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

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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
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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 factor$_1$ 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 ^{125}I-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.

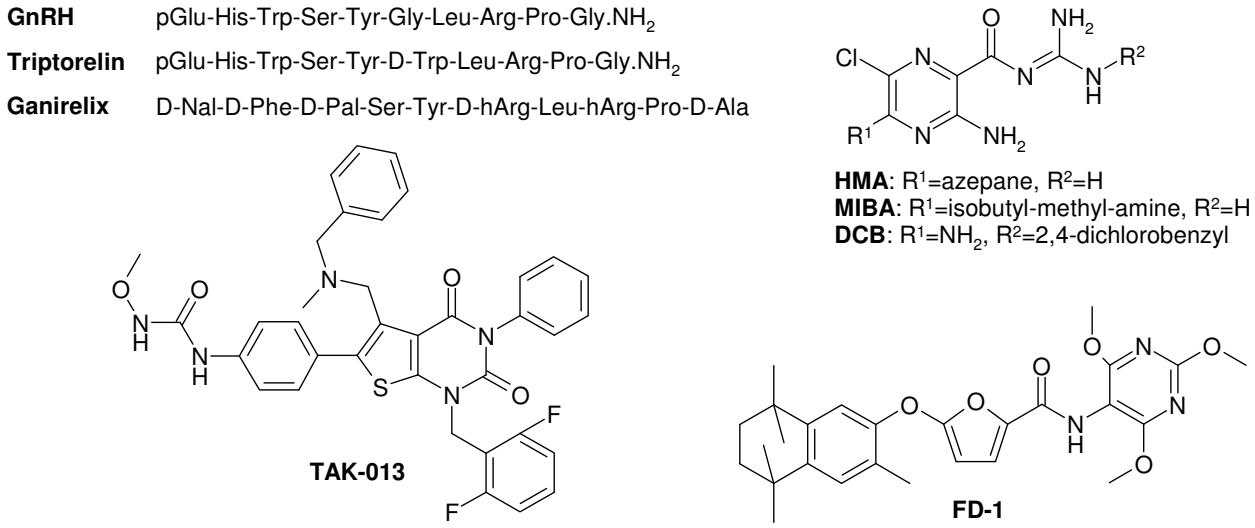

Figure 3.1 Sequences of GnRH, triptorelin (agonists), ganirelix (antagonist) and chemical structures of FD-1 (antagonist and allosteric inhibitor), HMA, MIBA, DCB (allosteric inhibitors) and TAK-013 (antagonist).

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(1H,3H)-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.). ^{125}I-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% CO_2.[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 $MgCl_2$, pH 7.4. An UltraThurrax (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 $MgCl_2$ and 0.1% (w/v) BSA) at 22 °C for 45 min. For saturation experiments unlabeled triptorelin was spiked with 20% ^{125}I-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) ^{125}I-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 $MgCl_2$ 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 °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 °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

CHOhGnRH_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 µg/mL insulin and 5 µg/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 luclite (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 $K_i = IC_{50}/(1 + [C]/K_d)$ where [C] is the concentration of the radioligand and K_d its dissociation constant.[245] The K_d value of ^{125}I-triptorelin at CHOhGnRH 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 $k_{on} = (k_{obs} - k_{off})/[L]$, where k_{obs} was obtained by computer analysis of the exponential association of the percentage of ^{125}I-triptorelin bound to the receptor and [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]

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

In Equation 3.1; EC_{50}^{HMA} is the observed EC_{50} of HMA in enhancing ^{125}I-triptorelin binding. $K_{FD\text{-}1}^{Triptorelin}$ 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.

