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Trapping of a Nonpeptide Ligand by the Extracellular Domains of the Gonadotropin-Releasing Hormone Receptor Results in Insurmountable Antagonism

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ABSTRACT

Drugs that exhibit insurmountable antagonism are proposed to provide improved clinical efficacy through extended receptor blockade. Long-term suppression of the gonadotropin-releasing hormone receptor (GnRHR) is an important therapeutic approach for a number of sex hormone-dependent diseases. In this study, we describe the mechanism and structural components required for insurmountable activity of a GnRHR antagonist. TAK-013 behaves as an insurmountable antagonist at the human receptor (hGnRHR) but as a surmountable antagonist at the macaque receptor (mGnRHR). Mutation of the eight residues that differ between hGnRHR and mGnRHR identified Ser-203 and Leu-300 in extracellular loops (ECL) 2 and 3 of hGnRHR as essential for the insurmountability of TAK-013. Substitution of the corresponding residues in mGnRHR with Ser and Leu (mGnRHR-P203S/V300L) converts TAK-013 to an insurmountable antagonist. In addition, mutation of Met-24 to Leu in the amino terminus of hGnRHR also ablates the insurmountable antagonism of TAK-013. The mechanism of insurmountability of TAK-013 was determined to be governed by its rate of dissociation from the receptor. Although the association rates of TAK-013 to hGnRHR, mGnRHR, and mGnRHR-P203S/V300L do not differ, the dissociation rate half-life correlates closely with the degree of insurmountability observed (169, 9, and 55 min, respectively). Taken together, these data suggest a model of the GnRHR in which ECL2, ECL3, and the amino terminus engage with TAK-013 upon its binding to the transmembrane region of the receptor. These additional interactions form a "trap door" above TAK-013, restricting its dissociation and thus resulting in its insurmountability.

As key regulators of sex hormone production, GnRH and its receptor (Millar et al., 2004) are important targets for clinical intervention in a variety of sex hormone-dependent disorders, including endometriosis, uterine fibroids, and prostate and breast cancers, where effective treatment requires reduction of gonadal sex steroid biosynthesis (Huirne and Lambalk, 2001). The currently available GnRH antagonists for clinical use are peptide analogs of the endogenous agonist that are typically administered by parenteral injection. However, injection site reactions and the limited ability to vary dosages once therapy has begun are important disadvantages of the peptide depots. To circumvent these disadvantages several nonpeptide small molecule antagonists have been developed. The antagonist NBI-42902 was shown to inhibit up to 55% of baseline luteinizing hormone in postmenopausal women after oral administration (Struthers et al., 2006). Another antagonist, TAK-013 (Sufugolix), developed by Takeda Chemical Industries (Osaka, Japan) (Sasaki et al., 2002; Sasaki et al., 2003), was also shown to suppress the menstrual cycle in studies in female cynomolgus macaques (Hara et al., 2003) and, more recently, has been evaluated in human clinical trials. Thus, the development of orally active small molecule GnRH antagonists with high potency and extended activity may be an important future therapeutic class for reproductive, hormone-related disorders.

Competitive antagonists of G protein-coupled receptors (GPCRs) can be classified as either surmountable or insurmountable, depending on whether their inhibitory actions can be overcome with an excess of competing ligand (Vauquelin et al., 2002; Kenakin et al., 2006). An antagonist is

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ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; TM, transmembrane domain; ECL, extracellular loop; GnRHR, gonadotropin-releasing hormone receptor; hGnRHR, human GnRHR; mGnRHR, macaque GnRHR; HEK, human embryonic kidney; RBL, rat basophilic leukemia; WT, wild type.

surmountable if, in a receptor function assay, increasing concentrations of antagonist cause the rightward shift of the agonist dose-response curve without altering the maximal response that can be achieved with high concentrations of agonist. In contrast, an insurmountable antagonist can reduce the maximal functional response even in the presence of very high concentrations of agonist.

The pharmacological mechanism for insurmountable but competitive antagonism is the slow kinetics of the dissociation of the antagonist-receptor complex, so that within the timeframe of the assay, there is little free receptor available for agonist binding (Lew et al., 2000; Kenakin et al., 2006). Thus, in the presence of an insurmountable antagonist, a proper equilibrium cannot be reached between the agonist, antagonist, and receptor. Nonequilibrium systems display increased efficacy at lower concentrations of the antagonist because the prolonged binding of the antagonist delays its metabolism and precludes binding of the agonist (Swinney, 2004). Furthermore, despite clearance of the drug from the general circulation, the prolonged binding of an insurmountable antagonist to its receptor could also be reflected in a longer duration of action with decreased dosing, which enhances its therapeutic window (Asmar and Lacourciere, 2000; Copeland et al., 2006).

Although many insurmountable compounds with slow dissociation kinetics of the antagonist-receptor complex have been described previously (Keith et al., 1989; Cabré et al., 2002; Jakubík et al., 2002; Rashid et al., 2002), only a few of these have been studied at the molecular level (Cucchi et al., 2005; Mathiesen et al., 2006; Sullivan et al., 2006). Moreover, only the dissociation kinetics of the insurmountable antagonist candesartan from its target receptor, the angiotensin II type 1 receptor, has been investigated at the level of specific molecular interactions between the chemical moieties of the compound and the residues composing its receptor binding pocket (Takezako et al., 2004).

The sites of interaction of the GnRHR antagonist TAK-013 with its target receptor have been mapped to a region composed of the extracellular domains of transmembrane domains (TM) 6 and 7 with a significant contribution of the ECL3 and the NH_2 terminus (Betz et al., 2006a,b). Because of the detailed understanding of this small molecule and its clinical relevance, we chose to use it as a tool to explore the underlying mechanism of insurmountability at the GnRH receptor. Furthermore, our goal in the present study is to identify and model the structural components of the receptor required to produce an insurmountable antagonist-receptor interaction.

Materials and Methods

Synthesis of Small Molecule Compounds. The compounds shown in Fig. 5 were synthesized as described previously (Guo et al., 2003; Sasaki et al., 2003). To prepare the radiolabel [^{3}H]TAK-013, the precursor 1-{4-[5-(benzylamino-methyl)-1-(2,6-difluoro-benzyl)-2,4-dioxo-3-phenyl-1,2,3,4-tetrahydro-thieno[2,3-*d*]pyrimidin-6-yl]-phenyl}-3-methoxyl-urea was synthesized by a method described previously (Sasaki et al., 2003). The labeling protocol was carried out by American Radiolabeled Chemicals, Inc (St. Louis, MO) as follows. The precursor to TAK-013 was treated with tritiated methyl iodide in dimethylformamide in the presence of potassium carbonate to yield the desired product, which was purified by reversed-phase high-performance liquid chromatography to 95% purity. The final product, [^{3}H]TAK-013, had a specific activity of 80 Ci/mmol and was stored in ethanol at −80°C.

