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

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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 [^{3}H]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

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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 M_1, M_4 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, [^{3}H]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 [^{3}H]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 [^{3}H]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.). [^{3}H]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.

6.2.2 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 µg/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*.

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 $MgCl_2$ 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 [^{3}H]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 $MgCl_2$ 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 $MgCl_2$ 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 [^{3}H]Org 43553. Non-specific binding was determined in the presence of 10 µM Org 43553 and represented approximately 30% of the total binding. [^{3}H]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 ^{125}I-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 $MgCl_2$ and 0.1% BSA) at 30 °C for 120 min with 4.5 nM [^{3}H]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 $MgCl_2$ and 0.1% BSA) with 4.5 nM [^{3}H]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 [^{3}H]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

CHOhLHr_luc cells were grown as described above. 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 µg/mL insulin, 5 µg/mL apo-transferrin, 100 µg/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 [^{3}H]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 [^{3}H]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 µM 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 sites[62] 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 [^{3}H]Org 43553 dissociation.

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

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

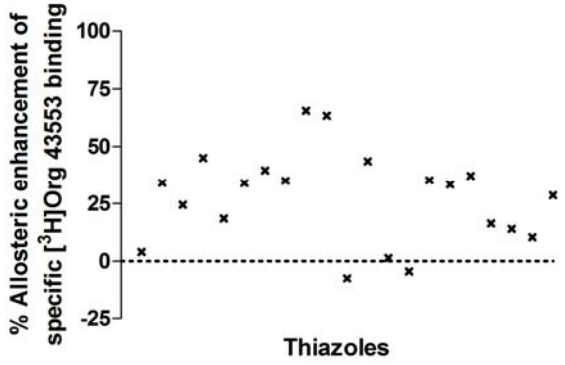
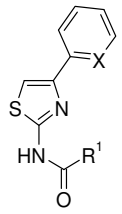


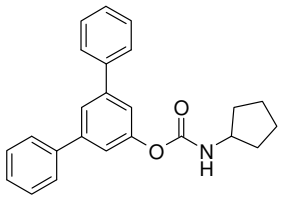
Figure 6.1 Effect of 21 compounds from a thiazole library[292,293] on single point dissociation of [^{3}H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes.

From Figure 6.1 it follows that these 21 thiazole compounds have different modulating potencies, ranging from no effect to almost 50% attenuation of [^{3}H]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 [^{3}H]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

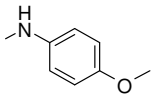
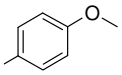
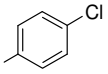
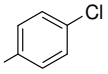
Table 6.1. Displacement and allosteric modulation of [^{3}H]Org 43553 binding at the human luteinizing hormone receptor by 10 µM of compounds **1**-**5** and **LUF5771**.



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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]Org 43553 was examined. As

shown in Table 6.1, LUF5419 (**4**) and compound **5** were also able to increase the total amount of [^{3}H]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 µM LUF5771 resulted in an 88% increased dissociation of [^{3}H]Org 43553 from the human LH receptor.

6.3.2 Radioligand Saturation Experiments

Although two other thiazoles were more potent allosteric enhancers of [^{3}H]Org 43553 binding (Figure 6.1), LUF5419 (**4**) was selected for further experiments due to ample availability. Saturation binding assays with [^{3}H]Org 43553 were performed in the absence (control) and presence of 10 µM LUF5419. The results of a representative saturation experiment are shown in Figure 6.2. In both conditions the saturation of [^{3}H]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 [^{3}H]Org 43553. The presence of 10 µM 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 [^{3}H]Org 43553 binding in the absence (control) and presence of 10 µM 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 µM LUF5419	3.1 ± 0.2 **	798 ± 71 *