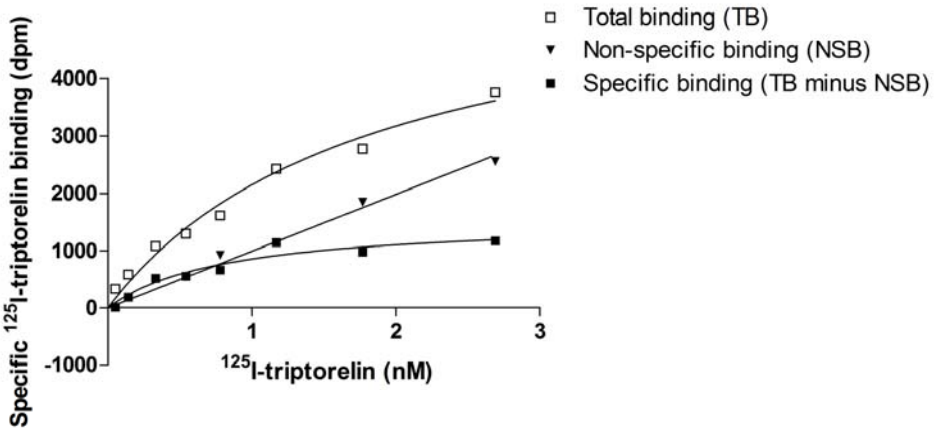

Figure 3.2 Saturation of ^{125}I-triptorelin binding to human gonadotropin-releasing hormone receptors. The specific binding was determined by subtracting the non-specific binding from the total binding. The K_D value was 0.35 (0.33 -0.37) nM and the B_{max} value was 217 (207 - 227) fmol/mg protein. 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 CHOhGnRH cell membranes. The endogenous agonist, GnRH, a derivative, triptorelin, a peptidic antagonist, ganirelix and two non-peptidic antagonists,

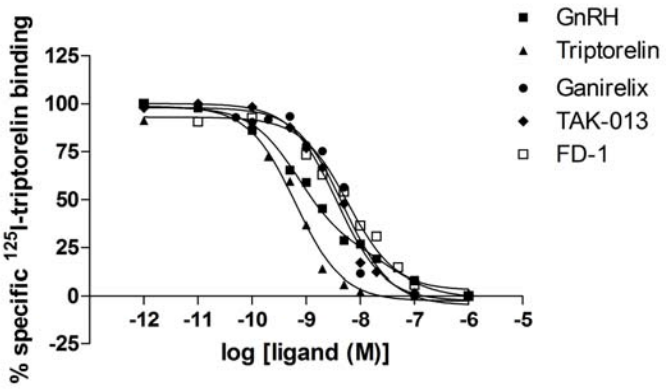

Figure 3.3 Displacement of ^{125}I-triptorelin from human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes by GnRH, triptorelin, ganirelix, TAK-013 and FD-1. Representative graphs from one experiment performed in duplicate (see Table 3.1 for affinity values).

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 ± 0.004 nM, K_L = 21 ± 10 nM and % R_H = 69 ± 3%.

Values are means (± 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 ± SEM, n = 3), respectively, with 69 ± 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

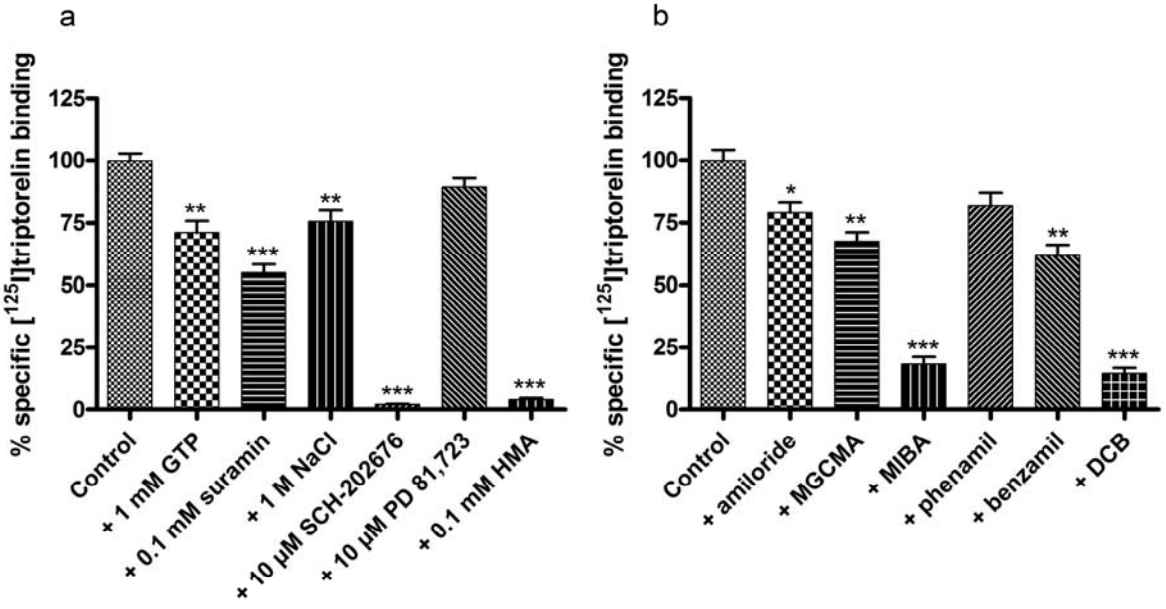

Figure 3.4 ^{125}I-Triptorelin equilibrium binding to human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes in the absence (control, 100%) or presence of a) GTP, suramin, sodium, SCH-202676, PD 81,723 and HMA and b) 0.1 mM amiloride derivatives. Values are means (± S.E.M.) 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 (IC_{50} = 29 ± 3 µM), MIBA (IC_{50} = 39 ± 7 µM) and DCB (IC_{50} = 30 ± 3 µM) with a pseudo-Hill coefficient of 1.4 ± 0.06, 1.3 ± 0.02 and 1.6 ± 0.2, respectively (Table 3.2).

