line. Addition of ADA inhibitor EHNA (10 μM) did not increase the adenosine effect in the LNCaP cell line (data not shown), although it significantly increased the effect of adenosine on the N1S1 cells, yielding almost full growth inhibition (data not shown; available with the published article as Online Resource 1). Addition of EHNA but not adenosine to N1S1 cells also revealed a 22% basal growth inhibition, probably mediated by adenosine excreted by the tumor cells.

Effect of IPA on the proliferation of LNCaP cells in the presence of the A_3R antagonist MRS1523

In a final proliferation assay we investigated whether MRS1523, an antagonist with appreciable affinity for both human and rat A_3R, was capable of blocking the effect of IPA on LNCaP cell proliferation (figure 5). The antagonist (at 0.1 and 1 μM) prevented the anti-proliferative effect of 0.01 and 0.1 μM IPA. When IPA was added at 1 μM, the inhibition by MRES1523 was small but still significant at higher concentrations. At 10 μM IPA, the antagonist had no effect.

Determination of IPA in muscle tissue by HPLC

Since IPA is a naturally occurring nucleoside we investigated its occurrence in muscle

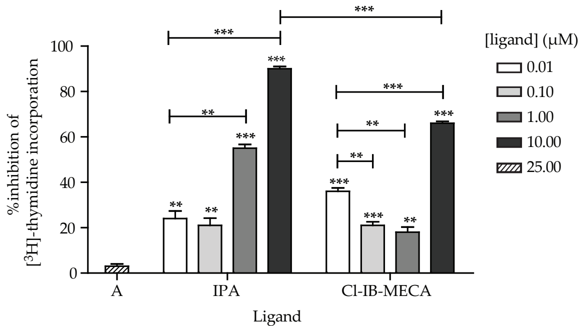


Figure 3. IPA and Cl-IB-MECA have similar antiproliferative effects on LNCaP tumor cells. IPA and Cl-IB-MECA (0.01-10 μM) both inhibited proliferation of the human prostate carcinoma cell line LNCaP. Adenosine (A) (25 μM) had virtually no effect. IPA and Cl-IB-MECA had significantly larger antiproliferative effects than adenosine at all concentrations (P<0.01). The effect of IPA increased significantly at 1 μM compared to the lower concentrations (P<0.01), and the effect increased further at 10 μM. The effect of Cl-IB-MECA showed a significant decrease from 0.01 μM to 1 μM (P<0.01), and then significantly increased again at 10 μM (P<0.001). At 10 μM, IPA had a significantly larger effect than Cl-IB-MECA (P<0.001). Proliferation was evaluated by incubating a monolayer of serum-starved cells for 24h with the compound of interest. For the last 18 h, 1 μCi of [3H]-thymidine was included in the buffer. Data are means (± S.E.M.) of three experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t-test. ** P<0.01; *** P<0.001. Stars without brackets indicate the significance of the difference to adenosine treatment.

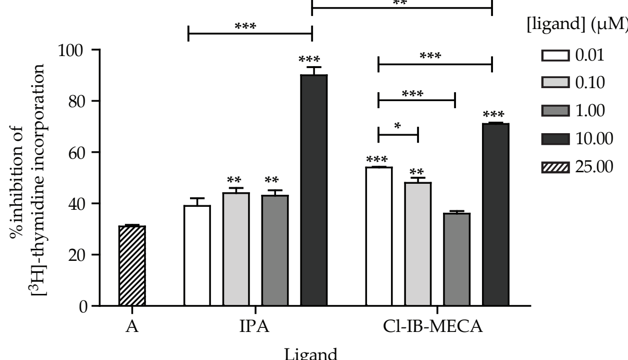


Figure 4. IPA and Cl-IB-MECA have similar antiproliferative effects on N1S1 tumor cells. IPA and Cl-IB-MECA (0.01-10 μM) both inhibited proliferation of the rat hepatocellular carcinoma cell line N1S1. Adenosine (A) (25 μM) had only a modest effect. IPA and Cl-IB-MECA had significantly larger antiproliferative effects than adenosine (P<0.01), with the exception of 0.01 μM IPA and 1 μM Cl-IB-MECA. The effect of IPA increased significantly at 10 μM compared to the other concentrations (P<0.001). The effect of Cl-IB-MECA showed a significant decrease from 0.01 μM to 1 μM, and then significantly increased again at 10 μM (both P<0.001). At 10 μM, IPA had a significantly larger effect than Cl-IB-MECA (P<0.01). Proliferation was evaluated by incubating a monolayer of serum-starved cells for 24h with the compound of interest. For the last 18 h, 1 μCi of [3H]-thymidine was included in the buffer. Data are means (± S.E.M.) of three experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t-test. * P<0.05; ** P<0.01; *** P<0.001

tissue. We therefore analyzed extracts from Wistar rat muscle. The spectra of spiked and unspiked tissue extracts are shown in figures 6A and 6B, respectively. From the spiked tissue, 23% of the added IPA was recovered in the extract. In the unspiked spectrum no IPA peak was observed.

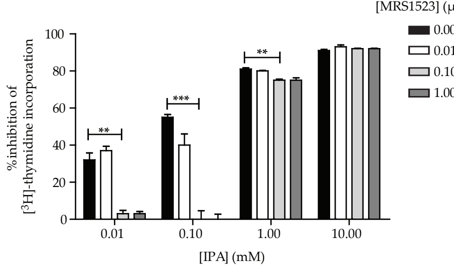


Figure 5. The antiproliferative effect of IPA is partly blocked by A_3R antagonist MRS1523. The selective A_3R antagonist MRS1523 (0.01-1 μM) blocked the effect of low concentrations (0.01-0.1 μM) of IPA on human prostate carcinoma (LNCaP) cells. MRS1523 did not inhibit the effect of high concentrations of IPA (1-10 μM). Proliferation was evaluated by incubating a monolayer of serum-starved cells for 24h with the compound of interest. For the last 18 h, 1 μCi of [^{3}H]-thymidine was included in the buffer. Data are means (± S.E.M.) of three experiments performed in triplicate. Statistical evaluation was done using an unpaired Student's t-test. ** P<0.01; *** P<0.001

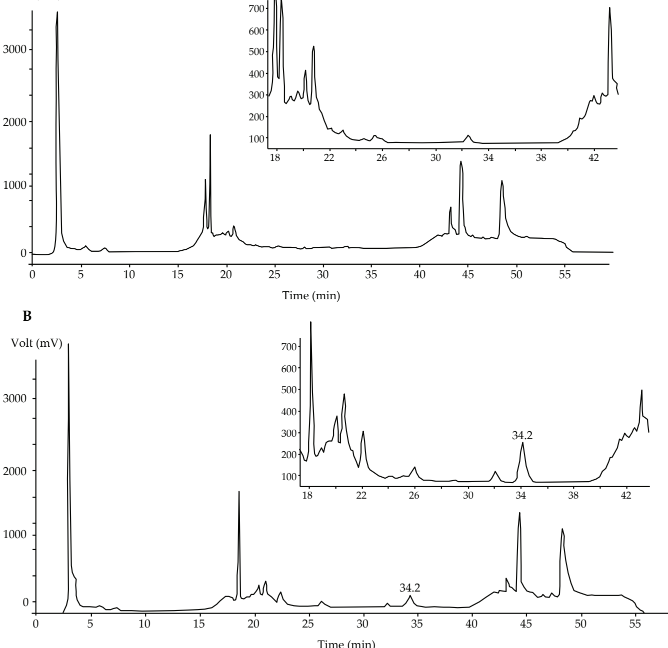


Figure 6. HPLC analysis of rat muscle extract detects no IPA. Comparison of rat muscle tissue spiked with IPA (B) and unspiked muscle extract (A) tissue shows that IPA, which is eluted at 34.2 min in the spiked tissue, cannot be detected in the blank tissue.

Discussion

In vitro evaluation of IPA activity

We have shown that zeatin riboside and particularly IPA are ligands for the A_3R. These naturally occurring modified nucleosides bind with submicromolar affinity to the hA_3R. Moreover, the affinity of IPA for A_3R is not restricted to the human orthologue, but extends to the rat A_3R. In line with previous findings on N^6-substituted adenosine derivatives with small alkyl substituents, IPA discriminates between the two A_3R homologues and has a 30-fold higher affinity for the human A_3R [17]. Our next step was the evaluation of receptor activation by IPA, zeatin riboside and adenosine in cAMP generation assays. The compounds had similar potencies in the low micromolar range, with zeatin riboside 2- or 3-fold less potent than IPA and adenosine.

The potency of adenosine towards the A_3R was classically considered to be in the

micromolar range and rather lower than the potency towards the adenosine A_1 and A_{2A} receptors [18]. In contrast, several more recent publications report similar potencies of adenosine towards the A_1, A_{2B} and A_3 subtypes, in the submicromolar range [19-21]. The affinity and potency that we observed for A_3R activation by adenosine are clearly in better agreement with the more recent reports. Some care should be taken in the interpretation of cAMP assay results, however, as receptor expression levels may have a strong impact on the EC_{50} values of tested agonists.

Anti-proliferative effects of IPA

Interestingly, IPA has antitumor activity both in vitro and in vivo [22-23]. In 1975 this compound has been tested in a pilot clinical trial for leukemia, where it caused remission in three patients out of twenty [24]. Currently, there is a renewed interest in the compound; in a recent review IPA was defined as an emerging anticancer drug [25]. The molecular mechanism of the anti-tumor activity of IPA has not been elucidated so far.

In view of the well-known role of the A_3R in cancer, we decided to investigate whether the antiproliferative effects of IPA are mediated by this receptor. IPA inhibits the proliferation of human prostate cancer cells (LNCaP) and rat hepatocellular cancer cells (N1S1) to a similar extent as the A_3R reference agonist Cl-IB-MECA, which has nanomolar affinity at the receptor. These effects may be mediated by the A_3R which is highly expressed in N1S1 cells and probably also in LNCaP cells [12, 26]. The potency of IPA in this assay seems somewhat lower in the rat cell line than in the human cell line; this would be in line with the difference in affinity of IPA towards the rat and human A_3R. At 10 μM, IPA is significantly more active than Cl-IB-MECA in both cell lines.

The A_3R antagonist MRS1523 was able to completely inhibit the effect of low concentrations of IPA (10 and 100 nM) but not higher concentrations (1 and 10 μM). The affinity of MRS1523 for the hA_3R is approximately 6-fold higher compared to IPA [27]. Therefore, it may be that the antiproliferative effect of higher concentrations of IPA is not mediated by the A_3R, but by a different mechanism. A_3R-independent antiproliferative effects of high concentrations of A_3R agonists, including Cl-IB-MECA and IB-MECA, have previously been observed [28-30]. For example, 30 μM Cl-IB-MECA inhibited growth of leukemia cell lines HL-60 and MOLT-4 in the presence of antagonists MRS1523 (10 μM) or MRS1220 (5 μM) [28]. Furthermore, Cl-IB-MECA and IB-MECA, but also adenosine, 2-chloro-adenosine and 3'-deoxyadenosine had antiproliferative effects on breast cancer cell lines lacking A_3R mRNA [29]. Involvement of other adenosine receptor subtypes was excluded. Suggested pathways for these effects include downregulation of the estrogen receptor α [29], upregulation of death receptor Fas [28] and downregulation of cyclins D1 and E2 together with dephosphorylation of ERK1/2 [30]. It should be noted that the concentrations used in these reports are between 10 and 100 μM (even 500 μM for adenosine). The A_3R-independent effect of IPA seems to occur even at 1 μM, so it may be a significantly more potent anti-cancer agent than the other agonists that have been assessed. At 10 and 100 nM IPA, the antiproliferative effect was entirely blocked by antagonist MRS1523, so at these low IPA concentrations the effect seems entirely A_3R-dependent. Plasma concentrations of IPA were not reported in the pilot clinical trial on leukemia patients [24, 31]. However, they

are expected to be very low since IPA is metabolized very fast [31]. After IPA enters the circulation, more than 50% is excreted in urine in the first 4 hours, mostly in metabolized form. Metabolites include N^6-(3-methyl-hydroxybutylamino)purine, hypoxanthine, adenine and several N^6-alkylated adenines and N-alkylated xanthines [31].

Origin and levels of endogenously occurring IPA

IPA and zeatin riboside are widely studied plant cytokinins controlling various processes in plant growth and development [32-33]. The nucleosides are also components of tRNA, but only IPA has been found in mammalian tRNA [33-34]. Free IPA is probably present in the mammalian cytoplasm, since its precursor N^6-(2-isopentenyl)adenosine-3-monophosphate has been identified in several cell lines [35]. Unchanged IPA has been detected in human urine at an average amount of 50 μg per day [36]. Although levels of IPA and zeatin riboside in grains, vegetables and fruits at the moment of consumption could not readily be extracted from the literature, these foods might be exogenous sources of these compounds. Tuberous roots, such as (sweet) potatoes, seem especially likely sources [37-38]. Furthermore, ingested or residential bacteria in the gut may provide additional cytokinins [33]. In plants, free zeatin riboside is in the trans conformation, but tRNA is a source of cis-zeatin riboside. Most likely, the cis-isomer has the higher affinity for the A_3R, since the trans-isomer is less active than the racemate (table 1). However, cis-zeatin riboside is not commercially available and was therefore not tested in our study.
Colocalization and thus interaction of the proposed nucleoside ligands and the A_3R may occur at the cellular membrane or, keeping in mind that A_3R has a nuclear localization signal, in or at the nucleus [39]. A_3R localized in colon could interact with IPA and zeatin riboside from exogenous sources [40].

