

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/23237416>

Identification of Tyr 290(6.58) of the Human Gonadotropin-Releasing Hormone (GnRH) Receptor as a Contact Residue for Both GnRH I and GnRH II: Importance for High-Affinity Binding a...

ARTICLE in BIOCHEMISTRY · OCTOBER 2008

Impact Factor: 3.02 · DOI: 10.1021/bi800911z · Source: PubMed

CITATIONS

8

READS

19

4 AUTHORS, INCLUDING:



Robert P Millar

University of Pretoria

334 PUBLICATIONS 13,650 CITATIONS

SEE PROFILE



Colleen A Flanagan

University of the Witwatersrand

48 PUBLICATIONS 2,374 CITATIONS

SEE PROFILE



Zhi-Liang Lu

Xi'an Jiaotong-Liverpool University

41 PUBLICATIONS 1,381 CITATIONS

SEE PROFILE

Identification of Tyr^{290(6.58)} of the Human Gonadotropin-Releasing Hormone (GnRH) Receptor as a Contact Residue for Both GnRH I and GnRH II: Importance for High-Affinity Binding and Receptor Activation[†]

Marla Coetsee,^{‡,§} Robert P. Millar,^{‡,§} Colleen A. Flanagan,^{§,||} and Zhi-Liang Lu^{*,‡}

MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, Scotland, United Kingdom, Research Group for Receptor Biology, Institute for Infectious Diseases and Molecular Medicine, The University of Cape Town, Anzio Road, Observatory 7925, South Africa, and School of Physiology, University of the Witwatersrand Medical School, 7 York Road, Parktown, 2193, South Africa

Received May 15, 2008; Revised Manuscript Received July 30, 2008

ABSTRACT: Molecular modeling showed interactions of Tyr^{290(6.58)} in transmembrane domain 6 of the GnRH receptor with Tyr⁵ of GnRH I, and His⁵ of GnRH II. The wild-type receptor exhibited high affinity for [Phe⁵]GnRH I and [Tyr⁵]GnRH II, but 127- and 177-fold decreased affinity for [Ala⁵]GnRH I and [Ala⁵]GnRH II, indicating that the aromatic ring in position 5 is crucial for receptor binding. The receptor mutation Y290F decreased affinity for GnRH I, [Phe⁵]GnRH I, GnRH II and [Tyr⁵]GnRH II, while Y290A and Y290L caused larger decreases, suggesting that both the *para*-OH and aromatic ring of Tyr^{290(6.58)} are important for binding of ligands with aromatic residues in position 5. Mutating Tyr^{290(6.58)} to Gln increased affinity for Tyr⁵-containing GnRH analogues 3–12-fold compared with the Y290A and Y290L mutants, suggesting a hydrogen-bond between Gln of the Y290Q mutant and Tyr⁵ of GnRH analogues. All mutations had small effects on affinity of GnRH analogues that lack an aromatic residue in position 5. These results support direct interactions of the Tyr^{290(6.58)} side chain with Tyr⁵ of GnRH I and His⁵ of GnRH II. Tyr^{290(6.58)} mutations, except for Y290F, caused larger decreases in GnRH potency than affinity, indicating that an aromatic ring is important for the agonist-induced receptor conformational switch.

Hypothalamic gonadotropin-releasing hormone I (GnRH I¹) is an important regulator of the mammalian reproductive system. GnRH I is released in a pulsatile manner from the hypothalamus and binds to the GnRH receptor in the pituitary gonadotrope cells. GnRH I binding to the receptor leads to the activation of G_{q/11} and downstream signaling cascades which control release of luteinizing hormone and follicle-stimulating hormone. The latter in turn regulate steroidogenesis and gametogenesis (1). Thus GnRH analogues are extensively applied clinically in the treatment of hormone-dependent diseases such as infertility, precocious puberty,

endometriosis, uterine fibroids, benign prostate hyperplasia and breast and prostate cancers (1–3).

The GnRH receptor is a member of the rhodopsin-like family of G protein-coupled receptors (GPCRs). There is one functional GnRH receptor type but two endogenous ligands (hypothalamic GnRH I and extra-hypothalamic GnRH II) in humans. This indicates that the single human GnRH receptor mediates all activities of both GnRH I and GnRH II, although they display differential physiological and pharmacological profiles (4). The distinct pharmacological and signaling profiles of GnRH I and GnRH II at the GnRH receptor are proposed to be mediated by different receptor active conformations, induced by differential ligand–receptor interactions, which we have termed GnRH ligand-induced selective signaling (LiSS) (1, 5). GnRH II differs from GnRH I by three amino acids in which Tyr⁵, Leu⁷ and Arg⁸ of GnRH I are replaced by His⁵, Trp⁷ and Tyr⁸. Delineation of differential ligand–receptor contacts made by GnRH I and GnRH II will enable identification of ligand contacts that steer LiSS and facilitate rational development of drugs with improved specificity at desired target signaling pathways. Homology and molecular docking models of GPCRs, especially those supported and refined by experimental data, can also be used for *in silico* screenings of lead compounds and structure–activity relationship studies (6).

The extracellular end of transmembrane domain (TM) 6 of GPCRs has been shown to play a major role in ligand binding and resultant receptor activation (7–12), but the roles of individual residues of the GnRH receptor in this segment

[†] This work was supported by the Medical Research Council, United Kingdom (to M.C., R.P.M. and Z.L.L.), and Ardana Biosciences (to M.C.), and the Commonwealth Scholarship (to M.C.), the South African National Research Foundation (to M.C.) and the Medical Research Council of South Africa (to C.A.F. and R.P.M.).

* To whom correspondence should be addressed: MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, Scotland, United Kingdom. Phone: 44-131-242-6218. Fax: 44-131-242-6197. E-mail: z.lu@hrcsu.mrc.ac.uk.

[‡] MRC Human Reproductive Sciences Unit, Edinburgh.

[§] Institute for Infectious Diseases and Molecular Medicine, The University of Cape Town.

^{||} School of Physiology, University of the Witwatersrand Medical School.