Preparation of Receptor Clones and Mutants. The human and macaque GnRH receptors were cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) as described previously (Reinhart et al., 2004). All point mutations were introduced into the wild-type human and macaque GnRH receptors using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Amino-terminal deletions were constructed by polymerase chain reaction amplification using primers to incorporate the Kozak consensus site and start Met 3′ to the sequence to be deleted. The correct constructs were identified by DNA sequence analysis. The wild-type human GnRHR (hGnRHR), wild-type macaque GnRHR (mGnRHR), hGnRHR-S203P, hGnRHR-L300V, hGnRHR-S203P/L300V, mGnRHR-P203S, mGnRHR-V300L, and mGnRHR-P203S/V300L receptors were subcloned into a vector generated from pBSII and placed under the transcriptional control of the spleen-focus-forming virus long terminal repeat (Heise et al., 2005) for the purpose of generating stably expressing rat basophilic leukemia RBL cell lines.

Cell Lines and Transfections. All cell lines were obtained from the American Type Culture Collection (Manassas, VA). Human embryonic kidney (HEK) 293 cells and RBL-2H3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, and 50 IU/ml penicillin, and 50 μg/ml streptomycin.

The recombinant vectors containing the GnRH receptor mutants described above were transfected into RBL-2H3 cells using Lipofectamine2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The stable transfectant clonal populations were selected in 1 mg/ml G418 (Calbiochem/EMD Biosciences, San Diego, CA). Transient transfections of HEK293 cells were performed with 10 μg of plasmid and 30 μl of FuGENE 6 (Roche Applied Science, Indianapolis, IN), according to the manufacturer's protocol. HEK293 cells transiently expressing the various receptors were plated onto 96-well poly(D-lysine)-coated plates (Biocoat, Fort Washington, PA) the day after transfection.

Inositol Phosphate Accumulation Assay. The various RBL-GnRHR stable cells and the HEK293 cells transiently expressing the various GnRHR constructs were labeled overnight with 2 μCi/ml [*myo*-2-^{3}H]inositol (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) in inositol-free Dulbecco's modified Eagle's medium (Millipore, Billerica, MA) supplemented with 10% dialyzed fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, and 50 IU/ml penicillin, and 50 μg/ml streptomycin). Cells were washed twice with buffer I (140 mM NaCl, 4 mM KCl, 20 mM HEPES, 0.1% bovine serum albumin, 8.3 mM D-glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 15 mM LiCl, pH 7.4). The cells were preincubated with the appropriate concentrations of TAK-013 (or its analogs) for 20 min at 37°C before the addition of GnRH to stimulate inositol phosphate accumulation for 60 min. The inositol phosphates were then measured as described previously (Guo et al., 2005).

Preparation of Cell Membranes. Membranes were prepared from RBL-hGnRHR, RBL-mGnRHR, hGnRHR-L300V, and RBL-mGnRHR-P203S/V300L cell lines, as described previously (Betz et al., 2006b). Membranes were resuspended in kinetic assay buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM EDTA, pH 7.5) at 1 mg/ml and stored at −80°C.

Association and Dissociation Kinetics Studies. Radioligand binding experiments were performed as described previously (Sullivan et al., 2006) using membranes prepared from RBL-hGnRHR, RBL-mGnRHR, RBL-hGnRHR-L300V, and RBL-mGnRHR-P203S/V300L. In brief, saturation binding experiments were performed with 25 μg of membranes from hGnRHR, mGnRHR, hGnRHR-L300V, and 50 μg of membranes from mGnRHR-P203S/V300L in the presence of various concentrations of [^{3}H]TAK-013 (ranging from 10 pM to 3 nM) for 8 h at 37°C. Nonspecific binding was measured using 1 μM TAK-013 and subtracted from the total binding to yield the

specific binding. Association of [^{3}H]TAK-013 was determined using conditions similar to those above, except that a single concentration of radiolabel was used (0.6–2.4 nM [^{3}H]TAK-013) and incubations were carried out for 1 h.

Dissociation kinetic experiments were preformed by preincubating the cell membranes (same membrane protein concentrations as above) with 2 nM [^{3}H]TAK-013 for 1 h at 37°C. The dissociation of the radiolabel was initiated by the addition of 1 μM unlabeled TAK-013 and incubated for 1 to 6 h at 37°C. Total binding remained stable for at least 6 h.

Data Analysis. Dose response data were plotted and analyzed using GraphPad Prism 4.01 software (San Diego, CA). The functional binding affinity (K_b) of TAK-013 to the various GnRHRs was determined from the inositol phosphate accumulation assays. When the inhibition of inositol phosphates by TAK-013 was surmountable, the K_b was calculated from a Schild regression plot, which is a plot of log(Dose Ratio − 1) at the ordinate versus the log(concentration of TAK-013) at the abscissa. The regression has a slope of unity and the absissal intercept is log(K_b).

When TAK-013 caused a depression of the E_{max}, then the estimate of the antagonist affinity was calculated by a method described for noncompetitive antagonists by Gaddum (1957) and Kenakin (2003). In brief, the equieffective concentrations of agonist A in the absence and the presence of antagonist B were calculated. A regression is constructed of 1/[A] at the ordinate versus 1/[A′] at the abscissa, where [A] is the concentration of agonist for the control curve and [A′] is the equieffective concentrations of the agonist in the presence of antagonist B. From this plot the K_b is derived from the slope using the equation K_b = [B]/slope − 1. Association and dissociation rate constants were generated by analyzing the kinetic binding data using GraphPad Prism 4.01. Association binding data were fit to the one-phase exponential association equation: $Y = Y_{max}(1 - e^{-k_{obs} \cdot x})$, where Y_{max} is specific binding at infinite time, k_{obs} is the observed rate constant, and x is the association time expressed in minutes.

The dissociation rate constant was fitted by the exponential decay equation: Y = span $\cdot e^{-k_{off} \cdot x}$ + plateau, where span is the difference between the binding at time 0 and the plateau, which is routinely set at zero. k_{off} is the dissociation rate constant. The dissociation half-life was calculated from the following equation: $t_{1/2} = 0.693/k_{off}$. The association rate constant, k_{on}, was then generated from the following equation: $k_{on} = k_{obs} - k_{off}$/[radioligand].