Detection of endogenous IPA

We have previously reported that an unknown A_3R agonist with anti-tumor properties is excreted by muscle cells [13]. Since IPA activates A_3R and has anti-proliferative properties, the compound might qualify as this unknown factor. We used HPLC to investigate its presence in commercial cow, pork and chicken meat, but detected no IPA. Since the freshness and storage conditions of commercial meat may not be ideal, we then switched to fresh rat muscle tissue. Again, IPA could not be detected, nor did we detect peaks of IPA metabolites. It is therefore unlikely that IPA is the muscle-derived A_3R agonist we observed previously [13].

Conclusion

In conclusion, we have shown that both IPA and zeatin riboside bind selectively to the A_3R. Moreover, we provide evidence that the antiproliferative effect of low concentrations of IPA is mediated by the A_3R. Higher concentrations of the compound seem to have a potent A_3R- independent antitumor effect. It is however unlikely that IPA is the previously reported muscle-derived antiproliferative A_3R agonist, since it could not be detected in rat muscle. More research is needed to elucidate the putative physiological roles of IPA and the importance of the A_3R for the antitumor action of IPA.

Acknowledgement

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Chapter 7

Compound screen on orphan G protein-coupled receptor GPR88

The work described in this chapter was done at the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France, under supervision of Prof. Dr. B. L. Kieffer and Dr. J.A. Becker; and at AstraZeneca R&D, Montréal, Québec, under supervision of Dr. E. Grazzini and Dr. P. LaPlante.

Abstract

GPR88 is a G protein-coupled receptor without known endogenous or synthetic ligands. However, multiple genetic and expression studies suggest that GPR88 may be implicated in psychiatric disorders such as schizophrenia, depression and bipolar disorder. We performed a ligand identification screen testing over 4000 small molecules and peptides. This was done in a 384-well format, using changes in intracellular calcium levels detected with a Fluorescent Imaging Plate Reader (FLIPR) as the readout. Since the G protein coupling specificity of GPR88 is unknown, HEK293s cell lines stably co-expressing the receptor with $G\alpha_{16}$ or $G\alpha_{qi5}$ were used, as well as a native HEK-GPR88 cell line. In the calcium screen 46 hits were identified, with responses occurring in different GPR88-expressing cell lines giving no clue as to the G protein coupling specificity. In validation assays, dose-response curves could be obtained for papaverine, an opium alkaloid. However, this compound also provoked calcium responses in HEK cells that were not transfected with GPR88. Thus, identification of a ligand, either endogenous or synthetic, for GPR88 remains an unmet challenge.

Introduction

Since approximately 30% of all drugs currently on the market target rhodopsin-like G protein-coupled receptors (GPCRs), this protein family is of great interest for drug research. However, from a total of 284 human non-olfactory class A receptors, only about 50 are currently targets of marketed drugs. Even more strikingly, 63 class A GPCRs have no known endogenous ligand or function (see also Chapter 1). Unlocking the full therapeutic potential of all these receptors could bring highly significant advances to the treatment of a variety of diseases. A first step towards this goal can be the identification of (endogenous) ligands for orphan receptors.

This study focused on the orphan receptor GPR88, which was initially identified as a striatum-specific receptor [1-2]. We have also reported it as a gene with enriched expression in the central extended amygdala [3]. GPR88 is of special interest, since it may be implicated in several psychiatric and neurological disorders. Mice lacking this receptor, which is predominantly expressed in the brain, show a schizophrenia-like phenotype with impaired prepulse inhibition of startle (PPI), increased apomorphine-induced climbing and stereotypy, and increased amphetamine-stimulated locomotor hyperactivity [4]. These phenotypes were sensitive to treatment with antipsychotics. Lack of GPR88 modulated the striatal dopaminergic system by increasing the functional sensitivity of dopamine D_2 receptors and increasing DARPP-32 Thr-34 phosphorylation. Other hints regarding the role of GPR88 come from gene expression studies. We and others have reported regulation of the rodent GPR88 gene in several brain regions after treatment regimens with addictive drugs, antidepressants and mood regulators [5-10]. For example, a 13-fold upregulation was seen in nucleus accumbens dopaminergic neurons after chronic treatment of mice with the tricyclic antidepressant amitriptyline [6]. Other antidepressant treatments, including fluoxetine, sleep deprivation and electro-convulsive therapy, were shown to cause changes in GPR88 expression in the hypothalamus [8]. In our laboratory, GPR88 upregulation was seen in mouse central extended amygdala after chronic morphine treatment [5]. Changes in GPR88 expression levels were also observed in lactating rats [11], in rodent models of depression [12] and Parkinson's disease [13], and in human Huntington's disease brains [14].

GPR88 is most closely related to the biogenic amine receptors, especially the β_1 and β_3 adrenergic receptors, and the 5-HT1D receptor. However, even to these receptors the sequence homology is relatively low (up to 27% amino acid identity) and it lacks the aspartic acid in the third transmembrane (TM) domain that is the hallmark of biogenic amine receptors. A phylogenetic analysis using only 30 residues predicted to line the TM binding cavity of GPCRs detected similarities between the class A receptor GPR88 and the class C receptors $GABA_B$, taste and metabotropic glutamate receptors [15]. In this analysis GPR88 was the only receptor clustering to a different receptor class. Possibly, GPR88 and certain class C receptors bind structurally related ligands in their TM binding pockets (which is the allosteric pocket for class C receptors).

In our ligand identification campaign, we screened over 4000 compounds, including naturally occurring and synthetic small molecules and peptides, for activation of human

GPR88. Elevation of intracellular calcium concentrations was the functional readout in our screen. The G protein coupling specificity of GPR88 is unknown, so to obtain calcium responses we co-expressed the receptor with the chimeric G protein $G\alpha_{qi5}$ or the promiscuous $G\alpha_q$-family protein $G\alpha_{16}$, thus forcing GPR88 to couple to the G_q pathway, regardless of the native coupling preference [16-18].

Materials and Methods

Generation and characterization of HEK293s-hGPR88 cell lines

Construction of a hGPR88 - N-Flag - signal-sequence plasmid

Human GPR88 cDNA cloned in pcDNA 3.1+ (Neomycin) was bought from the cDNA Resource Center from the University of Missouri-Rolla. A modified influenza hemaglutinin signal sequence [19] and a Flag tag were inserted upstream of the receptor sequence. The ~240 N-terminal bases of the GPR88 gene were amplified by PCR using a Signal sequence-Flag tag sense primer and Expand High Fidelity polymerase (Roche). The sequences of the primers were: Sense: GCGAATTCCACCATGAAGACGATCATCGCCCTGAGCTACATCTTCTGC CTGGTATTCGCCGACTACAAGGACGATGATGACGCCACCAACTCCTCCTCCACATCCACC; Reverse: GGACGACACGAGATAGATGACCATGCCGTT. In total 50 ng of matrix was used with 100 pmol of each primer in a total volume of 100 µl. An initial denaturation step of 3 min at 94 °C was followed by 20 amplification cycles (30s 94 °C; 30s 55 °C; 20s 72 °C). A phenol-chloroform precipitation was carried out after which the product was taken up in 10 mM Tris pH 7.4 / 1 mM EDTA. Then, 2 µg was digested with 20 IU of EcoRI and AgeI and the resulting product was inserted into the pcDNA3.1+ plasmid bearing the cDNA of GPR88 that was pre-digested with the same enzymes. The ligation was carried out with 50 ng linearized plasmid and 2 ng fragment in 10 µl of commercial buffer with 3 IU ligase (Biolabs) for 4h at 4 °C. The product was used to transform competent Escherichia Coli XLM Blue cells and the plasmid was isolated from an ampicillin-resistant colony. Sequencing confirmed that the plasmid contained the tagged human GPR88 sequence.

Generation of stable cell lines expressing hGPR88- Flag-signal sequence

Transfection of HEK293s cells

The day before transfection $2.5\text{-}4.10^6$ human embryonic kidney (HEK) 293s cells were seeded in a 10 cm diameter cell culture plate. For transfection, 16 µl JetPEI transfection reagent (Polyplus transfection) in 500 µl NaCl 150 mM was added to a solution of 8 µg pcDNA3+/GPR88 in 500 µl NaCl 150 mM, and incubated for 15-30 min at RT. The empty vector pcDNA3 was used as a negative control. Fresh cell culture medium was added to the cells and 1 ml of the transfection mixture was added to each plate. To obtain a stable cell line, Geneticin 418 was added to the medium on day 4.

Cell lines and cell culture conditions

Three HEK293s cell lines from AstraZeneca R&D Montréal were used: HEK293s nontransfected cells, HEK293s stably expressing chimeric $G\alpha_{qi5}$ and HEK293s stably expressing $G\alpha_{16}$. Plasmids used for construction of these cell lines were pCEP-$G\alpha_{qi5}$-HA from Molecular Devices and pcDNA3.1-$G\alpha_{16}$ from AstraZeneca Montréal.

Cells were grown at 37 °C and 5% CO_2, in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1000 units/ml penicillin G and 1 mg/ml streptomycin. The HEK-Gα_{qi5} cells were grown in the presence of 300 μg/ml Hygromycin B and HEK-Gα_{16} cells were grown in the presence of 350 μg/ml Zeocin. Geneticin 418 sulfate was used at a concentration of 500 μg/ml for selection of the cells stably expressing GPR88.

Enrichment of hGPR88-Flag expressing cells by fluorescent automated cell sorting (FACS)

Cells were detached using 10 mM EDTA and washed two times in PBS, followed by 30 min incubation on ice with the monoclonal antibody anti-Flag M2 (Sigma Aldrich) (1:20000 in PBS 1% BSA, filter sterilized). Cells were washed two times with PBS 1% BSA and incubated 30 min on ice with the secondary antibody goat F(ab')2 fragment antimouse IgG (H+L)-FITC (Beckman Coulter) (1:200 in PBS 1% BSA, filter sterilized). After two final washes in PBS the cells were diluted at 5.10^5 cells/ml in culture medium. Cell clumps were removed with a sterile 50 μm Filcon filter (Consul T.S.). Cells were sorted on a Becton Dickinson FACS DiVa using a 100 μm gauge. FITC-positive cells with fluorescence ≥ 1000 units were deflected into tubes containing 0.5 ml culture medium.

Assessment of GPR88-Flag expression in HEK293s cell lines

Flow cytometric analysis

Preparation of the cells for flow cytometric analysis was performed as described for FACS, but not under sterile conditions. The amount of fixed FITC was analyzed on a Becton Dickinson FACS DiVa as described, or on a Becton Dickinson FACSCalibur using essentially the same settings. A total of 3.10^4 single cells were included in each analysis.

Immunocytochemistry

Cells were seeded in 6-well plates containing glass coverslips coated in poly-L-ornithine or poly-D-lysine at 2.10^5 cells per well. After 1-2 days cells were washed three times in PBS and fixed for 15-60 min with 4% PFA in PBS at room temperature. Cells were washed three times with PBS and incubated overnight at 4 °C with the monoclonal anti-Flag M2 antibody (Sigma Aldrich) (1:20000 in PBS 1% BSA 0.1% Tween20). Cells were washed three times with PBS 0.1% Tween20. The glass slides were covered with the secondary antibody goat F(ab')2 fragment antimouse IgG (H+L)-FITC (Beckman Coulter) (1:200 in PBS 1% BSA 0.1% Tween20). Alternatively, the secondary antibody goat anti mouse Alexa fluor 488 (1:800 in PBS 1% BSA 0.1% Tween20) was used. After two washes in PBS the cells were DAPI stained (1:10000 in PBS 5 min at RT). After final washing (3xPBS, 1xH_2O) the glass slides were air dried and mounted on microscope slides using Mowiol. Alternatively the wet slides were mounted with Aqua Poly/Mount (Polysciences) and sealed with nail polish.

