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.