Allosteric and Orthosteric Binding Modes of Two Nonpeptide Human Gonadotropin-Releasing Hormone Receptor Antagonists

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ABSTRACT: Nonpeptide antagonists of the human gonadotropin-releasing hormone receptor (GnRH-R) have been the subject of considerable interest because of their potential as a new class of oral therapeutics for the treatment of sex hormone-dependent diseases and infertility. While many classes of competitive GnRH-R antagonists have been described, we present here the first characterization of an allosteric nonpeptide GnRH-R antagonist. Previously, 5-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-ylmethyl)furan-2-carboxylic acid (2,4,6-trimethoxyphenyl)amide (here called Furan-1) had been demonstrated to be a potent GnRH-R antagonist both in vitro and in vivo. Using mutagenesis, the binding sites for Furan-1 and another potent nonpeptide antagonist (NBI-42902) have been mapped and are shown to be adjacent but nonoverlapping. Furan-1 is shown to affect the binding kinetics of radiolabeled peptide agonists as well as radiolabeled NBI-42902, and the kinetic data fit the allosteric ternary complex model. Furan-1 is considerably negatively cooperative with the nonpeptide antagonist and extremely negatively cooperative with the peptide agonist [^{125}I-His5,D-Tyr6]GnRH so that it is nearly indistinguishable from an orthosteric competitive compound. Taken together, these data were used to develop a model of the nonpeptides bound to the GnRH-R binding site consistent with the current data.

Gonadotropin-releasing hormone (GnRH,[1] also known as luteinizing hormone-releasing hormone or LH-RH) is a linear decapeptide secreted from the hypothalamus into the hypophyseal portal circulation. GnRH binds to and activates the GnRH receptor (GnRH-R) on gonadotropes located in the anterior pituitary. GnRH-R activation stimulates the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which bind to gonadal receptors, stimulating the synthesis of the sex steroid hormones estrogen and testosterone (*1*, *2*). GnRH-R is a seven-transmembrane domain G protein-coupled receptor (GPCR) belonging to the class A family of GPCRs (*3*). GnRH-R signals predominantly through the GTP binding proteins $G_{\alpha q}$ and $G_{\alpha 11}$, stimulating phosphatidylinositol turnover by phospholipase C, mobilization of Ca^{2+}, diacylglycerol formation, protein kinase C activation, and arachidonic acid release (*4*).

Gonadal suppression can be achieved with GnRH-R agonist peptide therapy, a consequence of desensitization and downregulation of pituitary GnRH-Rs (*5*, *6*). Accordingly, peptidyl GnRH-R agonists have been used therapeutically to modulate the reproductive endocrine axis in a variety of disorders, including precocious puberty, endometriosis, prostate cancer, uterine fibroids, breast cancer, and fertility disorders (*5*, *7*−*13*). Although GnRH agonist peptides have proven to be clinically effective, there are important drawbacks to this approach, including initial pituitary stimulation and subsequent increases in LH, FSH, and sex steroids prior to GnRH-R downregulation (*6*, *14*). This hormonal "flare" can be prevented by using peptide antagonists (e.g., abarelix and cetrorelix) which bind pituitary GnRH-Rs without stimulating FSH and LH release (*12*, *15*−*18*). However, agonist and antagonist peptides require parenteral administration typically employing depot formulation. Consequently, considerable effort has been directed toward development of orally active nonpeptide GnRH-R antagonists (*19*, *20*). Using medicinal chemistry, mutagenesis, and molecular modeling, a number of antagonists have been identified that are proposed to bind GnRH-R at sites that at least partially overlap the binding site of the native peptide ligand and are therefore orthosteric antagonists of human GnRH-R (*3*, *21*, *22*).

The orthosteric binding site for a GPCR has been defined as the site at which the endogenous agonist binds the receptor and initiates signaling (*23*). Most currently marketed GPCR-based therapeutics bind the orthosteric site to regulate receptor activation, as either agonists, inverse agonists, or neutral antagonists (*24*). For several GPCRs, additional binding sites that do not overlap the orthosteric site (allosteric sites) that can modulate orthosteric ligand receptor activity have been identified (*23*).

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[1] Abbreviations: GnRH, gonadotropin-releasing hormone; GnRH-R, gonadotropin-releasing hormone receptor; GPCR, G protein-coupled receptor; LH, luteinizing hormone; FSH, follicle stimulating hormone; ATCM, allosteric ternary complex model; sem, standard error of the mean.

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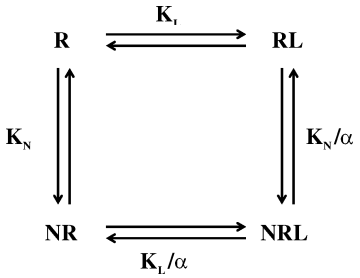

FIGURE 1: Allosteric ternary complex model (ATCM) which describes the interaction among a receptor (R), its ligand (L), and an allosteric modulator (N). RL is the receptor−radioligand complex, NR the receptor−allosteric modulator complex, NRL the receptor−radioligand−allosteric modulator complex, K_L the equilibrium dissociation constant of the ligand, K_N the equilibrium dissociation constant of the allosteric modulator, and α the cooperativity factor. For a competitive interaction, $\alpha = 0$.

The allosteric ternary complex model (ATCM) (Figure 1) is the most basic model used to describe the interaction of the orthosteric ligand, the allosteric ligand, and the receptor (*25*). In this model, the two binding sites are conformationally linked such that binding to one site changes the ligand affinity of the other site (*26*). The change in affinity can result from the alteration of the binding kinetics of the orthosteric ligand in the presence of the allosteric ligand (and vice versa). This cannot occur if the two ligands possess overlapping binding sites which would prevent them from simultaneously binding to the receptor. The binding of the allosteric ligand can affect the affinity of the orthosteric ligand in a positive, negative, or neutral manner. Accordingly, a positive allosteric modulator increases the affinity of the orthosteric ligand for the receptor. A negative allosteric modulator decreases the binding affinity of the othosteric ligand, and a neutral allosteric modulator has no effect on the affinity of the orthosteric ligand for the receptor. Allosteric sites have been characterized well for adenosine A1 (*27*−*30*), muscarinic (*25*, *31*−*35*), calcium-sensing (*36*, *37*), and metabotropic glutamate receptors (*38*−*41*). More recent reports of the modulation of the CB-1 (*42*), CRF-1 (*43*), and CCR-5 (*44*) receptors by allosteric ligands have characterized positive, negative, and neutral allosteric modulators of these receptors. Allosteric ligands that modify the signaling properties of the receptor instead of, or in addition to, affecting orthosteric ligand affinity have also been identified (*31*, *38*, *45*−*47*), and on the basis of these types of data, more complex models of allosteric−receptor interactions have been proposed (*23*, *48*).