Compound library screening for activation of GPR88

Calcium mobilization experiments using Fluorescent Imaging Plate Reader (FLIPR)

Cells were detached with Accutase (Innovative Cell Technologies) and resuspended in 10 ml complete medium without selection agents. Cells were seeded in 384-well black, clear-bottom poly-D-lysine coated plates (Becton Dickinson BioCoat) at approximately 10^4 cells/well in 50 µl, titrated to obtain a confluence of 80% the following day. Then, Fluo-4 AM (Invitrogen) (8 µM) and Pluronic F-127 (Molecular Probes) (0.08%), both from DMSO stocks, were dissolved in the assay buffer (HBSS containing calcium and magnesium and 5 mM glucose (Wisent) with 0.1% BSA and 20 mM Hepes added fresh, pH 7.4). The final concentration of DMSO in the loading solution was 0.8%. Before loading, part of the medium was removed with an Embla 384 cell washer (Molecular Devices) so that 25 µl residual medium was left in each well. Then, 25 µl loading buffer was added to each well using a Multidrop 384 (Thermo Scientific). The plates were incubated 1 h at 37 °C with 5% CO_2. After the incubation period the cells were washed four times in assay buffer using the Embla cell washer, leaving 25 µl buffer per well after the wash protocol. Then the cells were allowed to rest at least 5 min at room temperature in the dark. The plates were read in a $FLIPR^{384}$ system (Molecular Devices) using the following settings: pipettor height: 30 µl; dispense speed: 15 µl/s; fluid volume: 12.5 µl; exposure length: 0.4 s; filter #1 (510-570 nm). The baseline was read for 10 seconds, and then 12.5 µl of the ligands (final concentration 1 µM) was automatically added to the cell plate. The DMSO concentration at the cells never exceeded 2%. The total reading time was 3 min. The data was analysed using the SoftMax Pro software (Molecular Devices).

Calcium mobilization experiments using Flexstation 3

The experiments were carried out essentially as for FLIPR with the following exceptions. Cells were plated in 96-well black, clear-bottom poly-D-lysine coated plates (Becton Dickinson BioCoat) at about 5.10^4 cells/well in 200 µl. The assay buffer composition was HBSS (Sigma) with 0.1% BSA and 20 mM Hepes added fresh. The loading solution contained 4 µM Fluo-4 AM (Invitrogen) and 0.04% Pluronic F-127 (Molecular Probes) in a mixture of 50% assay buffer and 50% of the usual culture medium without selection antibiotics. Before loading, the medium was removed by decanting. Then, 100 µL loading buffer was added to each well using a multichannel pipette. After the incubation period the cells were washed three times in assay buffer (200 µl/well), and then 40 µl assay buffer was added per well. The plates were analyzed in a Flexstation 3 system (Molecular Devices). Fluo-4 was excited at 488 nm and the emission at 520 nm was detected. The 'Flex' reading mode was used and the detection sensitivity was set to 'high'. The baseline was read during 25 s and then 20 µl of the ligands was automatically added to the selected wells of the cell plate (pipetting speed 1; pipette height 50 µl). For compounds cherry-picked from the compound plates this resulted in a final compound concentration of 1 µM.

Compound collection

The compounds tested in the ligand identification campaign were stored at 3 μM in aqueous solution at -80 °C, in 96-well compound plates. Prior to the experiments four 96 well plates were combined into one 384 well plate using a Biomek FX robot (Beckman Coulter). The compound libraries tested were the Sigma-RBI LOPAC^{640} ™ ligand collection (640 compounds), the Prestwick Chemical Library (880 compounds), a library of endogenous and predicted peptides (1611 compounds) and the Jerini (JPT) RF-amide collection (1000 compounds), yielding a total of 4131 compounds.

Results

Generation and characterization of HEK293s-hGPR88 cell lines

Construction of a hGPR88 - N-Flag - signal-sequence plasmid

In order to facilitate immunostaining and membrane expression of GPR88 in HEK293s cell lines we inserted a Flag-tag and a modified influenza hemaglutinin signal sequence [19] upstream of the receptor sequence. The full GPR88 sequence was resistant to PCR amplification, so a cloning strategy was developed where only the 240 N-terminal bases of the GPR88 genes were amplified by PCR to insert the Flag-tag and signal sequence. Cloning of the product into pcDNA3.1+ bearing the cDNA of GPR88 yielded the correct plasmid as verified by digestion and sequencing.

Subcellular localization of transiently expressed GPR88

We verified by immunocytochemistry that hGPR88 with Flag tag and signal sequence was expressed on the plasma membrane in transiently transfected HEK293s cells. We used transient transfection with a Flag-tagged human delta opioid receptor (DOR-Flag) as a positive control. Cells transfected with GPR88-Signal-Flag and DOR-Flag showed both intracellular and membrane staining in about 50% of the cells 48 h after transfection (data not shown).

Generation of stable cell lines expressing hGPR88- Flag-signal sequence

Since the G protein coupling specificity of GPR88 is unknown, co-transfection with promiscuous $G\alpha_{16}$ or with chimeric $G\alpha_{qi5}$ was employed to facilitate coupling to the

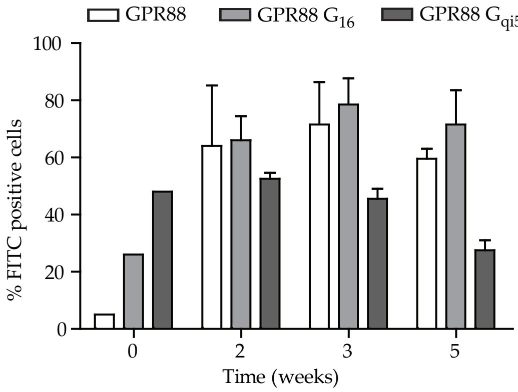

Figure 1. Expression of GPR88-Flag in the three stable cell lines estimated by flow cytometric analysis of 30000 cells (N=2). Timepoint 0 represents the values before FACS sorting. All cells with fluorescence above 1000 units were considered FITC-positive.

calcium pathway. Native HEK293s cells and HEK293s cell lines stably expressing either $G\alpha_{16}$ or $G\alpha_{qi5}$ were transfected with the pcDNA3+/hGPR88/Flag/signal sequence plasmid. Control cell lines were generated by transfection with empty pcDNA3 vector. Four days after transfection the appropriate selection agents were added. Per cell line, twelve resistant colonies were isolated and stored, whereas all other resistant colonies (>100 per cell line) were pooled. Six weeks after transfection the expression level of GPR88-Flag in the resistant cell pools was determined by labeling with a primary anti-Flag antibody and a secondary antibody labeled with FITC, followed by flow cytometry. At the first analysis the transfected HEK293s and HEK293s $G\alpha_{qi5}$ cell lines had approximately 5% FITC-positive cells, whereas the transfected HEK293s $G\alpha_{16}$ cell line had 25% FITC-positive cells.

Enrichment of GPR88-Flag expressing cells by fluorescent automated cell sorting

In order to obtain higher GPR88 expression, FITC positive cells were selected by fluorescent automated cell sorting (FACS). HEK cells transfected with the empty vector showed fluorescence below 800 units, so cells with fluorescence above 1000 units were considered FITC-positive. The negative control cell lines HEK293s, HEK293s-$G\alpha_{16}$ and HEK293s-$G\alpha_{qi5}$

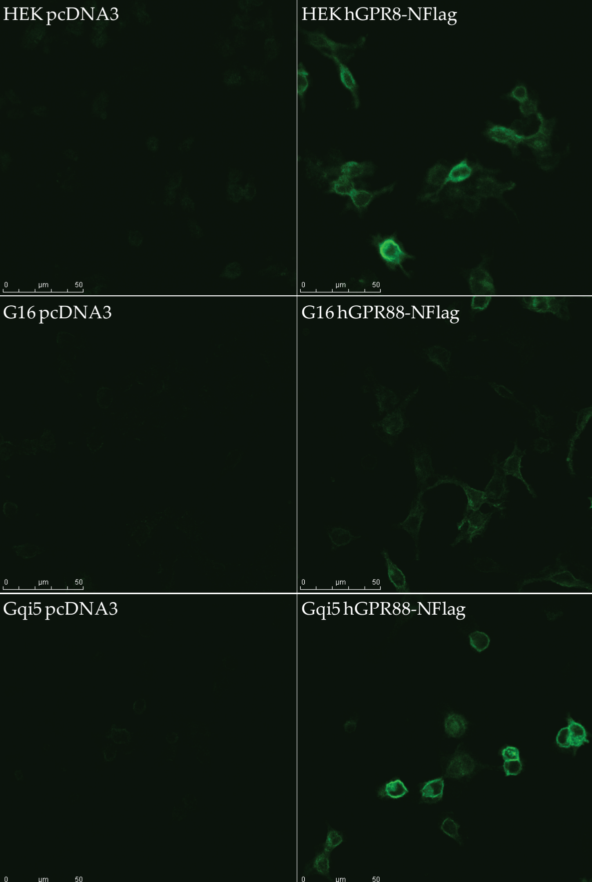

Figure 2. Immunocytochemical staining with an anti-Flag antibody in order to demonstrate expression of GPR88-Flag at the cell membrane of HEK293 cells. The same staining was performed on control cell lines transfected with empty pcDNA3 vector (left panels).

transfected with pcDNA3 were also passed through FACS as a mock-sorting.

The enriched HEK-Gα_{qi5}-GPR88 cells lost expression of GPR88 in the first week after sorting. We therefore analyzed the 12 clones isolated after the Geneticin selection, of which two had a moderate percentage of GPR88 expressing cells (14 and 17% FITC positives) and one a low percentage (0.5%). The three positive colonies were pooled and the positive cells were isolated by FACS sorting.

The stability of the GPR88-Flag expression over time was monitored by flow cytometric analysis (figure 1). The expression of GPR88 was fairly stable, although the percentage of expressing cells diminished in the Gα_{qi5} GPR88 cell lines.

Monitoring of GPR88-Flag expression by immunocytochemistry

The expression of GPR88-Flag over time was also assessed by immunocytochemistry using a primary anti-Flag antibody and a fluorescent secondary antibody. Three weeks after FACS enrichment, the percentage of positive cells was estimated at 45%, 50% and 40% for the HEK cells containing GPR88 alone, GPR88-G_{16}, and GPR88-G_{qi5}, respectively. Prior to the start of experiments on a new batch of cells the expression level of GPR88-Flag was checked by immunocytochemistry, an example of which is shown in figure 2. The expression levels were checked again at the end of each series of experiments. Typically, membrane staining was observed in approximately 50% of the cells stably transfected with GPR88-Flag. In some cases, especially in the cells transfected with Gα_{qi5}, a strong signal was observed around the nucleus.

Compound library screening for activation of GPR88

We performed a ligand identification campaign for GPR88. The compounds we tested in the screen were stored in ready-for-use compound plates at -80°C. In total, 4131 compounds were tested at least in duplicate at a final concentration of 1 μM. The compound libraries we tested were the Sigma-RBI LOPAC^{640} ™ ligand collection, the Prestwick Chemical Library, an in-house library of endogenous and predicted peptides and the Jerini (JPT) RF-amide collection.

Calcium mobilization primary screen using FLIPR

The collection of 4131 compounds was tested for the capability of provoking a calcium response through activation of GPR88 in native HEK cells or cells co-expressing Gα_{16} or Gα_{qi5}. Corresponding cell lines transfected with empty vector pcDNA3 were used as negative controls. The experiments were conducted on attached cells in 384 well plates, using a FLIPR system (Molecular Devices) for detection of the fluorescent signal of the calcium indicator Fluo-4, and for robotic addition of the test compounds. The DMSO concentration never exceeded 2% and controls showed that this amount did not change the fluorescence signal. The fluorescence maxima and the shape of the curves were examined by eye to conclude whether GPCR activation took place. A maximum fluorescence below 1000 fluorescence counts was considered background. On each compound plate positive and negative (buffer only) controls were included. As positive controls, the agonists endothelin-1, vasoactive intestinal protein (VIP) and pituitary adenylate cyclase-

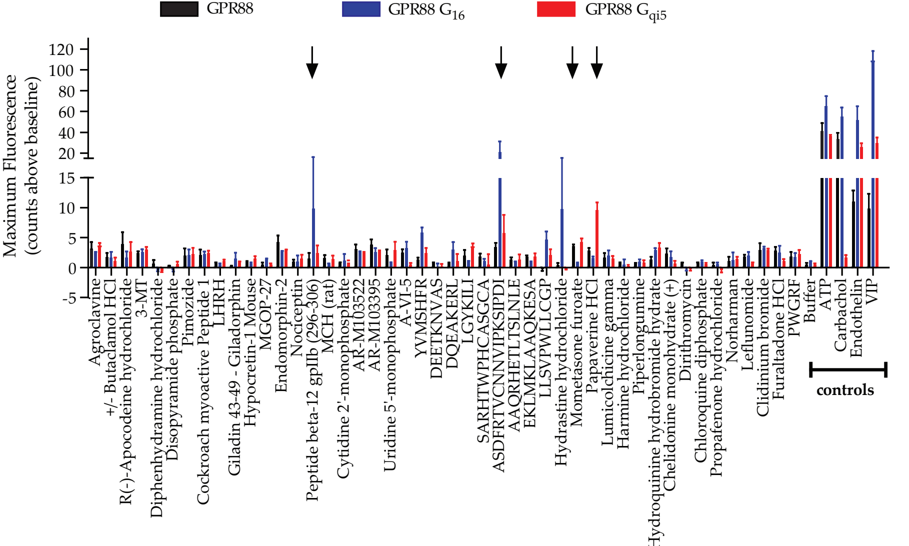

Figure 3. Maximum fluorescence responses in a calcium mobilization assay for 46 hits from the primary screen. The compound solutions were cherry-picked from the screening plates (final concentration 1 μM). Average values (± SEM) (N≥2) are shown. The arrows indicate compounds yielding a robust response in this assay.

activating peptide (PACAP), capable of activating endogenous HEK293s GPCRs, were used. All compounds were tested in two independent single-point experiments at a final concentration of 1 μM.

