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

Heitman, L.H.

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Allosteric Modulation of ‘Reproductive’ GPCRs

A Case for the Human GnRH and LH Receptors

Allosteric Modulation of ‘Reproductive’ GPCRs

A Case for the Human GnRH and LH Receptors

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Laura Helena Heitman

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Promotor: Prof. Dr. A. P. IJzerman

Overige Leden: Prof. Dr. A. Christopoulos (University of Melbourne, Australia)
Dr. M. W. Beukers
Prof. Dr. R. Leurs
Prof. Dr. C. A. A. van Boeckel
Prof. Dr. M. Danhof

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LIST OF ABBREVIATIONS

7-TM	Seven-transmembrane
AID	Assay identifier number in PubChem data repository
AMP	Adenosine-5'-monophosphate
ATP	adenosine 5'-triphosphate
δ	Cooperativity factor of two allosteric modulators
$B_{\max}$	Maximal specific radioligand binding
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-5'-monophosphate
CHO	Chinese hamster ovary
CID	Compound identifier number in PubChem data repository
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EDAC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA	Ethylene diamine tetraacetic acid
EC ₅₀	Half-maximal effective concentration (potency)
E _{max}	Maximal effect (efficacy)
EtOAc	Ethyl acetate
FD-1	5-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-naphthalen-2-yloxy)-furan-2-carboxylic acid (2,4,6-trimethoxy-pyrimidin-5-yl)-amide
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
GPCR(s)	G protein-coupled receptor(s)
GTP	Guanosine-5'-triphosphate
hCG	Human chorionic gonadotropin
HMA	5-(N,N-hexamethylene)amiloride
HMW	High molecular weight
HOBt	1-hydroxybenzotriazole
IC ₅₀	Half-maximal inhibitory concentration (affinity);

K_D	Equilibrium dissociation constant
K_i	Equilibrium inhibition constant (absolute affinity)
K_M	Substrate concentration at half maximal reaction rate
K_{off}	Dissociation rate
K_{on}	Association rate
LMW	Low molecular weight
logD	Logarithm of octanol-water distribution coefficient
LUF5419	4-Chloro-N-(4-pyridin-2-yl-thiazol-2-yl)-benzamide
LUF5771	Cyclopentyl-carbamic acid [1,1';3',1'']terphenyl-5'-yl ester
MIBA	5-(N-methyl-N-isobutyl)amiloride
NFAT-luc	Nuclear Factor Activated T-cell luciferase reporter gene
Org 43553	5-Amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno[2,3-d]pyrimidine-6-carboxylic acid tert-butylamide
PBS	Phosphate-buffered saline
(Ph ₃ P) ₄ Pd	Tetrakis(triphenylphosphine)palladium
rec-hCG	Recombinant human chorionic gonadotropin
recLH	Recombinant luteinizing hormone
RMSD	Root mean square deviation
SAR	Structure-activity relationship
SEM	Standard error of the mean
TLC	Thin-layer chromatography
TSH	Thyroid-stimulating hormone
V_{max}	Maximal reaction rate



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, [³H]Org 43553, at the LH receptor is characterized. Equilibrium saturation and displacement assays were developed and optimized. Specific binding of [³H]Org 43553 to human LH receptor was saturable with a high affinity ($K_D = 2.4 \pm 0.4$ 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 ¹²⁵I-hCG, is not suitable for screening for LMW ligands, as they do not compete for the same binding site. This new radioligand, [³H]Org 43553, is therefore a welcome addition in the field of drug research for the LH receptor.

In *Chapter 5* and *Chapter 6*, [³H]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 [³H]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 [³H]Org 43553 by 3.3-fold (at 10 μ M). Secondly, several allosteric enhancers of [³H]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 [³H]Org 43553 by 2.4-fold (at 10 μ M). 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 $0.069 \pm 0.01 \mu\text{M}$. 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.

CHAPTER

1

GENERAL INTRODUCTION

This chapter introduces G protein-coupled receptors (GPCRs) and the recent developments in drug research involving these proteins. Allosteric modulation will be discussed in more detail and especially its therapeutic potential for the ‘reproductive’ GPCRs, the GnRH and LH receptor. Finally, the scope and content of this thesis will be introduced.

1.1 G PROTEIN-COUPLED RECEPTORS

1.1.1 Introduction

The first primary structure (i.e. the protein sequence) of a G protein-coupled receptor (GPCR) was described twenty-five years ago, for bovine rhodopsin.¹ It was shown that activation of this membrane protein by light, an extracellular signal, resulted in an intracellular response. Since that time the structural elucidation of the GPCR receptor family further expanded with the aid of different molecular biological techniques. Completion of the human genome project provided a list of possible GPCR family members.² In 2007, it was

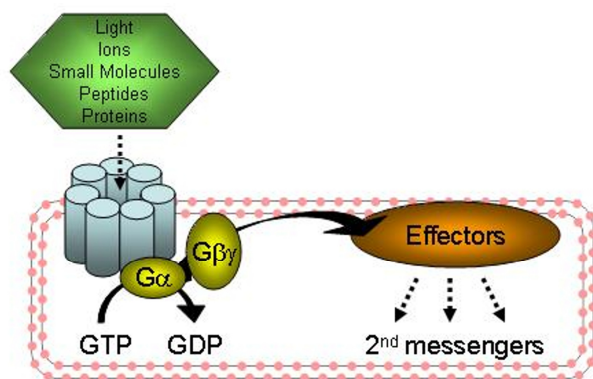


Figure 1.1 A simplified model of ligand binding to and activation of a GPCR.

reported that the human GPCR family consisted of 799 unique full-length members.³

The endogenous ligands for these receptors are very divergent, consisting of light (rhodopsin), cations (e.g. calcium-sensing receptor), small organic compounds (e.g. adenosine receptor), peptides [e.g. gonadotropin-releasing hormone (GnRH) receptor] or proteins [e.g. luteinizing hormone (LH) receptor] as shown in Figure 1.1.⁴

Activation of the receptor by an extracellular ligand induces a conformational change that is followed by G protein binding to the intracellular loops and C-terminus of the receptor (Figure 1.1).⁵ GTP replaces GDP on the G α subunit, the G protein subunits (α and $\beta\gamma$) dissociate from the receptor and bind to their downstream effector proteins, e.g. adenylate cyclase (via G α_s or G α_i) or phospholipase C (via G α_q). In this way different cellular functions are controlled, such as growth, movement and gene expression. However, more and more evidence is accumulating that GPCRs can also signal independently from the G protein, which will be discussed in Chapter 1.1.3.⁶

1.1.2 GPCR Structure and Classification

All GPCRs contain seven transmembrane (7-TM) helices connected by three extracellular and three intracellular loops with an extracellular N-terminus and an intracellular C-terminus.

GPCRs can be divided in five groups according to a phylogenetic classification; Rhodopsin-like (class A), Secretin receptor-like (class B), Glutamate receptor-like (class C), Frizzled receptors and Adhesion-like receptors.⁷ However, usually a division into four groups is made; class A, B, C (Figure 1.2), where Adhesion-like receptors are part of class B, and a fourth Frizzled receptor class.

The class A family of GPCRs is the largest and consists of approximately 670 members.³ More than half of class A GPCRs are olfactory receptors, which are activated by a broad range of odorants.⁸ Although, they are interesting targets for the fragrance industry, these receptors are usually not considered as drug targets. In general, the N-terminus of class A GPCRs is relatively short and an additional eighth helix is present at the C-terminus of the receptor (Figure 1.2). The binding site of the endogenous ligand, termed the orthosteric binding site, is often located within the 7-TM domain of the receptor. Ligands for these receptors are widely varied, consisting of amines, peptides and lipids. The secretin-like GPCR family (class B) is much smaller than class A consisting of nearly 50 members.⁴ These receptors have a large extracellular N-terminus containing several conserved cysteine bridges, which results in a rigid structure (Figure 1.2). The endogenous ligands are peptide hormones, such as calcitonin, glucagon and parathyroid hormone that bind to the extracellular domain of the receptor. Class C or glutamate-family of GPCRs contains a large N-terminus, similar to class B receptors (Figure 1.2). However, an additional unique motif is present, named the Venus flytrap, consisting of two domains that form a cavity where the endogenous ligand binds.⁹

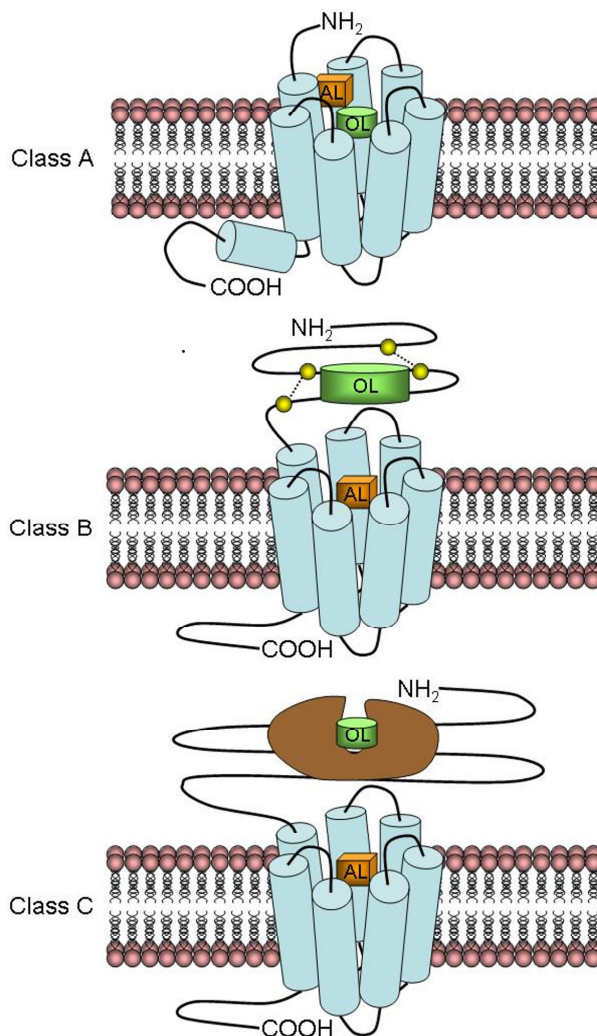


Figure 1.2 Schematic representations of the structure and binding pocket of orthosteric (OL) and allosteric ligands (AL) for class A, B and C GPCRs.

1.1.3 ‘Reproductive’ GPCRs

GnRH Receptor. The GnRH receptor is classified as a class A receptor based on its sequence homology of the 7-TM domain.⁴ It contains the typical short N-terminus followed by seven α -helical bundles. However, a unique feature of the human GnRH receptor is that it lacks the C-terminus.¹⁰ The GnRH receptor is predominantly coupled to $G\alpha_q$, however, interactions with other G proteins have been reported.¹¹ As the human GnRH receptor does not have an intracellular C-terminus, interactions with multiple G proteins should occur via the intracellular loops.¹⁰ Class A GPCRs are further divided into four groups, α , β , γ and δ , in which the GnRH receptor belongs to the β -group.¹² All known ligands for this group of receptors are peptides. In this case the endogenous ligand is GnRH, a decapeptide that is produced in the hypothalamus.¹⁰ As shown in Figure 1.2 for class A GPCRs, the binding pocket of the orthosteric ligand, GnRH, is located within the 7-TM domain. Most peptidic ligands for GPCRs also interact with amino acids in the extracellular loops and exofacial parts of the 7-TM domain.¹³ In the past decade, several non-peptidic and low molecular weight (LMW) antagonists for the human GnRH receptor have been reported.¹⁴ These (and other) compounds and their putative binding pocket will be discussed in *Chapter 2*.

LH Receptor. Based on the GPCR classification described in Chapter 1.1.2 and as shown in Figure 1.2 the human LH receptor could easily be classified as a class B GPCR. Like class B GPCRs, the LH receptor contains a large N-terminus which binds the endogenous ligand,

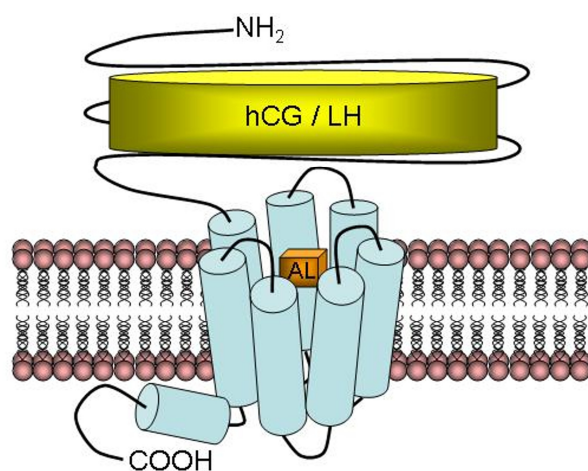


Figure 1.3 Schematic representations of the structure and binding pockets of orthosteric (hCG and LH) and allosteric ligands (AL) of the LH receptor.

the glycoprotein hormone LH. This receptor, however, has been classified as a class A GPCR based on conserved amino acid motifs in its 7-TM domain.⁴ Most rhodopsin-like GPCRs have a short N-terminus without any conserved domains. However, there are some exceptions within class A, namely the leucine-rich-repeat-containing GPCRs (LGRs)¹⁵⁻¹⁷ and the glycoprotein hormone receptors.¹⁸ These eight receptors, LGR4-8, follicle-stimulating hormone (FSH) receptor, luteinizing hormone (LH) receptor and thyroid-stimulating hormone (TSH) receptor, have in common that they contain several leucine-rich domains

in their large N-terminus. These receptors belong to the δ -group of class A GPCRs and their endogenous ligands are peptide hormones, relaxin (LGR7 and 8), or glycoprotein hormones (FSH, LH and TSH receptor) that bind to this N-terminal domain (Figure 1.3). Notably, LGR4, 5 and 6 are still orphan receptors according to the IUPHAR database, i.e. the endogenous ligand is not known.^{4,19} Activation of the LH receptor predominantly results in cAMP production via $G\alpha_s$. Activation of the LH receptor is thought to occur when hCG or LH bind to the N-terminus, which causes the ‘hinge-region’ (i.e. amino-acids connecting 7-TM to N-terminal domain) to interact with the 7-TM domain, leading to receptor activation.²⁰ Recently, it was shown that not only the high molecular weight (HMW) endogenous ligands are able to do that, but also LMW ligands.²¹ These LMW ligands bind to the 7-TM domain (the allosteric site) of the receptor similar to where most other ligands of class A GPCRs bind (Figure 1.3). These (and other) LMW ligands will be discussed in *Chapter 2*.

1.1.4 Current Developments in Drug Discovery for GPCRs

Currently more than 30% of the marketed drugs target GPCRs.²² However, the general trend in drug development has been towards increasing research and development (R&D) costs and decreasing output; the number of novel drugs approved by the FDA is depressingly low, especially from 1996 onward (Figure 1.4).^{23,24} Therefore, it is necessary that the GPCR research field develops novel approaches for drug discovery. One of these so-called ‘hot topics’ is *allosteric modulation*, which will be discussed in more detail in Chapter 1.2. First, other important developments in the field dealing with novel concepts, novel targets and a basic understanding of receptor structure will be briefly discussed.

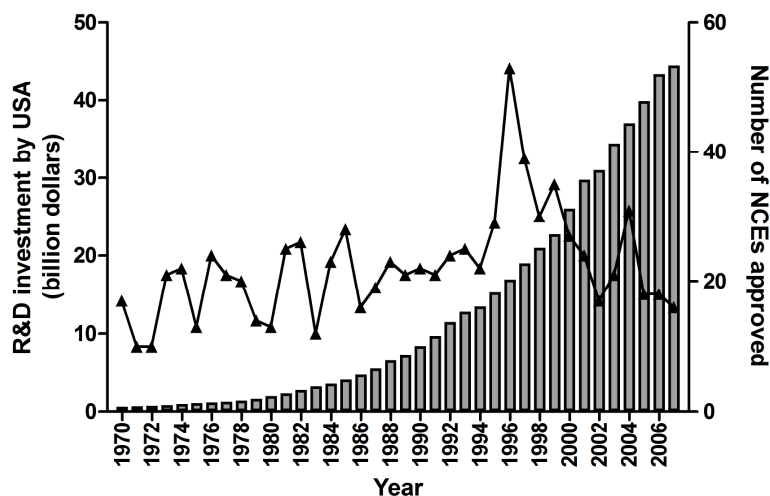


Figure 1.4 The bargraph and line show the R&D investments and number of NCEs approved by the FDA from 1970 to 2007, respectively.

Constitutive Activity. The first report of a constitutively active receptor, i.e. a receptor that is active in the absence of an agonist, already dates back to the late eighties.²⁵ Somewhat later the group of Lefkowitz showed that a single mutation in the α_{1b} -adrenergic receptor resulted in a constitutively active receptor.²⁶ Since then many mutation-induced constitutively active receptors have been reported and the therapeutic potential of inverse agonists, i.e. ligands with negative intrinsic efficacy, has been reviewed.^{27,28} However, the importance of constitutive activity *in vivo* is still poorly understood, as the presence of an endogenous (orthosteric or allosteric) ligand can often not be ruled out.²⁹

Receptor Dimerization. Rhodopsin is arranged as oligomers in native disc membranes.³⁰ This has been taken as evidence that class A GPCRs can occur in dimers or oligomers of higher order. However, the expression levels of rhodopsin are this high that oligomerization might be inevitable. There is conclusive evidence for other class A GPCRs, such as the GABA_B receptor,³¹ and mu and delta opioid receptors,³² where the formation of heterodimers can result in distinct pharmacology. Most reports on homo- or heterodimerization of GPCRs result from co-expression of receptors in heterologous cell systems, and can thus not be taken as proof for the physiological occurrence of receptor dimers. The field of receptor dimerization could have great potential in drug discovery, especially if ligands could be developed that are dimer-selective.³³

Ligand-Directed Signaling. Recently, it was shown that GPCRs can function G protein-independently. For example, β -arrestin, which was already known for its role in agonist-induced receptor internalization, or tyrosine kinase Src can have important direct signaling functions.^{34,35} GPCRs are, therefore, more often referred to as 7TM or serpentine receptors. Another development is the possibility of functional selectivity or biased-agonism. In this case, the activation of a certain signaling pathway is directed by the ligand that activates the receptor. For example, binding of the endogenous ligand, LH, to its receptor activates both the cAMP pathway and the PLC pathway. However, the LMW agonist, Org 43553, only activates the cAMP pathway.³⁶ In addition, it was shown for the β_2 -adrenoceptor different stereo-isomers of a ligand can activate a different signaling pathway.³⁷ This shows the need for analyses of multiple pathways in order to find new ligands or to understand the effects of a certain ligand.

'Deorphanization'. In class A-C of GPCRs there are receptors that have unknown endogenous ligands (orphan receptors). According to the IUPHAR database, approximately 120 orphan receptors still need to be linked to a ligand.¹⁹ It is thought that some of these

receptors might not even have an endogenous ligand, but modulate the functions of other proteins by dimerization, constitutive activity or other mechanisms.³⁸ For example, GPR50 was shown to form a heterodimer with the melatonin MT1-receptor, resulting in strongly decreased melatonin binding.³⁹ ‘Deorphanization’ or elucidation of ligand-independent functions of these receptors could possibly yield new drug targets.

Receptor Crystallization. The first GPCR crystal structure, rhodopsin, was obtained in 2000.⁴⁰ It proved to be much more difficult to crystallize other GPCRs and it took seven years before another crystal structure, the human β_2 -adrenergic receptor, was published.^{41,42} Two different methods were used to obtain conformational stability next to the presence of the partial inverse agonist carazolol with high affinity. Firstly, a monoclonal antibody (Mab5)⁴² was generated against the third intracellular loop or secondly, T4 lysozyme (T4L)⁴¹ was inserted in the third intracellular loop. The presence of the inverse agonist in combination with either Mab5 or T4L resulted in a less flexible receptor, thereby facilitating crystallization. Another method of crystallization, i.e. constraining the receptor, was reported a year later for the (turkey) β_1 -adrenergic receptor.⁴³ Random mutagenesis was performed on the receptor to increase the thermostability of the receptor. In the presence of an antagonist, cyanopindolol, the engineered thermostable β_1 -adrenergic receptor was stabilized to a single conformation, a prerequisite for crystallization. Similarly, two classes of engineered thermostable adenosine A_{2A} receptors were reported for either agonist or antagonist occupancy.⁴⁴ Comparison of the applied mutations does not point to a general amino acid pattern to increase thermostability. More recently, the human adenosine A_{2A} receptor crystal structure was obtained in combination with a high affinity antagonist, ZM241385, and the T4L method.⁴⁵ Surprisingly, the binding pocket of this antagonist differed greatly from carazolol in the β_2 -adrenergic receptor. This indicates that one should be cautious in interpreting results from molecular modeling and ligand docking studies based upon existing (inactive) crystal structures. Notably, a welcome addition to these ‘inactive’ crystal structures would be the crystal structure of an active conformation of a GPCR (i.e. a receptor with an agonist). Until then several questions remain, such as what the active state of the receptor looks like and if this state is the same for each type of ligand (e.g. protein or LMW).

1.2 ALLOSTERIC MODULATION

1.2.1 Allosteric Modulation of GPCRs

Allosteric modulation was first reported for enzymes. In the field of enzymology it was noted that the chemical structure of inhibitors was often very different from the substrate of the enzyme. Therefore, it was suggested that another binding site accommodated these inhibitors through which they transmitted their effect to the substrate site.⁴⁶ GPCRs, however, are naturally modulated by the presence of the (allosteric) G protein.⁴⁷ GPCRs can interact with a variety of other cellular proteins that, for example, influence receptor activation.⁴⁸ Moreover, much smaller molecular entities have been reported as endogenous allosteric modulators for GPCRs, such as (cat)ions, peptides, and lipids.⁴⁹ For example, Zn^{2+} and anandamide have been shown to allosterically inhibit dopamine D_2 receptors⁵⁰ and M_1 muscarinic acetylcholine receptors,⁵¹ respectively. Interestingly, endogenous allosteric modulators also play a role in some autoimmune diseases. For example in Sjögren's syndrome in which (allosteric) autoantibodies are raised against M_3 muscarinic acetylcholine receptors, thereby enhancing their activity.⁵²

For class A GPCRs the classical orthosteric site is accommodated by helices III, V and VI, and to some extent helix VII. This pocket can be accessed by low molecular weight ligands, which is supported by the recent crystal structures of the β -adrenergic receptors^{41,43} and adenosine A_{2a} receptor⁴⁵ with ligand bound. As schematically represented in Figure 1.2, the allosteric binding site for class A GPCRs is most likely located in the 7-TM domain as well. For the GnRH receptor, it was shown recently that the allosteric site partially overlaps with the orthosteric site.⁵³ The orthosteric site of the glycoprotein hormone receptors is located at the large N-terminus, which results in an essentially unoccupied 7-TM domain (Figure 1.3). Experiments with chimeric receptor constructs for the LH³⁶ and FSH⁵⁴ receptor have indeed shown that allosteric ligands bind in that domain. However, it seems that two different sites are occupied here, i.e. the classical class A orthosteric site and a second smaller pocket that is formed by helices I, II, III and IV.⁵⁵

1.2.2 Detecting and Describing Allosteric Modulation

In the last two decades, methods for the identification of new ligands were based on equilibrium displacement assays using a radiolabeled or otherwise tagged (orthosteric)

ligand. As a consequence, new ligands were often orthosteric of nature as (true) allosteric ligands do not compete with the radioligand. For clarity, the orthosteric binding site is referred to as the site which binds the endogenous ligand, while the allosteric site is a topographically distinct binding site (Figure 1.2).⁵⁶ Nowadays, functional assays are used in high-throughput screens (HTS) to find new allosteric ligands for certain drug targets, e.g. GPCRs.⁵⁷ In addition, kinetic association and dissociation assays of the (radio)ligand-receptor interaction are often used. The binding of an allosteric ligand induces a conformational change in the receptor, thereby altering the rates at which the orthosteric ligand associates or dissociates from its binding site.⁵⁸ With the aid of these different screening methods, allosteric modulators have been reported for all classes (A-C) of GPCRs.⁴⁹ Therefore, allosteric modulation of GPCRs seems to be a rule rather than an exception.

Several mathematical models have been developed that describe different ligand-receptor interactions.⁵⁸ One of the first and most simple models is the linear two-state model (Figure 1.5a).⁵⁹ This model uses an equilibrium dissociation constant (K_A) to describe the interaction between a ligand (A) and a receptor (R). It proposes that ligand binding results in a conformational change of the receptor from an inactive to an active state. The active receptor

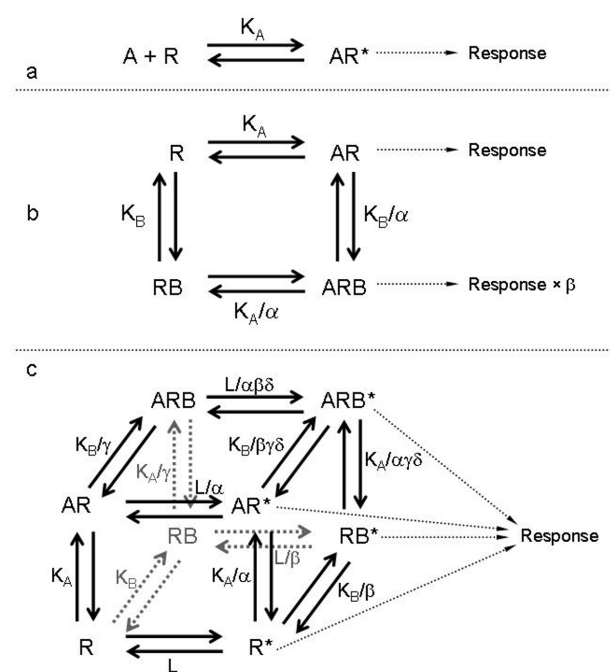


Figure 1.5 Mathematical models that describe ligand-receptor interactions. a) The linear two-state model. b) The allosteric ternary complex model. c) The allosteric two-state model.

conformation will ultimately elicit a biological response. This model was not sufficient to explain experimentally obtained data on allosteric modulation. Therefore, the allosteric ternary complex model was developed (Figure 1.5b).⁶⁰ In this model the effect of the binding of an allosteric ligand (B) on the affinity (α) and efficacy (β) of an orthosteric ligand (A) is described. However, next to ‘true’ allosteric modulators, i.e. compounds that do not have an intrinsic activity on their own, allosteric agonists have been identified. These compounds are able to activate the receptor by binding at an allosteric site. Addition of this possibility and constitutive receptor activity into a new model resulted in the allosteric two state

model (Figure 1.5c).⁶¹ In this model orthosteric and allosteric ligands can bind and activate the receptor, the extent of which is described by the cooperativity factors α and β , respectively. The ability of the ligands to modulate the binding of and activation by each other is described by cooperativity factors γ and δ , respectively. Moreover, constitutive activity of the receptor is taken into account (L). Extension of these models is possible for accommodation of multiple allosteric sites,⁶² G protein-coupling⁵⁸ or allosteric modulation across dimeric receptors.⁶³ Notably, the cooperativity factors shown in Figure 1.5 can be different for each orthosteric-allosteric ligand pair at a given receptor, also referred to as probe dependence.⁴⁹ Hence, the physiological relevance of a certain allosteric effect should always, when possible, be examined with a physiologically relevant probe, the endogenous ligand.

1.2.3 Therapeutic Potential – Allosteric Modulation

Most drugs targeting GPCRs that are currently on the market are orthosteric in nature.⁶⁴ For a therapeutic effect these compounds must have a high affinity for the orthosteric site and a high local concentration should be maintained. The resulting disadvantage of synthetic orthosteric ligands are therefore effects such as, toxicity, desensitization and long-term changes in receptor up/down regulation.⁴⁹ Allosteric modulators have the potential to overcome these negative effects. Moreover, these compounds have other advantages that orthosteric ligands do not possess, which will be described in more detail below. Therefore, allosteric modulation of GPCRs has fuelled further interest by scientists from academia and industry.

Only two allosteric modulators of GPCRs are currently on the market, Cinacalcet and Maraviroc, which are an allosteric enhancer of the calcium-sensing receptor⁶⁵ and an allosteric inhibitor of the CCR5 receptor,⁶⁶ respectively. LMW allosteric ligands potentially have several advantages over orthosteric ligands.⁶⁷ The main advantage results from the fact that allosteric ligands target a different binding site than the endogenous (orthosteric) site. This site has not been conserved through evolution, which results in increased receptor subtype selectivity. For example, the adenosine receptors consist of four subtypes (A_1 , A_{2a} , A_{2b} , A_3) that all bind the endogenous ligand adenosine due to a conserved (orthosteric) binding pocket. For these (and other) receptors it was shown that allosteric modulators can be more selective than synthetic orthosteric ligands.⁶⁸ Allosteric modulators are characterized as

compounds that only have an effect in the presence of the endogenous ligand. The latter yields two additional advantages, saturability of the effect and preservation of physiological patterns. Firstly, increasing the dosage of an allosteric modulator per se will not produce an increased effect, also known as the ‘ceiling-effect’. The cooperativity factor of the allosteric modulator and the orthosteric ligand determines the effect. In addition, allosteric modulators with a lower affinity can be administered at a higher dose with less safety or toxicity problems. Secondly, the allosteric modulator will only exert an effect where and when the endogenous ligand is produced. This results in tissue selectivity and the duration of an effect remains physiologically relevant.

1.2.4 Therapeutic Potential – GnRH and LH Receptor

An additional advantage of allosteric modulation is worth mentioning for GPCRs (e.g. GnRH and LH receptor) that have peptide or protein hormones as endogenous ligands, which lack oral bioavailability. Synthetic ligands for these receptors (both orthosteric and allosteric) can be made drug-like, i.e. LMW, orally bioavailable, metabolically stable and with an acceptable safety profile. In addition, pure synthetic ligands lack batch variability and contamination with other proteins, when compared to proteins obtained from urine or recombinant production.⁶⁹ Examples of drug-like ligands for the GnRH and LH receptor are shown in Figure 1.6. The chemical structures and size of these LMW ligands are compared to the crystal structures of GnRH (PDB entry: 1yy1) and hCG (PDB entry: 1hrp). NBI-42902 is an (orthosteric) antagonist for the GnRH receptor (Figure 1.6a), which has been shown to suppress plasma LH levels after oral administration in post-menopausal women.⁷⁰ For the LH receptor, Org 43553 was introduced as the first potent and orally active allosteric agonist that induced ovulation in mice and rats.⁶⁹

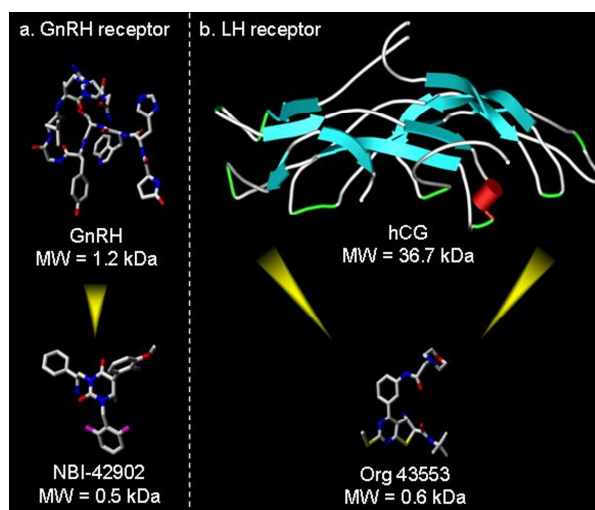


Figure 1.6 Structural comparisons of the endogenous HMW ligand and a LMW ligand for the (a) GnRH and (b) LH receptor, respectively.

The GnRH receptor has been described as a potential target for different diseases. So far, orthosteric (peptide) agonists and antagonists are well-characterized. Allosteric enhancers and inhibitors could be beneficial in similar treatments. GnRH receptor agonists and antagonists have been shown to be efficacious in IVF procedures. It should be noted that (peptide) agonists are used to desensitize the receptor, which in turn also results in a decreased gonadotrope function.⁷¹ In addition, GnRH receptor ligands may also be applied in a number of sex hormone-dependent conditions.^{72,73} Notably, various peptide GnRH receptor agonists and antagonists are marketed for the treatment of prostate, breast, uterine and ovarian cancer, leiomyomas, infertility, benign prostatic hyperplasia (BPH), IVF, premenstrual syndrome and endometriosis.^{74,75}

The LH receptor is an important regulator of reproductive functions in humans. Currently, recombinant LH (recLH) is used for the treatment of female hypogonadism.⁷¹ In addition, recLH was approved for the use in the late follicular phase of IVF treatment to enhance oocyte maturation and pregnancy outcome.⁷⁶ Similarly, recombinant hCG and urinary hCG are used for ovulation induction and oocyte maturation. However, these hormone preparations need to be administered daily by subcutaneous injection. Therefore, efforts are made to develop more patient friendly formulations, such as gonadotropins with longer half-lives^{77,78} and orally bioavailable drugs.⁵⁵ Another important goal is to eliminate ovarian hyperstimulation syndrome (OHSS), a side effect resulting from the hormonal treatments.⁷⁹ GnRH receptor agonists already show improvement, however, they are peptidic in nature. Therefore, LMW LH receptor agonists are of interest here. Recently, it was shown that such a compound, the allosteric agonist Org 43553 can possibly be used for ovulation induction or final oocyte maturation in IVF therapy with reduced side effects (e.g. OHSS).⁶⁹ Gonadotropins (hCG) have also been shown to promote ovarian tumor cell growth⁸⁰ and inhibit primary breast tumor growth.⁸¹ Thus, negative and positive allosteric modulators could be beneficial in these cases, respectively. Notably, several naturally occurring mutations have been described for the LH receptors that are involved in male and female fertility.⁸² These data indicate that LH receptor antagonists or allosteric inhibitors could be useful as contraceptives.

1.3 THIS THESIS

In the previous paragraphs GPCRs, the concept of allosteric modulation and the therapeutic potential thereof for the human GnRH and LH receptor were presented. In earlier work we have demonstrated that other class A GPCRs (adenosine A₁, A_{2A} and A₃ receptors) can be allosterically modulated.⁶⁸ In this thesis we have expanded our knowledge on allosteric modulation of class A GPCRs to two of the ‘reproductive’ family members, the GnRH and LH receptor. As both these receptors have protein hormones as endogenous ligands, new LMW ligands (either allosteric or orthosteric) are of general interest. Therefore, a review of the current literature on LMW ligands for GPCRs of the hypothalamic-pituitary-gonadal axis (HPG) (e.g. GnRH and LH receptor) is presented in *Chapter 2*.

At the start of this project, no allosteric modulators of GnRH and LH receptors were known. Therefore, the effect of general allosteric modulators (e.g. GTP, sodium ions and amiloride derivatives) on dissociation kinetics of either GnRH or LH receptor ligands was examined. For the GnRH receptor we found that an amiloride analog (HMA) was a potent allosteric inhibitor. Meanwhile, Sullivan and coworkers reported that a non-peptidic antagonist for the human GnRH receptor also displayed allosteric effects.⁵³ Intrigued by the availability of two allosteric ligands [HMA and FD-1 (an analog of Sullivan’s compound)], the studies described in *Chapter 3* were performed. Figure 1.7 schematically represents the question that needs to be answered when three structurally different ligands are available for one receptor. In this case the green, red and cyan ligands represent GnRH, HMA and FD-1 binding at the human GnRH receptor (blue), respectively.

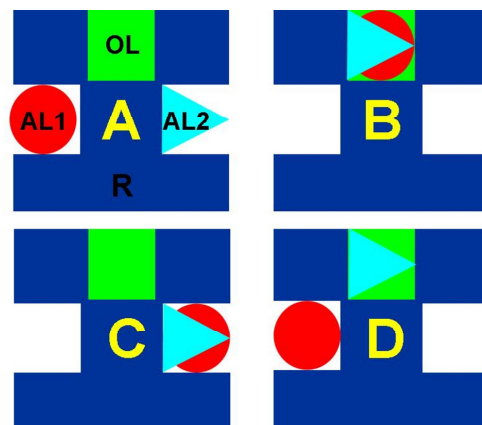


Figure 1.7 Four different possibilities of ligand distribution (A-D) in the receptor (R), when the orthosteric ligand (OL; square) and two other (allosteric) ligands (AL1; circle and AL2; triangle) are present.

In 2002, the first orally active LMW agonist for the LH receptor was reported.²¹ A more potent analog was labeled with tritium and *Chapter 4* introduces this first LMW radiolabeled agonist for the human LH receptor, [³H]Org 43553. We hypothesized that similar to other class A GPCRs, the LH receptor could possibly also contain two binding sites in the 7-TM domain. Therefore, a screen was performed in which allosteric modulation of [³H]Org 43553 was examined. This resulted in the first class of allosteric inhibitors (e.g. LUF5771; *Chapter*

5) and allosteric enhancers (e.g. LUF5419; *Chapter 6*). When Figure 1.7 is used to describe the LH receptor (blue), the green, red and cyan ligands represent LH (or hCG), Org 43553 and a LUF compound (e.g. LUF5419 or LUF5771), respectively. Moreover, the same library of compounds was screened for an inhibitory effect in a reporter gene assay, more specifically a luciferase assay (*Chapter 7*). This resulted in a surprising amount of apparent LH receptor antagonists. We felt that this deserved some more attention and it appeared that some of these compounds were competitive inhibitors of the enzyme rather than of the receptor.

In conclusion, in this thesis I present the evidence that ‘reproductive’ GPCRs, like most other class A GPCRs, can be allosterically modulated by LMW ligands. *Chapter 8* will conclude this work, describing the general conclusions and future perspectives for this field of research.

CHAPTER

2

G PROTEIN-COUPLED RECEPTORS OF THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS; A CASE FOR GNRH, LH, FSH AND GPR54 RECEPTOR LIGANDS

The hypothalamic-pituitary-gonadal (HPG) axis, important in reproduction and sex hormone-dependent diseases, is regulated by a number of G protein-coupled receptors. The recently 'deorphanized' GPR54 receptor activated by the peptide metastin is thought to be the key regulator of the axis, mainly by releasing gonadotropin-releasing hormone (GnRH) from the hypothalamus. The latter decapeptide, through the activation of the GnRH receptor in the anterior pituitary, causes the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which subsequently activate their respective receptors on the gonadotrope cells. In this review we will discuss the small molecule agonists and antagonists that are currently being developed to intervene with the action of these four receptors. For GnRH receptors, fourteen different chemical classes of non-peptidic antagonists have been reported, while for the LH receptor three classes of agonists have been described. Both agonists and antagonists have been introduced for the FSH receptor. Recently, the first non-peptidic agonist for GPR54 was reported.

This chapter is an update of a recent publication:

Heitman, L. H.; IJzerman, A. P. G Protein-Coupled Receptors of the Hypothalamic-Pituitary-Gonadal Axis; a case for GnRH, LH, FSH and GPR54 Receptor Ligands. *Med Res Rev* **2008**, 28, 975-1011

2.1 INTRODUCTION

The receptors of the hypothalamic-pituitary-gonadal-axis (HPG-axis) that will be discussed in this review all belong to the rhodopsin-like subfamily of G protein-coupled receptors (GPCRs). The human genes of the gonadotropin-releasing hormone (GnRH)^{83,84} luteinizing hormone (LH)⁸⁵ and follicle-stimulating hormone receptors (FSH)⁸⁶ were cloned in the early nineties, whereas human GPR54 cDNA was isolated in 1999.⁸⁷ The GnRH receptor is predominantly coupled to the G_q-protein, through which it regulates the biosynthesis and secretion of the gonadotropins, FSH and LH.¹⁰ The FSH and LH receptor belong to the glycoprotein-hormone receptor family together with the thyroid-stimulating hormone (TSH) receptor.⁸⁸ These receptors contain a large N-terminus to which the endogenous hormone binds. Activation of the LH and FSH receptor mainly results in the production of intracellular cAMP *via* G_s proteins. These hormones stimulate germ cell development and hormone (estrogen and progesterone) secretion in the ovaries.⁸⁹ In addition, LH and FSH to some extent, stimulate the testis to produce testosterone. GnRH secretion in turn is inhibited by estrogen and progesterone, allowing a negative feedback loop in the HPG-axis. Recently, it was shown that a placental peptide, kisspeptin-54 (metastin), activates GPR54, which results in the activation of phospholipase C *via* G_q.⁹⁰ GPR54 has been shown to stimulate the hypothalamic secretion of GnRH.⁹¹

The endogenous ligands for the GnRH, LH, FSH and GPR54 receptor are either peptide or protein hormones, and can be administered parenterally, also in their recombinant form, if available. However, it would be very desirable to have orally available, non-peptidic, chemical entities as well, which is the focus of intensive research efforts especially in industry. As ligands for the receptors of the HPG-axis have similar clinical applications, this review gives a detailed overview of the search for non-peptidic ligands that have been identified for these receptors. The identification of selective and high affinity ligands for these receptors could be beneficial in the treatment of several sex-hormone dependent diseases, ovarian, prostate, or breast cancer, infertility or as non-steroidal contraceptives.^{71,72,92,93} In this review we will first address non-peptidic antagonists for the GnRH receptor, followed by non-peptidic agonists for the LH receptor. Then non-peptidic agonists and antagonists for the FSH receptor will be reviewed, and we conclude by discussing the first non-peptidic agonist for GPR54.

2.2 GNRH RECEPTOR ANTAGONISTS

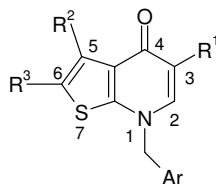
GnRH or its agonist analogs need to be administered in a pulsatile fashion to result in physiologic gonadotropin secretion.⁹⁴ A continuous administration of GnRH (agonists) will initially lead to gonadotropin release followed however by antagonism of the HPG axis by subsequent desensitization of GnRH receptors. Initially, analogues of the endogenous ligand GnRH were prepared as agonists and antagonists for this receptor.⁹⁵⁻⁹⁷ However, peptidic ligands are not preferred as drugs in chronic treatments as they have to be administered by injection due to their susceptibility for biological degradation. Therefore, intensive efforts were undertaken to develop non-peptidic GnRH receptor ligands, which have the potential to be orally bioavailable. To date only non-peptidic antagonists have been identified, which can be classified into fourteen chemical classes. Each of them will be discussed separately, where only the most potent compounds of each class are highlighted. Furthermore, this paragraph includes additional patented compound classes that have not been published (yet). These compounds are classified based on the presence of a mono-, bi- or tricyclic scaffold.

2.2.1 Thieno[2,3-d]pyridin-4-one Derivatives

The first class of non-peptidic antagonists for the human GnRH receptor was described by a research team at Takeda in 1998.⁹⁸ Structure-activity relationships (SARs) of peptide agonists and antagonists showed that the type II β -turn involving residues 5-8 (Tyr-Gly-Leu-Arg) of GnRH is important for binding affinity.⁹⁹ A compound library was selected that consisted of general GPCR antagonistic structures and screening resulted in a thieno[2,3-d]pyridin-4-one derivative as a lead. Structural similarity was found with the β -turn moiety of GnRH, where the Tyr-, Gly- and Leu-residues were mimicked by the substituents at positions 6, 1 and 3, respectively. Introduction of a basic amino moiety at the 5-position added similarity to the Arg-residue and further optimization resulted in compound **1** (T-98475) (Table 2.1). Compound **1** had a high affinity for the human GnRH receptor ($IC_{50} = 0.2$ nM) and showed selectivity over other GPCRs interacting with peptide ligands.⁹⁸ Although **1** was 20-fold less potent on the monkey GnRH receptor, oral administration in monkeys showed over 70% inhibition of plasma LH-levels *in vivo*. In an extension of this study, Imada and coworkers aimed to further optimize each substituent to improve *in vivo* antagonism.¹⁰⁰ It appeared that all substituents, except on the 6-position, were already optimal. Introduction of the 1-hydroxycyclopropanecarboxamide group yielded compound **2**, which increased the

potency on human receptors by 2-fold, while displaying a 9-fold lower potency on monkey receptors. Oral administration of 60 mg/kg of compound **1** to monkeys resulted in a duration of action of 8 h,⁹⁸ whereas 10 mg/kg of **2** suppressed LH-levels for 24 h.¹⁰⁰

Table 2.1 Binding affinities of thieno[2,3-*b*]pyridin-4-one derivatives (**1-2**) at the human GnRH receptor.



Compound	R ¹	R ²	R ³	Ar	IC ₅₀ (nM) ^a	Ref
1					0.2	98
2					0.1	100

^a The ability to inhibit binding of ¹²⁵I-leuporelin to the cloned human GnRH receptor stably expressed in CHO cells.⁹⁸

2.2.2 Quinolin-2-one Derivatives

In 1999 researchers at Merck introduced quinolin-2-one derivatives as a novel class of non-peptidic GnRH receptor antagonists (Table 2.2).¹⁰¹ The lead compound (**3**), which had micromolar affinity for the rat GnRH receptor (IC₅₀ = 10 μM), was identified by screening an in-house compound library. At first the 2-pyridyl substituent at position 4 was replaced by other (nitrogen-containing) ring systems. An alkyl cyclic amine with a 3-carbon spacer between the basic amine and the 4-quinolone oxygen provided the highest binding affinity. The SAR of the 3-aryl group was also described by the same group.¹⁰² As a consequence a 3,5-dimethylgroup was incorporated. Subsequent optimization of the quinolone ring substituents showed that a chlorine atom at the 7-position was important for high affinity. A 10-fold increase in potency was obtained when a 6-nitro group was incorporated resulting in the first nanomolar-affinity compound of this class (**4**; IC₅₀ = 32 nM).¹⁰¹ The chirality and ring size of the alkyl cyclic amine substituent at position 4 was further investigated.¹⁰³ It was determinant in binding affinity. Together with the removal of the N-methyl group of this

Table 2.2 Binding affinities of quinolin-2-one derivatives (**3-10**) at the rat or human GnRH receptor.

Compound	R ¹	R ²	Ar	IC ₅₀ (nM) ^a	Ref
3		H		10,000 ^b	101
4		NO ₂		32 ^b	101
5		NO ₂		10 ^b	103
6				0.9	104
7				0.3	102
8				0.44	105
9				0.1	106

^a The ability to inhibit the binding of ¹²⁵I-buserelin to the cloned human GnRH receptor stably expressed in CHO cells.¹⁰⁵

^b The ability to inhibit the binding of ¹²⁵I-buserelin to the rat pituitary GnRH receptor.¹⁰¹

substituent, compound **5** was obtained with an IC₅₀-value of 10 nM at the rat GnRH receptor.¹⁰³ At Merck parallel efforts were undertaken to replace the 6-nitro group by different substituted amide groups.^{102,104} In both papers, the pyrimidine-carboxamide was the superior substituent (**6**). In addition, a 3,4,5-trimethylphenyl substituent at position 3 further increased the potency by 3-fold (**7**).¹⁰² Due to the availability of a binding assay for the

human GnRH receptor, it appeared that these quinolone analogs had a somewhat higher affinity than at the rat GnRH receptor. In addition, for compound **8** it was shown that its affinity at monkey GnRH receptors was equal to the human receptor ($IC_{50} = 0.44$ nM). However, at the rat GnRH receptor a 10-fold lower affinity was found.¹⁰⁵ Compound **8** was also characterized in *in vivo* studies. Intravenous administration of 3 mg/kg of **8** resulted in 79% suppression of LH and 92% suppression of testosterone blood levels in primates. In 2004, an improved synthetic route was published.¹⁰⁶ In this study, it was shown that also other heterocyclic rings at position 4 yielded a high potency. Replacement of the pyrimidine with a thiadiazole ring only slightly improved the affinity, while changing the cyclic amine for a cyclic amide, such as a γ -lactam moiety (**9**), improved the affinity 4-fold compared to **8**.

2.2.3 Indole Derivatives

Another class of non-peptidic GnRH antagonists was described by Chu and coworkers (Table 2.3).¹⁰⁷ An indole-based lead (**10**) was identified after in-house screening, having micromolar binding affinity at the rat GnRH receptor ($IC_{50} = 3$ μ M). In a first attempt to increase the affinity, the substituents at positions 2 and 3 were optimized. It appeared that neither the stereochemistry nor the ether linkage in lead compound **10** were important for GnRH receptor affinity. In addition, replacement of the aryl group at position 2 for a 3,5-dimethylphenyl resulted in a 60-fold increase in receptor affinity (**11**; $IC_{50} = 50$ nM).¹⁰⁷ In an extension of this study, the effect of substituents at position 5 on receptor affinity was studied.¹⁰⁸ It was shown that a functionalized piperazinyl group, especially when it was sulfonylated, increased the binding affinity over 10-fold (**12**; $IC_{50} = 4$ nM). Since compounds **10-12** were phenol derivatives, and therefore metabolically unstable, the Merck group continued to study phenol ring surrogates.^{109,110} It appeared that a hydrogen bond donating group in combination with a four-carbon spacer resulted in the most active compounds. The methanesulfonamide group in compound **13** resulted in a ligand with a similar affinity.¹⁰⁹ Notably, the affinity of compound **13** was almost 25-fold lower on the human GnRH receptor ($IC_{50} = 170$ nM). It was shown that the introduction of a heterocyclic 4-pyridyl substituent also resulted in a potent compound (**14**) with an affinity between that of **11** and **13**.¹¹⁰ At the same time, the substituents at the 5-position of the indole were further explored.¹¹¹ Carboxamide groups, particularly those derived from secondary amines, increased receptor affinity. Interestingly, the affinity of these compounds for the human receptor increased (**15**;

Table 2.3 Binding affinities of indole derivatives (**10-19**) at the rat or human GnRH receptor.

Compound	R ¹	R ²	Ar	IC ₅₀ (nM) ^a	Ref
10		H		3,000 ^b	107
11		H		50 ^b	107
12				4 ^b	108
13		H		7 ^b	109
14		H		16 ^b	110
15				5.7	111
16				1.4	112
17				0.6	113
18				0.6	114
19				0.3	115

^a The ability to inhibit the binding of ¹²⁵I-buserelin to the cloned human GnRH receptor stably expressed in CHO cells.¹⁰⁵^b The ability to inhibit the binding of ¹²⁵I-buserelin to the rat pituitary GnRH receptor.⁹⁸

IC₅₀ = 5.7 nM). Furthermore, these compounds were also most effective in antagonizing LH release from pituitary cells. Combining the optimal substituents of compounds **14** and **15** resulted in compound **16**, which had the highest affinity for the human GnRH receptor so far (IC₅₀ = 1.4 nM).¹¹² In addition, the 5-substituent was *gem*-dimethylated, which was favorable to reduce metabolic cleavage. Several attempts were made to improve the pharmacodynamic and pharmacokinetic properties of this class of compounds. Introduction of a chiral β -methyl group at the 3-substituent and reducing the four-carbon to a two-carbon spacer, resulted in higher potency and oral bioavailability (**17**).¹¹³ Oral administration of 10 mg/kg of compound **17** in castrated male rats completely suppressed plasma LH levels for 13 h. Notably, the affinity of **17** was almost 3-fold lower for the rat GnRH receptor than the human receptor. The pyridine portion of **17** was modified in two separate studies.^{114,115} Firstly, the introduction of a benzotriazole group (**18**) resulted in a two-fold increase in the potency (IC₅₀ = 0.6 nM), and maintained oral bioavailability and low plasma clearance.¹¹⁴ In addition, the cytochrome P450 3A4 inhibition that was found for some analogues of **18** was substantially decreased. Secondly, the introduction of an *ortho* methyl to the pyridine portion of **17** and oxidation of the pyridine nitrogen resulted in compound **19**.¹¹⁵ Compound **19** had a lower oral bioavailability in dogs (25% compared to 37%), but a longer terminal half-life (5 h compared to 2.7 h) and a 2-fold higher affinity than compound **18**.

