Structure-Activity Relations of Successful Pharmacologic Chaperones for Rescue of Naturally Occurring and Manufactured Mutants of the Gonadotropin-Releasing Hormone Receptor

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ABSTRACT

We expressed a test system of wild-type (WT) rat (r) and human (h) gonadotropin-releasing hormone (GnRH) receptors (GnRHRs), including naturally occurring (13) and manufactured (five) “loss-of-function” mutants of the GnRHR. These were used to assess the ability of different GnRH peptidomimetics to rescue defective GnRHR mutants and determine their effect on the level of membrane expression of the WT receptors. Among the manufactured mutants were the shortest rGnRHR C-terminal truncation mutant that resulted in receptor loss-of-function (des235–327-rGnRHR), two nonfunctional deletion mutants (des237–241-rGnRHR and des260–265-rGnRHR), two nonfunctional Cys mutants (C229A-rGnRHR and C278A-rGnRHR); the naturally occurring mutants included all 13 full-length GnRHR point mutations reported to date that result in full or partial human hypogonadotropic hypogonadism. The 10 peptidomimetics assessed as potential rescue molecules (“pharmacoperones”) are from three differing chemical pedigrees (indoles, quinolones, and erythromycin-derived macrolides) and were originally developed as GnRH peptidomimetic antagonists. These structures were selected for this study because of their predicted ability to permeate the cell membrane and interact with a defined affinity with the GnRHR receptor. All peptidomimetics studied with an IC50 value (for hGnRHR) ≤2.3 nM had measurable efficacy in rescuing GnRHR mutants, and within a single chemical class, this ability correlated to these IC50 values. Erythromycin-derived macrolides with IC50 values as high as 669.5 nM showed efficacy as rescue compounds. The ability to rescue a particular receptor was a reasonable predictor of the ability to rescue others, even across species lines, although particular mutants could not be rescued by any of the drugs tested.

Disease-causing receptor mutations are widely believed to lose function as a result of inability to engage in receptor-ligand or receptor-effector binding interactions. Recent observations challenge this view and suggest that receptor misfolding and subsequent misrouting is a mechanism that results in loss of receptor function (Conn et al., 2002; Janovick et al., 2002; Lean˜os-Miranda et al., 2002). We recently reported (Janovick et al., 2002) a pharmacologic stratagem relying on an antagonistic peptidomimetic to correct the routing of a naturally occurring mutant GnRHR and correctly route it to the plasma membrane. This “pharmacoperone” could then be removed and the rescued GnRHR was shown to bind ligand with similar specificity and affinity as the WT receptor and couple to its effector protein. Subsequently, the arresting observation was made (Lean˜os-Miranda et al., 2002) that 11 of the 13 reported human mutants of the GnRHR that cause hypogonadotropic hypogonadism (Janovick et al., 2002; Lean˜os-Miranda et al., 2002) could be rescued by this approach, as could many “manufactured” mutants (truncations, deletions, and Cys substitutions), suggesting the generality of this approach. Based on this and a similar observation for the V2 receptor (Morello et al., 2000), we proposed (Conn et al., 2002) that this approach might be generally applicable to correcting diseases for which the etiology is misrouted or misfolded proteins, such as cystic fibrosis, nephrogenic diabetes insipidus, hypercholesterolemia, cataracts, Alzheimer’s, retinitis pigmentosa, and others (Belotti et al., 1999; Brooks, 1999; Radford and Dobson, 1999; Deen et al., 2000; Kopito and Ron, 2000; Morello et al., 2000; Sanders and Nagy, 2000; Dobson, 2001; Ellgaard and Heleneius, 2001; Gregersen et al., 2001; Hammarstrom et al.,

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ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; h, human; r, rat; WT, wild-type; IP, inositol phosphates.
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Materials and Methods