As part of an overall effort to understand the interactions of nonpeptide GnRH-R ligands, we report here the first pharmacological characterization of a negative allosteric modulator of human GnRH-R, Furan-1 (*49*) (Figure 2). Furan-1 was derived from a series of compounds that have been shown to be good GnRH-R antagonists, exhibiting nanomolar affinity and efficacy in vitro (*50*, *51*). Furan-1 has also been shown to be orally available and efficacious in rodent models, suppressing GnRH-dependent increases in the LH level in castrated male rats and super-agonist-dependent increases in LH and testosterone levels in gonad-intact male rats and lowering the level of circulating testosterone in gonad-intact male rats (*49*). In addition to it being allosteric to agonist peptide binding, we have determined that Furan-1 is also allosteric with NBI-42902, a

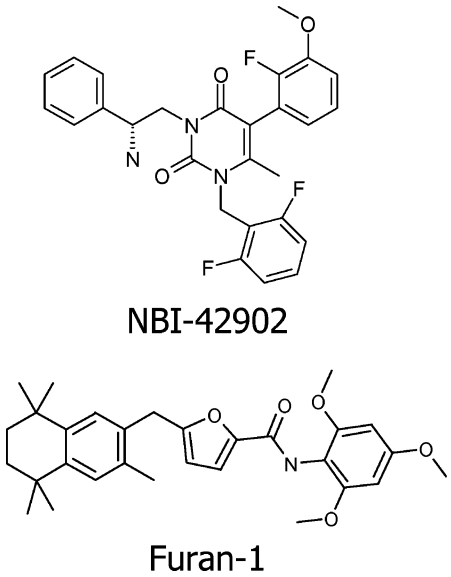

FIGURE 2: Chemical structures of nonpeptide GnRH-R antagonists described in this study.

potent, orthosteric nonpeptide GnRH-R antagonist. NBI-42902 is a highly potent inhibitor of binding of peptide radioligand to human GnRH-R (*52*, *53*), and [^{3}H]NBI-42902 binds with high affinity to a single class of binding sites and can be displaced by a range of peptide and nonpeptide GnRH-R ligands (*54*). In addition to excellent in vitro potency, NBI-42902 has exhibited clinical efficacy in lowering the serum LH level in postmenopausal women using oral administration (*55*). Finally, we determine that these two nonpeptide antagonists possess nonoverlapping binding sites and propose a docked model that satisfies both molecules' simultaneous occupation of the receptor's binding cavity.

EXPERIMENTAL PROCEDURES

Nomenclature. GnRH peptides are named by the residue's three-letter abbreviation, with its sequence position in the peptide superscripted (e.g., His^5 denotes a His substitution at position 5 of the GnRH peptide). Receptor residues are named by the Ballesteros−Weinstein numbering method (*56*), using a residue's one-letter abbreviation and primary sequence position, with its transmembrane helix and number position within that helix based on its relative position to the most evolutionarily conserved residue in that helix superscripted [e.g., H306$^{(7.36)}$]. GnRH-R mutants are denoted as the wild-type residue, residue number, and mutant residue [e.g., H306$^{(7.36)}$A denotes a histidine to alanine mutation at residue 306].

Materials. All chemicals and reagents were purchased from either Sigma Chemical (St. Louis, MO) or Fisher Scientific (Los Angeles, CA) unless otherwise stated. The synthesis of NBI-42902, 1-(2,6-difluorobenzyl)-3-[(2*R*)-amino-2-phenethyl]-5-(2-fluoro-3-methoxyphenyl)-6-methyluracil, is described elsewhere (*52*). Synthesis of Furan-1, 5-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-ylmethyl)furan-2-carboxylic acid (2,4,6-trimethoxyphenyl)-amide, was performed in house and is described elsewhere (*57*). Preparation of [^{3}H]NBI-42902 is described elsewhere (*54*) and was conducted under contract by American Radiolabeled Chemical Inc. (St. Louis, MO). The product was purified by HPLC to a purity of >99%. The final material

had a specific activity of 80 Ci/mmol and was stored at −20 °C until it was used.

[His^5,D-Tyr^6]GnRH (*58*) was synthesized in house by solid state methods. Iodination was done under contract by Perkin-Elmer Life Sciences, using a modification of a chloramine T procedure, and purified by HPLC to a purity of >95%. The final material had a specific acitivity of 2200 Ci/mmol and was stored lyophilized at −80 °C until it was used. Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Dialyzed FBS was from Gibco (Carlsbad, CA). Geneticin (G418 sulfate) was from Cellgo (Herndon, VA). Inositol-free medium was obtained from Specialty Media (Phillipsburg, NJ). Myo-2-[^{3}H]inositol was purchased from Amersham Biosciences (Newark, NJ). UniFilter 350 and UniFilter GF/C plates were both from Whatman (Clifton, NJ); 96-well low binding plates were obtained from Corning (Palo Alto, CA), and Lumaplates were purchased from Perkin-Elmer Life Sciences (Boston, MA). AG1-X8 Dowex Resin was obtained from Bio-Rad (Hercules, CA).

Receptor Cloning and Mutagenesis. Cloning of GnRH-R into RBL-1 and COS-7 cells has been described elsewhere (*21*, *54*, *59*). The wild-type receptor was stably transfected into RBL-1 cells using lipofectamine. Vectors containing cloned mutant and wild-type receptors were transiently transfected into COS-7 cells using electroporation. Both were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μg/mL penicillin/streptomycin, and 250 μg/mL Geneticin (G418 sulfate). Site-directed mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene). All mutant receptors were made in the F272$^{(6.40)}$L background mutant (*21*, *60*). The complete coding region for each mutant receptor was confirmed by DNA sequence analysis (ABI Prism 377 DNA sequencer, Applied Biosystems Inc., Foster City, CA).