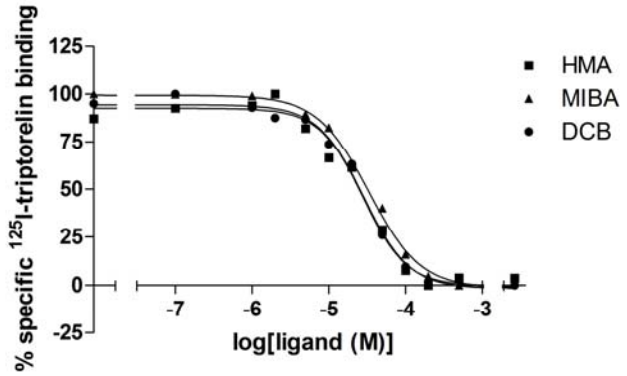

Figure 3.5 Inhibition of ^{125}I-triptorelin equilibrium binding to human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes by HMA, DCB and MIBA. Representative graphs from one experiment performed in duplicate (see Table 3.2 for affinity values).

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 ± 0.08 nM^{-1} min^{-1}. Under control conditions the radioligand dissociated from the receptor with a dissociation rate constant of 0.021 ± 0.002 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 CHOhGnRH 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

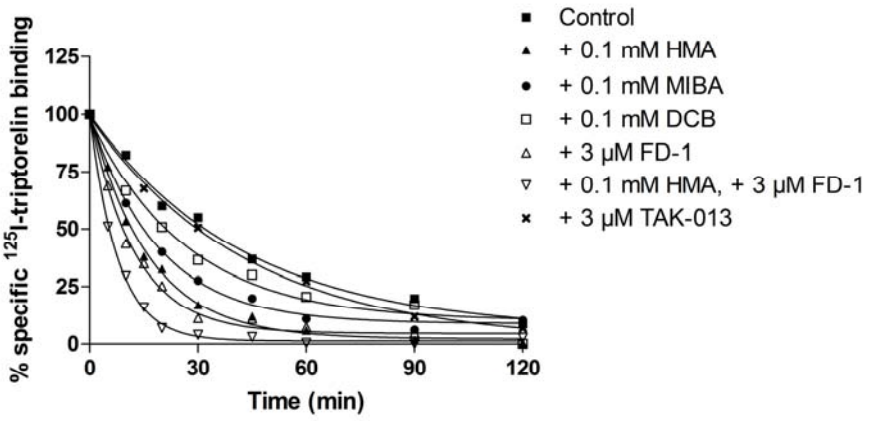

Figure 3.6 Dissociation kinetics of ^{125}I-triptorelin binding to human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes. Dissociation was either initiated by the addition 1 µM ganirelix mixed with buffer (control) or modulator. Representative graphs from one experiment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

	Inhibitory potency[a]			**Modulatory potency[d]**
Condition	**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 (± 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 ± 0.006 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 ± 0.009 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_{off} = 0.11 ± 0.01 min^{-1}).

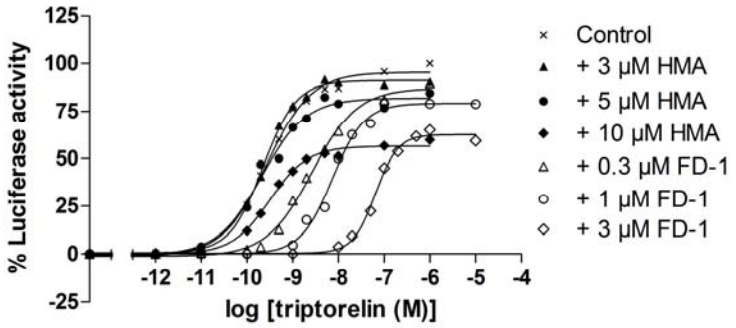

Figure 3.7 Concentration-effect curves of triporelin on NFAT-induced luciferase production through human gonadotropin-releasing hormone receptors in the presence and absence (control) of different concentrations HMA or FD-1. Representative graphs from one experiment performed in duplicate (see Table 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 (EC_{50} = 0.24 ± 0.02 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 ± 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 Emax value, indicative for its allosteric nature. For example, addition of 3 µM FD-1 decreased the Emax value to 72 ± 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.