Natural sequence GnRH (NIDDK National Hormone and Peptide Program, Bethesda, MD) and the GnRH agonist buserelin ([3α,4α,5α]-des-Gly10, Pro2, ethylamide-GnRH (Hoescht-Roussel Pharmaceuticals, Somerville, NJ)) were obtained as specified. The following chemical structures (collectively referenced as “the drugs”) were used: those of the quinolone class are prefaced by the letter “Q” and those of the indole class by the letters “In” and were produced by Merck and Company ( Rahway, NJ) (Ashton et al., 2001a, b); Q98 [7-chloro-2-oxo-4-[2-[(2S)-piperidin-2-yl]ethoxy]-N-pyrimidin-4-yl-3-(3,4,5-trimethylphenyl)-1,2-dihydroquinoline-6-carboxamide], Q76 [N-[7-chloro-3-(3,5-dimethylphenyl)-2-oxo-4-[(2S)-piperidin-2-yl]ethoxy]-1,2-dihydroquinolin-6-yl]-N-cyclopropylurea], Q80 [(2S)-2-(2-[7-chloro-6-(6,7-dimethoxy-3,4-dihydroisquinolin-2(1H)-yl]carbonyl]-3-(3,5-dimethylphenyl)-2-oxo-1,2-dihydroquinolin-4-yl]oxyl(ethyl)piperidin trifluoroacetate], In30 [(2S)-2-[(2-[2-azabicyclo[2.2.2]oct-7-yl]-2-methyl-2-oxoethyl)-2-[3,5-(dimethylphenyl)]-1H-indol-3-yl]-N-[2-(4-methylsulfonyl)phenyl]ethyl]propan-1-amine], In31b [(2S)-N-[2-(4-methylphenyl)ethyl]-2-[5,1-dimethyl-2-(4-methylisopropenyl-1-yl)-2-oxoethyl]-2-[3,5-(dimethylphenyl)]-1H-indol-3-yl]-N-(2-pyridin-4-ylethyl)propan-1-amine). Erythromycin-derived macrolides were prepared by Abbott Laboratories (Bush et al., 1999; Diaz et al., 1999) and are prefaced by the letter “A”. They are as follows: A-7662.0 (erythromycin A), A-6755.0 [11-deoxy-11-[carboxy-phenylethylamino]-6-O-methyl-erythromycin A 11,12-(cyclic carbamate)]; A-17777.5 [3′N-desmethyl-3′,N-cyclopentyl-11-deoxy-11-[carboxy-(3,4-dichloro-phenylethylamino)]-6-O-methyl-erythromycin A 11,12-(cyclic carbamate)], A-22590.9 [3′,3′-N-desmethyl-3′,3′-N-cyclopentylmethyl-11-deoxy-11-[carboxy-(3,4-chloro,4-fluoro-phenylethylamino)]-6-O-methyl-erythromycin A 11,12-(cyclic carbamate)], Dulbecco's modified Eagle's medium, OPTI-minimal essential medium, lipofectamine, and polymerase chain reaction reagents (Invitrogen, Carlsbad, CA) were obtained as indicated. Restriction enzymes, modified enzymes, and competent cells for subcloning were purchased from Promega (Madison, WI). Other reagents were of the highest degree of purity available from commercial sources.

Vector Construction. WT hGnRHR cDNA in pcDNA3 was subcloned into pcDNA3.1 at KpnI and XbaI restriction enzymes sites. All the GnRH mutants were constructed, sequenced, and prepared as previously described (Janovick et al., 2002). The WT human and rat GnRH receptor (Janovick et al., 2002; Leanos-Miranda et al., 2002) and naturally occurring mutants of the hGnRHR, associated with human hypogonadotropic hypogonadism (Achermann and Jameson, 2001), were N148K, T217I, E38K, Q108R, A259D, R238H, S168R, C206Y, S217R, R228Q, L266R, C277Y, and Y284C. Manufactured mutants of the GnRH receptor include the shortest rat GnRH c-terminal truncation mutant that results in receptor loss of function (des235-327-GnRH) (Brothers et al., 2002), two deletion mutants [des237-241-GnRH, which has been rescueable previously (Janovick et al., 2002), and des260-265-rGnRH, which has not been rescueable previously (Janovick et al., 2002)], and two Cys mutants [C229A-GnRH, which was not rescueable previously with the indole IN3, and C277A-GnRH, which was rescued with this agent (Janovick et al., 2002).]