¹ Abbreviations: GnRH, gonadotropin-releasing hormone; TM, transmembrane domain; GPCR, G protein-coupled receptor; H-bond, hydrogen-bond; LiSS, ligand-induced selective signaling; IP, inositol phosphates; E_{max}, maximal agonist-elicited inositol phosphate responses; MD, molecular dynamics; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PDB, Protein Data Base.

are poorly understood as mutations of residues such as Tyr^{283(6.51)}, Tyr^{284(6.52)} and Trp^{291(6.59)} result in receptors that are inactive, probably due to abolished cell surface receptor expression, reduced ligand binding affinity or both (13). Molecular docking of GnRH I (14) in our laboratory and of [D-Trp⁶]GnRH (13) combined with an Ala mutation of Tyr^{290(6.58)} identified putative intermolecular interactions between Tyr^{290(6.58)} of the receptor and Tyr⁵ of GnRH I (14) or [D-Trp⁶]GnRH (13). The equivalent residue at position 6.58 is also suggested to play an important role in ligand binding and/or receptor activation of other peptide GPCRs, such as the NK2 tachykinin receptor (15), AT_{1A} angiotensin receptor (16) and CXCR1 chemokine receptor (17). However, the ligand–receptor intermolecular contacts, predicted by molecular modeling, have not been verified experimentally. It is essential to validate the models for use in the rational design of novel functional ligands. For example, previous site-directed mutagenesis and molecular modeling data suggested that the Trp³ side chain of [D-Trp⁶]GnRH interacts with Trp^{280(6.48)} of the receptor (13, 18). However, the Trp³ side chain in this model has an opposite orientation to the recently available NMR structure of GnRH I (PDB code: 1YY1) (19). Furthermore, our detailed mutagenesis studies (20) show that Trp³ of GnRH I does not interact with Trp^{280(6.48)} of the GnRH receptor, but faces away toward TM 4 where it makes other intermolecular interactions (14). This emphasizes the importance of verifying the intermolecular interactions suggested by molecular modeling and site-directed mutagenesis of the receptor by using single amino acid modified peptides. In addition, there are few data for the human GnRH receptor binding of GnRH II (14) and the interaction between His⁵ of GnRH II and the receptor has not been investigated. Because the Tyr^{290(6.58)} side chain is proposed to interact with the Tyr⁵ residue of GnRH I, which is His⁵ in GnRH II, it is potentially a differential ligand–receptor interaction that may underlie receptor conformational selection and LiSS of GnRH I and GnRH II. Hence, a more detailed definition of the role of Tyr^{290(6.58)} in binding of GnRH I and GnRH II would potentially elucidate intermolecular interactions that initiate LiSS. We have used GnRH analogues with substitutions of Tyr⁵ or His⁵ combined with site-directed mutagenesis of the Tyr^{290(6.58)} residue of the receptor to provide experimental evidence that Tyr^{290(6.58)} is able to interact with both Tyr⁵ of GnRH I and His⁵ of GnRH II. Our refined GnRH receptor molecular docking analyses, incorporating a recent NMR structure of GnRH I and docking of GnRH II, support these interactions.

EXPERIMENTAL PROCEDURES

Receptor Amino Acid Residue Numbering. Receptor residues are identified by the amino acid sequence number of the receptor followed by nomenclature of Ballesteros and Weinstein (7) in which the position of the most conserved amino acids in the TM domain N is designated N.50 in parentheses. This distinguishes receptor residues from that of the decapeptide GnRH analogues labeled with the sequence number only.

Materials. GnRH I (pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰-NH₂) and GnRH II ([His⁵,Trp⁷,Tyr⁸]GnRH) were purchased from Sigma-Aldrich (Dorset, U.K.) and Bachem (Bubendorf, Switzerland). Cetrorelix (Ac-D-Nap-

Ala¹-D-CIPh-Ala²-D-Pyr-Ala³-Ser⁴-Tyr⁵-D-Cit⁶-Leu⁷-Arg⁸-Pro⁹-D-Ala¹⁰-NH₂) and [His⁵,D-Tyr⁶]GnRH I were synthesized and iodinated as described previously (21). [Phe⁵]GnRH I, [Ala⁵]GnRH I, [Tyr⁵]GnRH II and [Ala⁵]GnRH II were purchased from EZBiolab Inc. (Westfield, IN). DeepVent polymerase was from New England Biolabs (Hertfordshire, U.K.). *Eco*RI, *Bsr*GI and *Xho*I restriction endonucleases and T4 ligase were from Promega UK Ltd. (Southampton, U.K.). D-[myo-³H]Inositol was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, U.K.).

Site-Directed Mutagenesis and Receptor Expression. The GnRH receptor was cloned into the pcDNA1 expression vector. Mutant sequences were constructed using a PCR method and validated by dideoxy sequencing (22). Wild-type and mutant receptors were transiently expressed in COS-7 cells by transfection using a Bio-Rad Gene Pulser at 230 V, 960 μ F with 15 μ g of DNA/0.4 cm cuvette (1.5 \times 10⁷ cells; 0.7 mL). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, antibiotics and 2 mM glutamine for 48 h to allow receptor expression.

Ligand Binding Assays. Radioligand binding assays were performed on intact cells 48 h after transfection as described previously (5, 14, 19). Transfected cells in 12-well culture plates were incubated with ¹²⁵I-Cetrorelix (150000 cpm/0.5 mL/well) or ¹²⁵I-[His⁵,D-Tyr⁶]GnRH I (100000 cpm/0.5 mL/well) and various concentrations of unlabeled GnRH analogues in 0.1% BSA/HEPES/DMEM for 4 h at 4 °C. Nonspecific binding was determined in the presence of 1 μ M unlabeled [His⁵,D-Tyr⁶]GnRH I for the ¹²⁵I-[His⁵,D-Tyr⁶]GnRH I binding assays, resulting in 0.4–0.8% of the total radioactivity. For ¹²⁵I-Cetrorelix binding assays, the nonspecific binding was measured in the presence of 1 μ M unlabeled Cetrorelix. After incubation, the cells were washed twice with ice-cold phosphate-buffered saline, pH 7.4, and solubilized in 0.5 mL of 0.1 M NaOH. Radioactivity was counted with a gamma counter.

Inositol Phosphates (IP) Assays. Measurements of GnRH-elicited IP accumulation were performed as described previously (5, 14, 19). Transfected COS-7 cells were seeded onto 12-well plates, and after 36 h, they were labeled overnight with 1 μ Ci/mL D-[myo-³H]inositol in inositol-free DMEM containing 1% dialyzed serum. At 48 h after transfection, the cells were preincubated with 0.5 mL of buffer (140 mM NaCl, 20 mM HEPES, 8 mM Glucose, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mg/mL BSA containing 10 mM LiCl) at 37 °C for 30 min, followed by the addition of GnRH analogues for an additional 30 min. This was shown to be within the linear period of the assay. The stimulation was terminated by the removal of the media and the addition of 0.5 mL of 10 mM formic acid. The ³H-labeled IPs were isolated from the formic acid extracts using Dowex AG 1-X8 ion-exchange resin, collected with 1 M ammonium formate/0.1 M formic acid, and quantified by liquid scintillation counting.