2.2.4 Pyrrolo[1,2-*a*]pyrimidin-7-one Derivatives

Pyrrolo[1,2-*a*]pyrimidones, as a novel class of heterocyclic non-peptidic antagonists for the human GnRH receptor were introduced by Neurocrine Biosciences (Table 2.4).¹¹⁶ All non-basic compounds were inactive. At position 2 a hydrophobic aromatic ring with an extra hydrogen bond acceptor was preferred and at position 4 a 2-fluorobenzyl group was most potent. The potency was increased when the *para*-substituent at the 2-aromatic ring was replaced with the more lipophilic isobutoxy group, yielding nanomolar affinity. Introduction of a medium-sized lipophilic ester group at position 6 resulted in high binding affinity at the human GnRH receptor (**20**; K_i = 25 nM).¹¹⁶ Compound **20** was highly selective for the human receptor, as the affinity at the rat GnRH receptor was almost 300-fold lower. Further optimization by Zhu *et al.* proved that removal of the cyano-group at the 3-position resulted in more potent compounds.¹¹⁷ At position 2, a hydrogen bond acceptor together with a lipophilic group and a linear, rather than branched, alkyl group provided a drastically

increased affinity (**21**; $K_i = 1.2$ nM). For other compounds in this class functional antagonism was shown by their inhibition of GnRH-stimulated calcium flux. The removal of the cyano group in compound **20** resulted in a compound that was relatively unstable under acidic conditions, but more potent. Therefore, Tucci *et al.* introduced a fluoro substituent as a smaller electron-withdrawing group at position 3.¹¹⁸ As a result the core was stabilized, while maintaining a high affinity for the human GnRH receptor (**22**; $K_i = 9$ nM). It appeared that introduction of the 3-fluoro group in compound **22** resulted in an electron-poor density of the 4-(2-fluorobenzyl) moiety, while the cyano group of **20** yielded an electron-rich moiety. In this study, compound **22** was docked into a 3D-model of the human GnRH receptor. It was believed that the 2-fluorobenzyl group interacts with one of two tyrosine residues in transmembrane domain (TM) VI (Y283 and Y284) of the receptor. A ligand with an electron-poor aromatic ring, like **22**, could therefore interact with the electron-rich aromatic ring of the tyrosine residues by π stacking, resulting in a higher binding affinity.

Table 2.4 Binding affinities of pyrrolo[1,2-a]pyrimidin-7-one derivatives (**20-22**) at the human GnRH receptor.

Compound	R ¹	R ²	R ³	X	K _i (nM) ^a	Ref
20				CN	25	116
21				H	1.2	117
22				F	9	118

^a The ability to inhibit the binding of des-Gly¹⁰[¹²⁵I-Tyr⁵,DLeu⁶,NMeLeu⁷,Pro⁹-NEt]-GnRH to the cloned human GnRH receptor stably expressed in HEK293 cells.¹¹⁹

2.2.5 Imidazolo[1,2-a]pyrimidin-5-one Derivatives

In 2002 Takeda¹²⁰ and Neurocrine Biosciences¹²¹ both introduced imidazolo[1,2-a]pyrimidin-5-one derivatives. The thiophene ring of the thieno[2,3-b]pyridin-4-one derivatives (**1-2**) and the pyrrole ring of the pyrrolo[1,2-a]pyrimidin-7-one derivatives (**20-22**) were replaced by an imidazole ring, respectively (Table 2.5). These replacements resulted in potent GnRH receptor antagonists with improved pharmacokinetic profiles and increased stability under acidic conditions. Sasaki *et al.* showed that similar substituents as at compound **1** resulted in a functional antagonist at the human GnRH receptor with comparable binding affinities (**23**; IC₅₀ = 0.3 nM).¹²⁰ Wilcoxon *et al.* confirmed the importance of the basic nitrogen and the attached benzyl group at position 3.¹²¹ The substitution pattern of their most potent compound (**24**) was similar as **23** with a K_i value of 7.5 nM. Modeling of this compound showed that the basic tertiary amine possibly interacts with an aspartic acid in TM

Table 2.5 Binding affinities of imidazolo[1,2-a]pyrimidin-5-one derivatives (**23-26**) at the human GnRH receptor.

Compound	R ¹	R ²	R ³	Ar	X	K _i (nM) ^a	Ref
23					H	0.3 ^b	120
24					H	7.5	121
25					CH ₃	4.6	122
26					CH ₃	1.2	123

^a The ability to inhibit the binding of des-Gly¹⁰[¹²⁵I-Tyr⁵,DLeu⁶,NMeLeu⁷,Pro⁹-NEt]-GnRH to the cloned human GnRH receptor stably expressed in HEK293 cells.¹¹⁹

^b IC₅₀ value, determined by the ability to inhibit binding of ¹²⁵I-leuporelin to the cloned human GnRH receptor stably expressed in CHO cells.⁹⁸

VII (Asp302) and the pyridine ring could provide π - π interaction with an aromatic residue in the receptor.¹²³ Although this class of compounds was more stable, Neurocrine Biosciences focused on replacing the ester group at position 6, as it is hydrolyzed *in vivo*.¹²² This group may function as both a lipophilic group and a hydrogen bond acceptor, and was therefore replaced by a phenyl group bearing a hydrogen-bond accepting group. Replacement of the *n*-butyroylamidophenyl (**24**) by a 4-methoxyphenyl group at position 2 resulted in a 30-fold decreased binding affinity (data not shown). However, in combination with the 3-methoxyphenyl and a methyl group at position 6 and 7, respectively, compound **25** emerged with a K_i -value of 4.6 nM.¹²² The 7-methyl was speculated to force the 5-phenyl ring into a perpendicular conformation, which may be preferred for π - π interaction with an aromatic residue in the GnRH receptor. Furthermore, it was postulated that the *para*-substituted phenyl group at position 2 might play a less important role in binding. Introduction of a *tert*-butyl moiety indeed resulted in similar binding affinity as compound **25**. Some potency was gained upon the introduction of a 1-methyl-1-methoxycarbonyl ethyl group at position 2 (**26**; K_i = 1.2 nM). This modification led to high-affinity functional GnRH receptor antagonists with a reduced molecular weight.¹²³ However, the affinity of this novel class of antagonists for the rat GnRH receptor was almost 100-fold lower,^{121,122} in agreement with earlier reports by Takeda and Merck on their low molecular weight antagonists.^{113,116}

2.2.6 Thieno[2,3-d]pyrimidin-2,4-dione Derivatives

The thieno[2,3-d]pyridin-4-one derivative **1** showed high binding affinity *in vitro*. However, *in vivo* antagonism was not as effective, due to its low oral bioavailability. Therefore, another research program to identify non-peptidic GnRH receptor antagonists was conducted.¹²⁴ A novel class of ligands was introduced with a thieno[2,3-d]pyrimidin-2,4-dione core (Table 2.6). Previous studies showed that the *N*-benzyl-*N*-methylaminomethyl and 2,6-difluorobenzyl substituents were important for receptor binding. Only the 3- and 6-substituents were therefore investigated. A phenyl group at position 3 resulted in the highest affinity when a 4-methoxyphenyl group was present at position 6 (data not shown). Since the methoxyphenyl group is a metabolic target, different *para*-acylaminophenyl substituents were investigated. These compounds showed high receptor affinity and it was therefore hypothesized that the 6-substituent should contain a hydrogen-bond donor and a small alkyl group. Compound **27** (TAK-013), with a 6-(4-methoxyurea-phenyl) group had an IC_{50} -value

of 0.1 nM and similar binding affinity at the monkey GnRH receptor ($IC_{50} = 0.6$ nM). Compound **27** showed effective *in vitro* functional antagonism and *in vivo* efficacy after oral administration. Oral administration of 10 mg/kg of compound **27** completely suppressed plasma LH levels for more than 24 h in monkeys.¹²⁴ An urea moiety is widely acknowledged to cause low oral adsorption due to its strong hydrogen bonding. However, molecular modeling studies suggested that through the introduction of the methoxy oxygen, an intramolecular hydrogen bond is formed. This would result in an increased apparent lipophilicity and therefore a higher oral bioavailability. Further effort was put into making more water-soluble analogues of compound **27**. As was already shown by Wilcoxon *et al.*, replacement of the benzyl by a pyridine ring at position 5 did not affect the binding affinity.¹²¹ The advantage of incorporation of a pyridine ring is that it is slightly basic, which reduces the lipophilicity and increases the water solubility. Although introduction of a nitro group at position 6 resulted in a lower affinity for the *N*-benzyl analogue, in combination with a 2-pyridylethyl group an affinity comparable to **27** was obtained (**28**; $K_i = 0.6$ nM).¹²⁵ This

Table 2.6 Binding affinities of thieno[2,3-b]pyrimidin-2,4-dione derivatives (**27-28**) at the human GnRH receptor.

Compound	R ²	R ³	K _i (nM) ^a	Ref
27			0.1 ^b	124
28			0.6	125

^a The ability to inhibit binding of des-Gly¹⁰[¹²⁵I-Tyr⁵,DLeu⁶,NMeLeu⁷,Pro⁹-NET]-GnRH to the cloned human GnRH receptor expressed in HEK293 cells.¹¹⁹

^b IC_{50} value, determined by the ability to inhibit binding of ¹²⁵I-leuporelin to the cloned human GnRH receptor stably expressed in CHO cells.⁹⁸

finding is in line with the previously mentioned receptor model suggested by Takeda (section 2.2.5), where the pyridyl side chain is in close proximity to Asp302 in TM VII.¹²³

2.2.7 Furamide Derivatives

In 2002 Pfizer identified the first class of non-peptidic GnRH receptor antagonists without a 5-6 membered heterocyclic core (Table 2.7).¹²⁶ Compound **29** was identified through screening of the in-house libraries. Although **29** was a potent and functional GnRH receptor antagonist ($K_i = 40$ nM), the guanidine moiety was suspected to cause potential absorption problems.

Table 2.7 Binding affinities of furamide derivatives (**29-33**) at the human GnRH receptor.

Compound	R ¹	R ²	K _i (nM) ^a	Ref
29			40 ± 5	126
30			13 ± 4	126
31			9.3 ± 0.9	127
32			6.0 ± 0.8	128
33			0.4 ± 0.1	129

^a The ability to inhibit [¹²⁵I]GnRH-A to the cloned human GnRH receptor stably expressed in HEK293 cells.¹²⁸

Therefore, a variety of other functional groups were examined. The SAR study revealed that the guanidine moiety on the cyclohexyl ring could be replaced by different substituents, resulting in species as well as potency differences. Introduction of a carboxylic acid amide resulted in the most potent compound (**30**) with a 3-fold increase in receptor affinity.¹²⁶ Both compound **29** and **30** preferred the human over the rat GnRH receptor by approximately 20-fold. The guanidine moiety was also modified to a 'caged' form with mono- or diaminopyrimidine substituents, and the cyclohexyl ring was replaced for a benzyl ring (e.g. **31**).¹²⁷ Compound **31**, a diaminopyrimidine derivative, was a potent and functionally active antagonist *in vitro* and *in vivo*. Intravenous administration of 20 mg/kg of **31** suppressed LH levels for at least 6 h in castrated rats, whereas intramuscular administration significantly lowered testosterone blood levels for up to 24 h in intact rats.¹²⁷ In addition, compound **31** was profiled against other drug targets and showed 50- to > 100-fold selectivity for human GnRH receptors, except for dopamine D₂ receptors and sodium channels. Based on the previous SAR studies,^{126,127} compound **32** was developed, which contains a 2,4,6-trimethoxyphenyl group at the amide bond.¹²⁸ Anderes and coworkers evaluated **32** for its bioavailability and *in vivo* activity. Compound **32** had a high affinity for the human GnRH receptor ($K_i = 6.0$ nM) and showed functional antagonism *in vitro*. In addition, **31** had a similar affinity at the rat GnRH receptor and showed over a 1000-fold selectivity against other drug targets, except for dopamine D₂, 5-HT_{2a} serotonin receptors and calcium channels. In contrast to compound **31**, this compound was orally active. Although at a relatively high concentration (100 mg/kg), oral administration of **32** completely suppressed LH and testosterone blood levels for up to 8 h in castrated rats and 24 h in intact rats, respectively.¹²⁸ Notably, Neurocrine Biosciences reported that **32** acts as a negative allosteric modulator rather than as an orthosteric ligand for both a peptide GnRH agonist and a non-peptidic antagonist.⁵³ Hence, since *in vivo* activity was previously shown,¹²⁸ the allosteric mechanism is proven to be effective. Interestingly, a close analog of **33** was shown to be a negative allosteric modulator as well (*Chapter 3*). Moreover, in the same study it was shown that the GnRH receptor contains another allosteric site to which amiloride analogues bind. These compounds have been shown to modulate several GPCRs. Some additional structural changes (e.g. addition of a morpholino group) resulted in **33**, which was highly potent with improved oral activity.¹²⁹ Compound **33** had identical affinity values at rat and human GnRH receptors ($K_i = 0.4$ nM). Oral administration of 50 mg/kg of **33** completely suppressed LH and testosterone blood levels for up to 24 h in castrated rats and 12 h in intact rats, respectively.

Importantly, **33** was highly selective over other drug targets and had low potential for interaction with various cytochrome P450s.

2.2.8 Pyrimidin-2,4-dione Derivatives

Previously, Neurocrine Biosciences had introduced the pyrrolo[1,2-a]pyrimidin-7-one (**20-22**) and imidazolo[1,2-a]pyrimidin-5-one (**23-26**) derivatives as potent human GnRH receptor antagonists.^{116,121} Based on the SAR of these compounds, it was postulated that the five-membered ring of the scaffold was not necessary for receptor binding. The cyano- or imidazole-nitrogen was replaced by a carbonyl moiety resulting in pyrimidine-2,4-dione derivatives, which were also referred to as uracil-based GnRH receptor antagonists (Table 2.8).¹³⁰ Similar substituents as on the previous scaffolds resulted in a GnRH receptor antagonist with reasonably high affinity (**34**; $K_i = 34$ nM). The bioavailability of **34** was only 1.6% due to the high lipophilicity and poor metabolic stability. Further efforts were, therefore, undertaken to improve the metabolic stability. Introduction of an α - (**36**) or β -methyl (**35**) at the N-3 position increased the affinity, with the (*R*)-isomers being more active than the (*S*)-isomers.^{131,132} The increase in binding affinity was explained by a receptor model

Table 2.8 Binding affinities of pyrimidin-2,4-dione derivatives (**34-45**) at the human GnRH receptor.

Compound	R ¹	R ²	Ar	X	K _i (nM) ^a	Ref
34				CH ₃	34	130
35				CH ₃	5.5	131
36				CH ₃	20	132

(Continued)

37				CH ₃	1.1	133
38				CH ₃	0.6 ± 0.1	134
39				CH ₃	2 ^b	135
40				CH ₃	0.7	136
41				CH ₃	0.56 ± 0.03	137
42				H	0.45	138
43				CH ₃	0.30	139
44				H	2.2	140
45				CH ₃	1.2	141

^a The ability to inhibit the binding of des-Gly¹⁰[¹²⁵I-Tyr⁵,DLeu⁶,NMeLeu⁷,Pro⁹-NEt]-GnRH to the cloned human GnRH receptor stably expressed in HEK293 cells.¹¹⁹

^b The ability to inhibit the binding of ¹²⁵I-[His⁵, D-Tyr⁶]GnRH to the cloned human GnRH receptor stably expressed in RBL cells.¹⁴²

in which the pyridyl side chain of the R-isomer is oriented towards the aspartic acid in TM VII (Asp302) and the ring itself towards the phenylalanine in TM VII (Phe309) of the receptor. When the rotational freedom of the 5-(3-methoxy)phenyl group was restrained with the introduction of a 2-fluoro substituent a significant increase in binding affinity was obtained (**37**; K_i = 1.1 nM).¹³³ Furthermore, **37** was a highly potent functional antagonist with

an IC_{50} -value of 0.5 nM. The metabolic stability of these compounds was still poor and therefore branched primary amines were introduced at the N-3 side chain.¹³⁴ Compound **38** had the highest affinity ($K_i = 0.6$ nM) reported thus far and showed increased metabolic stability in an *in vitro* liver microsomes assay. However, the half-life was still too short for acceptable pharmacokinetics. In another attempt to improve pharmacokinetic and metabolic properties, the 5-phenylgroup was substituted with a thiazole or thienyl ring.¹³⁵ In addition, one of the nitrogen-carbon bonds from the N-3 substituent was removed, which was known to be easily cleaved and oxidized by liver enzymes, by replacing it with (*R*)-phenylglycinol. With these modifications the potency was maintained (see **39**) and the intrinsic clearance drastically decreased. Subsequently, the N-1 substituent was optimized. Although the substituent at position 5 was replaced by a (fluoro) substituted phenyl again, the substitution of a 2,6-difluorobenzyl for a 2-chloro-6-fluorobenzyl group resulted in an increased binding affinity (**40**; $K_i = 0.7$ nM).¹³⁶ In this study it was indicated once more that the electron-deficient N-1 benzyl group possibly interacts with a tyrosine residue in TM VI (Tyr283 or Tyr284). These two amino acids have also been implicated in binding of the endogenous ligand GnRH as the mutated receptor could not be activated by GnRH.¹⁴³ Combination of the optimized substituents resulted in the highly potent ($K_i = 0.56$ nM) and orally available compound **41**, which is also referred to as NBI 42902.¹³⁷ Compound **41** was pharmacologically characterized in a second study, where it was shown to provide novel opportunities to control the hypothalamic-pituitary-gonadal axis.¹⁴⁴ Similar to the compounds described above, the affinity of compound **41** was very low for the rat GnRH receptor ($IC_{50} < 10$ μ M). *In vivo* studies were, therefore, performed in monkeys, where the affinity for the GnRH receptor was only 6-fold lower ($K_i = 3.5$ nM).^{137,144} Oral administration of 100 mg/kg **41** completely suppressed LH blood levels over 24 h in castrated male macaques. A final optimization study was performed to improve the manufacturing reproducibility, as **41** showed atropisomerism (rotational stereoisomerism) of the 5-aryl group due to the 6-methyl.¹³⁸ Therefore another study was conducted to modify 1- and 5-substituents of the desmethyl analogs (**42** and **44**), while maintaining a high potency. The resulting compound **42** had a high receptor affinity ($K_i = 0.45$ nM) and did not possess stereo isomeric properties. Molecular modeling indicated that the 5-phenyl ring could interact with a tyrosine residue in TM V (Tyr211) through π - π stacking. In addition, an asparagine residue in TM V (Asn212) could form a hydrogen-bond with the 3-methoxy group on the phenyl ring. Both residues have also been implicated in GnRH binding.¹⁴⁵ Further studies were performed to obtain

thermally stable single isomers.¹³⁹ Previously, pyrimidin-2,4-dione derivatives with an *N*-alkyl aminoalkyl side chain (e.g. **38**) were reported as potent GnRH receptor antagonists.¹³⁴ Therefore, compound **43** was synthesized and its isomers separated.¹³⁹ The *R*-isomer of **43** was stable at room temperature, either dry or in DMSO solution, and 15-fold more potent than the *S*-isomer. X-ray crystallography of another derivative confirmed that the *R*-isomer is preferred for receptor interactions. Although compounds **41-43** had high affinity and potency for the human GnRH receptor, their metabolic profile was poor. Therefore, additional efforts were made to decrease CYP3A4 metabolism of these compounds. A close analog of **42** (2-F instead of 2-Cl) inhibited this metabolic liver enzyme with an IC₅₀ of 0.1 μM.¹⁴⁰ Neurocrine Biosciences reasoned that introduction of polar groups would be tolerated, due to the presence of several basic residues in the ligand binding pocket of the human GnRH receptor.¹⁴⁶ The attachment of an acidic group to either the amine (**44**)¹⁴⁰ or to the 3-methoxyphenyl group (**45**)¹⁴¹ was further investigated. The location of the acidic group proved important. Both compound **44** and **45** had over 100-fold decreased CYP3A4 potency, with an IC₅₀ value of 36 μM and 13 μM, respectively. The pharmacokinetic profiles were investigated and it was shown that bioavailability was low in rats. However, reduction of the amount of hydrogen bond donors by methylation of the basic amine group increased oral bioavailability (**44**: %F = 2; **45**: %F = 13). Notably, oral bioavailability of **44** was 10-fold higher in monkeys than in rats.¹⁴⁰

2.2.9 Benzimidazole Derivatives

In 2005, Bayer reported benzimidazole derivatives as a new class of non-peptidic GnRH receptor antagonists (Table 2.9).¹⁴⁷ A screen of approximately one million compounds resulted in a first hit, compound **46**. This compound had low micromolar potency at both rat and human GnRH receptors (hIC₅₀ = 3.4 μM). Electron withdrawing *para*-substituents on the sulfonamide phenyl ring improved the potency of this compound. In addition, introduction of a flexible side chain with a basic moiety, as seen in most GnRH receptor antagonists, resulted in compound **47** (hIC₅₀ = 120 nM). Notably, the potency at the rat receptor was retained. For further studies, *in vivo* low nanomolar antagonists were a prerequisite. Therefore, compound **47** was further optimized to compound **48** that had an almost 30-fold increased potency and showed no selectivity against the rat GnRH receptor. It was shown that two hydrogen-bond

Table 2.9 Binding affinities of benzimidazole derivatives (**46-50**) at the human GnRH receptor.

Compound	R ¹	R ²	R ³	R ⁴	IC ₅₀ (nM) ^a	Ref
46			H		3400	147
47			H		120	147
48			H		4.2	148
49	H	CF ₃		H	1540 ^b	149
50	H			H	1.7 ± 0.65 ^b	149

^a IC₅₀ in a CHO-hGnRHR-Ca²⁺ assay.¹⁴⁷^b The ability to inhibit the binding of [¹²⁵I-(D-trp⁶)LHRH to the cloned human GnRH receptor in recombinant cells.¹⁴⁹

donors at the 2-position increased the potency, for example by introducing an urea-linker. In addition, the spatial orientation of the bulky aliphatic group attached to the linker was of great importance.¹⁴⁸ The findings were in correspondence with Sasaki and coworkers for the thieno[2,3-d]pyrimidin-2,4-dione derivatives.¹²⁴ Recently, Wyeth published on a screen of a library of approximately 2200 compounds that was rich in GPCR ligands.¹⁴⁹ This resulted in two lead compounds (e.g. **49**) that were also potent in binding the human serotonin (5-HT-1) receptor subtypes. Compound **49** had low micromolar affinity for the human GnRH receptor (IC₅₀ = 1.54 μM) and a much higher affinity at the 5-HT-1 receptor subtypes (e.g. IC₅₀ = 0.55 nM at 5-HT_{1D}). Therefore, efforts were made to optimize binding potency for the human and rat GnRH receptors, as well as selectivity over the 5-HT-1 receptor subtypes. Optimization of the piperazine linker and one of the benzimidazole groups resulted in compound **50** that had a

900-fold increased affinity for the human GnRH receptor, a greatly enhanced rat receptor affinity and selectivity over other drug-targets.¹⁴⁹ Compound **50** had a high oral bioavailability in rats (%F = 74) and oral administration of 30 mg/kg completely suppressed serum LH levels over 6 h in castrated rats.

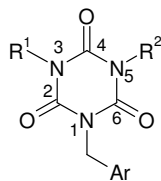
2.2.10 1,3,5-Triazine-2,4,6-trione Derivatives

In 2005 Neurocrine Biosciences introduced another monocyclic class of non-peptidic GnRH receptor antagonists, the 1,3,5-triazine-2,4,6-trione derivatives (Table 2.10).¹⁵⁰ Replacement of the 6-methyl group of **34** with a carbonyl moiety resulted in a compound with micromolar affinity (**51**; $K_i = 4.2 \mu\text{M}$). Introduction of (*R*)-phenylglycinol at the 3-position yielded compound **52**, which exhibited a substantially higher affinity, $K_i = 37 \text{ nM}$. This proved that the 6-methyl group is not essential for human GnRH receptor binding. The advantage of this novel scaffold was that the synthetic route was a convenient two-step, one-pot cyclization procedure with high yields.¹⁵⁰ Receptor affinity of **52** was 5-fold improved by the introduction of a 2-fluoro substituent at the 5-phenyl group.¹⁵¹ Substitution of the 2-fluoro by a bromo substituent at the N-1 benzyl group resulted in a 20-fold overall increase in potency (**53**; $K_i = 2 \text{ nM}$). Compound **53** showed functional antagonism in an inositol phosphate accumulation assay ($\text{IC}_{50} = 33 \text{ nM}$) and its metabolic stability was comparable to that of compound **39**. These compounds also showed species selectivity with a 10- and 1000-fold lower binding affinity at the monkey and rat GnRH receptor for **53**, respectively.

2.2.11 Various

Neurocrine Biosciences has recently published four other classes of non-peptidic compounds as human GnRH receptor antagonists (Figure 2.1). The scaffolds of compounds **54-56** are derived from the pyrimidin-2,4-dione class (**34-45**).

In case of **54** a thiazole ring was introduced, resulting in thiazolino[3,2-*c*]pyrimidin-5,7-dione derivatives. Also different compounds with an oxazole instead of a thiazole ring were tested. The sulfur derivatives were equipotent to the oxo-derivatives. A bicyclic system rigidifies the N-1 benzyl substituent and apparently yielded more potent ligands, such as compound **54**, which showed high receptor affinity ($K_i = 4.5 \text{ nM}$).¹⁵² Similar to 1,3,5-triazine-2,4,6-trione

Table 2.10 Binding affinities of 1,3,5-triazine-2,4,6-trione derivatives (**51-53**) at the human GnRH receptor.

Compound	R ¹	R ²	Ar	K _i (nM) ^a	Ref
51				4200	150
52				37	150
53				2	151

^a The ability to inhibit the binding of des-Gly¹⁰[¹²⁵I-Tyr⁵,DLeu⁶,NMeLeu⁷,Pro⁹-NEt]-GnRH to the cloned human GnRH receptor stably expressed in HEK293 cells.¹¹⁹

derivatives (**51-53**), the synthesis of this class of compounds was straightforward and proceeded with high yields. Pontillo *et al.* showed that the presence of a 6-carbonyl moiety was more important than the 2-carbonyl moiety of the triazinedione derivatives.¹⁵³ Removal of the 6-carbonyl resulted in a reduction of receptor affinity that could be recovered by the introduction of a 2-chloro substituent at the 3-methoxyphenyl ring and replacement of a fluoro by a trifluoromethyl group at the benzyl ring (**55**; K_i = 2.3 nM). Although **55** had the same substituents as **42**, the latter had a 5-fold higher affinity, which indicates that the third nitrogen in the ring is unfavorable. Another scaffold with similar substituents was introduced by Lanier *et al.*, the tetrahydropyrido[4,3-d]pyrimidin-2,4-dione derivatives.¹⁵⁴ Compounds with low nanomolar binding affinity emerged in this class (**56**; K_i = 5 nM).

Chen and coworkers studied a series of tetrahydropyrrolo[3,2-*c*]pyridines as GnRH receptor ligands.¹⁵⁵ These compounds were based on the indole derivatives (**10-19**) that were described previously.¹¹⁵ The indole-based ligands were less species-selective, and could therefore be tested on the more convenient and cost effective *in vivo* castrated rat model.

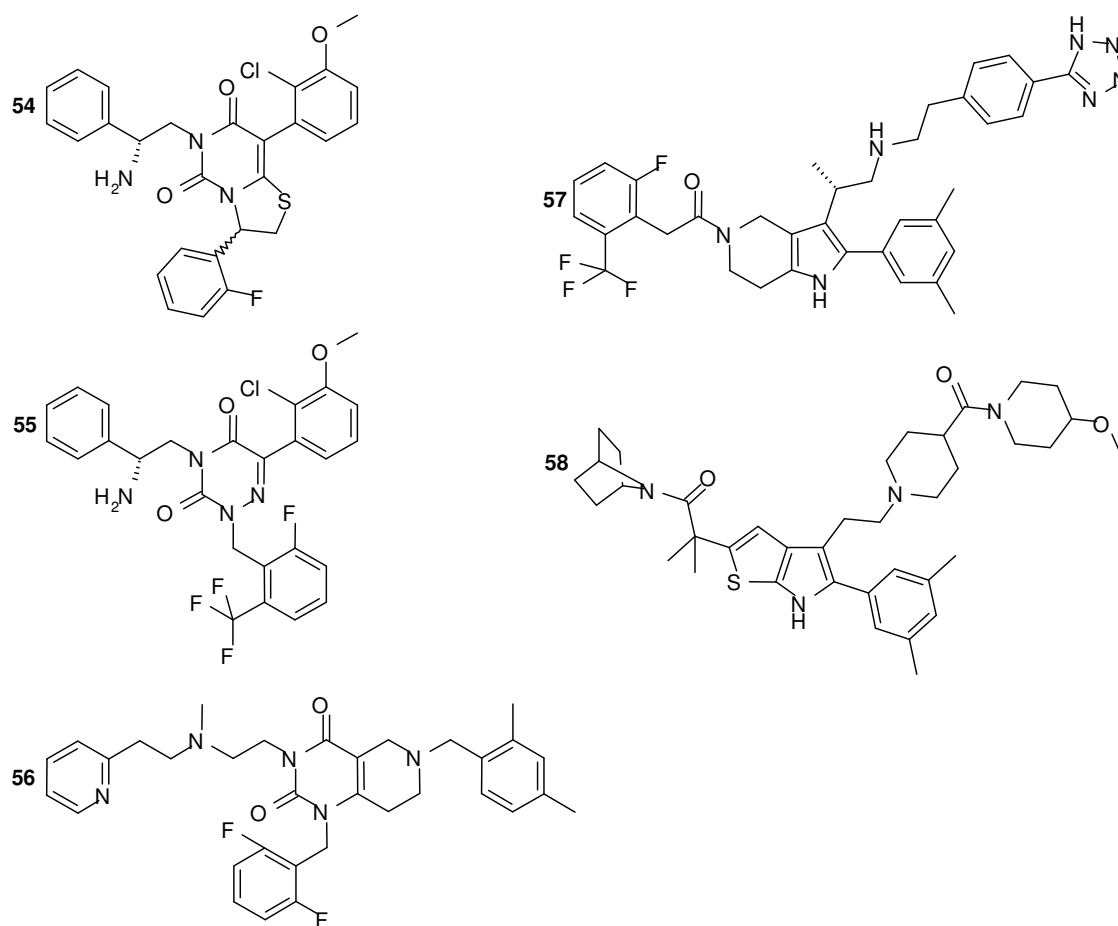


Figure 2.1 Chemical structures of the most potent analogues of thiazolino[3,2-c]pyrimidin-5,7-dione (**54**)¹⁵², triazinedione (**55**)¹⁵³, tetrahydropyrido[4,3-d]pyrimidin-2,4-dione (**56**)¹⁵⁴, pyrrolo[3,2-c]pyridine (**57**)¹⁵⁵ and thieno[2,3-b]pyrrole (**58**)¹⁵⁶ derivatives as antagonists at the human GnRH receptor.

Compound **57** had a high rat and human GnRH receptor affinity ($\text{hIC}_{50} = 1.5 \text{ nM}$; $\text{rK}_i = 0.2 \text{ nM}$) and intraperitoneal administration of 15 mg/kg suppressed LH blood levels for up to 8 h in castrated rats.

Very recently, AstraZeneca reported a new class of human GnRH receptor antagonists, thieno[2,3-*b*]pyrrole derivatives (e.g. **58**).¹⁵⁶ This study was particularly directed towards potent orally bioavailable non-peptidic antagonists, based on previously described orally active compounds, thieno[2,3-*d*]pyrimidin-2,4-dione,⁹⁸ indole¹¹⁵ and pyrimidin-2,4-dione derivatives.¹³⁷ Although the affinity of **58** was lower ($\text{IC}_{50} = 184 \text{ nM}$) than reported for the other classes, it had a similar affinity value for the rat GnRH receptor ($\text{IC}_{50} = 76 \text{ nM}$). The

pharmacokinetic profile of **58** was therefore obtained, which showed that the plasma clearance was slower ($CL = 0.1 \text{ mL/min/kg}$) and this translated into better oral bioavailability ($\%F = 35$) than that of previous ligands. In addition, the CYP3A4 activity was much lower for these compounds.¹⁵⁶

2.2.12 Patented Ligands

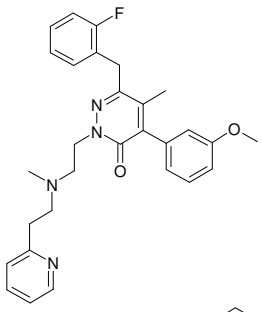
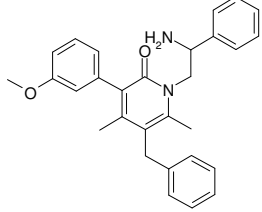
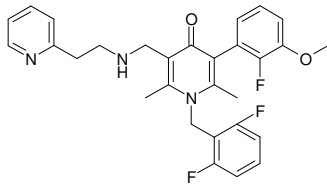
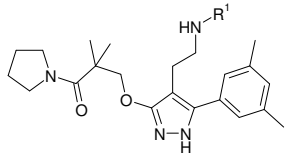
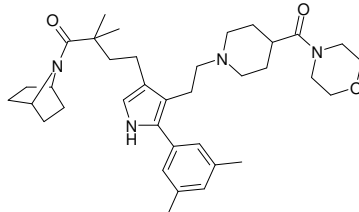
Patent literature revealed several other non-peptidic compounds classes that have been described as human GnRH receptor antagonists. In this review, these were divided into mono-, bi- and tricyclic antagonists, as shown in Table 2.11-2.13, respectively.

Neurocrine Biosciences reported three monocyclic compound classes that showed antagonistic activity at the human GnRH receptor (Table 2.11), namely 3-pyrazinone,¹⁵⁷ pyrid-2-one¹⁵⁷ and pyrid-4-one derivatives.¹⁵⁸ AstraZeneca similarly patented pyrazole^{159,160} and pyrrole derivatives,¹⁶¹ while Paradigm Therapeutics showed that oxazole- and thiazole-4-carboxamide derivatives¹⁶² are potent GnRH antagonists. Based on their substituents the latter two could be classified with the furamide analogues described above (Table 2.7).

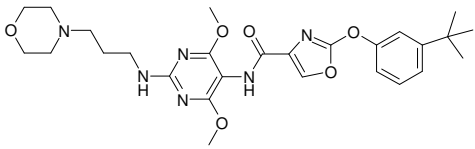
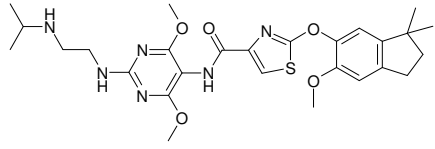
Table 2.12 summarizes all non-peptidic bicyclic GnRH receptor antagonist classes that have been patented. A wide variety of scaffolds have been contributed by several companies. Tetrahydroisoquinoline derivatives^{163,164} were described by Abbott, while 1,3-dihydrobenzimidazole derivatives were patented by Yamanouchi¹⁶⁵ and Astellas^{166,167} as potent GnRH receptor antagonists. GSK have patented bicyclic pyrrolidines^{168,169} and Ortho-McNeill¹⁷⁰ and Schering^{171,172} both described quinoline derivatives. AstraZeneca^{173,174} and Wyeth¹⁷⁵ have both patented imidazo[1,2-*a*]pyridine-based ligands. In addition, Wyeth also described imidazo[4,5-*c*]pyridine^{176,177} and triazolo[1,5-*a*]pyridine¹⁷⁵ derivatives as GnRH receptor antagonists. SCRAS¹⁷⁸ has patented benzimidazoles, where Wyeth has substituted an imidazole-nitrogen for sulfur or oxygen, resulting in two other compound classes, namely benzoxazoles and benzothiazoles.¹⁷⁹ Following thieno[2,3-*b*]pyrimidin-2,4-dione derivatives (Table 2.6), Takeda also described quinazoline-2,4-dione-based¹⁸⁰ and thieno[3,2-*d*]pyrimidin-2,4-dione¹⁸¹ antagonists. Recently, a slightly modified class of GnRH receptor antagonists was described by Kissei Pharmaceutical, thieno[3,4-*d*]pyrimidin-2,4-dione derivatives.¹⁸²

Tricyclic GnRH receptor antagonists have been made by GSK and Zentaris, which patented a series of tricyclic pyrrolidine^{168,169} and tetrahydrocarbazole derivatives,^{183,184} respectively (Table 2.13).

Table 2.11 Binding affinities of patented monocyclic antagonists at the human GnRH receptor.

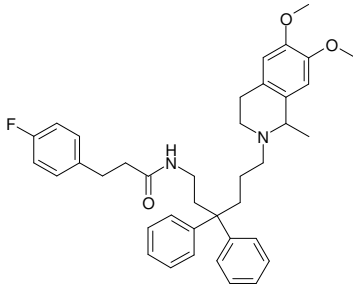
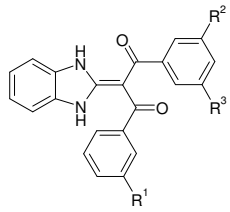
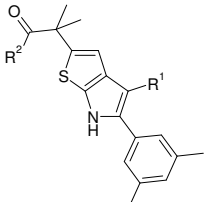
Class	Company	Structure	Potency	Ref
3-Pyrazinone	Neurocrine		n.p.	157
Pyrid-2-one	Neurocrine		n.p.	157
Pyrid-4-one	Neurocrine		$K_i < 100 \mu\text{M}$	158
Pyrazole	AstraZeneca		$\text{IC}_{50} = 1\text{-}5,000 \text{ nM}$	159,160
Pyrrole*	AstraZeneca		$\text{IC}_{50} = 1\text{-}5,000 \text{ nM}$	161

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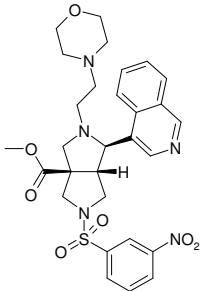
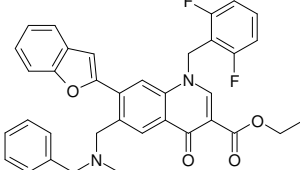
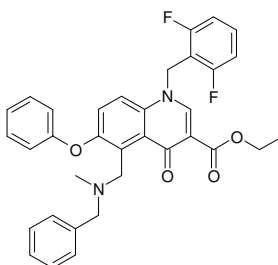
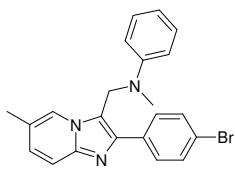
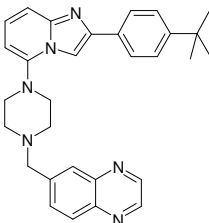
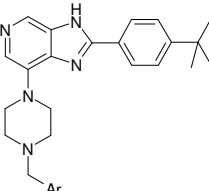
Oxazole-4-carboxamide	Paradigm		n.p.	162
Thiazole-4-carboxamide	Paradigm		IC ₅₀ = 4 nM	162

* The depicted chemical structure is an example from the patent. n.p. = not published

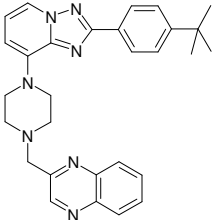
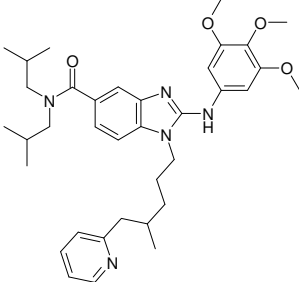
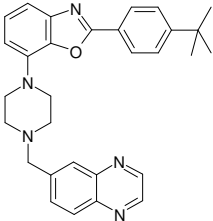
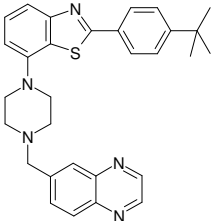
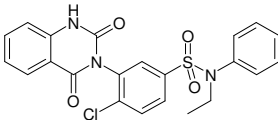
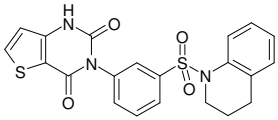
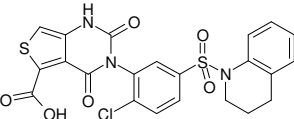
Table 2.12 Binding affinities of patented bicyclic antagonists at the human GnRH receptor.

Class	Company	Structure	Potency	Ref
Tetrahydro-isoquinoline	Abbott		K _i = 1.6 nM	163,164
1,3-Dihydrobenzimidazoles	Yamanouchi		IC ₅₀ = 0.1-1 nM	165
1,3-Dihydrobenzimidazoles	Astellas		IC ₅₀ = 0.058-0.24 nM	166,167

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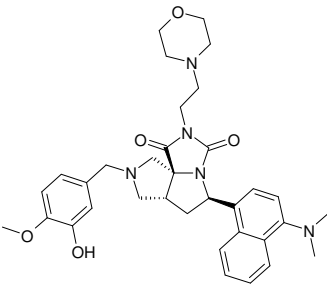
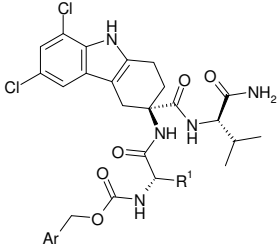
Bicyclic pyrrolidines*	GSK		IC ₅₀ = 35–1,500 nM	168,169
Quinolines*	Ortho-McNeill		IC ₅₀ = 1–32 μM	170
Quinolines	Schering		n.p.	171,172
Imidazo[1,2- a]pyridines*	AstraZeneca		IC ₅₀ = 1-30,000 nM	173,174
Imidazo[1,2- a]pyridines*	Wyeth		IC ₅₀ = 1–1,000 nM	175
Imidazo[4,5- c]pyridines	Wyeth		IC ₅₀ < 10 μM	176,177

(Continued)

Triazolo[1,5-a]pyridines*	Wyeth		IC ₅₀ = 1–1,000 nM	175
Benzimidazoles	SCRAS		n.p.	178
Benzoxazoles*	Wyeth		IC ₅₀ = 25–100,000 nM	179
Benzothiazoles*	Wyeth		IC ₅₀ = 25–100,000 nM	179
Quinazoline-2,4-diones	Takeda		IC ₅₀ < 10 μM	180
Thieno[3,2-d]pyrimid-2,4-diones	Takeda		> 86% inhibition	181
Thieno[3,4-d]pyrimid-2,4-diones	Kissei		IC ₅₀ = 2 nM	182

* The depicted chemical structure is an example from the patent. n.p. = not published

Table 2.13 Binding affinities of patented tricyclic antagonists at the human GnRH receptor.

Class	Company	Structure	Potency	Ref
Tricyclic pyrrolidines*	GSK		IC ₅₀ = 35–1,500 nM	168,169
1,2,3,4-Tetrahydro-carbazoles	Zentaris		IC ₅₀ = 0.1-15 nM	183,184

* The depicted chemical structure is an example from the patent.

2.2.13 Overview GnRH receptor antagonists

A comparison of the GnRH receptor antagonists presented here shows that within each structural class high-affinity and bioavailable compounds have been designed. This indicates that the substituents rather than the scaffolds are the key determinants, which is not surprising as similar substituents often prove optimal for each class. For four compound classes possible interaction sites were predicted and/or visualized by molecular modeling using the architecture of rhodopsin as a template for the GnRH receptor structure. It seems that the binding sites of non-peptidic GnRH receptor antagonists overlap with each other, but also (in part) with the orthosteric binding site of GnRH itself. Figure 2.2 represents a summary of the optimal pharmacophore extracted from the data described above, including the possible interaction sites in the receptor pocket. 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, species differences and the interaction with cytochrome P450 activity of these compounds add to this uncertainty.

Although a wide range of GnRH receptor antagonists has been described, non-peptidic agonists have not thus far. The only agonistic 'starting point' currently available is the

endogenous peptide ligand and some of its analogues. As the binding site of non-peptidic ligands seems to overlap with GnRH, a pharmacophore model of the peptide may give further clues. Moreover high-throughput screening of large compound libraries may yield lead compounds too, as there is no upfront reason why this successful approach would not work in this specific situation.

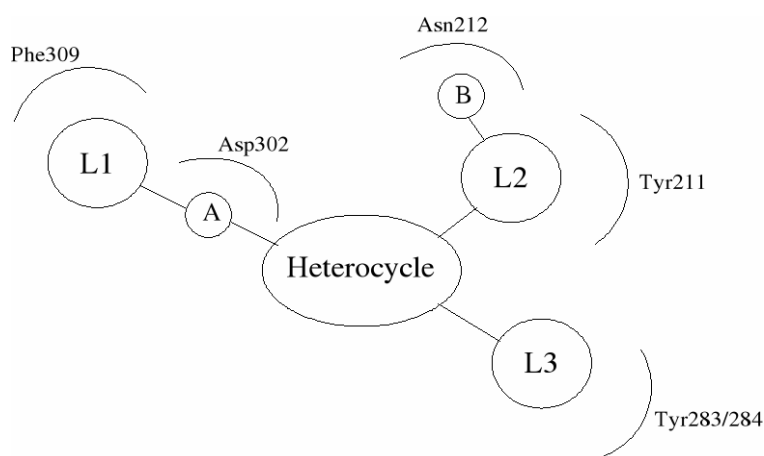


Figure 2.2 Schematic diagram that summarizes the non-peptidic GnRH receptor antagonist pharmacophore obtained from literature survey, including the possible interactions with the receptor pocket. A and B represent a basic nitrogen (H-bond donor) and an ester (H-bond acceptor), respectively. L₁₋₃ represent (heterocyclic) aromatic rings.

2.3 LH RECEPTOR AGONISTS

The LH receptor recognizes two endogenous ligands, namely LH and human chorionic gonadotropin (hCG). These gonadotropins are closely related in structure and consist of a conserved α -subunit and a hormone specific β -subunit.¹⁸⁵ It has been shown that these hormones bind to the large N-terminal domain of the LH receptor.¹⁸⁶ Infertility treatment has traditionally been based on the administration of urine-derived gonadotropins. More recently recombinant hormones have also been developed with a similar activity profile.¹⁸⁷ Deglycosylated hCG has been described as an antagonist of the LH receptor.¹⁸⁸ Recently, another hormonal antagonist was identified, which was generated by fusing two hCG β -subunits.¹⁸⁹ Antagonists of the LH receptor could lead to novel contraceptive treatments. However, protein hormones need to be administered by intramuscular or subcutaneous injection. Research is therefore aimed at the development of non-peptide, orally active gonadotropins. For the LH receptor, the first low molecular weight agonists have now been identified by Organon Biosciences (currently Schering-Plough) and Merck Serono, respectively, which will be described here.

2.3.1 Thieno[2,3-b]pyri(mi)dine Derivatives

In 2002, Van Straten and coworkers revealed the first two classes of orally active non-peptidic LH receptor agonists.¹⁹⁰ Both thienopyridines and -pyrimidines were able to activate the receptor (Table 2.14). Substitution of the amide group at position 6 with a branched alkyl group resulted in a dramatic increase in potency. The potency was further enhanced by the introduction of a 3-methoxyphenyl moiety at position 4. The most potent thienopyridine and -pyrimidine compounds were **59** and **60**, with EC₅₀-values of 160 nM and 20 nM, respectively. Although the two compound classes showed similar SAR, the overall potency of thienopyrimidines was higher, which indicates the importance of the additional nitrogen atom in the ring. Compound **60** showed *in vivo* efficacy, namely oral administration of 50 mg/kg resulted in 40% ovulating FSH-primed mice.¹⁹⁰ Notably, these ligands were not able to displace ¹²⁵I-LH binding.²¹ This indicates that the binding site of low molecular weight ligands is located in the 7-TM domain. Therefore these compounds can be considered as allosteric agonists. Jäschke and coworkers further examined the binding pocket of **60** by molecular modeling.¹⁹¹ Compound **60** was shown to be a partial agonist at the thyroid-stimulating hormone (TSH) receptor (EC₅₀ = 7700 nM). Based on the predicted binding

pocket different mutant receptors were constructed, where residues in the binding pocket of the LH receptor were introduced at corresponding positions in the TSH receptor. As expected, **60** was a full agonist with increased potency in some of these constructs. As a

Table 2.14 Potencies of thieno[2,3-b]pyri(mi)dines (**59-61**) at the human LH receptor.

Compound	X	R ₁	R ₂	R ₃	EC ₅₀ (nM) ^a	Ref
59	CH				160 ± 20	21
60	N				20 ± 5	21
61	N				1.7 ± 0.2	Chapter 4

^a EC₅₀ in the CHO-hLHR-luciferase assay.^{192,193}

* Position of the tritium substitution in [³H]Org 43553 (*Chapter 4*).

follow-up, Moore and colleagues examined a key residue for receptor-ligand interaction, Glu3.37, which is conserved in LH and TSH receptors.¹⁹⁴ Different analogues of **60** were prepared, which were aimed at increasing the selectivity toward the LH receptor. Compounds with increased steric bulk at the amide portion of **60** showed enhanced selectivity, while maintaining their potency and efficacy at the LH receptor. This corresponded to the finding that the binding pocket of the TSH receptor is smaller than the pocket of the LH receptor.^{191,194} Interestingly, compound **60** was shown to act as a chaperone molecule by increasing the membrane expression of wild-type FSH receptors, while it did not have intrinsic efficacy at this receptor.¹⁹⁵ Recently, a few other thienopyrimidines were reported as described in *Chapter 4*. The most potent compound (**61**; EC₅₀ = 1.7 nM) was labeled with tritium and characterized for its receptor binding. It was shown that there was a high correlation between the affinity and potency of low molecular weight LH receptor agonists. Notably, a high concentration of the endogenous hormone LH only modestly displaced the

radioligand, confirming an allosteric binding site for the thienopyridimines. Furthermore, Organon Biosciences (currently Schering-Plough) recently published some additional data on compound **61** (i.e. Org 43553).³⁶ Chimeric TSH/LH receptors were prepared and it was confirmed that **61** activates the LH receptor through the 7-TM domain. In addition, it was shown that this low molecular weight agonist is signaling-selective, as it only stimulates the cAMP pathway and not the phospholipase C pathway, unlike LH.

2.3.2 Pyrazole Derivatives

Recently, Merck Serono reported a new class of low molecular weight LH receptor agonists (Figure 2.3).¹⁹⁶ Pyrazole derivatives were identified from an in-house library screen. Subsequently, a new pyrazole library was synthesized to examine the SAR of these new ligands. The most potent agonist (**62**) had an EC₅₀ value of 20 nM in an *in vitro* cAMP assay and was able to partially activate the receptor ($E_{\max}$ = 70% of maximal LH response). Notably, compound **62** did not compete for ¹²⁵I-hCG binding to the LH receptor,¹⁹⁶ as was also described for the thienopyrimidine derivatives.²¹ In an *in vivo* testosterone-induction model in rats, **62** was able to dose-dependently increase testosterone levels after subcutaneous injection.

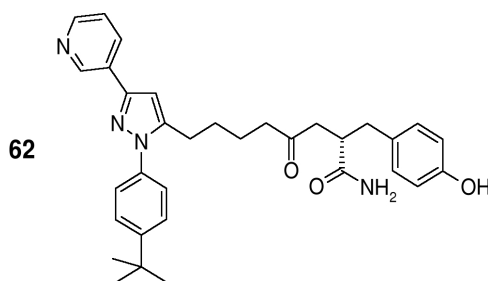


Figure 2.3 Chemical structure of the most potent pyrazole (**62**) derivative as an agonist at the human LH receptor.¹⁹⁶

2.3.3 Patented ligands

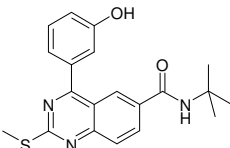
Organon Biosciences (currently Schering-Plough) patented another class of LH receptor agonists, the quinazoline-6-carboxamides (Table 2.15).¹⁹⁷ The apparent similarity with the

thienopyrimidine-based ligand is due to the presence of a pyrimidine ring and the substituted amide group.