Membrane Preparation. Membrane preparation of COS-7 and RBL-1 cells expressing mutant or wild-type GnRH-Rs has been described elsewhere (*21*, *54*). Briefly, cells were harvested from culture, resuspended in tissue buffer [DPBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 138 mM NaCl) supplemented with 10 mM $MgCl_2$ and 2 mM EGTA (pH 7.4)], and transferred to a pressure cell. Nitrogen gas was applied to the cell at 4 °C, and cells were lysed by release of pressure. Unbroken cells and other debris were removed by low-speed centrifugation. The supernatant was centrifuged at 40000*g* for 45 min at 4 °C and the resulting membrane pellet resuspended in tissue buffer using a tissue homogenizer. Protein concentrations were determined with the Coomassie Plus Protein Reagent kit (Pierce, Rockford, IL), and membrane aliquots were stored at −80 °C until they were used. Titration analysis using a range of protein concentrations determined the optimal protein concentration of the GnRH-R in RBL-1 membranes to be 15 and 30 μg per well for [^{125}I-His^5,D-Tyr^6]GnRH and [^{3}H]NBI-42902 assays, respectively.

Radioligand Inhibition Binding Assays. Radioligand binding assays were performed in 96-well filter plates (Multiscreen 1.2 μm glass-fiber plates, Millipore, Bedford, MA). Each assay point consisted of a 100 μL cocktail of cell membrane containing the mutant GnRH-R of interest (5−40 μg), 300 pM [^{125}I-His5,D-Tyr6]GnRH, and varying concentrations of nonpeptide, all prepared in assay buffer [10 mM HEPES (pH 7.45), 150 mM NaCl, and 0.1% bovine serum albumin (fraction V)]. Assay plates were shaken at 100 rpm for 2 h at room temperature and then vacuum filtered. The filter plates were washed twice with PBS and then dried completely. Scintillation fluid (Scint20, Packard Instruments, Downers Grove, IL) was added to each well prior to detection in a TopCount NXT counter (Packard Instruments).

Radioligand Dissociation Assays. Radioligand dissociation assays utilizing [^{125}I-His^5,D-Tyr^6]GnRH were performed in buffer containing 10 mM HEPES, 150 mM NaCl, and 0.1% fraction V BSA. Radioligand dissociation assays utilizing [^{3}H]NBI-42902 were performed in buffer containing 50 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM EDTA (pH 7.5). Radioligands were equilibrated with GnRH-R RBL-1 membranes for 90 min at 21 °C in low binding plates, by which time the radioligand had reached steady state binding. Dissociation was initiated by the addition of an excess of unlabeled [His^5,D-Tyr^6]GnRH or NBI-42902 (final concentration of 10 μM) in a miminal volume in the presence or absence of various concentrations of Furan-1 at final concentrations ranging from 100 nM to 3 μM. The assay was terminated by rapid vacuum filtration onto Unifilter GF/C filter plates at time points ranging from 0 min to 5 h. GF/C filter plates were pretreated with a solution of 0.5% polyethyleneimine in distilled water for 30 min. Plates used for [^{125}I-His^5,D-Tyr^6]GnRH binding were additionally rinsed with a 1% BSA/PBS solution. After filtration, membranes were washed twice with 250 μL of ice-cold buffer [PBS and 0.01% Tween 20 (pH 7.4)]. Filter plates were air-dried; 50 μL of scintillation fluid (Microscint 20, Packard) was added, and the plate was monitored for radioactivity using a TopCount NXT device. The total amount of radioligand added to the assay was measured using a Perkin-Elmer Life Sciences 1600TR liquid scintillation counter for [^{3}H]NBI-42902 and a Cobra II gamma counter for [^{125}I-His^5,D-Tyr^6]-GnRH. Nonspecific binding was assessed in the presence of 1 μM unlabeled [His^5,D-Tyr^6]GnRH or NBI-42902. In each set of experiments, total binding over time was monitored to ensure assay viability. In both assay systems, total binding was stable for the duration of the experiment. The total binding of radioligand did not exceed 10% of the radioligand added to the assays. The nonspecific binding accounted for 5% of the total binding in the [^{125}I-His^5,D-Tyr^6]GnRH assays and 25% of the total binding in the [^{3}H]-NBI-42902 assays. Specific binding was calculated by subtracting the nonspecific binding from the total binding of radioligand at each time point for each concentration of Furan-1. Duplicate determinations were made for each time point.

Radioligand Binding Saturation Assays. Radioligand saturation binding experiments were performed by adding GnRH-R membranes to low protein binding 96-well pates containing varying concentrations of [^{125}I-His^5,D-Tyr^6]GnRH or [^{3}H]NBI-42902 ranging from 50 pM to 3 nM in the absence or presence of 1 μM [His^5,D-Tyr^6]GnRH or 10 μM NBI-42902 (to define nonspecific binding), or varying concentrations of Furan-1 ranging from 3.16 nM to 1 μM. The assay mixture was incubated for 4 h at 21 °C, and the experiment was terminated by rapid vacuum filtration onto GF/C filter plates. The total amount of radioligand added to

the assay was measured using the same technique that was employed for the dissociation binding experiments (see above).

Inositol Phosphate (IP) Accumulation Assays. Inositol phosphate accumulation was assessed by a modified version of published protocols (*54*, *61*). Briefly, GnRH-R RBL-1 cells were seeded into 96-well plates in inositol-free DMEM containing 10% dialyzed FBS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μg/mL penicillin/streptomycin and labeled for 48 h with 0.2 μCi of myo-2-[^{3}H]inositol. The cells were washed once in assay buffer containing 140 mM NaCl, 4 mM KCl, 20 mM HEPES, 8.3 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM LiCl, and 0.1% BSA. Cells were incubated with native GnRH, [His^5,D-Tyr^6]GnRH, or [D-Ala^6,proethylamide^9,des-Gly^{10}]GnRH (i.e., leuprolide) at concentrations ranging from 31.6 pM to 10 μM in the presence or absence of Furan-1 at seven concentrations ranging from 31.6 nM to 31.6 μM for 1 h. Cells were extracted with 10 mM formic acid at 4 °C for 30 min, and the lysate was loaded onto Whatman plates containing 20 μg of Dowex AG1-X8 resin. The plate was washed once in H_2O and once in a 60 mM ammonium formate/5 mM sodium tetraborate solution, and inositol phosphates eluted with a 1 M ammonium formate/0.1 M formic acid solution. The eluate was transferred to a Lumaplate, dried at 37 °C, and counted in a TopCount NXT device.