GnRH Docking and Molecular Dynamics (MD) Simulations. The human GnRH receptor model was built by homology modeling through MODELER within DS Modeling (version 1.6, Accelrys, San Diego) as described previously (4, 14, 19) using the crystal structure of a photoactivated deprotonated intermediate state of bovine rhodopsin (PDB code 2I37) (23) as a template. A β II'-type

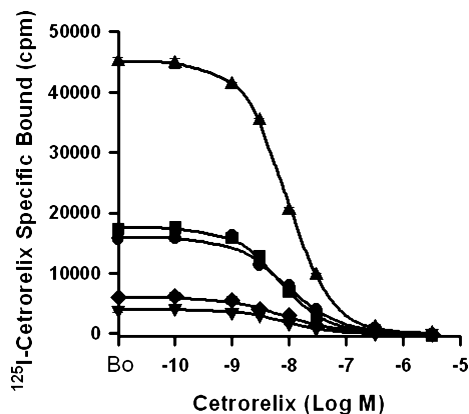


FIGURE 1: Homologous competition binding of Cetorelix. Binding curves showing homologous competitive binding of the peptide antagonist Cetorelix in wild-type and Tyr^{290(6.58)} mutant receptors transiently expressed in COS-7 cells. Results are representative experiments, which were repeated at least three times with essentially the same results. The binding curves show little difference in receptor affinity for Cetorelix between wild-type and mutant receptors. ●, wild-type; ▼, Y290F; ■, Y290A; ◆, Y290L; ▲, Y290Q. Points, mean \pm standard error of triplicate measurements.

turn conformation of GnRH I (derived from an NMR structure, PDB code 1YY1) and of GnRH II was docked into the model (1, 4, 14, 24) according to the experimentally identified or putative contact points between GnRH and receptor, i.e. pGlu¹ with Asn^{212(5.39)} (25), His² with Asp^{98(2.61)}/Lys^{121(3.32)} (21), and Pro⁹-Gly¹⁰NH₂ with Arg^{38(1.35)}/Asn^{102(2.65)} (14, 26). The GnRH-receptor complex was then optimized by energy-minimization and MD simulations of 150 ps by means of the CHARMM program (27) using a similar setup as described for the oxytocin receptor (28) with harmonic restraints on the receptor backbone atoms, except for extracellular loop 2 and its covalently linked N-terminal domain (4, 19).

Data Analysis. All experiments were performed in triplicate and repeated at least three times. Binding curves were fitted to the Hill equation or to the one-site model of the binding using Sigmaplot 9.0 (Systat Software, Inc., Point Richmond, CA) or GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA), yielding an IC₅₀ value (14, 19). The mutant receptor expression levels (R_{exp}) were expressed relative to a wild-type control included in each transfection. IP dose-response curves were fitted to a four-parameter logistic function, yielding a basal activity, a maximum response (E_{max}), an EC₅₀ value and slope factor that was characteristically near 1.0. Statistical analysis was performed with Student's *t* test.

RESULTS

Homologous Binding of Radiolabeled Ligands and Receptor Expression. The IC₅₀ value for binding of the peptide antagonist, Cetorelix, to the wild-type human GnRH receptor was 5.7 ± 1.9 nM, as measured by homologous competitive binding assays using ¹²⁵I-Cetorelix as a radiolabeled ligand on the intact cells (Figure 1). Mutation of Tyr^{290(6.58)} of the GnRH receptor to Phe, Ala, Leu or Gln had little effect on the binding affinity of Cetorelix, thus the B_0 values measured in the absence of unlabeled ligand by this tracer reflect relative receptor expression levels (R_{exp}) on the cell surface.

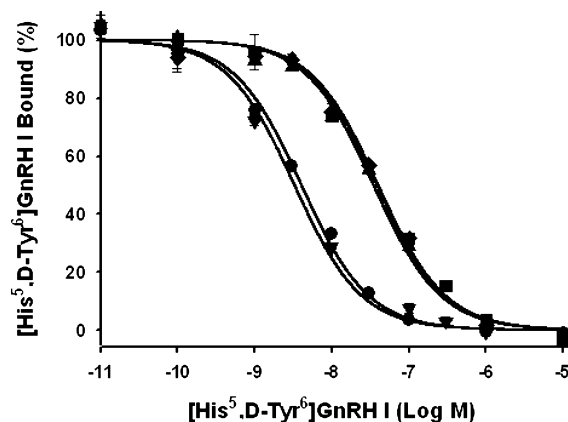


FIGURE 2: Homologous competition binding of [His⁵,D-Tyr⁶]GnRH I in wild-type and Tyr^{290(6.58)} mutant receptors. Results are representative experiments, which were repeated at least three times with essentially the same results. ●, wild-type; ▼, Y290F; ■, Y290A; ◆, Y290L; ▲, Y290Q.

Mutation of Tyr^{290(6.58)} of the GnRH receptor to Phe or Leu decreased receptor expression to 30% and 32% of the wild-type level, respectively (Figure 1). In contrast, mutation of Tyr^{290(6.58)} to Gln markedly increased the mutant receptor expression to 228% of wild-type, while Ala mutation did not affect expression. These results indicate that mutations of Tyr^{290(6.58)} have differential effects on cell surface expression of receptor, as binding was measured using intact cells, but no effect on binding affinity for the peptide antagonist, Cetorelix. Because of high nonspecific binding of ¹²⁵I-Cetorelix, we used ¹²⁵I-[His⁵,D-Tyr⁶]GnRH I as the tracer in the subsequent assays to measure receptor binding affinities for GnRH analogues. The mutants, Y290A, Y290L and Y290Q, had 8–11-fold reductions in affinity for [His⁵,D-Tyr⁶]GnRH I (Figure 2).

Importance of Tyr⁵ of GnRH I in Receptor Binding. Previous molecular modeling studies suggested that the side chain of Tyr^{290(6.58)} of the GnRH receptor may interact with Tyr⁵ of GnRH I (1, 14). To assess the contribution of Tyr⁵ to the affinity of GnRH I binding to the wild-type receptor, we examined changes in affinity of the wild-type human GnRH receptor for Tyr⁵-substituted analogues of GnRH I. [Phe⁵]GnRH I exhibited only a 2-fold reduction, while [Ala⁵]GnRH I gave a 127-fold decrease in affinity at the wild-type receptor compared with GnRH I (Table 1). These results indicate that the aromatic ring and, to a much lesser degree, the *para*-OH of Tyr⁵ of GnRH I contribute to high affinity GnRH I binding at the receptor.