2.3.4 Overview LH receptor agonists

Research of non-peptidic LH receptor agonists has started only recently. With four reported compound classes, there is still a large field to be explored. In addition, non-peptidic antagonists would be of great interest, as they could possibly be used as non-hormonal contraceptives. Hopefully, the first thienopyrimidine radioligand, [^3H]Org 43553 (**56**), will aid in the discovery of other allosteric ligands for the LH receptor (*Chapter 4*).

Table 2.15 Potency of a patented agonist at the human LH receptor

Class	Company	Structure	Potency	Ref
Quinazoline-6-carboxamides*	N.V. Organon		$\text{IC}_{50} =$ 10-100 nM	197

* The depicted chemical structure is an example from the patent.

2.4 FSH RECEPTOR LIGANDS

FSH is a glycoprotein hormone that consists of an α - and a β -subunit, like LH and hCG.¹⁸ In 2005 the crystal structure of FSH bound to the large extracellular domain of the receptor was reported.¹⁹⁸ Hence, it was shown that FSH could form a stable complex with the N-terminal domain of the receptor in the absence of the 7-TM domain. Urinary and recombinant FSH is also used in the treatment of infertility.¹⁸⁷ Recombinant gonadotropins need to be administered daily and therefore effort was made to develop gonadotropins with a longer half-life. Long-acting FSH may be produced by fusing the C-terminal extension of the β -subunit of hCG to the β -subunit of FSH or by additional glycosylation of FSH.^{77,78} Sustained-release formulations of FSH are also in development that could result in less frequent dosing.¹⁹⁹ In contrast to the LH receptor, both low molecular weight agonists and antagonists have been identified for the FSH receptor. FSH receptor antagonists may be used as non-hormonal contraceptives. The different compound classes will be discussed separately, including patented ligands.

Table 2.16 Potencies of biphenyl derivatives (**63-65**) at the human FSH receptor.

Compound	R ¹	R ²	R ³	EC ₅₀ (nM) ^a	Ref
63			H	3900	200
64		H		1700	200
65		H		1.2	201

^a EC₅₀ in the CHO-hFSHR-luciferase assay.^{192,193}

2.4.1 Biphenyl-based agonists

A high-throughput screen at Pharmacopeia of two million compounds yielded the first non-peptidic FSH receptor agonist.²⁰⁰ Compound **63** consists of a biphenyl scaffold (Table 2.16) with low micromolar potency ($EC_{50} = 3.9 \mu M$). A combinatorial chemistry approach was used to synthesize a wide variety of compounds. Different substituents were placed on the R^1 - R^3 groups, where the amide function in R^1 was retained. Through this approach over 30,000 distinct compounds were obtained of which 72 were active ($EC_{50} < 10 \mu M$), for example **64**.²⁰⁰ The actives were generally the R^3 -*meta*-substituted compounds. Guo *et al.* further optimized the biphenyl compounds into potent FSH receptor agonists via parallel synthesis.²⁰¹ Removal of the R^1 amide function proved beneficial and an octyl substituent on the N-4 position appeared optimal for high potency. In addition, introduction of a second (*S*)-methyl group at the diketopiperazine ring resulted in a dramatic increase in potency. The most potent compound in this series was **65** ($EC_{50} = 1.2 \text{ nM}$; luciferase-reporter-gene assay), which displayed similar (partial) agonistic activity in a cAMP accumulation assay.

2.4.2 Thiazolidin-4-one-based agonists

A second low molecular weight FSH receptor agonist class was described by Affymax.²⁰² From a screen, a thiazolidinone derivative (**66**) was discovered, which had only moderate potency ($EC_{50} = 5 \mu M$) in a luciferase reporter gene assay (Table 2.17). A similar combinatorial chemistry approach was used as described for the biphenyl-based ligands, which resulted in a library of more than 42,000 compounds with a thiazolidinone scaffold. A modification of the R^3 substituent, addition of a phenyl ring, resulted in a more than 150-fold increase in potency (**67**; $EC_{50} = 32 \text{ nM}$). This thiazolidinone library described by Maclean and coworkers also resulted in several hits against other biological targets (data not shown).²⁰² Another (encoded) library of thiazolidinone analogs (e.g. **66**) was prepared by Yanofsky and coworkers, consisting of more than 40,000 compounds.⁵⁴ The library was screened in an FSH receptor reporter gene assay, as none of the compounds was able to displace [^{125}I]FSH. Further optimization by parallel synthesis resulted in the identification of a highly potent FSH receptor agonist (**68**; $EC_{50} = 2 \text{ nM}$).⁵⁴ Compound **68** fully activated the native FSH receptor in rat ovarian granulosa cells with an EC_{50} value of 10.5 nM. In addition, chimeric human FSH/TSH receptors were constructed. It appeared that the thiazolidinone

Table 2.17 Potencies of thiazolidinone derivatives (**66-70**) at the human FSH receptor.

Compound	R ¹	R ²	R ³	X	EC ₅₀ (nM) ^a	Ref
66				H	5,000	202
67				H	32	202
68				H	2	54
69				CH ₃	51 ± 42	203
70				H	1,700 ^b	204

^a EC₅₀ in the CHO-hFSHR-luciferase assay.²⁰⁵^b IC₅₀ in a phFSH-induced rat aromatase assay.²⁰⁶

compounds activated the FSH receptor through an allosteric site located in the region formed by TM I/II and the first extracellular loop. Wyeth continued the optimization of the thiazolidinone compounds to facilitate the synthesis of the active trans isomer.²⁰³ The addition of a 5-methyl substituent locked both scaffold side chains into the trans orientation. FSH receptor activity of compound **69** was similar to the 5-hydrogen analog **67**. Recently, four thiazolidinone compounds were pharmacologically characterized by Arey and coworkers.²⁰⁴ Among these four compounds were **68** and a demethylated analog of **69** (X = H), which were shown to be a full and partial agonist in a cAMP assay, respectively. Another derivative (**70**) was shown to be an antagonist in this assay. Together, these data demonstrated that thiazolidinone compounds can have different pharmacological effects,

ranging from full agonists to partial agonists and antagonists. In addition, it was shown that compound **70** was capable of inducing association of the human FSH receptor to the G_i , next to the G_s signaling pathway, unlike the endogenous hormone FSH.²⁰⁴ These LMW ligands are able to activate additional G protein-signaling pathways, providing another way of modulation of the action of glycoprotein hormones.

2.4.3 Various Agonists

Recently, Wyeth described an analog of **69** as a potent FSH receptor agonist, where the thiazolidinone scaffold was replaced by a γ -lactam ring (Figure 2.4).²⁰⁷ The γ -lactam derivative **71** showed similar FSH receptor potency ($EC_{50} = 24.8 \pm 11.8$ nM) and selectivity over the TSH receptor.

Van Straten *et al.* identified compound **72** as an FSH receptor agonist through a high-throughput screen in a luciferase reporter gene assay (Figure 2.4).²⁰⁸ Compound **72** was a selective agonist with an EC_{50} of 4.4 μ M. In an optimization study, the pharmacological profile of analogues of **72** shifted from functional agonists to antagonists, which was further exploited and will be discussed in one of the next paragraphs.

2.4.4 Patented Agonists

Several companies have patented FSH receptor agonists containing different scaffolds (Table 2.18). ARS Holding has patented carbazole derivatives²⁰⁹ that show low nanomolar potency on the receptor, while Serono has patented pyrazole-based agonists.²¹⁰ Besides tetrahydroquinoline-based agonists, Organon (currently Schering-Plough) has described two additional low molecular weight compound classes, namely thieno[2,3-*b*]pyrimidine²¹¹ and hexahydroquinoline derivatives.²¹²⁻²¹⁴ Notably, the former class was also identified as LH receptor agonists by this company.²¹ Lastly, Arena patented isoxazolythiazole-based compounds as FSH receptor agonists.²¹⁵

2.4.5 Various Antagonists

More than a decade ago, it was shown that suramin inhibited FSH binding to its receptor.²¹⁶ However, suramin *per se* is a non-selective agent that also interacts with other

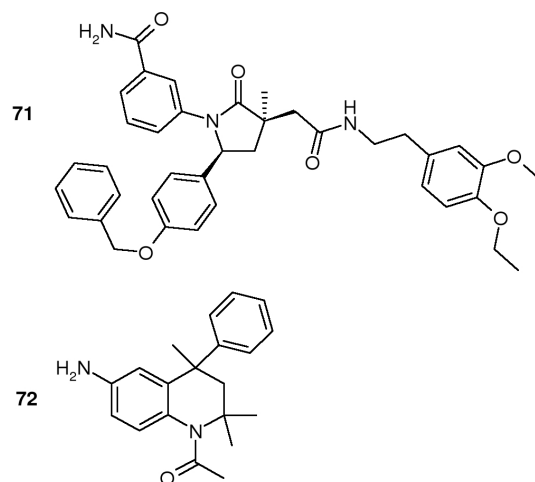


Figure 2.4 Chemical structure of the most potent γ -lactam (**71**)²⁰⁷ and tetrahydroquinoline (**72**)²⁰⁸ derivative as an agonist at the human FSH receptor.

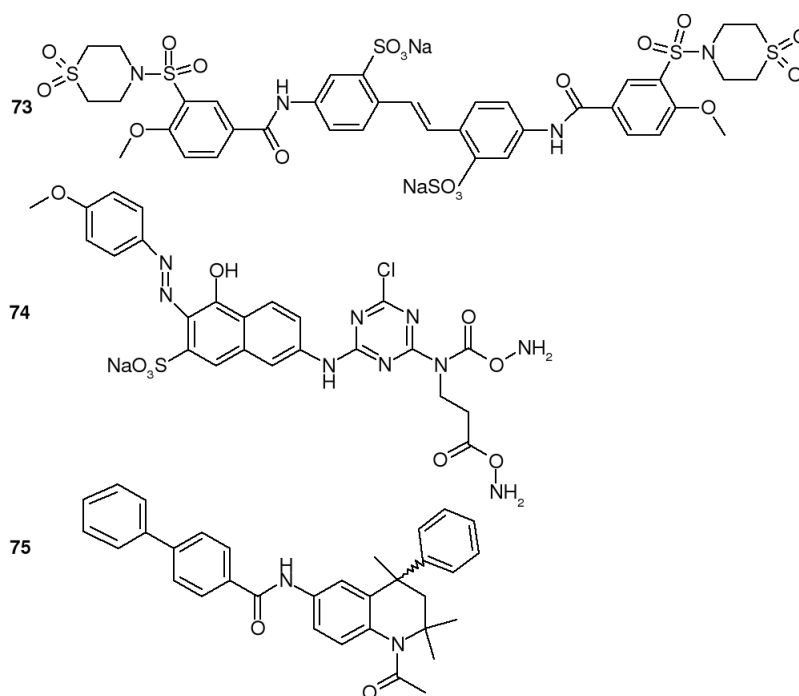
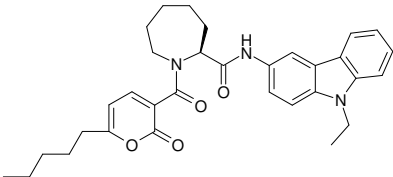
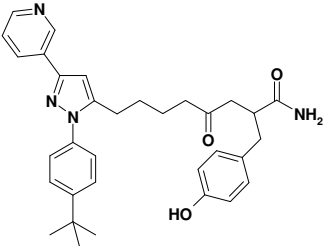
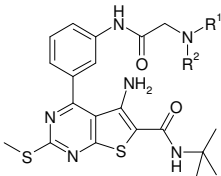
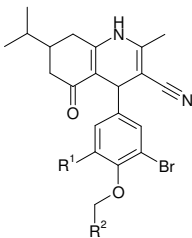
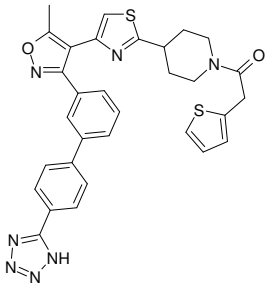


Figure 2.5 Chemical structures of the most potent analogues of suramin (**73**)²¹⁷, diazonaphthylsulfonic acid (**74**)²¹⁸ and c) tetrahydroquinoline (**75**)²⁰⁸ derivatives as antagonists at the human FSH receptor.

Table 2.18 Potencies of patented agonists at the human FSH receptor.

Class	Company	Structure	Potency	Ref
Carbazoles	ARS Holding		EC ₅₀ = 3 nM	209
Pyrazoles	Serono RBI		EC ₅₀ = 62 nM	210
Thieno[2,3-d]-pyrimidines	N.V. Organon		EC ₅₀ < 10 μM	211,219
Hexahydro-quinolines	N.V. Organon		EC ₅₀ < 10 nM	212-214
Isoxazolyl-thiazoles	Arena		EC ₅₀ = 1,100 nM	215

(glycoprotein hormone) receptors. Therefore, Wrobel and coworkers developed suramin derivatives as selective FSH receptor antagonists.²¹⁷ Compound **73** (Figure 2.5) was shown to inhibit the binding of [¹²⁵I]FSH (IC₅₀ = 2.0 ± 0.21 μM) and antagonized the production of

cAMP ($EC_{50} = 1.5 \pm 0.1 \mu\text{M}$). It should be noted, however, that suramin has also been shown to inhibit agonist receptor binding by disturbing the receptor's interaction with the G protein.^{220,221} A large compound library was screened for displacement of [^{125}I]FSH from the FSH receptor expressed in 3D2 cell membranes, which resulted in compound **74** ($IC_{50} = 10 \pm 2.8 \text{ nM}$; Figure 2.5), a diazonaphthylsulfonic acid derivative.²¹⁸ Scatchard analysis of the binding of hFSH in the presence of **74** showed that inhibition of binding was non-competitive in nature. In addition, this compound was selective for the FSH receptor, as no effect was detected on other glycoprotein hormone receptors and other unrelated GPCRs.

Organon Biosciences (currently Schering-Plough) identified another class of non-peptidic FSH receptor antagonists.²⁰⁸ The lead tetrahydroquinoline compound (**72**) was identified as a selective agonist. Introduction of a 6-phenyl substituent via an amide linkage yielded antagonistic compounds. Especially, compounds with phenyl substituents with electron-donating groups showed high potencies. Compound **75** (Figure 2.5) was the most potent ($IC_{50} = 5 \text{ nM}$) and contained a biphenyl substituent, indicating the presence of a large lipophilic pocket in the receptor.²⁰⁸ None of the reported compounds was able to displace ^{125}I -FSH. The authors therefore postulated that the lipophilic non-peptidic antagonists bind in the seven transmembrane (7-TM) domain of the FSH receptor.

2.4.6 Patented Antagonists

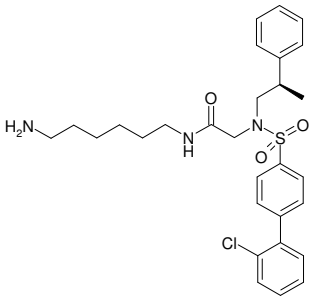
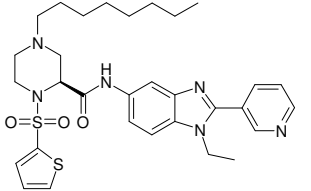
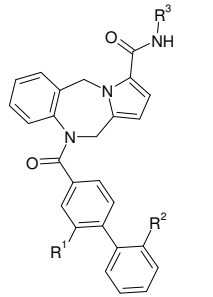
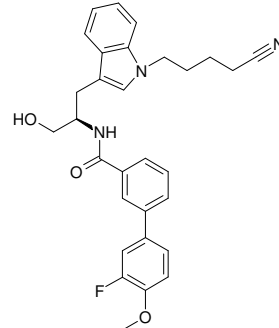
Four additional compounds classes have been reported in patent literature as non-peptidic human FSH receptor antagonists (Table 2.19). Ortho-McNeill and ARS Holding have described aminoalkylamides²²² and piperazines²²³ as antagonists, respectively. Pyrrolo[2,1-*c*]benzodiazepine²²⁴⁻²²⁷ and indole derivatives²²⁸ have been patented by Wyeth en Schering. Notably, all compounds classes, except for piperazines, contain the biphenyl moiety that has been described as a scaffold for FSH receptor agonists.

2.4.7 Overview FSH Receptor Ligands

In comparison with the LH receptor, more compound classes, including agonists and antagonists, have been described. However, the potency of these ligands needs to be improved. Further insight into the high affinity ligand binding criteria may be obtained from a molecular pharmacophore study. It appeared from the tetrahydroquinoline derivatives that

there is a delicate balance whether a compound behaves like an agonist or antagonist. This should be further investigated using the tetrahydroquinoline-based ligands as a starting point. In addition, data concerning oral bioavailability of these non-peptidic ligands is lacking.

Table 2.19 Potencies of patented antagonists at the human FSH receptor.

Class	Company	Structure	Potency	Ref
Aminoalkyl- amide	Ortho-McNeill		IC ₅₀ = 50 nM	222
Piperazines	ARS Holding		IC ₅₀ = 13 nM	223
Pyrrolo[2,1-c]- benzodiazepine	Wyeth		IC ₅₀ = 41-6,820 nM	224-227
Indole	Schering		IC ₅₀ = 100 nM	228

2.5 GPR54 RECEPTOR LIGANDS

In 1999 the cDNA of a novel GPCR was isolated and named GPR54.⁸⁷ Only very recently, the endogenous ligand for GPR54 was discovered, metastin.⁹⁰ Ohtaki *et al.* showed that the biological activity of metastin was retained when all but ten residues (45-54) were removed.²²⁹ In the past two years, this peptide ligand, also referred to as KP-10 or KiSS-1, has been investigated. Niida and coworkers performed both an alanine and D-amino acid scan of KP-10 to identify important residues for GPR54 agonistic activity.²³⁰ It was shown that five amino acids (50-54) were important for receptor binding and activation. Incorporation of a basic group, e.g. guanidine, and substitution of Phe with Trp resulted in the first significantly downsized peptide GPR54 agonist with similar potency as KP-10 ($EC_{50} = 1.4$ nM). A 2-fold improvement in potency was obtained when Phe-50 was replaced with the more lipophilic 3-(2-naphthyl)alanine.²³¹ A similar approach was used by Orsini *et al.*, which led to the development of three metastin pharmacophore models.²³² In short, the models consisted of four-point queries; two phenyl/hydrophobic and one isopropyl feature in combination with either a positively charged or amide contribution. Virtual screening of the in-house library led to the identification of the first non-peptidic (partial) agonist, **76** (Figure 2.6) with a K_i value of 708 nM.

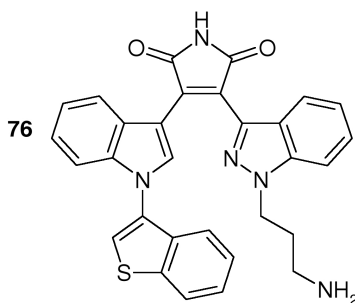


Figure 2.6 Chemical structure of the most potent non-peptidic GPR54 agonist (**76**).²³²

2.6 PERSPECTIVES

For the GnRH receptor large progress has been made over the past decade in the identification of potent non-peptidic antagonists. Different scaffolds yield compounds with high affinity, functionality and oral bioavailability. In several cases, it has been proven possible to activate the LH and FSH receptor (and antagonize the latter) via an allosteric site with non-peptidic ligands. However, only little SAR-knowledge has been obtained for these receptors, in comparison to LMW ligands for the GnRH receptor. In addition, the first non-peptidic agonist for GPR54 was identified very recently. Possibly with the help of this pharmacophore, novel agonists and antagonists will be reported in the near future.

With several series of ligands available, the different receptors of the HPG-axis can be studied more extensively. Although species (rat/mouse vs. human) and target selectivity, metabolic stability (cytochrome P450) and bioavailability data is often lacking, non-peptidic ligands offer the promise of oral bioavailability. These compounds may therefore emerge as useful drugs for these receptors, mainly for the treatment of infertility and for contraception.

CHAPTER

3

AMILORIDE DERIVATIVES AND A NON-PEPTIDIC ANTAGONIST BIND AT TWO DISTINCT ALLOSTERIC SITES IN THE HUMAN GONADOTROPIN-RELEASING HORMONE RECEPTOR

The interest in the allosteric modulation of G protein-coupled receptors has grown during the past decade. It has been shown that ligands acting at allosteric sites present in these important drug targets have the ability to modulate receptor conformations and fine-tune pharmacological responses to the orthosteric ligand. In the present study, allosteric modulation of the human gonadotropin-releasing hormone (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 stably expressed in CHO cell membranes was investigated. HMA and FD-1, but not TAK-013, another non-peptide antagonist, were shown to increase the dissociation rate of ^{125}I -triptorelin, revealing their allosteric
(Continued)

(Continued)

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. Equilibrium displacement studies showed that the inhibition of ^{125}I -triptorelin binding by FD-1 was not affected by HMA. 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. This presence of a second allosteric site may provide yet another opportunity for the discovery of new ligands for the human GnRH receptor.

This chapter is an adjusted version of a recent publication:

Heitman, L. H.; Ye, K.; Oosterom, J.; IJzerman, A. P. Amiloride derivatives and a non-peptidic antagonist bind at two distinct allosteric sites in the human gonadotropin-releasing hormone receptor. *Mol Pharmacol* **2008**, 73, 1808-1815

3.1 INTRODUCTION

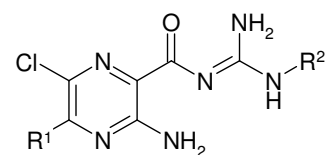
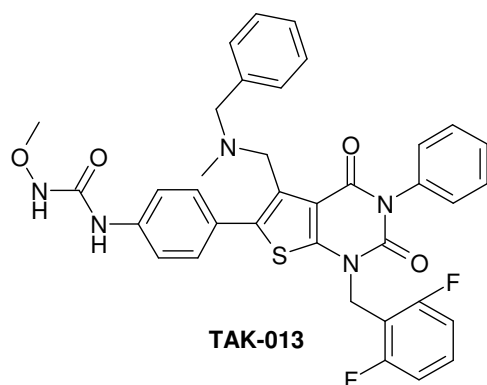
The gonadotropin-releasing hormone (GnRH) receptor belongs to the rhodopsin-like subfamily (class A) of G protein-coupled receptors (GPCRs).¹⁰ Activation of the GnRH receptor results in the biosynthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The gonadotropins bind to their respective receptors on the gonadal cells, which stimulates germ cell development and hormone secretion in the ovaries.⁸⁹ GnRH, also named luteinizing hormone-releasing hormone (LHRH), is a linear hypothalamic decapeptide (Figure 3.1) and was first isolated and characterized by Schally *et al.*²³³ Several peptidic agonists and antagonists for the GnRH receptor have been approved for the treatment of a variety of sex-hormone dependent diseases, such as prostate and breast cancer and endometriosis.^{72,95} Superagonists, a somewhat ambiguous term for chronically administered peptidic agonists, are used to desensitize and downregulate the GnRH receptor, resulting in gonadal suppression. Such use of agonists, however, produces an initial hormonal ‘flare’, resulting in a temporary activation of the pituitary, which can be prevented by giving peptidic antagonists instead. Peptidic compounds often need to be administered by parenteral (subcutaneous or intramuscular) injection.⁷² Therefore intensive efforts have been initiated to develop non-peptidic antagonists, which have the potential to become orally available drugs.²³⁴

In the past decade several classes of non-peptidic GnRH receptor antagonists have been reported (see *Chapter 2* for review). These ligands compete with a peptidic agonist for the same binding site on the receptor, providing evidence that they can be classified as orthosteric ligands. In addition, mutational analysis of the GnRH receptor has shown that these non-peptidic antagonists have overlapping, but non-identical binding sites.²³⁵ The orthosteric binding site of a GPCR has been defined as the site that is recognized by the endogenous ligand.⁴⁹ For several GPCRs, however, another (allosteric) binding site has been identified, e.g. for muscarinic receptors (class A), the corticotropin-releasing factor₁ receptor (class B) and glutamate receptors (class C).^{58,236} In comparison to conventional orthosteric ligands, allosteric modulators can have the therapeutic advantage of greater selectivity and tissue-specificity. In addition, the risk of over-dosing is diminished by their saturability.

In the present study, the allosteric modulation of the human GnRH receptor was examined. Equilibrium and kinetic radioligand binding experiments were performed in the presence and absence of both non-specific [e.g. 5-(*N,N*-hexamethylene)amiloride (HMA)] and GnRH receptor-selective allosteric modulators [furan derivative-1 (FD-1)] (Figure 3.1).

Amiloride derivatives have been well described as allosteric inhibitors for different GPCRs at concentrations in the high micromolar range,²³⁷ while FD-1 is a derivative of a recently described allosteric inhibitor for the GnRH receptor.⁵³ The ability of a compound to modulate the dissociation rate of ¹²⁵I-triptorelin was used as a measure for allosteric modulation. This revealed that there are two rather than one allosteric binding site on this receptor. This emerging concept of multiple allosteric sites may offer further options to modulate GPCR activity.

GnRH pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.NH₂
Triptorelin pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly.NH₂
Ganirelix D-Nal-D-Phe-D-Pal-Ser-Tyr-D-hArg-Leu-hArg-Pro-D-Ala



HMA: R¹=azepane, R²=H

MIBA: R¹=isobutyl-methyl-amine, R²=H

DCB: R¹=NH₂, R²=2,4-dichlorobenzyl

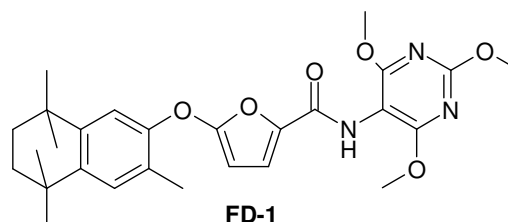


Figure 3.1 Sequences of GnRH, triptorelin (agonists), ganirelix (antagonist) and chemical structures of FD-1 (antagonist and allosteric inhibitor), HMA, MIBA, DCB (allosteric inhibitors) and TAK-013 (antagonist).

3.2 MATERIALS AND METHODS

3.2.1 Materials

GnRH, triptorelin, guanosine-5'-triphosphate (GTP) and HMA were purchased from Sigma Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Amiloride, 5-(*N*-methyl-*N*-guanidinocarbonylmethyl)amiloride (MGCMA), 5-(*N*-methyl-*N*-isobutyl)amiloride (MIBA), Phenamil, Benzamil and dichlorobenzamil (DCB) were kindly provided by Dr EJ Cragoe (Lansdale, USA) and were synthesized as described previously.²³⁸ Suramin was a generous gift from Bayer AG (Wuppertal, Germany). (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD81,723) and (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2*H*)-ylidene)methanamine (SCH-202676) were synthesized in our own laboratory as described by Van der Klein *et al.*²³⁹ and Van den Nieuwendijk *et al.*²⁴⁰ Ganirelix was provided by Schering-Plough (Oss, The Netherlands). 5-(*N*-benzyl-*N*-methylaminomethyl)-1-(2,6-difluorobenzyl)-6-[4-(3-methoxyureido)phenyl]-3-phenylthieno[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)-dione (TAK-013) and 5-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-naphthalen-2-yloxy)-furan-2-carboxylic acid (2,4,6-trimethoxy-pyrimidin-5-yl)-amide (FD-1) were prepared according to literature procedures.^{241,242} Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, U.S.A.), whereas BCA (bicinchoninic acid) protein assay reagent was from Pierce Chemical Company (Rockford, IL, U.S.A.). ¹²⁵I-triptorelin (specific activity 2200 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Groningen, The Netherlands). CHO (Chinese hamster ovary) cells stably expressing the human gonadotropin releasing-hormone receptor was obtained from Euroscreen (Brussels, Belgium). The CHO-K1 cells expressing the wild-type human GnRH receptor and Nuclear Factor Activated T-cell luciferase reporter gene (NFAT-luc) were provided by Schering-Plough (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

3.2.2 Cell Culture

CHO cells stably expressing the human GnRH receptor (CHOhGnRH) were grown in Ham's F12 medium containing 10% (v/v) normal adult bovine serum, streptomycin (100 µg/mL), penicillin (100 IU/mL) and G418 (0.4 mg/mL) at 37 °C in 5% CO₂.²⁴³ The cells

were subcultured twice weekly at a ratio of 1:20. For membrane preparation the cells were subcultured 1:10 and transferred to large 15-cm diameter plates.

3.2.3 Membrane Preparation

Cells were detached from the plates by scraping them into 5 mL PBS, collected and centrifuged at 700 *g* (3000 rpm) for 5 min. Pellets derived from 30 plates were pooled and resuspended in 20 mL of ice-cold 50 mM Tris-HCl buffer containing 2 mM MgCl₂, pH 7.4. An UltraThurax (Heidolph Instruments, Germany) was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 *g* (31,000 rpm) in an Optima LE-80K ultracentrifuge (Beckman Coulter, Fullerton, CA) at 4 °C for 20 min. The pellet was resuspended in 10 mL of the Tris buffer and the homogenization and centrifugation step was repeated. Tris buffer (10 mL) was used to resuspend the pellet and the membranes were stored in 250 and 500 µL aliquots at –80 °C. Membrane protein concentrations were measured using the BCA method with BSA as a standard.²⁴⁴

3.2.4 Radioligand Displacement and Saturation Assays

Membrane aliquots containing 5 - 7.5 µg protein were incubated in a total volume of 100 µL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% (w/v) BSA) at 22 °C for 45 min. For saturation experiments unlabeled triptorelin was spiked with 20% ¹²⁵I-triptorelin resulting in final concentrations of 0.1 to 3 nM. Non-specific binding was determined at three concentrations of radioligand in the presence of 100 µM ganirelix. Displacement experiments were performed using eleven concentrations of competing ligand in the presence of 30,000 cpm (~ 0.1 nM) ¹²⁵I-triptorelin. Here, non-specific binding was determined in the presence of 1 µM ganirelix and represented approximately 15% of the total binding. Incubations were terminated by dilution with ice-cold Tris-HCl buffer. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters pre-soaked with 0.25% poly(ethylenimine) (PEI) for 1 h using a Brandel harvester. Filters were subsequently washed three times with 2 mL ice-cold wash buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.05% BSA). Filter-bound radioactivity was determined in a γ-counter (Wizard 1470, PerkinElmer Life Sciences).

3.2.5 Radioligand Kinetic Association and Dissociation Assays

Association experiments were performed by incubating membrane aliquots containing 5 - 7.5 μg protein in a total volume of 100 μL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl_2 and 0.1% BSA) at 22 $^{\circ}\text{C}$ with 30,000 cpm of ^{125}I -triptorelin. The amount of radioligand bound to the receptor was measured at different time intervals during incubation for 90 min. Dissociation experiments were performed by preincubating membrane aliquots containing 5 – 7.5 μg protein in a total volume of 100 μL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl_2 and 0.1% BSA) at 22 $^{\circ}\text{C}$ for 45 min with 30,000 cpm (~ 0.1 nM) ^{125}I -triptorelin. After preincubation, dissociation was initiated by addition of 1 μM ganirelix in the presence or absence (control) of HMA, MIBA, DCB, FD-1 or TAK-013 in a total volume of 5 μL . The amount of radioligand still bound to the receptor was measured at various time intervals for a total of 2 h. Incubations were terminated and samples were obtained and analyzed as described under *Radioligand Displacement and Saturation Assays*.

3.2.6 ‘Competitive’ Kinetic Radioligand Dissociation Assays

Dissociation experiments were mainly performed as described above. After preincubation, dissociation was initiated by addition of 1 μM ganirelix in the presence or absence (control) of different concentrations FD-1 (1, 3 or 10 μM) and in the presence or absence (control) of six different concentrations of HMA (5 – 100 μM) in a total volume of 5 μL . The amount of radioligand still bound to the receptor was measured after 30 min. Incubations were terminated and samples were obtained and analyzed as described under *Radioligand Displacement and Saturation Assays*.

3.2.7 Luciferase Assays

CHO_hGnRH_{luc} cells were cultured as described under *Cell Culture*. However, Dulbecco’s Modified Eagle’s Medium (DMEM) was added to the culture medium (1:1 with F12). On the day of the assay, cells were washed with PBS and then harvested using trypsol (0.25% (w/v) in PBS containing 4.4 mM EDTA). Cells were resuspended in assay medium consisting of DMEM and F12 (1:1) supplemented with 1 $\mu\text{g}/\text{mL}$ insulin and 5 $\mu\text{g}/\text{mL}$ apo-transferrin. Typically, a well contained 30 μL of a certain concentration of triptorelin, 30 μL

of modulator (HMA or FD-1) or assay medium (control) and 30 μL cell suspension containing 7.5×10^5 cells/mL. After 4 h stimulation, 50 μL of luciferase (PerkinElmer Life Sciences) was added to each well for detection of luciferase protein and plates were left for 30 minutes at room temperature in the dark. Finally, the luminescence signal was quantified on a Microbeta Trilux 1450 Luminescence Counter (PerkinElmer Life Sciences).

3.2.8 Data Analysis

All binding data was analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 5.00 (GraphPad Software Inc, San Diego, CA). EC_{50} values were directly obtained from the dose-response curves and inhibitory binding constants (K_i values) were derived from the IC_{50} values according to $K_i = \text{IC}_{50}/(1 + [\text{C}]/K_d)$ where $[\text{C}]$ is the concentration of the radioligand and K_d its dissociation constant.²⁴⁵ The K_d value of ^{125}I -triptorelin at CHO hGnRH membranes was obtained by computer analysis of saturation curves. Dissociation constants, k_{off} , were obtained by computer analysis of the exponential decay of the percentage of ^{125}I -triptorelin bound to the receptor. Association rates were calculated according to the equation $k_{\text{on}} = (k_{\text{obs}} - k_{\text{off}})/[\text{L}]$, where k_{obs} was obtained by computer analysis of the exponential association of the percentage of ^{125}I -triptorelin bound to the receptor and $[\text{L}]$ is the amount of radioligand used for the association experiments. The EC_{50} from competitive dissociation experiments was obtained from dose response-curves of enhanced dissociation by different concentrations of HMA, where the non-specific binding was set at 0% and either the true control (buffer) or own control binding (1, 3 or 10 μM FD-1) after 30 min was set at 100%. All values obtained are means of at least three independent experiments performed in duplicate.

3.2.9 Simulation of Cooperativity Between FD-1 and HMA

A mathematical model (Eq. 3.1) for two distinct allosteric sites was implemented in MatLab R to simulate the effects of different cooperativities between HMA and FD-1 on the EC_{50} of HMA in enhancing ^{125}I -triptorelin dissociation.⁶²

$$\text{EC}_{50}^{\text{HMA}} = \frac{1 + [\text{FD-1}] \cdot K_{\text{FD-1}}^{\text{Triptorelin}}}{K_{\text{HMA}}^{\text{Triptorelin}} \cdot (1 + [\text{FD-1}] \cdot K_{\text{FD-1}}^{\text{Triptorelin}} \cdot \delta)} \quad \text{Eq. 3.1}$$

In Equation 3.1; EC_{50}^{HMA} is the observed EC_{50} of HMA in enhancing ^{125}I -triptorelin binding. $K_{FD-1}^{Triptorelin}$ and $K_{HMA}^{Triptorelin}$ are the affinities on the triptorelin-occupied receptor for FD-1 and HMA, respectively. δ is the parameter defining the cooperativity between HMA and FD-1.

3.3 RESULTS

3.3.1 Radioligand Saturation Experiments

Saturation experiments were performed with unlabeled triptorelin spiked with 20% ^{125}I -triptorelin on CHO cells expressing the human GnRH receptor. The results of a representative saturation experiment are shown in Figure 3.2. Although the non-specific binding was high, the receptor binding of ^{125}I -triptorelin was saturable and best characterized by a one-site receptor model. A K_D and $B_{\max}$ value of 0.35 (0.33 - 0.37) nM and 217 (207 - 227) fmol/mg protein, respectively, were obtained from two independent saturation experiments. The K_D value for ^{125}I -triptorelin obtained with these experiments was used to derive K_i rather than IC_{50} values in the following paragraph.

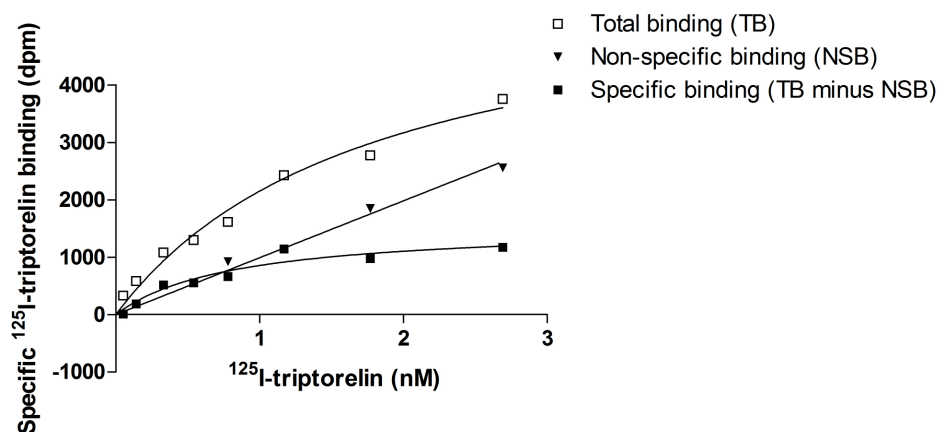


Figure 3.2 Saturation of ^{125}I -triptorelin binding to human gonadotropin-releasing hormone receptors. The specific binding was determined by subtracting the non-specific binding from the total binding. The K_D value was 0.35 (0.33 - 0.37) nM and the $B_{\max}$ value was 217 (207 - 227) fmol/mg protein. Representative graphs from one experiment performed in duplicate.

3.3.2 Radioligand Displacement Assays

Experiments were performed to assess the ability of various ligands to compete with the binding of ^{125}I -triptorelin to CHO_hGnRH cell membranes. The endogenous agonist, GnRH, a derivative, triptorelin, a peptidic antagonist, ganirelix and two non-peptidic antagonists,

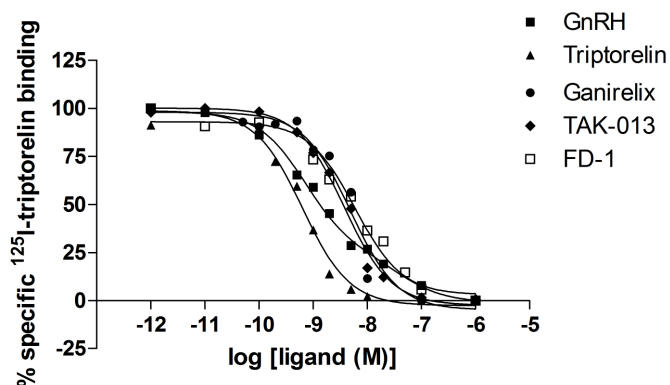


Figure 3.3 Displacement of ^{125}I -triptorelin from human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes by GnRH, triptorelin, ganirelix, TAK-013 and FD-1. Representative graphs from one experiment performed in duplicate (see Table 3.1 for affinity values).

Table 3.1 Receptor affinity of peptidic agonists, GnRH and triptorelin, peptidic antagonist, ganirelix, and non-peptidic antagonists, TAK-013 and FD-1, expressed as K_i values.

Compound	K_i (nM) ^a
GnRH*	1.2 ± 0.1
Triptorelin	0.42 ± 0.07
Ganirelix	3.8 ± 0.4
TAK-013	1.9 ± 0.7
FD-1	4.9 ± 1

^a Displacement of specific ^{125}I -triptorelin binding from human gonadotropin-releasing hormone receptors stably expressed in CHO cell membranes.

* According to computer analysis of the binding curve of GnRH, a two-site competition model of higher (H) and lower (L) affinity was statistically preferred with $K_H = 0.54 \pm 0.004$ nM, $K_L = 21 \pm 10$ nM and $\% R_H = 69 \pm 3\%$.

Values are means ($\pm$ S.E.M.) of at least three separate assays performed in duplicate.

TAK-013 and FD-1 (Figure 3.1), were used to displace radioligand binding. The displacement curves and affinity values are shown in Figure 3.3 and Table 3.1, respectively. All ligands were able to fully displace ^{125}I -triptorelin with affinities ranging from 0.42 nM for triptorelin to 4.9 nM for FD-1. From Figure 3.3 it follows that the curve of GnRH had a smaller Hill coefficient than that of the other ligands. Computational analysis indeed showed that it was best described by a two-site competition model with a higher (K_H) and a lower

affinity (K_L) of 0.54 ± 0.004 nM and 21 ± 10 nM (mean $\pm$ SEM, $n = 3$), respectively, with $69 \pm 3\%$ of high-affinity receptors (R_H).

3.3.3 Allosteric Modulation 125 I-Triptorelin Binding

The effect of some allosteric modulators was tested on equilibrium binding of 125 I-triptorelin. As shown in Figure 3.4a, PD81,723, a selective adenosine A_1 receptor modulator, had no effect on radioligand binding to the GnRH receptor. The addition of GTP, suramin and sodium ions had a modest effect on the binding of 125 I-triptorelin. Both SCH-202676 and HMA (Figure 3.1), however, had a detrimental effect on radioligand binding, as almost no radioactivity was detected after incubation with these agents. To investigate whether the effects of HMA could be extended to other amiloride derivatives, a similar experiment was performed with amiloride, MGCMA, MIBA, phenamil, benzamil and DCB. From Figure

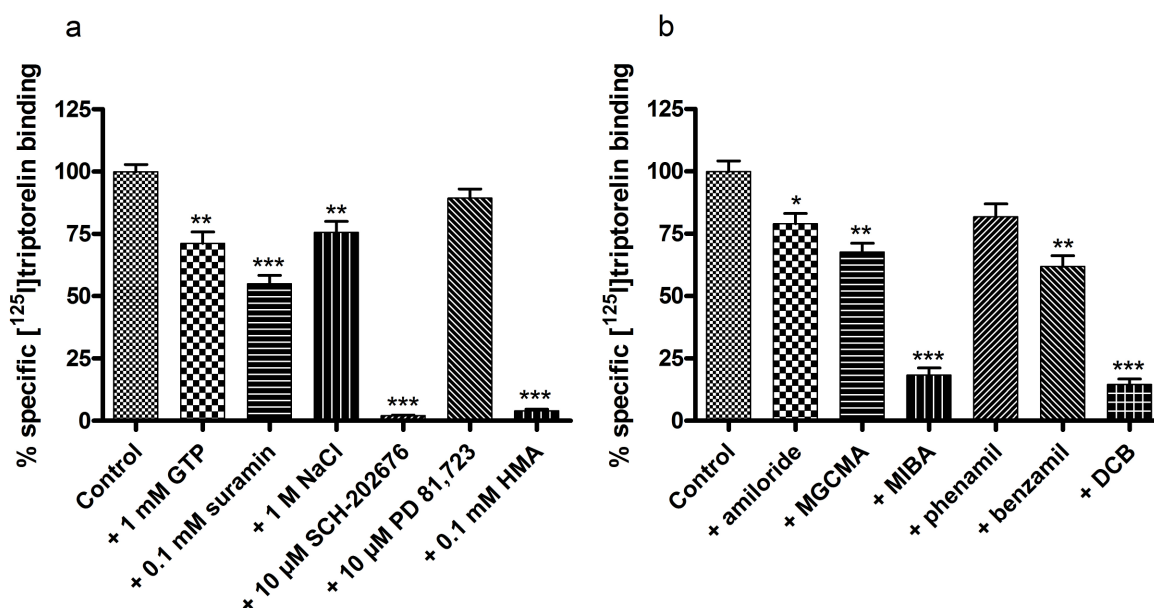


Figure 3.4 125 I-Triptorelin equilibrium binding to human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes in the absence (control, 100%) or presence of a) GTP, suramin, sodium, SCH-202676, PD 81,723 and HMA and b) 0.1 mM amiloride derivatives. Values are means ($\pm$ S.E.M.) from at least three independent experiments, performed in duplicate. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control)

3.4b follows that most amilorides had little effect and that only MIBA and DCB were able to inhibit ^{125}I -triptorelin binding. Therefore, displacement of ^{125}I -triptorelin equilibrium binding by HMA, MIBA and DCB at different concentrations was determined (Figure 3.5). The obtained inhibition curves were best described by a one-site receptor model and resulted in similar potencies for HMA ($\text{IC}_{50} = 29 \pm 3 \mu\text{M}$), MIBA ($\text{IC}_{50} = 39 \pm 7 \mu\text{M}$) and DCB ($\text{IC}_{50} = 30 \pm 3 \mu\text{M}$) with a pseudo-Hill coefficient of 1.4 ± 0.06 , 1.3 ± 0.02 and 1.6 ± 0.2 , respectively (Table 3.2).

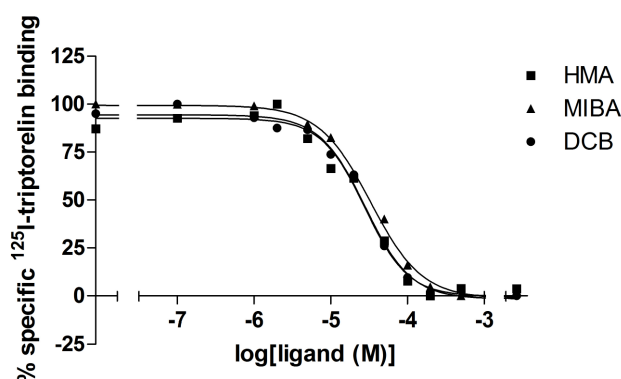


Figure 3.5 Inhibition of ^{125}I -triptorelin equilibrium binding to human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes by HMA, DCB and MIBA. Representative graphs from one experiment performed in duplicate (see Table 3.2 for affinity values).

3.3.4 Kinetic Association and Dissociation Experiments

The dissociation constant (K_D) of ^{125}I -triptorelin in the absence of modulators was also derived from kinetic experiments and the resulting dissociation and association rate constants. Equilibrium binding was reached after approximately 45 min with an association rate constant of $0.28 \pm 0.08 \text{ nM}^{-1} \text{ min}^{-1}$. Under control conditions the radioligand dissociated from the receptor with a dissociation rate constant of $0.021 \pm 0.002 \text{ min}^{-1}$. Together this resulted in a 'kinetic' K_D value of 0.74 nM, which was in good agreement with the K_D value (0.35 nM) obtained in the 'spiked' saturation analysis. Next, the dissociation kinetics of ^{125}I -triptorelin from CHO_hGnRH receptor membranes was determined in the presence of modulator (Figure 3.6 and Table 3.2). All compounds, except TAK-013 (values not shown), increased the

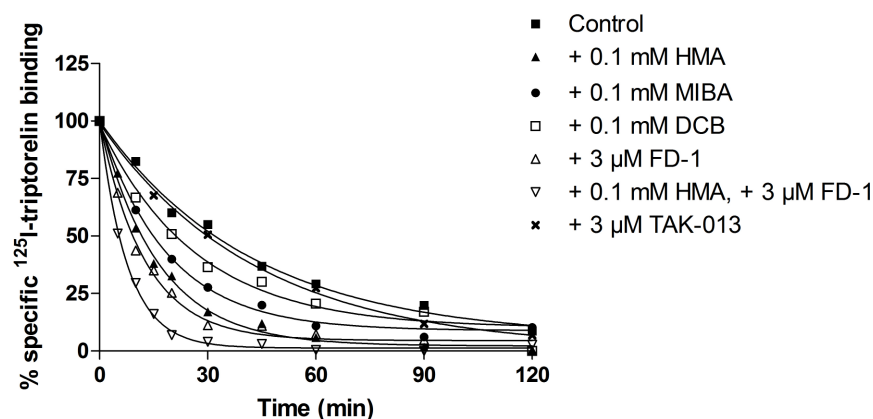


Figure 3.6 Dissociation kinetics of ^{125}I -triptorelin binding to human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes. Dissociation was either initiated by the addition 1 μM ganirelix mixed with buffer (control) or modulator. Representative graphs from one experiment performed in duplicate (see Table 3.2 for kinetic parameters).

Table 3.2 Displacement, dissociation and allosteric modulation of ^{125}I -triptorelin binding by HMA, MIBA, DCB and FD-1, expressed as IC_{50} , k_{off} and EC_{50} values.

Condition	Inhibitory potency ^a		Modulatory potency ^d	
	IC_{50} (μM)	k_{off} (min^{-1}) ^b	Shift k_{off} ^c	EC_{50} (μM)
Control	-	0.021 ± 0.002	-	-
+ HMA	29 ± 3	0.053 ± 0.006	2.5	49 ± 7
+ MIBA	39 ± 7	0.045 ± 0.006	2.1	ND
+ DCB	30 ± 3	0.035 ± 0.006	1.7	ND
+ FD-1	-	0.068 ± 0.009	3.2	5.0 ± 1
+ HMA, + FD-1	-	0.11 ± 0.01	5.2	-

^a Displacement of specific ^{125}I -triptorelin binding from human gonadotropin-releasing hormone receptors stably expressed in CHO cell membranes.

^b The value of the kinetic dissociation rate constant was obtained by analysis of the exponential dissociation curve of ^{125}I -triptorelin bound to human gonadotropin-releasing hormone receptors in the presence of buffer (control), 0.1 mM HMA, MIBA, DCB, 3 μM FD-1 or 0.1 mM HMA and 3 μM FD-1.

^c The shift is defined as the ratio of k_{off} values in the presence and absence (control) of modulator, respectively.

^d The value for the concentration at half-maximal enhancement of dissociation kinetics.

Values are means ($\pm$ S.E.M.) of at least three separate assays performed in duplicate.

ND, not determined

dissociation rate of ^{125}I -triptorelin in comparison to the control off-rate, indicative of their allosteric nature and negative modulation of the receptor. The dissociation rate constant of ^{125}I -triptorelin was increased 2.5-fold to $0.053 \pm 0.006 \text{ min}^{-1}$ with the addition of 0.1 mM HMA, which was a more potent allosteric inhibitor than MIBA and DCB, although their effect on the equilibrium binding was similar (Table 3.2). Similarly, the addition of 3 μM of the non-peptidic antagonist FD-1 resulted in a 3.2-fold increase of the dissociation rate constant, $0.068 \pm 0.009 \text{ min}^{-1}$. The simultaneous addition of HMA and FD-1 in the above concentrations resulted in an additive effect on the dissociation rate constant, which increased 5.2-fold under this condition ($k_{\text{off}} = 0.11 \pm 0.01 \text{ min}^{-1}$).

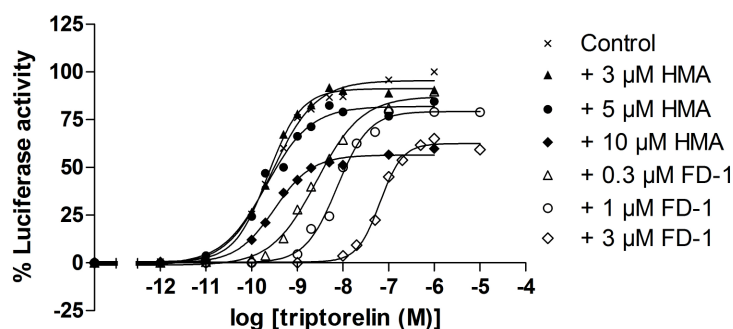


Figure 3.7 Concentration-effect curves of triptorelin on NFAT-induced luciferase production through human gonadotropin-releasing hormone receptors in the presence and absence (control) of different concentrations HMA or FD-1. Representative graphs from one experiment performed in duplicate (see Table 3.3 for EC_{50} and E_{max} values).