The calcium responses obtained from potential hits were low to moderate. Out of 71 calcium responses to the most promising compounds only 5 responses were above 3000 fluorescence counts, and 47 reached their maximum below 2000 fluorescence counts. Some compounds provoked calcium responses in the cell line HEK293s-Gα_{qi5}-GPR88, some in the cell line HEK293s-Gα_{16}-GPR88 and yet others in the cell line HEK293s-GPR88, whereas several compounds stimulated more than one cell line. Only the hits peptide beta-12,

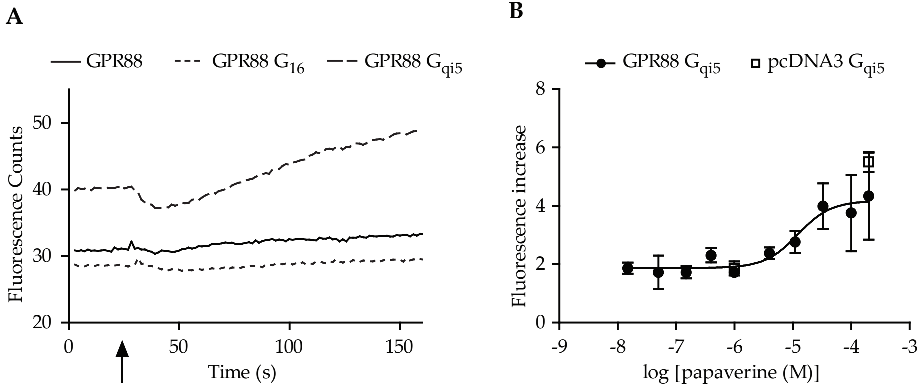

Figure 4. A. Calcium mobilization responses to papaverine in HEK cells expressing GPR88-Flag. This experiment was performed with compound solution cherry-picked from the screening plates (final concentration 1 μM). The arrow indicates the time of compound addition. **B.** Dose-response curves of papaverine from calcium mobilization assays. Mean data from two independent experiments is shown. The compounds were also tested on nontransfected cell lines at 1 and 200 μM.

chelidonine monohydrate (+), chloroquine diphosphate, clidinium bromide and PWGRF could be confirmed in two independent experiments. When the average responses over the two experiments were taken into account, 26 compounds qualified as hits. Several other compounds that provoked a robust calcium response, but only in one of the two experiments were also selected for further study, bringing the total to 46 hits.

Hit validation assays

The compounds that were selected from the screen performed on FLIPR were entered in a secondary screening round that was performed in a similar manner but using a Flexstation 3 (Molecular Devices). First, the ideal conditions for the calcium experiments at the Flexstation 3 were determined. In order to keep the compound use as low as possible a working volume of 60 μl was selected instead of the recommended 150 μl, which did not affect the reproducibility and the signal-to-noise ratio.
In total, 46 promising compounds were tested twice at the Flexstation 3 on the three cell lines expressing GPR88. Figure 3 shows the maximum responses that were observed in these experiments. Most compounds did not provoke a calcium response. Small responses were seen in one experiment for papaverine (Figure 4A) and mometasone furoate and moderate responses were seen for peptide beta-12 and the peptide ASDFRTVCNNVIPKSIPDI. The responses to peptide beta-12 administration could not be reproduced in two further experiments using this compound from the same and a different compound plate. Fresh solutions were prepared for testing mometasone furoate, papaverine hydrochloride and the peptide ASDFRTVCNNVIPKSIPDI at higher concentrations. Mometasone furoate and papaverine were tested at ten concentrations ranging between 15 nM and 200 μM, and the peptide was tested at 1 and 10 μM (N=2). No responses were seen to either concentration of ASDFRTVCNNVIPKSIPDI. For mometasone furoate small responses were observed in all GPR88 expressing cell lines at the highest compound concentration (200 μM), but the responses were also observed in the HEK293s pcDNA3 cells and no dose-response curve could be obtained. For papaverine, a dose-response curve was observed in the cell lines containing $G\alpha_{qi5}$ (figure 4B) and $G\alpha_{16}$ (not shown), but the small responses observed were similar in the pcDNA3-transfected cells. Therefore we must conclude that no GPR88-mediated responses could be demonstrated.

$GABA_B$ allosteric modulators as putative GPR88 ligands

As described in the introduction, phylogenetic analysis suggests that ligands for GPR88 might bear a certain resemblance to allosteric modulators of the $GABA_B$ receptor. Therefore, we tested three commercially available compounds that are known to modulate $GABA_B$ (CGP7930, CGP13501 and GS39783) on the GPR88 expressing cell lines, using the Flexstation 3. The compounds did not provoke a calcium response, even at a concentration of 10 μM (data not shown).

Discussion

Despite a decade of academic and private research efforts, GPR88 remains an orphan receptor with no known synthetic or endogenous ligands. The only hint at compounds

activating GPR88 was found in the patent literature, where ligands for GPR84 were described with limited selectivity towards this receptor over GPR88 [20]. The ligand identification screen we performed yielded 46 hits out of 4131 compounds tested, but none of the hits could be confirmed as a GPR88 agonist.

Our failure to identify an agonist for GPR88 may be due to the limited number of compounds we tested. Compound collections of tens to hundreds of thousands in size are no exception [21], and Johnson and coworkers even screened over a million molecules [22]. Major GPCR ligand classes were represented in our compound collection but GPR88 may display specific binding to a ligand or ligand class that was not included. It is possible that this receptor, which does not show high sequence similarity to any GPCR subfamily [23-25], binds to an unusual or unknown signaling molecule. Testing at a higher concentration could also increase the chances of identifying a ligand. Even a low potency agonist could give valuable clues regarding downstream signaling, and at the same time serve as a starting point in the search for a more potent ligand. Furthermore, some GPCRs may not bind with a high affinity to their endogenous ligands, as seems to be the case for the recently deorphanized hydroxy-carboxylic acid receptor family [26]. Despite the added cost, screening at a higher concentration may be worth it.

A major difficulty in deorphanization screens is the inherent absence of real positive control conditions to test the detection of receptor activation by an agonist. Accessory proteins potentially required for GPR88 function could be absent in HEK293s cells. Although we confirmed that the receptor was expressed at the membrane and that the signals from endogenous GPCRs on HEK cells were detected correctly, it is also conceivable that our screening methods were not ideal for the detection of GPR88 activation. We cast our net wider by performing a second screen, where the read-out was a membrane depolarization instead of a change in intracellular calcium concentration. This screen, which is not further described here, yielded 77 hits of which none could be confirmed in validation experiments.

GPR88 so far resisted attempts at deorphanization, not only by us but by several other groups as well, judging from personal communications and the website of the Michael J. Fox Foundation, which awarded a grant for exploring GPR88 as a target for the treatment of Parkinson's disease in 2008, which did not yield any ligands (http://www.michaeljfox.org/research_MJFFfundingPortfolio_searchableAwardedGrants_3.cfm?ID=432). It could be that GPR88 is one of the 'real' orphans that do not have an endogenous ligand, nor need one to function. The physiological relevance of such a GPCR could be as a chaperone for another receptor, similar to $GABA_{B2}$ [27] or as a constitutively signaling protein, like GPR3 (GPR12), a G_s-coupled orphan receptor with an apparently ligand-independent role in the female reproductive system [28-29].

Another example of an orphan receptor functioning independently from a ligand is GPR50 dimerizing with the MT_1 and MT_2 melatonin receptors, which prevents melatonin signaling in the case of MT_1 [30]. In fact, unpublished data from R. Massart, P. Sokoloff et al. (briefly summarized in this editorial: http://scielo.isciii.es/pdf/ejpen/v24n1/editorial.pdf) suggest an unusual and possibly ligand-independent function for GPR88: the receptor may have a role as an intranuclear, chromatin-associated protein modulating

developmental nuclear transportation in differentiating cortical neurons. However, the same group also reported localization of GPR88 outside of the nucleus in mature striatal medium spiny neurons, preferentially in post-synaptic domains [13].

A first step towards future deorphanization of GPR88 could be the elucidation of its downstream signaling. Receptor overexpression or mutagenesis could be used to trigger enhanced basal or constitutive signaling. Further screening could then be limited to one GPR88-expressing cell line and one assay only, while we screened on three cell lines in two assays. Screening in a striatal, neuronal cell line [see for example 31, 32] expressing GPR88 naturally would further increase the chances of success, since all necessary machinery for receptor signaling is presumably expressed in these cells.

In GPCR deorphanization, the identification of the first ligand is an enormous hurdle. When this is overcome, further ligands can be identified with relative ease, and the role of the receptor in (patho-)physiology can be explored in more detail. In my opinion, new ligands for orphan GPCRs, even if they have a low potency, should be made public as soon as possible after discovery. This would be in the interest of the scientific community as a whole, and the pharmaceutical industry in particular.

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Chapter 8

General discussion and future perspectives

For the preparation of this thesis a ligand-based approach was used to investigate three different G protein-coupled receptors from class A, that are all (potentially) important drug targets. The most well characterized is the adenosine A_3 receptor, which was discovered by homology cloning in 1992 and readily recognized as an adenosine receptor [1]. The adenosine A_3 receptor has received attention as a potential target for anti-cancer medication. Approximately a decade later, the second receptor of interest, GPR109A, was deorphanized by pairing to endogenous ligand 3-hydroxybutyrate, prompting re-naming as the hydroxy carboxylic acid receptor 2 (HCA_2). This receptor is a target for anti-atherosclerotic drugs. The focus of the last research chapter is on the orphan receptor GPR88, which is still a mystery in many aspects, but it may play a role in the pathophysiology of the brain.

The hydroxy-carboxylic acid receptor 2 (HCA_2)

In **chapter 2**, an overview is presented of the literature on the 3-hydroxybuyrate receptor HCA_2 and the two other members of the subfamily, namely HCA_1 (for lactate) and HCA_3 (for β-oxydation intermediates). As the target of nicotinic acid, HCA_2 has been most widely studied, although HCA_1 and HCA_3 may present interesting future drug targets, for instance for the treatment of obesity and dyslipidemia, respectively. After their deorphanization, the physiological roles of the HCA receptors on adipocytes are now coming into focus: HCA_1 boosts the antilipolytic effect of insulin, and HCA_2 and HCA_3 inhibit excessive lipolysis during starvation. Especially for HCA_2, but also HCA_3, many synthetic agonists have been developed, with the aim of introducing a new and improved successor for nicotinic acid in the clinic. Hopes were high for MK-0354, a biased HCA_2 agonists with very low ERK1/2 activation *in vitro* and almost no flushing *in vivo*, but in phase II clinical trials the lipid modification targets were not reached [2]. Another HCA_2 agonist, MK-1903, followed a similar path. However, new findings concerning the mechanism of action of nicotinic acid beg the question whether other clinical endpoints are needed for clinical trials of HCA_2 agonists. A significant part of the anti-atherosclerotic effect of nicotinic acid appears to be mediated by activation of HCA_2 on immune cells, decreasing endothelial dysfunction and vascular inflammation, and increasing cholesterol efflux from plaque macrophages [3]. Therefore, instead of a complete focus on lipid modification, more attention should be paid to the anti-inflammatory effects of new HCA_2 agonists in (pre)clinical drug testing.

The skin flushing side-effect of nicotinic acid was recently shown to be partly mediated by HCA_2 on keratinocytes (through COX-2 and PGE_2), next to the well-known involvement of Langerhans cells [4]. Better understanding of flushing could lead to better prevention in the future, although advances have already been made. The use of extended-release formulations of nicotinic acid [5] and combination with the DP_1 prostaglandin receptor antagonist laropiprant [6] have significantly reduced the occurrence and severity of nicotinic acid-induced flushing.

In **chapter 3**, the *in vitro* and *in vivo* effects of two partial agonists for HCA_2, both pyrazole compounds, are presented. We have shown that these ligands have lower relative potency for ERK1/2 phosphorylation compared to nicotinic acid, a property that has been linked

to decreased skin flushing [7]. In mice, we confirmed that the pyrazoles did not increase cutaneous temperature. Although less precise than laser doppler velocimetry, we believe surface temperature recording is a good alternative for measuring blood flow in the skin, and thus flushing. The absence of a skin flushing response may be due to the moderate shift in the relative potencies on the G protein pathway and the ERK phosphorylation pathway, to the reduced intrinsic efficacy of these ligands, or to a combination of these two factors. Nicotinic acid and both partial agonists significantly reduced plasma VLDL concentrations. In the case of nicotinic acid, a 2-fold reduction of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) in the adipose tissue seemed mainly responsible for this effect. Interestingly, the two pyrazole compounds did not affect gene expression in the adipose tissue. Instead, these compounds triggered a >40% reduction of apolipoprotein B (ApoB) in the liver, which indicates decreased hepatic VLDL production. The reduced ApoB expression could be downstream from suppressed hepatic PGC-1β expression, which has been previously reported as an effect of nicotinic acid [8]. No effect was seen on HDL, but this is expected in wildtype mice that naturally lack a homolog to the human cholesterylester transfer protein (CETP), responsible for transfer of cholesterol from HDL to less dense particles [9].