Mutation of Tyr^{290(6.58)} of the Human GnRH Receptor Decreased Affinity for GnRH I. To determine whether the *para*-OH group and aromatic ring of Tyr^{290(6.58)} of the GnRH receptor are important for binding of GnRH I, Tyr^{290(6.58)} was mutated to Phe, Ala, Leu and Gln, and the affinities of wild-type and mutant receptors for GnRH I were measured by competition binding assays. In agreement with previous reports (5, 14, 19), GnRH I binds to the wild-type human GnRH receptor expressed in COS-7 cells with high affinity, giving an IC₅₀ value of 3.8 nM (Table 1). Mutation of Tyr^{290(6.58)} to Phe, which deletes the *para*-OH group, resulted in a 4.9-fold decrease in affinity for GnRH I, while mutation of Tyr^{290(6.58)} to Ala, which deletes the side chain beyond the β -carbon, led to a 332-fold reduction in affinity for GnRH I, with an IC₅₀ value of 1262 nM (Table 1). Mutation of

Table 1: Binding of GnRH I and Tyr⁵-Substituted Analogues to the Wild-Type and Tyr^{290(6.58)} Mutant Human GnRH Receptors^a

mutants	GnRH I		[Phe ⁵]GnRH I		[Ala ⁵]GnRH I	
	IC ₅₀ , ^b nM	fold change ^{c,d}	IC ₅₀ , nM	fold change	IC ₅₀ , nM	fold change
wild-type	3.8 ± 0.14	1 (1)	7.6 ± 0.4	1 (2)	481 ± 34	1 (127)
Y290F	18.6 ± 3.0	4.9 (1)	32.2 ± 4.0	4.2 (1.7)	458 ± 60	1 (25)
Y290A	1262 ± 113	332 (1)	1817 ± 111	239 (1.4)	3998 ± 729	8.3 (3.2)
Y290L	1094 ± 232	288 (1)	3799 ± 752	500 (3.5)	2682 ± 1212	5.6 (2.4)
Y290Q	382 ± 84	100 (1)	1703 ± 192	224 (4.5)	3753 ± 1221	7.8 (9.8)

^a Receptor binding affinity (IC₅₀) was measured on the intact COS-7 cells, 48 h after transient transfection of the wild-type and mutant receptors, by competition binding assays using ¹²⁵I-[His⁵,D-Tyr⁶]GnRH I as a radiolabeled ligand with increasing concentrations of unlabeled GnRH analogues.

^b Values are means ± SE of three or more independent experiments. ^c Fold change relative to the wild-type receptor. ^d Fold change relative to GnRH I in parentheses.

Table 2: Binding of GnRH II and His⁵-Substituted Analogues to the Wild-Type and Tyr^{290(6.58)} Mutant Receptors^a

mutants	GnRH II		[Tyr ⁵]GnRH II		[Ala ⁵]GnRH II	
	IC ₅₀ , ^b nM	fold change ^{c,d}	IC ₅₀ , nM	fold change	IC ₅₀ , nM	fold change
wild-type	25 ± 2	1 (1)	46 ± 4	1 (1.8)	4458 ± 122	1 (177)
Y290F	201 ± 14	8 (1)	506 ± 21	11 (2.5)	2752 ± 258	0.6 (14)
Y290A	2057 ± 291	82 (1)	2078 ± 137	45 (1)	9177 ± 1423	2.1 (4.5)
Y290L	963 ± 176	38 (1)	3207 ± 336	69 (3.3)	12800 ± 3236	2.9 (13)
Y290Q	846 ± 103	34 (1)	268 ± 19	5.8 (0.3)	8000 ± 240	1.8 (9.5)

^a Ligand binding affinity (IC₅₀) was measured on the intact COS-7 cells, 48 h after transient transfection of the wild-type and mutant receptors, by competition binding assays using ¹²⁵I-[His⁵,D-Tyr⁶]GnRH I as a radiolabeled ligand with increasing concentrations of unlabeled GnRH analogues.

^b Values are means ± SE of three or more independent experiments. ^c Fold change relative to the wild-type receptor. ^d Fold change relative to GnRH II in parentheses.

Tyr^{290(6.58)} of the GnRH receptor to a smaller, nonpolar residue (Leu) or a polar residue (Gln) also exhibited a marked reduction in affinity for GnRH I by 288- and 100-fold (Table 1). These results indicate that both the *para*-OH and the aromatic ring of Tyr^{290(6.58)} of the GnRH receptor play an important role in the binding of GnRH I.

Effects of Mutations of Tyr^{290(6.58)} of the Human GnRH Receptor on Binding Affinity for Tyr⁵-Substituted Analogues of GnRH I. To determine whether the side chain of Tyr^{290(6.58)} of the receptor has a role in interaction with Tyr⁵ of GnRH I, we examined the effects of mutations of Tyr^{290(6.58)} of the human GnRH receptor on the binding affinity for Tyr⁵-substituted analogues of GnRH I. Mutation of Tyr^{290(6.58)} of the GnRH receptor to Phe had similar decreases in affinity for GnRH I (4.9-fold) and [Phe⁵]GnRH I (4.2-fold) affinity, but had little effect on the affinity for [Ala⁵]GnRH I (Table 1). This shows that the *para*-OH of Tyr^{290(6.58)} is important for binding of GnRH analogues with an aromatic residue in position 5, but not for binding of [Ala⁵]GnRH I, which lacks the aromatic side-chain. Mutation of Tyr^{290(6.58)} to Ala and Leu led to 288- to 332-fold reductions in affinity for GnRH I and 239- to 500-fold for [Phe⁵]GnRH I, but had much smaller effects on mutant receptor binding affinity for [Ala⁵]GnRH I (5.6- to 8.3-fold) (Table 1). Interestingly, the Y290Q mutant receptor, which was designed to reconstitute the hydrogen-bond (H-bond) potential of the *para*-OH group of Tyr^{290(6.58)} in the absence of the aromatic ring, exhibited a 3-fold higher affinity than Y290A and Y290L mutant receptors for GnRH I (Table 1). This indicates that the Gln

side chain of the Y290Q mutant receptor enhances affinity for GnRH I, but not [Phe⁵]GnRH I or [Ala⁵]GnRH I, and suggests that Gln may form an H-bond with the *para*-OH of Tyr⁵ of GnRH I.

Effects of Mutations of Tyr^{290(6.58)} on Receptor Binding Affinity for His⁵-Substituted Analogues of GnRH II. Replacement of His⁵ of GnRH II with Tyr⁵, which is present in GnRH I, caused only a minor reduction (<2-fold) of the binding affinity at the wild-type receptor (Table 2). These results show that substitution of His⁵ of GnRH II with Tyr is well tolerated. Replacement of His⁵ of GnRH II with Ala led to a marked reduction (177-fold) of the peptide affinity at the wild-type receptor (Table 2). These data indicate that the aromatic ring in position 5 of GnRH II plays an important role in high affinity binding of the peptide to the receptor.

