Allosteric Modulation of 'Reproductive' GPCRs : a case for the GnRH and LH receptors

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CHAPTER 8

GENERAL CONCLUSIONS AND PERSPECTIVES

The research described in this thesis has provided novel insights in the allosteric modulation of 'reproductive' GPCRs. First, allosteric modulation of the human GnRH receptor by a more common allosteric modulator (HMA) and an apparent competitive antagonist (FD-1) was described in *Chapter 3*. Moreover, these two allosteric inhibitors modulated the receptor by binding at two distinct allosteric sites. Secondly, the first non-peptidic allosteric agonist, Org 43553, for the human LH receptor was labeled with tritium and characterized (*Chapter 4*). Finally, this novel pharmacological tool and a luciferase-based reporter gene assay were used (*Chapters 5-7*) to identify the first allosteric inhibitor (LUF5771) and allosteric enhancer (LUF5419) of [^{3}H]Org 43553 binding at the human LH receptor, respectively. The discovery of the latter compounds proves that the LH receptor, like the GnRH receptor, contains three binding sites. In short, the human GnRH and LH receptor, like several other (class A) GPCRs, can be allosterically modulated by non-peptidic ligands.

8.1 CONCLUSIONS FROM THIS THESIS

Several allosteric modulators for (class A) GPCRs have been described. However, at the start of this project allosteric modulation of so-called 'reproductive' GPCRs was still unknown. Combined molecular pharmacology techniques, such as radioligand binding, both equilibrium and kinetic, and functional assays have resulted in convincing data regarding allosteric modulation of these GPCRs, in particular for the human GnRH and LH receptor.

8.1.1 GnRH Receptor

For the GnRH receptor extensive SAR studies have resulted in several classes of low-molecular weight (LMW) (i.e. non-peptidic) antagonists, while LMW agonists have not been reported so far for this receptor. These compounds have been reviewed in *Chapter 2*, which shows that within each structural class high-affinity and bioavailable compounds have been designed. All of these compounds are described as orthosteric ligands, and for some of them this has been predicted and/or visualized by molecular modeling using the crystal structure of rhodopsin as a template for the GnRH receptor structure. The clinical use of these GnRH receptor antagonists is uncertain as selectivity data with respect to other drug targets (e.g. GPCRs) is often lacking. In addition, the interaction with cytochrome P450 activity of these compounds adds to this uncertainty.

Allosteric modulation of the GnRH receptor might, therefore, be another opportunity to target this receptor with selective, orally available, LMW compounds. In *Chapter 3* two allosteric inhibitors of both (radio-)ligand binding (^{125}I-triptorelin) and function (Ca^{2+}-induced luciferase activity) of the GnRH receptor have been described. Firstly, amiloride analogs, in particular HMA, were shown to allosterically inhibit the GnRH receptor. These compounds have also been shown to allosterically modulate other GPCRs. Secondly, another compound, FD-1, was shown to be a competitive antagonist and (at higher concentrations) an allosteric inhibitor. Molecular modeling of an analog of FD-1 was shown to bind at a site that partially overlaps with the orthosteric site.[53] With these three types of ligands at hand; 1) orthosteric peptide agonist, 2) non-peptidic allosteric inhibitor, and 3) non-peptidic orthosteric/allosteric antagonist, the binding sites were examined performing additional kinetic binding assays. In *Chapter 3*, we coin the term 'competitive radioligand dissociation assays' for this type of experiments, which are also utilized in *Chapter 5*. With these assays competition of two compounds for one allosteric site can be examined. A combination of the

results obtained with these assays and mathematical modeling thereof, demonstrate that these three chemically unrelated compounds have three distinct binding sites (i.e. one orthosteric and two allosteric) on the human GnRH receptor.