Chapters 4 and **5** describe medicinal chemistry programs in which potential new ligands for HCA_2 were devised, synthetized and characterized in our laboratory. In **chapter 4** a series of propenoic acid derivatives and their affinity for HCA_2 is presented. Two agonists of this compound class, monomethyl- and monoethyl-fumarate (MMF and MEF), are used in the clinic as anti-psoriatic drugs [10]. We confirmed binding of these compounds and of cinnamic acid to HCA_2, and describe synthesis and affinity testing of a series of fumaric acid esters and amides, as well as cinnamic acid derivatives. The binding pocket for these ligands seems very restricted: in a series of 55 related compounds, only 15 had a K_i of ≤10 μM, and none could be identified with higher affinity than MMF and MEF. On the basis of molecular modeling and analysis of the structure-activity relationships, we propose a pharmacophore featuring a planar *trans*-propenoic acid with a maximum length of 8 Å, with larger substituents oriented out-of-plane.

Chapter 5 revolves around a very different type of ligands, the pyrazolopyrimidines, which are much larger and bind at an allosteric site on the HCA_2 receptor protein. Starting from a previously published ligand series [11], we prepared a number of novel compounds and characterized them in radioligand binding and functional assays. Several compounds were potent and efficacious agonists on HCA_2 when tested alone, even matching nicotinic acid's potency, while at the same time enhancing the potency (up to 20-fold) and/or efficacy (up to +50%) of nicotinic acid when simultaneously present in the assay. We observed that the agonistic and potency-enhancing properties of the pyrazolopyrimidines were correlated, suggesting that the ability to stabilize an active receptor conformation contributed to the modulating properties of these ligands. Of special interest is our finding that some derivatives markedly enhance the efficacy and potency of the endogenous HCA_2 ligand 3-hydroxybutyrate, since this highlights the potential of this type of ligands for future drug development.

The adenosine A_3 receptor (A_3R)

In **chapter 6**, we report that the naturally occurring nucleosides N^6-(2-isopentenyl)-adenosine (IPA) and racemic zeatin riboside are selective human A_3R ligands with affinities in the high nanomolar range, which is comparable with the affinity of adenosine. IPA also bound to the rat A_3R which good affinity. Both ligands could inhibit forskolin-induced cAMP formation with micromolar potency, and in the case of IPA we showed that an A_3R antagonist could block this effect. IPA was previously reported to inhibit tumor growth, but the A_3R was not implicated in this effect until now. We observed strong and similar anti-proliferative effects of IPA and reference A_3R agonist Cl-IB-MECA in two tumor cell lines. At low concentrations, the effect of IPA could be blocked by a selective A_3R antagonist, but at higher concentrations this was not the case. The anti-proliferative effect at higher concentrations of IPA seems to be independent of A_3R, which was previously observed for high concentrations of other A_3R agonists, for example for Cl-IB-MECA [12]. The existence of an additional endogenous ligand for A_3R, next to adenosine, with protective anti-cancer properties, was previously predicted [13]. Although IPA could fit this profile, we were unable to detect the ligand in fresh rat muscle using HPLC analysis. Thus, no definite proof could be found for a role of IPA as an endogenous agonist for A_3R.

Orphan receptor GPR88

Chapter 7 focuses on GPR88, which is one of the dozens of G protein-coupled receptors for which no ligand or function is known to date. Analysis of its expression pattern and changes in expression levels, as well as the phenotype of a mouse model lacking the receptor, suggest that GPR88 may be implicated in several diseases including schizophrenia, depression and bipolar disorder. We attempted to identify a ligand for this receptor by testing a library of 4131 small molecules and naturally occurring peptides, using changes in intracellular calcium levels as the readout. In native HEK293 cells, GPR88 may stimulate the calcium pathway via $G\alpha_q$, but since the coupling preference of the receptor is unknown, cell lines co-expressing chimeric $G\alpha_{qi5}$ or $G\alpha_{16}$ were also used (in case of natural G_i or G_s coupling, respectively). Our primary screen, performed using the Fluorescent Imaging Platereader (FLIPR) in 384-well format, yielded 46 hits, with responses in all three cell lines but with poor reproducibility. In validation assays, dose-response curves could only be obtained for papaverine, but these seemed independent of GPR88, since calcium mobilization was also observed in control cell lines lacking the receptor. Thus, our efforts mainly served to confirm the challenging nature of receptor deorphanization programs.

Future perspectives

G protein-coupled receptors have become an important field of study within the life sciences. We now know that they make up the largest gene family in our genome, and are prominently involved in most major physiological processes in the body [14]. Since their discovery, new concepts have regularly emerged that refined our understanding of this fascinating protein family in general, or of specific receptors. Recent advances include,

but are not limited to, structural data on a number of receptors (reviewed by [15]), the realization that GPCRs function not only as receptors for transmitters, taste and odorants, but also as sensors for nutrients and intermediates of metabolism [16-18], and receptor-ligand pairing of a number of orphan receptors, including the three hydroxy-carboxylic acid receptors (reviewed by [19]). However, many unresolved questions remain in GPCR research. The current thesis focuses on three receptors: the hydroxy-carboxylic acid receptor 2 (HCA_2) (chapters 2-5), the adenosine A_3 receptor (chapter 6), and orphan receptor GPR88 (chapter 7). In this section I will discuss the open questions regarding these 3 receptors and possible routes towards their resolution. Some general remarks regarding the future of GPCR research will also be made.

Hydroxy-carboxylic acid receptors

All three hydroxy-carboxylic acid receptors have been deorphanized in the last few years [20-23]. It has been proposed that their roles in physiology are the conservation of fat stores during periods of starvation (HCA_2 and HCA_3), and under influence of insulin (HCA_1). In the case of HCA_2, the negative feedback loop which is formed when 3-hydroxy butyrate counteracts its own production by activating HCA_2, thus reducing lipolysis, may also be important as a break on the levels of acidifying ketone bodies in the blood. In primates HCA_3 may also play a role here, since it also forms a negative feedback loop on lipolysis with its ligands produced by β-oxydation of free fatty acid [20]. As yet, the feedback mechanism I propose has not been verified. It is well known that insulin keeps the plasma levels of ketone bodies in check, and even if the HCA_2 loop has a role here as well, it is apparently, in certain cases, not sufficient to prevent diabetic ketoacidosis when the insulin loop is non-functional. However, 3-hydroxy butyrate may not be a full agonist for HCA_2 (chapter 5), and thus have a limited effect even at high concentrations. Full agonists or allosteric enhancers of HCA_2 or HCA_3 may have therapeutic potential in acidosis. Acute nicotinic acid administration also diminishes gluconeogenesis in the liver [24], which would be of further benefit to counteract the hyperglycaemia in diabetic ketoacidosis. In addition, a recent publication suggests that elevation of 3-hydroxy butyrate in diabetic patients, with concomitant upregulation of HCA_2 expression, is a mechanism to protect the tissues from the damaging effects of the inflammation associated with the disease [25]. Even in absence of ketoacidosis, HCA_2 agonists may thus be valuable drugs in the treatment of diabetes. However, it has also been reported that nicotinic acid may decrease insulin sensitivity [26] and promote the onset of diabetes [27] so more research is needed into the role of HCA_2 and its ligands in this disease.

In intestine and skin, HCA_2 appears to prevent excessive inflammation and tumor formation [28-29]. In the intestine, the major ligand is likely not 3-hydroxy butyrate, but non-hydroxylated butyrate produced by bacterial fermentation. By comparing potency [23] and local concentration from the literature [30], it seems that pentanoate and hexanoate are also likely to activate HCA_2 in the intestine, but no published experimental work proves this. Although ligands derived from nutrition and bacterial fermentation in the intestine are not produced by the body itself, they could still be regarded endogenous ligands of GPCRs in the intestinal wall. Functionally they could be compared to the

receptors involved in taste, smell and vision, which also detect compounds and signals from the outside world. Several other GPCRs, for example the sweet taste receptor T1R2/T1R3, have already been implicated in nutrient sensing in the bowel [31].

In certain inflammatory diseases, like psoriasis and multiple sclerosis, HCA_2 has also been implicated. Psoriasis is already treated with topical application of HCA_2 agonist monomethylfumarate (MMF) [32], and a phase III trial has recently been favourably concluded with oral dimethylfumarate (BG-12) for multiple sclerosis (MS) [33-34]. *In vivo*, dimethylfumarate is rapidly metabolized to HCA_2 agonist MMF, and the side effect profile of BG-12, featuring mainly flushing and gastrointestinal complaints, is typical for a HCA_2 agonist. Inhibition of nuclear translocation of NF-κB has been reported as the mechanism of action, and this is a known downstream effect of HCA_2 activation [29]. However, it has not been clearly demonstrated if HCA_2 is a key player in the therapeutic action of fumarates, so experimental confirmation in an animal model lacking HCA_2 would be valuable. Nicotinic acid itself has also been proposed as a potential anti-inflammatory therapy for MS [35], as well as sepsis [36], chronic renal failure [37] and arthritis [38]. For such acutely life-threatening or debilitating indications, the harmless side effect of skin flushing is less likely to affect patient compliance, and the side effect may be more acceptable if more efficacious or safer treatments are not available.

Adenosine A_3 receptor and isopentenyl adenosine

We identified N^6-isopentenyl-adenosine (IPA) as a ligand for the adenosine A_3 receptor (A_3R). We also investigated whether IPA is an endogenous ligand for the A_3R. No evidence could be found for significant concentrations of IPA in muscle tissue, but it is still a possibility that IPA binds to the A_3R in certain (patho-) physiological conditions. The idea that one receptor can have more than one orthosteric ligand is well-known, especially when synthetic ligands are considered. In recent deorphanization efforts, receptors have regularly been found to have multiple endogenous ligands, that may or may not be chemically related (for example GPR17 with uracil nucleotides and cysteinyl-leukotrienes [39], and several receptors binding a range of free fatty acids (for a review see [40]). In some cases this could be a sign of erroneous receptor-ligand pairing (for example for GPR65/TDAG8, a proton sensor but not lysophosphatidylcholine receptor [41]), but in other cases the double or multiple pairing may be correct. It would be interesting to subject receptors that have known ligands (discovered by classical pharmacology or early screens) to a state-of-the-art receptor deorphanization screen. The possibility of additional endogenous ligands should also be kept in mind when investigating unexplained receptor activation or transmitter effects. Examples of GPCRs that were 'partial orphans' are the P2Y1 receptor which binds not only ADP but also ADP ribose [42] and β-NAD [43], and the CXCR2 receptor that binds 3 chemokines but was later shown to bind macrophage-derived lectin MNCF as well [44]. For adenosine receptors it was observed that endogenous adenosine concentrations could not always account for the activation levels [45-46], thus raising the question of alternative endogenous ligands. In the case of the A_3R, it has already been shown that it binds to inosine next to adenosine [47].

GPR88 and other orphan GPCRs

Since no endogenous ligand is known for GPR88, it is possible that this receptor functions independently from a ligand. Even if this is the case, the receptor remains a potential therapeutic target. Drugs targeting a ligand-less receptor could aim at changing its expression level, at blocking its interaction with other proteins in the cell, or synthetic ligands could be devised just as for a liganded receptor, even in absence of an endogenous ligand. For example, if a lower expression level or loss-of-function of GPR88 could be demonstrated in patients with schizophrenia, in parallel to the 'schizophrenic' GPR88 knock-out mice [48], treatment with synthetic GPR88 agonists or GPR88 up-regulating drugs could be effective.

The reported similarity of the GPR88 binding pocket to the class C glutamate and $GABA_B$ receptor binding pockets [49] can be a starting point for the identification of a synthetic GPR88 ligand. An *in silico* prediction of GPR88 ligands (thesis J.K. Bray 2010 [50]) identified the lipids FFA, LPA and S1P as potential ligands. Functional or binding assays either confirming or refuting this prediction have not been reported. In the patent literature, ligands for medium-chain fatty acid receptor GPR84 were described with limited selectivity towards this receptor over GPR88 (see table 1) [51]. From the text it could be deduced that these ligands have a potency on GPR88 between 10 and 100 μM (compounds 2 and 3), or between 100 μM and 1 mM (compound 1), although this is not clearly stated. The reported potencies on GPR84 are in the nanomolar range, so these ligands are clearly not selective GPR88 ligands, but if their action on GPR88 were confirmed, they could be a starting point for further ligand optimization.

Table 1. Ligands with (high) micromolar potency at GPR88, next to nanomolar potency at GPR84. Adapted from (WO2007027661).