To assess the roles of Tyr^{290(6.58)} of the human GnRH receptor in high affinity binding of GnRH II, we examined the effects of its mutation on receptor binding affinity for GnRH II and His⁵-substituted GnRH II analogues, [Tyr⁵]GnRH II and [Ala⁵]GnRH II. The mutation Y290F decreased affinity for GnRH II relative to the wild-type receptor (IC₅₀ value 25 nM) by 8-fold, while the mutation Y290A had a much larger effect, producing an 82-fold reduction in affinity. Mutation of Tyr^{290(6.58)} to Leu and Gln also decreased affinity for GnRH II (38- and 34-fold, Table 2). These results indicate that Tyr^{290(6.58)} of the GnRH receptor is also important for high affinity binding of GnRH II.

Table 3: GnRH-Elicited IP Responses at the Wild-Type and Tyr^{290(6.58)} Mutant Human GnRH Receptors

mutants	R_{exp}^a	GnRH I		GnRH II		[His ⁵ ,D-Tyr ⁶]GnRH I	
		EC ₅₀ , ^b nM	E_{max} , % wt	EC ₅₀ , nM	E_{max} , % wt	EC ₅₀ , nM	E_{max} , % wt
wild-type	100	0.9 ± 0.2	100	9.6 ± 2	100	0.7 ± 0.1	100
Y290F	30 ± 5	51 ± 10	94 ± 7	254 ± 27	87 ± 12	4.7 ± 0.4	107 ± 3
Y290A	102 ± 5	1724 ± 146	72 ± 6	1940 ± 175	82 ± 18	73 ± 10	88 ± 6
Y290L	32 ± 3	u.d.	<10	1674 ± 208	85 ± 10		
Y290Q	228 ± 13	227 ± 26	156 ± 7	1607 ± 162	167 ± 24		

^a Cell surface receptor expression levels (R_{exp}) were measured on intact cells using ¹²⁵I-Cetrorelix, which showed no changes in affinity for Tyr^{290(6.58)} mutant receptors, and were expressed relative to the wild-type control included in each transfection. ^b IP responses were determined in COS-7 cells transfected with wild-type and mutant receptors. Values are the mean ± SE of three or more independent experiments. u.d., undetectable.

Mutation of Tyr^{290(6.58)} to Phe, Ala and Leu had essentially similar effects on receptor binding affinity for [Tyr⁵]GnRH II as observed for GnRH II. Specifically, Y290F exhibited an 11-fold reduction for [Tyr⁵]GnRH II, while Y290A and Y290L gave 45- and 69-fold reductions respectively (Table 2). This suggests that Tyr⁵ can efficiently substitute for the interactions of His⁵ of GnRH II with the GnRH receptor. Interestingly, the Y290Q mutant receptor exhibited a smaller reduction in affinity (5.8-fold) for [Tyr⁵]GnRH II compared with wild-type. Y290Q also exhibited higher affinity for [Tyr⁵]GnRH II, compared with GnRH II (3-fold), and with Y290A and Y290L mutants (8–12-fold, Table 2). These data suggest that substitution of His⁵ of GnRH II with Tyr⁵ may lead to the acquisition of a new H-bond interaction with the Gln side chain of the Y290Q mutant. Compared with the wild-type receptor, mutations of Tyr^{290(6.58)} to Phe, Ala, Leu or Gln had only marginal effects (<3-fold) on receptor binding affinity for [Ala⁵]GnRH II, which lacks an aromatic ring at position 5 of the peptide to interact with Tyr^{290(6.58)} of the receptor. These results suggest that the *para*-OH and the aromatic ring of Tyr^{290(6.58)} of the human GnRH receptor may make direct contacts with His⁵ of GnRH II.

Effects of Mutations of Tyr^{290(6.58)} on GnRH-Elicited IP Responses. Consistent with previous experiments (14, 19), GnRH I and GnRH II elicited robust IP responses from COS-7 cells expressing wild-type GnRH receptors with EC₅₀ values of 0.9 nM and 9.6 nM (Table 3). The maximum IP responses evoked by the wild-type receptor were typically 5 times the basal activity. The effects of mutations of Tyr^{290(6.58)} of the GnRH receptor on the IP responses are summarized in Table 3.

GnRH I and GnRH II elicited IP responses at the Y290F mutant receptor with increased EC₅₀ values, by 57- and 26-fold relative to the wild-type respectively, and slightly decreased maxima (94% and 87% of the wild-type E_{max}). This may be caused by the decreased mutant receptor expression level (Figure 1 and Table 3) and reduced binding affinity for GnRH I (Table 1) and GnRH II (Table 2). [His⁵,D-Tyr⁶]GnRH I, which has no change in receptor binding affinity for the Y290F mutant (Figure 2), exhibited a 6.7-fold increase in its EC₅₀ value with a slightly enhanced E_{max} relative to the wild-type (Figure 3). These data suggest that the intermolecular interaction between the *para*-OH group of Tyr^{290(6.58)} and the GnRH peptides may not play a major role in the GnRH-induced receptor conformational switch associated with receptor activation.

The Y290A mutant, which had similar receptor expression as the wild-type (Figure 1 and Table 3), exhibited 1915- and 202-fold reductions in potency of GnRH I and GnRH II in stimulating IP responses, with decreased E_{max} to 72% and

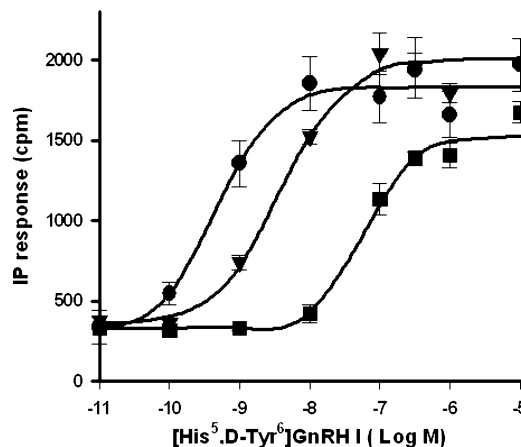


FIGURE 3: [His⁵,D-Tyr⁶]GnRH I elicited IP responses in human GnRH wild-type, Y290F, and Y290A mutant receptors. Results are representative experiments, which were repeated at least three times with essentially the same results. ●, wild-type; ▼, Y290F; ■, Y290A.