3.3.5 Allosteric Modulation of Receptor Activation.

The effect of HMA and FD-1 on receptor activation by triptorelin was measured using a NFAT-induced luciferase assay (Figure 3.7 and Table 3.3). HMA at three concentrations did not cause a shift in potency of triptorelin ($\text{EC}_{50} = 0.24 \pm 0.02 \text{ nM}$). However, increasing concentrations of HMA resulted in a dose-dependent lowering of the maximal effect (E_{max}). For example, the presence of 10 μM HMA resulted in an E_{max} value of $58 \pm 1\%$ compared to control (100%). This indicated non-competitive antagonism, which agrees with the allosteric inhibition seen in the kinetic dissociation experiments. FD-1 at three concentrations caused parallel rightward shifts in the dose-response curves of triptorelin, proof rather of competitive

antagonism. However, addition of FD-1 also resulted in a suppression of the $E_{\max}$ value, indicative for its allosteric nature. For example, addition of 3 μM FD-1 decreased the $E_{\max}$ value to $72 \pm 5\%$ of the control value.

Table 3.3 Receptor activation by triptorelin in the presence or absence of different concentrations of HMA or FD-1, expressed as EC_{50} and $E_{\max}$ values.

Compound	Activity in luciferase assay ^a	
	EC_{50} (nM)	$E_{\max}$ (%)
Triptorelin	0.24 ± 0.02	100 ± 2
+ 3 μM HMA	0.21 ± 0.03	95 ± 5
+ 5 μM HMA	0.24 ± 0.03	$89 \pm 3^{***}$
+ 10 μM HMA	$0.29 \pm 0.02^*$	$58 \pm 1^{***}$
+ 0.3 μM FD-1	$2.7 \pm 0.04^{***}$	$93 \pm 1^{***}$
+ 1 μM FD-1	$10 \pm 2^{***}$	$84 \pm 5^{***}$
+ 3 μM FD-1	$44 \pm 12^{***}$	$72 \pm 5^{***}$

^a Ca^{2+} -mediated luciferase activity in CHO cells that stably express the human gonadotropin-releasing hormone receptor and NFAT-luciferase reporter gene.

Values are means ($\pm$ S.E.M.) of at least three separate assays performed in duplicate. (* $p < 0.05$, *** $p < 0.001$ versus control)

3.3.6 Effect of HMA on FD-1 Binding.

In order to determine whether the allosteric effects described above occurred through an interaction at different allosteric sites, displacement of ^{125}I -triptorelin by different concentrations of FD-1 was determined in the presence and absence of three concentrations of HMA (Figure 3.8 and Table 3.4). It follows from Figure 3.8 that the addition of HMA alone (data points on Y-axis) inhibited the binding of ^{125}I -triptorelin dose-dependently, as shown by the decrease in $B_{\max}$ in Table 3.4 and corresponding to the results shown in Figure 3.5. FD-1 potently displaced the binding of the radioligand in a concentration-dependent manner. The addition of HMA, however, did not impede the displacement by FD-1. Interestingly, at 30 μM HMA, the affinity of FD-1 was significantly increased (Table 3.4), indicating a possible allosteric interaction between these compounds.

3.3.7 Competitive Dissociation Experiments.

Another series of experiments were performed to determine if FD-1 and HMA bound at a different allosteric site. As FD-1 also acts as an orthosteric antagonist (Figure 3.8), ‘competitive dissociation’ experiments were performed to solely study allosteric interactions. The concentration-dependent effect of HMA on ^{125}I -triptorelin dissociation was studied in the absence and presence of three concentrations of FD-1 (Figure 3.9). The data obtained are represented in two formats. In Figure 3.9a is shown that the addition of FD-1 enhanced the dissociation and under every condition HMA dose-dependently further enhanced that dissociation. Figure 3.9b shows that the addition of FD-1 did not affect the modulating potency of HMA ($\text{EC}_{50} = 49 \pm 7 \mu\text{M}$), which indicates a non-competitive interaction of these two compounds. It is noteworthy that FD-1 has a 10-fold higher modulatory potency than HMA, namely $5.0 \pm 1 \mu\text{M}$ (Table 3.2).

Table 3.4 Receptor affinity of FD-1 and radioligand binding capacity (in the absence of FD-1) in the presence or absence of different concentrations of HMA, expressed as K_i and B_{max} values, respectively.

Compound	^{125}I -triptorelin displacement ^a	
	K_i (nM)	B_{max} (%)
FD-1	4.9 ± 1	100 ± 5
+ 3 μM HMA	5.9 ± 2	$87 \pm 5^*$
+ 10 μM HMA	5.3 ± 1	$75 \pm 6^{**}$
+ 30 μM HMA	$2.3 \pm 0.7^*$	$47 \pm 5^{***}$

^a Displacement of specific ^{125}I -triptorelin binding from human gonadotropin-releasing hormone receptors stably expressed in CHO cell membranes.

Values are means ($\pm$ S.E.M.) of at least three separate assays performed in duplicate. (* $p < 0.05$, *** $p < 0.001$ versus control)

3.3.8 Simulation of Cooperativity between FD-1 and HMA.

Equation 1 in the Materials & Methods section, taken from Lazareno *et al.*,⁶² was used to simulate the effects of different cooperativities between HMA and FD-1 on the potency of HMA in enhancing the ^{125}I -triptorelin dissociation. When $\delta = 1$, the binding of two allosteric modulators is noninteracting (neutral cooperativity). When $\delta < 1$ or $\delta > 1$, they exhibit either negative (competitive) or positive (enhancement) cooperativity. These simulations, shown in Figure 3.10, demonstrate that the data points we had gathered comply with a δ value of 1, thus indicating a neutral cooperativity between the binding of HMA and FD-1.

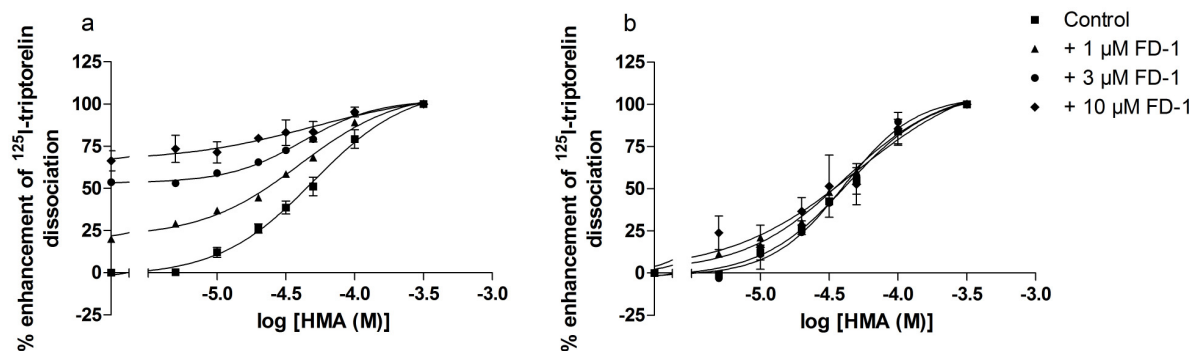


Figure 3.9 Effect of HMA on single point dissociation of ^{125}I -triptorelin from human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes in the presence or absence (control) of three concentrations of FD-1. Graph (a) shows data normalized to the control measured in the absence of FD-1 and graph (b) shows data normalized to the four conditions in the absence of HMA. Graphs are mean $\pm$ SEM from at least four independent experiments, performed in duplicate.

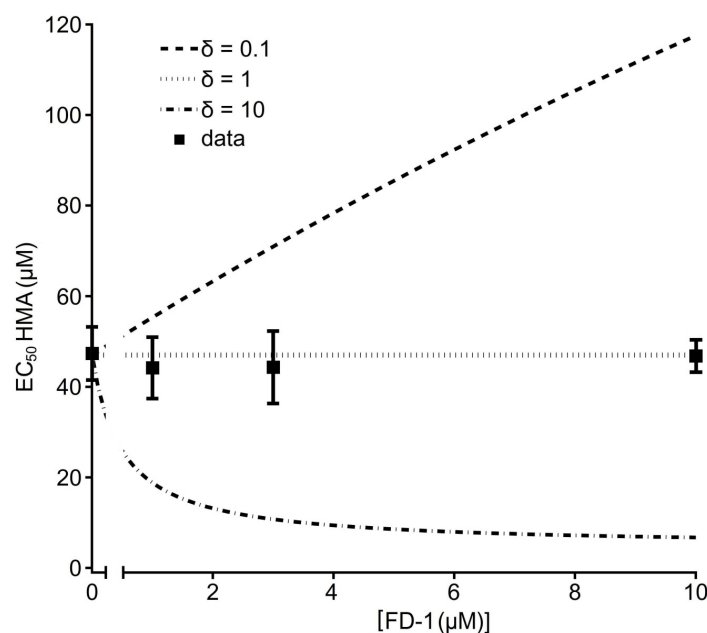


Figure 3.10 Neutral cooperativity between HMA and FD-1 in enhancing ^{125}I -triptorelin dissociation. The experimental data of different concentrations of FD-1 affecting the modulating potency of HMA is displayed with standard deviation. The lines show the fit of the data to eq. 1 (see material and methods), where the situations are simulated that two compounds exhibit positive ($\delta > 1$), neutral ($\delta = 1$) and negative cooperativity ($\delta < 1$).

3.4 DISCUSSION

In the present study it was demonstrated that human GnRH receptors are allosterically modulated by amiloride derivatives and a non-peptidic antagonist (FD-1). Radioligand displacement assays were performed, where four reference compounds were tested (Figure 3.3 and Table 3.1). For GnRH, a shallow displacement curve was obtained, which was best fitted with a two-site competition model. In the presence of 1 mM GTP the favored mode of binding for GnRH shifted towards a one-site competition model with a K_i value of 18 ± 0.6 nM (data not shown). Note that the latter affinity equals the affinity found for the low-affinity receptors in the absence of GTP ($K_L = 21 \pm 10$ nM). This can be explained by the ternary complex model, in which the presence of GTP causes a shift to a higher K_i value through uncoupling of the receptor from the G protein.²⁴⁶ Notably, triptorelin binding was best described by a one-site competition model, although the presence of GTP did decrease radioligand binding (Figure 3.4a). Beckers *et al.* reported the affinity of GnRH obtained in a ^{125}I -triptorelin displacement assay, where they used whole LTK cells transfected with the human GnRH receptor.²⁴⁷ A 5-fold lower affinity (5.4 ± 1.8 nM) was found, which may be caused by a higher amount of endogenous GTP present in whole cells. The affinities reported for triptorelin and ganirelix, however, were in good agreement with the affinities reported here (Table 3.1). For TAK-013, an IC_{50} value of 2.5 nM was reported,¹²⁴ while we found a K_i value of 1.9 ± 0.7 nM. Lastly, FD-1 was tested, which belongs to a different class of non-peptidic antagonists (Table 3.1). FD-1 had a K_i -value of 4.9 ± 1 nM, which was comparable to the affinity reported for an analogue of this compound, 5-[(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthalenyl)methyl]-*N*-(2,4,6-trimethoxyphenyl)-2-furamide (CMPD-1) having a K_i value of 6.0 ± 0.8 nM.¹²⁸

The modulation of ^{125}I -triptorelin binding was explored in the absence and presence of different generally known modulators (Figure 3.4a). GTP and suramin are compounds that have an effect on G protein coupling. It was shown that they had only a modest effect on ^{125}I -triptorelin binding. The effect of PD81,723 on the adenosine A_1 receptor has been extensively studied.²⁴⁸ It has been shown to be a selective allosteric enhancer at the adenosine A_1 receptor, and as might be expected, it did not affect ^{125}I -triptorelin equilibrium binding to the GnRH receptor. The influence of a high concentration of sodium ions was also examined at the human GnRH receptor. On other GPCRs, for example adenosine A_{2A} , α_2 -adrenergic and dopamine D_2 receptors, it has been shown that sodium ions regulate ligand binding.^{237,249,250} However, on the GnRH receptor, sodium ions do not have such a profound effect. In contrast,

HMA, which has been shown at higher micromolar concentrations to modulate the same receptor subtypes as sodium ions,^{237,251,252} was able to fully inhibit radioligand binding. In addition, SCH-202676 was shown to have a similar effect on equilibrium binding as HMA. However, this compound was recently shown to be a protein modifier rather than an allosteric modulator.²⁵³ To further explore the modulation of ¹²⁵I-triptorelin binding by HMA, other amiloride derivatives were tested (Figure 3.4b). Two other amiloride derivatives, MIBA and DCB, showed inhibition of equilibrium binding. It had been shown previously that MIBA was the most potent of this class of inhibitors next to HMA.^{237,252}

Allosteric inhibition of ¹²⁵I-triptorelin binding was shown by the increase in its dissociation rate from human GnRH receptors in the presence of HMA or MIBA (Figure 3.6). CMPD-1 has recently been shown to be an allosteric inhibitor for the GnRH receptor too.⁵³ Previously, that same compound (named Furan-1 or CMPD-1) had been demonstrated to be a potent non-peptidic antagonist,¹²⁸ whereas its allosteric effects occur at higher concentrations. FD-1 and CMPD-1 belong to the same class of non-peptidic antagonists with only some small structural differences (see Figure 3.1 for FD-1). It was demonstrated that, as for HMA, MIBA and DCB, FD-1 was also able to increase the dissociation rate (Figure 3.6). As mentioned above HMA was shown to be an allosteric inhibitor on different GPCRs, e.g. at the adenosine A_{2A} receptor.²³⁷ The selectivity of FD-1 was therefore tested on this receptor; FD-1 did not modulate the dissociation rate of the A_{2A} receptor radioligand (data not shown). FD-1 is therefore a more selective allosteric inhibitor, compared to HMA. The simultaneous addition of HMA and FD-1 resulted in an additive effect on the dissociation rate. However, addition of a high concentration (10 μM) of FD-1 further enhanced the dissociation (Figure 3.9a). Therefore, this did not indicate *per se* that the observed additive effect was due to the presence of two allosteric binding sites, although the two compounds are structurally different. The effect on *in vitro* functional efficacy was also determined (Figure 3.7 and Table 3.3). The functional data showed that HMA is a pure non-competitive antagonist (allosteric inhibitor) of the effects of triptorelin. On the other hand, FD-1 showed a mixed type of antagonism, indicating both orthosteric and allosteric characteristics. In this assay HMA and FD-1 showed the same effects when the endogenous ligand GnRH was used (data not shown), even though the binding sites of triptorelin and GnRH are not identical.²⁵⁴ Furthermore, FD-1 seemed to be a more potent allosteric inhibitor than Furan-1.⁵³ To prove that the allosteric characteristics of FD-1 were specific for this non-peptidic antagonist, TAK-013 was also examined. It was shown that TAK-013 had no effect on the dissociation rate of

¹²⁵I-triptorelin (Figure 3.6). This suggests that the allosteric nature of FD-1 is not a general feature of all non-peptidic antagonists, but due to structural aspects of FD-1 itself. Importantly, trypan blue exclusion tests showed that cell viability always exceeded 95%, which ruled out that the decrease in maximal response was caused by any cytotoxic effects of the relatively high concentrations of HMA or FD-1. In addition, reversible binding was shown in a luciferase assay where the cells were pre-incubated with the highest concentrations used of HMA and FD-1. After washing of the cells according to a method described by Lu and coworkers,²⁵⁵ full agonist responses were obtained, while unwashed pre-incubated cells still showed a decreased maximal response.

Lastly we examined whether HMA and FD-1 exert their effect through two distinct allosteric sites on the GnRH receptor. Lazareno *et al.*⁶² and Lanzafame *et al.*²⁵⁶ have reported two allosteric sites for the M₁ and M₄ muscarinic receptor, respectively. In addition, three distinct allosteric sites were reported by Schetz and Sibley²⁵⁷ for the dopamine D₄ receptor. To explore if HMA and FD-1 compete for the same allosteric binding site further experiments were conducted. Firstly, the effect of HMA on the displacement of ¹²⁵I-triptorelin by FD-1 was determined (Figure 3.8 and Table 3.4). It was shown that HMA has no competitive interaction with FD-1. However, the allosteric nature of FD-1 only occurs at high concentrations (micromolar range), which makes it difficult to observe an effect of HMA. Secondly, a competitive dissociation assay was performed, in which the effect of FD-1 on HMA-induced dissociation was examined. Especially from Figure 3.9b it follows that the presence of FD-1 had no effect on the modulatory potency of HMA. This suggests that FD-1 acts at a site distinct from the binding site of HMA and that there is no interaction between the binding of FD-1 and HMA. To strengthen this, a simulation was performed using a model according to Lazareno *et al.*⁶² As demonstrated in Figure 3.10, HMA and FD-1 indeed have neutral cooperativity ($\delta = 1$). It is quite feasible that other GPCRs modulated by amilorides, can also be modulated by a receptor-specific modulator from a second allosteric site. For example, the dopamine D₂ receptor, which was earlier shown to be modulated by amiloride analogues²⁵² is also influenced allosterically by the tripeptide L-prolyl-L-leucyl-glycinamide (PLG).²⁵⁸

In conclusion, we have demonstrated that the GnRH receptor can be allosterically modulated by amiloride analogues. In addition, FD-1 was shown to have both orthosteric and allosteric binding properties. Furthermore, we demonstrate that these two chemically unrelated compounds have two distinct allosteric binding sites on the human GnRH receptor, and that

these sites show neutral cooperativity. The allosteric sites revealed in this study may provide novel targets at the GnRH receptor for orally available, low molecular weight compounds.

CHAPTER

4

[³H]ORG 43553, THE FIRST LOW MOLECULAR WEIGHT AGONISTIC AND ALLOSTERIC RADIOLIGAND FOR THE HUMAN LUTEINIZING HORMONE RECEPTOR

The luteinizing hormone (LH) receptor plays a pivotal role in reproduction. The high-molecular-weight (HMW) hCG and LH are the endogenous ligands of this receptor and bind to its large N-terminus. The present study characterizes the binding of a new low-molecular-weight (LMW) radioligand, [³H]Org 43553, at the LH receptor. Equilibrium saturation and displacement assays were developed and optimized. Specific binding of [³H]Org 43553 to CHO-K1 cell membranes expressing the human LH receptor and a CRE-luciferase reporter gene was saturable with a K_D value of 2.4 ± 0.4 nM and a B_{max} value of 1.6 ± 0.2 pmol/mg protein. Affinities of five LMW analogues of Org 43553 were determined. All displaced the radioligand competitively with K_i values ranging from 3.3 - 100 nM. Lastly, the potency of these compounds in a cAMP-induced luciferase assay was also determined. There
(Continued)

(Continued)

was a high correlation between affinity and potency ($r^2 = 0.99$; $p < 0.0001$) of these compounds. In the search for LMW ligands, which bind allosterically to the seven-transmembrane (7-TM) domain of the LH receptor, a HMW radioligand (e.g. ^{125}I -hCG) is not suitable as it is not displaced by a LMW compound. Therefore, [^3H]Org 43553, a new radioligand with good binding properties, allows screening for new LMW ligands that mimic the action of the endogenous hormone at the LH receptor.

This chapter is an adjusted version of a recent publication:

Heitman, L. H.; Oosterom, J.; Bongers, K. M.; Timmers, C. M.; Wiegerinck, P. H.; IJzerman, A. P. [^3H]Org 43553, the first low molecular weight agonistic and allosteric radioligand for the human luteinizing hormone receptor. *Mol Pharmacol* **2008**, 73, 518-524

4.1 INTRODUCTION

The luteinizing hormone (LH) receptor is a member of the glycoprotein hormone receptor family within class A of G protein-coupled receptors.¹⁸ A unique feature of the LH receptor is that it recognizes two endogenous ligands with high molecular weight, namely human chorionic gonadotropin (hCG) and LH. Both hormones bind with high affinity and selectivity to the large N-terminus of the receptor.²⁵⁹ Together with other gonadotropins, LH and hCG play a pivotal role in reproduction in which LH is responsible for ovulation induction in women and control of testosterone production in men, whereas hCG maintains the early stages of pregnancy.²⁶⁰ Gonadotropins are currently used clinically in infertility treatment. Here, either urinary or recombinant gonadotropins are used, which need to be administered by parenteral (subcutaneous or intramuscular) injection.¹⁸⁷ The advantage of low molecular weight (LMW) agonists is that they have the potential to become orally available drugs.²¹ This will alleviate the necessity of parenteral administration, which may result in enhanced patient compliance and convenience in comparison to current methods.

In the past few years, medicinal chemists have therefore been challenged to find LMW ligands for receptors that have high molecular weight (HMW) endogenous ligands (e.g. polypeptides and protein hormones). Although LMW ligands have already been described for the gonadotropin hormone receptors (for review see *Chapter 2*), radioligands have not thus far. Small molecule radioligands have been reported for other receptors with HMW endogenous ligands, for example, an antagonist for the corticotropin-releasing factor₁ receptor,²⁶¹ an agonist for the insulin receptor²⁶² and an agonist for the glucagon-like peptide₁ receptor.²⁶³

This paper describes for the first time the pharmacological characterization of the human luteinizing hormone receptor transfected in Chinese hamster ovary (CHO) cells using a tritium-labeled form of a small molecule ligand, [³H]Org 43553 (see Table 4.2 for its chemical structure). Org 43553 is one of a series of thieno[2,3-d]pyrimidine derivatives that showed agonistic LH activity in a functional assay.²¹¹ Org 43553 was chosen to be labeled with tritium, because it was one of the more potent compounds from this screen. The kinetic and equilibrium binding characteristics of the new radioligand were determined, and compared to those of the radiolabeled natural hormone hCG. [³H]Org 43553 was also used in a displacement assay with four other thienopyrimidines and one quinazoline derivative. Their affinities correspond well to their potency in generating a cAMP response. Thus, this LMW

radioligand provides a useful tool to further understand the interactions of the LH receptor with small molecule ligands.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Org 41841, Org 41247, Org 42619, Org 43311, Org 43553, Org 43983, (compounds **1-6**, respectively) and recLH were provided by Schering-Plough (Oss, The Netherlands), where the Org-compounds were synthesized as described previously.^{197,211,219,264} Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, U.S.A.), whereas BCA protein assay reagent was from Pierce Chemical Company (Rockford, IL, U.S.A.). ¹²⁵I-hCG (5966 Ci/mmol) was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). Chinese Hamster Ovary (CHO-K1) cells stably expressing the human luteinizing hormone (LH) receptor and cAMP-response-element luciferase reporter gene (CRE-luc) were kindly provided by Schering-Plough (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

4.2.2 Cell Culture

CHO cells with stable expression of the human LH receptor and CRE-luc (CHO^hLH_r_luc) were grown in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (1:1) supplemented with 5% normal adult bovine serum, streptomycin (100 µg/mL), penicillin (100 IU/ mL) at 37 °C in 5% CO₂. The cells were subcultured twice weekly at a ratio of 1:15. For membrane preparation the cells were subcultured 1:10 and transferred to large 15-cm diameter plates.

4.2.3 Membrane Preparation

Cells were detached from the plates by scraping them into 5 mL phosphate-buffered saline (PBS), collected and centrifuged at 700 g (3000 rpm) for 5 min. Pellets derived from 30 plates were pooled and resuspended in 20 mL of ice-cold 50 mM Tris-HCl buffer containing 2 mM MgCl₂, pH 7.4. An UltraThurrax was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 g (31,000 rpm) in a Beckman Optima LE-80K ultracentrifuge at 4 °C for 20 min. The pellet was resuspended in 10 mL of the Tris buffer and the homogenization and centrifugation step was repeated. Tris buffer (12 mL) was used to resuspend the pellet and the

membranes were stored in 500 μL aliquots at $-80\text{ }^{\circ}\text{C}$. Membrane protein concentrations were measured using the BCA (bicinchoninic acid) method with BSA as a standard.²⁴⁴

4.2.4 Preparation of [^3H]Org 43553

The tritiation of Org 43553 was carried out by RC Tritec AG (Teufen, Switzerland). In short, 10 mg of 5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno[2,3-d]pyrimidine-6-carboxylic acid tert-butylamide, Org 43553, was dissolved in 250 μL THF containing 0.1% (v/v) water under nitrogen atmosphere. This solution was stirred for about 20 min, while cooling the flask to $-78\text{ }^{\circ}\text{C}$. Then 120 μL of 1.3 M sec-butyllithium in hexane/cyclohexane (final concentration 10 eq.) was added drop wise. The color of the solution changed from yellow to dark red/brown, indicative for the molecule to be deprotonated. The solution was stirred for another 2 h at $-78\text{ }^{\circ}\text{C}$. Next, the reaction was quenched with 20 Ci (i.e. an excess) of tritiated water at $-78\text{ }^{\circ}\text{C}$. The color of the solution changed back to yellow, showing that the deprotonated molecule was tritiated. The reaction mixture was stirred for another 1.5 h at $-78\text{ }^{\circ}\text{C}$ after which the reaction mixture was allowed to warm up to room temperature in about 30 min. Next a large excess of diethyl ether (50 mL) was added. The organic layer was washed twice with water and once with brine, dried and concentrated under reduced pressure. The residue was dissolved in ethanol to give a solution of 250 mCi crude [^3H]Org 43553 in 25 mL ethanol. This solution was then purified by high-performance liquid chromatography on a Symmetry C_{18} column eluting with acetonitrile/water (40/60 (v/v) containing 0.1% TFA) at Schering-Plough (Oss, The Netherlands). After purification 140 mCi [^3H]Org 43553 with a radiochemical purity $\geq 95\%$ and a specific activity of 16.6 Ci/mmol was obtained.

4.2.5 Radioligand Displacement and Saturation Assays

[^3H]Org 43553 Membrane aliquots containing 20 μg protein were incubated in a total volume of 100 μL assay buffer (25 mM Tris-HCl, pH 7.4, supplemented with 2 mM MgCl_2 and 0.1% BSA) at $30\text{ }^{\circ}\text{C}$ for 1 h. For saturation experiments, total binding was determined at increasing concentrations (0.2-20 nM) of [^3H]Org 43553, whereas nonspecific binding was determined at three concentrations of radioligand in the presence of 10 μM Org 43553 and analyzed by linear regression. Displacement experiments were performed using ten

concentrations of competing ligand in the presence of 20 nM [³H]Org 43553. Non-specific binding was determined in the presence of 10 μ M Org 43553 and represented approximately 50% of the total binding. [³H]Org 43553 did not bind specifically to membranes prepared from CHO cells lacking the LH receptor. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. Incubations were terminated by dilution with 1 mL ice-cold Tris-HCl buffer. Bound from free radioligand was immediately separated by rapid filtration through Whatman GF/B filters using a Millipore manifold. Filters were subsequently washed three times with ice-cold wash buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.05% BSA). Filter-bound radioactivity was determined by scintillation spectrometry (Packard Tri-Carb 2900TR) after addition of 3.5 mL Packard Emulsifier Safe.

¹²⁵I-hCG Membrane aliquots containing 15 μ g protein were incubated for 2½ h at 30 °C in a total volume of 100 μ L assay buffer (25 mM Tris-HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA). For ¹²⁵I-hCG, displacement experiments were performed using ten concentrations of recLH in the presence of 80,000 cpm (~0.1 nM) radioligand. Non-specific binding was determined in the presence of 50 U/mL (~70 nM) recLH and represented approximately 50% of the total binding. ¹²⁵I-hCG did not bind specifically to membranes prepared from CHO cells lacking the LH receptor. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. Incubations were terminated by dilution with 1 mL ice-cold Tris-HCl buffer. Bound from free radioligand was immediately separated by rapid filtration through Whatman GF/C filters pre-soaked with 0.25% poly(ethyleneimine) (PEI) for 1 h using a Millipore manifold. Filters were subsequently washed three times with ice-cold wash buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.05% BSA). Filter-bound radioactivity was determined in a γ -counter (Wallac, Wizard 1470).

4.2.6 Radioligand Association and Dissociation Assays

Association experiments were performed by incubating membrane aliquots containing 20 μ g protein in a total volume of 100 μ L assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA) at 30 °C for 3 h with 80,000 cpm of ¹²⁵I-hCG or for 90 min with 20 nM [³H]Org 43553. The amount of radioligand bound to the receptor was measured at various time intervals during incubation. Dissociation experiments were

performed by preincubating membrane aliquots containing 20 µg protein in a total volume of 100 µL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA) at 30 °C for 2½ h with 80,000 cpm ¹²⁵I-hCG or for 1 h with 20 nM [³H]Org 43553. After preincubation, dissociation was initiated by addition of 50 U/mL recLH for ¹²⁵I-hCG. For [³H]Org 43553 dissociation was initiated by addition of 10 µM Org 43553 (control), 10 mL assay buffer (100-fold dilution) or 50 U/mL recLH or combinations thereof, as explained in the Results section. The amount of radioligand still bound to the receptor was measured at various time intervals for a total of 240 min (¹²⁵I-hCG) or 180 min ([³H]Org 43553). Incubations were terminated and samples were obtained and analyzed as described above.

4.2.7 Luciferase Assays

CHO_hLHr_{luc} cells were grown as described above. On the day of the assay, cells were washed with PBS and then harvested using trypsin (0.25% (w/v) in PBS containing 4.4 mM EDTA). Cells were resuspended in assay medium consisting of DMEM and F12 (1:1) supplemented with 1 µg/mL insulin and 5 µg/mL apo-transferrin. Typically, a well contained 30 µL of test compound, 30 µL of assay medium and 30 µL cell suspension containing 7.5×10^5 cells/mL. Luciferase assays were performed using ten concentrations of test compound. Basal activity was determined in the presence of assay medium and represented approximately 10% of the maximal activity. Maximal receptor activity was determined in the presence of 1 nM recLH and was set at 100% in all experiments, whereas basal activity was set at 0% in all experiments. After 4 h stimulation, 50 µL of luciferase (PerkinElmer, Groningen, The Netherlands) was added to each well for detection of luciferase protein and plates were left at room temperature for 30 minutes in the dark. Finally, the luminescence signal was quantified on a Microbeta Trilux 1450 Luminescence Counter (PerkinElmer, Groningen, The Netherlands).

4.2.8 Data Analysis

All binding data were analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 4.02 (GraphPad Software Inc, San Diego, CA, U.S.A.). EC₅₀ values were directly obtained from the dose-response curves and inhibitory binding constants (K_i values) were derived from the IC₅₀ values according to $K_i = IC_{50}/(1 + [C]/K_d)$ where [C] is the

concentration of the radioligand and K_d its dissociation constant.²⁴⁵ The K_d value of [³H]Org 43553 at CHO_hLHr_{luc} membranes was obtained by computer analysis of saturation curves. Dissociation constants, k_{off} , were obtained by computer analysis of the exponential decay of either ¹²⁵I-hCG or [³H]Org 43553 bound to the receptor. Association rates were calculated according to the equation $k_{on} = (k_{obs} - k_{off})/[L]$, where k_{obs} was obtained by computer analysis of the exponential association of either ¹²⁵I-hCG or [³H]Org 43553 bound to the receptor and $[L]$ is the amount of radioligand used for the association experiments. All values obtained are means of at least three independent experiments performed in duplicate.

4.3 RESULTS

4.3.1 Binding Assay Optimization

The assay conditions for [^3H]Org 43553 binding to CHO hLHR_{luc} membranes were optimized according to a general radioligand binding protocol in our laboratory.²⁶⁵ We started our optimization efforts with [^3H]Org 43553 and 15 μg of protein in a simple buffer of low ionic strength (25 mM Tris HCl, pH 7.4) to which 2 mM MgCl_2 was added as is often done with agonist radioligands. Figure 4.1 summarizes the results of these experiments in which we studied buffer components, filters and filter pretreatment, and membrane concentration.

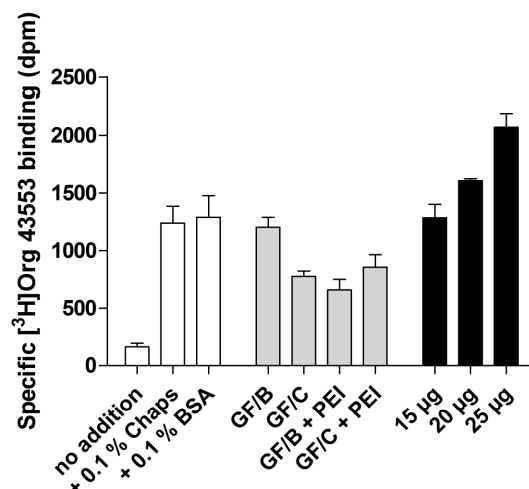


Figure 4.1 Optimization of specific [^3H]Org 43553 binding to CHO hLHR_{luc} membranes. The effect of buffer composition, filter and filter treatment (with or without 0.25% PEI), and protein amount on the amount of specific radioligand binding was surveyed. Bar graph presentation of the results from a single experiment performed in duplicate. These experiments were repeated one more time with similar outcome. White bars: 15 μg of protein, 20 nM [^3H]Org 43553, grey bars: 15 μg of protein, 20 nM [^3H]Org 43553 and 0.1% BSA, black bars: 20 nM [^3H]Org 43553, 0.1% BSA, GF/B filters.

Firstly, a relatively high concentration (20 nM) of [^3H]Org 43553 was needed for an appreciable window of specific binding, which was greatly improved by the addition of BSA or CHAPS. It was decided to continue with 0.1% BSA in the assay buffer. Secondly, the initial choice to use uncoated GF/B glass fiber filters to separate free from membrane-bound

radioligand resulted in the highest specific binding; coating with PEI was not favorable. Next, the amount of protein used was increased to 20 µg, as this yielded a desired window of more than 1500 dpm. Initial kinetic association experiments taught us that the optimal incubation time was 60 min at 30 °C.

4.3.2 Radioligand Saturation Experiments

Saturation binding assays were performed with [³H]Org 43553. The results of a representative saturation experiment are shown in Figure 4.2. Binding of [³H]Org 43553 to membranes of CHO cells expressing the human LH receptor was saturable and best described by a one-site model. The K_D value and B_{max} value obtained from the saturation experiments were 2.4 ± 0.4 nM and 1.6 ± 0.2 pmol/mg protein, respectively. The K_D value for [³H]Org 43553 obtained with these experiments was used to derive K_i rather than IC_{50} values for analogues of Org43553 (see below). In the presence of 70 nM recLH, the K_D value was unaffected, while the B_{max} value was decreased by $24 \pm 2\%$ (Figure 4.2), indicative for a non-competitive interaction.

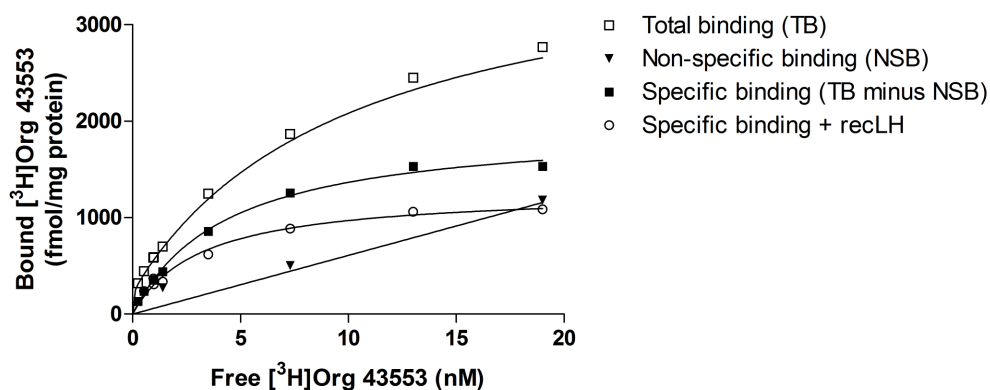


Figure 4.2 Saturation of [³H]Org 43553 to luteinizing hormone receptors in the absence (control) or presence of 70 nM recLH. The control specific binding was determined by subtracting the non-specific binding from the total binding curve. The control K_D value was 2.4 ± 0.4 nM and the B_{max} value was 1.6 ± 0.2 pmol/mg protein ($n = 5$). A similar experiment was performed in the presence of 70 nM recLH, of which only the specific binding is shown. Representative graphs from one experiment performed in duplicate.

Specific binding for ^{125}I -hCG was also demonstrated on these cell membranes. A consistent difference between total and non-specific binding was observed, but a plateau of specific binding was never reached at the radioligand concentrations used. The use of higher concentrations was considered prohibitively expensive, and spiking the samples with cold ligand yielded ambiguous results. Thus, it appeared impossible to determine K_D and $B_{\max}$ values for ^{125}I -hCG from these equilibrium studies. Therefore, the K_D value of ^{125}I -hCG was derived from its kinetic parameters as described in the next paragraph.

4.3.3 Kinetic Association and Dissociation Experiments

Subsequently, the kinetic behavior of $[^3\text{H}]\text{Org 43553}$ was studied and compared to that of ^{125}I -hCG. The kinetics of both radioligands were determined at 30 °C on CHO_{hLHR}_luc cell membranes. The binding of $[^3\text{H}]\text{Org 43553}$ and ^{125}I -hCG reached equilibrium after approximately 60 min and 150 min, respectively (Figures 4.3 and 4.4). Notably, equilibrium binding remained stable for both radioligands for at least 4 h (data not shown). $[^3\text{H}]\text{Org 43553}$ binding was reversible after the addition of 10 μM Org 43553 and complete dissociation was achieved after approximately 2 h as shown in Figure 4.3. The addition of an excess of recLH after association only resulted in a partial dissociation of $[^3\text{H}]\text{Org 43553}$. When recLH was already present during the preincubation, the $B_{\max}$ value of $[^3\text{H}]\text{Org 43553}$ was decreased by approximately 25% (cf. Figure 4.2) and the dissociation rate was 1.7 ± 0.1 -fold increased. The dissociation rate of the radioligand obtained by the ‘infinite dilution’ method ($k_{\text{off}} = 0.020 \pm 0.006 \text{ min}^{-1}$), however, was equal to the rate obtained in the control experiment in which dissociation was initiated by the addition of cold ligand (Table 4.1). The further addition of 10 μM Org 43553 or 70 nM recLH in the infinite dilution experiment did not alter the dissociation rate of $[^3\text{H}]\text{Org 43553}$, as examined at 60 min of dissociation. For ^{125}I -hCG this was somewhat different: the addition of 70 nM recLH resulted in dissociation of this radioligand, however, after 4 h only 50% was displaced (Figure 4.4). In Table 4.1, the association (k_{on}) and dissociation (k_{off}) constants are listed, as well as the apparent equilibrium dissociation constant (K_D), obtained from the control experiments represented in Figures 4.3 and 4.4. ^{125}I -hCG had a lower dissociation rate constant than $[^3\text{H}]\text{Org 43553}$, namely $0.0084 \pm 0.001 \text{ min}^{-1}$ compared to $0.020 \pm 0.001 \text{ min}^{-1}$, respectively. The k_{obs} values obtained by analysis of the exponential association curves of both radioligands together with the k_{off} values, allowed determination of the kinetic association rate constants, k_{on} (Table 4.1).

The dissociation binding constants (K_D) of the radioligands were derived from the dissociation and association rates. ¹²⁵I-hCG had a 60-fold higher affinity for the LH receptor than [³H]Org 43553, 0.064 nM compared to 4.1 nM, respectively. The latter is in good agreement with the K_D value (2.4 nM) obtained in the saturation analysis.

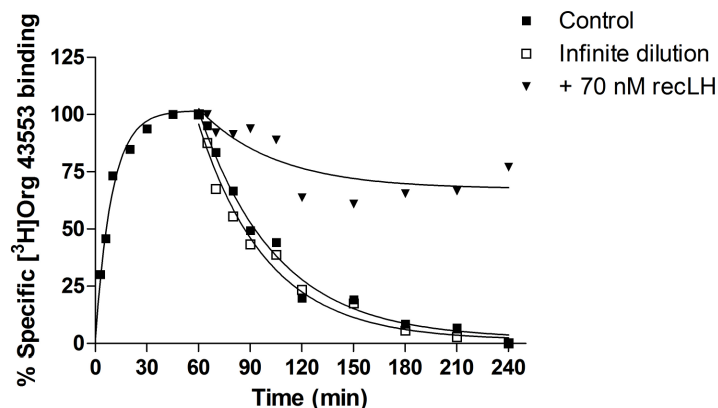


Figure 4.3 Association and dissociation kinetics of [³H]Org 43553 binding to CHO-K1 membranes expressing the human luteinizing hormone receptor at 30°C. Dissociation was either initialized by the addition of 10 μ M Org 43553 (control) or 70 nM recLH ($\blacktriangledown$) or dilution in 100 volumes of assay buffer ($\square$). Representative graphs from one experiment performed in duplicate (see Table 4.1 for kinetic parameters of control experiment).

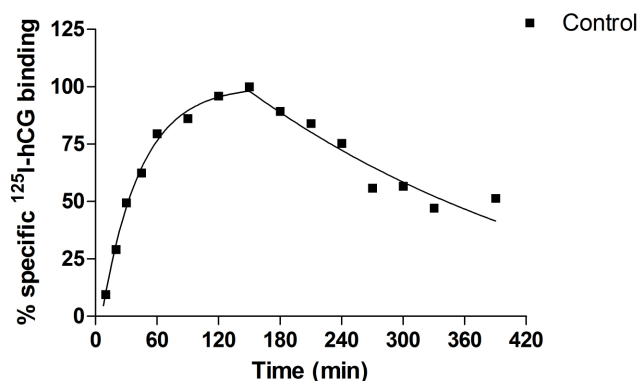


Figure 4.4 Association and dissociation kinetics of ¹²⁵I-hCG binding to CHO-K1 membranes expressing the human luteinizing hormone receptor at 30 °C. Dissociation was initialized by the addition of 70 nM recLH (control). Representative graphs from one experiment performed in duplicate (see Table 4.1 for kinetic parameters).

Table 4.1 Association (k_{on}) rate, dissociation (k_{off}) rate constants and the apparent dissociation constant (K_D) of radiolabeled hCG and Org 43553.

Radioligand	k_{on} ($\text{nM}^{-1} \text{min}^{-1}$) ^a	k_{off} (min^{-1}) ^a	K_D (nM) ^b
^{125}I -hCG	0.13 ± 0.006	0.0084 ± 0.0006	0.064
$[^3\text{H}]$ Org 43553	0.0051 ± 0.0007	0.021 ± 0.001	4.1

^a The values of the kinetic association and dissociation rate constants were obtained by analysis of the exponential association and dissociation of either ^{125}I -hCG or $[^3\text{H}]$ Org 43553 bound to human luteinizing hormone receptors.

^b The dissociation constant was defined as the ratio of k_{off} - and k_{on} -values.

Values are means ($\pm$ S.E.M.) of at least three separate assays performed in duplicate.

4.3.4 Radioligand Displacement Assays

After this characterization of the radioligand, the affinities of non-labeled compounds **1-6** for the human luteinizing hormone receptor were determined (Table 4.2 and Figure 4.5). Full displacement curves were carried out with $[^3\text{H}]$ Org 43553 on CHO_hLHr_{luc} membranes, since the small molecule compounds were not able to displace ^{125}I -hCG from the human luteinizing hormone receptor (Table 4.2). Compound **1** (Org 41841) was potent, displacing $[^3\text{H}]$ Org 43553 with a K_i value of 17 ± 5 nM. Replacement of the S-Me group of **1** with a phenyl group had a negative effect on receptor affinity (**2**, Org 41247). Enlargement of

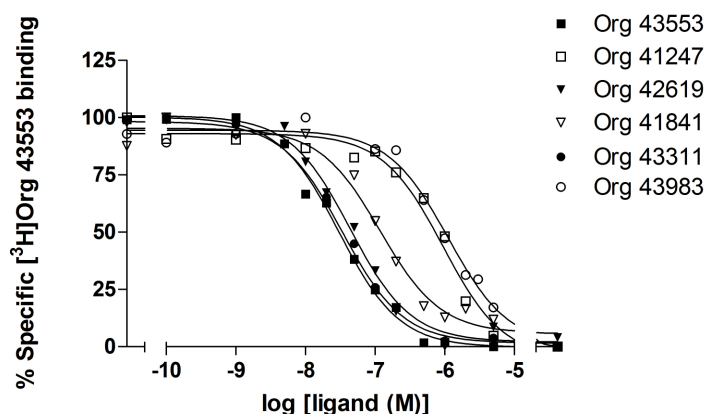
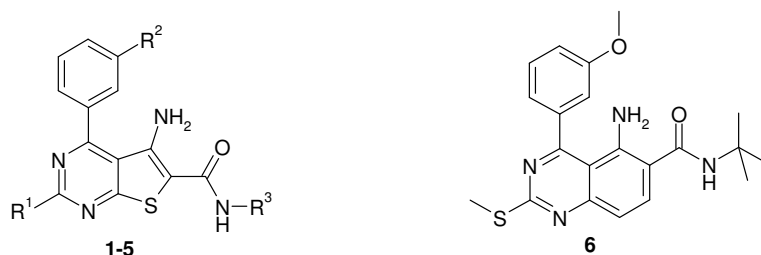


Figure 4.5 Displacement of $[^3\text{H}]$ Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes. Representative graphs from one experiment performed in duplicate (see Table 4.2 for affinity values).

Table 4.2 Receptor affinity assessed with either [³H]Org 43553 or ¹²⁵I-hCG, and receptor activity of compounds **1-6** and recombinant LH at the human luteinizing hormone receptor.

Compound	R ¹	R ²	R ³	[³ H]Org 43553 binding ^a	¹²⁵ I-hCG binding ^b	EC ₅₀ (nM) ^c
1 (Org 41841)	SMe	OMe	<i>t</i> Bu	17 ± 5	1 (0-1)	13 ± 1
2 (Org 41247)	Ph	OMe	<i>i</i> Pr	100 ± 6	4 (0-8)	119 ± 18
3 (Org 42619)	SMe		<i>t</i> Bu	4.7 ± 0.7	0 (0-0)	3.0 ± 0.5
4 (Org 43311)	SMe		<i>t</i> Bu	4.1 ± 0.7	4 (0-7)	1.3 ± 0.4
5 (Org 43553)	SMe		<i>t</i> Bu	3.3 ± 0.3	7 (6-8)	1.7 ± 0.2
6 (Org 43983)	-	-	-	82 ± 17	0 (0-0)	111 ± 3
recLH	-	-	-	32 (31-33) ^d	0.61 ± 0.1 ^e	0.078 ± 0.002

* Position of the tritium substitution in Org 43553.

^a Displacement of specific [³H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes (K_i ± S.E.M. (nM), n ≥ 3, duplicate) or ^d % displacement of specific [³H]Org 43553 binding at 70 nM recLH (n = 4, duplicate).^b % Displacement of specific ¹²⁵I-hCG binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes at 10 μM concentrations (n = 2, duplicate) or ^e displacement of specific ¹²⁵I-hCG binding (K_i ± S.E.M. (nM), n = 3, duplicate)^c cAMP-mediated luciferase activity in CHO-K1 cells that stably express the human luteinizing hormone receptor and CRE-luciferase reporter gene (mean ± S.E.M., n ≥ 3, duplicate).^d % Displacement of specific [³H]Org 43553 binding at 70 nM recLH (n = 4, duplicate)^e Displacement of specific ¹²⁵I-hCG binding [K_i ± S.E.M. (nM), n = 3, duplicate]

the meta-oriented substituent on the 4-phenylgroup had a positive impact, resulting in compounds **3-5** (Org 42619, 43311 and 43553, respectively) with a 4- to 5- fold higher affinity than compound **1**. Replacement of the thienyl moiety in the scaffold of **1** (Org 41841) by a phenyl ring (**6**, Org 43983) resulted in an approximately five-fold lower affinity. This value was comparable to the affinity of compound **2** (Org 41247). Org 43553 was chosen to be labeled with tritium, because it was one of the compounds with a nanomolar potency in

the initial functional assay screen. Accordingly, it was assumed to also exhibit a nanomolar binding affinity, which appeared to be the case (Table 4.2). In comparison, the endogenous ligand, recLH, was able to displace ^{125}I -hCG with a 3-fold higher affinity of 0.61 ± 0.1 nM, whereas it only partially displaced [^3H]Org 43553 at a high concentration (Table 4.2). Labeling Org 43553 also had a practical reason, because the relatively acidic proton next to the morpholino-group was easily exchanged with tritium without a need for the synthesis of precursor molecules.

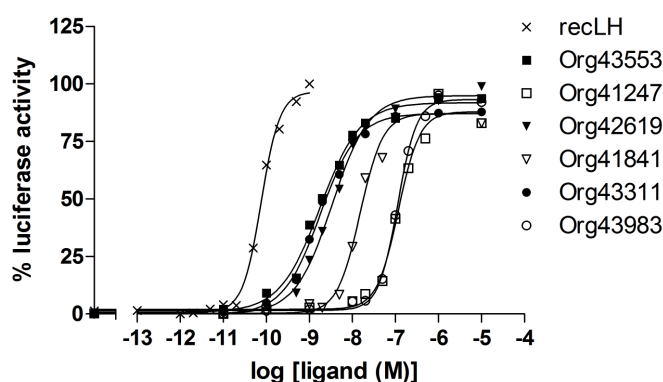


Figure 4.6 Concentration-effect curves of recLH and low molecular weight ligands for cAMP-mediated luciferase production through human luteinizing hormone receptors. Representative graphs from one experiment performed in duplicate (see Table 4.2 for EC₅₀ values).

4.3.5 Agonistic Activity and Selectivity at the LH Receptor

In addition to radioligand displacement experiments, cAMP-induced luciferase assays were performed with compounds **1-6**. It follows from Figure 4.6 that all compounds were able to almost fully activate the receptor in comparison to recLH (87-95%). Their potencies were determined from the relatively steep dose-response curves and ranged from 1.3 nM (**4**) to 119 ± 18 nM (**2**) (Table 4.2). In comparison, the endogenous ligand, recLH, had an EC₅₀ value of 78 ± 2 pM in this cellular assay. There was a high correlation between data obtained from binding and functional assays ($r^2 = 0.99$; $P < 0.0001$) (Figure 4.7). To obtain a full selectivity profile, Org 43553 was examined on 59 different drug targets, including 39 GPCRs. Org 43553 was at least ten-fold selective for the LH receptor versus the other

glycoprotein hormone receptors (data not shown). LH receptor selectivity was more than 3000-fold for all other targets.

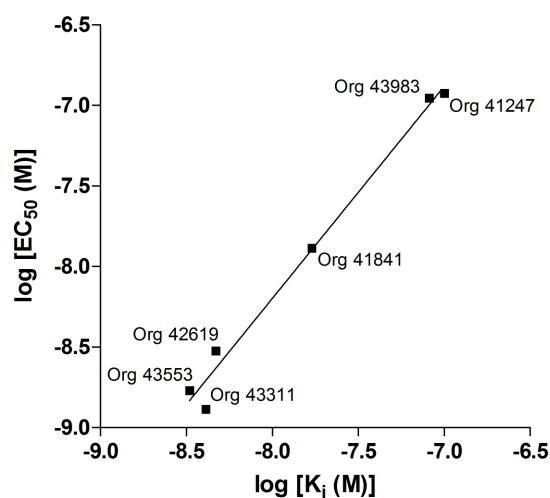


Figure 4.7 Comparison between the logarithms of affinity values (M) of [³H]Org 43553 binding and of EC₅₀ values (M) obtained in luciferase assays for human luteinizing hormone receptors stably expressed on CHO-K1 cells ($r^2 = 0.99$; $P < 0.0001$).

4.4 DISCUSSION

The present study describes the first small molecule radioligand for the LH receptor. [³H]Org 43553 has a thienopyrimidine core and literature survey reveals that this scaffold is a widely used pharmacophore, for example, as a serotonin receptor ligand,²⁶⁶ kinase inhibitor,²⁶⁷ and antimicrobial agent.²⁶⁸ The substitution pattern can be diverse, however, resulting in selectivity towards different targets. Org 43553 is selective for the LH receptor though, at least tenfold when compared with other glycoprotein hormone receptors, and more than 3000-fold selective for a whole panel of drug targets, including serotonin receptors.