Compound	Chemical structure
1	OH, O, O, OH
2	O, OH
3	HO, O, HO, OH

Ligand pairing of GPR88 and the other remaining orphan receptors will be a challenging task. A promising technique that may increase the chances of success is the use of a label-free biosensor [52]. When this is used as the screening assay, no knowledge is needed of the downstream coupling of the orphan receptor, because any change in the cells is captured [53]. This is useful even when a downstream signalling pathway is known, as

for constitutively active orphans, because binding of an agonist may activate a completely different pathway. Another advantage of label-free assays is the high sensitivity, which facilitates the use of a cell line endogenously expressing the GPCR of interest [54], instead of using heterologous receptor expression, ensuring that all components necessary for receptor function are present, next to the native receptor.

Final note

The work presented in this thesis revolves around the identification and characterization of novel ligands for G protein-coupled receptors: small molecule (partial) agonists and ago-allosteric modulators for HCA_2, naturally occurring nucleosides for A_3R, and a wide medium-throughput screen at GPR88. The model of lock and key, one receptor plus one ligand, opening the door to the same pathway every time, is too simple for GPCR reality. The receptor could be seen as a magical door, with multiple locks (orthosteric and allosteric sites), where different keys (ligands) in the same lock can open the door to different pathways, and where simultaneously inserting keys in multiple locks gives even more possible outcomes. In the body, a plethora of compounds is present at the receptor, and if there is sufficient affinity between receptor and ligand, binding will occur, possibly followed by an effect. The expectation that the physiological role of a GPCR is mainly determined by its interaction with a single ligand, may thus be somewhat simplistic. Even for receptors that are not orphans anymore, additional endogenous ligands may still be found. The interest in receptor deorphanization has prompted the development of highly sophisticated screening methods, and it could be profitable to use them on non-orphan receptors as well. Even though GPCRs can be considered established drug targets, novel concepts, such as those presented in this thesis, are needed to unravel their function in physiology, and to optimise their exploitation in medicine.

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Summary

In this thesis, several orthosteric and allosteric agonists are presented for the newly discovered hydroxy-carboxylic acid (HCA) receptor 2, and their in vivo activity or in vitro structure-activity relationships are described. The literature on HCA receptors was also thoroughly reviewed, providing some insight into the future of this receptor family as drug targets. The anti-cancer drug N^6-(2-isopentenyl)adenosine (IPA) was shown to be a specific ligand for the adenosine A_3 receptor, and its antiproliferative effect seems to be mediated by this receptor at low concentrations. A ligand discovery screen for orphan receptor GPR88 was performed, in which over 4000 compounds were tested.

Most G protein-coupled receptors (GPCRs) can function only in concord with their (endogenous) ligand(s). When the ligand concentrations are disturbed, or when the receptor has a defect that precludes ligand-dependent activation or signaling, correct receptor function becomes impossible, and often this causes a disease. Knowing which endogenous ligand binds to a GPCR in the body is therefore considered essential for understanding its function. On the other hand, synthetic ligands for GPCRs can be highly effective drugs for correcting a pathophysiological imbalance. This thesis is about three receptors, the hydroxy-carboxylic acid receptor 2 (HCA_2), the adenosine A_3 receptor, and GPR88, and the quest for ligands that may influence these proteins in the body, either naturally or as drugs.

In **chapter 1**, GPCRs are introduced, and special attention is given to 'orphan' GPCRs, and the research focusing on the identification of their endogenous ligands. GPR88 is still an orphan receptor today, whereas the HCA receptors were deorphanized recently.

Chapter 2 is a review of our current knowledge on HCA receptors 1, 2 and 3, with special attention for HCA_2 as the receptor activated by the anti-atherosclerosis drug nicotinic acid. These three receptors were deorphanized in recent years as the receptors for lactate, 3-hydroxybutyrate and β-oxidation intermediates like 3-hydroxyoctanoate. Their physiological roles are most likely the fine-tuning of free fatty acid release from the adipose tissue (lipolysis) in conditions of food shortage (HCA_2 and HCA_3) or abundance (HCA_1). The importance of HCA_2 receptor activation for the therapeutic effects of nicotinic acid is still under discussion. Anti-inflammatory effects of HCA_2 agonists have been found in multiple studies, and may be of relevance to other diseases like multiple sclerosis.

In **chapter 3**, in vitro and in vivo studies into the pharmacological effects of two partial agonists for HCA_2, compared to nicotinic acid, are described. These partial agonists of the pyrazole class, LUF6281 and LUF6283, were shown to have affinities of 3 μM and 0.55 μM for HCA_2. In $[^{35}S]$-GTPγS functional studies, the rank order of potency was nicotinic acid>LUF6283>LUF6281, and the efficacies of the partial agonists were ~75% for LUF6283 and 50% for LUF6281. The partial agonists, like nicotinic acid, were more potent in an ERK 1/2-phosphorylation assay, but the EC_{50} ratio $[^{35}S]$-GTPγS/pERK was 2-3x higher for nicotinic acid. In mice, the pyrazoles reduced VLDL levels to a similar extent as nicotinic acid, but these compounds did not elicit a flushing response as measured by skin temperature increase. Whereas nicotinic acid halved the expression of pro-lipolytic enzymes HSL and

ATGL in the adipose tissue, LUF6281 and LUF6283 did not affect their expression levels. On the other hand, the pyrazoles increased the expression of ApoB in the liver by ~40%. These findings show that LUF6281 and LUF6283, although activating the same receptor as nicotinic acid, have different in vivo effects, retaining the lipid-lowering properties, while the flushing effect was not detected.

In **chapters 4** and **5**, the identification and in vitro characterization of new ligands for HCA_2 is described. **Chapter 4** focuses on derivatives of trans-propenoic acid, which are related to the anti-psoriasis drug monomethylfumarate. In a series of cinnamic acid derivatives and fumaric acid esters and amides, ligands with affinities in the high nanomolar to high micromolar range were indentified. The binding pocket seemed rather restricted, and trans-cinnamic acid was the longest planar ligand. Taking into account the structure-activity relationships (SAR) of this compound series, molecular modeling suggests a planar trans-propenoic acid pharmacophore of 8 Å in length, where any larger substituents may only be oriented out-of-plane.

In **chapter 5** ligands for the allosteric binding site of HCA_2 are explored using both functional and radioligand binding assays. Several compounds were found to be positive allosteric enhancers of HCA_2 activation by nicotinic acid, and in addition most of the modulators were (allosteric) agonists when tested alone. Several different parameters for ago-allosteric action were used to reveal the ligand texture and the multi-dimensional SAR. Since allosteric modulation is dependent on the orthosteric ligand that is used, we also investigated the enhancing properties of several compounds with regard to the endogenous HCA_2 ligand 3-hydroxybutyrate. Interestingly, several pyrazolopyrimidine ligands were found to increase the efficacy of 3-hydroxybutyrate and enhance its potency up to 10-fold.

Chapter 6 describes the discovery that the naturally occurring nucleosides N^6-(2-isopentenyl)adenosine (IPA) and zeatin riboside are selective ligands for the human adenosine A_3 receptor (A_3R), with affinities of 159 and 649 nM, respectively. IPA also bound with micromolar affinity to the rat A_3R. In a cAMP accumulation assay in CHO cells stably expressing hA_3R, IPA and zeatin riboside exhibited potencies in the micromolar range. The effect of IPA could be blocked by the selective A_3R antagonist VUF5574. Like the reference A_3R agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide (Cl-IB-MECA), IPA has known activity against tumor growth. In vitro antiproliferative effects on human and rat tumor cell lines LNCaP and N1S1 were highly similar for IPA and Cl-IB-MECA, and at low concentrations the effect could be blocked by the selective A_3R antagonist MRS1523. Higher concentrations of IPA seemed to inhibit tumor cell growth by an A_3R-independent mechanism. A similar phenomenon has previously been observed for other A_3R agonists. Since it is a natural compound, we hypothesized that IPA might activate the A_3R in vivo under physiological conditions. However, IPA could not be detected in fresh rat striated muscle preparation using HPLC.

In **chapter 7**, a ligand discovery screen on the human orphan receptor GPR88, which may be implicated in several psychiatric disorders, is described. Over 4000 compounds, including small molecules and peptides, were tested in a medium-throughput, 384-well format Fluorescent Imaging Platereader (FLIPR) screen using increases in Ca^{2+} concentrations as

the readout. Next to a native HEK cell line stably expressing GPR88, stable co-transfection of $G\alpha_{16}$ and $G\alpha_{qi5}$ was also done, since the natural coupling preference of GPR88 is unknown. In the primary screen, a total of 47 hits was identified, and in validation assays, dose-response curves could be obtained for papaverine, an opioid alkaloid. However, the effects of papaverine seemed independent of GPR88, since similar responses were seen in non-transfected cells. This research was finalized in 2009, and even today no ligand has been published for GPR88.

Chapter 8 provides a general discussion of the work presented in this thesis, and future perspectives in this area of research are discussed. The relevance of HCA_2, the A_3 adenosine receptor and GPR88 as targets for drugs is still to be confirmed. In the case of HCA_2, the identification of an antagonist, especially one that can be used in humans, could clarify the role of the receptor in the action of nicotinic acid. Anti-inflammatory effects of HCA_2 agonists should be more thoroughly explored in drug discovery programs, since this could yield therapies for diseases like psoriasis and multiple sclerosis. A_3 ligand IPA could become a valuable anticancer drug, either alone or as add-on to other drugs, especially since it may protect against common side effects on bone marrow cells. GPR88 is still an orphan receptor, but sensitive techniques to monitor the activation of endogenously expressed receptors, for example through impedance measurement, could be of value for ligand pairing of the remaining orphan receptors.

Samenvatting

In dit proefschrift worden verschillende orthostere en allostere agonisten voor de recent ontdekte hydroxy-carbonzuur (HCA) receptor 2 gepresenteerd, en hun in vivo activiteit of in vitro structuur-activiteits relaties worden beschreven. De literatuur met betrekking tot de HCA receptoren wordt ook uitgebreid besproken, en geeft inzicht op de toekomst van deze receptorfamilie als aangrijpingspunten voor medicijnen. Het wordt aangetoond dat het anti-kanker medicijn N^6-(2-isopentenyl)adenosine (IPA) een specifiek ligand is voor de adenosine A_3 receptor, en aannemelijk gemaakt dat het celgroei-remmende effect van deze stof, bij lage concentraties, afhankelijk is van deze receptor. Tevens is er een ligand-identificatiescreen voor de wees-receptor GPR88 uitgevoerd, waarin meer dan 4000 stoffen werden getest.

De meeste G eiwit-gekoppelde receptoren (GPCRs) kunnen alleen functioneren in samenspel met hun (endogene) ligand(en). Als de ligandconcentraties verstoord zijn, of als de receptor, of de intracellulaire signaaltransductie, niet geactiveerd kunnen worden onder invloed van het ligand, dan kan de receptor niet functioneren, en vaak leidt dit tot ziekte. De identificatie van het endogene ligand is dus essentieel om de functie van een receptor te begrijpen. Tegelijkertijd kunnen synthetische liganden voor GPCRs zeer effectieve medicijnen zijn, omdat ze kunnen worden ingezet om pathofysiologische verstoringen te corrigeren. In dit proefschrift staan drie receptoren centraal, namelijk de hydroxy-carbonzuur receptor 2 (HCA_2), de adenosine A_3 receptor, en GPR88, en in het bijzonder de zoektocht naar liganden die deze eiwitten kunnen beïnvloeden in het lichaam, op natuurlijke wijze of als medicijn.

Hoofdstuk 1 is een inleidend hoofdstuk, waarin GPCRs worden geïntroduceerd, met speciale aandacht voor zogenaamde wees-GPCRs, en het onderzoek dat de identificatie van hun endogene liganden tot doel heeft. GPR88 is nog steeds een wees-receptor, en de natuurlijke liganden van de HCA receptoren zijn recent ontdekt.

Hoofdstuk 2 geeft een overzicht van de huidige kennis over de HCA receptoren HCA_1, HCA_2 en HCA_3, waarbij de nadruk ligt op HCA_2, de receptor voor het atherosclerose-medicijn nicotinezuur. De natuurlijke liganden voor deze drie receptoren zijn in het laatste decennium geïdentificeerd, en dit zijn respectievelijk lactaat, 3-hydroxybutyraat en tussenproducten van β-oxidatie, zoals 3-hydroxyoctanoaat. De rol van de HCA receptoren in het lichaam is waarschijnlijk het reguleren van het vrijkomen van vetzuren uit het vetweefsel (lipolyse) wanneer er gebrek is aan voeding (HCA_2 en HCA_3), of juist in tijden van overvloed (HCA_1). Het belang van HCA_2 activatie voor de atherosclerose-remmende werking van nicotinezuur staat nog ter discussie. Ontstekingsremmende effecten van HCA_2 agonisten zijn gevonden in een aantal studies, en dit zou gebruikt kunnen worden in de strijd tegen andere ziektes als multiple sclerosis.