Data and Statistical Analysis. In all radioligand binding assays, specific binding was calculated by subtracting the nonspecific binding from the total binding. IC_{50} values from inhibition binding experiments were calculated using a three-parameter logistic equation in GraphPad Prism (version 4.01, GraphPad, San Diego, CA). Each experiment was performed at least three times. For dissociation experiments, the noncompeted control minus the nonspecific binding was set to 100% specific binding at each time point. Radioligand dissociation data were fit to one- and two-phase exponential dissociation equations using Prism 4.01 with the plateau of the specific binding set to zero, and the best fit was determined using a partial F-test. In all cases, using either [^{125}I-His^5,D-Tyr^6]GnRH or [^{3}H]NBI-42902, a one-phase equation provided the best fit. A one-way analysis of variance followed by a Dunnett's post test was used to determine if the dissociation rate constants and dissociation half-life in the presence of Furan-1 were statistically different from the dissociation rate constant and the dissociation half-life of the radioligand alone. The dissociation rate constant of each radioligand (k_{off}) was determined using the equation

$$Y = \text{span} \times e^{-k_{off}x} + \text{plateau} \quad (1)$$

where Y is the total binding, x the time in minutes, Span the difference between the binding at time zero and the plateau, and k_{off} the dissociation rate constant. The plateau was set to zero. For the determination of the half-life ($t_{1/2}$) of dissociation, the following equation was employed:

$$t_{1/2} = \frac{0.693}{k_{off}} \quad (2)$$

Radioligand saturation data were analyzed by adjusting for ligand depletion, as previously described (*43*). Initial saturation binding data of each radioligand in the absence of Furan-1 were fit to one- and two-site saturation equations, and the best fit was determined using a partial F-test. A single-site binding model fit best for all saturation binding experiments.

Specific binding of the radioligand in the presence of multiple concentrations of Furan-1 was globally fit to the following equation to determine the K_d of the radioligand (K_L), the K_d of the allosteric modulator (K_N), and the cooperativity factor (α) (*25*).

$$[RL] + [NRL] = R_{total}\frac{[L]\frac{1}{K_L}\left(1 + \alpha[N]\frac{1}{K_N}\right)}{1 + [L]\frac{1}{K_L}\left(1 + \alpha[N]\frac{1}{K_N}\right) + [N]\frac{1}{K_N}} \quad (3)$$

where [RL] is the receptor−radioligand complex concentration, [NRL] the receptor−radioligand−allosteric modulator complex concentration, R_{total} the total receptor concentration, [L] the concentration of free radioligand, K_L the equilibrium dissociation constant of the radioligand, N the concentration of Furan-1, K_N the equilibrium dissociation constant of Furan-1, and α the cooperativity factor. For a competitive interaction, $\alpha = 0$.

Data were globally fit to both competitive ($\alpha = 0$) and allosteric ($\alpha > 0$) models of interaction with the receptor using Prism 4.01, and the best fit was determined using a partial F-test. For [^{3}H]NBI-42902 binding in the presence of Furan-1, an allosteric model fit best for all experiments. For [^{125}I-His^5,D-Tyr^6]GnRH binding in the presence of Furan-1, a competitive model fit best for all experiments. Computer simulation of the ATCM (Figure 1) determined that for the range of radioligand concentrations used in our assay, the limit of detection of α for Furan-1 using the [^{125}I-His^5,D-Tyr^6]GnRH radioligand is an α of 0.005. Values of α of <0.005 cannot be distinguished from a competitive binding model. Error calculations on model parameters were performed using logarithmic data, as these parameters are normally distributed. Scatchard transformation of the global fit of the data was performed by plotting the ratio of the bound radioligand over the free radioligand concentration versus the bound radioligand. The resultant data were plotted and fit to a standard linear regression.

For agonist IP accumulation assays, data were analyzed using a three-parameter logistic equation with slope values fixed to 1. The log of the concentration of compound was plotted against the log of the dose response minus one [log(DR − 1)], and the data were analyzed by linear regression to obtain the x-intercept (pA_2 value). The slope of the linear regression ranged from 0.92 to 1.1. The r^2 for the linear regression fits was >0.95 for all data sets. Data are the mean of at least four independent experiments.

Molecular Modeling. The model of GnRH-R was built as previously described (*21*). Diverse docking poses with various low-energy conformations of NBI-42902 within the binding site were generated using the Triangle matcher method in MOE (Chemical Computing Group, Montreal, PQ) with the MMFF94x force field (*62*, *63*). The receptor was rigid, and NBI-42902 was allowed to be flexible. Seventy-eight diverse docking solutions were generated. These

solutions were manually assessed for those that were consistent with the experimental mutagenesis constraints.

The entire system (excluding the exterior lipid-spanning region) was then solvated with explicit water. The whole system was minimized with all atoms fixed except water hydrogen followed by a 5 ps MD run at 400 K and minimization to reorient the water hydrogens. All water atoms were then unfixed and equilibrated with another 5 ps MD trajectory at 400 K. A wall function was applied to the water molecules (weight of 20) to prevent them from "escaping" during simulation. MD simulation was carried out with transmembrane helix backbone atoms and phenylglycine side chain weakly tethered ($K_1 = 5$), as were conserved residue side chains ($K_2 = 1$) using the MMFF94x force field at 400 K for 75 ps followed by simulated annealing at 300, 200, and 100 K and minimization. The MD trajectory was then repeated with only weak tethering ($K_1 = 5$) of the transmembrane helix backbone atoms with all other atoms free.

To introduce Furan-1, a stochastic conformation search was performed; the lowest-energy conformation of Furan-1 was then manually placed into the receptor−NBI-42902 complex with the cyclohexylphenyl side chain toward F313$^{(7.43)}$ and the trimethoxylphenyl group toward H306$^{(7.36)}$. The system was then fixed (except H_2O protons) followed by short MD (3 ps) and minimization. Finally, all atoms in the system other than 7TM backbone atoms (weight of 5) were allowed to move freely; routine MD simulation was carried out for 150 ps at 400 K before simulated annealing and minimization to produce the final model with NBI-42902 and Furan-1 complexed.

RESULTS

Mutagenesis Data. The interaction of Furan-1 with GnRH-R was probed using site-directed mutagenesis. The ability of Furan-1 to inhibit the binding of [^{125}I-His^5,D-Tyr^6]GnRH was tested in membranes expressing the wild-type receptor and a panel of mutants. Several residues critical for the binding of NBI-42902 have been described previously (*21*). The mutant receptors tested here have all been previously shown to have little to no impact on the binding of [His^5,D-Tyr^6]-GnRH to GnRH-R. IC_{50} values for the wild-type receptor and comparison of the fold change values [IC_{50}(mutant)/IC_{50}-(wild-type)] for different mutants of both Furan-1 and NBI-42902 are presented in Table 1.