8.1.2 LH Receptor

The endogenous ligand of the human LH receptor is a large protein hormone that binds to the extraordinary long N-terminal domain. Unlike most other class A GPCRs the 7-TM domain is therefore 'free' to be targeted with LMW ligands. SAR studies of LMW (allosteric) ligands for the LH receptor are, however, very limited to date (*Chapter 2*). One of the first classes of LMW agonists for the LH receptor, thienopyrimidines, also emerged as the first radioligand, [^{3}H]Org 43553. *Chapter 4* describes the characterization of this new radioligand that can be used for the discovery of novel allosteric ligands for the LH receptor. Equilibrium saturation and displacement, and kinetic association and dissociation assays with [^{3}H]Org 43553 showed that this radioligand is a highly potent and useful tool. Comparison of binding data with functional data showed that a high correlation exists between affinity and activity of low molecular weight ligands, respectively. Based upon the data obtained in *Chapter 4*, we used this radioligand in a screening campaign for new LMW ligands for the LH receptor. A diverse selection of our in-house compound library (LUF-compounds) was tested in both equilibrium displacement (competitive hits) and kinetic dissociation assays (allosteric hits). Interestingly, competitive hits were not found, while several allosteric hits were obtained (i.e. compounds affecting the dissociation rate of [^{3}H]Org 43553). The SAR of terphenyl-containing compounds as allosteric inhibitors was further explored (*Chapter 5*). Although these compounds were rather lipophilic (high logD value), a specific and potent allosteric inhibitor, LUF5771, was further examined. Interestingly, LUF5771 also inhibited activation of the receptor by the endogenous ligand (LH), while it was discovered using [^{3}H]Org 43553. Furthermore, allosteric enhancers were also picked up in the screen (*Chapter 6*). This thiazole derivative (LUF5419) was only able to modulate Org 43553 binding and activation, while no effect was observed on the hormone. Similar to *Chapter 3* we now had three structurally different LMW ligands for the LH receptor at hand. Competitive dissociation assays and mathematical modeling was therefore performed to elucidate the number of binding sites within the 7-TM domain, next to the N-terminal orthosteric binding site of LH. Based on this data we hypothesize that the LH receptor contains two allosteric

sites within the 7-TM domain that bind compounds like LUF5419 and LUF5771 and one allosteric binding site that recognizes compounds such as Org 43553. In the future, this second allosteric site may allow for the development of new LMW ligands that target the LH receptor. Notably, a screen for allosteric modulators of the LH receptor proved to be more fruitful using a more elaborate radioligand dissociation screen (*Chapter 5* and *6*) than a luciferase reporter gene assay (*Chapter 7*). Interestingly, we identified and synthesized luciferase enzyme inhibitors in that reporter gene assay, demonstrating the power of medicinal chemistry in discriminating between receptor-mediated and later-stage effects in a ligand screen.

8.2 FUTURE PERSPECTIVES

Identification of novel LMW ligands and a better understanding of their binding properties remain vital for improved effects *in vivo* and better prediction thereof. Moreover, ensuing mutagenesis studies (combined with molecular modeling) and receptor crystallography may further elucidate the exact receptor pockets in which orthosteric and allosteric ligands bind. This will result in positive feedback for the development of new chemical entities; typical for the cycle of medicinal chemistry. Both of these topics will be discussed in more detail below.

8.2.1 Novel Ligands and Their Characteristics

For the four receptors of the HPG-axis, the same applies: non-peptidic ligands are welcome (reviewed in *Chapter 2*). While for the GnRH receptor a multitude of non-peptidic antagonists have been developed, none of them have entered the market yet, even though they offer the promise of oral bioavailability. Recently, the first *peptidic* antagonist, Degarelix, for the GnRH receptor was approved by the FDA for the treatment of prostate cancer.[330] The advantage over agonistic ligands such as leuprolide is that there is no initial flare-up effect. However, due to its peptidic nature Degarelix needs to be administered via parenteral injection, which causes patient inconvenience as shown in the clinical report. This emphasizes once more, that LMW ligands with oral bioavailability for receptors with peptidic (orthosteric) ligands are highly desirable. The difficulty, however, remains to predict pharmacological effects in humans from *in vitro* and *in vivo* animal data, as was shown for the GnRH receptor.[14] Therefore, *in vitro* research should be extended, for example, by examining the (dissociation) kinetics of newly developed ligands, which was recently referred to as receptor residence time of ligands.[331,332] For two classes of non-peptidic GnRH receptor ligands, it appeared that the obtained affinities from a standard radioligand binding assay were incorrect, due to (unexpected) slow dissociation kinetics.[146,333] Slowly dissociating ligands could have enhanced clinical efficacy due to a longer duration of action. It is, therefore, important to understand the SAR of ligands not only when it comes to their affinity, but also with respect to their (dissociation) kinetics at a certain target.

For the glycoprotein hormone receptors (i.e. LH and FSH receptor) and GPR54, there are only few reported non-peptidic ligand classes (*Chapter 2*). For GPR54, the first (and currently only) LMW ligand was reported recently based on a pharmacophore study.[232]

Hopefully more LMW ligands will be developed in the near future, as this receptor is thought to be the 'gatekeeper' of the HPG-axis in reproductive functions.[334] For the glycoprotein hormone receptors, an important recent development was the discovery of the first LMW TSH receptor antagonist.[297] This ligand was based on a LMW agonist originally developed for the LH receptor (Org 41841). LMW antagonists for the LH receptor have not been reported so far (*Chapter 2*) and this new compound could potentially give a clue. In addition, the allosteric modulators of the LH receptor described in *Chapter 5* and *Chapter 6* could be used as a starting point for the development of other or improved allosteric modulators for the LH receptor. Moreover, it would be interesting to examine whether these compounds also bind/modulate the FSH or TSH receptor, since LH receptor ligands have been shown to act on the TSH[191] and FSH[195] receptor.