Hoofdstuk 3 beschrijft in vitro en in vivo onderzoek naar de farmacologische effecten van twee partiële agonisten voor HCA_2, in vergelijking met nicotinezuur. Deze partiële agonisten, LUF6281 en LUF6283, hebben structuren gebaseerd op de pyrazol-ring, en hun affiniteit voor HCA_2 was repectievelijk 3 μM en 0.55 μM. In [^{35}S]-GTPγS experimenten

was de rangorde van activiteit (potency) nicotinezuur > LUF6283 > LUF6281, en het maximale effect van de partiele agonisten was ~75% voor LUF6283 en ~50% voor LUF6281. De partiële agonisten waren, net als nicotinezuur, actiever in een ERK 1/2 fosforylatie experiment, maar de EC_{50} ratio [^{35}S]-GTPγS / pERK was 2-3x hoger voor nicotinezuur. In muizen verlaagden de pyrazolen het VLDL-gehalte in vergelijkbare mate als nicotinezuur, maar deze stoffen gaven geen 'flushing' (bloedvatverwijding in de huid), aangezien de huidtemperatuur niet toenam. Nicotinezuur verlaagde de expressie van de pro-lipolyse enzymen HSL en ATGL met de helft, terwijl LUF6281 en LUF6283 geen effect hadden op hun expressie. Aan de andere kant verhoogden de pyrazolen de expressie van ApoB in de lever met 40%. Dit suggereert dat LUF6281 en LUF6283, hoewel ze dezelfde receptor activeren als nicotinezuur, andere in vivo effecten hebben, waarbij de cholesterol-verlagende werking behouden blijft, terwijl het flush-effect niet werd waargenomen.

In **hoofdstuk 4** en **5** wordt de identificatie en in vitro karakterisatie van nieuwe HCA_2-liganden beschreven. In **hoofdstuk 4** staan derivaten van trans-propeenzuur centraal. Deze stoffen zijn chemisch verwant aan het psoriasis medicijn monomethylfumaraat. Uit een serie kaneelzuurderivaten en fumaarzuuresters en –amides konden liganden worden geïdentificeerd met een affiniteit in het hoge nanomolaire tot hoge micromolaire bereik. De bindingsplek leek vrij beperkt, en trans-kaneelzuur was het langste vlakke ligand. Een moleculair computermodel op basis van de structuur-activiteits relatie van deze serie stoffen, suggereert een vlakke trans-propeenzuur farmacofoor van 8 Å lang, waarin grotere substituenten alleen buiten het vlak kunnen worden aangebracht.

In **hoofdstuk 5** zijn liganden voor de allostere bindingsplek van HCA_2 onderzocht met behulp van functionele en radioligand-binding analyses. Verschillende stoffen bleken positieve allostere modulatoren te zijn voor de activatie van HCA_2 door nicotinezuur, en bovendien waren de meeste modulatoren ook (allostere) agonisten wanneer ze afzonderlijk getest werden. Verschillende parameters werden bepaald om de details van de ago-allostere werking in kaart te brengen, en de multi-dimensionale structuur-activiteits relaties te kunnen beschrijven. Omdat allostere modulatie afhankelijk is van het gebruikte orthostere ligand, hebben we van een aantal stoffen ook de versterkende werking op de HCA_2 activatie door het endogene ligand 3-hydroxybutyraat getest. Verschillende pyrazolopyrimidines bleken in staat om het maximale effect van 3-hydroxybutyraat te versterken, en de EC_{50} tot tien maal te verlagen.

In **hoofdstuk 6** is beschreven hoe de natuurlijke nucleosiden N^6-(2-isopentenyl)adenosine (IPA) en zeatine riboside geïdentificeerd worden als selectieve liganden voor de humane adenosine A_3 receptor (A_3R), met affiniteiten van respectievelijk 159 en 649 nM. IPA bond ook met micromolaire affiniteit aan de rat A_3R. In een cAMP accumulatie experiment in CHO cellen met stabiele expressie van de hA_3R, hadden IPA en zeatine riboside een activiteit (EC_{50}) in het micromolaire bereik. Het effect van IPA kon worden geblokkeerd door de selectieve A_3R antagonist VUF5574. Het is bekend dat IPA effectief tumorgroei kan tegengaan, net als de referentie A_3R agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide (Cl-IB-MECA). De groeiremmende eigenschappen van IPA en Cl-IB-MECA op de kanker cellijnen LNCaP en N1S1 waren zeer vergelijkbaar, en bij lage concentraties kon de A_3R antagonist MRS1523 dit effect blokkeren. Hogere concentraties

IPA leken de celgroei tegen te gaan via een mechanisme dat onafhankelijk was van de A_3R. Vergelijkbare observaties zijn in het verleden ook gedaan voor andere A_3R agonisten. Aangezien IPA in de natuur voorkomt, zou deze stof, in theorie, de A_3R in vivo kunnen activeren onder fysiologische condities. IPA kon echter niet worden gedetecteerd door middel van HPLC in vers dwarsgestreept spierweefsel van de rat.

Hoofdstuk 7 behandelt een ligand-identificatiescreen met de wees-receptor GPR88, die mogelijk een rol speelt bij verschillende psychiatrische aandoeningen. Meer dan 4000 stoffen, waaronder kleine moleculen en peptiden, werden getest in een medium-throughput screen in 384-well platen, met verhoging van Ca^{2+} concentratie gemeten door middel van een Fluorescent Imaging Plate Reader (FLIPR). Naast de constructie van een ongemodificeerde HEK cellijn die GPR88 stabiel tot expressie bracht, werden de $G\alpha_{16}$ en $G\alpha_{qi5}$ eiwitten ook samen met GPR88 getransfecteerd, omdat de natuurlijke G eiwitkoppeling van GPR88 onbekend is. In een primaire screen werden 47 hits gevonden, en in bevestigingsexperimenten konden dosis-effect curves worden gemeten voor papaverine, een opiumalkaloide. Het effect van papaverine leek echter onafhankelijk te zijn van GPR88, want vergelijkbare uitkomsten werden gemeten in niet-getransfecteerde cellen. Dit deel van het onderzoek werd in 2009 afgerond, en er is in de literatuur ook nu nog geen ligand voor GPR88 gepubliceerd.

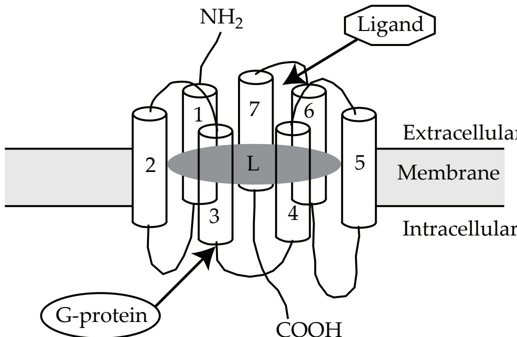
In **hoofdstuk 8** wordt een algemene discussie van het hier beschreven onderzoek gepresenteerd, en ook worden de toekomstperspectieven besproken. Het belang van HCA_2, de adenosine A_3 receptor en GPR88 als aangrijpingspunten voor medicijnen moet nog worden bevestigd. In het geval van HCA_2 zou de identificatie van een antagonist, vooral één die aan mensen kan worden toegediend, de rol van de receptor in de werking van nicotinezuur kunnen verhelderen. Ontstekingsremmende effecten van HCA_2 agonisten zouden nog verder kunnen worden onderzocht in het kader van de geneesmiddel-ontwikkeling, want dit zou therapieën kunnen opleveren voor ziektes as psoriasis en multiple sclerosis. A_3 ligand IPA zou een waardevol antikanker middel kunnen worden, als monotherapie of in combinatie met andere cytostatica, vooral aangezien het zou kunnen beschermen tegen de gangbare bijwerkingen op beenmerg cellen. GPR88 is nog steeds een wees-receptor, maar gevoelige technieken om de activatie van endogeen aanwezige receptoren te meten, bij voorbeeld door middel van impedantie-bepalingen, zouden waardevol kunnen zijn voor het vinden van liganden voor de resterende wees-receptoren.

Samenvatting voor algemeen publiek

Anno 2012 begrijpen we tamelijk veel van het menselijk lichaam: we hebben het erfelijk materiaal in kaart gebracht, we kunnen met geavanceerde instrumenten de levende organen in beeld brengen, en we hebben een heel scala aan medicijnen, die in veel gevallen ook helpen tegen de aandoening in kwestie. We weten nu ook dat ongeveer een derde van deze medicijnen werken via een bepaald soort ontvanger-eiwitten, die G-eiwit-gekoppelde receptoren (GPCR) genoemd worden.

G-eiwit-gekoppelde receptoren

De mens heeft zo'n 800 verschillende GPCRs, die betrokken zijn bij vrijwel alle lichaamsfuncties. Medicijnen maken gebruik van deze receptoren, maar hun originele functie is communicatie tussen verschillende cellen van het lichaam. Hierbij maken de verzendende cellen een stof aan, en vangen andere cellen het signaal op via receptoren. Bekende stoffen die via receptoren werken zijn bijvoorbeeld endorfinen, smaakstoffen, dopamine, adrenaline, THC (de werkzame stof in marihuana) en cafeïne. Nicotinezuur is ook een stof die op een GPCR inwerkt, en dit is belangrijk voor dit proefschrift.



Figuur 1: G-eiwit-gekoppelde receptor (GPCR). Deze receptoren gaan zeven keer door de celmembraan (nummers 1-7). Het ligand komt van de buitenkant van de cel, en het G-eiwit neemt het signaal over aan de binnenkant.

De ontdekking van nicotinezuur

In de jaren '50 van de twinstigste eeuw waren receptor-eiwitten al bekend, maar er was nog niet zo veel kennis van de individuele receptoren die specifieke signaalstoffen opvangen. Meer dan nu berustte het ontdekken van nieuwe medicijnen toen op *trial and error*. Het medicijn nicotinezuur, dat aderverkalking (atherosclerose) kan voorkomen of vertragen, werd bij toeval ontdekt in de vijftiger jaren door de Canadese psychiater dr. Hoffer. Hij zag namelijk dat de schizofrene patienten die hij ermee behandelde, lagere cholesterolwaarden kregen. Daarna zette zijn vroegere professor, dr. Altschul, het onderzoek voort, en hij wordt nu meestal genoemd als de ontdekker van nicotinezuur als medicijn. Behandeling van hoog cholesterol met nicotinezuur verkleint de kans op een hartinfarct of een beroerte. Waarschijnlijk werkt dit medicijn zo goed omdat het een drievoudige werking heeft: 1) verlaging van slecht cholesterol (LDL), 2) verhoging van goed cholesterol (HDL),

en 3) remming van ontstekingsreacties in de bloedvaten. Een minpunt is dat nicotinezuur een huidreactie veroorzaakt, die lijkt op verbranding door de zon.

De nicotinezuur-receptor

Pas veel later, begin twinstigste eeuw, werd ontdekt dat nicotinezuur een GPCR activeert, die daarna ook meteen bekend werd als de nicotinezuur-receptor. Intussen zijn er meer cholesterolverlagende middelen op de markt, maar nicotinezuur is nog steeds het beste middel om het goede (HDL) cholesterol te verhogen. Helaas is de bijwerking op de huid, ook wel *flushing* genoemd, zo vervelend dat veel patienten al snel hun pillen niet meer innemen. Een mogelijke oplossing zou kunnen zijn, om een nieuw medicijn te ontwikkelen dat de nicotinezuur-receptor ook activeert, maar geen huidreactie geeft.

Op zoek naar de klik

Het grootste gedeelte van mijn proefschrift gaat over de zoektocht naar nieuwe activators, ook wel liganden of agonisten genoemd, voor de nicotinezuur-receptor. Hiervoor maken we gebruik van menselijke cellen die onder laboratoriumcondities kunnen groeien in een petrischaal, als producenten en 'huisvesting' voor de receptor-eiwitten. We hebben het gen voor de nicotinezuur-receptor in flinke hoeveelheden in deze cellen gestopt, waardoor ze veel receptor-eiwitten aanmaken. Vervolgens kunnen we de cellen blootstellen aan stoffen, die bij ons in het laboratorium zijn gemaakt (of, in sommige gevallen, kant en klaar gekocht). We kunnen daarbij meten of de nicotinezuur receptor wordt geactiveerd door deze stoffen, en zo ja, hoe sterk ze op de receptor inwerken.

Halfzachte liganden

Twee van deze stoffen, zogenaamde partiële agonisten, hadden een minder sterke werking op de receptor in vergelijking met nicotinezuur, maar verlaagden wel het slechte cholesterol in muizen. Daarnaast hebben we gekeken naar een verandering in de huidtemperatuur, wat een maat is voor *flushing*. Als nicotinezuur werd toegediend aan muizen ging de huidtemperatuur ongeveer drie graden omhoog, terwijl de partiële agonisten geen effect hadden. Er zijn twee mogelijke verklaringen voor het verbeterde werkingsprofiel van de partiële agonisten. Het zou kunnen dat er minder activatie van de receptor nodig is om cholesterolverlaging te krijgen, en relatief meer om de bijwerking op de huid in gang te zetten. Hierdoor zouden partiële agonisten minder *flushing* veroorzaken door hun subtielere werking op de receptor. De tweede mogelijkheid is dat de partiële agonisten de receptor in een andere 'stand' zetten, waardoor het uiteindelijke effect op de (huid)cellen anders is. De balans tussen de activatie van twee andere eiwitten in de cel, namelijk het G-eiwit en ERK, zou daarbij betrokken kunnen zijn. We hebben laten zien dat nicotinezuur via de receptor twintig keer krachtiger inwerkt op ERK dan op het G-eiwit, terwijl dit verschil veel kleiner is voor de partiële agonisten (vijf tot tien keer). Of dit de reden is voor de verminderde *flushing*-reactie is echter op dit moment nog niet duidelijk.