Results

Antagonism of TAK-013 at the Human and Macaque GnRH Receptors. It has been previously shown that the antagonist TAK-013 can completely displace the radiolabeled peptide ligands ^{125}I-[Tyr^5]Leuprorelin (Sasaki et al., 2003) and ^{125}I-[His^5-D-Tyr^6]GnRH (Betz et al., 2006b) from the human GnRH receptor and the macaque GnRH receptor. This is consistent with a competitive interaction between the labeled peptide and TAK-013 at both receptors. Yet TAK-013 displayed a different antagonism profile at the human receptor than at the macaque receptor in a GnRH dose-response assay measuring inositol phosphate accumulation (Fig. 1). Stimulation of RBL cells stably expressing either the human or the macaque GnRH receptors with increasing concentrations of TAK-013 (from 10 nM to 1 μM) resulted in the rightward shift of the GnRH dose-response curve at both receptors. However, at the human receptor, a depression of the maximal response, E_{max}, was also observed (Fig. 1A), whereas at the macaque receptor GnRH was able to completely overcome the inhibition by TAK-013 (Fig. 1B). Hence, TAK-013 behaves as a surmountable antagonist at the macaque GnRH receptor but as an insurmountable antagonist at the human GnRH receptor.

Mechanism of Antagonism of TAK-013 at the Human and Macaque GnRH Receptors. To determine whether the kinetics of TAK-013 are responsible for its mode of binding at the two species of GnRH receptor, the association and dissociation rates at the hGnRHR and mGnRHR were determined (Fig. 1). Although there is a small 2-fold difference in the association rates (k_{on}) between the receptors (k_{on} are $1.2 \pm 0.6 \times 10^8$ and $2.4 \pm 0.2 \times 10^8$ $M^{-1}min^{-1}$ for human and macaque GnRHRs, respectively) (Fig. 1C), the dissociation rate constant (k_{off}) of TAK-013 at the human GnRHR (half-life of 169 min) is 18-fold slower than its dissociation from the macaque receptor (half-life of 9 min) (Fig. 1D). These data suggest that the slow rate of dissociation of the antagonist from the human receptor relative to the macaque receptor is responsible for its insurmountable characteristic in the functional assay.

Residues Involved in Insurmountable Binding of TAK-013. Because the same compound is insurmountable at the human receptor but surmountable at the macaque receptor, the explanation for the different antagonist profiles must reside within the receptor and its ability to interact with the TAK-013. A comparison of the human and macaque receptor sequences reveals only eight residues that differ (Fig. 2); thus, one or a combination of several of these residues must determine the type of antagonism observed with TAK-013. To identify which residues are important for insurmountability, each of the eight amino acids in the human receptor was mutated to the corresponding residues in the macaque. The reciprocal mutations were also made in the macaque receptor. To facilitate screening large numbers of receptor mutants, the initial screening of the pharmacology of TAK-013 was performed in a HEK293 transient expression system. However, once the important residues were identified, RBL-2H3 stable cell lines were generated with the selected receptor mutants to assist in their in-depth analysis. Each mutant receptor was subjected to a GnRH dose response in an inositol phosphate accumulation assay in the absence and presence of increasing concentrations of TAK-013. The insurmountable antagonism of TAK-013 was quantified as the reduction of the E_{max} in the presence of the highest concentration of antagonist.

Of the eight amino acid differences, substitutions of six of them (A16V, L112F, N152S, V155L, F225L, and E248K) from the human receptor to the macaque counterpart had no effect on the insurmountable antagonism of TAK-013 (data not shown). The converse was also true: mutation of those same residues in the macaque receptor to the human residues did not alter the surmountable property of TAK-013 (data not shown). However, mutation of Ser-203 in ECL2 of the human receptor to the macaque counterpart, Pro, resulted in a significant loss of TAK-013 insurmountability. TAK-013 displays a 19.1% suppression of E_{max} at the S203P mutant compared with 56.6% suppression at the wild-type (WT) receptor (Table 1, Fig. 3, A and B). Substitution of the human Leu-300 to the macaque Val alone, or in combination with S203P, converts TAK-013 to a surmountable compound (Table 1, Fig. 3, C and D). Therefore, both Ser-203 and Leu-300 are necessary, but each alone is not sufficient for the insurmountable behavior of TAK-013.

The reciprocal mutations at the macaque receptor of Pro-203 to the corresponding human residue Ser does not change the surmountability of TAK-013 (Table 1 and Fig. 3F), but

substitution of Val-300 to Leu results in a slight reduction in the E_{max} in the presence of drug (Table 1 and Fig. 3G). However, TAK-013 displays insurmountable properties at the macaque receptor similar to the hGnRHR only when the P203S and V300L mutations are both present (Table 1 and Fig. 3H).

Mutational Analysis of the Second and Third Extracellular Loops of the Human GnRHR. To further map the interactions responsible for tight binding and insurmountable antagonism of TAK-013, Ser-203 and Leu-300 were mutated to a variety of residues with nonpolar, polar, and charged side chains (Table 2) and expressed transiently in HEK293 cells. When Ser-203 was changed to a nonpolar residue (alanine) or to an uncharged polar residue (threonine or asparagine), TAK-013 retains its insurmountable property at the receptor. However, when Ser-203 is mutated to aspartic acid or glutamic acid, both

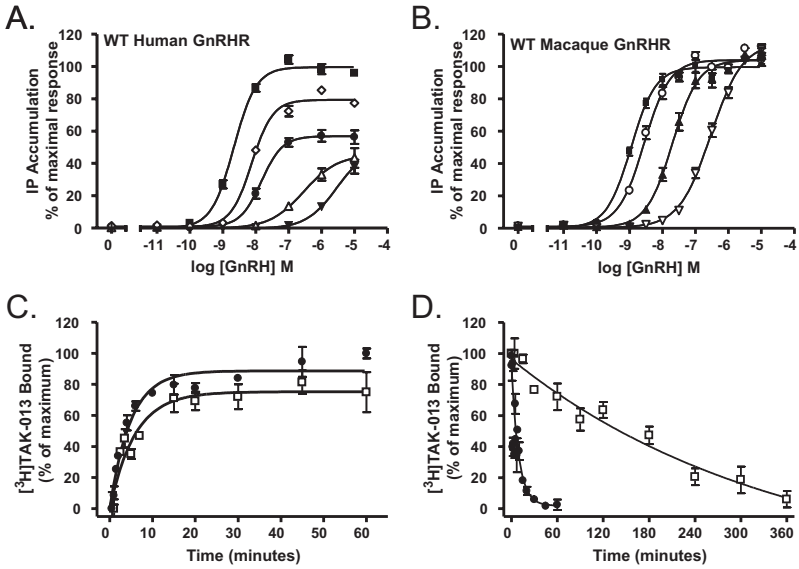


Fig. 1. Pharmacological characterization of TAK-013 at the human and macaque GnRH receptors. A, RBL cells stably expressing the WT human GnRHR were incubated with 0 (■), 1 nM (◇), 3 nM (●), 30 nM (△), or 300 nM (▼) TAK-013 for 20 min. B, RBL cells stably expressing WT macaque GnRHR were preincubated with 0 (■), 10 nM (○), 100 nM (▲), or 1 μM (▽) TAK-013 for 20 min. Cells were then stimulated with various concentrations of GnRH and accumulation of inositol phosphates measured. C, association of [^{3}H]TAK-013 to the human (□) and macaque (●) GnRH receptors was determined by a radioligand binding assay with approximately 1 nM [^{3}H]TAK-013 and 25 to 50 μg of membrane protein and incubated for 0.5 to 60 min at 37°C. Nonspecific binding was determined with 1 μM unlabeled TAK-013. The graph shows data with mean ± S.E. of duplicates from three independent experiments. D, dissociation of [^{3}H]TAK-013 from the human (□) and macaque (●) GnRH receptors was performed by incubating 25 to 50 μg of membrane protein with 2 nM [^{3}H]TAK-013 for 1 h at 37°C. Dissociation was initiated by the addition of 1 μM unlabeled TAK-013 for the times indicated on the graph. The data shown are the mean ± S.E. of three independent experiments. Human and macaque GnRHR expression in the stable cell lines are 922 ± 115 and 958 ± 61 fmol/mg, respectively. Data are the mean ± S.E. of three independent experiments.