The results of this paper show that [³H]Org 43553 is a highly potent and selective agonistic radioligand that represents a novel tool for the screening of low molecular weight ligands for the LH receptor. The radioligand's receptor binding was saturable with a high affinity ($K_D = 2.4 \pm 0.4$ nM). Kinetic experiments showed that both association and dissociation were much faster for [³H]Org 43553 than for ¹²⁵I-hCG (Table 4.1). In addition, ¹²⁵I-hCG did not fully dissociate from the receptor. This has been described before for ¹²⁵I-hCG, where dissociation of specific binding was only 40% after 8 h at 37 °C.²⁶⁹ This pseudo-irreversibility of dissociation most likely precluded the determination of the radioligand's K_D and B_{max} values from equilibrium saturation studies in the present experimental set-up. Others have reported a K_D value of 0.1 nM for ¹²⁵I-hCG, when binding to intact COS-7 cells transiently expressing the rat LH receptor was measured.^{270,271} This value is in good agreement with the K_D -value of 0.064 nM obtained from the kinetic experiments presented here. The dissociation of another glycoprotein hormone, thyroid-stimulating hormone (TSH), however, was shown to be complete and much faster, already at 22 °C.²⁷² In the kinetic comparison in the present study the binding of [³H]Org 43553, unlike that of ¹²⁵I-hCG, was fully reversible. This feature rendered [³H]Org 43553 a more suitable radioligand for further displacement studies.

To further explore the binding characteristics of this novel radioligand, assays were performed where the dissociation was initiated by the 'infinite dilution' method.²⁷³ A 100-fold ('infinite') dilution gave a comparable dissociation rate as the rate obtained by the addition of excess cold Org 43553 (Figure 4.3), which suggests that maximal radioligand dissociation was achieved and that Org 43553 binds to a single non-interacting site. Infinite dilution in the presence of excess cold Org 43553 or recLH did not alter the dissociation rate of the radioligand; further proof of Org 43553's binding to a single, non-interacting site. To get more insight into the fact that recLH showed some displacement of Org 43553, the effect

of recLH on the dissociation of [³H]Org 43553 was monitored. As shown in Figure 4.3, recLH induces some dissociation of the radioligand, which correlates with the effect seen on the saturation (Figure 4.2) and displacement equilibrium binding of [³H]Org 43553 (Table 4.2).

Characterization of the new radioligand was continued by radioligand displacement assays with non-labeled Org 43553 and a number of derivatives. Recently, a high throughput screen identified compounds with a thieno[2,3-*d*]pyrimidine core as potent and selective receptor agonists for the LH receptor.²¹ It was shown that the amide group at position 6 of the heterocyclic core was crucial for low nanomolar activity. From this series Org 41841 (**1**) was the most potent. In addition, a more bulky meta-substituent on the phenyl group at position 4 resulted in more potent compounds.²¹¹ The brief structure-activity relationship (SAR) study presented here shows that the affinity of Org 41841 can be improved 5-fold through meta substitution, e.g. resulting in Org 43553 (Table 4.2). When compound **1** is compared to compound **3**, it becomes clear that the introduction of an additional H-bond donor by the amide-group in combination with steric bulk from the benzoyl group is favorable for receptor affinity. Introduction of the ethanolamine- (**4**) and morpholinogroup (**5**) was allowed, and helped to increase water solubility. The compounds were not able to displace ¹²⁵I-hCG in a binding assay (Table 4.2), which is in accordance with similar experiments on the FSH receptor.²⁰⁸ Recently, it was shown in docking and mutational studies that Org 41841 has its putative binding site in the seven-transmembrane (7-TM) part of the receptor,^{191,194} unlike the presumed binding of hCG and LH to the N-terminus of the receptor. This is in line with many other class A GPCRs, for example the adenosine A_{2A},²⁷⁴ D₃ dopamine²⁷⁵ and the GnRH receptor,²⁷⁶ which bind their cognate ligands in the 7-TM domain as retinal in the rhodopsin receptor.⁴⁰ Therefore, we assume that all small molecule ligands reported here also bind in this domain of the LH receptor, explaining why the endogenous ligand, ¹²⁵I-hCG, is not displaced. Conversely, recLH has some effect on [³H]Org 43553 displacement. It is feasible that the recLH-occupied rather than ‘empty’ receptor is seen differently by LMW compounds such as Org 43553.

In the cAMP-mediated luciferase assay, the compounds studied exhibited a rank order of potency similar to that observed in binding experiments (Figure 4.5). Although the endogenous ligand, recLH, was more potent, the small molecule ligands are potent in binding (K_i-values ranging from 3.3 – 100 nM) and in functional assays (EC₅₀-values ranging from 1.3 – 119 nM).

In conclusion, equilibrium saturation and displacement, and kinetic association and dissociation assays have been performed to elucidate the binding characteristics of the first small molecule radioligand, [^3H]Org 43553, at the human LH receptor. It was shown that a high correlation exists between affinity and activity of this series of low molecular weight ligands. Moreover, the development of this binding assay will aid in the identification and elucidation of the SAR of newly synthesized small molecule LH receptor ligands.

CHAPTER

5

SUBSTITUTED TERPHENYL COMPOUNDS AS THE FIRST CLASS OF LOW MOLECULAR WEIGHT ALLOSTERIC INHIBITORS OF THE LUTEINIZING HORMONE RECEPTOR

The luteinizing hormone (LH) receptor plays an important role in fertility and certain cancers. The endogenous ligands, human chorionic gonadotropin (hCG) and LH bind to the large N terminal domain of the receptor. We recently reported on the first radiolabeled low molecular weight (LMW) agonist for this receptor, [³H]Org 43553, which was now used to screen for new LMW ligands. We identified a terphenyl derivative that inhibited [³H]Org 43553 binding to the receptor, which led us to synthesize a number of derivatives. The most potent compound of this terphenyl series **24** (LUF5771) was able to increase the dissociation rate of [³H]Org 43553 by 3.3-fold (at 10 μM). In a functional assay, the presence of **24** resulted in a 2- to 3-fold lower potency of both Org 43553 and LH. Thus, the compounds presented in this paper are the first LMW ligands that allosterically inhibit the LH receptor.

5.1 INTRODUCTION

The luteinizing hormone (LH) receptor is a member of the glycoprotein hormone receptor family within the class A subfamily of G protein-coupled receptors (GPCRs).¹⁸ While most class A GPCRs recognize low molecular weight (LMW) endogenous ligands that bind in the seven transmembrane (7-TM) domain, the LH receptor has two high molecular weight endogenous ligands, human chorionic gonadotropin (hCG) and LH. Both hormones bind with high affinity and selectivity to the N terminus of the LH receptor and thereby activate the receptor.²⁵⁹ In the clinic, these so-called gonadotropins are currently used in infertility treatment. The hormones need to be administered by parenteral (subcutaneous or intramuscular) injection.¹⁸⁷

To increase patient convenience and compliance, efforts are made to develop non-peptide orally active gonadotropins as drugs. For the LH receptor only a few compound classes have been described as LMW receptor agonists, such as thienopyrimidine, Org 43553. This compound was shown to have *in vivo* efficacy upon oral administration.⁶⁹ Recently the first high molecular weight antagonist was reported for the LH receptor, two fused beta-subunits of hCG.¹⁸⁹ However, LMW antagonists have not been reported so far (*Chapter 2*). Antagonists for the LH receptor may be novel contraceptive agents. In addition, antagonists could be used against ovarian cancer related to menopause.⁸⁰ Therefore, next to LMW agonists, antagonists would be very beneficial as well.

Recently, we reported on the first radiolabeled LMW agonist for the LH receptor, [³H]Org 43553 (described in *Chapter 4*). Here, we used this radioligand to screen for new LMW ligands at the LH receptor. Initially, 50 compounds were screened for displacement of [³H]Org 43553. Subsequently, the same library was screened in a kinetic radioligand binding assay, where a change in dissociation rate is indicative for allosteric modulation of the radioligand used.²⁷⁷ The latter resulted in a few hits including the terphenyl compound **4**, which we anticipated to be an allosteric inhibitor as it increased the dissociation rate of [³H]Org 43553. Subsequently, several analogues of **4** were synthesized and tested for their effect on the dissociation rate of [³H]Org 43553. This yielded an even more potent allosteric inhibitor, compound **24** (LUF5771) that was further characterized in radioligand dissociation experiments and functional assays. As a consequence, the present study is the first to report LMW allosteric inhibitors of the LH receptor.

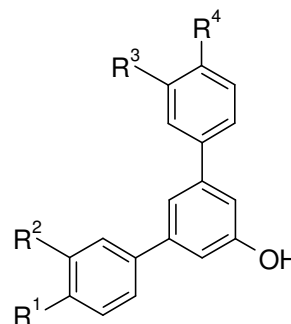
5.2 MATERIALS AND METHODS

5.2.1 Chemistry - Materials

All reagents used were obtained from commercial sources and all solvents were of analytical grade. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 400 (^1H NMR, 400 MHz; ^{13}C NMR, 100 MHz) and Bruker AC 200 (^1H NMR, 200 MHz; ^{13}C NMR, 50 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in δ (ppm). Melting points were determined by Büchi melting point apparatus and are uncorrected. Elemental analyses were performed by Leiden Institute of Chemistry and are within 0.4% of theoretical values unless otherwise stated. Reactions were routinely monitored by TLC using Merck silica gel F254 plates. Microwave reactions were performed on an Emrys Optimizer (Biotage AB). Wattage was automatically adjusted to maintain the desired temperature.

5.2.2 General Methods for Microwave Assisted Suzuki–Miyaura Cross Coupling Reaction (6-10)

To a solution of 3,5-dibromophenol (1 eq) in toluene, $(\text{Ph}_3\text{P})_4\text{Pd}$ (5 mol.%), substituted phenylboronic acid (3 eq), 2 M aq. Na_2CO_3 (2 eq) was added and the resulting mixture was heated in microwave at 150 °C for 10 min. Upon completion (TLC), the reaction mixture was diluted with EtOAc (100 mL), washed with H_2O , brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:9) to afford the desired product.



[1,1',3',1'']Terphenyl-5'-ol (6). Low melting colorless solid, yield 71%. ^1H NMR (400 MHz, CDCl_3): δ = 7.69–7.58 (m, 5H), 7.44–7.33 (m, 5H), 7.03 (d, J = 1.6 Hz, 2H), 5.29 (brs, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 156.3, 143.5, 140.8, 129.4, 128.9, 128.0, 127.7, 127.3, 117.4, 113.0 ppm.

4,4''-Dichloro-1,1',3',1''-terphenyl-5'-ol (7). Yield: 130 mg, 34%. ^1H NMR (400 MHz, CDCl_3): δ = 7.56 (d, J = 8.1 Hz, 4H), 7.41 (d, J = 8.4 Hz, 4H), 7.23 (s, 1H), 7.03 (s, 2H), 4.65 (brs, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 157.7, 141.8, 139.4, 133.3, 128.6, 128.2, 117.0, 112.0 ppm.

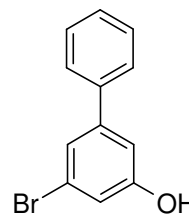
3,3'',4,4''-Tetrachloro-1,1',3',1''-terphenyl-5'-ol (8). Yield: 280 mg, 61%. ^1H NMR (400 MHz, CDCl_3): δ = 7.78 (s, 2H), 7.53–7.48 (m, 4H), 7.25 (s, 1H), 7.03 (s, 2H), 4.63 (brs, 1H) ppm.

4,4''-Dimethoxy-1,1',3',1''-terphenyl-5'-ol (9). Yield: 150 mg, 41%. ^1H NMR (400 MHz, CDCl_3): δ = 7.51 (d, J = 8.8 Hz, 4H), 7.29 (s, 1H), 6.98–6.93 (m, 6H), 5.35 (brs, 1H) 3.82 (m, 6H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 159.5, 156.4, 143.2, 133.6, 128.5, 118.3, 114.5, 111.4, 55.7 ppm.

4,4''-Dimethyl-1,1',3',1''-terphenyl-5'-ol (10). Yield: 220 mg, 67%. ^1H NMR (400 MHz, CDCl_3): δ = 7.51 (d, J = 8.0 Hz, 4H), 7.27 (s, 1H), 7.24 (d, J = 8.0 Hz, 4H), 6.99 (s, 2H), 4.90 (brs, 1H), 2.38 (s, 6H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 145.7, 141.0, 139.6, 131.8, 129.2, 129.1, 119.3, 114.8 22.5 ppm.

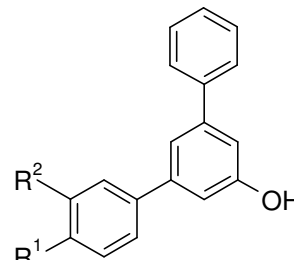
5.2.3 Synthesis of 5-Bromo-biphenyl-3-ol (11)

To a stirred solution of 3,5-dibromophenol (11.95 mmol, 3 g) in toluene (50 mL), phenylboronic acid (12.55 mmol, 1.53 g), $(\text{Ph}_3\text{P})_4\text{Pd}$ (0.6 mmol, 0.69 g), and 2 M aqueous solution of Na_2CO_3 (23.91 mmol, 2.53 g) was added and refluxed for 3 h. The progress of the reaction was monitored by TLC. Upon completion, the reaction was cooled to ambient temperature, diluted with EtOAc (150 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by column chromatography using 10% EtOAc in petroleum ether to obtain compound **11** as colorless oil (Yield: 0.89 g, 30%). ^1H NMR (400 MHz, CDCl_3): δ = 7.52 (d, J = 7.2 Hz, 2H), 7.43 (t, J = 7.2 Hz, 2H), 7.37 (d, J = 7.2 Hz, 1H), 7.31 (s, 1H), 6.98 (d, J = 2.8 Hz, 2H), 5.06 (brs, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 154.5, 141.2, 135.1, 129.2, 128.4, 127.4, 125.1, 123.3, 117.8, 113.5 ppm.



5.2.4 General Methods for Microwave Assisted Suzuki–Miyaura Cross Coupling Reaction (12-15)

To a solution of 5-bromo-biphenyl-3-ol (**11**) in toluene, $(\text{Ph}_3\text{P})_4\text{Pd}$ (5 mol.%), substituted phenylboronic acid (1.5 eq) and 2 M aq. Na_2CO_3 (2 eq) were added and the resulting mixture was heated in microwave at 150 °C for 10 min. Upon completion (TLC), the reaction mixture was diluted with EtOAc (100 mL), washed with H_2O , brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:9) to yield the desired product.



4-Chloro-[1,1',3',1'']terphenyl-5'-ol (12). Yield: 200 mg, 67%. ^1H NMR (400 MHz, CDCl_3): δ = 7.65 (d, J = 8.0 Hz, 2H), 7.58 (d, J = 7.6 Hz, 2H), 7.49–7.36 (m, 5H), 7.34 (s, 1H), 7.03 (s, 1H), 6.98 (s, 1H), 5.14 (brs, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 156.5, 143.9, 142.4, 140.8, 139.4, 129.3, 129.2, 129.1, 128.7, 128.0, 127.5, 119.1, 113.7, 113.2 ppm.

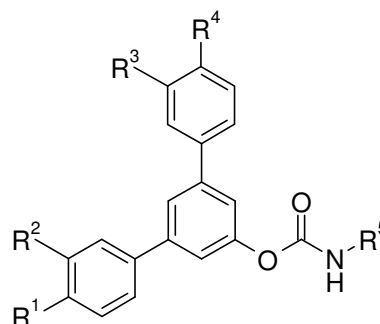
3,4-Dichloro-[1,1',3',1'']terphenyl-5'-ol (13). Yield: 265 mg, 70%. ^1H NMR (400 MHz, CDCl_3): δ = 7.69 (d, J = 2.0 Hz, 2H), 7.53–7.36 (m, 6H), 7.25 (s, 1H), 7.07 (s, 1H), 6.98 (s, 1H), 5.04 (brs, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 155.5, 143.3, 141.1, 140.2, 139.1, 134.3, 132.2, 131.8, 129.8, 129.4, 128.5, 127.5, 126.7, 119.0, 114.2, 113.9 ppm.

4-Methoxy-[1,1',3',1'']terphenyl-5'-ol (14). Yield: 300 mg, 64%. ^1H NMR (400 MHz, CDCl_3): δ = 7.59 (d, J = 7.6 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H), 7.42 (t, J = 7.4 Hz, 2H), 7.36–7.33 (m, 2H), 6.99 (s, 2H), 6.96 (d, J = 8.4 Hz, 2H), 5.27 (brs, 1H), 3.83 (s, 3H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 159.6, 156.5, 143.7, 143.3, 141.2, 133.6, 129.1, 128.5, 127.9, 127.5, 118.8, 114.6, 113.0, 112.8, 55.7 ppm.

4-Methyl-[1,1',3',1'']terphenyl-5'-ol (15). Yield: 194 mg, 62%. ^1H NMR (400 MHz, CDCl_3): δ = 7.60 (d, J = 7.6 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.44 (t, J = 7.8 Hz, 2H), 7.38–7.34 (m, 2H), 7.25 (d, J = 7.2 Hz, 2H), 7.01 (s, 2H), 4.99 (brs, 1H), 2.42 (s, 3H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 155.6, 141.7, 140.3, 139.7, 139.1, 133.6, 129.8, 129.7, 129.1, 127.9, 127.5, 127.3, 119.1, 113.2, 113.1, 21.4 ppm.

5.2.5 General Procedure for Carbamate Synthesis (4, 16-25, 34-41)

Triethylamine (1.5 eq) was added to a stirred solution of phenol (1 eq) in anhydrous dichloromethane and stirred at ambient temperature for 15 min. Isocyanate (1.5 eq) was added and stirred for another 12 to 18 h at ambient temperature. Upon completion (TLC), the reaction mixture was diluted with dichloromethane (100 mL), washed with water, brine, dried and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (dichloromethane/petroleum ether, 4:1) to get the carbamate. The column purified carbamate was crystallized using dichloromethane and petroleum ether.



Phenyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (4). Yield: 259 mg, 87%. M.p.: 165–166 °C. ^1H NMR (200 MHz, CDCl_3): δ = 7.68–7.61 (m, 5H), 7.51–7.31 (m, 12H), 7.17–7.05 (m, 2H), 4.08 (brs, 1H), 3.85 (s, 3H) ppm. ^{13}C NMR (50 MHz, CDCl_3): δ = 164.1, 151.1, 143.0, 137.2, 129.1, 128.7, 127.6, 127.2, 123.9, 123.3, 119.1, 118.8 ppm. MS (ES^+): 366 (MH^+). Anal. ($\text{C}_{25}\text{H}_{19}\text{NO}_2$) C, H, N.

(4-Chloro-phenyl)-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (16). Yield: 290 mg, 89%. ^1H NMR (400 MHz, CDCl_3): δ = 7.67 (t, J = 1.6 Hz, 1H), 7.63–7.57 (m, 4H), 7.48–7.35 (m, 10H), 6.88 (td, J = 8.8 Hz, J = 2.4 Hz, 2H), 7.04 (brs, 1H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 151.5, 143.6, 140.5, 129.5, 129.2, 128.1, 127.6, 123.9, 119.5, 114.3 ppm. Anal. ($\text{C}_{25}\text{H}_{18}\text{ClNO}_2$) C, H, N.

(4-Methoxy-phenyl)-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (17). Yield: 300 mg, 93%. ^1H NMR (400 MHz, CDCl_3): δ = 7.67–7.59 (m, 5H), 7.47–7.35 (m, 10H), 6.88 (d, J = 8.8 Hz, 2H), 4.08 (brs, 1H), 3.85 (s, 3H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 151.6, 143.1, 140.3, 128.8, 127.8, 127.2, 123.1, 120.8, 119.3, 114.3, 55.5 ppm. Anal. ($\text{C}_{26}\text{H}_{21}\text{NO}_3$) C, H, N.

Ethyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (18). Yield: 240 mg, 93%. ^1H NMR (400 MHz, CDCl_3): δ = 7.62–7.55 (m, 5H), 7.48–7.40 (m, 4H), 7.39–7.32 (m, 4H), 5.08 (brs, 1H), 3.38–3.29 (m, 2H), 1.24 (t, J = 7.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 152.1, 143.2, 140.7, 129.1, 129.0, 127.9, 127.8, 127.6, 127.5, 123.3, 119.6, 36.5, 15.4 ppm. Anal. ($\text{C}_{21}\text{H}_{19}\text{NO}_2 \cdot 0.5 \text{H}_2\text{O}$) C, H, N.

Propyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (19). Yield: 180 mg, 66%. ^1H NMR (400 MHz, CDCl_3): δ = 7.65–7.60 (m, 5H), 7.46–7.41 (m, 4H), 7.38–7.32 (m, 4H),

5.12 (brs, 1H), 3.27–3.20 (m, 2H), 1.64–1.55 (m, 2H), 0.95 (t, $J = 8.3$ Hz, 3H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 152.1, 143.2, 140.7, 129.1, 128.0, 127.6, 127.5, 123.3, 119.6, 43.3, 23.4, 11.6$ ppm. Anal. ($\text{C}_{22}\text{H}_{21}\text{NO}_2 \cdot 0.5 \text{H}_2\text{O}$) C, H, N.

Butyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (20). Yield: 240 mg, 85%. ^1H NMR (400 MHz, CDCl_3): $\delta = 7.63\text{--}7.60$ (m, 5H), 7.46–7.39 (m, 4H), 7.38–7.33 (m, 4H), 5.08 (brs, 1H), 3.31–3.24 (m, 2H), 1.60–1.53 (m, 2H), 1.44–1.35 (m, 2H), 0.95 (t, $J = 7.2$ Hz, 3H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 154.6, 143.0, 140.4, 129.5, 128.8, 127.7, 127.3, 127.2, 124.0, 123.0, 119.2, 41.0, 31.9, 19.9, 13.7$ ppm. Anal. ($\text{C}_{23}\text{H}_{23}\text{NO}_2 \cdot 0.5 \text{H}_2\text{O}$) C, H, N.

Pentyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (21). Yield: 130 mg, 89%. ^1H NMR (400 MHz, CDCl_3): $\delta = 7.66\text{--}7.61$ (m, 5H), 7.45–7.40 (m, 4H), 7.38–7.31 (m, 4H), 5.10 (brs, 1H), 3.30–3.21 (m, 2H), 1.63–1.52 (m, 2H), 1.41–1.28 (m, 4H), 0.91 (t, $J = 6.4$ Hz, 3H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 152.0, 143.2, 140.7, 129.0, 128.1, 127.9, 127.5, 127.2, 123.2, 123.0, 119.5, 41.6, 31.9, 29.8, 22.6, 14.2$ ppm. Anal. ($\text{C}_{24}\text{H}_{25}\text{NO}_2 \cdot 0.5 \text{H}_2\text{O}$) C, H, N.

Isopropyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (22). Yield: 174 mg, 65%. M.p.: 160 °C. ^1H NMR (200 MHz, CDCl_3): $\delta = 7.67\text{--}7.64$ (m, 4H), 7.50–7.36 (m, 9H), 7.39–7.32 (m, 4H), 4.97 (m, 1H), 4.00–3.90 (m, 1H), 1.27 (d, $J = 6.6$ Hz, 6H) ppm. ^{13}C NMR (50 MHz, CDCl_3): $\delta = 151.6, 142.8, 140.3, 130.0, 128.7, 127.6, 127.2, 122.9, 119.2, 98.0, 43.4, 22.8$ ppm. MS (ES^+): 332 (MH^+). Anal. ($\text{C}_{22}\text{H}_{21}\text{NO}_2$) C, H, N.

tert-Butyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (23). Yield: 200 mg, 71%. M.p.: 139 °C. ^1H NMR (200 MHz, CDCl_3): $\delta = 7.66\text{--}7.63$ (m, 5H), 7.49–7.35 (m, 8H), 5.06 (m, 1H), 1.43 (s, 9H) ppm. ^{13}C NMR (50 MHz, CDCl_3): $\delta = 151.5, 142.8, 140.4, 128.7, 127.5, 127.2, 122.8, 119.3, 28.7$ ppm. Anal. ($\text{C}_{23}\text{H}_{21}\text{NO}_2 \cdot 0.1 \text{H}_2\text{O}$) C, H, N.

Cyclopentyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (24). Yield: 197 mg, 68%. M.p.: 136 °C. ^1H NMR (200 MHz, CDCl_3): $\delta = 7.67\text{--}7.64$ (m, 4H), 7.51–7.38 (m, 9H), 5.18–5.14 (m, 1H), 4.16–4.10 (m, 1H), 2.07–2.04 (m, 2H), 1.70–1.30 (m, 6H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 153.9, 151.7, 142.8, 140.3, 128.7, 127.6, 127.2, 122.9, 119.2, 53.0, 33.1, 23.9$ ppm. Anal. ($\text{C}_{24}\text{H}_{23}\text{NO}_2$) C, H, N.

Cyclohexyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (25). Yield: 139 mg, 70%. ^1H NMR (400 MHz, CDCl_3): $\delta = 7.74\text{--}7.64$ (m, 5H), 7.54–7.47 (m, 4H), 7.45–7.39 (m, 4H), 5.13 (t, $J = 7.6$ Hz, 1H), 3.74–3.60 (m, 1H), 2.17–2.02 (m, 2H), 1.85–1.53 (m, 3H), 1.48–1.20 (m, 5H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 154.0, 152.1, 143.2, 140.7, 129.7,$

129.0, 128.2, 127.9, 127.5, 123.1, 119.6, 50.5, 33.5, 25.7, 25.0 ppm. Anal. ($C_{25}H_{25}NO_2 \cdot 0.5 H_2O$) C, H, N.

4-Chloro-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (34). Yield: 149 mg, 73%. 1H NMR (400 MHz, $CDCl_3$): δ = 7.60 (d, J = 7.6 Hz, 2H), 7.56 (s, 1H), 7.54 (d, J = 8.4 Hz, 2H), 7.47–7.38 (m, 5H), 7.34 (s, 1H), 7.30 (s, 1H), 5.06 (d, J = 7.2 Hz, 1H), 4.12–4.04 (m, 2H), 2.08–1.98 (m, 2H), 1.78–1.61 (m, 4H), 1.57–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.2, 144.5, 141.2, 139.7, 137.7, 133.0, 129.2, 129.1, 128.8, 128.1, 127.6, 123.1, 119.9, 119.5, 53.4, 33.5, 23.9 ppm. Anal. ($C_{22}H_{22}ClNO_2$) C, H, N.

4,4''-Dichloro-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (35). Yield: 118 mg, 88%. 1H NMR (400 MHz, $CDCl_3$): δ = 7.54 (s, 1H), 7.52 (d, J = 8.0 Hz, 4H), 7.40 (d, J = 8.0 Hz, 4H), 7.30 (s, 2H), 5.06 (d, J = 7.2 Hz, 1H), 4.12–4.04 (m, 2H), 2.10–1.99 (m, 2H), 1.78–1.60 (m, 4H), 1.57–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.2, 142.2, 139.0, 134.2, 129.3, 128.8, 122.8, 119.8, 53.4, 33.5, 23.9 ppm. Anal. ($C_{24}H_{21}Cl_2NO_2 \cdot 0.5 H_2O$) C, H, N.

3,4-dichloro-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (36). Yield: 130 mg, 87%. 1H NMR (400 MHz, $CDCl_3$): δ = 7.45 (d, J = 7.6 Hz, 1H), 7.65–7.52 (m, 3H), 7.50–7.34 (m, 6H), 7.28 (s, 1H), 5.07 (d, J = 6.4 Hz, 1H), 4.10–4.01 (m, 2H), 2.10–1.98 (m, 2H), 1.75–1.62 (m, 4H), 1.56–1.46 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.2, 140.5, 139.2, 137.7, 136.7, 132.5, 132.0, 131.1, 130.6, 129.7, 129.3, 128.5, 127.5, 123.1, 119.5, 119.4, 53.4, 33.5, 23.9 ppm. Anal. ($C_{24}H_{21}Cl_2NO_2 \cdot 0.5 H_2O$) C, H, N.

3,3'',4,4''-tetrachloro-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (37). Yield: 200 mg, 70%. 1H NMR (400 MHz, $CDCl_3$): δ = 7.68 (d, J = 2.0 Hz, 2H), 7.54–7.49 (m, 2H), 7.46 (s, 1H), 7.43–7.38 (m, 2H), 7.34 (d, J = 1.0 Hz, 2H), 5.07 (d, J = 7.2 Hz, 1H), 4.12–4.03 (m, 2H), 2.09–2.01 (m, 2H), 1.78–1.62 (m, 4H), 1.57–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.4, 141.1, 140.3, 133.3, 132.4, 131.1, 129.3, 126.8, 122.7, 120.3, 53.5, 33.5, 23.9 ppm. Anal. ($C_{24}H_{19}Cl_4NO_2 \cdot 0.5 H_2O$) C, H, N.

4-Methoxy-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (38). Yield: 220 mg, 68%. 1H NMR (400 MHz, $CDCl_3$): δ = 7.66 (d, J = 7.2 Hz, 2H), 7.62 (s, 1H), 7.60 (d, J = 8.8 Hz, 2H), 7.48 (t, J = 7.2 Hz, 2H), 7.39 (t, J = 7.2 Hz, 1H), 7.33 (s, 2H), 7.01 (d, J = 8.8 Hz, 2H), 5.09 (d, J = 7.6 Hz, 1H), 4.17–4.10 (m, 2H), 3.92 (s, 3H), 2.09–1.99 (m, 2H), 1.76–1.62 (m, 4H), 1.56–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.5, 152.5, 141.5, 140.2, 138.7, 136.7, 129.0, 128.6, 127.9, 127.6, 122.9, 119.1, 119.0, 114.5, 55.7, 53.4, 33.5, 23.9 ppm. Anal. ($C_{25}H_{25}NO_3$) C, H, N.

4,4''-Dimethoxy-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (39). Yield: 142 mg, 86%. ^1H NMR (400 MHz, CDCl_3): δ = 7.56 (s, 1H), 7.54 (d, J = 8.8 Hz, 4H), 7.24 (s, 2H), 6.90 (d, J = 8.8 Hz, 4H), 5.02 (d, J = 7.6 Hz, 1H), 4.12–4.04 (m, 2H), 3.85 (s, 6H), 2.09–2.01 (m, 2H), 1.77–1.62 (m, 4H), 1.57–1.48 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 158.2, 152.7, 140.2, 138.0, 132.2, 131.3, 128.7, 118.6, 114.5, 55.6, 53.5, 33.5, 23.9 ppm. Anal. ($\text{C}_{26}\text{H}_{27}\text{NO}_4$) C, H, N.

4-Methyl-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (40). Yield: 140 mg, 75%. ^1H NMR (400 MHz, CDCl_3): δ = 7.65–7.58 (m, 3H), 7.52 (d, J = 8.0 Hz, 2H), 7.43 (t, J = 7.6 Hz, 2H), 7.37–7.30 (m, 3H), 7.24 (d, J = 8.0 Hz, 2H), 5.06 (d, J = 7.6 Hz, 1H), 4.12–4.05 (m, 2H), 2.38 (s, 3H), 2.08–1.99 (m, 2H), 1.75–1.59 (m, 4H), 1.54–1.46 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 156.5, 152.0, 143.1, 140.8, 137.8, 137.7, 129.8, 129.0, 127.9, 127.6, 127.4, 123.0, 119.4, 119.3, 53.3, 33.5, 23.8, 21.4 ppm. Anal. ($\text{C}_{25}\text{H}_{25}\text{NO}_2$) C, H, N.

4,4''-Dimethyl-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (41). Yield: 215 mg, 82%. ^1H NMR (400 MHz, CDCl_3): δ = 7.59 (s, 1H), 7.51 (d, J = 8.0 Hz, 4H), 7.29 (s, 2H), 7.24 (d, J = 8.0 Hz, 4H), 5.04 (d, J = 6.8 Hz, 1H), 4.12–4.03 (m, 2H), 2.39 (s, 6H), 2.08–1.98 (m, 2H), 1.77–1.60 (m, 4H), 1.57–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 152.0, 143.1, 137.9, 129.8, 127.4, 127.3, 122.9, 119.1, 53.3, 33.5, 23.9, 21.4 ppm. Anal. ($\text{C}_{26}\text{H}_{27}\text{NO}_2 \cdot 0.5 \text{H}_2\text{O}$) C, H, N.

5.2.6 Synthesis of Cyclopentanecarboxylic acid [1,1',3',1'']terphenyl-5'-yl ester (26)

To a solution of cyclopentanecarboxylic acid (1.95 mmol, 0.18 mL) in dichloromethane, Et_3N (1.95 mmol, 0.27 mL) was added and stirred for 15 min. To it, EDAC (0.21 mmol, 400 mg) and HOBt (0.21 mmol, 290 mg) were added followed by [1,1',3',1'']terphenyl-5'-ol (**6**) (1.62 mmol, 400 mg) and Et_3N (1.62 mmol, 0.23 mL). The reaction mixture was then stirred at ambient temperature for 24 h. Upon completion (TLC), the reaction mixture was diluted with dichloromethane (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc /petroleum ether, 1:5) to afford the desired product as colorless solid (130 mg, 24%). ^1H NMR (400 MHz, CDCl_3): δ = 7.65 (s, 1H), 7.62 (d, J = 7.6 Hz, 4H), 7.44 (t, J = 7.6 Hz, 4H), 7.36 (t, J = 7.6 Hz, 2H), 7.28 (s, 2H), 3.07–2.98 (m, 1H), 2.08–1.96 (m, 4H), 1.83–1.75 (m, 2H), 1.71–1.62 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 152.0, 143.4, 140.6, 129.1, 128.0, 127.6, 123.7, 119.5, 44.2, 30.4, 26.2 ppm.

5.2.7 Synthesis of 1,3-Dibromo-5-nitro-benzene (**28**)

To a solution of sodium nitrite (2.07 g, 30 mmol) in concentrated sulfuric acid (20 mL) was added slowly a suspension of 2,6-dibromo-4-nitroaniline (**27**) (6 g, 20 mmol) in glacial acetic acid (74 mL), maintaining an internal temperature below 20 °C. After stirring for 30 min, the resulting diazonium salt was then slowly added to a suspension of cuprous oxide (0.24 g, 3 mmol) in 95% ethanol (55 mL). After stirring overnight, the mixture was quenched with water (10 mL). The organic layer was separated, washed with saturated NaHCO₃, dried over anhydrous Na₂SO₄, filtered, and concentrated to afford a brown solid. The aqueous layer from the extracts was diluted, in portions, into 4 times the volume of water, causing precipitation of additional product which was collected by suction filtration. The combined lot of crude product was purified by flash column chromatography (dichloromethane/petroleum ether, 3:2) to afford analytically pure compound **28** as pale yellow solid (2.4 g, 42%). ¹H NMR (400 MHz, CDCl₃): δ = 8.40 (s, 2H), 8.00 (s, 1H) ¹³C NMR (100 MHz, CDCl₃): δ = 151.2, 140.4, 125.9, 123.8 ppm.

5.2.8 Synthesis of 5'-Nitro-[1,1',3',1'']terphenyl (**29**)

To a stirred solution of 1,3-dibromo-5-nitro-benzene (**28**) (5.33 mmol, 1.5 g) in toluene (50 mL), phenylboronic acid (16 mmol, 1.95 g), (Ph₃P)₄Pd (5 mol.%, 310 mg), and 2 M aq. solution of Na₂CO₃ (16 mmol, 1.7 g) was added and refluxed for 16 h. The progress of the reaction was monitored by TLC. Upon completion, the reaction was cooled to ambient temperature, diluted with EtOAc (400 mL), washed with water, brine, dried over anhydrous Na₂SO₄ and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:19) to yield compound **29** as lemon colored fluffy solid (800 mg, 54%).

5.2.9 Synthesis of [1,1',3',1'']Terphenyl-5'-ylamine (**30**)

A homogeneous solution of 3,5-diphenyl-nitrobenzene (**29**) (500 mg, 1.81 mmol) and anhydrous stannous chloride (1.72 g, 9 mmol) in EtOH (5 mL) was heated at 70 °C for 30 min under N₂. Upon cooling, the solvent was removed under reduced pressure and the residue diluted with excess 20–30% aqueous NaOH. The aqueous layer was extracted with dichloromethane (3 x 50 mL). The combined organic extracts were washed with brine, dried

over anhydrous NaSO_4 , filtered, and concentrated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:5) to afford colorless solid of analytically pure compound **30** (250 mg, 56%). ^1H NMR (400 MHz, CDCl_3): δ = 7.61 (d, J = 7.6 Hz, 4H), 7.42 (t, J = 7.2 Hz, 4H), 7.33 (t, J = 7.2 Hz, 2H), 7.20 (s, 1H), 6.95 (s, 2H), 3.78 (brs, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 147.4, 143.2, 141.7, 129.0, 127.7, 127.5, 117.3, 113.2 ppm.

5.2.10 Synthesis of Cyclopentanecarboxylic acid [1,1',3',1'']terphenyl-5'-yl amide (**31**)

To a solution of cyclopentanecarboxylic acid (1.47 mmol, 0.16 mL) in dichloromethane, Et_3N (1.47 mmol, 0.21 mL) and ethyl chloroformate (1.47 mmol, 0.14 mL) was added and stirred for 30 min at 0 °C. To it, [1,1',3',1'']terphenyl-5'-yl amine (**30**) (1.22 mmol, 300 mg) and Et_3N (1.22 mmol, 0.17 mL) was added and stirred at ambient temperature for 6 h. Upon completion (TLC), the reaction mixture was diluted with dichloromethane (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:4) to afford the desired product as colorless solid (205 mg, 60%). ^1H NMR (400 MHz, CDCl_3): δ = 7.81 (s, 2H), 7.61 (d, J = 7.2 Hz, 4H), 7.53 (s, 1H), 7.41 (t, J = 7.4 Hz, 4H), 7.34 (t, J = 7.2 Hz, 2H), 2.75–2.69 (m, 1H), 2.01–1.86 (m, 4H), 1.84–1.72 (m, 2H), 1.64–1.55 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): δ = 142.8, 141.0, 139.3, 129.1, 127.9, 127.6, 122.1, 117.8, 47.2, 30.9, 26.4 ppm.

5.2.11 Synthesis of Cyclopentyl-3-[1,1',3',1'']terphenyl-5'-yl-urea (**32**)

Triethylamine (0.98 mmol, 0.13 μL) was added to a stirred solution of [1,1',3',1'']terphenyl-5'-yl amine (**30**) (0.81 mmol, 200 mg) in anhydrous dichloromethane and stirred at ambient temperature for 15 min. To it, cyclopentyl isocyanate (1.22 mmol, 138 μL) was added and stirred for another 18 h at ambient temperature. Upon completion (TLC), the reaction mixture was diluted with dichloromethane (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:5) to obtain compound **32** (200 mg, 69%). The column purified carbamate was crystallized using dichloromethane and petroleum ether. ^1H NMR (400 MHz, CDCl_3): δ = 7.72–7.58 (m, 6H), 7.49–7.31 (m, 7H),

5.82 (d, $J = 6.4$ Hz, 1H), 4.18–4.10 (m, 1H), 2.07–1.91 (m, 2H), 1.74–1.57 (m, 4H), 1.51–1.39 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 156.5, 142.5, 141.1, 138.2, 128.8, 127.5, 127.2, 120.2, 116.8, 49.0, 33.4, 23.6$ ppm.

5.2.12 Synthesis of Cyclopentyl-carbamic acid biphenyl-3-yl ester (33)

This compound was synthesized from commercially available 3-phenylphenol using the general method for carbamate synthesis. Yield: 279 mg, 81%. ^1H NMR (400 MHz, CDCl_3): $\delta = 7.57$ (d, $J = 7.2$ Hz, 2H), 7.47–7.39 (m, 4H), 7.37–7.31 (m, 2H), 7.11 (s, 1H), 4.10–4.02 (m, 1H), 2.08–1.98 (m, 2H), 1.70–1.56 (m, 4H), 1.52–1.46 (m, 2H) ppm. ^{13}C NMR (100 MHz, CDCl_3): $\delta = 142.8, 138.2, 129.7, 129.0, 128.9, 127.8, 127.7, 127.4, 124.1, 120.6, 53.2, 33.4, 23.8$ ppm.

5.2.13 Biology - Materials

Org 43553, recLH and rec-hCG were provided by Schering Plough (Oss, The Netherlands); Org 43553 was synthesized as described previously.²¹¹ Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, U.S.A.), whereas BCA protein assay reagent was from Pierce Chemical Company (Rockford, IL, U.S.A.). [^3H]Org 43553 (16.6 Ci/mmol) was labeled as described in *Chapter 4*. ^{125}I -hCG (4408 Ci/mmol) was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). Chinese Hamster Ovary (CHO-K1) cells stably expressing the human luteinizing hormone (LH) receptor and cAMP-response-element luciferase reporter gene (CRE-luc) were kindly provided by Schering-Plough (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

5.2.14 Cell Culture and Membrane Preparation

CHO cells with stable expression of the human LH receptor and CRE-luc (CHOhLHr_luc) were grown in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (1:1) supplemented with 7.5% normal adult bovine serum, streptomycin (100 $\mu\text{g}/\text{mL}$), penicillin (100 IU/ mL) at 37 °C in 5% CO_2 . The

cells were subcultured twice weekly at a ratio of 1:20. Cell membranes were prepared as described in *Chapter 4*.

5.2.15 Radioligand Displacement Assays

¹²⁵I-hCG displacement assays were performed as described in *Chapter 4*. For [³H]Org 43553, membrane aliquots containing 50 µg protein were incubated in a total volume of 100 µL assay buffer (25 mM Tris-HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA) at 30 °C for 90 min. Displacement experiments were performed using 10 µM or a range of concentrations of competing ligand in the presence of 4.5 nM [³H]Org 43553. Non-specific binding was determined in the presence of 10 µM Org 43553 and represented approximately 35% of the total binding. [³H]Org 43553 did not bind specifically to membranes prepared from CHO cells lacking the LH receptor. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. Incubations were terminated by dilution with 1 mL ice-cold Tris-HCl buffer. Bound from free radioligand was immediately separated by rapid filtration through Whatman GF/B filters using a Millipore manifold. Filters were subsequently washed three times with ice-cold wash buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.05% BSA). Filter-bound radioactivity was determined by scintillation spectrometry (Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences) after addition of 3.5 mL of PerkinElmer Emulsifier Safe.

5.2.16 Radioligand Dissociation Assays

Dissociation assays with ¹²⁵I-hCG were performed as described in *Chapter 4*. The amount of radioligand still bound to the receptor was measured after 4 h of dissociation. The obtained amount of radioligand binding determined at control conditions (t = 0) was set at 100%. For [³H]Org 43553, dissociation experiments were performed by preincubating membrane aliquots containing 50 µg protein in a total volume of 100 µL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA) with 4.5 nM [³H]Org 43553 at 30 °C for 90 min. After preincubation, dissociation was initiated by addition of 10 µM Org 43553 in the absence (control) or presence of allosteric modulators in a total volume of 5 µL of which was 50% (v/v) DMSO. The amount of radioligand still bound to the receptor was

measured after 30 min of dissociation. The obtained amount of radioligand binding determined at control conditions was set at 100%. In addition, the amount of [³H]Org 43553 still bound to the receptor was measured at various time intervals for a total of 120 min in the absence (control) and presence of 1 and 10 μ M **24**. Incubations were terminated and samples were obtained and analyzed as described under *Radioligand Displacement Assays*.

5.2.17 Luciferase Assays

CHOhLHr_luc cells were grown as described above. On the day of the assay, cells were washed with PBS and then harvested using trypsin (0.25% (w/v) in PBS containing 4.4 mM EDTA). Cells were resuspended in assay medium consisting of DMEM and F12 (1:1) supplemented with 1 μ g/mL insulin and 5 μ g/mL apo-transferrin. Typically, a well contained 30 μ L of ligand, 30 μ L of assay medium with or without 10 μ M **24** and 30 μ L cell suspension containing 7.5×10^5 cells/mL. Luciferase assays were performed using ten concentrations of recLH or Org 43553. Basal activity was determined in the presence of assay medium and represented approximately 10% of the maximal activity. Maximal receptor activity was determined in the presence of 1 nM recLH and was set at 100% in all experiments, whereas basal activity was set at 0% in all experiments. After 4 h of stimulation, 45 μ L of Britelite® (PerkinElmer, Groningen, The Netherlands) was added to each well for detection of luciferase protein. Finally, the luminescence signal was quantified on a Microbeta Trilux 1450 Luminescence Counter (PerkinElmer, Groningen, The Netherlands).

5.2.18 LogD Determination by HPLC

Distribution coefficients (log D) were determined as described by Lombardo and coworkers.²⁷⁸ In short, retention times of the compounds were determined in an HPLC system with three different methanol percentages. These retention times were converted to k' values by using the formula $k' = (t_r - t_0)/t_0$ in which t_r is the retention time and t_0 the retention time of an 'non-delayed' compound (pure methanol). The calculated k' values were plotted against the methanol percentage and extrapolated to a 0% methanol situation which yielded the k'w value (y axis cutoff). In a standard curve, the known logD values of the reference compounds were plotted against their k'w values found in the HPLC system used. From this standard curve the logD values of the compounds described in this paper were determined.

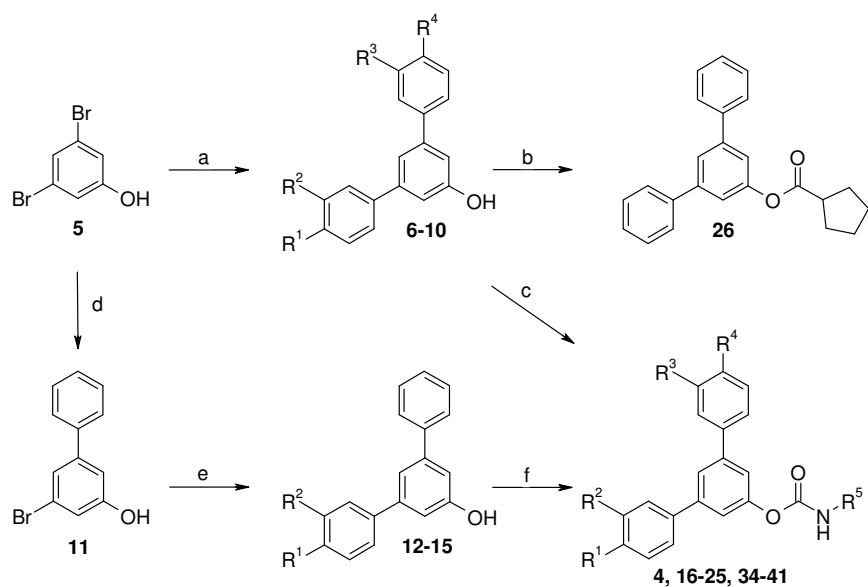
5.2.19 Data Analysis

All binding data were analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 5.00 (GraphPad Software Inc, San Diego, CA, U.S.A.). Dissociation constants, k_{off} , were obtained by computer analysis of the exponential decay of [^3H]Org 43553 bound to the receptor. All values obtained are means of at least three independent experiments performed in duplicate.

5.3 RESULTS AND DISCUSSION

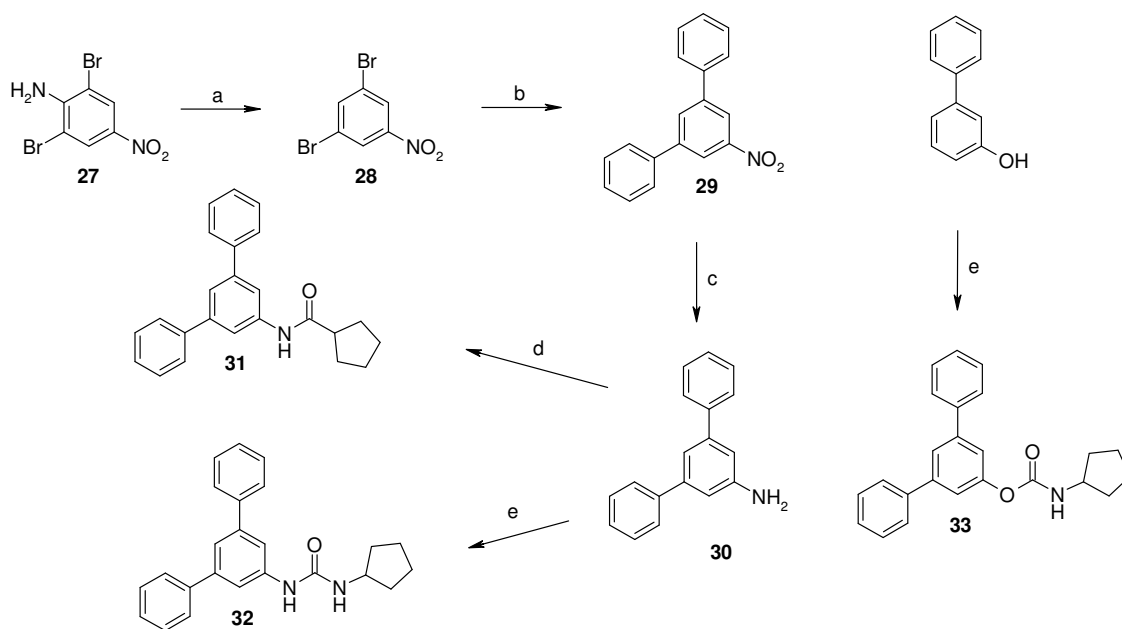
5.3.1 Chemistry

A series of symmetrical and unsymmetrical terphenyl carbamates was synthesized as depicted in Scheme 5.1. The synthesis was started from commercially available 3,5-dibromophenol **5**. The microwave assisted Suzuki-Miyaura cross coupling of phenol **5** with phenylboronic acid and substituted phenylboronic acid using the catalyst $[(\text{Ph}_3\text{P})_4]\text{Pd}$ gave terphenylphenols **6–10**.²⁷⁹ These compounds (**6–10**) were then treated with various isocyanates and Et_3N in anhydrous dichloromethane to furnish the terphenyl carbamates (**4**, **16–25**, **35**, **37**, **39**, **41**). The esterification of terphenylphenol **6** was achieved using EDAC and HOBt to afford the cyclopentyl ester **26**. The unsymmetrical terphenylphenols (**12–15**) were synthesized via the sequential Suzuki-Miyaura cross coupling of phenol **6** with phenylboronic acid followed by substituted phenylboronic acid. The intermediates were treated with cyclopentyl isocyanate and Et_3N in anhydrous dichloromethane to afford the terphenyl carbamates (**34**, **36**, **38**, **40**).



Scheme 5.1 Synthetic route to compounds **4**, **6**, **16–26**, **34–41**. a) $\text{PhB}(\text{OH})_2$, aq. Na_2CO_3 , toluene, MW, 10 min., 70–90%; b) EDAC, HOBt, cyclopentanecarboxylic acid, Et_3N , CH_2Cl_2 , RT, 24 h, 40%; c) R^5NCO , Et_3N , CH_2Cl_2 , RT, overnight, 70–90%; d) $\text{PhB}(\text{OH})_2$, aq. Na_2CO_3 , toluene, reflux, 3h, 30%; e) substituted phenylboronic acid, aq. Na_2CO_3 , toluene, MW, 10 min., 35–52%; f) R^5NCO , Et_3N , CH_2Cl_2 , RT, overnight, 60–82%.