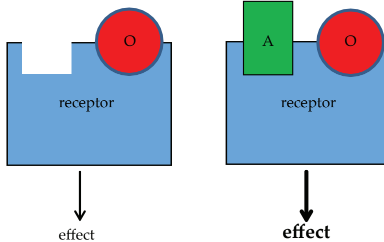
Samenwerken

Ik beschrijf ook een serie stoffen die de nicotinezuur-receptor zelfstandig kunnen activ-

eren, maar daarnaast kunnen samenwerken met andere agonisten, en hun werking versterken. Deze 'allostere' agonisten binden op een andere plek aan het receptor-eiwit, en daardoor blijft de plek voor nicotinezuur en andere 'orthostere' agonisten vrij en kunnen ze tegelijk aankoppelen aan de receptor. Als dit soort allostere agonisten geschikt gemaakt kunnen worden voor gebruik bij mensen, dan zouden ze gecombineerd kunnen worden met nicotinezuur. De dosis nicotinezuur zou dan tien keer lager kunnen zijn, omdat de werking op de receptor zo veel sterker is. Nog veel interessanter is het idee, dat allostere agonisten de werking van lichaamseigen liganden voor de nicotinezuur-receptor zouden kunnen versterken. We hebben als eerste laten zien dat het lichaamseigen ligand beta-hydroxy-butyraat sterker werkt, en bij lagere concentraties, als een allostere agonist tegelijkertijd aanwezig is. Zo zou toediening van een allostere agonist als medicijn ervoor kunnen zorgen dat er geen nicotinezuur meer hoeft te worden toegediend, omdat de stoffen die het lichaam zelf produceert sterker gaan werken.

Andere receptoren

Aan het eind van mijn proefschrift beschrijf ik nog onderzoek naar twee andere GPCRs: de adenosine A_3 receptor en GPR88. Ik heb laten zien dat het experimentele anti-kanker medicijn isopentenyl adenosine zijn effect gedeeltelijk te danken heeft aan de activatie van de adenosine A_3 receptor. We hebben ook gekeken naar de aanwezigheid van dit ligand in spierweefsel, omdat het misschien een lichaamseigen ligand zou kunnen zijn, maar dit is waarschijnlijk niet het geval. GPR88 is een zogenaamde wees-receptor: het is nog niet bekend door welke stof(fen) dit eiwit wordt geactiveerd. Ik heb geprobeerd een agonist te vinden voor GPR88 door ruim 4000 stoffen te testen, maar helaas is het niet gelukt daartussen een ligand te ontdekken.



Figuur 2: Orthostere en allostere liganden. Het orthostere ligand (O) kan zelfstandig de receptor activeren, en dat geeft dan een bepaald effect. Als een allosteer ligand (A) tegelijk bindt, kan het effect veranderen, bijvoorbeeld sterker worden.

Toekomst

Er is vandaag de dag zeer veel informatie beschikbaar over de werking van het menselijk lichaam, en elke dag komt er meer bij. Maar er valt nog veel te ontdekken. Op het gebied van de nicotinezuur-receptor is het nog steeds niet helemaal duidelijk of het medicijn nicotinezuur met name via deze receptor werkt, of dat het effect van nicotinezuur op andere lichaamsprocessen belangrijker is voor de werking als geneesmiddel. Het zou kunnen dat activatie van de nicotinezuur-receptor een positief effect heeft op ontstekingsziekten als MS en psoriasis, maar bewijs ontbreekt nog. Naast agonisten, is het ook belangrijk om zogenaamde antagonisten, blokkerende stoffen, te ontdekken voor de nicotinezuur-

receptor. Die zijn namelijk nog niet bekend. De A_3 receptor agonist isopentenyl adenosine zou een goed anti-kanker medicijn kunnen worden, vooral omdat het geen negatief effect heeft op gezonde lichaamscellen (zoals veel bestaande anti-kanker middelen wel hebben). Het word nu nog niet gebruikt bij mensen. GPR88 is nog steeds een wees-receptor, maar nieuwe technieken zouden kunnen helpen eindelijk een ligand te vinden. Als men het gen voor deze receptor uitschakelt bij muizen, krijgen ze een soort schizofrene trekjes. Daarom denken we dat agonisten voor deze receptor interessant zouden kunnen zijn voor de behandeling van psychiatrische aandoeningen.

Nawoord

Het onderzoek voor dit proefschrift heeft zich op drie verschillende plekken afgespeeld. Om te beginnen met de ‚Leidse tijd': Ik wil Jaco en Ad graag als eersten noemen, die beiden een grote bijdrage hebben geleverd aan dit onderzoek. Jaco, jouw synthese-vaardigheden en chemisch inzicht hebben veel mooie stofjes opgeleverd, en vooral de allostere modulatoren zullen in de literatuur voortleven. Al onze discussies over HCA_2 hebben ook geweldig geholpen. Ad, je bent al sinds mijn derde BFW-jaar een mentor voor mij, en hebt me op wetenschappelijk en persoonlijk vlak geadviseerd, gemotiveerd, en uitgedaagd. Onze gedeelde belangstelling voor klassieke muziek heeft ook mooie momenten opgeleverd. Hartelijk dank voor alle steun en vertrouwen. Mijn kantoorgenoten, Miriam, Henk, Elisabeth, Annelien en Ann, jullie zorgden voor een gezellige sfeer, leuke discussies, en stonden altijd klaar om te helpen. Miriam, onze (soms lange) gesprekken over alle aspecten van het AIO-schap waren naar mijn idee zeker geen verloren tijd! Met jou als paranimf kan ik tijdens de verdediging met een gerust hart flauwvallen. Henk, aan jouw analytische vermogens heeft het niet gelegen dat we geen endogeen IPA hebben gevonden! Mijn studenten, Margot, Maha, Laurens, Lucienne en Maarten, jullie hebben allemaal je bijdrage geleverd aan het bestuderen van HCA_2. Het was erg leuk om met jullie samen te werken en ik hoop dat het net zo leerzaam was voor jullie als voor mij. De studenten die met Jaco liganden hebben gesynthetiseerd voor het HCA_2 project moeten hier natuurlijk ook genoemd worden, net als Marysa die HCA_2 in silico heeft bestudeerd. Rob, thank you for giving me a ‚jumpstart' at the beginning of the project, and for your help and suggestions. Munikumar en Gerard hebben onder andere in silico teststofjes geselecteerd en de (allostere) bindingsplek van HCA_2 voorspeld. Eigenlijk jammer dat we uiteindelijk niet meer bio/chem-informatics hebben kunnen koppelen aan dit project, dat zou interessant zijn geweest! Thea, jij hebt snel en vakkundig data gegenereerd voor het vehaal over de A_3 receptor. Outside our Medicinal Chemistry group, contributions to this thesis came from the division of Biopharmacy (especially Zhaosha and Menno), from Canfite BioPharma (Sara Bar-Yehuda and Pnina Fishman), from MPI Bad Nauheim (Stefan, Sorin and Julien), and from our sister group at Amsterdam University (especially Meritxell). Thank you all for inspiring collaborations, sound advice and excellent work! Margot, jij hebt me tijdens mijn eerste stages enthousiast gemaakt voor het GPCR-onderzoek, en zonder jou waren we hier misschien niet geweest. Jouw input was ook onmisbaar voor TI-Pharma, en dat *Public-private partnership* was verantwoordelijk voor de financiële ondersteuning van mijn werk. Helen en collega's van de 'keuken' hebben mijn leven praktisch gezien een stuk makkelijker gemaakt. Laura, bedankt voor jouw deskundige advies over allostere modulatoren en andere bio-zaken. All other colleagues at Medicinal Chemistry: I had a great time, and I miss the good atmosphere in the group and our twice-daily coffee breaks (and not principally because of the many excellent cakes).

A l'IGBMC (Strasbourg/Illkirch) et AstraZeneca (Montréal) j'ai été aidée dans le travail sur GPR88 par Jérôme, Brigitte, Pascal, Patricia et Eric. Un grand merci aussi à Manu, Domi, Dom, Aminah, Jack, Manon et à tous les autres collègues à Strasbourg et Montréal. Je suis

heureuse d'avoir travaillé dans un environnement où l'étude des RCPGs va plus loin que l'in vitro, et d'avoir eu l'occasion de voir le R&D industriel de tout près. Et de parler et comprendre le français est un grand cadeau que j'espère ne jamais perdre.
Mijn vrienden en huisgenoten, mes amiEs et colocataires, my friends and flatmates, these years would not have been as fun without you! Thank you for the laughter and the support. Cornelia, you were there for the infamous 'last 5%'. I could not have wished for a better thesis (and life-) coach. Like a real InDesign wizard you made the layout for my thesis book, and this was a great gift! Mijn familie, oma, mama, papa en Jochem, jullie zijn lichtende voorbeelden wat betreft energie, gevoel en verstand, en met jullie gaan pittige discussies en slappe lach hand in hand. Gelukkig zorgen telefoon, internet en hoge snelheidstrein voor korte lijntjes ongeacht de geografische situatie. Mr. Jochem (M.A.), ik ben blij dat ik je *in extremis* nog even actief kan betrekken in het promotie-proces als mijn paranimf. Wie weet worden de rollen in de toekomst nog een keer omgedraaid, maar zo niet dan weet ik zeker dat je een ander geweldig pad zult kiezen!

Curriculum vitae

Clara Catelijne Blad was born on December 5, 1982 in Rotterdam. She grew up mostly in Strijen, and in 1994 she started attending the Erasmiaans Gymnasium high school in Rotterdam, where she chose to study an eclectic mix of languages and science subjects. Furthermore, she was involved in a youth circus and played the flute. In 1998 she won the Gaudeamus prize for her rendering of a contemporary flute piece at the Prinses Christina Concours. She obtained her highschool diploma in Latin, geology and economics one year early in 1999, and in 11 other subjects including ancient Greek, chemistry, biology and physics in 2000. She chose to study bio-pharmaceutical sciences at Leiden University, with the goal of becoming a research scientist.

In 2003 she started her first internship, in which she studied the adenosine A_{2B} receptor expressed in yeast cells. This project was a collaboration of the divisions of Medicinal Chemistry and of Molecular Genetics of Leiden University, and was supervised by Margot Beukers. After this first experience with G protein-coupled receptors she was hooked. The adenosine A_{2B} receptor was also the subject of a second, nine-month internship in the same department. It included the presentation of a scientific poster at the Figon Dutch Medicines Days. From 2003 to 2005 Clara was also receiving music education, including weekly flute lessons, at the Conservatory of The Hague. She was selected for the National Student Orchestra in 2003 and 2005 and played in Berlin and Vienna with this ensemble.

In 2005 Clara moved to Strasbourg, France to study the Mu opiate receptor in the laboratory of Prof. Dr. Brigitte Kieffer at the Institut de Génétique et de Biologie Moléculaire et Cellulaire. There, she learnt about a future project on a receptor with unknown function, GPR88, and jumped at the chance to be involved. After obtaining her 'doctorandus' degree (Bachelor and Master combined) from Leiden University in August 2006, she worked with professor Kieffer a further two and a half years, including a 6-month stay at the AstraZeneca R&D division in Montréal, Canada. In 2009 she defended a thesis on this research to obtain a postgraduate Diplôme de Recherche Spécialisé of Strasbourg University.

Directly after this Clara returned to Leiden and to the division of Medicinal Chemistry of the Leiden/Amsterdam Center for Drug Research (LACDR), this time to study the hydroxy-carboxylic acid receptor 2 (HCA_2) and the adenosine A_3 receptor under supervision of Prof. Dr. Ad IJzerman. The research on the A_3 receptor was a collaboration with Canfite BioPharma in Israel, and the work on the HCA_2 receptor was partly a collaboration with the Division of Bio-Pharmaceutics within the LACDR, whereas the review chapter on HCA receptors was written in collaboration with Prof. Dr. Stefan Offermanns, MPI Bad Nauheim, Germany. Clara presented parts of the research on (inter-)national conferences, including an oral communication at the IUPHAR WorldCongress of Basis and Clinical Pharmacology in Copenhagen in 2010. This work, together with the project on GPR88, constitutes this PhD thesis.

In the final writing stage, Clara joined the Max Planck Institute for Heart and Lung Research in Bad Nauheim, Germany, as a postdoctoral fellow in the group of Prof. Dr. Stefan Offermanns.

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