Previously published analyses comparing the binding of nonpeptide GnRH-R antagonists have centered on compounds that share binding sites that at least partially overlap with the GnRH peptide binding site and those of each other (*3*, *21*, *22*). A comparison of the ability of different mutants to affect the binding of Furan-1 and NBI-42902 indicated that these two compounds were not affected by any mutations in common, with the possible exception of F313L$^{(7.43)}$. That mutation, which is located deep within the binding pocket of GnRH-R, causes low-level changes in the binding of several molecules which is consistent with a structural rearrangement that could cause an indirect effect on ligand binding (*21*). This mutation, however, greatly affects the binding of Furan-1 (>100-fold change) while slightly affecting the binding of NBI-42902. The effect on Furan-1 is on a scale that is most readily interpreted as being a direct interaction.

Table 1: Fold Changes in IC_{50} Values for Furan-1 and NBI-42902 from Inhibition Binding Experiments on GnRH-R-Containing COS-7 Membranes[a]

residue	location	Furan-1	NBI-42902
	NBI-42902 Selective Mutations		
M24I	N-terminus	1.3	2900
S118N	3.29	0.8	3500
Q208E	5.35	1.8	6.2
Y283F	6.51	2.0	9.6
Y284L	6.52	4.8	85
Y290L	6.58	0.8	110
Y290Q	6.58	1.1	310
L300A	6.68/ECL3	0.8	21
L300K	6.68/ECL3	0.3	55
L300V	6.68/ECL3	1.4	7.0
	Neutral Mutations		
N27E	N-terminus	2.0	5.2
L112A	3.23	1.6	1.0
S203P	5.30	2.5	4.4
W291F	6.59	1.0	2.2
D293A	6.61	1.2	2.2
E295A	6.63/ECL3	1.5	0.8
D302A	7.32	0.6	1.2
D302N	7.32	1.0	1.8
	Furan-1 Selective Mutations		
H306A	7.36	52	0.7
H306E	7.36	112	1.8
F309L	7.39	21	2.0
F309Q	7.39	72	4.6
F313L	7.43	182	12

[a] The IC_{50} values for wild-type Furan-1 and NBI-42902 are 14.8 and 1.45 nM, respectively. Fold change is defined as IC_{50}(mut)/IC_{50}(wt). N-terminus refers to the extracellular N-terminal domain that precedes the transmembrane region.

The binding site of NBI-42902 bridges several regions of the receptor, including the N-terminal extension, TM3, TM5, and TM6 (*21*). The binding site of Furan-1 is different, localized primarily along one face of TM7, interacting with residues spaced half-helical turns apart: H306$^{(7.36)}$, F309$^{(7.39)}$, and F313$^{(7.43)}$. A schematic indicating the relative positions of the two binding sites is depicted in Figure 3. This lack of overlap suggests that these compounds could occupy distinct binding sites within the receptor's binding pocket. To investigate this hypothesis more thoroughly, the effect of Furan-1 on the kinetics of radiolabeled peptide and nonpeptide binding was investigated.

Dissociation Kinetic Assays. Dissociation kinetics experiments with [^{125}I-His^5,D-Tyr^6]GnRH and [^{3}H]NBI-42902 from GnRH-R were performed in the presence of increasing concentrations of Furan-1 (Figure 4 and Table 2) in membranes from stably transfected RBL-1 cells. Furan-1 increased the dissociation rate constant of both [^{125}I-His^5,D-Tyr^6]GnRH and [^{3}H]NBI-42902 in a dose-dependent manner, indicative of allosteric binding of Furan-1 and negative modulation of the receptor. The dissociation rate constant of [^{125}I-His^5,D-Tyr^6]GnRH was statistically significantly increased from 0.0061 ± 0.0001 min^{-1} ($t_{1/2} = 113$ min) to 0.0200 ± 0.0040 min^{-1} ($t_{1/2} = 35$ min) ($p < 0.01$) with the addition of 3 μM Furan-1. Similarly, the dissociation rate constant of [^{3}H]NBI-42902 was statistically significantly increased from 0.0020 ± 0.0003 min^{-1} ($t_{1/2} = 369$ min) to 0.0088 ± 0.0008 min^{-1} ($t_{1/2} = 81$ min) ($p < 0.01$), a 4.4-fold increase under the same conditions. All dissociation rate constants were single-phase constants, indicating a single

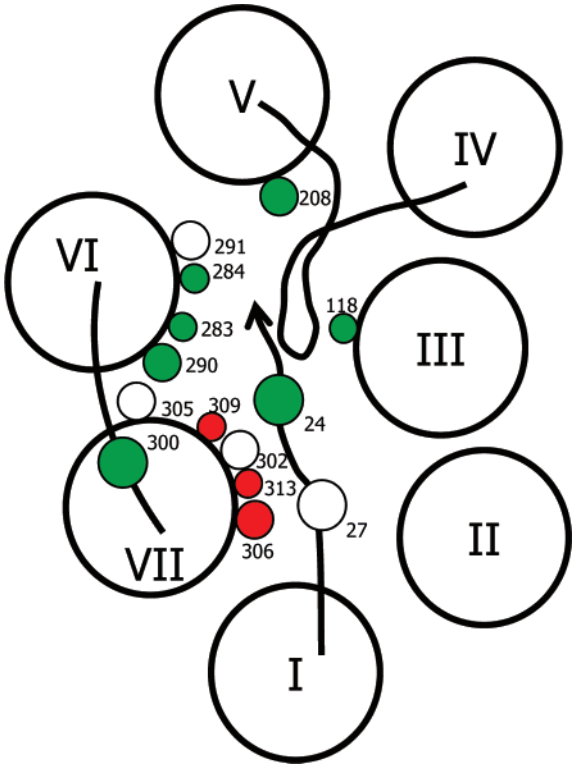

FIGURE 3: Comparison of the binding motifs of Furan-1 and NBI-42902. The schematic represents a projection of the structure of GnRH-R. Large circles with Roman numerals represent GnRH-R transmembrane helices. ECL1 is omitted, and the N-terminal extension is truncated for clarity. The positions of the N-terminal extension and ECL2 are approximate and based on the locations of disulfide linkages. Small circles indicate residue positions, and the diameter of the circle roughly corresponds to the proximity to the extracellular environment. Residues important for the binding of NBI-42902 are colored green: M24, S118$^{(3.29)}$, Q208$^{(5.35)}$, Y283$^{(6.51)}$, Y284$^{(6.52)}$, Y290$^{(6.58)}$, L300$^{(6.68)}$. Residues important for the binding of Furan-1 are colored red: H306$^{(7.36)}$, F309$^{(7.39)}$, and F313$^{(7.43)}$. Residues that did not affect the binding of either compound are colored white.