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

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

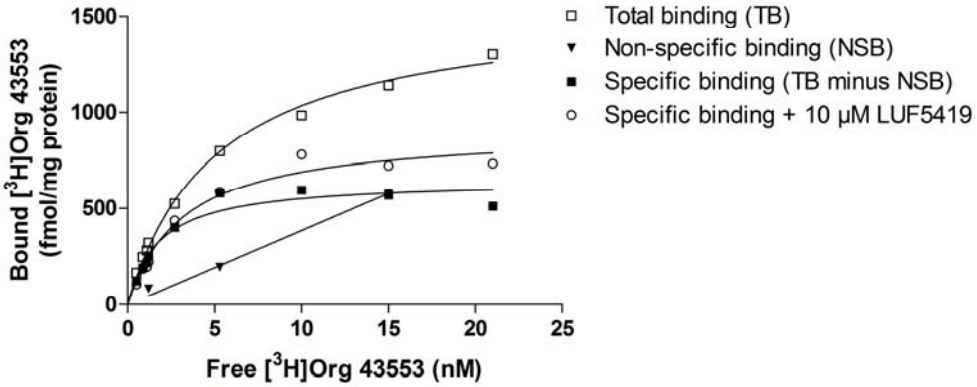


Figure 6.2 Saturation of [^{3}H]Org 43553 to luteinizing hormone receptors in the absence (control) or presence of 10 µM LUF5419. The control specific binding was determined by subtracting the non-specific binding from the total binding curve. A similar experiment was performed in the presence of 10 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 ± 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.

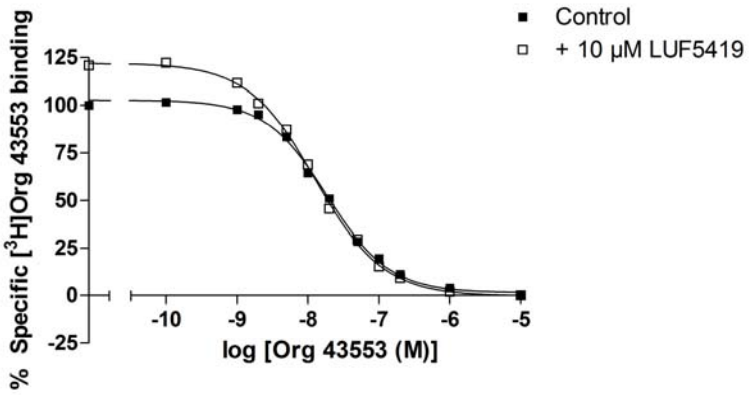


Figure 6.3 Displacement of [^{3}H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes by unlabeled Org 43553 in the absence (control) or presence of 10 µM LUF5419. Representative graphs from one experiment performed in duplicate.

6.3.4 Kinetic Association and Dissociation Experiments

Subsequently, the effect of LUF5419 on the kinetic association and dissociation parameters of [^{3}H]Org 43553 at CHOhLHr_luc membranes at 30 ºC were determined. As shown in Table 6.3 and Figure 6.4, [^{3}H]Org 43553 associated to the receptor within 120 min with a k_{on} value of 0.0082 ± 0.0004 nM^{-1} min^{-1}. In the presence of 10 µM LUF5419, the association rate was not significantly altered (k_{on} = 0.0092 ± 0.0003 nM^{-1} 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 [^{3}H]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_{off}/k_{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 µM LUF5419	0.0092 ± 0.0003	123 ± 3**	0.011 ± 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 [^{3}H]Org 43553 bound to human luteinizing hormone receptors.

