A quest for connections : ligands for the HCA2, adenosine A3 and GPR88 receptors

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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

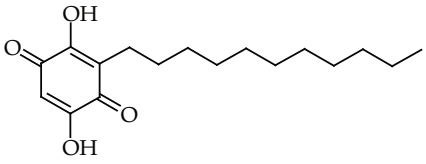
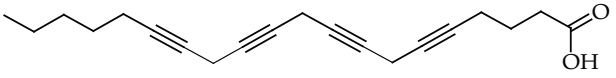
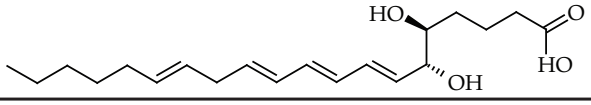
We identified N^6-isopentenyl-adenosine (IPA) as a ligand for the adenosine A_3 receptor (A_3R). We also investigated whether IPA is an endogenous ligand for the A_3R. No evidence could be found for significant concentrations of IPA in muscle tissue, but it is still a possibility that IPA binds to the 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	
2	
3	

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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