Terphenyl amide derivative **31** and terphenyl urea derivative **32** were synthesized as outlined in Scheme 5.2. Deamination of commercially available 2,6-dibromo-4-nitroaniline **27** was achieved using NaNO_2 and CuO to yield 3,5-dibromonitrobenzene **28**.²⁸⁰ The Suzuki-Miyaura cross coupling of **28** with phenylboronic acid using the catalyst $[(\text{Ph}_3\text{P})_4]\text{Pd}$ gave 3,5-diphenylnitrobenzene **29**. The nitro group was reduced using anhydrous SnCl_2 in EtOH at 70 °C to afford amine **30** which subsequently was treated with cyclopentanecarboxylic acid, ethyl chloroformate and Et_3N in anhydrous dichloromethane to afford the amide **31**. Terphenyl urea **32** was synthesized from amine **30** using cyclopentyl isocyanate and Et_3N in anhydrous dichloromethane. Biphenyl carbamate **33** was synthesized by treating commercially available 3-phenylphenol with isopentyl isocyanate and Et_3N in anhydrous dichloromethane.



Scheme 5.2 Synthetic route to compounds **30-33**. Reagents and conditions: a) NaNO_2 , H_2SO_4 , CH_3COOH , CuO , EtOH, overnight, 30%; b) $(\text{Ph}_3\text{P})_4\text{Pd}$, $\text{PhB}(\text{OH})_2$, aq. Na_2CO_3 , toluene, reflux, 16 h, 42%; c) anhydrous SnCl_2 , EtOH, 70 °C, 30 min., 40%; d) cyclopentanecarboxylic acid, EtOCOCl , Et_3N , CH_2Cl_2 , RT, 4 h, 50%; e) cyclopentyl isocyanate, Et_3N , CH_2Cl_2 , RT, overnight, 70%.

5.3.2 Structure-Activity Relationships

In an initial screen, 50 diverse low molecular weight compounds were tested for their ability to either increase or decrease the dissociation rate of [^3H]Org 43553 from the human LH receptor stably expressed on CHO cell membranes. Most compounds did not change the dissociation rate compared to control conditions. However, some hits were obtained that significantly increased the dissociation rate of the radioligand, indicative for allosteric inhibition. In Figure 5.1, four of these hits (**1-4**) are depicted that showed some resemblance in their chemical structures. The presence of these compounds resulted in an increase of the dissociation by 42, 16, 29 and 79% when compared to the dissociation of [^3H]Org 43553 by unlabeled Org 43553 alone, respectively.

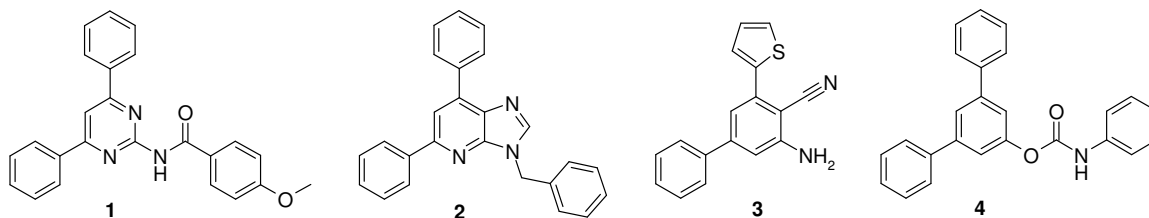
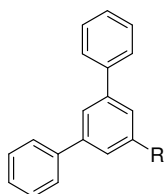
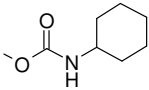
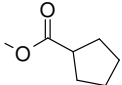
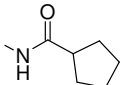
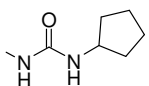


Figure 5.1 Chemical structures of the first hits (**1 – 4**) resulting from screening in the [^3H]Org 43553 dissociation assay. The presence of 10 μM of compounds **1 – 4** resulted in 42, 16, 29 and 79% enhanced radioligand dissociation compared to control conditions, respectively.

Subsequently, the structure-activity relationships around compound **4** were further explored for two reasons; 1) this was the most potent allosteric inhibitor (79% increased dissociation compared to control), and 2) compounds **1-3** have been reported as adenosine receptor antagonists, where **1** and **2** showed low affinity for the A_1 receptor subtype,^{281,282} while **3** displayed nanomolar affinity for both the A_1 and $\text{A}_{2\text{A}}$ receptor.²⁸³ Notably, **4** did not show any affinity for the adenosine receptor subtypes (data not shown). Compound **4** also caused displacement of [^3H]Org 43553 in equilibrium radioligand binding studies (see also Table 5.1). This probably results from non-competitive (allosteric) inhibition, as shown for other allosteric inhibitors, e.g. 5-(N,N-hexamethylene)amiloride (HMA) on the human GnRH receptor (*Chapter 3*).

Table 5.1 Displacement and allosteric modulation of [^3H]Org 43553 binding at the human luteinizing hormone receptors by 10 μM of compounds **4**, **6**, **16-26**, **30-32**

Compound	R	% Displacement ^a	% Allosteric Inhibition ^b
4		28 (23/33)	79 (77/81)
6		-2 (-4/2)	27 (26/27)
16		9 (8/9)	19 (12/26)
17		58 (56/59)	35 (23/47)
18		-1 (-1/-1)	61 (58/64)
19		47 (39/54)	72 (67/76)
20		61 (60/61)	65 (60/69)
21		30 (29/31)	80 (77/83)
22		14 (12/15)	55 (49/60)
23		68 (62/73)	82 (78/87)
24 (LUF5771)		91 (89/93)	88 (84/92)

25		83 (83/83)	51 (48/54)
26		9 (6/12)	41 (38/44)
30		9 (8/9)	42 (31/52)
31		30 (25/35)	56 (53/60)
32		49 (46/53)	70 (64/75)

^a % Displacement of specific [³H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes at 10 μ M concentrations (n = 2, duplicate)

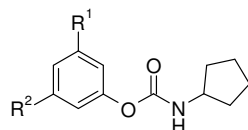
^b % Enhanced dissociation [³H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes in the absence (control; set at 0%) or presence of 10 μ M of the compounds (n = 2, duplicate)

Analogues of **4** were synthesized for further exploration of the structure-affinity relationships of this prototype allosteric inhibitor of the luteinizing hormone receptor; their behavior in radioligand binding studies is reported in Tables 5.1 and 5.2. First the phenyl carbamic acid of **4** was removed yielding a phenol analog (**6**) that had low modulating potency and did not cause any displacement. Addition of a 4-chloro (**16**) or 4-methoxy (**17**) also resulted in a lower modulating potency. Apparently substitution of the phenyl is not tolerated in that binding pocket. In addition, the carbamic acid was substituted with several alkyls, where an ethyl (**18**) and isopropyl substituent (**22**) were less potent than **4**, but compounds bearing a propyl (**19**), butyl (**20**) or pentyl (**21**) substituent were equally potent in modulating [³H]Org 43553 binding. Remarkably, compound **18** was not able to displace the radioligand. The first compounds that showed an increased potency (with respect to allosteric inhibition) possessed a tert-butyl (**23**) or cyclopentyl (**24**) substituent. The latter compound was able to increase the dissociation of [³H]Org 43553 by 88%. In addition, 10 μ M of **24** caused a similar amount of displacement compared to the percentage allosteric inhibition. The more bulky cyclohexyl group (**25**) resulted in a loss of modulatory potency, which indicated some steric hindrance in the binding pocket. Then the carbamic acid linker between the cyclopentyl and the terphenyl scaffold was examined. Replacement with an ester (**26**) or amide (**31**) resulted in a moderate potency. To examine whether this was due to the length of

the linker, a urea derivative was introduced (**32**). This compound had only a slightly decreased potency indicating that at least the length or the location of the nitrogen in the linker was important. The free amine (**30**) did not increase the dissociation rate, which was similar to the hydroxyl (**6**) analog.

It follows from Table 5.1 that a carbamate linker with a cyclopentyl substituent results in the most potent allosteric inhibitor. Therefore, other analogs were prepared where the terphenyl scaffold was substituted (Table 5.2). First, one of the phenyl rings was removed (**33**), still yielding an allosteric inhibitor, but much less lipophilic (Figure 5.2). Apparently, the third phenyl ring is not a prerequisite for high modulating potency. Subsequently, either one or both phenyl rings were substituted. As the molecule has a symmetry axis it does not matter where the single substituent is introduced. Introduction of one 4-chloro (**34**) results in a slightly reduced potency that is both reflected in the displacement and modulating potency compared to **24**. Substitution of the second phenyl ring with a 4-chloro atom (**35**) decreases its ability to displace the radioligand even further. The initial concentration of a compound in

Table 5.2 Displacement and allosteric modulation of [³H]Org 43553 binding at the human luteinizing hormone receptors by 10 μ M of compounds **33-41**



Compound	R ¹	R ²	% Displacement ^a	% Allosteric Inhibition ^b
33	H	phenyl	81 (77/84)	75 (70/79)
34	phenyl	4-Cl-phenyl	77 (71/82)	71 (65/77)
35	4-Cl-phenyl	4-Cl-phenyl	55 (50/60)	ND
36	phenyl	3,4-diCl-phenyl	69 (64/71)	64 (57/72)
37	3,4-diCl-phenyl	3,4-diCl-phenyl	7 (6/8)	ND
38	phenyl	4-MeO-phenyl	67 (58/76)	83 (82/83)
39	4-MeO-phenyl	4-MeO-phenyl	17 (7/26)	31 (27/34)
40	phenyl	4-Me-phenyl	72 (67/74)	102 (97/106)
41	4-Me-phenyl	4-Me-phenyl	62 (58/66)	59 (57/61)

^a % Displacement of specific [³H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes at 10 μ M concentrations (n = 2, duplicate)

^b % Enhanced dissociation [³H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes in the absence (control; 0%) or presence of 10 μ M of the compounds (n = 2, duplicate)

ND: not determined

a dissociation assay is 10 fold higher than in a displacement assay. For certain compounds (**35**, and **37**) this resulted in solubility problems and their modulating potency could therefore not be determined. In general, the modulatory potency of this series of compounds seems to parallel their displacement properties. Extrapolation of this notion would indicate that **35** will be a poor allosteric inhibitor. A similar observation was done for the single (**36**) and double 3,4-dichloro (**37**) substituted compounds. Compound **36** had an intermediate modulating potency, while that was almost completely lost for **37** (based on the displacement values due to solubility problems). The first substituted compound that did not lose a significant amount of potency was compound **38** with a 4-methoxy group. A second 4-methoxy substituent (**39**), however, resulted in a substantial loss of potency. Introduction of a smaller 4-methyl substituent (**40**) resulted in a significant gain of modulating potency, to the extent that in the presence of **40** the radioligand had fully dissociated from the receptor after 30 min in comparison to control conditions. Double substitution with a 4-methyl group (**41**) resulted in a loss of potency. In short, although a single substituent only results in a small loss or even a gain of potency, double substitution resulted in a significant loss of potency when compared to compound **24**. Apparently, only one of the pockets that accommodate a phenyl ring has some space for a substituent, where the smallest substituent, a 4-methyl (**40**) results in the highest potency.

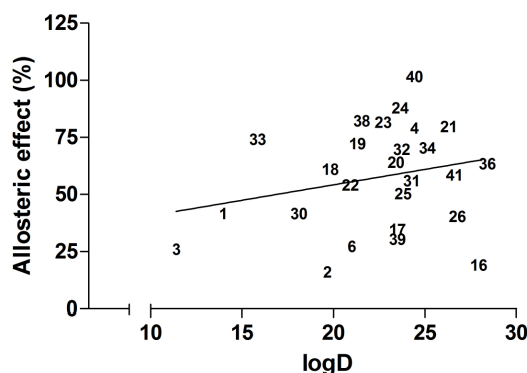


Figure 5.2 LogD of compounds **1-4**, **6**, **16-26**, **34-41** plotted against their allosteric effect on [³H]Org 43553 binding. Analysis of the plot by linear regression resulted in a poor correlation ($r^2 = 0.05791$). Compound **35** and **37** did not dissolve in the methanol-based eluent and are therefore not included in the graph.

Due to the terphenyl scaffold, these ligands are highly lipophilic and hard to dissolve at high concentrations in an aqueous buffer. Therefore, the logD of these compounds was determined to assess whether a correlation between the lipophilicity (logD) of these compounds and their modulating behavior existed (Figure 5.2), which could indicate a non-specific effect. However, a poor correlation between the logD and the allosteric effect was found for the terphenyl compounds. The observed effect is therefore most probably truly LH receptor-mediated (see also the functional assays described below). This selectivity was further corroborated by the observation that LUF5771 (**24**) did not allosterically modulate another class A GPCR, the adenosine A₃ receptor (data not shown).

Table 5.3 Dissociation (k_{off}) rate constants of [³H]Org 43553 in the presence of buffer (control), 1 μM or 10 μM LUF5771 (**24**).

Condition	k_{off} (min^{-1}) ^a	Shift ^b
Control	0.026 ± 0.0007	-
+ 1 μM LUF5771	0.054 ± 0.004	2.1
+ 10 μM LUF5771	0.087 ± 0.002	3.3

^a The values of the kinetic dissociation rate constants were obtained by analysis of the exponential dissociation of [³H]Org 43553 bound to human luteinizing hormone receptors.

^b The shift is defined as the ratio of k_{off} values in absence (control) and presence of LUF 5771 (**24**), respectively. Values are means ($\pm$ S.E.M.) of three separate assays performed in duplicate.

Further studies were undertaken to investigate the pharmacological characteristics of these novel allosteric inhibitors of the LH receptor. Based on the results of the first series of compounds (Table 5.1), the kinetic behavior of [³H]Org 43553 was studied by performing full dissociation experiments in the absence (control) and presence of 1 μM or 10 μM **24** (Table 5.3 and Figure 5.3). The dissociation rate of the radioligand obtained by the addition of unlabeled ligand alone was $0.026 \pm 0.0007 \text{ min}^{-1}$. This was slightly higher than the rate reported in *Chapter 4* (0.021 min^{-1}), due to the presence of a higher concentration of DMSO (also applied in the control experiment) that was necessary to dissolve these highly lipophilic compounds. In the presence of 1 μM **24** the dissociation rate was increased 2.1-fold to $0.054 \pm 0.004 \text{ min}^{-1}$. In addition, allosteric inhibition by **24** was concentration-dependent, as the presence of 10 μM of **24** increased the dissociation even further, by 3.3-fold (Table 5.3). As

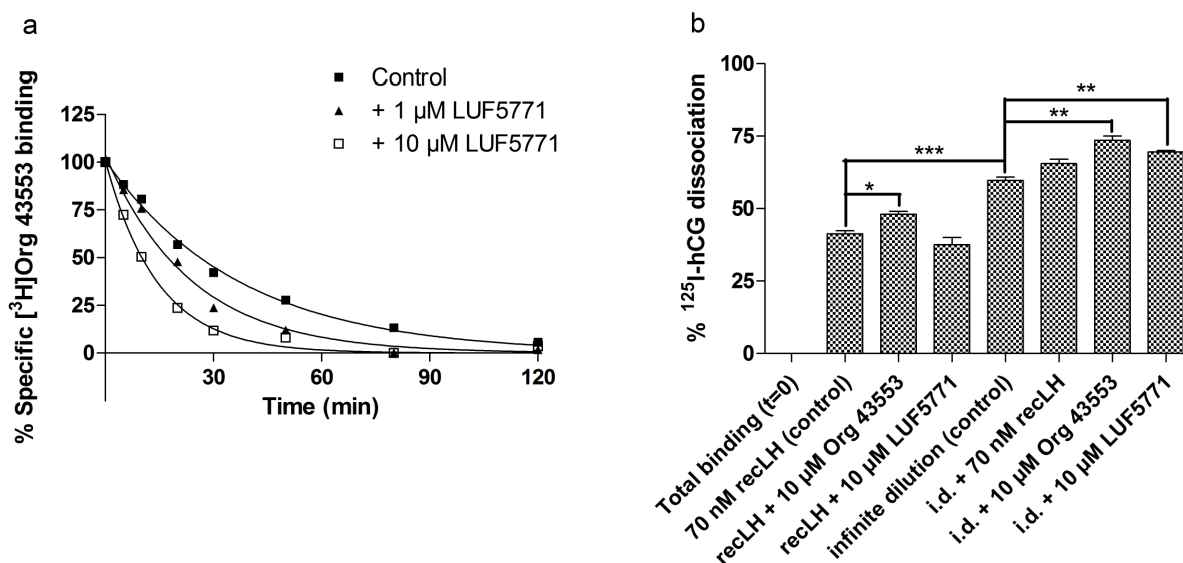


Figure 5.3 a) Dissociation kinetics of [³H]Org 43553 binding to human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes. Dissociation was either initialized by the addition of 10 μM Org 43553 mixed with buffer (control), 1 μM or 10 μM (final concentrations) of LUF5771 (**24**). Representative graphs are shown from one experiment performed in duplicate (see Table 5.3 for kinetic parameters). b) Dissociation kinetics of ¹²⁵I-hCG binding to human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes. Dissociation was either initialized by the addition of 70 nM recLH mixed with buffer (control), 10 μM (final concentrations) Org 43553 or LUF5771. In addition, dissociation was initialized by infinite dilution (i.d.) in absence (control) or presence of 70 nM recLH, 10 μM Org 43553 or LUF5771. Values are means (± S.E.M.) of at least two separate assays performed in triplicate (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ versus control).

shown in the single point experiments **24** also displaced [³H]Org 43553 in an equilibrium binding experiment by 91% at 10 μM (Table 5.1). Therefore, displacement of [³H]Org 43553 equilibrium binding at different concentrations of **24** was determined (Figure 5.4). The obtained inhibition curve was best described by a one-site receptor model and yielded an IC₅₀ value of 2.3 ± 0.4 μM with a pseudo-Hill coefficient of 1.1 ± 0.06 .

The question arose if the LH receptor could possibly contain two allosteric sites in the 7-TM domain, as its orthosteric ligand binding site is located on the large N terminal domain of the receptor. For several class A GPCRs two binding sites, one orthosteric and one allosteric, have been reported in the 7-TM domain. The adenosine A₁ and A₃ receptors and the

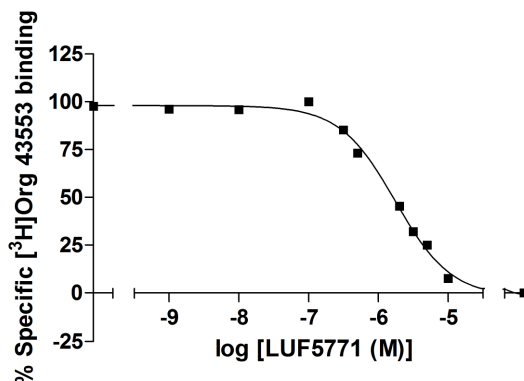


Figure 5.4 Displacement of [^3H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes by LUF 5771 (**24**). The IC_{50} value was $2.3 \pm 0.4 \mu\text{M}$. Representative graph is shown from one experiment performed in duplicate.

cannabinoid CB_1 receptor, where PD81,723, LUF6000 and Org27569 are selective allosteric enhancers, respectively, are typical examples.^{239,284,285} Therefore, the effect of **24** on the equilibrium binding and dissociation kinetics of the iodinated endogenous ligand, ^{125}I -hCG, was examined. Compound **24** was not able to displace the hormone (data not shown), similar to Org 43553's behavior (*Chapter 4*). This indicates that LUF5771 probably also binds to the 7-TM domain like Org 43553 does.³⁶ In addition, the dissociation kinetics of ^{125}I -hCG under several conditions were examined (Figure 5.3b). An excess (70 nM) of recLH was able to induce 40% dissociation of ^{125}I -hCG after 4h. In the presence of 10 μM Org 43553 the dissociation was significantly increased, in line with a recent observation by Van Koppen and coworkers.³⁶ However, when dissociation was induced by recLH in the presence of 10 μM LUF5771 (**24**), no significant increase was observed. We also studied ^{125}I -hCG dissociation induced by infinite dilution (Figure 5.3b). In this case, the presence of recLH did not alter the amount of dissociation, while the presence of Org 43553 or LUF5771 significantly increased radioligand dissociation. Taken together, this suggests that the high molecular weight ligand, hCG (and most likely recLH) and the low molecular weight ligands, Org 43553 and LUF5771, bind at three distinct sites, where both LMW ligands induce a conformational change that (negatively) modulates hCG binding to the receptor.

Finally, the effect of LUF5771 (**24**) on the activation of the LH receptor by both of its endogenous hormones was examined in cAMP-induced luciferase assays (Table 5.4 and Figure 5.5). RecLH had an EC_{50} value of $56 \pm 8 \text{ pM}$ in this functional assay, which is

comparable to data reported in *Chapter 4*. The other endogenous ligand, rec-hCG, had a similar potency as recLH ($EC_{50} = 97 \pm 10$ pM), while it had an approximately 25% lower efficacy in our hands. The presence of 10 μ M LUF5771 did not affect the efficacy, while an approximately 3-fold decrease in potency was observed for both recLH and rec-hCG. This indicates once more that LUF5771 induces a conformational change in the receptor that is

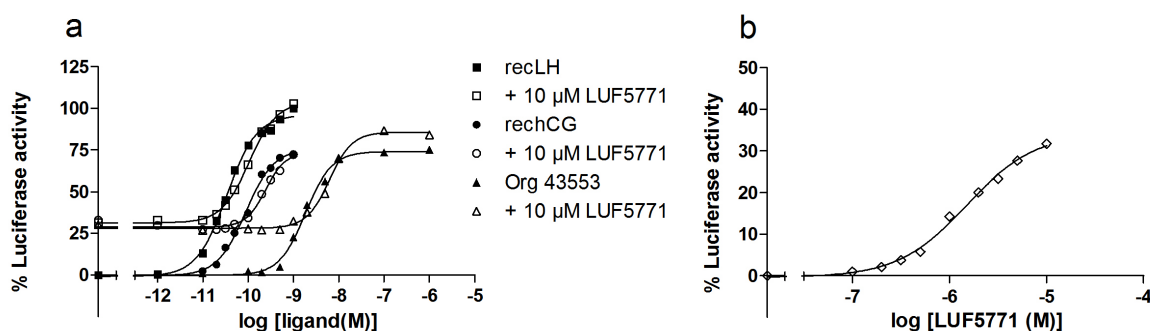


Figure 5.5 a) Concentration-effect curves of recLH (■), hCG (●) and Org 43553 (▲) in the absence (closed) or presence (open) of 10 μ M LUF5771 and b) of LUF5771 itself for cAMP-mediated luciferase production through human luteinizing hormone receptors. Representative graphs from one experiment performed in duplicate (see Table 5.4 for EC_{50} and E_{max} values).

Table 5.4 Receptor activation by recLH, rec-hCG or Org 43553 in the presence or absence of 10 μ M LUF5771 (**24**), expressed as EC_{50} and E_{max} values.

Compound	Activity in luciferase assay ^a		
	EC_{50} (nM)	Shift ^b	E_{max} (%) ^c
recLH	0.056 ± 0.008	-	100 ± 1
+ 10 μ M LUF 5771	$0.15 \pm 0.02^{**}$	2.7	108 ± 4
rec-hCG	0.097 ± 0.01	-	74 ± 2
+ 10 μ M LUF 5771	$0.33 \pm 0.05^*$	3.4	69 ± 3
Org 43553	1.9 ± 0.04	-	85 ± 4
+ 10 μ M LUF 5771	$4.6 \pm 0.8^*$	2.4	92 ± 5

^a cAMP-mediated luciferase activity in CHO-K1 cells that stably express the human luteinizing hormone receptor and CRE-luciferase reporter gene.

^b The shift is defined as the ratio of EC_{50} values in the presence or absence of LUF5771, respectively.

^c Maximal effect of either recLH or Org 43553 in the absence or presence of 10 μ M LUF5771, where recLH in the absence of LUF5771 was set at 100%.

Values are means ($\pm$ S.E.M.) of at least three separate assays performed in duplicate (* $p < 0.05$, ** $p < 0.005$ versus control).

disfavored by both endogenous hormones. As reported in *Chapter 4*, Org 43553 was a highly efficacious partial agonist in the cAMP-induced luciferase assay. Org 43553's efficacy was not affected by the presence of LUF5771, while its potency was decreased over 2-fold ($EC_{50} = 4.6 \pm 0.8$ nM). The effect of two other compounds (**33** and **40**) from the second series (Table 5.2) was also investigated in a luciferase assay. Both **33** and **40** decreased the potency of recLH and Org 43553, similar to **24** (data not shown). From Figure 5.5a, it also follows that 10 μ M of compound **24** alone was able to partially activate the LH receptor by $31 \pm 4\%$. This agonistic behavior was further analyzed, and it was shown that **24** had an EC_{50} value of 1.6 ± 0.1 μ M (Figure 5.5b). Some of the other terphenyl ligands also showed (low) intrinsic efficacy similar to compound LUF5771 (**24**; data not shown). It is noteworthy that the intrinsic activity of some of these terphenyl compounds might indicate that the second allosteric site is located close to or partially overlapping with the Org 43553 binding site. Interestingly, most allosteric modulators reported so far do not have an intrinsic efficacy at the receptor in the absence of an orthosteric agonist. However, there are some examples of (positive) allosteric modulators that can act as agonists by themselves.⁴⁹ For example, PD81,723 is an allosteric enhancer at the adenosine A_1 receptor as mentioned above, but it can also activate the receptor.²⁸⁶ For the GABA_B receptor CGP7930 was reported as an allosteric enhancer that is able to activate the receptor by interacting with the 7-TM domain of the GABA_{B2} subunit.²⁸⁷ Another example is AC-42 that was shown to activate the receptor in absence of the orthosteric agonist, but it is also a modest allosteric enhancer of the muscarinic M_1 receptor.²⁸⁸ For compounds that show allosteric agonism besides allosteric modulation of the orthosteric ligand, the term ago-allosteric modulator was proposed by Schwartz and coworkers.^{289,290} To our knowledge only allosteric enhancers have been reported with intrinsic activity and no allosteric inhibitors. Compound **24** (LUF5771) could therefore be characterized as the first ago-allosteric inhibitor of the human LH receptor.

In conclusion, this paper describes the first series of allosteric inhibitors of [³H]Org 43553 binding at the human LH receptor. In particular, LUF5771 (**24**) and **40** are highly potent. In addition, **24** inhibited the activation of the receptor by the endogenous ligand, recLH, and by Org 43553 in a functional assay. Although **24** is an allosteric inhibitor of recLH and Org 43553, it was also able to partially activate the LH receptor with low efficacy. The presence of a second allosteric site in the 7-TM domain, as demonstrated in this paper, may provide novel targets at the human luteinizing hormone receptor for low molecular weight allosteric modulators and allosteric agonists.

CHAPTER

6

IDENTIFICATION OF A SECOND ALLOSTERIC SITE AT THE HUMAN LUTEINIZING HORMONE RECEPTOR THAT RECOGNIZES BOTH LOW MOLECULAR WEIGHT ALLOSTERIC ENHANCERS AND INHIBITORS

Allosteric modulation of G protein-coupled receptors (GPCRs) has been of great interest in the past decade. Especially, for GPCRs with proteins or peptides as endogenous ligands, low molecular weight (LMW) allosteric modulators would provide additional benefits, such as oral bioavailability. In the present study, the first allosteric enhancer of the human luteinizing hormone (LH) receptor is described. Firstly, a compound library was screened on the ability to influence the dissociation [^3H]Org 43553 from the human LH receptor. This search yielded several thiazole-containing compounds (e.g. LUF5419) that were able to allosterically enhance the binding of the allosteric agonist, Org 43553, by decreasing its dissociation. Secondly, in a functional assay it was shown that LUF5419 increased the efficacy of Org 43553, while the potency and efficacy of (the orthosteric
(Continued)

(Continued)

agonist) recLH was unaffected. Furthermore, the potency to increase radioligand dissociation of the recently described allosteric inhibitor, LUF5771, was decreased by the presence of LUF5419. These results demonstrate that LUF5419 and LUF5771 are allosteric modulators that bind at the same allosteric site in the human LH receptor. Although LUF5419 was unable to allosterically modulate the endogenous hormone, the work presented in this paper proves that this second allosteric site can be targeted by different LMW ligands. This may provide yet another opportunity for the discovery of new LMW ligands for the human LH receptor.

6.1 INTRODUCTION

Most class A G protein-coupled receptors (GPCRs) have an endogenous ligand that binds within the seven transmembrane (7-TM) domain.²³⁶ A subfamily of receptors is formed by the luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) receptor, termed the glycoprotein hormone receptors.¹⁸ Although this receptor subfamily belongs to class A, their endogenous ligand binds to the large extracellular N-terminus. A unique feature of the LH receptor is that it recognizes two endogenous ligands, namely LH and human chorionic gonadotropin (hCG), both of which are large protein hormones.¹⁸

Several class A GPCRs have been shown to contain two binding sites, one orthosteric and one allosteric site, in the 7-TM domain.⁴⁹ For the muscarinic M₁, M₄ receptor and more recently for the GnRH receptor (*Chapter 3*) even three sites have been described.^{62,256} As the LH receptor is classified as a class A GPCR, the 7-TM domain could also contain two binding sites for low molecular weight (LMW) ligands, since its endogenous ligand binds to the extracellular N-terminus. The advantage of LMW ligands is that they have the potential of oral bioavailability, while hormonal therapeutics need to be administered by parenteral injection.¹⁸⁷

In the past few years, some LMW agonists and antagonists for the FSH and LH receptor have been reported (*Chapter 2*). For the LH receptor it was shown that certain thienopyrimidines activate the receptor and are orally available.²¹ Their selectivity is however less than hoped for. Recently, one of these LMW agonists (Org 41841) was reported as a low potency partial agonist for the TSH receptor too.¹⁹¹ Moreover, Org 41841 was shown to act as a pharmacological chaperone for the FSH receptor to increase FSH receptor presence at the cell membrane, while it did not have intrinsic efficacy per se.¹⁹⁵ Although the endogenous hormones contain a high degree of selectivity within the glycoprotein hormone receptor family,²⁹¹ the LMW thienopyrimidine compounds that were originally reported as LH receptor agonists, are thus less selective with divergent effects on each glycoprotein hormone receptor family member.

Recently, the first radiolabeled LMW agonist for the LH receptor was described, [³H]Org 43553 (*Chapter 4*), a more potent analogue of Org 41841. In the present study, we used this radioligand to examine whether the LH receptor contains a second binding site within the 7-TM domain, next to the (allosteric) Org 43553 binding site. To this end a selection of 50 compounds from the in-house compound library was screened for their effect on the

dissociation rate of [^3H]Org 43553 as a measure for allosteric modulation. Some compounds behaved as allosteric inhibitors by increasing the dissociation rate, such as LUF5771 in *Chapter 5*. From this screen two allosteric enhancers emerged as well that were both compounds containing a thiazole core. These thiazole-containing compounds and several derivatives have been reported as (competitive) adenosine A_1 and A_3 receptor antagonists.^{292,293} Subsequently, all thiazoles available in the in-house collection were screened, which resulted in a potent allosteric enhancer of [^3H]Org 43553 binding, LUF5419. In a functional assay, the presence of LUF5419 enhanced the efficacy of Org 43553 from a partial to a full agonistic response compared to the endogenous ligand, recLH. LUF5419 did not allosterically enhance recLH binding or function. Nevertheless, this paper shows that a second allosteric site is present in the human LH receptor that can be targeted by LMW ligands. We also demonstrated that the allosteric inhibitor LUF5771 and the allosteric enhancer LUF5419 both bind to this novel allosteric site. Through this site other, more selective and orally available ligands can possibly be developed for the human LH receptor.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Org 43553 and recLH were provided by Schering Plough (Oss, The Netherlands), where Org 43553 was synthesized as described previously by Hanssen and Timmers.²¹¹ Compound **1-5** (including LUF5419) and LUF5771 were synthesized in our own laboratory as described by Van Muijlwijk-Koezen et al.²⁹³ and in *Chapter 5*, respectively. Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, U.S.A.), whereas BCA protein assay reagent was from Pierce Chemical Company (Rockford, IL, U.S.A.). [³H]Org 43553 (16.6 Ci/mmol) was labeled as described in *Chapter 4*. ¹²⁵I-hCG (4408 Ci/mmol) was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). Chinese Hamster Ovary (CHO-K1) cells stably expressing the human luteinizing hormone (LH) receptor and cAMP-response-element luciferase reporter gene (CRE-luc) were kindly provided by Schering-Plough (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

6.2.2 Cell Culture and Membrane Preparation

CHO cells with stable expression of the human LH receptor and CRE-luc (CHO_{hLHr}_luc) were grown in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (1:1) supplemented with 7.5% normal adult bovine serum, streptomycin (100 µg/mL), penicillin (100 IU/ mL) at 37 °C in 5% CO₂. The cells were subcultured twice weekly at a ratio of 1:20. Cell membranes were prepared as described in *Chapter 4*.

6.2.3 Radioligand Saturation Assays

Membrane aliquots containing 50 µg protein were incubated in a total volume of 100 µL assay buffer (25 mM Tris-HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA) at 30 °C for 90 min. For saturation experiments, total binding was determined at increasing concentrations (0.25-25 nM) of [³H]Org 43553, whereas nonspecific binding was determined at three concentrations of radioligand in the presence of 10 µM Org 43553 and analyzed by linear regression. Incubations were terminated by dilution with 1 mL ice-cold Tris-HCl

buffer. Bound from free radioligand was immediately separated by rapid filtration through Whatman GF/B filters using a Millipore manifold. Filters were subsequently washed three times with ice-cold wash buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.05% BSA). Filter-bound radioactivity was determined by scintillation spectrometry (Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences) after addition of 3.5 mL of PerkinElmer Emulsifier Safe.

6.2.4 Radioligand Displacement Assays

Membrane aliquots containing 50 µg protein were incubated in a total volume of 100 µL assay buffer (25 mM Tris-HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA) at 30 °C for 90 min. Displacement experiments were performed using 10 µM of competing ligand in the presence of 4.5 nM [³H]Org 43553. Non-specific binding was determined in the presence of 10 µM Org 43553 and represented approximately 30% of the total binding. [³H]Org 43553 did not bind specifically to membranes prepared from CHO_{luc} cells lacking the LH receptor. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. Incubations were terminated and samples were obtained and analyzed as described under *Radioligand Saturation Assays*. Displacement assays with ¹²⁵I-hCG were performed as described in *Chapter 4*.

6.2.5 Kinetic Association and Dissociation Assays

Association experiments were performed by incubating membrane aliquots containing 50 µg protein in a total volume of 100 µL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA) at 30 °C for 120 min with 4.5 nM [³H]Org 43553 in the absence (control) or presence of 10 µM LUF5419. The amount of radioligand bound to the receptor was measured at various time intervals during incubation. Dissociation experiments were performed by preincubating membrane aliquots containing 50 µg protein in a total volume of 100 µL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM MgCl₂ and 0.1% BSA) with 4.5 nM [³H]Org 43553 at 30 °C for 90 min in the absence (control) or presence of 10 µM LUF5419. After preincubation, dissociation was initiated by addition of 10 µM Org 43553 in the absence (control) or presence of allosteric modulators in a total volume of 5 µL of which 25% (v/v) DMSO. The amount of radioligand still bound to the

receptor was measured after 30 min of dissociation. The amount of specific radioligand binding obtained under control conditions was set at 0% and the total binding ($t = 0$ min) was set at 100%. In addition, the amount of [^3H]Org 43553 still bound to the receptor was measured at various time intervals for a total of 120 min in the absence (control) and presence of 10 μM LUF5419. Incubations were terminated and samples were obtained and analyzed as described under *Radioligand Saturation Assays*. Dissociation assays with ^{125}I -hCG were performed as described in *Chapter 4*.

6.2.6 ‘Competitive’ Kinetic Radioligand Dissociation Assays

Dissociation experiments were mainly performed as described above. After preincubation, dissociation was initiated by addition of 10 μM Org 43553 in the presence or absence (control) of different concentrations LUF5419 (10 or 50 μM) and in the presence or absence (control) of seven different concentrations of LUF5771 (0.1 – 10 μM) in a total volume of 5 μL . For LUF5419 alone five different concentrations were used (5 - 100 μM). The amount of radioligand still bound to the receptor was measured after 30 min. Incubations were terminated and samples were obtained and analyzed as described under *Radioligand Saturation Assays*.

6.2.7 Luciferase Assays

CHO_hLHr_{luc} cells were grown as described above. On the day of the assay, cells were washed with PBS and then harvested using trypsin (0.25% (w/v) in PBS containing 4.4 mM EDTA). Cells were resuspended in assay medium consisting of DMEM and F12 (1:1) supplemented with 1 $\mu\text{g/mL}$ insulin, 5 $\mu\text{g/mL}$ apo-transferrin, 100 $\mu\text{g/mL}$ streptomycin and 100 IU/mL penicillin. Typically, a well contained 30 μL of test compound, 30 μL of assay medium and 30 μL of cell suspension containing 7.5×10^5 cells/mL. Luciferase assays were performed using ten concentrations of test compound. Basal activity was determined in the presence of assay medium and represented approximately 10% of the maximal activity. Maximal receptor activity was determined in the presence of 1 nM recLH and was set at 100% in all experiments, whereas basal activity was set at 0% in all experiments. After 4 h stimulation, 45 μL of Britelite® (PerkinElmer, Groningen, The Netherlands) was added to each well for detection of luciferase protein. Finally, the luminescence signal was quantified

on a Microbeta Trilux 1450 Luminescence Counter (PerkinElmer, Groningen, The Netherlands).

6.2.8 Data Analysis

All binding data were analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 5.00 (GraphPad Software Inc, San Diego, CA, U.S.A.). EC_{50} values were directly obtained from the dose-response curves and inhibitory binding constants (K_i values) were derived from the IC_{50} values according to $K_i = IC_{50}/(1 + [C]/K_d)$ where $[C]$ is the concentration of the radioligand and K_d its dissociation constant.²⁴⁵ Dissociation rate constants, k_{off} , were obtained by computer analysis of the exponential decay of [³H]Org 43553 bound to the receptor. Association rate constants were calculated according to the equation $k_{on} = (k_{obs} - k_{off})/[L]$, where k_{obs} was obtained by computer analysis of the exponential association of the percentage of [³H]Org 43553 bound to the receptor and $[L]$ is the amount of radioligand used for the association experiments. The EC_{50} from competitive dissociation experiments was obtained from dose response-curves of enhanced dissociation by different concentrations of LUF5771, where the non-specific binding was set at 0% and either the true control (buffer) or own control binding (10 or 50 μ M LUF5419) after 30 min was set at 100%. All values obtained are means of at least three independent experiments performed in duplicate.

6.2.9 Simulation of Cooperativity between LUF5419 and LUF5771

A mathematical model (Eq. 6.1) for two distinct allosteric sites⁶² was implemented in MatLab (version 7.1) to simulate the effects of different cooperativities between LUF5419 and LUF5771 on the EC_{50} of LUF5771 in enhancing [³H]Org 43553 dissociation.

$$EC_{50}^{LUF5771} = \frac{1 + [LUF5419] \times K_{LUF5419}^{Org43553}}{K_{LUF5771}^{Org43553} \times (1 + [LUF5419] \times K_{LUF5419}^{Org43553} \times \delta)} \quad \text{Eq. 6.1}$$

in which $EC_{50}^{LUF5771}$ is the observed EC_{50} of LUF5771 in enhancing [³H]Org 43553 binding.

$K_{LUF5419}^{Org43553}$ and $K_{LUF5771}^{Org43553}$ are the affinities on the Org 43553-occupied receptor for LUF5419 and LUF5771, respectively. δ is the parameter defining the cooperativity between LUF5419 and LUF5771.

6.3 RESULTS

6.3.1 Screen for Allosteric Modulation of [^3H]Org 43553 Binding

Fifty compounds were selected from our in-house library based on chemical diversity and availability. Subsequently, these compounds were screened for their effect on the dissociation rate of [^3H]Org 43553 from the human LH receptor in a single point ($t = 30$ min) dissociation assay. This protocol resulted in a few hits that increased (allosteric inhibitors) or decreased (allosteric enhancers) the dissociation rate of the radioligand. As two of these allosteric enhancers were thiazole derivatives, all thiazoles available in our laboratory were screened.

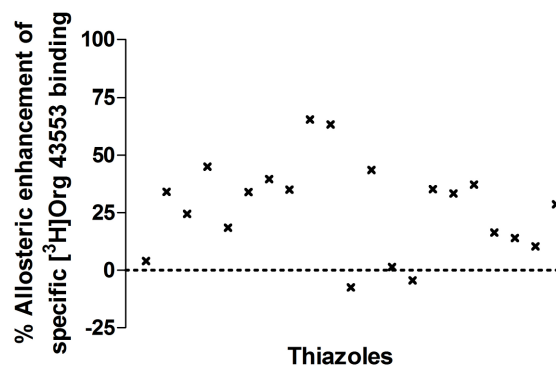
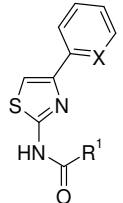


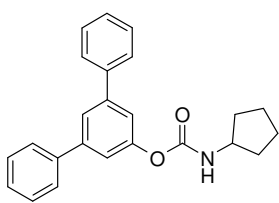
Figure 6.1 Effect of 21 compounds from a thiazole library^{292,293} on single point dissociation of [^3H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes.

From Figure 6.1 it follows that these 21 thiazole compounds have different modulating potencies, ranging from no effect to almost 50% attenuation of [^3H]Org 43553 dissociation. In Table 6.1, five derivatives with different modulating potencies are shown, including a potent allosteric enhancer that emerged from the screen, LUF5419. Compound **1** and **2** were two of the derivatives that were not able to modulate [^3H]Org 43553 dissociation. Apparently, an aliphatic substituent, such as a cyclopentyl ring, or a substituted aromatic ring on a urea linker to the thiazole scaffold, does not result in allosteric enhancement. When the urea was substituted for an amide, which resulted in a shorter linker and one possible hydrogen bond donor less (**3**), the modulating potency was significantly increased to 19% compared to

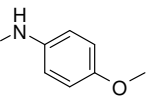
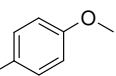
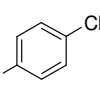
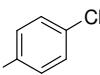
Table 6.1. Displacement and allosteric modulation of [^3H]Org 43553 binding at the human luteinizing hormone receptor by 10 μM of compounds **1-5** and **LUF5771**.



1-5



LUF5771

Compound	R ¹	X	% Displacement ^a	% Allosteric Enhancement ^b
1		N	-3 (-1/-5)	1 (-2/4)
2		N	-10 (-7/-13)	-5 (-11/3)
3		N	-1 (6/-7)	19 (17/20)
4 (LUF5419)		N	-22 (-21/-22)	45 (40/50)
5		CH	-18 (-11/-25)	34 (32/35)
LUF5771	-	-	91 (89/93) ^c	-88 (-84/-92) ^c

^a % Displacement of specific [^3H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes at 10 μM concentrations (n = 2, duplicate)

^b % Allosteric enhancement of [^3H]Org 43553 binding at human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes in the absence (control; 0%) or presence of 10 μM of the compounds (n = 2, duplicate)

^c Data taken from Chapter 5.

control conditions (0 %). Exchanging the methoxy-substituent with chlorine in the *para*-position yielded a more potent allosteric enhancer (**4**; LUF5419). Compound **4** was able to decrease the dissociation of [^3H]Org 43553 from the human LH receptor by 45 %. Incorporation of other electronegative, but bigger substituents in the *para*-position, such as iodine or isopropoxy, resulted in the most potent allosteric enhancers (63 % and 66 % enhancement, respectively; Figure 6.1; not shown in Table 6.1). A decrease in potency was observed when the pyridine-ring was replaced by a phenyl ring (**5**). In addition, the effect of compounds **1-5** in equilibrium displacement assays with [^3H]Org 43553 was examined. As

shown in Table 6.1, LUF5419 (**4**) and compound **5** were also able to increase the total amount of [³H]Org 43553 binding with approximately 20%. Table 6.1 also includes the data for the allosteric inhibitor LUF5771 that was reported in *Chapter 5*. The presence of 10 μ M LUF5771 resulted in an 88% increased dissociation of [³H]Org 43553 from the human LH receptor.

6.3.2 Radioligand Saturation Experiments

Although two other thiazoles were more potent allosteric enhancers of [³H]Org 43553 binding (Figure 6.1), LUF5419 (**4**) was selected for further experiments due to ample availability. Saturation binding assays with [³H]Org 43553 were performed in the absence (control) and presence of 10 μ M LUF5419. The results of a representative saturation experiment are shown in Figure 6.2. In both conditions the saturation of [³H]Org 43553 to membranes of CHO cells expressing the human LH receptor was saturable and best characterized by a one-site receptor model. The K_D and B_{max} values obtained from the saturation experiments are given in Table 6.2. Under control conditions a K_D and B_{max} value of 2.2 ± 0.4 nM and 601 ± 61 fmol/mg was obtained for [³H]Org 43553. The presence of 10 μ M LUF5419 resulted in a 33% increase in the B_{max} value (798 fmol/mg), while the K_D value was somewhat increased to 3.1 ± 0.2 nM. The K_D values obtained in the absence or presence of LUF5419 were used to derive K_i rather than IC_{50} values for Org 43553, as described in the next section.

Table 6.2 K_D and B_{max} values of specific [³H]Org 43553 binding in the absence (control) and presence of 10 μ M LUF5419 at human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes.

Condition	K_D (nM)	B_{max} (fmol/mg)
Control	2.2 ± 0.4	601 ± 61
+ 10 μ M LUF5419	3.1 ± 0.2 **	798 ± 71 *

The values of the saturation binding constants were obtained by analysis of increasing concentrations of [³H]Org 43553 bound to human luteinizing hormone receptors.

Values are means ($\pm$ SEM) of three separate assays each performed in duplicate (* $p < 0.05$, ** $p < 0.005$ versus control).

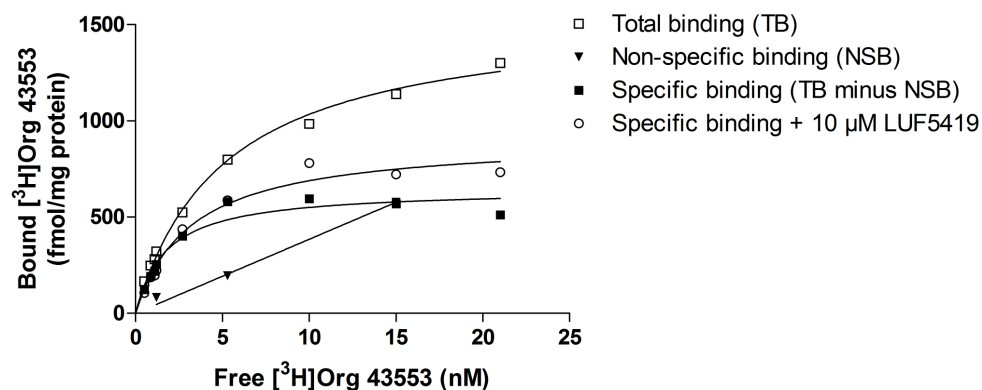


Figure 6.2 Saturation of [^3H]Org 43553 to luteinizing hormone receptors in the absence (control) or presence of 10 μM LUF5419. The control specific binding was determined by subtracting the non-specific binding from the total binding curve. A similar experiment was performed in the presence of 10 μM LUF5419, of which only the specific binding is shown. Representative graphs from one experiment performed in duplicate (see Table 6.2 for K_D and B_{max} values).

6.3.3 Radioligand Displacement Experiments

The affinity of Org 43553 in the absence and presence of 10 μM LUF5419 for the human luteinizing hormone receptor was determined (Figure 6.3). In the control condition Org 43553 had an affinity of 6.4 ± 1 nM. In the presence of 10 μM LUF5419, the affinity of Org 43553 was unchanged ($K_i = 6.8 \pm 1$ nM), whereas the B_{max} was enhanced, as already mentioned above for labeled Org 43553. In addition, the effect of LUF5419 on the equilibrium binding of the iodinated endogenous ligand, ^{125}I -hCG, was examined. LUF5419 was not able to displace or enhance ^{125}I -hCG binding (data not shown). Furthermore, the affinity of recLH was also unaffected by 10 μM LUF5419.

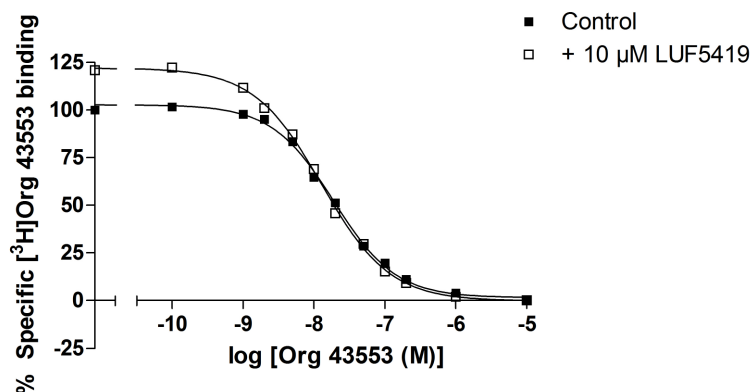


Figure 6.3 Displacement of [^3H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes by unlabeled Org 43553 in the absence (control) or presence of 10 μM LUF5419. Representative graphs from one experiment performed in duplicate.

6.3.4 Kinetic Association and Dissociation Experiments

Subsequently, the effect of LUF5419 on the kinetic association and dissociation parameters of [^3H]Org 43553 at CHO hLHr_{luc} membranes at 30 $^{\circ}\text{C}$ were determined. As shown in Table 6.3 and Figure 6.4, [^3H]Org 43553 associated to the receptor within 120 min with a k_{on} value of $0.0082 \pm 0.0004 \text{ nM}^{-1} \text{ min}^{-1}$. In the presence of 10 μM LUF5419, the association rate was not significantly altered ($k_{\text{on}} = 0.0092 \pm 0.0003 \text{ nM}^{-1} \text{ min}^{-1}$). As expected, the B_{max} was significantly increased by 23%, corresponding to the effect found in equilibrium saturation and displacement assays. The dissociation rate of [^3H]Org 43553 was almost two-fold decreased in the presence of 10 μM LUF5419 (Table 6.3). Taken together, this resulted in a ‘kinetic’ K_{D} ($k_{\text{off}}/k_{\text{on}}$) value of 2.4 nM for control conditions, which was in good agreement with the K_{D} value (2.1 nM) obtained by saturation analysis. In the presence of 10 μM LUF5419, a ‘kinetic’ K_{D} value of 1.2 nM was obtained, which was somewhat lower than the K_{D} value obtained in the equilibrium saturation experiments. Similar to the results in equilibrium binding assays, the dissociation rate of ^{125}I -hCG was not changed by the presence of 10 μM LUF5419 (data not shown).

Table 6.3 Association (k_{on}) rate constants, dissociation (k_{off}) rate constants and the apparent (kinetic) dissociation constant (K_D) of radiolabeled Org 43553.

	k_{on} (nM ⁻¹ min ⁻¹) ^a	B_{max} (%) ^b	k_{off} (nM ⁻¹) ^a	K_D ^c
Control	0.0082 ± 0.0004	100 ± 1	0.020 ± 0.002	2.4
+ 10 μ M LUF5419	0.0092 ± 0.0003	$123 \pm 3^{**}$	$0.011 \pm 0.001^*$	1.2

^a The values of the kinetic association and dissociation rate constants were obtained by analysis of the exponential association and dissociation of [³H]Org 43553 bound to human luteinizing hormone receptors.

^b Maximal amount of [³H]Org 43553 bound to human luteinizing hormone receptors after association in the absence (control = 100%) or presence of 10 μ M LUF5419.

^c The dissociation constant was defined as the ratio of k_{off} - and k_{on} -values.

Values are means ($\pm$ S.E.M.) of at least three separate assays performed in duplicate (* $p < 0.05$, ** $p < 0.005$ versus control).

