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

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SUMMARY

G protein-coupled receptors (GPCRs) are currently targeted by more than 30% of the drugs on the market. In the past few years, however, a decline in newly marketed drugs (in general) is observed, stressing the importance of new approaches for drug therapy. One of these new approaches is the development of so-called allosteric modulators. Allosteric ligands bind at a site distinct from the site where the endogenous ligand binds and are capable of changing the receptor conformation. Thereby, a change in the pharmacological responses to the orthosteric ligand will occur. The resulting advantages of allosteric modulation are, for example, increased receptor-subtype selectivity and preservation of the physiological effects (with respect to duration and site of action). *Chapter 1* introduces GPCRs and the recent developments in drug research, such as allosteric modulation, involving these proteins.

The hypothalamic-pituitary-gonadal (HPG) axis is regulated by a number of G protein-coupled receptors that play an important role in reproduction and sex hormone-dependent diseases. These receptors are therefore referred to as 'reproductive' GPCRs. The main focus of this thesis is on the gonadotropin-releasing hormone (GnRH) (*Chapter 3*) and luteinizing hormone (LH) receptor (*Chapters 4-6*). These targets have been classified as class A GPCRs that are usually comprised of a short extracellular N-terminal domain, seven transmembrane (7-TM) α-helices, which are connected by three intra- and extracellular loops, and an intracellular C-terminus. In general, the endogenous ligands of this class of GPCRs bind within the 7-TM domain, referred to as the orthosteric binding site. The LH receptor, however, is an exceptional class A GPCR, because it has two endogenous ligands, LH and hCG, which both bind to the (unusually) large N-terminus. Both the GnRH and LH receptor have high molecular weight (HMW) endogenous ligands that are peptide/protein hormones. One of the advantages of allosteric modulation of these receptors is low molecular weight (LMW) allosteric ligands can be developed that are potentially orally bioavailable, unlike peptidic ligands such as GnRH and LH. *Chapter 2* reviews the LMW (orthosteric and allosteric) ligands for GPCRs of the HPG axis that have been reported so far.

In *Chapter 3*, allosteric modulation of the human GnRH receptor by amiloride analogues (e.g. HMA) and a non-peptide antagonistic furan derivative (FD-1) was studied. Firstly, the compounds' ability to influence the dissociation of a radiolabeled peptide agonist (^{125}I-triptorelin) from human GnRH receptors was investigated. HMA and FD-1 were shown to increase the dissociation rate of ^{125}I-triptorelin, revealing their allosteric inhibitory characteristics. The simultaneous addition of HMA and FD-1 resulted in an additive effect on the dissociation rate. Secondly, in a functional assay it was shown that HMA was a non-

competitive antagonist and that FD-1 had both competitive and non-competitive antagonistic properties. Furthermore, the potency of HMA to increase radioligand dissociation was not affected by the presence of FD-1. Simulation of the data obtained in the latter experiment also indicated neutral cooperativity between the binding of HMA and FD-1. Taken together, these results demonstrate that HMA and FD-1 are allosteric inhibitors that bind at two distinct, non-cooperative, allosteric sites.

In *Chapter 4*, the binding of a new low-molecular-weight (LMW) radioligand, [^{3}H]Org 43553, at the LH receptor is characterized. Equilibrium saturation and displacement assays were developed and optimized. Specific binding of [^{3}H]Org 43553 to human LH receptor was saturable with a high affinity (K_D = 2.4 ± 0.4 nM). Affinities and potencies of five LMW analogues of Org 43553 were determined, showing a high correlation between these values. A HMW radioligand, such as ^{125}I-hCG, is not suitable for screening for LMW ligands, as they do not compete for the same binding site. This new radioligand, [^{3}H]Org 43553, is therefore a welcome addition in the field of drug research for the LH receptor.

In *Chapter 5* and *Chapter 6*, [^{3}H]Org 43553 was used to screen a library of 50 compounds for possible new LMW ligands targeting the LH receptor. Especially, the kinetic radioligand dissociation screen (i.e. to identify allosteric modulators) resulted in the identification of both allosteric inhibitors (*Chapter 5*) and allosteric enhancers (*Chapter 6*) of Org 43553. Firstly, a terphenyl derivative was shown to (allosterically) inhibit [^{3}H]Org 43553 binding to the receptor. This led us to synthesize a series of 25 terphenyl derivatives. The most potent compound of this series was LUF5771, which was able to increase the dissociation rate of [^{3}H]Org 43553 by 3.3-fold (at 10 μM). Secondly, several allosteric enhancers of [^{3}H]Org 43553 were identified, each containing a thiazole core. In this case, LUF5419 was chosen to be characterized further as it was one of the most potent compounds, with an ability to decrease the dissociation rate of [^{3}H]Org 43553 by 2.4-fold (at 10 μM). Both LUF5771 and LUF5419 were also tested in a functional assay, where the presence of the first resulted in a 2.4-fold decreased potency of Org 43553, while the latter did not affect the potency. The efficacy of (the partial agonist) Org 43553, however, was unaffected by LUF5771, while LUF5419 caused an enhancement, resulting in full receptor activation when compared to recLH. Interestingly, LUF5771 was also able to allosterically inhibit the potency of recLH (and rec-hCG). LUF5419, however, did not affect the potency or efficacy of recLH. It is noteworthy, that LUF5771 is the first LMW antagonistic/inhibitory ligand reported for the LH receptor to date. Furthermore, the potency to increase radioligand dissociation of

LUF5771 was decreased by the presence of LUF5419. These results demonstrate that LUF5771 and LUF5419 are allosteric modulators that bind at the same allosteric site in the LH receptor.

In this thesis radioligand dissociation assays were used to identify new allosteric modulators in a more low-throughput fashion. In high-throughput-screening, however, new (allosteric) ligands are often searched for by functional assays (e.g. luciferase reporter-gene assay). In *Chapter 7*, we report a luciferase inhibitor, which emerged from a luciferase reporter-gene assay screen for LH receptor ligands. Instead of displaying receptor activity this compound was shown to potently inhibit luciferase (i.e. a false positive). Further characterization showed that it was a competitive inhibitor with respect to the substrate luciferin. When a database search yielded another a structurally similar inhibitor, we were triggered to prepare several analogs of the luciferase inhibitors. This yielded a very potent inhibitor with an IC_{50} value of 0.069 ± 0.01 μM. Further molecular modeling studies suggested that the latter compound can be accommodated in the luciferin binding site. *Chapter 7* should serve as an alert to users of luciferase reporter gene assays for possible false positive hits due to direct luciferase inhibition.

Finally, in *Chapter 8* the general conclusions from the research described in this thesis are presented and future perspectives in this field of research are given. In short, this thesis provides novel insights in the allosteric modulation of ‘reproductive’ GPCRs. The human GnRH and LH receptor, like several other (class A) GPCRs, can be allosterically modulated. Moreover, both receptors are shown to contain three binding sites of which at least two can be targeted by LMW ligands. The presence of these other allosteric sites may provide other opportunities for the discovery of LMW and orally available ligands for the human GnRH and LH receptor.