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

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

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

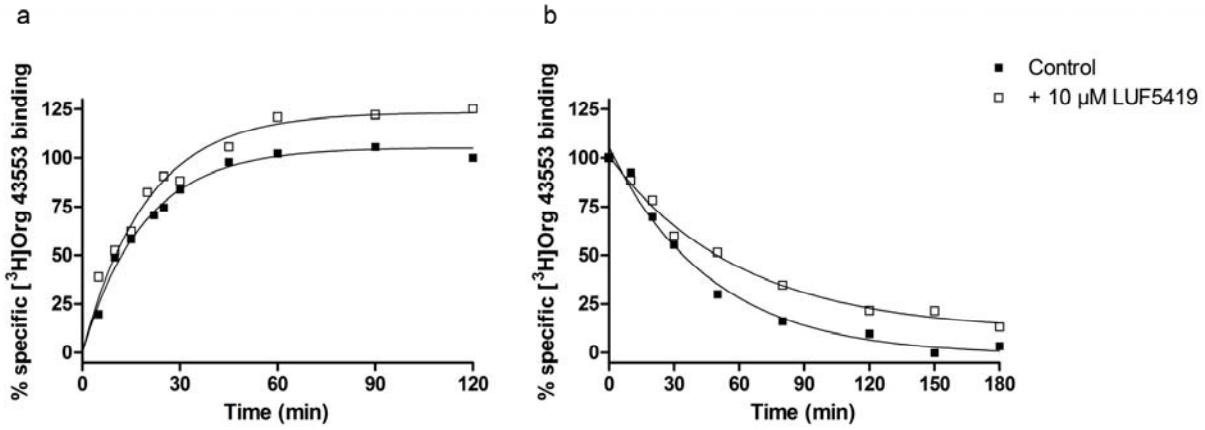


Figure 6.4 a) Association and b) dissociation kinetics of [^{3}H]Org 43553 binding to CHO-K1 membranes expressing the human luteinizing hormone receptor at 30ºC. Dissociation was either initiated by the addition of 10 µM Org 43553 in the absence (control) or presence of 10 µM LUF5419. Representative 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 ± 2%) the LH receptor with an EC_{50} value of 0.78 ± 0.2 nM. In the presence of 10 µM 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 µM 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 µM LUF5419 (E_{max} = 103 ± 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 µM 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 µM LUF5419	0.13 ± 0.03	64 ± 9**	90 ± 12
Org 43553	0.78 ± 0.2	79 ± 2	79 ± 2
+ 10 µM LUF5419	1.0 ± 0.2	73 ± 3	103 ± 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 µM LUF5419, where recLH in the absence of LUF5419 was set at 100%.

[c] Maximal effect corrected for the effect of 10 µM LUF5419 on forskolin-induced luciferase activity.

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

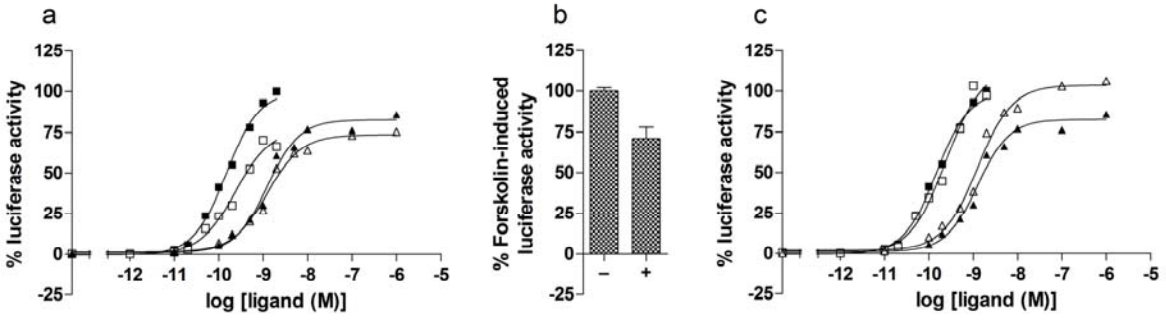


Figure 6.5 Concentration-effect curves of recLH and Org 43553 in the absence (■; recLH, ▲; Org 43553) or presence of 10 µM LUF5419 (□; recLH, Δ; Org 43553) for cAMP-mediated luciferase production through human luteinizing hormone receptors. a) curves of raw data, b) bargraph showing the effect of 10 µM LUF5419 on forskolin-induced (10 µM) 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 [^{3}H]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 EC_{50} value of 23 ± 4 µM for this compound. Subsequently, the effect of different concentrations of LUF5771 on [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]Org 43553 binding by LUF5419 and LUF5771, expressed as EC_{50} 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 [^{3}H]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 EC_{50} 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).

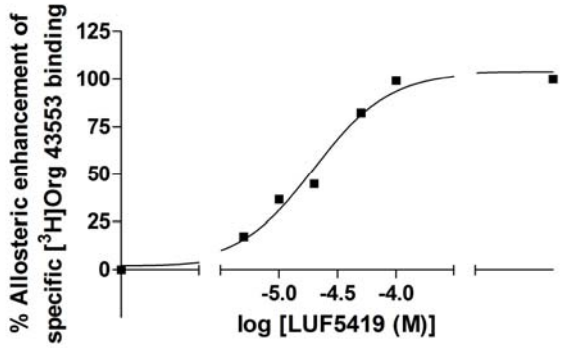


Figure 6.6 Concentration-dependent effect of LUF5419 on dissociation of [^{3}H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes measured at a single time point of 30 min. Data points corresponding to total binding (no dissociation) and control binding (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).