82% that of the wild-type. The larger effects of the mutation of Tyr^{290(6.58)} to Ala on potency than affinity for GnRH I (5.8-fold) and GnRH II (2.5-fold) indicate that the aromatic ring of Tyr^{290(6.58)} of the GnRH receptor is important for GnRH I- and GnRH II-induced receptor activation. This is further supported by the studies with [His⁵,D-Tyr⁶]GnRH I, which had only a 10-fold decrease in affinity (Figure 2), but a 104-fold reduction in potency at the Y290A mutant receptor (Figure 3 and Table 3). Mutation of Tyr^{290(6.58)} to Leu appears to be more detrimental to GnRH I than GnRH II in stimulating IP responses, giving an E_{max} less than 10% that of the wild-type for GnRH I, but 85% for GnRH II. In parallel with the increased receptor expression, Y290Q also evoked an increased E_{max} of 156% and 167% of that of the wild-type for GnRH I and GnRH II respectively.

GnRH Docking and MD Simulations. A human GnRH receptor model was built (19) using the crystal structure of a photoactivated deprotonated intermediate state of bovine rhodopsin (23) as a template. A β II'-turn conformation of GnRH I (derived from a recent NMR structure) and of GnRH II could be docked into the receptor model successfully, accommodating the experimentally confirmed or putative intermolecular interactions between GnRH and the receptor, followed by energy-minimization and MD simulations (4, 14). In the docking model of GnRH I, the side chain of Tyr^{290(6.58)} of the receptor forms a T-shaped stacking interaction with Tyr⁵ of GnRH I (Figure 4A). The *para*-OH group of Tyr⁵ of GnRH I points away from Tyr^{290(6.58)} of the receptor. The model shows no H-bond between the two *para*-OH moieties of the Tyr residues. This is consistent with the mutagenesis

I suggested by the molecular model. In our molecular docking of GnRH II (Figure 4B), the side chain of Tyr^{290(6.58)} makes an off-center parallel stacking contact with His⁵ of GnRH II, involving π - π , π -OH and π -NH interactions (31, 32). That the mutations of Tyr^{290(6.58)} have much smaller effects (<3-fold) on receptor binding affinity for [Ala⁵]GnRH II, which lacks the aromatic ring to interact with Tyr^{290(6.58)}, than the parent GnRH II supports the conclusion of a direct contact between the side chain of Tyr^{290(6.58)} and His⁵ of GnRH II. An interesting prediction from our molecular modeling is that, while both Tyr⁵ of GnRH I and His⁵ of GnRH II interact with Tyr^{290(6.58)}, they interact with different rotamer conformations of the Tyr^{290(6.58)} side chain. This is also supported by our previous result that substitution of Tyr⁵ of GnRH I with His⁵ leads to a 3-fold ($p < 0.01$) increase in receptor binding affinity (5). This subtle difference of the intermolecular interactions between the receptor and GnRH I and GnRH II may, together with the distinct intermolecular interactions made by Arg⁸ of GnRH I and Tyr⁸ GnRH II (5), underlie LiSS and supports our hypothesis that GnRH I and GnRH II stabilize different receptor active conformations (4, 5, 19).

The relative expression of the Tyr^{290(6.58)} mutant human GnRH receptors was calculated from the B_0 values in ¹²⁵I-Cetorelix competition binding assays on intact cells, as Cetorelix showed unchanged affinity for the mutant receptors (Figure 1). The mutation Y290A had no effect on receptor expression, while Y290F and Y290L decreased, but Y290Q increased, the mutant receptor expression on cell surfaces (Figure 1 and Table 3). This also indicates that both the *para*-OH and aromatic ring are involved in the proper packing of the Tyr^{290(6.58)} side chain and substitution of Tyr^{290(6.58)} with Phe, Leu and Gln is able to induce subtle and differential receptor conformational changes, also supporting the above hypothesis that the subtle difference of the interactions between Tyr^{290(6.58)} and Tyr⁵ of GnRH I and His⁵ of GnRH II may participate in ligand-induced receptor conformational selection and LiSS.

Our results, showing that mutations of Tyr^{290(6.58)} had no effect on receptor affinity for Cetorelix, indicate that it is unlikely that Tyr⁵ of Cetorelix interacts with Tyr^{290(6.58)}. Mutations of the GnRH receptor which affect peptide agonist binding affinity (14, 33, 34) often have little effect on the affinity of peptide antagonists, suggesting that the peptide antagonists, which possess bulky hydrophobic D-amino acids at the N-terminal domain and a D-amino acid at position 6 (1), have a different receptor binding mode from that of the native GnRH. A similar phenomenon was also observed in other peptide GPCRs (35). Similarly, substitution of Gly⁶ in GnRH I with a D-amino acid (e.g., [D-Trp⁶]GnRH) is thought to alter peptide conformations (36) and thus receptor binding mode, and therefore mutations of Tyr^{290(6.58)} (Figure 2) and other residues (26, 37, 38) of the GnRH receptor, which caused large decreases in mutant receptor affinity for GnRH I, often had a much smaller effect on [His⁵,D-Tyr⁶]GnRH I binding affinity (Figure 2). The central residues (Tyr⁵-Gly⁶-Leu⁷-Arg⁸) of GnRH I are thought to be involved in the formation of a β II'-turn conformation of the peptide (36). Substitution of Gly⁶ with a D-amino acid stabilizes the β II'-turn conformation and thus increases the receptor binding affinity (1). In addition to having this effect, the D-Tyr⁶ side chain of [His⁵,D-Tyr⁶]GnRH I may make an intermolecular

interaction with a receptor residue, thereby compensating for the loss of the interaction between Tyr⁵ of GnRH I and Tyr^{290(6.58)} of the receptor caused by Tyr^{290(6.58)} mutations. These results also indicate that the Tyr^{290(6.58)} mutations result in little disruption of the global structure of the receptor.