	Activity in luciferase assay[a]	
Compound	**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 ± 3***
+ 10 µM HMA	0.29 ± 0.02*	58 ± 1***
+ 0.3 µM FD-1	2.7 ± 0.04***	93 ± 1***
+ 1 µM FD-1	10 ± 2***	84 ± 5***
+ 3 µM FD-1	44 ± 12***	72 ± 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 (± 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 (EC_{50} = 49 ± 7 μM), 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 ± 1 μM (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.

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

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

Values are means (± 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.

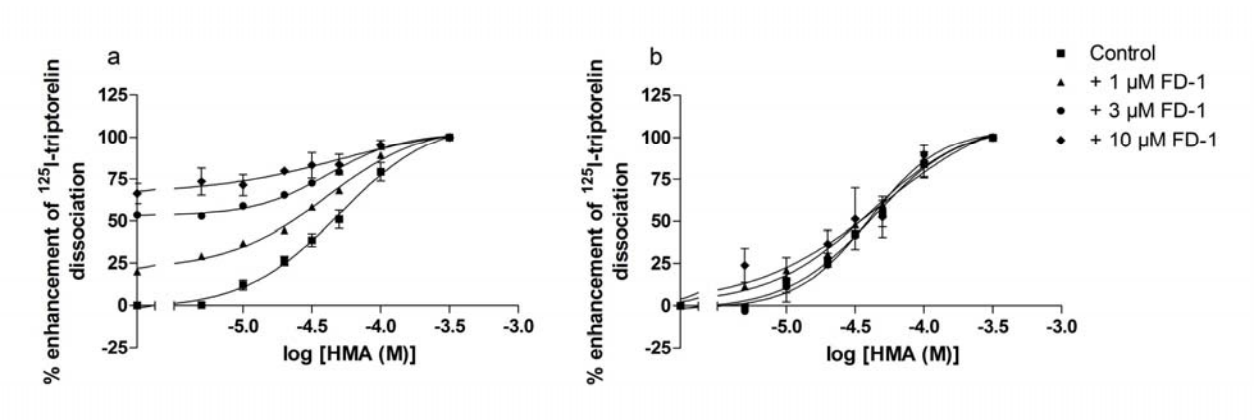

Figure 3.9 Effect of HMA on single point dissociation of ^{125}I-triptorelin from human gonadotropin-releasing hormone receptors stably expressed on CHO cell membranes in the presence or absence (control) of three concentrations of FD-1. Graph (a) shows data normalized to the control measured in the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

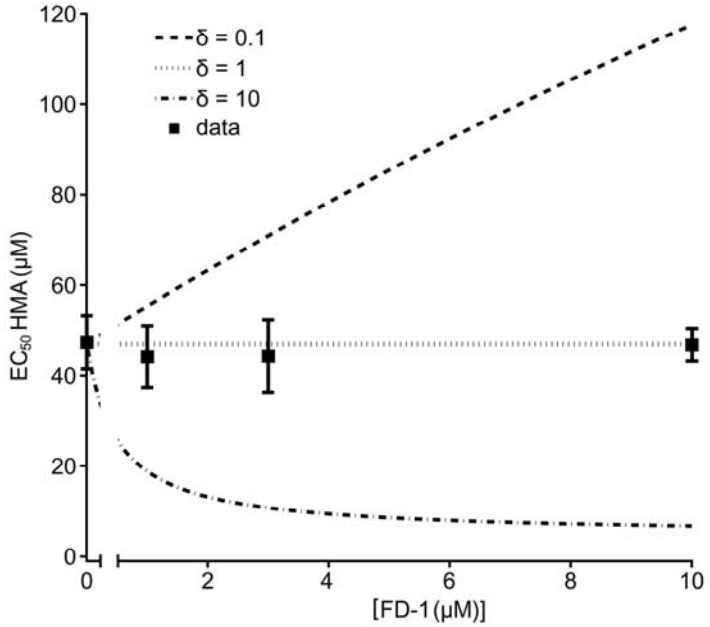

Figure 3.10 Neutral cooperativity between HMA and FD-1 in enhancing ^{125}I-triptorelin dissociation. The experimental data of different concentrations of FD-1 affecting the modulating potency of HMA is displayed with standard deviation. The lines show the fit of the data to eq. 1 (see material and methods), 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 ± 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-naphtalenyl)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 ^{125}I-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 ^{125}I-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

^{125}I-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 M_1 and M_4 muscarinic receptor, respectively. In addition, three distinct allosteric sites were reported by Schetz and Sibley[257] for the dopamine D_4 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 ^{125}I-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 (δ =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 D_2 receptor, which was earlier shown to be modulated by amiloride analogues[252] 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.