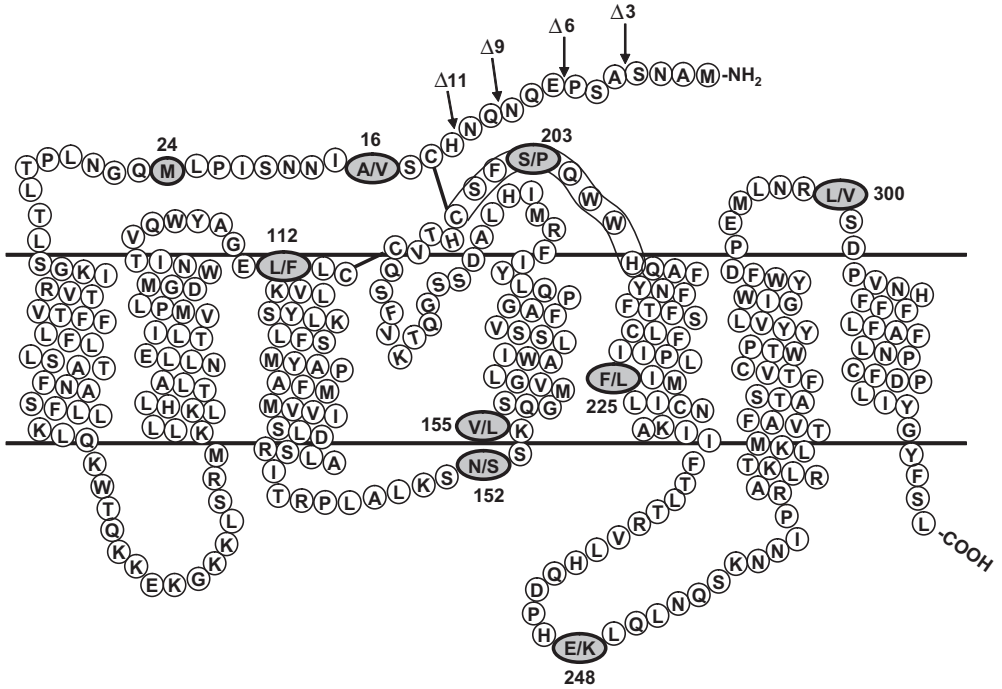


Fig. 2. Schematic representation of human and macaque GnRH receptor. The alignment of the helical regions of the receptor is based on conserved residues present throughout the class A GPCR family. Residues that are mutated in this study are highlighted, shaded in gray, and numbered. Residues that differ between the human and macaque receptors are in ovals (human/macaque). Arrows show sites at which the NH_2 terminus was truncated after Met-1. Disulfide bonds are shown between Cys-14 and Cys-200, and between Cys-114 and Cys-196.

charged residues, the insurmountability is disrupted, as it is with the inclusion of proline at this position. These results show that position 203 is permissive for the conformation necessary for insurmountability, because only a large disruption of the structure by proline or insertion of a charge alters TAK-013 insurmountable antagonism.

The valine at position 300 in the macaque receptor is structurally similar to the leucine found in the human receptor, yet this small change is sufficient for the complete loss of insurmountability, as shown in Tables 1 and 2 and Fig. 3C. A more conservative change is to the β-branched isoleucine instead of the γ-branched leucine at residue 300, yet this mutant also caused TAK-013 to become surmountable. Further mutations of Leu-300 included changes to an alanine and to a threonine, both of which resulted in a loss of insurmountability (Table 2). These results indicate that a leucine at 300 is critical to maintain the insurmountability of TAK-013, because even the smallest change in side-chain structure (from γ- to β-branched residues) ablates this property.

Because the L300V substitution at the hGnRHR and the P203S/V300L double substitution at the mGnRHR converts the type of antagonism elicited by TAK-013, experiments