Measurements of GnRH-elicited functional responses showed that the Y290F mutant gave markedly decreased potencies in stimulating IP accumulation, with increased EC₅₀ values for GnRH I and GnRH II by 57- and 26-fold (Table 3). This may be partially explained by the decreased receptor binding affinity and lower expression level. Nevertheless, the Y290F mutant was able to evoke an E_{\max} near the wild-type receptor for both GnRH I and GnRH II, despite its expression level being less than one-third of the wild-type. This shows that the Y290F mutant was well coupled to intracellular signaling via G_{q/11}. In contrast, the Y290A mutant receptor, which was expressed at the wild-type level, exhibited larger decreases in potency than affinity for GnRH I (5.8-fold, $p < 0.01$) (Tables 1 and 3), GnRH II (2.5-fold, $p < 0.01$) (Tables 2 and 3) and [His⁵,D-Tyr⁶]GnRH I (10-fold) (Figure 2 and 3) and maximum IP responses were also reduced to less than 88% that of the wild-type. We propose that Tyr^{290(6.58)} of the GnRH receptor is important for GnRH-induced receptor activation, and thus the interactions between Tyr^{290(6.58)} and Tyr⁵ of GnRH I and His⁵ of GnRH II may be involved in triggering receptor conformational switch/selection via "ligand-induced fit" (5), a process that has also been observed in other GPCRs such as the β_2 -adrenergic receptor (39). Consistent with this, we previously demonstrated that certain residues, located at the intracellular segment of the TM 6, make intramolecular interactions that stabilize the receptor in the inactive state and are broken during ligand-induced receptor conformational changes (5, 19). Interestingly, the mutation Y290L appears to be more detrimental to the functional responses for GnRH I compared with GnRH II. This suggests that substitution of Tyr^{290(6.58)} with Leu, a smaller nonpolar amino acid lacking aromatic and H-bonding capabilities, may create a new intramolecular interaction which alters the mutant receptor conformation, supported by the decreased receptor expression level (5), which becomes less efficient for GnRH I to trigger receptor activation in comparison with GnRH II (Table 3). In addition, Y290L showed a 2-fold higher affinity for GnRH II in comparison with Y290A (Table 2), suggesting that the side chain of Y290L mutant interacts with His⁵ of GnRH II to some extent. These data further support the prediction of our molecular models (Figure 4), which suggests that Tyr⁵ of GnRH I and His⁵ of GnRH II interact with different rotamer conformations of Tyr^{290(6.58)} and thus influence receptor conformational selection (40).

In summary, we have shown that the *para*-OH group and the aromatic ring of Tyr^{290(6.58)} of the human GnRH receptor are important for binding of both GnRH I and GnRH II. Our studies using molecular modeling and site-directed mutagenesis, in combination with position 5-modified GnRH I and GnRH II analogues, suggest that the side chain of Tyr^{290(6.58)} of the human GnRH receptor interacts directly with Tyr⁵ of GnRH I and His⁵ of GnRH II, and these interactions are essential for high affinity binding. We also show that the side chain of Tyr^{290(6.58)} of the human GnRH receptor is important for the switch between the inactive and activated states and may be involved in ligand-induced

receptor conformational selection. Together with the results obtained in other GPCRs (15–17), we suggest that the residues at position 6.58 may have a common role in peptide GPCR ligand binding and receptor activation.

ACKNOWLEDGMENT

We thank Robin Sellar for preparation of ^{125}I -labeled ligands and Laura Melville for technical support.