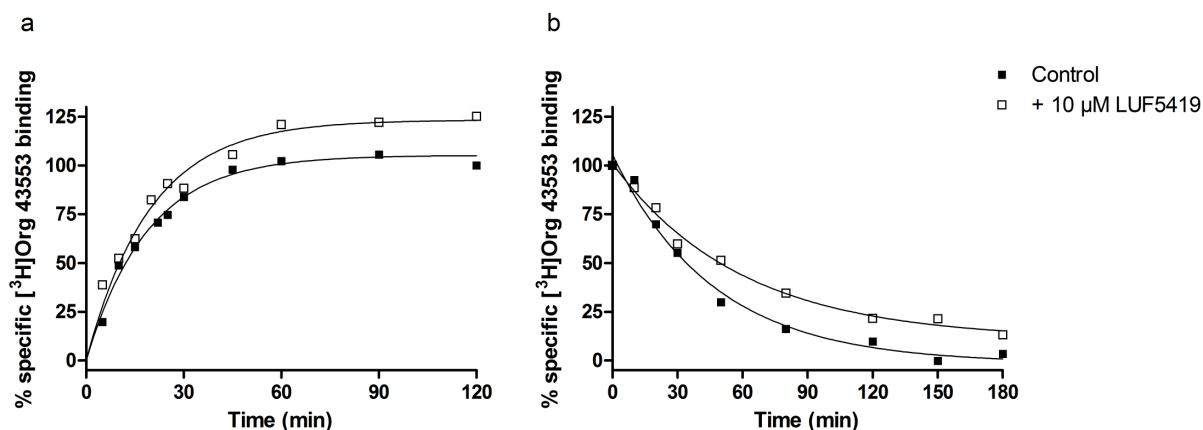


Figure 6.4 a) Association and b) dissociation kinetics of [³H]Org 43553 binding to CHO-K1 membranes expressing the human luteinizing hormone receptor at 30°C. Dissociation was either initiated by the addition of 10 μ M Org 43553 in the absence (control) or presence of 10 μ M LUF5419. Representative graphs from one experiment performed in duplicate (see Table 6.3 for kinetic parameters).

6.3.5 Allosteric Modulation of Receptor Activation

The effect of LUF5419 on receptor activation by the endogenous hormone, recLH, or the low molecular weight agonist, Org 43553, was measured using a CRE-induced luciferase assay (Figure 6.5 and Table 6.4). RecLH fully activated the LH receptor with a potency of 140 ± 30 pM, while Org 43553 partially activated ($E_{max} = 79 \pm 2\%$) the LH receptor with an EC_{50} value of 0.78 ± 0.2 nM. In the presence of 10 μ M LUF5419, the potencies of recLH and Org 43553 were not shifted. The efficacy, however, was decreased for recLH, while it was

unchanged for Org 43553. The decrease in luciferase activity with LUF5419 was also observed when the CRE-pathway was activated by 10 μ M forskolin (Figure 6.5). After correction for the forskolin-effect, an enhancement of the efficacy of Org 43553 was observed. As a consequence, it appeared that Org 43553 was able to fully activate the receptor in the presence of 10 μ M LUF5419 ($E_{\max} = 103 \pm 5\%$), similar to the effect by recLH alone.

Table 6.4 Receptor activation by recLH or Org 43553 in the presence or absence of 10 μ M LUF5419, expressed as EC_{50} and $E_{\max}$ values.

	Activity in luciferase assay ^a		
	EC_{50} (nM)	$E_{\max}$ (%) ^b	$E_{\max}$ (%) normalized ^c
RecLH	0.14 ± 0.03	100 ± 2	100 ± 2
+ 10 μ M LUF5419	0.13 ± 0.03	$64 \pm 9^{**}$	90 ± 12
Org 43553	0.78 ± 0.2	79 ± 2	79 ± 2
+ 10 μ M LUF5419	1.0 ± 0.2	73 ± 3	$103 \pm 5^{**}$

^a cAMP-mediated luciferase activity in CHO-K1 cells that stably express the human luteinizing hormone receptor and CRE-luciferase reporter gene.

^b Maximal effect of either recLH or Org 43553 in the absence or presence of 10 μ M LUF5419, where recLH in the absence of LUF5419 was set at 100%.

^c Maximal effect corrected for the effect of 10 μ M LUF5419 on forskolin-induced luciferase activity.

Values are means ($\pm$ S.E.M.) of at least three separate assays performed in duplicate (** $p < 0.005$ versus control).

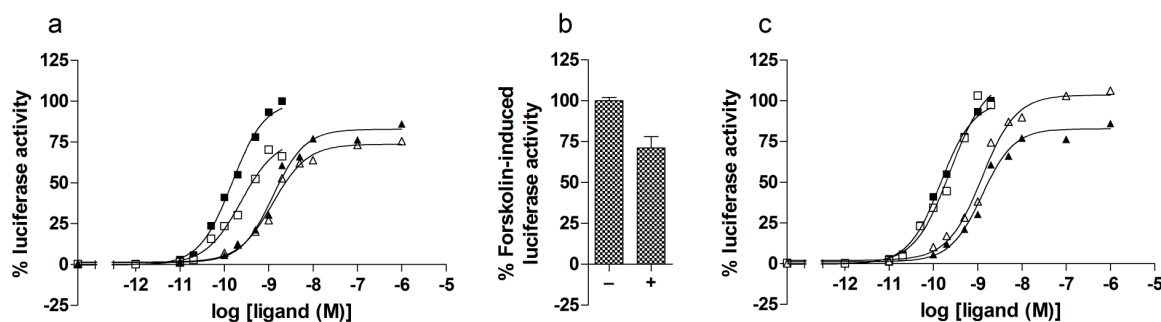


Figure 6.5 Concentration-effect curves of recLH and Org 43553 in the absence (■; recLH, ▲; Org 43553) or presence of 10 μ M LUF5419 (□; recLH, △; Org 43553) for cAMP-mediated luciferase production through human luteinizing hormone receptors. a) curves of raw data, b) bargraph showing the effect of 10 μ M LUF5419 on forskolin-induced (10 μ M) luciferase activity, c) curves corrected for forskolin-effect. Representative graphs from one experiment performed in duplicate (see Table 6.4 for EC_{50} and $E_{\max}$ values).

6.3.6 Competitive Dissociation Experiments

Subsequently, competitive dissociation experiments were performed to determine whether the allosteric inhibitor LUF5771 and the allosteric enhancer of [³H]Org 43553 binding, LUF5419, bound at the (same) allosteric site. First, the modulating potency of LUF5419 was determined (Figure 6.6 and Table 6.5). Therefore, dissociation was induced by an excess unlabelled Org 43553 in the presence of different concentrations of LUF5419, which resulted in an EC₅₀ value of 23 ± 4 μM for this compound. Subsequently, the effect of different concentrations of LUF5771 on [³H]Org 43553 dissociation was determined in the absence and presence of 10 or 50 μM LUF5419 (Table 6.5 and Figure 6.7). The obtained data are represented in two formats. Figure 6.7a shows that the addition of LUF5419 resulted in allosteric enhancement of [³H]Org 43553, i.e. increased percentage of radioligand binding after 30 min dissociation in comparison to control conditions. In addition, irrespective of the presence of LUF5419, LUF5771 dose-dependently increased the dissociation of [³H]Org 43553, i.e. allosteric inhibition. In Figure 6.7b, the data was normalized and this proved that the modulating potency of LUF5771 was decreased either two- or ten-fold by the presence of 10 μM or 50 μM LUF5419, respectively, indicative for a competitive interaction of these two compounds.

Table 6.5 Allosteric modulation of [³H]Org 43553 binding by LUF5419 and LUF5771, expressed as EC₅₀ values.

Compound	Modulatory potency EC ₅₀ ± SEM (μM) ^a	Shift ^b
LUF5419	23 ± 4	-
LUF5771	1.7 ± 0.3	-
+ 10 μM LUF5419	3.4 ± 0.4*	2.0
+ 50 μM LUF5419	17 ± 1***	10

^a The value for the concentration at half-maximal [³H]Org 43553 binding after 30 min of dissociation in the presence of LUF5771 or LUF5419 or a combination of these modulators.

^b The shift is defined as the ratio of EC₅₀ values in the absence (control; LUF5771) or presence of LUF5419, respectively.

Values are means (± S.E.M.) of at least three separate assays performed in duplicate (* p < 0.05, *** p < 0.001 versus control).

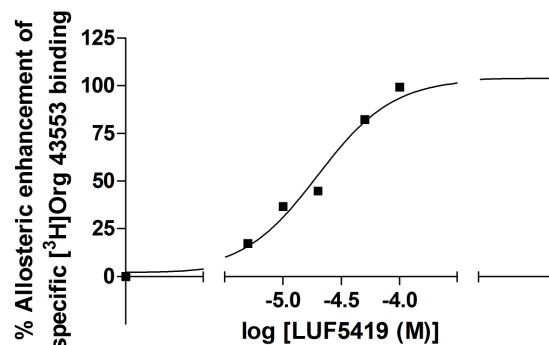


Figure 6.6 Concentration-dependent effect of LUF5419 on dissociation of [^3H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes measured at a single time point of 30 min. Data points corresponding to total binding (no dissociation) and control binding (dissociation after 30 min) are set at 100 and 0%, respectively. Representative graph from one experiment performed in duplicate (see Table 6.5 for EC_{50} values).

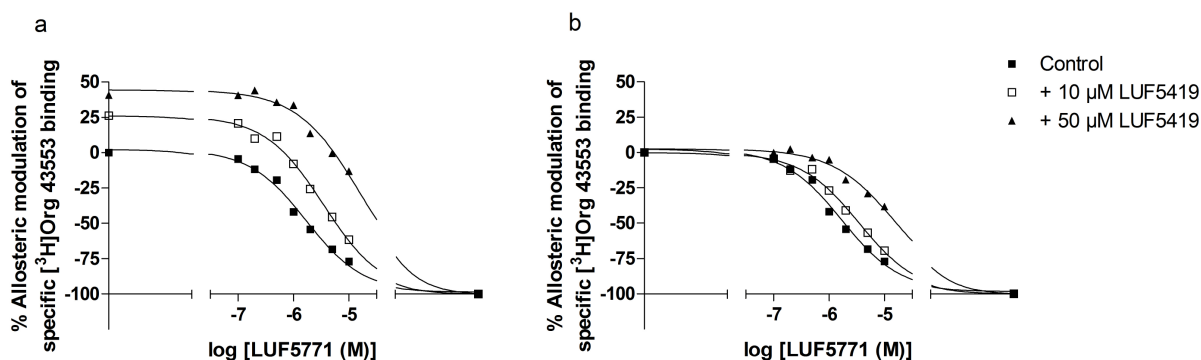


Figure 6.7 Concentration-dependent effect of LUF5771 on dissociation of [^3H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes in the absence (control) or presence of 10 μM or 50 μM LUF5419 measured at a single time point of 30 min. The top graph (a) shows data normalized to the control measured in the absence of LUF5419 and the middle graph (b) shows data normalized to the three conditions in the absence of LUF5771. Representative graph from one experiment performed in duplicate (see Table 6.5 for EC_{50} values).

6.3.7 Simulation of Cooperativity between LUF5419 and LUF5771

The effect of LUF5419 on the modulating potency of LUF5771 for [^3H]Org 43553 binding to the human LH receptor was simulated using Eq. 6.1 under *Materials and Methods*,

taken from Lazareno and coworkers.⁶² The different simulations in which $\delta = 1$ (neutral cooperativity), $\delta < 1$ (negative cooperativity) or $\delta > 1$ (positive cooperativity) are shown in Figure 6.8. The data points obtained with the competitive dissociation assays (Table 6.5) can not be fitted by this model that describes three separate binding sites. These three compounds, therefore, might occupy only two sites within the receptor.

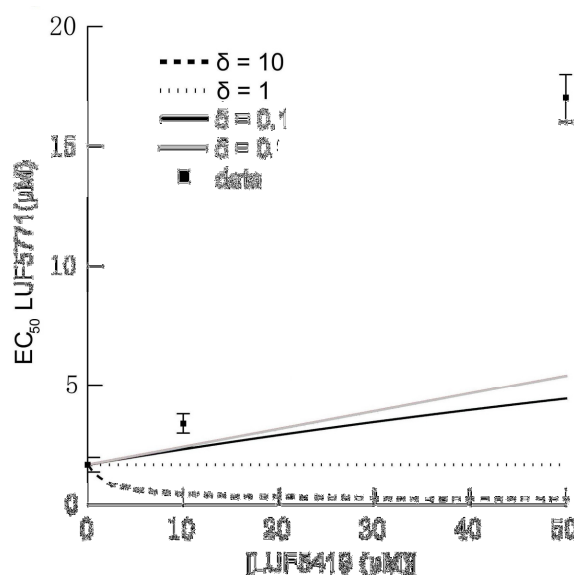


Figure 6.8 Negative cooperativity between LUF5771 and LUF5419 in modulating [^3H]Org 43553 dissociation. The experimental data of different concentrations of LUF5419 affecting the modulating potency of LUF5771 is displayed with standard deviation. The lines show the fit of the data to eq. 1 (see material and methods), where the situations are simulated that two compounds exhibit positive ($\delta > 1$), neutral ($\delta = 1$) and negative cooperativity ($\delta < 1$).

6.4 DISCUSSION

In the present study the first allosteric enhancer of [^3H]Org 43553 binding at the human LH receptor, LUF5419, is described. An initial screen for allosteric modulation of [^3H]Org 43553 resulted in a few hits, among which a thiazole-containing ligand. From Figure 6.1, it follows that the thiazole-analogs showed different modulating potencies, where LUF5419 was one of the more potent derivatives. Previously, thiazole analogs have been a subject for investigation as (competitive) antagonists at the adenosine A_1 and A_3 receptors.^{292,293} Indeed [^{125}I]AB-MECA, an agonist, was displaced from the human adenosine A_3 receptor for 69% by 10 μM LUF5419, while it was not from the human A_1 receptor (data not shown). Notably, LUF5419 did not affect the dissociation rate of that radioligand from either one of the receptor subtypes. In the present study, LUF5419 and the derivatives (**1-3**, **5**) did not compete with [^3H]Org 43553 for its binding site (Table 6.1). However, the dissociation rate was decreased and as a consequence the total binding of [^3H]Org 43553 was enhanced. The modulating potency of LUF5419 is therefore typical for [^3H]Org 43553 binding at the LH receptor.

Saturation assays in the absence and presence of LUF5419 were performed to investigate whether the increased [^3H]Org 43553 binding was due to an increase in the B_{max} or an increase in affinity. From Figure 6.2 and Table 6.2 it follows that the B_{max} was increased and the K_D slightly increased. An equilibrium displacement assay showed that the affinity of unlabelled Org 43553 was not affected by LUF5419. In a cAMP-induced luciferase assay, the efficacy of Org 43553 increased, while the potency was unchanged (Table 6.4). A similar observation was reported for LUF6000, an allosteric enhancer of the adenosine A_3 receptor.²⁸⁴ In this case, however, LUF6000 enhanced the effects of a ligand bound to the orthosteric site located within the 7-TM domain, as for most other class A GPCRs. Although the LH receptor has also been classified as a class A GPCR, the binding site for the endogenous hormones, recLH and hCG, is located at the large extracellular N-terminal domain.¹⁸ Org 43553 has been shown to bind an allosteric site located within the 7-TM domain.³⁶ The interaction between orthosteric and allosteric ligands of most class A GPCRs occurs across the 7-TM domain. It could be that the modulation of the N-terminal orthosteric site of the LH receptor is different. For this purpose, allosteric modulation of the LH receptor could possibly best be compared to class B or class C GPCRs, which also have a large N-terminal domain that binds the endogenous ligand.²³⁶ Noteworthy, is that we screened for allosteric modulation of the allosteric agonist, Org 43553, while in papers concerning

allosteric modulation of class B or C GPCRs, the read-out is the effect on the endogenous ligand. For example, in the case of the GABA_B receptor, a class C GPCR, two allosteric enhancers have been reported that modulate the potency and efficacy of GABA.^{294,295} In addition, allosteric modulation of GPCRs was recently reviewed by May *et al.*, where it was explained that the effect of an allosteric modulator can be divergent.⁴⁹ Moreover, allosteric modulation has also been shown to be probe-dependent.²⁹⁶ Altogether, this might explain that LUF5419 does not show enhancement of hormone affinity, potency or efficacy, while it does affect Org 43553 binding and efficacy at the human LH receptor.

Very recently, the first allosteric inhibitor for the human LH receptor was reported, as described in *Chapter 5* (Table 6.1). This LMW compound, LUF5771, was able to allosterically modulate both Org 43553 and recLH binding and function, unlike LUF5419. With these two allosteric modulators at hand, the question arose, whether these compounds bind to the same allosteric site (a site different from Org 43553). Therefore, competitive dissociation experiments were performed as described previously for two allosteric inhibitors at the GnRH receptor (*Chapter 3*). In this case, these experiments were performed with an allosteric enhancer (LUF5419) and an allosteric inhibitor (LUF5771) of [³H]Org 43553 binding at the human LH receptor. First, the modulating potencies of LUF5419 and LUF5771 were determined under control conditions. From Table 6.5 it follows that LUF5771 ($EC_{50} = 1.7 \pm 0.3 \mu\text{M}$) is over 10-fold more potent in modulating [³H]Org 43553 binding than LUF5419 ($EC_{50} = 23 \pm 4 \mu\text{M}$). Second, it was shown that the modulating potency of LUF5771 was decreased in the presence of LUF5419, in other words these allosteric modulators probably competed for the same allosteric site at the human LH receptor (Figure 6.7b and Table 6.5).

Recently, we reported that three diverse ligands for the human GnRH receptor bind to three distinct sites. In the present study, we also have three different classes of ligands for the human LH receptor (without taking the hormone into account). Therefore, the data obtained by competitive dissociation experiments (Table 6.5) was simulated using a model described by Lazareno and coworkers⁶² as applied previously to the GnRH receptor (*Chapter 3*). This model can simulate the cooperativities between ligands at three available binding sites. The data shown in Table 6.5, however, could not be simulated with this model (Figure 6.8), indicating that these ligands do not bind to three distinct binding sites. Even when the δ value was decreased to zero (i.e. a competitive interaction) the data points could not be fit. Analysis of these data by Schild-regression was not possible as the extent of the shift is limited due to

solubility of LUF5419. However, the (linear) rightward shift observed in Figure 6.7b does implicate competitive antagonism of LUF5419. In other words, the human LH receptor contains one binding site for [^3H]Org 43553 and a second site that accommodates both LUF5419 and LUF5771. The nature of allosteric modulation of the glycoprotein hormone receptors was addressed in a recent review.⁵⁵ In this paper, the presence of two separate allosteric sites within the 7-TM domain of these receptors was also hypothesized. First there is the classical class A GPCR ligand binding site, where most probably Org 43553-like compounds bind. A similar binding site was proposed recently for an analogue of Org 43553 when interacting with both the LH and TSH receptor.^{191,297} LMW ligands for the FSH receptor, however, are thought to bind to a second site within the 7-TM domain, as shown by studies with chimeric receptor constructs.⁵⁴ A further clue might be in the notion that LUF5419 is also an (orthosteric) adenosine A_3 receptor antagonist.²⁹³ We, therefore, examined whether LUF5771 may possibly also bind to the same site in the adenosine A_3 receptor. Interestingly, LUF5771 showed a (low) affinity at this receptor (52% displacement at 10 μM), similar to LUF5419. The (orthosteric) binding pocket of typical adenosine A_3 receptor agonists and antagonists has been investigated by Gao and coworkers using site-directed mutagenesis.²⁹⁸ Several residues that are important for ligand binding were identified. However, in a multiple sequence alignment of all class A GPCRs obtained from the GPCRDB none of these residues is conserved or homologous between the human adenosine A_3 and LH receptor.²⁹⁹ This can be regarded as yet another indication that LUF5419 and LUF5771 do not bind to this binding pocket.

In conclusion, this paper describes the first series of allosteric enhancers displaying different potencies at the human LH receptor. In particular LUF5419 was able to significantly decrease the dissociation rate of Org 43553. In addition, LUF5419 increased the maximum binding of [^3H]Org 43553, which was reflected in an increase of the efficacy of Org 43553 in a functional assay to levels similar as obtained by stimulation with the endogenous hormone, recLH. Furthermore, we hypothesize that the human LH receptor contains a second allosteric site in the 7TM domain, dissimilar to the (orthosteric) binding pocket of the human adenosine A_3 receptor. The site is recognized by two low molecular weight allosteric modulators, a positive (LUF5419) and a negative one (LUF5771), which therefore offers another perspective of developing orally available ligands for the human LH receptor.

CHAPTER

7

False Positives in a Reporter Gene Assay: Identification and Synthesis of Substituted N- Pyridin-2-yl-benzamides as Competitive Inhibitors of Firefly Luciferase

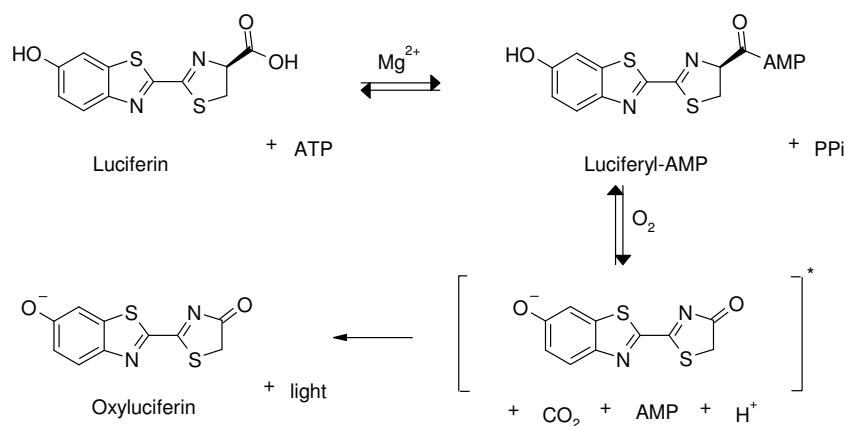
Luciferase reporter-gene assays are a commonly used technique in high-throughput screening campaigns. In this study, we report a luciferase inhibitor (**1**), which emerged from a luciferase reporter-gene assay screen. Instead of displaying receptor activity compound **1** was shown to potently inhibit luciferase in an *in vitro* enzymatic assay with an IC_{50} value of $1.7 \pm 0.1 \mu\text{M}$. In addition, **1** was a competitive inhibitor with respect to the substrate luciferin. A database search yielded another inhibitor (**3**) with a similar, N-pyridin-2-yl-benzamide core. Subsequently, several analogs were prepared to investigate the structure-activity relationships of these luciferase inhibitors. This yielded the most potent inhibitor of this series (**6**) with an IC_{50} value of $0.069 \pm 0.01 \mu\text{M}$. Further molecular modeling studies suggested that **6** can be accommodated in the luciferin binding site. This paper is meant to alert users of luciferase reporter gene assays for possible false positive hits including highly ‘drug-like’ molecules due to direct luciferase inhibition.

This chapter is an adjusted version of a recent publication:

Heitman, L. H; Veldhoven, J. P. D; Zweemer, A. M.; Ye, K.; Brussee, J.; IJzerman, A. P. False Positives in a Reporter Gene Assay: Identification and Synthesis of Substituted N-Pyridin-2-yl-benzamides as Competitive Inhibitors of Firefly Luciferase. *J Med Chem*, **2008**, *51*, 4724-4729

7.1 INTRODUCTION

Firefly luciferase has a long history of use in biology. This enzyme catalyses the formation of luciferyl-adenylate from the substrates luciferin and ATP (Scheme 7.1).³⁰⁰ The luciferyl-adenylate is oxidized and converted to an electronically excited state of oxyluciferin. Return to the ground state results in the emission of visible light with a wavelength of approximately 562 nm. The cloning of firefly luciferase in 1985 generated a great deal of interest in possible applications of the gene as a tool in scientific research.^{301,302} For example, luciferase has been proposed as a model for the μ -opioid receptor, as there are



Scheme 7.1 The luciferase-catalyzed reaction. In the presence of ATP, luciferin is activated to luciferyl-AMP, which is oxidized by O_2 to produce an excited state of oxyluciferin (*). On return to the ground state, light is emitted.

structural similarities between the catalytic site of the enzyme and the opioid binding site of the receptor.³⁰³ Nowadays, luciferase is applied widely as a reporter gene in high throughput screening processes, because of its high sensitivity and ease of use.³⁰⁴ Reporter assays couple the biological activity of a target to the expression of a variety of readily detected enzymes and thereby provide a highly amplified signal. It should be noted that the luciferin-luciferase reaction has been shown to be inhibited strongly by the products oxyluciferin and AMP.^{305,306} In addition, many substrate-like compounds such as ATP³⁰⁶ and luciferin-analogues,³⁰⁷ but also dissimilar compounds, such as pifithrin- α ³⁰⁸, lipoic acid³⁰⁹ and N-tosylphenylalanine chloromethyl ketone (TPCK)³¹⁰ have been shown to inhibit luciferase activity.

In an initial screen, several compounds were tested for activity at the human LH receptor using a radioligand dissociation assay (*Chapter 5* and *Chapter 6*) and the firefly luciferase reporter gene system (data not shown). In the latter case, this resulted in a high amount of false positive hits. In the present study, we report that compound **1**, which emerged from that screen, is a highly potent competitive luciferase inhibitor with respect to one of the substrates, luciferin. Very recently, luciferase inhibitors in the Pubchem database were described in a paper by Auld and coworkers.^{198,311} That library contained several structural analogues of **1** of which one compound was a highly potent luciferase inhibitor (**3**). Therefore, different N-pyridin-2-yl-benzamide analogs (**4-12**, **22-39**) were prepared to shed light on the molecular requirements for luciferase inhibition. In addition, the most potent inhibitor (**6**) was docked into the crystal structure of luciferase at the luciferin binding site, in agreement with its competitive nature. Since these compounds are drug-like molecules, it should be taken into account that ‘false positives’ can easily emerge when luciferase activity is used as a read-out in high-throughput screens.

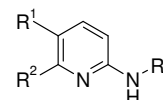
7.2 MATERIALS AND METHODS

7.2.1 Chemistry – Materials

All reagents used were obtained from commercial sources and all solvents were of analytical grade. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 200 (^1H NMR, 200 MHz; ^{13}C NMR, 50.29 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in δ (ppm) and the following abbreviations are used: s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, br = broad, Ar = aromatic protons. Elemental analyses were performed by the Leiden Institute of Chemistry and are within 0.4% of the theoretical values unless otherwise stated. Reactions were routinely monitored on TLC using Merck silica gel F₂₅₄ plates. Microwave reactions were performed in an Emrys Optimizer (Biotage AB, formerly Personal Chemistry). Wattage was automatically adjusted so as to maintain the desired temperature. The yields of the products were not optimized. All the final products were purified by column chromatography.

7.2.2 General Procedure for the Preparation of 1, 3-7, 9, 11 and 12.³¹²

The appropriate acid chloride (1.1 mmol) was added to a solution of 2-amino-(substituted)-pyridine (1.0 mmol) in pyridine (4 mL) at room temperature under a nitrogen atmosphere. According to TLC the reaction went to completion after 2 hours. The organic material was extracted with DCM, dried over MgSO_4 and evaporated under reduced pressure. The crude product was purified by column chromatography, eluting with a mixture of 0.5-2% methanol and chloroform or 0.5-2% methanol and dichloromethane.



N-Quinolin-2-yl-benzamide (1). Starting from 2-amino-quinoline.³¹³ Yield 31%; white solid, recrystallized from ethanol. ^1H -NMR δ (CDCl_3): 8.99 (br s, 1H, NH), 8.59 (d, 1H, J = 9.1 Hz, quinoline-H), 8.21 (d, 1H, J = 8.8 Hz, quinoline-H), 8.01-7.96 (m, 2H, phenyl-H), 7.84-7.77 (m, 2H, quinoline-H), 7.66 (dd, 1H, J¹ = 8.4 Hz, J² = 1.5 Hz, quinoline-H), 7.56-7.41 (m, 4H, phenyl-H + quinoline-H). ^{13}C -NMR δ (CDCl_3): 151.1, 146.5, 141.0, 138.5, 134.1, 132.3, 129.9, 129.2, 128.7, 127.5, 127.2, 125.1, 114.3, 114.1. Anal. ($\text{C}_{16}\text{H}_{12}\text{N}_2\text{O} \cdot 0.05\text{EtOH}$) C, H, N.

2,4-Dimethoxy-N-(5-methyl-pyridin-2-yl)benzamide (3). Starting from 2-amino-5-methyl-pyridine. Yield 54%; white solid. ^1H -NMR δ (CDCl_3): 10.17 (br s, 1H, NH), 8.31 (d,

1H, $J = 8.4$ Hz, pyridine-*H*), 8.24 (d, 1H, $J = 9.1$ Hz, Ar-*H*), 8.13 (s, 1H, pyridine-*H*), 7.52 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 6.63 (dd, 1H, $J^1 = 8.8$ Hz, $J^2 = 2.2$ Hz, Ar-*H*), 6.53 (d, 1H, $J = 2.2$ Hz, Ar-*H*), 4.04 (s, 3H, OCH₃), 3.86 (s, 3H, OCH₃), 2.29 (s, 3H, CH₃). ¹³C-NMR δ (CDCl₃): 163.7, 162.9, 158.6, 149.7, 147.5, 138.4, 133.8, 128.3, 114.0, 113.8, 105.0, 98.2, 55.9, 55.2, 17.5. Anal. (C₁₅H₁₆N₂O₃) C, H, N.

N-Pyridin-2-yl-benzamide (4). Starting from 2-amino-pyridine. Yield 69%; white solid. ¹H-NMR δ (DMSO): 10.81 (br s, 1H, NH), 8.41 (d, 1H, $J = 2.9$ Hz, pyridine-*H*), 8.22 (d, 1H, $J = 8.0$, pyridine-*H*), 8.07-8.03 (m, 2H, phenyl-*H*), 7.87 (dd, 1H, $J^1 = 7.3$ Hz, $J^2 = 1.5$ Hz, pyridine-*H*), 7.66-7.49 (m, 3H, phenyl-*H*), 7.19 (dd, 1H, $J^1 = 6.2$ Hz, $J^2 = 2.2$ Hz, pyridine-*H*). ¹³C-NMR δ (CDCl₃): 165.7, 151.4, 147.6, 138.2, 134.1, 132.0, 128.5, 127.1, 119.7, 114.0. Anal. (C₁₂H₁₀N₂O) C, H, N.

N-(6-Phenylpyridin-2-yl)benzamide (5). Starting from 2-amino-6-phenyl-pyridine. Yield 64%; white solid. ¹H-NMR δ (DMSO): 10.76 (br s, 1H, NH), 8.25-8.06 (m, 5H, phenyl-*H* + pyridine-*H*), 7.98-7.73 (m, 2H, pyridine-*H*), 7.60-7.43 (m, 6H, phenyl-*H*). ¹³C-NMR δ (CDCl₃): 165.7, 155.9, 151.2, 139.1, 138.6, 134.2, 132.1, 129.0, 128.7, 128.6, 127.1, 126.7, 116.6, 112.3. Anal. (C₁₂H₁₀N₂O) C, H, N.

N-(5-Phenylpyridin-2-yl)benzamide (6). Starting from 2-amino-5-phenyl-pyridine. Yield 46%; white solid. ¹H-NMR δ (CDCl₃): 8.83 (br s, 1H, NH), 8.50-8.46 (m, 2H, pyridine-*H*), 8.00-7.93 (m, 3H, pyridine-*H* + phenyl-*H*), 7.58-7.38 (m, 8H, phenyl-*H*). ¹³C-NMR δ (CDCl₃): 166.3, 150.9, 145.7, 137.0, 136.6, 134.5, 132.5, 131.9, 128.8, 128.5, 127.6, 127.4, 126.4, 114.1. Anal. (C₁₈H₁₄N₂O*0.04CHCl₃) C, H, N.

N-(5-Methylpyridin-2-yl)benzamide (7). Starting from 2-amino-5-methyl-pyridine.³¹⁴ Yield 49%; white solid. ¹H-NMR δ (CDCl₃): 8.93 (br s, 1H, NH), 8.29 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 7.98-7.89 (m, 3H, phenyl-*H* + pyridine-*H*), 7.58-7.43 (m, 4H, phenyl-*H* + pyridine-*H*), 2.28 (s, 3H, CH₃). ¹³C-NMR δ (CDCl₃): 166.2, 149.7, 147.3, 138.6, 134.6, 131.4, 128.6, 128.2, 127.3, 113.8, 17.4. Anal. (C₁₃H₁₂N₂O) C, H, N.

Phenyl N-(5-phenylpyridin-2-yl)carbamate (9). Starting from phenyl chloroformate. Yield 47%; white solid. ¹H-NMR δ (CDCl₃): 9.60 (br s, 1H, NH), 8.67 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 8.13 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 7.96 (dd, 1H, $J^1 = 8.7$ Hz, $J^2 = 2.2$ Hz, pyridine-*H*), 7.56-7.23 (m, 10H, phenyl-*H*). ¹³C-NMR δ (CDCl₃): 151.5, 150.6, 150.2, 145.8, 136.9, 132.1, 129.3, 128.8, 127.5, 126.4, 125.7, 121.4, 112.3. Anal. (C₁₈H₁₄N₂O₂*0.05CHCl₃) C, H, N.

N-(5-Phenylpyridin-2-yl)butanamide (11). Yield 66%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.49 (br s, 1H, NH), 8.33-8.29 (m, 2H, pyridine-*H*), 7.92 (dd, 1H, $J^1 = 8.8$ Hz, $J^2 = 2.2$ Hz, pyridine-*H*), 7.57-7.37 (m, 5H, phenyl-*H*), 2.40 (t, 2H, $J = 7.66$ Hz, $\text{COCH}_2\text{CH}_2\text{CH}_3$), 1.84-1.73 (m, 2H, $\text{COCH}_2\text{CH}_2\text{CH}_3$), 1.02 (t, 3H, $J = 7.3$ Hz, $\text{COCH}_2\text{CH}_2\text{CH}_3$). $^{13}\text{C-NMR}$ δ (DMSO): 172.2, 151.5, 145.7, 136.9, 136.1, 131.0, 129.1, 127.6, 126.3, 113.4, 38.1, 18.5, 13.6. Anal. ($\text{C}_{15}\text{H}_{16}\text{N}_2\text{O} \cdot 0.02\text{CHCl}_3$) C, H, N.

N-(5-Phenylpyridin-2-yl)cyclohexanecarboxamide (12). Yield 69%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.49 (d, 1H, $J = 2.2$ Hz, pyridine-*H*), 8.31 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 8.16 (br s, 1H, NH), 7.91 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.5$ Hz, pyridine-*H*), 7.58-7.37 (m, 5H, phenyl-*H*), 2.36-2.22 (m, 1H, $J = 7.66$ Hz, *CH*-cyclohexane), 2.02-1.25 (m, 10H, *CH*₂-cyclohexane). $^{13}\text{C-NMR}$ δ (CDCl_3): 174.9, 150.9, 145.4, 136.9, 137.2, 136.7, 132.4, 128.8, 127.5, 126.4, 114.0, 46.1, 29.3, 25.4. Anal. ($\text{C}_{18}\text{H}_{20}\text{N}_2\text{O} \cdot 0.02\text{CHCl}_3$) C, H, N.

7.2.3 Synthesis of N-(5-Phenylpyridin-2-yl)benzylamine (8).

A solution of 2-amino-5-phenyl-pyridine (300 mg, 1.76 mmol) and benzyl chloride (405 μL , 3.52 mmol) in pyridine (3.5 mL) was heated in a closed vessel in the microwave at 180 $^\circ\text{C}$ for 2 hours. The reaction mixture was poured in water, adjusted to pH = 10 with 1 M NaOH and extracted with DCM. The combined organic layers were dried over MgSO_4 , filtered and the solvents were evaporated under reduced pressure. The crude product was purified by silica gel column chromatography using DCM/methanol (99/1), followed by recrystallization from methanol, yielding 78 mg (17% yield) of the crystalline product. Yield 17%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.36 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.65 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.5$ Hz, pyridine-*H*), 7.52-7.27 (m, 10H, phenyl-*H*), 6.45 (d, 1H, $J = 8.8$ Hz, pyridine-*H*), 5.03 (br s, 1H, NH), 4.55 (d, 2H, $J = 5.9$ Hz, *CH*₂). $^{13}\text{C-NMR}$ δ (CDCl_3): 157.9, 146.3, 139.0, 138.3, 136.1, 128.7, 128.5, 127.3, 127.1, 126.5, 126.0, 106.4, 46.2. Anal. ($\text{C}_{18}\text{H}_{16}\text{N}_2$) C, H, N.

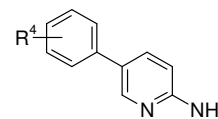
7.2.4 Synthesis of 1-Phenyl-3-(5-phenylpyridin-2-yl)urea (10).

The 2-amino-5-phenylpyridine (250 mg, 1.47 mmol) was dissolved in dioxane (2 mL) and phenyl isocyanate (160 μL , 1.47 mmol) was added at room temperature under a nitrogen atmosphere. After 4 hours the formed precipitate was collected by filtration and washed with

chloroform. Yield 84%; white solid. $^1\text{H-NMR}$ δ (DMSO): 10.41 (br s, 1H, NH), 9.55 (s, 1H, NH), 8.64 (d, 1H, $J = 1.8$ Hz, pyridine-*H*), 8.10 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.5$ Hz, pyridine-*H*), 7.73-7.30 (m, 10H, phenyl-*H*), 7.08-7.01 (m, 1H, pyridine-*H*). $^{13}\text{C-NMR}$ δ (DMSO): 152.2, 144.8, 139.1, 136.9, 136.7, 129.5, 129.1, 127.6, 126.3, 122.6, 118.9, 111.9. Anal. ($\text{C}_{18}\text{H}_{15}\text{N}_3\text{O} \cdot 0.12\text{CHCl}_3$) C, H, N.

7.2.5 General Procedure for the Preparation of 2a and 13-21.³¹⁵

Under a nitrogen atmosphere 2-amino-5-bromo-pyridine (1.0 equiv), the appropriate substituted boronic acid (1.3 equiv) and Na_2CO_3 (2.6 equiv) were dissolved in a mixture of water and DMF (1:4), followed by the addition of $\text{Pd}(\text{OAc})_2$ (0.01 equiv). The reaction mixture was heated at 110 °C for 3 hours. After cooling to room temperature, it was diluted with water and the organic materials extracted with ethyl acetate. The organic layer was washed with water and brine, dried over MgSO_4 and concentrated *in vacuo*. The crude products were purified by silica column chromatography, eluting with ethyl acetate or with a 0-3% methanol in dichloromethane mixture.



2-Amino-5-phenylpyridine (2a).³¹⁵ Yield 34%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.32 (d, 1H, $J = 2.5$ Hz, pyridine-*H*), 7.68 (dd, 1H, $J^1 = 8.4$ Hz, $J^2 = 2.5$ Hz, pyridine-*H*), 7.52-7.31 (m, 5H, phenyl-*H*), 6.58 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.51 (br s, 1H, NH_2).

5-(4-Chloro-phenyl)-pyridin-2ylamine (13). Yield 26%; off white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.28 (d, 1H, $J = 2.2$ Hz, pyridine-*H*), 7.63 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.2$ Hz, pyridine-*H*), 7.45-7.35 (m, 4H, Ar-*H*), 6.57 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.55 (br s, 1H, NH_2).

5-(4-Methoxy-phenyl)-pyridin-2ylamine (14). Yield 64%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.28 (d, 1H, $J = 2.5$ Hz, pyridine-*H*), 7.62 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.6$ Hz, pyridine-*H*), 7.42 (d, 2H, $J = 8.8$ Hz, Ar-*H*), 6.97 (d, 2H, $J = 8.4$ Hz, Ar-*H*), 6.56 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.50 (br s, 1H, NH_2), 3.84 (s, 3H, OCH_3).

5-(4-Methyl-phenyl)-pyridin-2ylamine (15). Yield 59%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.34 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.65 (dd, 1H, $J^1 = 8.4$ Hz, $J^2 = 2.6$ Hz, pyridine-*H*), 7.39 (d, 2H, $J = 8.1$ Hz, Ar-*H*), 7.23 (d, 2H, $J = 8.4$ Hz, Ar-*H*), 6.56 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.58 (br s, 1H, NH_2), 2.38 (s, 3H, CH_3).

5-(3-Chloro-phenyl)-pyridin-2ylamine (16). Yield 62%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.29 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.63 (dd, 1H, $J^1 = 8.7$ Hz, $J^2 = 2.6$ Hz, pyridine-*H*), 7.47 (s, 1H, Ar-*H*), 7.39-7.24 (m, 3H, Ar-*H*), 6.57 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.58 (br s, 1H, NH_2).

5-(4-Dimethylamino-phenyl)-pyridin-2ylamine (17). Yield 52%; yellowish solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.28 (s, 1H, pyridine-*H*), 7.62 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 7.39 (d, 2H, $J = 8.7$ Hz, Ar-*H*), 7.79 (d, 2H, $J = 9.1$ Hz, Ar-*H*), 6.55 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.42 (br s, 1H, NH_2), 2.95 (s, 6H, $\text{N}(\text{CH}_3)_2$).

5-(4-Isopropoxy-phenyl)-pyridin-2ylamine (18). Yield 65%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.30 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.61 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.6$ Hz, pyridine-*H*), 7.40 (d, 2H, $J = 8.4$ Hz, Ar-*H*), 6.93 (d, 2H, $J = 8.4$ Hz, Ar-*H*), 6.55 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.60-4.51 (m, 3H, $\text{CH}(\text{CH}_3)_2 + \text{NH}_2$), 1.37 (s, 3H, $\text{CH}(\text{CH}_3)_2$), 1.34 (s, 3H, $\text{CH}(\text{CH}_3)_2$).

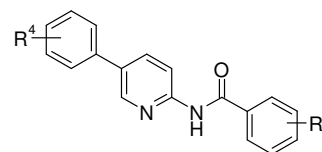
5-(4-*tert*-Butyl-phenyl)-pyridin-2ylamine (19). Yield 46%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.35 (d, 1H, $J = 2.2$ Hz, pyridine-*H*), 7.67 (dd, 1H, $J^1 = 8.5$ Hz, $J^2 = 2.4$ Hz, pyridine-*H*), 7.45 (m, 4H, Ar-*H*), 6.55 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.55 (s br, 2H, NH_2), 1.35 (s, 6H, $\text{C}(\text{CH}_3)_3$).

5-(4-Trifluoromethyl-phenyl)-pyridin-2ylamine (20). Yield 45%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.36 (d, 1H, $J = 2.2$ Hz, pyridine-*H*), 7.71-7.57 (m, 5H, pyridine-*H* + Ar-*H*), 6.60 (d, 1H, $J = 8.8$ Hz, pyridine-*H*), 4.73 (br s, 1H, NH_2).

5-(3,4-Dichloro-phenyl)-pyridin-2ylamine (21). Yield 62%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.30 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.65-7.56 (m, 2H, Ar-*H* + pyridine-*H*), 7.47 (d, 1H, $J = 8.4$ Hz, Ar-*H*), 7.30 (dd, 1H, $J^1 = 8.3$ Hz, $J^2 = 2.2$ Hz, Ar-*H*), 6.58 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 4.72 (br s, 2H, NH_2).

7.2.6 General Procedure for the Preparation of 22-39.³¹²

The appropriate acid chloride (1.1 mmol) was added to a solution of 2-amino-5-phenyl-pyridine (1.0 mmol) in pyridine (4 mL) at room temperature under a nitrogen atmosphere. According to TLC the reaction went to completion after 2 hours. The organic



material was extracted with DCM, dried over MgSO_4 and evaporated under reduced pressure. The crude product was purified by column chromatography, eluting with a mixture of 0.5-2% methanol and chloroform or 0.5-2% methanol and dichloromethane.

N-[5-(4-Chlorophenyl)pyridin-2-yl]benzamide (22). Yield 53%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.86 (br s, 1H, NH), 8.48 (d, 1H, $J = 8.76$ Hz, pyridine-*H*), 8.43 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.97-7.91 (m, 3H, pyridine-*H* + phenyl-*H*), 7.59-7.40 (m, 7H, phenyl-*H* + Ar-*H*). $^{13}\text{C-NMR}$ δ (CDCl_3): 165.8, 150.9, 145.6, 136.5, 135.5, 134.2, 133.8, 132.0, 131.4, 129.0, 128.6, 127.7, 127.2, 113.9. Anal. ($\text{C}_{18}\text{H}_{13}\text{ClN}_2\text{O} \cdot 0.04\text{CHCl}_3$) C, H, N.

N-[5-(4-Methoxyphenyl)pyridin-2-yl]benzamide (23). Yield 68%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.80 (br s, 1H, NH), 8.47-8.43 (m, 2H, pyridine-*H*), 7.97-7.90 (m, 3H, pyridine-*H* + phenyl-*H*), 7.62-7.48 (m, 5H, phenyl-*H* + Ar-*H*), 7.00 (d, 2H, $J = 8.7$ Hz, Ar-*H*), 3.86 (s, 3H, OCH_3). $^{13}\text{C-NMR}$ δ (CDCl_3): 165.8, 159.3, 150.2, 145.3, 136.1, 134.3, 132.4, 131.9, 129.5, 128.5, 127.5, 127.2, 114.3, 113.9, 55.1. Anal. ($\text{C}_{19}\text{H}_{16}\text{N}_2\text{O}_2 \cdot 0.06\text{CHCl}_3$) C, H, N.

N-[5-(4-Methylphenyl)pyridin-2-yl]benzamide (24). Yield 41%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.89 (br s, 1H, NH), 8.46-8.45 (m, 2H, pyridine-*H*), 7.97-7.94 (m, 3H, pyridine-*H* + phenyl-*H*), 7.59-7.56 (m, 1H, phenyl-*H*), 7.52-7.49 (m, 2H, phenyl-*H*), 7.46 (d, 2H, $J = 8.0$ Hz, Ar-*H*), 7.28 (d, 2H, $J = 8.0$ Hz, Ar-*H*), 2.28 (s, 3H, CH_3). $^{13}\text{C-NMR}$ δ (CDCl_3): 165.8, 150.5, 145.8, 137.7, 136.6, 134.4, 134.3, 133.0, 132.2, 129.7, 128.7, 127.3, 126.6, 114.0, 21.1. Anal. ($\text{C}_{19}\text{H}_{16}\text{N}_2\text{O}$) C, H, N.

N-[5-(3-Chlorophenyl)pyridin-2-yl]benzamide (25). Yield 43%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 9.07 (br s, 1H, NH), 8.50 (d, 1H, $J = 8.8$ Hz, pyridine-*H*), 8.35 (d, 1H, $J = 2.6$ Hz, pyridine-*H*), 7.96-7.91 (m, 3H, pyridine-*H* + phenyl-*H*), 7.63-7.32 (m, 7H, Ar-*H* + phenyl-*H*). $^{13}\text{C-NMR}$ δ (CDCl_3): 166.2, 151.3, 145.7, 138.8, 136.6, 134.7, 134.4, 132.1, 131.1, 130.0, 128.6, 127.5, 127.3, 126.4, 124.5, 113.9. Anal. ($\text{C}_{18}\text{H}_{13}\text{ClN}_2\text{O} \cdot 0.14\text{CH}_2\text{Cl}_2$) C, H, N.

N-[5-(4-Dimethylamino-phenyl)pyridin-2-yl]benzamide (26). Yield 75%; off-white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.86 (br s, 1H, NH), 8.43 (s, 1H, pyridine-*H*), 8.41 (d, 1H, $J = 4.7$ Hz, pyridine-*H*), 7.97-7.89 (m, 3H, pyridine-*H* + phenyl-*H*), 7.57-7.43 (m, 5H, Ar-*H* + phenyl-*H*), 5.85 (s, 6H, $\text{N}(\text{CH}_3)_2$). $^{13}\text{C-NMR}$ δ (CDCl_3): 165.6, 150.0, 149.5, 144.7, 135.7, 134.3, 132.9, 131.8, 128.5, 127.1, 124.8, 113.9, 112.6, 40.2. Anal. ($\text{C}_{20}\text{H}_{19}\text{N}_3\text{O} \cdot 0.02\text{CHCl}_3$) C, H, N.

N-[5-(4-Isopropoxy-phenyl)pyridin-2-yl]benzamide (27). Yield 65%; white solid. $^1\text{H-NMR}$ δ (CDCl_3): 8.87 (br s, 1H, NH), 8.46-8.42 (m, 2H, pyridine-*H*), 7.97-8.89 (m, 3H,

phenyl-*H* + pyridine-*H*), 7.58-7.43 (m, 5H, Ar-*H* + phenyl-*H*), 6.98 (d, 2H, $J = 9.1$ Hz, Ar-*H*), 4.60 (q, 1H, $J = 6.2$ Hz, OCH(CH₃)₂), 1.39 (s, 3H, CH₃), 1.36 (s, 3H, CH₃). ¹³C-NMR δ (CDCl₃): 166.2, 156.6, 150.4, 145.2, 135.9, 134.6, 132.2, 131.8, 129.1, 128.4, 127.5, 116.0, 114.2, 69.7, 21.8. Anal. (C₂₁H₂₀N₂O₂) C, H, N.

N-[5-(4-*tert*-Butylphenyl)pyridin-2-yl]benzamide (28). Yield 34%; white solid. ¹H-NMR δ (CDCl₃): 8.98 (br s, 1H, *NH*), 8.49-8.43 (m, 2H, pyridine-*H*), 7.99-7.92 (m, 3H, pyridine-*H* + phenyl-*H*), 7.57-7.45 (m, 7H, Ar-*H* + phenyl-*H*), 1.37 (s, 9H, *t*-butyl). ¹³C-NMR δ (CDCl₃): 166.1, 150.7, 145.6, 136.5, 134.4, 134.1, 132.4, 131.9, 128.5, 127.3, 126.1, 125.8, 114.1, 34.4, 31.1. Anal. (C₂₂H₂₂N₂O) C, H, N.

N-[5-[4-(Trifluoromethyl)phenyl]pyridin-2-yl]benzamide (29). Yield 52%; white solid. ¹H-NMR δ (CDCl₃): 9.11 (br s, 1H, *NH*), 8.52 (d, 1H, $J = 8.4$ Hz, pyridine-*H*), 8.40 (d, 1H, $J = 2.2$ Hz, pyridine-*H*), 8.00-7.93 (m, 3H, pyridine-*H* + phenyl-*H*), 7.73-7.47 (m, 7H, Ar-*H* + phenyl-*H*). ¹³C-NMR δ (CDCl₃): 165.9, 151.4, 145.9, 140.6, 136.7, 134.1, 132.1, 131.1, 128.6, 127.2, 126.7, 125.7, 116.0, 113.9. Anal. (C₁₉H₁₃F₃N₂O) C, H, N.

N-[5-(3,4-Dichlorophenyl)pyridin-2-yl]benzamide (30). Yield 49%; white solid. ¹H-NMR δ (CDCl₃): 8.74 (br s, 1H, *NH*), 8.51-8.45 (m, 2H, pyridine-*H*), 7.96-7.90 (m, 3H, pyridine-*H* + phenyl-*H*), 7.65-7.37 (m, 6H, Ar-*H* + phenyl-*H*). ¹³C-NMR δ (DMSO): 166.2, 152.2, 146.1, 137.5, 136.4, 134.0, 132.1, 132.0, 131.1, 130.5, 129.0, 128.4, 128.2, 128.1, 126.6, 114.4. Anal. (C₁₈H₁₂Cl₂N₂O * 0.15CH₂Cl₂) C, H, N.