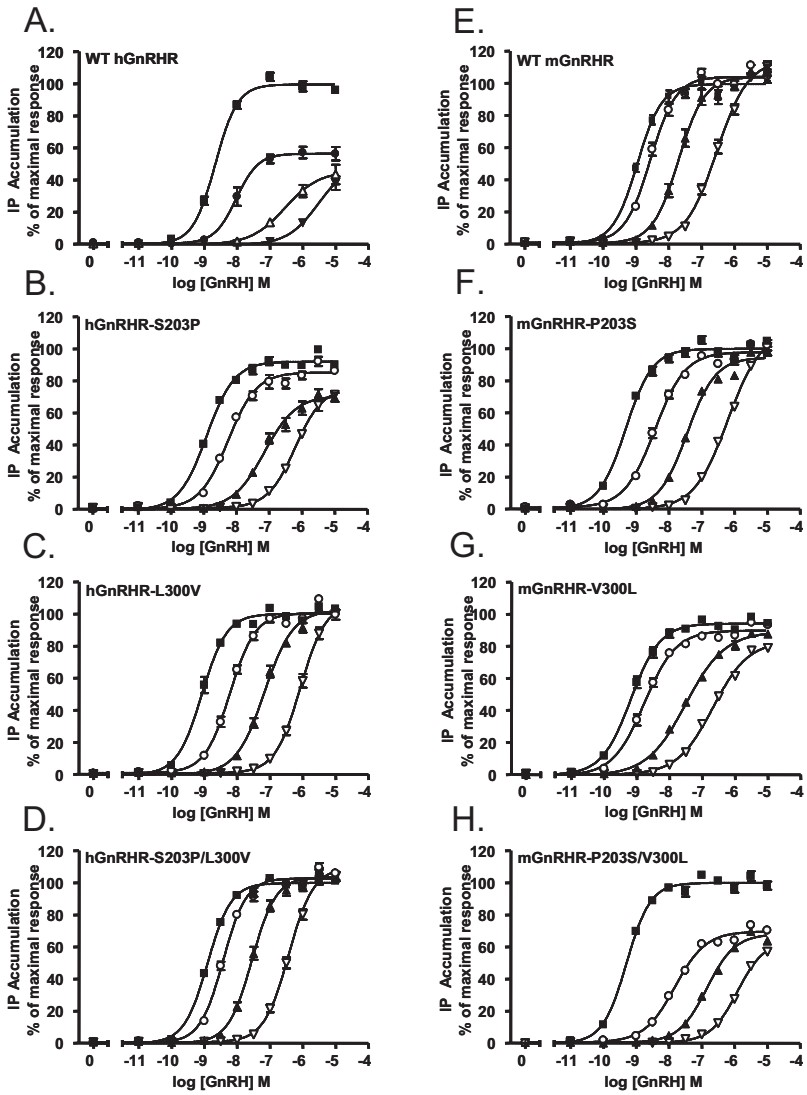


Fig. 3. Effects of TAK-013 on GnRH-stimulated inositol phosphate accumulation in various human and macaque GnRHR mutants. Dose-response curves of inositol phosphate (IP) accumulation stimulated with GnRH were generated in the presence of several concentrations of TAK-013. A, RBL cells stably expressing WT hGnRHR were preincubated with 0 (■), 3 nM (●), 30 nM (△), and 300 nM (▼) TAK-013 for 20 min. B–D, RBL cells stably expressing various mutant hGnRHRs were preincubated with 0 (■), 10 nM (○), 100 nM (▲), and 1 μM (▽) TAK-013 for 20 min. E–H, RBL cells stably expressing WT and various mutant mGnRHRs were preincubated with 0 (■), 10 nM (○), 100 nM(▲), or 1 μM (▽) TAK-013 for 20 min. All cell lines were then stimulated with varying concentrations of GnRH and IP accumulation measured after 1 h at 37°C. Data are the mean ± S.E. of three independent experiments.

TABLE 1

Suppression of maximal response (E_{max}) and functional potency of TAK-013 in human and macaque GnRH receptor mutants

Suppression of the maximal response (E_{max}) and K_b values were determined from inositol phosphate dose response assays performed in RBL cells stably expressing the above receptors. The percentage reduction in maximal response was calculated as the reduction in E_{max} in the presence of 1 μM TAK-013 compared with agonist alone. K_b values, a measure of functional TAK-013 potency, were calculated as described under *Materials and Methods*.

Human GnRHR	Reduction of Maximal Response	K_b	n	Macaque GnRHR	Reduction of Maximal Response	K_b	n
	%	*nM*			%	*nM*	
WT	56.6 ± 4.9	0.3 ± 0.04	6	WT	−2.7 ± 2.4	8.0 ± 1.0	3
S203P	19.1 ± 3.0	2.0 ± 0.09	4	P203S	2.3 ± 1.7	2.1 ± 0.4	3
L300V	−0.6 ± 1.3	2.0 ± 0.2	3	V300L	11.7 ± 1.8	5.5 ± 0.7	4
S203P/L300V	−2.6 ± 0.3	5.6 ± 0.6	3	P203S/V300L	36.6 ± 0.3	0.4 ± 0.05	3

were conducted to determine whether these mutations change the dissociation rate of the compound. The results show association rates of 0.9×10^8 and 1.0×10^8 $M^{-1}min^{-1}$ and dissociation rates of 0.053 ($t_{1/2}$ of 13 min) and 0.014 min^{-1} ($t_{1/2}$ of 55 min) for TAK-013 at the hGnRHR-L300V and the mGnRHR-P203S/V300L, respectively (Table 4 and Fig. 4). The association rates of TAK-013 to the mutants are very similar (Table 4); however, the dissociation rates parallel the change in the E_{max} in the functional assay (Table 1 and Fig. 3). Taken together, these data demonstrate that the insurmountable antagonism of TAK-013 is determined by the dissociation rate, and that, in turn, is dependent on the structure of the receptor created by the residues at positions 203 and 300.

TABLE 2

Mutational analysis of Leu-300 and Ser-203 residues in the human GnRH receptor

Suppression of the maximal response (E_{max}) and K_b values were determined from inositol phosphate dose response assays performed in HEK293 cells transiently transfected with various receptors. The percentage reduction in maximal response was calculated as the reduction in E_{max} in the presence of 1 μM TAK-013 compared with agonist alone. K_b values, a measure of functional TAK-013 potency, were calculated as described under *Materials and Methods*.

Human GnRHR	Reduction of Maximal Response	K_b	n
	%	*nM*	
WT	28.4 ± 1.1	0.7 ± 0.1	55
S203A	17.8 ± 4.9	1.0 ± 0.2	3
S203T	35.2 ± 2.5	0.8 ± 0.2	3
S203N	25.0 ± 2.7	1.1 ± 0.3	3
S203P	1.2 ± 4.3	4.2 ± 0.5	4
S203D	4.9 ± 3.9	1.4 ± 0.1	3
S203E	4.2 ± 7.6	2.2 ± 0.3	3
L300V	−2.2 ± 1.5	5.8 ± 0.7	7
L300I	3.1 ± 4.0	7.0 ± 0.9	4
L300A	5.4 ± 1.3	9.7 ± 0.6	4
L300T	−0.5 ± 2.8	28.0 ± 8.6	4

TABLE 3

Effect of deletions and mutation of the amino-terminus of the human GnRH receptor on E_{max} and functional affinity (K_b)

Suppression of the maximal response (E_{max}) and K_b values were determined from inositol phosphate dose response assays performed in HEK293 cells transiently transfected with various receptors. The percent reduction in maximal response was calculated as the reduction in E_{max} in the presence of 1 μM TAK-013 compared to agonist alone. K_b values, a measure of functional TAK-013 potency, were calculated as described under *Materials and Methods*.

Human GnRHR	Reduction of Maximal Response	K_b	n
	%	*nM*	
WT	28.4 ± 1.1	0.7 ± 0.1	55
N-Δ3	22.8 ± 7.3	1.0 ± 0.4	3
N-Δ6	24.5 ± 5.2	1.1 ± 0.7	3
N-Δ9	32.4 ± 5.6	0.8 ± 0.4	3
N-Δ11	43.6 ± 3.1	0.5 ± 0.1	3
M24L	−8.6 ± 2.1	36.8 ± 4.8	3

Mutational Analysis of the NH_2 Terminus of hGnRHR. The NH_2 terminus of the hGnRHR has been implicated in high-affinity binding of nonpeptides (Reinhart et al., 2004; Betz et al., 2006b). Thus, we carried out a mutational analysis of this region. Deletions of the NH_2 terminus of the human GnRH receptor were generated by the sequential removal of two or three residues to yield hGnRHR(N-Δ3), hGnRHR(N-Δ6), hGnRHR(N-Δ9), and hGnRHR(N-Δ11) (Fig. 2). Deletions beyond His-13 were not made because formation of a disulfide bridge between Cys-14 and Cys-200 has been shown to be important for the expression and function of the receptor (Cook and Eidne, 1997). Previous results have suggested that Met-24 is involved in nonpeptide binding (Betz et al., 2006b); thus, the M24L mutant in the human receptor was also examined. The mutants were transiently expressed in HEK293 cells and IP accumulation measured in the presence and absence of compound. The results displayed in Table 3 show that deletion of up to 11 residues from the NH_2 terminus does not affect insurmountability of TAK-013, but mutation of Met-24 converts it into a surmountable compound.