REFERENCES

1. Millar, R. P., Lu, Z. L., Pawson, A. J., Flanagan, C. A., Morgan, K., and Maudsley, S. R. (2004) Gonadotropin-releasing hormone receptors. *Endocr. Rev.* 25, 235–275.
2. Casper, R. F. (1991) Clinical uses of gonadotropin-releasing hormone analogs. *Can. Med. Assoc. J.* 144, 153–158.
3. Conn, P. M., and Crowley, W. F., Jr. (1994) Gonadotropin-releasing hormone and its analogs. *Annu. Rev. Med.* 45, 391–405.
4. Millar, R. P., Pawson, A. J., Morgan, K., Rissman, E. F., and Lu, Z. L. (2008) Diversity of actions of GnRHs mediated by ligand-induced selective signaling. *Front. Neuroendocrinol.* 29, 17–35.
5. Lu, Z. L., Gallagher, R., Sellar, R., Coetsee, M., and Millar, R. P. (2005) Mutations remote from the human gonadotropin-releasing hormone (GnRH) receptor binding sites specifically increase binding affinity for GnRH II, but not GnRH I: Evidence for ligand-selective receptor active conformations. *J. Biol. Chem.* 280, 29796–29803.
6. Costanzi, S. (2008) On the applicability of GPCR homology models to computer-aided drug discovery: A comparison between *in silico* and crystal structures of the beta2-adrenergic receptor. *J. Med. Chem.* 51, 2907–2914.
7. Ballesteros, J. A., Shi, L., and Javitch, J. A. (2001) Structural mimicry in G protein-coupled receptors: Implications of the high-resolution structure of rhodopsin for structure-function analysis of rhodopsin-like receptors. *Mol. Pharmacol.* 60, 1–19.
8. Lu, Z. L., Saldanha, J. W., and Hulme, E. C. (2002) Seven-transmembrane receptors: Crystals clarify. *Trends Pharmacol. Sci.* 23, 140–146.
9. Shi, L., and Javitch, J. A. (2002) The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu. Rev. Pharmacol. Toxicol.* 42, 437–467.
10. Schwartz, T. W., Frimurer, T. M., Holst, B., Rosenkilde, M. M., and Elling, C. E. (2006) Molecular mechanism of 7TM receptor activation—a global toggle switch model. *Annu. Rev. Pharmacol. Toxicol.* 46, 481–519.
11. Rosenbaum, D. M., Cherezov, V., Hanson, M. A., Rasmussen, S. G. F., Thian, F. S., Kobilka, T. S., Choi, H. J., Yao, X. J., Weis, W. I., Stevens, R. C., and Kobilka, B. K. (2007) GPCR engineering yields high-resolution structural insights into β -adrenergic receptor function. *Science* 318, 1266–1273.
12. Li, J. H., Hamdan, F. F., Kim, S.-K., Jacobson, K. A., Zhang, X., Han, S.-J., and Wess, J. (2008) Ligand-specific changes in M₃ muscarinic acetylcholine receptor structure detected by a disulfide scanning strategy. *Biochemistry* 47, 2776–2788.
13. Hovellmann, S., Hoffmann, S. H., Kuhne, R., ter Laak, T., Reilander, H., and Beckers, T. (2002) Impact of aromatic residues within transmembrane helix 6 of the human gonadotropin-releasing hormone receptor upon agonist and antagonist binding. *Biochemistry* 41, 1129–1136.
14. Stewart, A. J., Sellar, R., Wilson, D. J., Millar, R. P., and Lu, Z. L. (2008) Identification of a novel ligand binding residue Arg^{38(1.35)} in the human gonadotropin-releasing hormone receptor. *Mol. Pharmacol.* 73, 75–81.
15. Bhogal, N., Donnelly, D., and Findlay, J. B. (1994) The ligand binding site of the neurokinin 2 receptor: Site-directed mutagenesis and identification of neurokinin A binding residues in the human neurokinin 2 receptor. *J. Biol. Chem.* 269, 27269–27274.
16. Yamano, Y., Ohyama, K., Kikyo, M., Sano, T., Nakagomi, Y., Inoue, Y., Nakamura, N., Morishima, I., Guo, D. F., and Hamakubo, T. (1995) Mutagenesis and the molecular modeling of the rat angiotensin II receptor (AT1). *J. Biol. Chem.* 270, 14024–14030.
17. Leong, S. R., Kabakoff, R. C., and Hebert, C. A. (1994) Complete mutagenesis of the extracellular domain of interleukin-8 (IL-8) type A receptor identifies charged residues mediating IL-8 binding and signal transduction. *J. Biol. Chem.* 269, 19343–19348.
18. Chauvin, S., Berauld, A., Lerrant, Y., Hibert, M., and Counis, R. (2000) Functional importance of transmembrane helix 6 Trp(279) and exolooop 3 Val(299) of rat gonadotropin-releasing hormone receptor. *Mol. Pharmacol.* 57, 625–633.
19. Lu, Z. L., Coetsee, M., White, C. D., and Millar, R. P. (2007) Structural determinants for ligand-receptor conformational selection in a peptide G protein-coupled receptor. *J. Biol. Chem.* 282, 17921–17929.
20. Coetsee, M., Gallagher, R., Millar, R. P., Flanagan, C. A., and Lu, Z. L. (2006) Role of Trp280(6.48) in the gonadotropin-releasing hormone (GnRH) receptor. *BioScience* 2006, Glasgow, U.K., July 23–27, 2006, Abstract 0518, The Biochemical Society, London, U.K.
21. Flanagan, C. A., Rodic, V., Konvicka, K., Yuen, T., Chi, L., Rivier, J. E., Millar, R. P., Weinstein, H., and Sealfon, S. C. (2000) Multiple interactions of the Asp^{2.61(98)} side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction. *Biochemistry* 39, 8133–8141.
22. Lu, Z. L., Curtis, C. A., Jones, P. G., Pavia, J., and Hulme, E. C. (1997) The role of the aspartate-arginine tyrosine triad in the M₁ muscarinic receptor: mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signaling. *Mol. Pharmacol.* 51, 234–241.
23. Salom, D., Lodowski, D. T., Stenkamp, R. E., Le Trong, I., Golczak, M., Jastrzebska, B., Harris, T., Ballesteros, J. A., and Palczewski, K. (2006) Crystal structure of a photoactivated deprotonated intermediate of rhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16123–16128.
24. Mamputha, S., Lu, Z. L., Roeske, R. W., Millar, R. P., Katz, A. A., and Flanagan, C. A. (2007) Conserved amino acid residues that are important for ligand binding in the type I gonadotropin-releasing hormone (GnRH) receptor are required for high potency of GnRH II at the type II GnRH receptor. *Mol. Endocrinol.* 21, 281–292.
25. Hoffmann, S. H., ter Laak, T. T., Kuhne, R., Reilander, H., and Beckers, T. (2000) Residues within transmembrane helices 2 and 5 of the human gonadotropin-releasing hormone receptor contribute to agonist and antagonist binding. *Mol. Endocrinol.* 14, 1099–1115.
26. Davidson, J. S., McArdle, C. A., Davies, P. D., Elario, R., Flanagan, C. A., and Millar, R. P. (1996) Asn102 of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycineamide. *J. Biol. Chem.* 271, 15510–15514.
27. Brooks, B. R., Bruccoleri, R. E., Olason, B. D., States, D. J., Swaminathan, S., and Karplus, M. (1983) CHARMM: A program for macromolecular energy minimization and dynamics calculations. *J. Comput. Chem.* 4, 187–217.
28. Favre, N., Fanelli, F., Missotten, M., Nichols, A., Wilson, J., di Tiani, M., Rommel, C., and Scheer, A. (2005) The DRY motif as a molecular switch of the human oxytocin receptor. *Biochemistry* 44, 9990–10008.
29. Gervasio, F. L., Chelli, R., Procacci, P., and Schettino, V. (2002) The nature of intermolecular interactions between aromatic amino acid residues. *Proteins: Struct., Funct., Bioinformatics* 48, 117–125.
30. Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M., and Winter, G. (1985) Hydrogen bonding and biological specificity analysed by protein engineering. *Nature* 314, 235–238.
31. Meurisse, R., Brasseur, R., and Thomas, A. (2004) Aromatic side-chain interactions in proteins: Near- and far-sequence Tyr-X pairs. *Proteins: Structure, Function, and Bioinf.* 54, 478–490.
32. McGaughey, G. B., Gagne, M., and Rappe, A. K. (1998) π -stacking interactions: Alive and well in proteins. *J. Biol. Chem.* 273, 15458–15463.
33. Zhou, W., Rodic, V., Kitanovic, S., Flanagan, C. A., Chi, L., Weinstein, H., Maayani, S., Millar, R. P., and Sealfon, S. C. (1995) A locus of the gonadotropin-releasing hormone receptor which differentiates agonist and antagonist binding sites. *J. Biol. Chem.* 270, 18853–18857.
34. Fromme, B. J., Katz, A. A., Roeske, R. W., Millar, R. P., and Flanagan, C. A. (2001) Role of aspartate7.32(302) of the human gonadotropin-releasing hormone receptor in stabilizing a high-affinity ligand conformation. *Mol. Pharmacol.* 60, 1280–1287.
35. Hawtin, S. R., Wesley, V. J., Simms, J., Argent, C. C., Latif, K., and Wheatley, M. (2005) The N-terminal juxtamembrane segment of the V1a vasopressin receptor provides two independent epitopes required for high-affinity agonist binding and signaling. *Mol. Endocrinol.* 19, 2871–2881.

36. Sealfon, S. C., Weinstein, H., and Millar, R. P. (1997) Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocr. Rev.* 18, 180–205.
37. Flanagan, C. A., Becker, I. I., Davidson, J. S., Wakefield, I. K., Zhou, W., Sealfon, S. C., and Millar, R. P. (1994) Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for Arginine 8 of mammalian gonadotropin-releasing hormone. *J. Biol. Chem.* 269, 22636–22641.
38. Betz, S. F., Reinhart, G. J., Lio, F. M., Chen, C., and Struthers, R. S. (2006) Overlapping, nonidentical binding sites of different classes of nonpeptide antagonists for the human gonadotropin-releasing hormone receptor. *J. Med. Chem.* 49, 637–647.
39. Swaminath, G., Xiang, Y., Lee, T. W., Steenhuis, J., Parnot, C., and Kobilka, B. K. (2004) Sequential binding of agonists to the β_2 adrenoceptor: Kinetic evidence for intermediate conformational states. *J. Biol. Chem.* 279, 686–691.
40. Kenakin, T. (1997) Agonist-specific receptor conformations. *Trends Pharmacol. Sci.* 18, 416–417.

BI800911Z