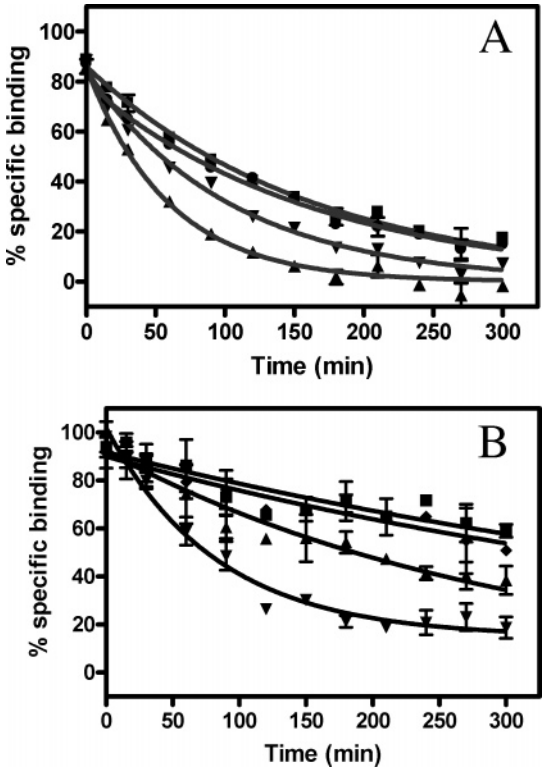

FIGURE 4: Dissociation of (A) [^{125}I-His^5,D-Tyr^6]GnRH and (B) [^{3}H]-NBI-42902 in the presence of increasing concentrations of Furan-1: (■) 0 M, (●) 100 nM, (◆) 300 nM, (▼) 1 μM, and (▲) 3 μM.

binding site for each of the radioligands and Furan-1 at the receptor.

Saturation Binding Assays. The level of cooperativity between Furan-1 and both [^{125}I-His^5,D-Tyr^6]GnRH and [^{3}H]-NBI-42902 was determined using saturation binding studies in the absence and presence of increasing concentrations of Furan-1 (Figure 5). Furan-1 was able to inhibit the binding of both [^{125}I-His^5,D-Tyr^6]GnRH and [^{3}H]NBI-42902 in a dose-dependent manner. The inhibition of binding of [^{3}H]-NBI-42902 approaches a plateau at 316 nM Furan-1, which can be more readily visualized by Scatchard transformation (Figure 5, inset), and is consistent with allosteric binding at the receptor and negative modulation of the orthosteric ligand. A plateau for binding inhibition was not able to be determined in the experiments using [^{125}I-His^5,D-Tyr^6]GnRH at concentrations of up to 1 μM (data not shown); however, the specific binding was nearly completely inhibited at this concentration of Furan-1, suggesting a very negatively cooperative interaction. Globally fitting the data to the ATCM (Figure 1) and eq 3 enabled the determination of the values of R_{total}, the K_L value for each radioligand, the K_N value for Furan-1, and the cooperativity factor (α) (Table 3). The R_{total} values of the two radioligands, while similar, were statistically significantly different [R_{total} for [^{125}I-His^5,D-Tyr^6]GnRH = 0.912 ± 0.017 pmol/mg; R_{total} for [^{3}H]NBI-42902 = 0.830 ± 0.009 ($p > 0.01$, Student's *t*-test)]. The K_L of [^{125}I-His^5,D-Tyr^6]GnRH determined by this method was 0.288 ± 0.011 nM, and the K_L of [^{3}H]NBI-42902 was 0.313 ± 0.029 nM, consistent with published results for direct determination of K_d by saturation binding (*54*). The values of K_N for Furan-1 were 5.7 ± 0.24 nM using [^{125}I-His^5,D-Tyr^6]GnRH and 14.8 ± 3.7 nM using [^{3}H]NBI-42902. These values are not statistically significantly different ($p > 0.05$, Student's *t*-test) and are consistent with inhibition binding data (Table 1 and ref *49*). The significant difference in the R_{total} values for the two radioligands is not due to G protein coupling effects as neither radioligand is sensitive to GTPγS (data not shown) and may be a result of the different experimental conditions required for each radioligand.

There were significant differences ($p < 0.001$) in the cooperativity factor of Furan-1 using the two radioligands. The cooperativity factor (α) of Furan-1 with [^{3}H]NBI-42902 was determined to be 0.054 ± 0.006, indicating considerable negative cooperativity. The cooperativity factor of Furan-1 with [^{125}I-His^5,D-Tyr^6]GnRH could not be determined due to limitations in the analysis but was at least 10-fold smaller than that determined using [^{3}H]NBI-42902 ($\alpha < 0.005$; see Experimental Procedures), indicating an even more negatively cooperative interaction that is indistinguishable from a competitive interaction in this system.

Measurement of in Vitro Functional Efficacy. The effect of Furan-1 on the modulation of multiple GnRH-R agonists was measured using inositol phosphate accumulation assays, which is used to assess the functional activation of $G_{\alpha q}$-coupled receptors. Furan-1 at seven concentrations caused parallel rightward shifts in the dose−response curves of GnRH, [His^5,D-Tyr^6]GnRH, and leuprolide (shown in Figure

Table 2: Allosteric Modulation of Radioligand Dissociation by Furan-1 versus Two Different Radioligands in GnRH-R-Containing RBL-1 Membranes[a]

radioligand	[Furan-1] (nM)	$k_{off} \pm$ sem (min^{-1})	dissociation $t_{1/2}$ (min)	ratio ($k_{off79480}/k_{off}$)
[^{125}I-His^5,D-Tyr^6]GnRH	0	0.0061 ± 0.0001	113 ± 2	1.00
	100	0.0066 ± 0.0002	105 ± 3	1.08
	1000	0.0140 ± 0.0024	49 ± 9[b]	2.30
	3000	0.0200 ± 0.0040[b]	35 ± 10[b]	3.28
[^{3}H]NBI-42902	0	0.0020 ± 0.0003	369 ± 56	1.00
	300	0.0024 ± 0.0004	317 ± 59	1.18
	1000	0.0044 ± 0.0004[c]	159 ± 13[b]	2.20
	3000	0.0088 ± 0.0008[b]	81 ± 8[b]	4.35

[a] A one-way ANOVA with a Dunnett's post test was used in the analysis to determine if the dissociation rate constant and the half-life of dissociation were statistically significantly different in the presence of Furan-1. [b] $p < 0.01$. [c] $p < 0.05$.