Structure-Activity Relationship of Insurmountable Antagonism of TAK-013 at the Human GnRH Receptor. Molecular modeling of the hGnRHR and TAK-013 has shown that Asp-302 and His-306 bind TAK-013 by hydrogen bonding to the urea moiety (Betz et al., 2006a,b). Because of the vicinity of these residues to Leu-300, the potential role of the urea substituent in TAK-013 in its insurmountable antagonism was explored. For this purpose five compounds were synthesized with alterations to the methoxyurea and are shown in Fig. 5. Urea-containing compounds, such as TAK-013 and compound A, are able to suppress E_{max}, but when the urea is replaced by amide substituents, as in compounds B, C, and D, or by an ethylamine, as in compound E, the insurmountable property of the compounds is lost (Fig. 5). Furthermore, there are also commensurate changes in the affinity of these compounds, suggesting that the surmountable compounds probably have a faster dissociation rate. Thus, the Leu-300 of the receptor and the urea of TAK-013 may interact in such a manner as to profoundly influence the insurmountable character of the compound.

Molecular Model of the Human GnRH Receptor and TAK-013 Complex. The docked model of TAK-013 bound to the human GnRH receptor was previously generated by Betz et al. (2006a) using reciprocal SAR between the compound and receptor mutants. Figure 6A shows the location of Leu-300 and TAK-013 in the docked model, and Fig. 6B shows the same model with the substitution of valine at position 300. A comparison of Fig. 6, A and B, reveals that the leucine, with its extra methyl group, spans the urea moiety of the TAK-013 molecule and may make more favorable contacts than valine.

TABLE 4

Association and dissociation kinetics of TAK-013 from the wild-type human GnRHR, wild type macaque GnRHR, human GnRHR-L300V, and macaque GnRHR-P203S/V300L

	WT hGnRHR	WT mGnRHR	hGnRHR-L300V	mGnRHR-P203S/V300L
k_{on} ($M^{-1}min^{-1}$)	$1.2 \pm 0.6 \times 10^8$	$2.4 \pm 0.2 \times 10^8$	$0.9 \pm 0.5 \times 10^8$	$1.0 \pm 0.6 \times 10^8$
log k_{on}	8.1	8.4	7.9	8.0
k_{off} (min^{-1})	0.0045 ± 0.0007	0.081 ± 0.008	0.053 ± 0.002	0.014 ± 0.002
pk_{off}	2.4	1.1	1.3	1.9
$t_{1/2}$ (min)	169	9	13	55
K_d (nM)	0.05 ± 0.02	0.34 ± 0.03	0.62 ± 0.05	0.14 ± 0.01

k_{on}, association rate constant; k_{off} , dissociation rate constant; $t_{1/2}$, dissociation half-life; K_d, k_{off}/k_{on}

In addition, valine side chains are more conformationally restricted than leucine, so leucine may be better suited to make adaptive interactions (Chamberlain and Bowie, 2004). Although ECL3 can be defined by homology modeling to rhodopsin, the NH_2 terminus and ECL2 of the human GnRHR are too different from rhodopsin to be accurately included in the docking model. An estimation of the location of the NH_2 terminus containing Met-24 and ECL2 containing Ser-203 was performed by analogy to the rhodopsin receptor (Fig. 6C) and is depicted in Fig. 6D. The ECL2 is held in place by two disulfide bonds: one between Cys-196 and Cys-114 of TM3, which is present in nearly all GPCRs; and one between Cys-200 and Cys-14 of the NH_2 terminus. These disulfide bonds serve to constrain ECL2 as a cap above the transmembrane domains that form a pocket where TAK-013 binds. Last, the NH_2 terminus is anchored to the ECL2 by the disulfide bond at Cys-14; however, its three-dimensional structure is unclear.

Discussion

In the present work, we explored the molecular mechanism and the structural components underlying insurmountability of nonpeptide compounds at the GnRH receptor. We initially observed that the nonpeptide antagonist TAK-013 had different patterns of competitive antagonism: insurmountable at the human GnRH receptor and surmountable at the macaque receptor. Measurement of the binding kinetics of TAK-013 to the GnRHR showed a direct correlation between the extent of insurmountability, the functional binding affinity (K_b), and the dissociation rate of TAK-013 from the receptor. The affinity of a compound (K_d) is defined as the association rate constant (k_{off}) divided by the association rate constant (k_{on}). Therefore, because k_{on} of TAK-013 remains constant among the different receptor mutants, a change in k_{off} can account for the different affinities. TAK-013 at the hGnRHR displays a 56.6% suppression of the E_{max} and has a K_b of 0.3 nM and a dissociation half-life ($t_{1/2}$) of 169 min, whereas, at the mGnRHR, TAK-013 is surmountable and has a K_b of 8.0 nM and a dissociation $t_{1/2}$ of 9 min.

It is striking that the conservative change of L300V at the hGnRHR converts the compound from insurmountable to surmountable and decreases its dissociation $t_{1/2}$ from 169 to 13 min. Mutation of V300L as well as P203S, however, was required for the analogous gain of TAK-013 insurmountability and increase in its dissociation $t_{1/2}$ at the macaque receptor. These data indicate that the slow dissociation and insurmountability of the molecule require the presence of both Ser-203 (in ECL2) and Leu-300 (in ECL3), but mutation of either one of these residues disrupts the receptor conformation responsible for tight binding.