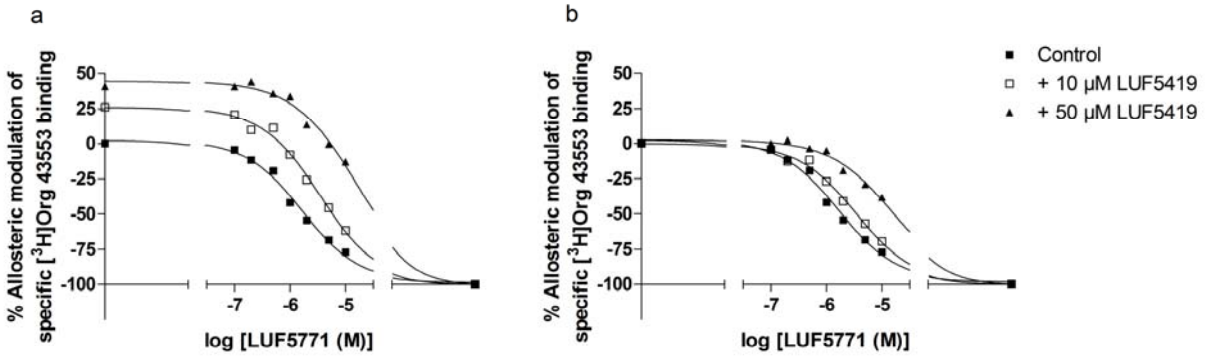


Figure 6.7 Concentration-dependent effect of LUF5771 on dissociation of [^{3}H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes in the absence (control) or presence of 10 µM or 50 µM LUF5419 measured at a single time point of 30 min. The top graph (a) 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 [^{3}H]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 δ = 1 (neutral cooperativity), δ < 1 (negative cooperativity) or δ > 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.

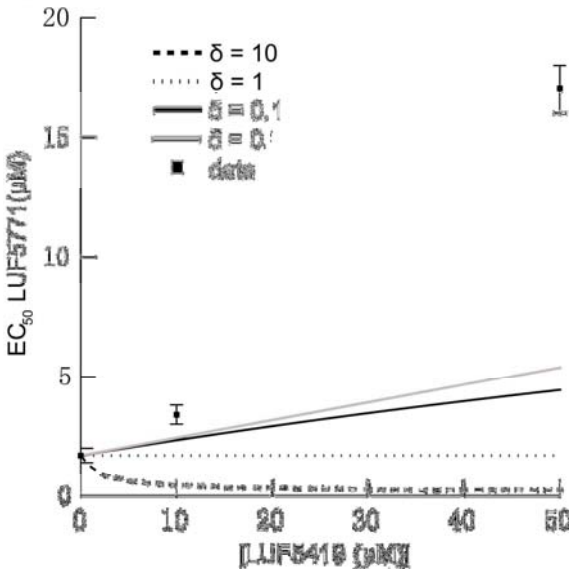


Figure 6.8 Negative cooperativity between LUF5771 and LUF5419 in modulating [^{3}H]Org 43553 dissociation. The experimental data of different concentrations of LUF5419 affecting the modulating potency of LUF5771 is displayed with standard deviation. The lines show the fit of the data to eq. 1 (see material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

6.4 DISCUSSION

In the present study the first allosteric enhancer of [^{3}H]Org 43553 binding at the human LH receptor, LUF5419, is described. An initial screen for allosteric modulation of [^{3}H]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 [^{3}H]Org 43553 for its binding site (Table 6.1). However, the dissociation rate was decreased and as a consequence the total binding of [^{3}H]Org 43553 was enhanced. The modulating potency of LUF5419 is therefore typical for [^{3}H]Org 43553 binding at the LH receptor.

Saturation assays in the absence and presence of LUF5419 were performed to investigate whether the increased [^{3}H]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 [^{3}H]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 ± 0.3 μM) is over 10-fold more potent in modulating [^{3}H]Org 43553 binding than LUF5419 (EC_{50} = 23 ± 4 μM). 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 coworkers[62] 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 [^{3}H]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 [^{3}H]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.