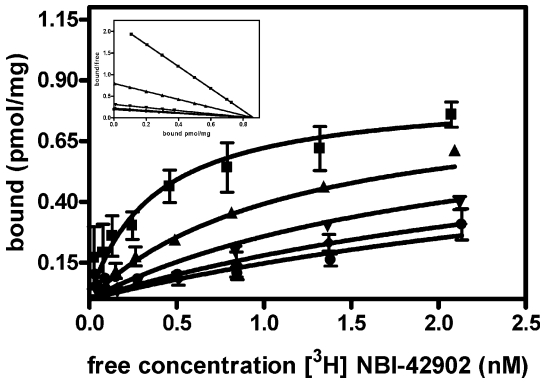

FIGURE 5: Saturation binding of [^{3}H]NBI-42902 in the presence of increasing concentrations of Furan-1: (■) 0 M, (▲) 31 nM, (▼) 100 nM, (◆) 316 nM, and (●) 1 μM. The inset is a Scatchard transformation of the globally fit saturation data.

6) with no apparent saturation of the maximal response in the Schild regression up to Furan-1 concentrations of 10 μM. Schild regression (Figure 6, inset) of the data was linear, with slope values ranging from 1.1 to 0.92. The pA_2 values (a measure of the affinity of an antagonist in a functional assay) calculated from the Schild regression of the three agonists gave values of 7.80 ± 0.13 (16.0 nM) for GnRH, 7.82 ± 0.10 (15.1 nM) for [His^5,D-Tyr^6]GnRH, and 8.14 ± 0.13 (7.3 nM) for leuprolide which are not statistically different from each other ($p > 0.05$) and are similar to published data (*49*). Also, the E_{max} values for each of the agonists in the absence of antagonist are not statistically different. Interestingly at 31.6 μM Furan-1, the leuprolide curve exhibited a suppression of E_{max} (34.7 ± 7.0%), which was not evident using the other agonists.

Modeling of NBI-42902 and Furan-1 with GnRH-R. Knowing the sites of interactions for both nonpeptide antagonists and having demonstrated that Furan-1 is allosteric for [^{3}H]NBI-42902 permitted the production of a docked model of both compounds bound to the receptor using molecular dynamics calculations and energy minimization. The model refinement was facilitated by requiring that both ligands bind simultaneously to the receptor. Orientations of NBI-42902 were selected that had the phenylglycine near Y290$^{(6.58)}$ because a close analogue without that substituent is affected by the same group of residues as NBI-42902, with the exception of Y290$^{(6.58)}$ (see the Supporting Information). Similarly, orientations of Furan-1 were selected for those in which the trimethoxyphenyl ring was in the proximity of H306$^{(7.36)}$. An analogue of Furan-1 in which that ring was replaced with a 4-methylphenyl ring is less sensitive to mutations at that position (see the Supporting Information). This orientation also places the very hydrophobic tetramethylcyclohexylphenyl substituent in the most hydrophobic area of its binding site, which is intuitively satisfying. The orientations of the two molecules after final refinement and minimization are displayed in Figure 7.

Figure 7 illustrates the two nonpeptide orientations that are consistent with the observations of their respective allosteric and orthosteric behavior. Furan-1 is bound deep within a pocket formed by TM7 and TM1, the most hydrophobic part of the molecule being close to F313$^{(7.43)}$. The furan ring is closest to F309$^{(7.39)}$, and the trimethoxyphenyl ring is in the proximity of H306$^{(7.36)}$. NBI-42902 appears to "straddle" TM6. The difluorobenzyl substituent is bound in an aryl arrangement with Y283$^{(6.51)}$ and Y284$^{(6.52)}$. The phenylglycine substituent is in the proximity of Y290$^{(6.58)}$, with the amine pointing toward residues in TM3 [e.g., S118$^{(3.29)}$] that have the potential for charged and/or hydrogen bonding-based interactions, though these do not appear to be close enough for direct interaction without invoking water-bridged interactions, or large-scale movement of TM3 and/or TM6. Comparisons of these antagonists to models of GnRH peptide binding (*3*, *64*) have been avoided because of likely differences between the antagonist- and agonist-bound states (*65*).

DISCUSSION

Site-directed mutagenesis and pharmacology have been used to identify the first example of an allosteric nonpeptide antagonist of human GnRH-R. Binding experiments indicate that NBI-42902 and Furan-1 possess nonoverlapping binding sites, and dissociation and saturation experiments using [^{3}H]-NBI-42902 indicated that Furan-1 is allosteric relative to that radioligand. Saturation binding experiments show that the cooperativity factor of Furan-1 varies depending on the radioligand used, which is not surprising because the two radioligands do not interact with the receptor in the same fashion: the two radioligands have overlapping, though distinct, binding pockets (*21*), and [^{3}H]NBI-42902 is an antagonist while [^{125}I-His^5,D-Tyr^6]GnRH is an agonist. The cooperativity factor of Furan-1 approaches zero and is so small when it competes with the radiolabeled peptide agonist ($\alpha < 0.005$) that in most assay systems, Furan-1 behaves in a manner consistent with a competitive orthosteric compound. However, the change in the dissociation rate constant of both radioligands in the presence of Furan-1 demonstrates that Furan-1 binds the receptor at a site that is allosteric for both interactions.

The observation that Furan-1 is allosteric for [^{125}I-His^5,D-Tyr^6]GnRH is somewhat surprising because there is evidence

Table 3: Characterization of Allosteric Ternary Model Complex (ATCM) Parameters of Furan-1 with Different Radioligands

radioligand	$R_{total} \pm$ sem (pmol/mg)	$K_L \pm$ sem (nM)	$K_N \pm$ sem (nM)	$\alpha \pm$ sem
[^{125}I-His^5,D-Tyr^6]GnRH	0.912 ± 0.017	0.288 ± 0.011	5.72 ± 0.24	<0.005
[^{3}H]NBI-42902	0.830 ± 0.009	0.313 ± 0.029	14.75 ± 3.7	0.054 ± 0.006

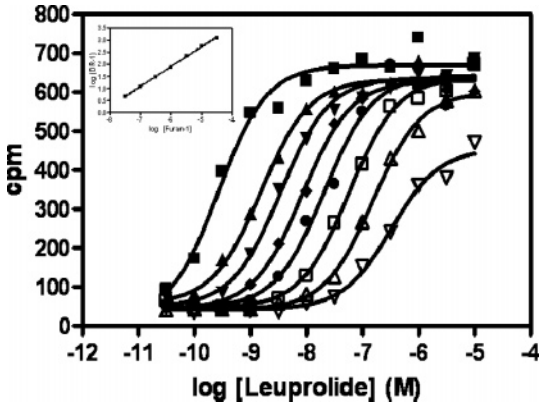

FIGURE 6: Leuprolide-induced inositol phosphate accumulation of RBL-1 cells transfected with GnRH-R in the presence of increasing concentrations of Furan-1: (■) 0 M, (▲) 31 nM, (▼) 100 nM, (◆) 316 nM, (●) 1 μM, (□) 3.16 μM, (△) 10 μM, and (▽) 31.6 μM. The inset is a Schild regression of inositol phosphate data.