The work presented here, together with previous data on the structure of GnRHR and other GPCRs, begins to suggest a structural basis of the insurmountable antagonism and slow receptor-ligand dissociation kinetics. Results presented

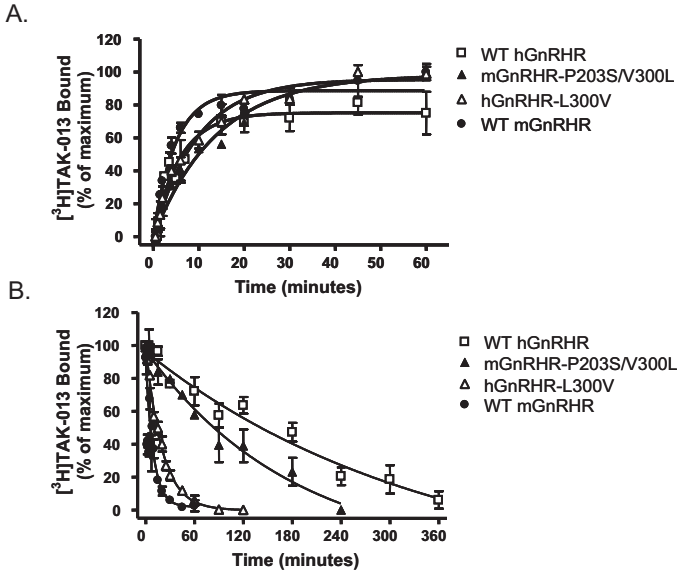
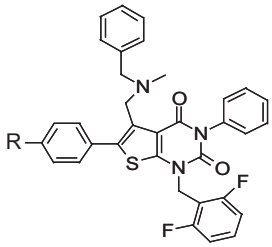


Fig. 4. Association and dissociation kinetics of TAK-013 from WT human and macaque GnRHR, hGnRHR-L300V, and mGnRHR-P203S/V300L mutants. Membranes were prepared from RBL cells stably expressing the various GnRHRs. A, association of [^{3}H]TAK-013 to the various GnRH receptors was determined by a radioligand binding assay with approximately 1 nM [^{3}H]TAK-013 and 25 to 50 μg of membrane protein and incubated for 0.5 to 60 min at 37°C. Nonspecific binding was determined with 1 μM unlabeled TAK-013. The graph shows representative data with mean ± S.E. of duplicates from three independent experiments. B, dissociation of [^{3}H]TAK-013 from the various GnRH receptors was performed by incubating 25 to 50 μg of membrane protein with 2 nM [^{3}H]TAK-013 for 1 h at 37°C. Dissociation was initiated by the addition of 1 μM unlabeled TAK-013 for the times indicated on the graph. The data shown are the mean ± S.E. of three independent experiments.



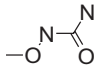
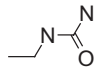
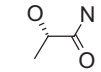
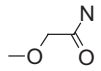
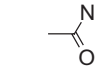
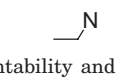
Compound	R group	% reduction of maximal response	K_b (nM)
TAK-013		28.4 ± 1.1	0.7 ± 0.1
A		47.0 ± 3.5	0.3 ± 0.04
B		3.4 ± 4.8	21.3 ± 4.9
C		1.3 ± 1.2	336 ± 71
D		-1.0 ± 2.8	169 ± 9
E		-4.6 ± 2.6	639 ± 137

Fig. 5. Insurmountability and functional binding affinities of TAK-013 and selected analogs. The core chemical structure of TAK-013 is shown with the various R group substitutions that generate the analogs used in this study. The percentage reduction of maximal response (insurmountability) was determined from inositol accumulation assays by comparing the maximal response in the absence and presence of 1 μM TAK-013, 1 μM analogs A and B, or 3 μM analogs C, D, and E. The functional affinities (K_b) were calculated as described under *Materials and Methods*.

in this study suggest that the NH_2 terminus (Met-24), ECL2 (Ser-203), and ECL3 (Leu-300) are critical contributors to the insurmountability and high-affinity binding of TAK-013. Previous work by Betz et al. (2006a) has shown that TAK-013 interacts with the NH_2-terminal end of TM7 and the COOH-terminal end of TM6. More precisely, the *N*-benzyl-*N*-methylamino substituent interacts predominantly with Tyr-290, the difluorobenzyl ring is inserted into the aromatic pocket adjacent to Tyr-284, and the methoxyurea interacts with both Asp-302 and His-306. In that study, it is noteworthy that the Leu-300 was not found to interact with the urea moiety, yet the results presented here clearly suggest that it is a requirement for the insurmountability of TAK-013. It is possible that Asp-302 and His-306 anchor the urea and provide the majority of the binding energy, and Leu-300 can then span the urea moiety (Fig. 6A) to stabilize the interaction, thereby decreasing the TAK-013 dissociation rate.

The other two residues implicated in the insurmountable property of TAK-013 are Ser-203, located in ECL2, and Met-24, located in the NH_2 terminus. Although both Ser-203 and Leu-300 are required for maximal insurmountability, Leu-300 seems to contribute the majority of the interaction necessary for insurmountable antagonism because the hGnRHR-S203P mutant still maintains the ability to suppress the E_{max} by 19.1%. We are unable to specifically model the interactions of Ser-203 and Met-24 with TAK-013 because they are located in ECL2 and the NH_2 terminus, which are too divergent from the structure of rhodopsin. In summary, these data suggest the hypothesis that whereas binding of TAK-013 to the GnRHR occurs at the TM regions, the NH_2-terminus, ECL2, and ECL3 provide the extra binding energy required for high-affinity binding and insurmountability.

Data from several studies provide evidence to integrate the role of the extracellular domains into an overall hypothesis of the structure of the hGnRHR and TAK-013 complex (Fig. 6D). The three-dimensional structure of bovine rhodopsin shows that the NH_2 terminus, ECL2, and ECL3 form a domain covering the extracellular entrance to the retinal binding site (Fig. 6C) (Palczewski et al., 2000). In addition, interactions between the extracellular domains and bound ligands have been shown for the dopamine D_2 (Shi and Javitch, 2004) and the V_{1a} vasopressin (Hawtin et al., 2003) receptors. Davidson et al. (1997) showed that the two disulfide bonds in the GnRHR (between TM3 and ECL2 and between ECL2 and the NH_2 terminus) introduce covalent constraints holding these regions together in the proximity of the transmembrane helical bundle. Furthermore, the Arg^8 in the native GnRH peptide can interact with the Asp-302 in ECL3 of the human GnRHR to stabilize its binding (Fromme et al., 2001). Finally, cross-linking experiments using photoreactive peptide ligands (both agonists and antagonists) have shown that they are in close proximity to the NH_2 terminus (Assefa et al., 1999). Thus, these studies suggest that the NH_2 terminus, ECL2, and ECL3 are in proximity to the ligand pocket as proposed in Fig. 6D.