4-Chloro-N-(5-phenylpyridin-2-yl)benzamide (31). Yield 41%; white solid. ¹H-NMR δ (CDCl₃): 8.85 (br s, 1H, *NH*), 8.47-8.43 (m, 2H, pyridine-*H*), 7.98 (d, 1H, $J = 8.0$ Hz, pyridine-*H*), 7.90 (dd, 2H, $J^1 = 8.8$ Hz, $J^2 = 1.5$ Hz, Ar-*H*), 7.58-7.35 (m, 7H, Ar-*H* + phenyl-*H*). ¹³C-NMR δ (DMSO): 165.1, 151.4, 146.2, 136.9, 136.8, 136.3, 132.9, 131.7, 130.1, 129.2, 128.5, 127.9, 126.5, 114.6, 126.6. Anal. (C₁₈H₁₃ClN₂O * 0.06CHCl₃) C, H, N.

4-Methoxy-N-(5-phenylpyridin-2-yl)benzamide (32). Yield 69%; white solid. ¹H-NMR δ (CDCl₃): 8.93 (br s, 1H, *NH*), 8.49-8.41 (m, 2H, pyridine-*H*), 7.99-7.90 (m, 3H, pyridine-*H* + Ar-*H*), 7.56-7.37 (m, 5H, phenyl-*H*), 6.98 (d, 2H, $J = 8.8$ Hz, Ar-*H*), 3.90 (s, 3H, OCH₃). ¹³C-NMR δ (CDCl₃): 165.8, 162.5, 151.2, 145.7, 137.0, 136.5, 132.1, 129.4, 128.8, 127.5, 126.7, 126.4, 114.0, 113.7, 55.2. Anal. (C₁₉H₁₆N₂O₂ * 0.03 CHCl₃) C, H, N.

4-Methyl-N-(5-phenylpyridin-2-yl)benzamide (33). Yield 70%; white solid. ¹H-NMR δ (CDCl₃): 8.96 (br s, 1H, *NH*), 8.47 (dd, 1H, $J^1 = 8.8$ Hz, $J^2 = 0.8$ Hz, pyridine-*H*), 8.41 (d, 1H, $J = 2.2$ Hz, pyridine-*H*), 7.96 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.6$ Hz, pyridine-*H*), 7.84 (d, 2H, $J = 8.0$ Hz, Ar-*H*), 7.57-7.27 (m, 7H, Ar-*H* + phenyl-*H*), 2.41 (s, 3H, CH₃). ¹³C-NMR δ (CDCl₃):

166.2, 151.0, 145.8, 142.5, 137.1, 136.5, 132.3, 131.7, 129.1, 128.8, 127.4, 127.3, 126.4, 113.9, 21.2. Anal. (C₁₉H₁₆N₂O) C, H, N.

3-Chloro-N-(5-phenylpyridin-2-yl)benzamide (34). Yield 59%; white solid. ¹H-NMR δ (CDCl₃): 8.74 (br s, 1H, NH), 8.51 (dd, 1H, J¹ = 2.2 Hz, J² = 0.7 Hz, pyridine-*H*), 8.44 (dd, 1H, J¹ = 8.4 Hz, J² = 0.7 Hz, pyridine-*H*), 8.01 (d, 1H, J = 2.6 Hz, pyridine-*H*), 7.96 (dd, 1H, J¹ = 3.5 Hz, J² = 1.8 Hz, Ar-*H*), 7.83-7.78 (m, 1H, Ar-*H*), 7.60-7.35 (m, 7H, Ar-*H* + phenyl-*H*). ¹³C-NMR δ (DMSO): 164.7, 151.4, 145.8, 136.8, 133.3, 131.7, 130.3, 129.1, 128.0, 127.8, 126.8, 126.5, 114.7. Anal. (C₁₈H₁₃ClN₂O) C, H, N.

3,4-Dichloro-N-(5-phenylpyridin-2-yl)benzamide (35). Yield 45%; white solid. ¹H-NMR δ (DMSO): 11.17 (br s, 1H, NH), 8.75 (s, 1H, pyridine-*H*), 8.32-8.17 (m, 3H, Ar-*H* + pyridine-*H*), 8.02 (d, 1H, J = 8.8 Hz, Ar-*H*), 7.83-7.74 (m, 3H, Ar-*H* + phenyl-*H*), 7.55-7.41 (m, 3H, phenyl-*H*). ¹³C-NMR δ (DMSO): 163.8, 151.2, 145.8, 136.7, 136.1, 134.8, 134.4, 131.8, 131.4, 130.6, 130.1, 129.1, 128.3, 127.8, 126.5, 114.6. Anal. (C₁₈H₁₂Cl₂N₂O) C, H, N.

2,4-Dimethoxy-N-(5-phenylpyridin-2-yl)benzamide (36). Yield 44%; white solid. ¹H-NMR δ (CDCl₃): 10.32 (br s, 1H, NH), 8.55 (d, 1H, J = 2.2 Hz, pyridine-*H*), 8.50 (d, 1H, J¹ = 8.8 Hz, pyridine-*H*), 8.26 (d, 1H, J = 8.8 Hz, Ar-*H*), 7.93 (dd, 1H, J¹ = 8.6 Hz, J² = 2.6 Hz, pyridine-*H*), 7.60-7.37 (m, 5H, phenyl-*H*), 6.65 (dd, 1H, J¹ = 8.9 Hz, J² = 2.6 Hz, Ar-*H*), 6.53 (d, 1H, J = 2.2 Hz, Ar-*H*), 4.06 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃). ¹³C-NMR δ (CDCl₃): 163.8, 163.0, 158.7, 151.2, 145.7, 137.3, 136.2, 133.8, 131.9, 128.8, 127.3, 126.4, 114.1, 113.9, 105.5, 98.2, 55.8, 55.2. Anal. (C₂₀H₁₈N₂O₃) C, H, N.

4-Amino-N-(5-phenylpyridin-2-yl)benzamide (37). Yield 33%; off-white solid. ¹H-NMR δ (CDCl₃): 8.67 (br s, 1H, NH), 8.49-8.43 (m, 2H, pyridine-*H*), 7.95 (dd, 1H, J¹ = 8.6 Hz, J² = 2.2 Hz, pyridine-*H*), 7.78 (d, 2H, J = 8.4 Hz, Ar-*H*), 7.50-7.33 (m, 5H, phenyl-*H*), 6.70 (d, 2H, J = 8.8 Hz, Ar-*H*), 4.09 (br s, 2H, NH₂). ¹³C-NMR δ (DMSO): 165.7, 152.6, 152.1, 145.5, 137.0, 136.0, 130.9, 129.9, 129.2, 127.7, 126.4, 120.2, 114.3, 112.7. Anal. (C₁₈H₁₅N₃O) C, H, N.

4-Isopropoxy-N-(5-phenylpyridin-2-yl)benzamide (38). Starting from 4-isopropoxybenzoyl chloride.³¹⁶ Yield 32%; white solid. ¹H-NMR δ (CDCl₃): 8.86 (br s, 1H, NH), 8.48-8.44 (m, 2H, pyridine-*H*), 7.98-7.87 (m, 3H, Ar-*H* + pyridine-*H*), 7.57-7.37 (m, 5H, phenyl-*H*), 6.95 (d, 2H, J = 8.8 Hz, Ar-*H*), 4.72-4.54 (m, 1H, OCH(CH₃)₂), 1.38 (s, 3H, OCH(CH₃)₂), 1.34 (s, 3H, OCH(CH₃)₂). ¹³C-NMR δ (CDCl₃): 165.8, 161.0, 151.3, 145.7, 137.1, 136.5, 132.1, 129.4, 128.8, 127.4, 126.4, 126.2, 115.1, 114.0, 69.8, 21.6. Anal. (C₂₁H₂₀N₂O₂) C, H, N.

4-Dimethylamino-N-(5-phenylpyridin-2-yl)benzamide (39). Starting from the *tert*-butyloxycarbonyl protected 4-amino-benzoyl chloride.^{317,318} Yield 52%; white solid. ¹H-NMR δ (CDCl₃): 8.64 (br s, 1H, NH), 8.49-8.46 (m, 2H, pyridine-*H*), 7.95 (dd, 1H, $J^1 = 8.6$ Hz, $J^2 = 2.2$ Hz, pyridine-*H*), 7.85 (d, 2H, $J = 9.1$ Hz, Ar-*H*), 7.60-7.37 (m, 5H, phenyl-*H*), 6.71 (d, 2H, $J = 9.1$ Hz, Ar-*H*), 3.05 (s, 6H, N(CH₃)₂). ¹³C-NMR δ (CDCl₃): 165.9, 152.6, 151.5, 145.8, 137.3, 136.4, 131.8, 129.0, 128.8, 127.4, 126.4, 120.6, 113.7, 110.8, 39.7. Anal. (C₂₀H₁₉N₃O*0.10CHCl₃) C, H, N.

7.2.7 Biology – Materials

D-Luciferin was purchased from Duchefa (Haarlem, The Netherlands). Adenosine 5'-triphosphate (ATP), luciferase (*Luciola mingrelica*)³¹⁹ and bovine serum albumin (BSA, fraction V) were bought from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). All other chemicals were obtained from standard commercial sources.

7.2.8 Luciferase Inhibition and Saturation Assays

All luciferase assays were performed according to a slightly adjusted protocol from Sigma-Aldrich (EC 1.13.12.7). In short, inhibition assays were performed using 32 pM FireFly Luciferase in buffer A (1 M Glycine-Tris buffer containing 10 mM EDTA and 100 mM MgSO₄, pH 7.8) and 50 μ M D-luciferin and 90 μ M ATP in buffer B (50 mM Glycine-Tris buffer supplemented with 5 mM MgSO₄, 0.5 mM EDTA, 0.1% (w/v) BSA and 0.1% (w/v) sodium azide, pH 7.8), either incubated with a single concentration of inhibitor (10 μ M) or eleven concentrations of inhibitor. Non-specific luciferase activity was determined in the presence of 100 μ M **1**. Saturation assays with respect to D-luciferin were performed using different luciferin concentrations (1 - 200 μ M). Saturation assays with respect to ATP were performed under equal conditions, where the ATP concentration varied from 1 - 400 μ M and the concentration luciferin was fixed at 150 μ M. Typically, a well contained 80 μ L luciferin/ATP solution, 10 μ L buffer B or inhibitor and 10 μ L luciferase solution. After 30 min of incubation in the dark at room temperature, the luminescence signal was quantified on a Microbeta Trilux 1450 Luminescence Counter (PerkinElmer, Groningen, The Netherlands).

7.2.9 Data Analysis

All enzymatic data were analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 5.00 (GraphPad Software Inc, San Diego, CA, U.S.A.). Inhibitory binding constants (IC_{50}) were directly obtained from the concentration-effect curves. The K_M and V_{max} values of luciferin and ATP in the absence or presence of **1** were obtained by computer analysis of one- or two-site saturation curves, respectively. All values obtained are means of three independent experiments performed in duplicate.

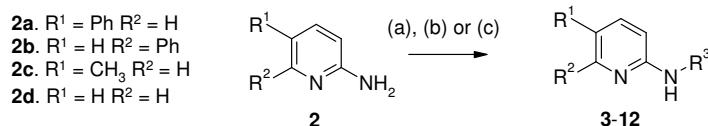
7.2.10 Docking Studies

The crystal structure of *Luciola cruciata* complexed with oxyluciferin and AMP was retrieved from the Brookhaven Protein Databank (PDB entry 2D1R).³²⁰ Sequence alignment of *Luciola cruciata* and *Luciola mingrelica* was performed using CLUSTALW.³²¹ The structural homology models were created using InsightII 98 (San Diego, CA, USA). Docking simulations were performed with AutoDock 3.³²² Grid maps of 20x20x20 Ångstrom representing the protein were calculated with AutoGrid. Docking simulations were carried out using the Lamarckian genetic algorithm, with an initial population of 100 individuals, a maximum number of 10,000,000 energy evaluations and a maximum number of 50,000 generations.³²² Resulting orientations lying within 1.5 Ångstrom in the RMSD were clustered together. Finally the configuration with the most favorable free energy of binding was further optimized by 1500 energy minimization steps with InsightII. PyMOL v. 1.0 (DeLano Scientific, Palo Alto, CA, USA) was used to superimpose and visualize the model.

7.3 RESULTS AND DISCUSSION

7.3.1 Chemistry

The initial active compound, N-quinolin-2-yl-benzamide (**1**), was obtained from the reaction of 2-aminoquinoline with benzoyl chloride in pyridine.³¹³ Based on the interesting behavior of quinoline **1** and the more active pyridine **3**¹⁹⁸ in the luciferase assay, N-pyridin-2-yl-benzamide analogs (**3-12**, **22-39**) were synthesized (Scheme 7.2 and 7.3). A Suzuki-coupling between phenylboronic acid and 2-amino-5-bromo-pyridine gave the 2-amino-5-phenyl-pyridine (**2a**).³¹⁵ The 2-amino-5-phenyl-pyridine (**2a**), the commercially available 2-amino-6-phenyl-pyridine (**2b**), 2-amino-5-methyl-pyridine (**2c**) and 2-amino-pyridine (**2d**) were benzoylated in pyridine at room temperature to the desired N-pyridin-2-yl-benzamides (**3-7**). A small library was designed around the privileged N-(5-phenylpyridin-2-yl)benzamide (**6**). The benzamide function of **6** was replaced by a benzyl amine (**8**), phenyl carbamate (**9**), phenyl urea (**10**),³²³ butanamide (**11**) and cyclohexanecarboxamide (**12**). The benzyl amine **8** was obtained from the reaction of 2-aminopyridine **2a** and benzyl chloride in the microwave reactor at 180 °C. A reaction with phenyl isocyanate and **2a** yielded the phenyl urea analogue (**10**). Compounds **9**, **11** and **12** were obtained by the reaction of **2a** with the appropriate acid chlorides. Introduction of substituents on the phenyl rings of **6** according to the Topliss system of substitution³²⁴ was done by Suzuki-coupling and acylation as described above, resulting in compounds **22-39**.

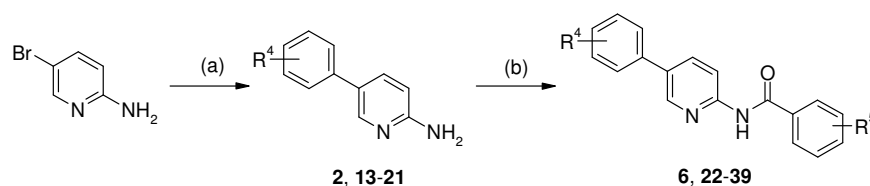


Scheme 7.2 Synthetic route to the compounds (a) **3-7**, **9**, **11** and **12**: acid chlorides, pyridine, (b) **8**: benzyl chloride, pyridine, MW, 180 °C, 2 h, (c) **10**: phenyl isocyanate, dioxane.

7.3.2 Structure-Activity Relationships

A small library of compounds was tested in an in-house luciferase reporter-gene assay screen for antagonistic activity at the human LH receptor (data not shown). This resulted in a high amount of apparent receptor antagonists. We wondered whether some of these

compounds were luciferase inhibitors rather than receptor antagonists, yielding false positive hits, an observation that has also been reported in a high-throughput screen for antibacterials with the same reporter gene.³²⁵ Therefore, we tested the ‘active’ compounds in an *in vitro* enzymatic luciferase assay as described in the *Experimental Section*. This led to the discovery of **1** that displayed significant enzyme inhibition with an IC_{50} value of $1.7 \pm 0.1 \mu\text{M}$ (Figure 7.1 and 7.2).



Scheme 7.3 Synthetic route to the N-(5-phenylpyridin-2-yl)benzamides **22-39**. (a) (subst.) phenylboronic acid, Na_2CO_3 , $\text{Pd}(\text{OAc})_2$, DMF, H_2O , 110°C ; (b) (subst.) benzoyl chloride, pyridine.

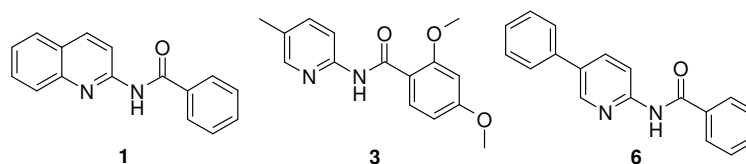


Figure 7.1 Chemical structures of compounds **1**, **3** and **6**.

Table 7.1 Saturation of luciferase activity by luciferin and ATP in the absence and presence of 0.1 or 1 μM compound **1**, represented by K_M - and $V_{\max}$ -values.

Condition	Luciferin ^a		ATP ^b			
	K_M (μM)	$V_{\max}$ (%)	K_{M1} (μM)	$V_{\max1}$ (%)	K_{M2} (μM)	$V_{\max2}$ (%)
Control	12 ± 1	100 ± 2	3.1 ± 0.3	100 ± 39	138 ± 16	100 ± 22
+ 0.1 μM 1	$16 \pm 0.8^*$	96 ± 0.8	ND	ND	ND	ND
+ 1 μM 1	$53 \pm 4^{***}$	95 ± 6	3.8 ± 0.5	87 ± 29	$94 \pm 4^{**}$	$56 \pm 13^*$

^a Saturation of luciferase activity by increasing concentrations of luciferin at 90 μM ATP.

^b Saturation of luciferase activity by increasing concentrations of ATP at 150 μM luciferin.

Values are means ($\pm$ S.E.M.) of three separate assays performed in duplicate (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control).

ND, not determined

As the luciferase assay is based upon a read-out of light production at 562 nm, quenching could cause false positive hits. Therefore, the absorbance spectra (240 – 600 nm) were determined of D-luciferin, **1** and 'Nile Red', which was identified as a luciferase inhibitor in the antibacterial screen mentioned above.³²⁵ As expected from its structure (and naming), the latter compound was a quencher of the luciferase signal with an absorption peak around 550 nm. On the other hand, both the endogenous substrate and compound **1** did not show any absorption at 350 nm and higher (data not shown).

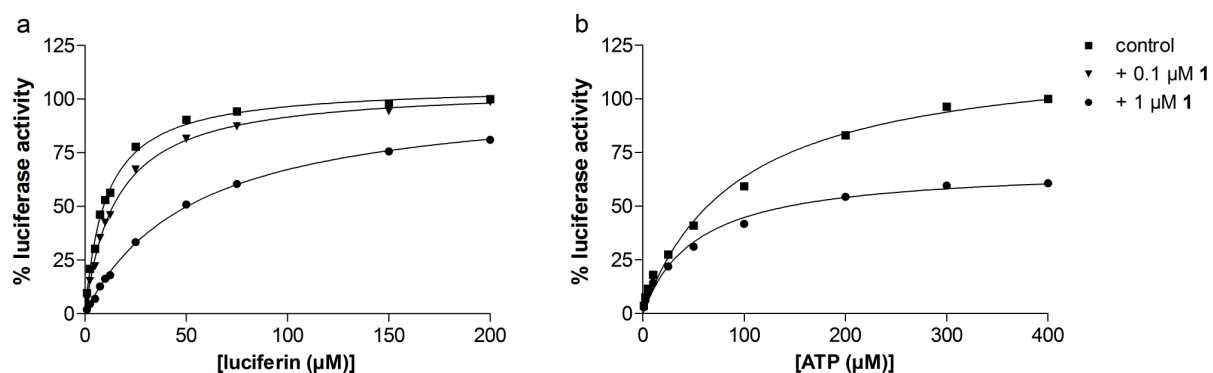


Figure 7.2 Saturation of luciferase activity by a) luciferin or b) ATP in the absence (control) or presence of 0.1 or 1 μM **1**. Representative graphs from one experiment performed in duplicate (see Table 7.1 for K_M - and V_{Max} -values).

Further studies were undertaken to investigate the pharmacological characteristics of compound **1**. Therefore, the Michaelis-Menten kinetics of luciferin in the absence and presence of two concentrations of **1** were examined (Figure 7.2a). Saturation of luciferase activity by increasing concentrations of luciferin resulted in a K_M -value of $12 \pm 2 \mu\text{M}$ in the absence of inhibitor (Table 7.2). In the presence of 0.1 or 1 μM of **1**, the K_M -value significantly increased, while the V_{max} was unchanged. This indicated that compound **1** competitively inhibited the action of luciferin. As luciferase has a second catalytic site for ATP, the Michaelis-Menten kinetics of ATP in the absence and presence of compound **1** were examined as well (Figure 7.2b). The presence of 1 μM of **1** resulted in a significantly decreased V_{max} -value (Table 7.1), proof for a non-competitive inhibition of ATP. Hence, **1** appears to solely compete for the luciferin binding site at luciferase. Notably,

Table 7.2 Inhibition of luciferase activity by compounds **4-12**, expressed as IC₅₀-values or as % inhibition at 10 μ M.

Compound	R ¹	R ²	R ³	IC ₅₀ (μ M) or % inh. ^a
4	H	H		27% (26-28)
5	H	Ph		30% (29-31)
6	Ph	H		0.069 $\pm$ 0.01
7	Me	H		6.4 $\pm$ 0.4
8	Ph	H		20% (19-21)
9	Ph	H		0% (-3-3)
10	Ph	H		20% (16-24)
11	Ph	H		22% (22-23)
12	Ph	H		2.4 $\pm$ 0.1

^a Inhibition of luciferase activity (IC₅₀ $\pm$ S.E.M. (μ M), n = 3, duplicate) or % inhibition at 10 μ M concentrations (n = 2, duplicate).

the ATP saturation curves were best analyzed by a two-site binding model ($p < 0.0001$). These results support earlier reports of two different ATP binding sites in the enzyme.^{306,326} At one of these sites the Mg-ATP complex is bound and at the other ATP, where the latter is thought to promote the release of product.³²⁷ The second site was therefore described as an allosteric site with positive cooperativity.

In our search for further evidence of direct luciferase inhibitors we analyzed screening data deposited at the PubChem database (luciferase profiling assay AID 411).¹⁹⁸ Interestingly,

one of the most active compounds (CID: 649849) also contained an *N*-pyridin-2-yl-benzamide core and had an IC_{50} -value of 0.25 μ M in their assay (**3**; Figure 7.1). This compound was synthesized by us and tested in the luciferase assay described here, which yielded a similar IC_{50} -value of 0.61 ± 0.09 μ M (Figure 7.3). For compound **1** and **3** the Hill coefficients of the inhibition curves were 1.07 ± 0.02 and 1.02 ± 0.02 , respectively, which indicates that the binding of these ligands is independent of the presence of any other substrates.

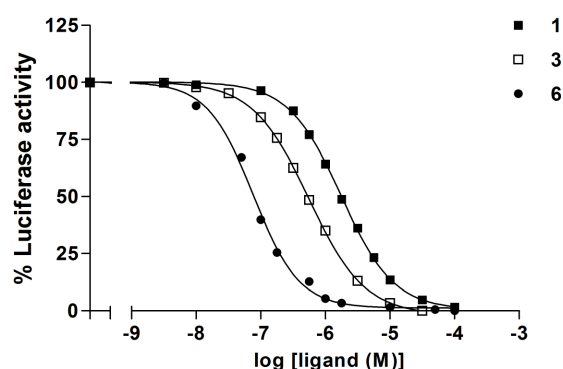
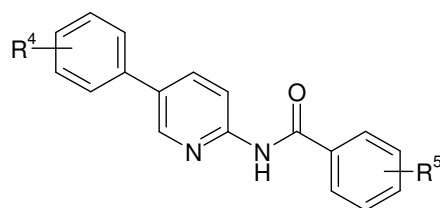


Figure 7.3 Inhibition of luciferase activity by compound **1**, **3** and **6**. The IC_{50} -values of **1**, **3** and **6** were 1.7 ± 0.1 μ M, 0.61 ± 0.09 μ M and 0.069 ± 0.01 μ M, respectively. Representative graphs from one of three experiments performed in duplicate.

Subsequently, analogs of *N*-pyridin-2-yl-benzamide (**4-12**) were synthesized and tested for luciferase inhibition to explore the structure-activity relationships of this compound class (Table 7.2). Replacement of the quinoline ring system of **1** for a pyridine ring (**4**) resulted in a dramatic loss of potency. Subsequent introduction of a 2-phenyl ring (**5**) did not improve the potency. Substitution with a 3-phenyl ring, however, resulted in a highly potent luciferase inhibitor (**6**) (Figure 7.3). This compound had an IC_{50} -value of 0.069 ± 0.01 μ M that was 10-fold lower than that of compound **3**. Interestingly, introduction of a *para*-methyl substituent as found in **3** decreased the potency by 100-fold (**7**; $IC_{50} = 6.4 \pm 0.4$ μ M). Apparently, either the 2,4-dimethoxy substituted phenyl ring on the right-hand side or the phenyl-substituted pyridine ring was important for high potency. In addition, the effect of different linkers between the pyridine and the phenyl ring was studied. As follows from Table 7.2, neither the

amine (**8**) nor the carbamic acid ester (**9**) nor the urea (**10**) linker resulted in an increase in potency. On the contrary, compounds **8-10** showed negligible inhibition, if any. With the amide as the preferred linker, two alkyl substituents were tested. A *cyclo*-hexyl (**12**), but not an *n*-propyl group (**11**), also resulted in a potent luciferase inhibitor, although approximately 4-fold less potent than compound **3** (Table 7.2). Apparently, a larger substituent, either alkyl (**12**) or aryl (**6**), is preferred in the binding pocket of the enzyme.

Table 7.3 Inhibition of luciferase activity by compounds **6, 22-39**, expressed as IC_{50} -values or as % inhibition at 10 μ M.



Compound	R ⁴	R ⁵	IC ₅₀ (μ M) or % inh. ^a
6	H	H	0.069 $\pm$ 0.01
22	4-Cl	H	0.56 $\pm$ 0.02
23	4-OMe	H	0.31 $\pm$ 0.02
24	4-Me	H	51% (47-55)
25	3-Cl	H	38% (34-42)
26	4-N(Me) ₂	H	14% (12-16)
27	4-OiPr	H	38% (27-48)
28	4- <i>t</i> Bu	H	49% (46-53)
29	4-CF ₃	H	39% (35-42)
30	3,4-diCl	H	4% (0-9)
31	H	4-Cl	0% (0-0)
32	H	4-OMe	28% (27-28)
33	H	4-Me	18% (17-19)
34	H	3-Cl	0.16 $\pm$ 0.01
35	H	3,4-diCl	13% (11-16)
36	H	2,4-diOMe	26% (22-30)
37	H	4-NH ₂	1.2 $\pm$ 0.05
38	H	4-OiPr	12% (8-17)
39	H	4-N(Me) ₂	35% (29-42)

^a Inhibition of luciferase activity ($IC_{50} \pm$ S.E.M. (μ M), n = 3, duplicate) or % inhibition at 10 μ M concentrations (n = 2, duplicate).

The design of other analogs was based upon a Topliss approach in which either one of the aromatic rings was modified with various substituents (Table 7.3).³²⁴ In general, modifications both on the 5-phenyl ring (**22-30**) and the 2-phenylamide group (**31-39**) of the pyridine ring resulted in a loss of potency in comparison to **6**. Apparently, the binding pocket does not tolerate any substituents on either one of the phenyl rings, indicating steric hindrance. Four compounds, however, showed inhibition of luciferase in the high nanomolar to low micromolar concentration range. On the 5-phenyl ring, introduction of a 4-chloro (**22**) or 4-methoxy (**23**) substituent resulted in IC₅₀-values of $0.56 \pm 0.02 \mu\text{M}$ or $0.31 \pm 0.02 \mu\text{M}$, respectively. Interestingly, the same substituents on the other side of the pyridine ring (almost) completely abolished the activity. This may indicate that these luciferase inhibitors bind in a certain pocket with specific sites of interaction. In addition, introduction of a 3-chloro (**34**) or 4-amino (**37**) substituent on the 2-phenylamide group resulted in IC₅₀-values of $0.16 \pm 0.01 \mu\text{M}$ or $1.2 \pm 0.05 \mu\text{M}$, respectively.

Finally, compound **6** was docked into a homology model of firefly luciferase based upon its crystal structure including AMP and oxyluciferin.³²⁰ From Figure 7.4, it becomes clear that AMP and oxyluciferin bind in two different pockets at the enzyme. Compound **6** was docked both into the AMP and the oxyluciferin pocket. Interestingly, the best model was obtained when the binding pocket of compound **6** overlapped with that of oxyluciferin. There are two reasons for that. Firstly, the binding pocket of AMP is curved, while the pocket of oxyluciferin is flat. A largely planar compound as **6** is therefore accommodated best by the latter pocket. Secondly, when the phenyl ring attached to the amide group is superimposed on the thiazole ring of oxyluciferin, the ‘other’ phenyl ring of **6** extends into an available pocket in the enzyme. The results obtained with this docking study therefore correspond with the competitive and non-competitive inhibition of **1** that was found with respect to luciferin and ATP, respectively (Figure 7.2). In addition, from Figure 7.4 it follows that the further introduction of substituents on both phenyl rings would cause steric hindrance, which may explain the fact that the unsubstituted compound **6** is the most potent inhibitor.

In conclusion, we have shown that (drug-like) compounds, such as **1** or **6**, are competitive inhibitors of luciferase with respect to luciferin. In addition, these compounds are non-competitive inhibitors with respect to ATP. The inclusion of similar compounds would result in a high number of ‘false positives’ in screening campaigns that rely on luciferase reporter-gene assays, an otherwise robust screening approach with good signal-to-noise ratio. Notably,

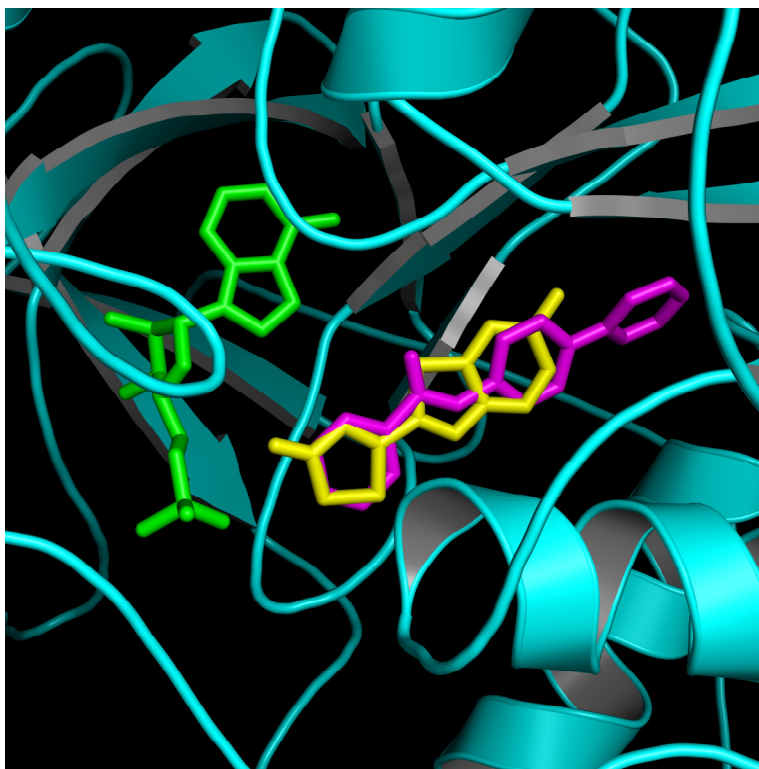


Figure 7.4 Docking results obtained using Autodock. Firefly luciferase homology model (cyan) with AMP (green), oxyluciferin (yellow) and compound **6** (magenta). For details of the modeling procedure see the Experimental Section.

we learned that compound **1** has been patented for osteoporosis treatment.^{328,329} However, also in these disclosures a luciferase reporter gene assay was used to identify the compounds. The data presented in this paper is therefore meant to warn researchers for direct luciferase inhibitors that also have drug-like properties. Such compounds may be ‘flagged’ after their evaluation in an assay with e.g., purified luciferase.

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 [³H]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, [³H]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 [³H]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 [³H]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 [³H]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 TSH¹⁹¹ and FSH¹⁹⁵ 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} receptor⁴⁵ 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 M₁ 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 M₂ 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, III 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.

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SAMENVATTING

Op dit moment zijn aan G eiwitten gekoppelde receptoren (GPCRs) aangrijpingspunt voor meer dan 30% van de geneesmiddelen op de markt. In de afgelopen jaren is er echter een afname van het aantal nieuw geïntroduceerde geneesmiddelen (niet alleen voor GPCRs), wat aangeeft hoe belangrijk het is om met nieuwe strategieën voor geneesmiddeltherapie te komen. Eén van deze nieuwe strategieën is de ontwikkeling van zogenaamde allosterische modulatoren. Allosterische liganden binden op een andere plaats dan het endogene ligand en zijn in staat om de vorm van de receptor te veranderen. Hierdoor wordt het farmacologische effect van het endogene ligand veranderd. De voordelen van allosterische modulatie zijn bijvoorbeeld een verbeterde receptor selectiviteit en het behouden van de fysiologie (duur en plaats) van een effect. In *Hoofdstuk 1* worden GPCRs en de recente ontwikkelingen in geneesmiddelenonderzoek rondom GPCRs, zoals allosterische modulatie verder toegelicht.

De verbinding tussen de hypothalamus, hypofyse en geslachtsdelen (de HPG-as) wordt gereguleerd door een aantal GPCRs, die een belangrijke rol spelen in de voortplanting en in geslachtshormoon-afhankelijke ziektes. Deze receptoren worden daarom ook wel ‘reproductieve’ GPCRs genoemd. In dit proefschrift spelen voornamelijk de gonadotrofine-releasing hormoon (GnRH) receptor en luteïniserend hormoon (LH) receptor een rol. Beide receptoren behoren tot klasse A GPCRs, die meestal opgebouwd zijn uit een korte extracellulaire amino-terminus (N-terminus), zeven transmembraan (7-TM) domeinen (α -helixen) die aan elkaar gekoppeld zijn door drie intracellulaire en drie extracellulaire lussen, eindigend met een intracellulaire carboxyl-terminus. Over het algemeen binden de endogene liganden van deze klasse GPCRs in het 7-TM domein, wat ook wel de orthostere bindingsplaats wordt genoemd. De LH receptor is echter een uitzondering, omdat het twee endogene liganden, LH en humaan choriogonadotrofine (hCG), heeft, welke aan de uitzonderlijk lange N-terminus binden. De endogene liganden van zowel de GnRH als de LH receptor zijn peptiden/eiwit hormonen met een hoog molecuul gewicht (HMW). Eén van de voordelen van allosterische modulatie van deze GPCRs is dat er liganden gemaakt kunnen worden met een laag molecuul gewicht (LMW) die oraal toegediend kunnen worden, wat niet het geval is voor peptiden en eiwitten, zoals GnRH en LH. *Hoofdstuk 2* geeft een overzicht van alle LMW liganden die tot nu toe ontwikkeld zijn voor de GPCRs van de HPG-as.

In *Hoofdstuk 3* komt allosterische modulatie van de GnRH receptor door amiloride derivaten (zoals HMA) en een niet-peptide antagonist (FD-1) aan de orde. Allereerst werd onderzocht of deze verbindingen een invloed hadden op de dissociatiesnelheid van een radioactief gemerkte peptide agonist (^{125}I -triptorelin) voor de GnRH receptor. Zowel HMA als FD-1

verhoogden de dissociatiesnelheid van ^{125}I -triptorelin, een indicatie voor allosterische remming. Het tegelijkertijd toevoegen van HMA en FD-1 resulteerde in een extra verhoging van de dissociatiesnelheid. Ten tweede werd het in een functionele analyse duidelijk dat HMA een niet-competitieve antagonist was, terwijl FD-1 zowel competitieve als niet-competitieve eigenschappen had. Bovendien werd de potentie van HMA om de dissociatiesnelheid te verhogen niet beïnvloed door de aanwezigheid van FD-1. Simulatie van deze data impliceerden dat de binding van HMA en FD-1 een neutrale coöperativiteit had. Samengenomen betekent dit dat HMA en FD-1 beide allosterische remmers zijn die op twee verschillende allosterische plaatsen binden in de GnRH receptor.

De binding van een nieuw LMW radioligand, $[^3\text{H}]\text{Org 43553}$, voor de LH receptor werd gekarakteriseerd in *Hoofdstuk 4*. Experimenten voor verzadiging en verdringing in een evenwichtsituatie werden ontworpen en geoptimaliseerd. De specifieke binding van $[^3\text{H}]\text{Org 43553}$ aan de LH receptor was verzadigbaar en had een hoge affiniteit ($K_D = 2.4 \pm 0.4 \text{ nM}$). Affiniteiten en potenties van vijf laag moleculaire derivaten van Org 43553 werden bepaald; een hoge correlatie tussen deze waarden werd vastgesteld. Een HMW radioligand, zoals ^{125}I -hCG, is niet bruikbaar voor het identificeren van nieuwe LMW verbindingen, omdat ze geen competitie met elkaar hebben voor dezelfde bindingsplaats. Dit nieuwe radioligand, $[^3\text{H}]\text{Org 43553}$, is daarom een belangrijke aanvulling voor het geneesmiddelenonderzoek voor de LH receptor.

In *Hoofdstuk 5* en *Hoofdstuk 6* werd $[^3\text{H}]\text{Org 43553}$ gebruikt om in een bibliotheek van 50 verbindingen te zoeken naar mogelijk nieuwe LMW liganden voor de LH receptor. Hierbij resulteerde het gebruik van radioligand-dissociatieexperimenten (om allosterische modulators te identificeren) in het vinden van zowel allosterische remmers (*Hoofdstuk 5*) als stimulators van Org 43553. Allereerst bleek een terphenyl derivaat een allosterische remmer van $[^3\text{H}]\text{Org 43553}$ binding aan de receptor te zijn. We besloten daarom om een serie van 25 terphenyl derivaten te synthetiseren. De meest potente allosterische remmer van deze serie was LUF5771, die bij een concentratie van $10 \mu\text{M}$ de dissociatiesnelheid van $[^3\text{H}]\text{Org 43553}$ 3.3-maal kon verhogen. Ten tweede werden er meerdere thiazolderivaten geïdentificeerd, die allosterische stimulators van $[^3\text{H}]\text{Org 43553}$ waren. In dit geval werd LUF5419 gekozen om verder te karakteriseren en het bleek dat deze verbinding de dissociatiesnelheid van $[^3\text{H}]\text{Org 43553}$ 2.4-maal kon vertragen bij $10 \mu\text{M}$. Beide verbindingen werden getest in een functioneel protocol, waar de aanwezigheid van LUF5771 resulteerde in een 2.4-maal verlaagde potentie van Org 43553, terwijl LUF5419 hierop geen effect had. Het maximale effect van (de partiële agonist) Org

43553 werd niet beïnvloed door LUF5771, maar LUF5419 verhoogde dit tot een volledige receptoractivatie, volkomen vergelijkbaar met recombinant LH (recLH). Interessant genoeg zorgde de aanwezigheid van LUF5771 ook in een verlaging van de potentie van recLH (en rec-hCG). LUF5419 had echter geen effect op receptoractivatie door recLH. LUF5771 is tot nu toe het eerste antagonistische/remmende LMW ligand voor de LH receptor. Vervolgens werd de potentie van LUF5771 om de dissociatiesnelheid van het radioligand te verhogen, verlaagd door de aanwezigheid van LUF5419. Dit betekent dat deze allosterische modulatoren op dezelfde allosterische plaats binden in de LH receptor.

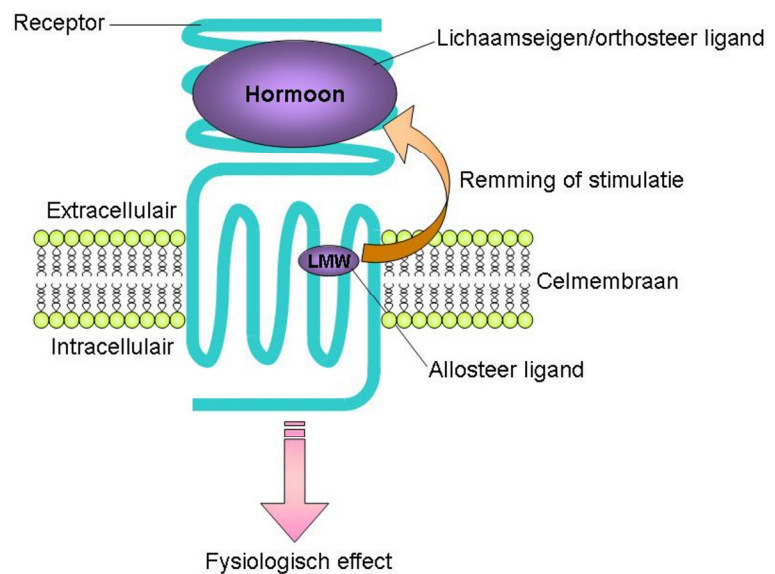
In dit proefschrift werden radioligand-dissociatieexperimenten gebruikt om nieuwe allosterische modulatoren te identificeren. Dit werd echter op een kleinere schaal gedaan dan wat meestal in de industrie gebeurt. Hier wordt vooral gebruik van zogenaamde ‘high-throughput-screening (HTS)’ experimenten, waarvoor functionele analyses (zoals een luciferase reporter-gen experiment) zich beter lenen. In *Hoofdstuk 7* laten we echter zien dat een dergelijk experiment kan resulteren in vals-positieven. Het bleek dat één van de geteste verbindingen een potente luciferaseremmer was en dus geen ligand voor de LH receptor. Verdere karakterisatie liet zien dat het een competitieve remmer was van het enzym luciferase via de bindingsplaats van het substraat voor dit enzym, luciferine. Een zoektocht in een databank leverde een luciferaseremmer met een soortgelijke structuur. Daarom werd er een serie van verbindingen gesynthetiseerd, en de meest potente luciferaseremmer in die reeks had een IC_{50} waarde van $0.069 \pm 0.01 \mu M$. Om de bindingsplaats in luciferase van deze laatste verbinding te visualiseren, werden er moleculaire modellen gemaakt. Deze bevestigden inderdaad dat de luciferaseremmers op dezelfde plaats binden als luciferine. *Hoofdstuk 7* zou moeten dienen als een waarschuwing voor gebruikers van luciferase-reportergen-experimenten voor vals-positieven door inhibitie van het enzym in plaats van de receptor.

Tot slot worden in *Hoofdstuk 8* algemene conclusies getrokken over het onderzoek dat in dit proefschrift beschreven is. Ook worden de toekomstperspectieven voor dit onderzoek geschetst. In het kort levert dit proefschrift nieuwe inzichten op in de allosterische modulatie van ‘reproductieve’ GPCRs. De humane GnRH en LH receptor kunnen allosteer gemoduleerd worden, net zoals andere klasse A GPCRs. Tevens wordt duidelijk dat beide receptoren drie ligand-bindingsplaatsen bevatten, waarvan tenminste twee een aangrijpingspunt kunnen zijn voor LMW liganden. De aanwezigheid van deze allosterische bindingsplaatsen biedt nieuwe mogelijkheden voor de ontwikkeling van LMW en oraal toepasbare liganden voor de GnRH en LH receptor.

SAMENVATTING VOOR EEN LEEK

De afgelopen vier jaar is het onderzoek uitgevoerd dat in dit proefschrift beschreven staat. Het doel van dit onderzoek was een nieuwe therapie te vinden voor ziektes/aandoeningen op het gebied van vruchtbaarheid/voortplanting, maar ook voor bepaalde kankersoorten. In de huidige therapieën worden meestal de lichaamseigen stoffen, hormonen, als geneesmiddel gebruikt, bijvoorbeeld bij IVF behandelingen. Deze stoffen zijn grote eiwitten en moeten daarom via een injectie direct in de bloedbaan gebracht worden, omdat ze anders in de maag zouden worden afgebroken door het maagzuur. Het zou dus erg interessant zijn om stoffen te ontwikkelen die als geneesmiddel oraal (via een tablet) toegediend kunnen worden. Dit zal een aanzienlijke verbetering zijn voor patiënten.

Het menselijke lichaam is opgebouwd uit cellen. De inhoud van een cel wordt omgeven door een celmembraan. In dit celmembraan zitten verschillende eiwitten, waaronder receptoren ('ontvangers'). De functie van een receptor is het ontvangen en doorgeven van een signaal van buiten de cel naar binnen. Op het moment dat een specifieke stof (het ligand) aan de receptor bindt, wordt een cellulaire respons op gang gebracht. Deze heeft een fysiologisch effect tot gevolg, zoals celdeling (zie figuur). Een receptor kan geactiveerd worden door een lichaamseigen ligand of door een ontworpen ligand, een geneesmiddel. De plaats waar deze stoffen binden wordt ook wel de orthostere plaats genoemd. Het is echter ook mogelijk dat er liganden op een andere plaats in de receptor binden. Deze wordt de allosterische bindingsplaats genoemd (afgeleid van de Griekse woorden 'allos' en 'stereos' = andere ruimte). Een allosterische ligand kan vanaf deze plaats het orthostere ligand positief of negatief beïnvloeden, ook wel allosterische modulatie genoemd. Hierdoor treedt er een stimulatie of een remming van het fysiologische effect op, wat gewenst zou kunnen zijn bij een bepaald ziektebeeld.



Voor dit proefschrift is onderzoek gedaan naar allosterische modulatie van de gonadotrofine-releasing-hormoon (GnRH) receptor en de luteïniserend hormoon (LH) receptor. De lichaamseigen liganden die aangrijpen op deze receptoren zijn GnRH en LH, zoals de receptornaam al doet vermoeden. Deze liganden zijn grote peptiden of eiwitten (hormonen), die een belangrijke rol spelen in vruchtbaarheid en voortplanting, maar ook bij bepaalde ziektes zoals prostaatkanker. Eén van de voordelen van allosterische modulatie van deze receptoren is dat allosterische liganden zo ontworpen kunnen worden, dat ze klein zijn en daarom als geneesmiddel oraal (via een tablet) toegediend kunnen worden. De GnRH en LH receptoren behoren tot de familie van aan G eiwitten gekoppelde receptoren (GPCRs), waarbij het G eiwit verantwoordelijk is voor het verder doorgeven van het signaal. Daarom worden ze ook wel ‘reproductieve’ GPCRs genoemd. GPCRs worden gekenmerkt door zeven aan elkaar verbonden ketens (α -helixen) die door het celmembraan steken (zie figuur). In het geval van de LH receptor bevindt de orthostere bindingsplaats (extracellulair) zich duidelijk op een andere locatie dan de allosterische bindingsplaats (tussen de α -helixen). Voor de GnRH receptor is dit minder duidelijk, want beide bindingsplaatsen bevinden zich tussen de α -helixen.

Aan het begin van dit project waren er nog geen allosterische modulatoren beschreven voor de GnRH en LH receptor. De bevindingen van het onderzoek beschreven in dit proefschrift bewijzen dat beide receptoren allosteer gemoduleerd kunnen worden door kleine liganden. Het is zelfs voor beide receptoren aangetoond dat er niet één, maar twee allosterische bindingsplaatsen zijn (naast de orthostere plaats). Deze bieden de mogelijkheid om door kleine liganden, die oraal toegediend kunnen worden, bezet te worden en zo het lichaamseigen ligand naar wens (positief of negatief) te moduleren. In de toekomst zou het mogelijk kunnen zijn, dat patiënten die bijvoorbeeld IVF behandelingen ondergaan behandeld worden met (genees)middelen in tabletvorm in plaats van met injecties.

CURRICULUM VITAE

Laura Helena Heitman werd geboren op 1 april 1981 te Alphen aan den Rijn. Na het behalen van het VWO-diploma aan het Christelijk Lyceum te Alphen aan den Rijn in 1999, werd in september van dat jaar begonnen met de studie Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden. In maart 2001 behaalde zij het propedeutisch examen, gevolgd door het doctoraal examen in oktober 2004. Tijdens haar eerste stage van september 2002 tot en met juni 2003 op de afdeling Farmacochemie van het Leiden/Amsterdam Center for Drug Research (LACDR) heeft zij onder begeleiding van Dr. J. Brussee gewerkt aan “Synthesis and Biological Evaluation of Fluorescent Agonists for the Adenosine A₁ Receptor”. Van september 2003 tot en met januari 2004 werd tijdens een tweede stage bij deze afdeling gewerkt aan “Pharmacology of the Adenosine A₁ Receptor – a New Partial Agonist” onder begeleiding van Prof. Dr. A.P. IJzerman. Aansluitend heeft zij een stage in het buitenland gedaan bij de vakgroep Biological and Pharmaceutical Chemistry van The School of Pharmacy (Londen, UK). Hier heeft zij 6 maanden gewerkt aan “Pharmacology of the NMDA Receptor Subtypes and Their Associated Proteins” onder begeleiding van Prof. Dr. F. A. Stephenson.

Vanaf oktober 2004 tot en met september 2008 werd als Assistent in Opleiding het in dit proefschrift beschreven onderzoek uitgevoerd bij de vakgroep Farmacochemie (LACDR) onder begeleiding van Prof. Dr. A.P. IJzerman en in samenwerking met Schering-Plough (Oss, NL) als onderdeel van het Top Institute Pharma (TI Pharma) consortium. Delen van haar onderzoek werden op een poster, dan wel mondeling gepresenteerd op verschillende (inter)nationale symposia en congressen. In 2008 ontving zij twee keer de eerste prijs voor een mondeling presentatie getiteld: “False Positives in a Reporter Gene Assay: Identification and Synthesis of Competitive Inhibitors of Firefly Luciferase” op het LACDR Spring symposium en de FIGON Dutch Medicines Days. Tevens ontving zij de eerste prijs voor de poster getiteld: “One Receptor, Three Binding Sites - A Case for the GnRH Receptor” tijdens de bijeenkomst van de Koninklijke Nederlandse Chemie Vereniging (KNCV) in 2008 te Lunteren.

Sinds oktober 2008 was zij werkzaam als postdoc gevolgd door een tenure track-positie sinds januari 2009 bij dezelfde vakgroep. Hier zal zij zich voornamelijk gaan bezig houden met onderzoek naar “Receptor Residence-Time of Ligands”.

CURRICULUM VITAE

Laura Helena Heitman was born on April 1st 1981 in Alphen aan den Rijn. After graduating from secondary school at the “Christelijk Lyceum” in Alphen aan den Rijn in 1999, she started studying Bio-Pharmaceutical Sciences at Leiden University. In March 2001, she passed the propaedeutic exam, followed by the Masters degree in October 2004. From September 2002 until June 2003, she did an internship involving “Synthesis and Biological Evaluation of Fluorescent Agonists for the Adenosine A₁ Receptor” at the division of Medicinal Chemistry of the Leiden/Amsterdam Centre for Drug Research (LACDR) under the supervision of Dr. J. Brussee. A second internship was performed at this division from September 2003 until January 2004 involving “Pharmacology of the Adenosine A₁ Receptor – a New Partial Agonist” under the supervision of Prof. Dr. A. P. IJzerman. Subsequently, a third internship was performed at the division of Biological and Pharmaceutical Chemistry van The School of Pharmacy (London, UK), where she worked for six months on the “Pharmacology of the NMDA Receptor Subtypes and Their Associated Proteins” under the supervision of Prof. Dr. F. A. Stephenson.

From October 2004 until September 2008, she was employed as a PhD student at the division of Medicinal Chemistry of the LACDR under supervision of Prof. Dr. A. P. IJzerman and in collaboration with Schering Plough (Oss, NL) as part of the Top Institute Pharma (TI Pharma) consortium. During her PhD, she presented her research at several (inter)national symposia and congresses. In 2008 she received two first prizes for an oral communication entitled: “False Positives in a Reporter Gene Assay: Identification and Synthesis of Competitive Inhibitors of Firefly Luciferase” at the LACDR Spring symposium and the FIGON Dutch Medicines Days. In addition, she received the first prize for the poster entitled: “One Receptor, Three Binding Sites - A Case for the GnRH Receptor” at the meeting of the Royal Dutch Chemistry Society (KNCV) in 2008 in Lunteren.

Since October 2008 was employed as a PostDoc followed by a tenure track-position since January 2009 at the same division. She will mainly be working on “Receptor Residence-Time of Ligands”.

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