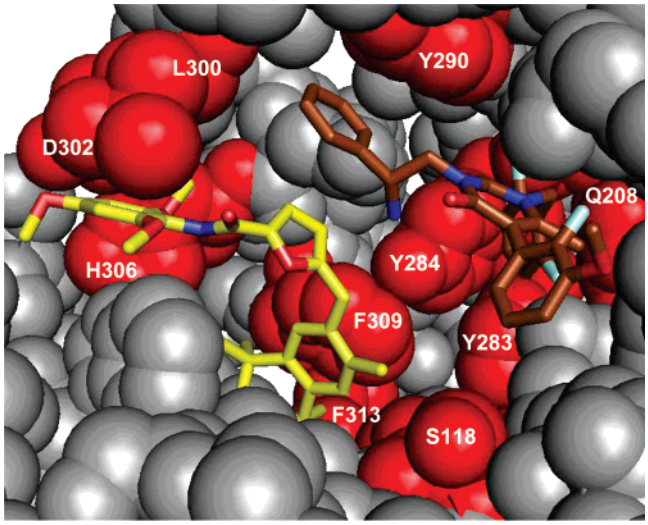

FIGURE 7: Homology model of human GnRH-R with Furan-1 and NBI-42902 bound. Residues important for the binding of these nonpeptide antagonists are colored red and labeled without their Ballesteros−Weinstein annotation for clarity. Furan-1 is on the left, and its carbon atoms are colored yellow. NBI-42902 is on the right, and its carbon atoms are colored brown.

that Arg^8 of GnRH interacts with D302$^{(7.32)}$ of the receptor (*66*), and the proximity of this interaction to H306$^{(7.36)}$ would suggest that these interactions might be orthosteric. However, it is known that for conformationally constrained versions of GnRH in which the active, high-affinity structure is preformed (e.g., peptides with D-amino acids at position 6), the Arg^8−D302$^{(7.32)}$ interaction is no longer required and may not be present (*67*). The lack of a requirement of this interaction is consistent with Furan-1 and [^{125}I-His^5,D-Tyr^6]-GnRH possessing distinct binding modes, with the action of the nonpeptide antagonist being nearly indistinguishable from that of a competitive, orthosteric interaction.

The functional data are consistent with strong negative cooperativity: the lack of saturation of the effect on agonist-induced IP accumulation (slope of the Schild regression close to 1) and the fact that the pA_2 values of Furan-1 with different agonists are not statistically different (*68*). If the ATCM is invoked (Figure 1), the insurmountability of Furan-1 in the leuprolide Schild assay is indicative of a compound that is noncompetitive under these conditions which is consistent with an allosteric compound. In addition, a slowly dissociating compound can also produce insurmountable behavior in a functional assay (*54*), but direct determination of the kinetic rate constants at 37 °C is required to test this hypothesis. Alternatively, a more complex model of allosteric compound−agonist interaction such as the allosteric two-state model, or the cubic quaternary complex model (*23*, *48*, *69*), can be used to explain the data in terms of the distribution of active and inactive receptor states and the effect of the allosteric modulator on them.

There are a number of potential advantages in using allosteric modulators as therapeutic agents over classic orthosteric ligands (*26*, *70*). Negative allosteric modulation of GnRH-R may offer advantages over competitive antagonists at the receptor and may increase the amount of chemical space available for negative modulation and antagonism of GnRH-R. Allosteric sites have faced less evolutionary pressure than orthosteric sites that accommodate an endogenous ligand and so may offer greater receptor selectivity, as observed with both muscarinic acetylcholine and adenosine family GPCRs (*71*, *72*). Normal patterns of neurohumoral signaling are also preserved with allosteric ligands such that a modulation of agonist signaling can be observed without a concomitant change in agonist concentration, and the effect is only observed in the presence of agonist (*23*). Last, allosteric modulators may have a decreased potential for toxic effects, since modulators with limited cooperativity will have a saturating level of effect regardless of the dose that is administered, potentially providing a greater therapeutic window (*26*).

In addition, the development of a model that satisfies the constraints for interactions of both nonpeptide antagonists with the receptor provides the opportunity for the structure-guided and hypothesis-driven development of new compounds. New antagonists could be designed that are hybrid molecules of Furan-1 and NBI-42902 [or other orthosteric antagonist classes (*22*)]. It is important to note that these representations present a snapshot of the simultaneous binding of these molecules to a static representation of GnRH-R. The observation that these nonoverlapping nonpeptide antagonists affect each other's binding kinetics ensures that upon binding one nonpeptide there is a conformational change in the receptor binding pocket that disfavors the binding of the other molecule. The determination that the cooperativity factor values approach zero indicates how strongly the co-occupation of the receptor is disfavored. The current information does not permit the development of a data-driven model of the changes the binding of either nonpeptide antagonist induces in the receptor binding pocket or the structural dependence of the degree of cooperativity between ligands. Higher-resolution experiments, perhaps with

spectroscopic probes and a purified receptor, will be immensely beneficial in defining the similarities and differences in those receptor states.

In summary, we have shown that Furan-1 is allosteric for both a GnRH agonist peptide and a potent nonpeptide antagonist (NBI-42902). It is important to note that both of these nonpeptides have exhibited activity in vivo, proving that either mechanism (allosteric or orthosteric antagonism) can be effective. These results also reinforce the idea that GnRH-R, and likely other peptide GPCRs, can be modulated effectively by nonpeptides that bind in significantly different ways. In fact, these antagonists do not have to be orthosteric to one another to have both in vitro potency and in vivo efficacy. Finally, these results underscore the fact that there are often multiple solutions to discovery problems and that targets once thought to be intractable to treatment from orally available, small organic molecules (e.g., peptide GPCRs) are eminently "druggable".

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SUPPORTING INFORMATION AVAILABLE

Two tables that detail ligand binding data at mutant GnRH-Rs for analogues of Furan-1 and NBI-42902 that help define their orientations within the receptor binding pocket and the chemical structures of the analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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