In light of some characteristics of Org 43553, namely its partial agonism and functional selectivity,[36] it would be interesting to investigate why this LMW ligand only partially activates the $G\alpha_s$ pathway and no other pathways, like LH does. In general, partial agonism could have the advantage of tissue selectivity, when a receptor is differentially expressed. In case of the LH receptor, it has been shown that overstimulation of the receptor can result in ovarian hyper-stimulation syndrome (OHSS). A partial agonist, like Org 43553, has the advantage of a ceiling effect and due to its faster kinetics (*Chapter 4*) and shorter half-life, compared to hCG, it could reduce the risk for OHSS.[69]

8.2.2 Ligand Binding Sites

A major breakthrough in the GPCR field has been the crystallization of the first two human (class A) GPCRs; β_2-adrenergic receptor,[41] adenosine A_{2A} receptor[45] and the turkey β_1-adrenergic receptor.[43] Up to that moment the crystal structure of bovine rhodopsin was used to understand receptor structure and ligand binding.[40] The latest crystal structures could give a better impression of (at least) class A GPCRs. However, a comparison of the ligand binding site in these crystallized receptors shows unexpected differences. Moreover, these three receptors were crystallized with an antagonist/inverse agonist. This raises some interesting questions. Can these crystallized receptors be used for molecular modeling studies and subsequent ligand design? What does the binding site of another type of ligand, such as an agonist or even an allosteric modulator look like? The current GPCR crystals were obtained using either of two methods; increasing thermostability by several (point-)mutations

or co-crystallizing with T4-lysozyme. Can we expect more crystal structures of GPCRs in the near future using these methods?

The data presented in *Chapter 3* (GnRH receptor) and *Chapter 6* (LH receptor) shows that GPCRs can contain (at least) two allosteric sites in the 7-TM domain. Similar data has been reported for muscarinic acetylcholine receptors. For example, there is strong experimental evidence that the M_1 muscarinic receptor contains two allosteric sites,[62] which was also visualized by molecular modeling.[335,336] Taken together, these papers actually describe the presence of three allosteric sites, two located at the extracellular face of the receptor and one at the intracellular domain of the receptor. One of the extracellular sites was also reported for the M_2 muscarinic receptor.[337] In this study, it was shown that the second extracellular loop was important for binding of both orthosteric and allosteric ligands, where allosteric ligands were located more at the surface of the receptor. The presence of the intracellular allosteric site close to TM 8 and the third intracellular loop at muscarinic receptors has not yet been confirmed by experimental data. However, a similar intracellular allosteric site has been recently reported for several chemokine receptors (CXCR1 and 2; CCR4 and 5).[338,339]

Focussing on the glycoprotein hormone family, two different allosteric sites have been described.[55] For LH and TSH receptors the allosteric site is thought to overlap with the general class A orthosteric binding site (TM III, IV, V and VI) and for the FSH receptor another site (TM I, II, II1 and VII) was determined. It would be interesting to investigate whether the additional allosteric sites described in *Chapter 3* and *Chapter 6* for the GnRH and LH receptor, respectively, are located at any of the positions described in literature.

8.3 FINAL NOTE

In short, the research described in this thesis proves that allosteric modulation of 'reproductive' GPCRs is possible, as is the case for several other (class A) GPCRs. This is a major step forward, as these findings allow for a more diverse set of LMW compounds to interact with these receptors. They may eventually replace or improve the action of the high molecular weight (peptidic) endogenous ligands (e.g. GnRH and LH), as they show better drug-like properties such as oral bioavailability.

Projects dealing with allosteric modulation of GPCRs will most certainly get a boost after a highly desired first report of a (class A) GPCR structure co-crystallized with an allosteric modulator. In this way at least one of the allosteric sites will be visualized and would aid tremendously in a better understanding of the process of allosteric modulation. Subsequently, structure-based (allosteric) ligand design could improve modulating potencies. Similarly we are also waiting impatiently for the first crystal structures of class B and class C GPCRs, which are also highly amenable to allosteric modulation. Hopefully, the methods that have been used for the currently available crystal structures will soon result in several others.