If the extracellular region in the GnRH receptor (NH_2 terminus, ECL2, and ECL3) covers the extracellular entrance to the binding site as is observed in rhodopsin (Palczewski et al., 2000), then clearly, some open state of the receptor must also exist that allows ligand entry and exit. We hypothesize that this extracellular domain may undergo a

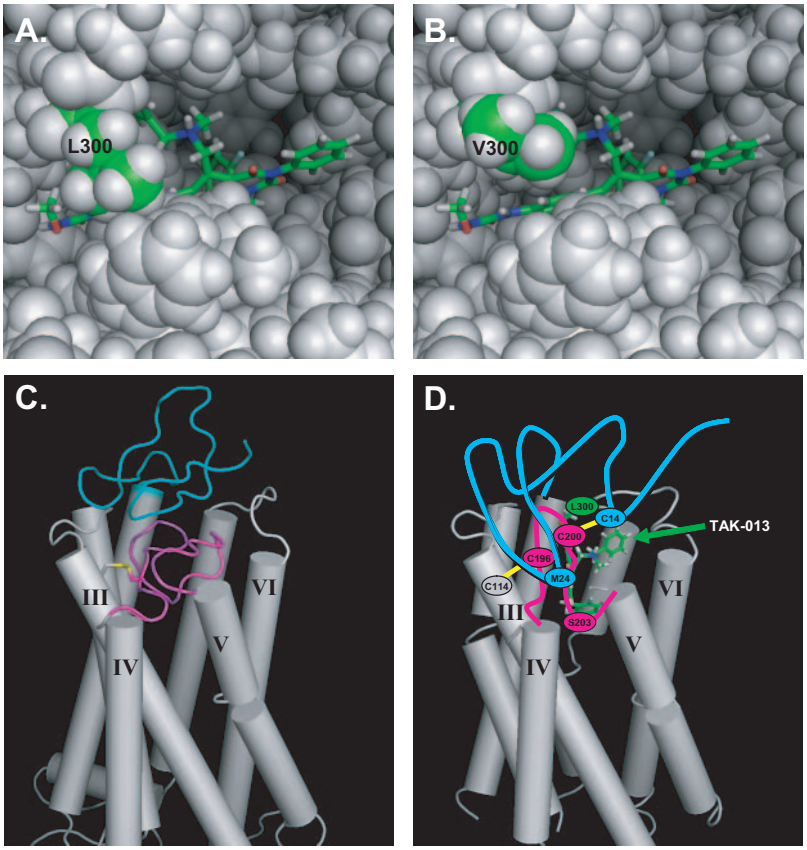


Fig. 6. Molecular modeling of human GnRH receptor with docking of TAK-013. A, homology model of hGnRHR with bound TAK-013. Diverse docking poses with varied conformations of TAK-013 within the binding site were generated using the MMFF94x force field implemented in MOE. The docking solution selected was consistent with experimental mutagenesis constraints (Betz et al., 2006a). Residue Leu-300 is shown with side chains colored green, and TAK-013 is shown as a stick representation. B, homology model of hGnRHR with Leu substituted with Val at position 300. C, three-dimensional structure of bovine rhodopsin (Palczewski et al., 2000). D, model of hGnRH receptor and its extracellular domains based on analogy to rhodopsin. The NH_2-terminal domain is shown in cyan, ECL2 in magenta, and disulfide bonds in yellow. TAK-013 is shown as a green stick representation, and the location of Leu-300 at ECL3 is shown in green.

conformational rearrangement, thus opening the active site to allow ligand binding. It has been shown previously that ligand binding to the 5-HT_4 receptor results in a conformational change in the conserved disulfide bond between TM3 and ECL2, the extent of which is dependent upon the amount of agonism or inverse agonism of the ligand (Banères et al., 2005). However, direct biophysical data for a conformational change in this region of the GnRH receptor is still lacking.

Applying this conceptual model to the present data, we view the extracellular domain as a "trap door" to the ligand binding site that must be open for TAK-013 to enter (Fig. 7). Upon TAK-013 binding to residues in the helical domain, the trap door closes and engages in additional interactions with the antagonist. Thus, the door is held closed by TAK-013. The strength of those interactions with the extracellular domain is in part determined by residues Ser-203 and Leu-300 in the human receptor and these interactions are weaker in the macaque receptor containing the Pro-203 and Val-300 residues. The energetic cost of opening the door in the ligand-bound structure results in a slow dissociation rate for the ligand and insurmountable antagonism. The lack of effect of these mutations on the association rate, is consistent with these residues being important for stabilizing the ligand bound state, not the open door state. Mutations that cause the trap door to be held less tightly closed in the presence of ligand (such as S203P or L300V) result in a more rapid dissociation and surmountable antagonism. Likewise, changes in the ligand, such as the urea moiety that interacts with His-306 and Asp-302 in TM7 and ECL3, would cause the door to be held less tightly to the TM region resulting in more rapid dissociation and surmountable antagonism.

It remains to be seen whether insurmountable characteristics of GnRH antagonists translate into important enhancements in clinical efficacy. It is reasonable, however, to expect that longer receptor occupancy provided by an insurmountable antagonist could contribute to the sustained reduction of the GnRH-mediated gonadotropin secretion. Moreover, the pulsatile nature of GnRH release results in transiently high concentrations of agonist that could effectively compete with a surmountable antagonist for receptor binding, but not with an insurmountable antagonist with prolonged receptor occupancy. It is important to note that several other receptor-based mechanisms may also play a role in a GnRH receptor antagonist's clinical efficacy. For example, the long-lasting effects of the peptide antagonist cetrorelix have been correlated with its down-regulation of receptor expression (Kovacs et al., 2001), and several small-molecule antagonists have been shown to enhance GnRH receptor expression by promoting the correct folding of intracellular receptors and trafficking them to the cell surface (Ulloa-Aguirre et al., 2004). Taken together, the work presented here could form the basis for a better understanding of the structural and mechanistic requirements for drug-insurmountable antagonism that could lead to the rational design of a new generation of highly efficacious GnRH receptor antagonists for the treatment of endometriosis, uterine fibroids, breast and prostate cancers.

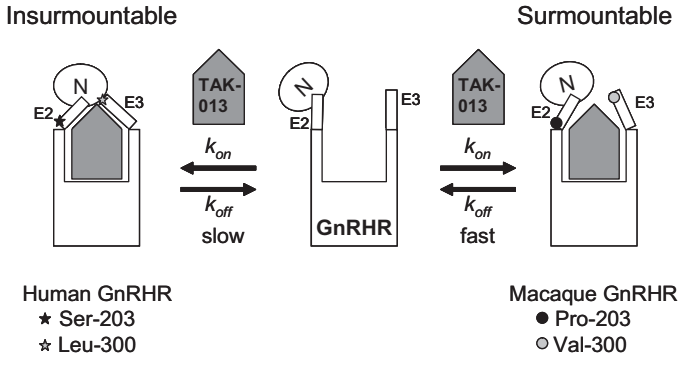


Fig. 7. The "trap door" model of GnRHR that leads to the insurmountable antagonism of TAK-013. The unbound GnRHR is shown in the open state, which is available for ligand entry. Evidence suggests that upon binding of TAK-013 to the helical transmembrane domain, the NH_2 terminus, ECL2, and ECL3 of the human GnRHR form a "trap door" by engaging additional interactions of Ser-203 and Leu-300 with TAK-013. These additional interactions result in the slow dissociation rate (k_{off}) of TAK-013 from the receptor and its functional insurmountability. The "trap door" in the macaque GnRHR contains Pro-203 and Val-300, which are unable to tightly interact with TAK-013, thus resulting in a looser conformation that is more permissive for the dissociation of the compound and surmountable antagonism.

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