Transient transfection of COS-7 cells. Wild-type (WT) hGnRHR and altered receptors were transiently expressed in COS-7 cells as described (Leanos-Miranda et al., 2002). Cells were transfected with 0.05 μg of DNA/well (for inositol phosphates (IP)) or 0.1 μg of DNA/well (for saturation binding studies) containing 1% DMSO (vehicle) or 1 μg/ml of each indole, quinolone or erythromycin macrolide, prepared in the vehicle. The quantification of IP production and saturation binding were made 27 h after the start of transfection. From transfection until 18 h before assay, drug was present; during the last 18 h, drug was not present. The quantification of IP production and saturation binding were made as described (Huckle and Conn, 1987).

Statistics. The data shown are the means ± S.E.M. from triplicate (IP) determinations. For clarity in three-dimensional graphs, the S.E.M. bars were omitted. In all experiments, the standard deviation was typically less than 10% of the corresponding mean. Each experiment was repeated three or more times with similar results. The results shown are from a single experiment.

Results

Figures 1 (indoles), 2 (quinolones), and 3 (erythromycin macrolides) show the efficacy (assessed by IP production) of each of the drugs tested as pharmacoperones of a single mutant, E38K. This mutant was selected because of its low basal expression in the absence of the first drug examined, IN3, and its pronounced response (both IP production and radioligand binding) to rescue by IN3 (Janovick et al., 2002). For each chemical class, the data are presented with the lowest IC50 value (for the hGnRHR, shown in figures) first. The data indicate that a concentration of 1 μg/ml of DNA is necessary for IP production and saturation binding were made as described (Huckle and Conn, 1987).

Figures 4 (indoles), 5 (quinolones), and 6 (erythromycin macrolides) show the effect of the 1 μg/ml concentration of each of the drugs in rescuing each component of the receptor. In each figure, the data shows IP production in the presence of drug and 10−7 M buserelin. For reference, Fig. 7 shows the unrescued coupling of the receptor in the absence (Fig. 7, upper image) or presence (Fig. 7, lower image) of 10−7 M buserelin. In the absence of buserelin none of the rescue agents produced a response greater than basal levels for any vector. The member of each drug class with the lowest affinity for the human GnRHR is oriented closest to the viewer. This data allows the following conclusions to be made: 1) The efficacy of these drugs (measured by the ability of a fixed dose to rescue receptor) is consistent with the binding affinity of each class of molecule for the WT receptor; 2) peptidomimetics that were successful in rescuing one mutant usually rescued all mutants (that could be rescued by any of the peptidomimetics); and 3) particular mutants (human: S168R and S217R; rat des260-265 -GnRH and C229A-GnRH) could not be rescued by any drug tried, suggesting that these were...
either grossly deformed or that loss-of-function was due to inability to bind ligand.

**Discussion**

In the present study, we used a palette of WT and loss-of-function mutants of the GnRHR. These were selected as representatives of receptor truncations, sequence deletions, and point mutations at Cys residues, as well as all point mutants that have been reported to cause hypogonadotropic hypogonadism in humans (Achermann and Jameson, 2001; Janovick et al., 2002; Leaños-Miranda et al., 2002). This palette was used to assess the efficacy of chemically distinct drugs as pharmacoperones, structures that serve as molecular scaffolding, cause mutants to -fold correctly, and thereby avoid detection by the cellular quality control apparatuses. Our data indicate that structures from all three different chemical classes can rescue defective mutant folding and allow defective mutants to route to the plasma membrane, bind ligand, and couple to effectors. Accordingly, exploitation
of this approach is not a unique feature of the indole class, a member of which class provided the first proof of principle for rescue of hypogonadotropic hypogonadism-causing mutations (Janovick et al., 2002). Efficacy of these drugs (measured by the ability of a fixed dose to rescue receptor) is proportional to the binding affinity of the molecules for the WT receptor. Particular mutants (human: S168R and S217R; rat des\textsuperscript{260–265}GnRHR, and C\textsuperscript{229}A-GnRHR) could not be rescued by any drug tried, suggesting that these were either grossly deformed or that loss-of-function resulted from the inability to bind ligand. Because addition of green fluorescent protein or HA-tags in themselves result in rescue, we have not been able to determine whether these particular loss-of-function mutants reside at the plasma membrane.

We were initially surprised by the lack of rescue specificity for different drugs (that is, all effective agents rescued virtually the same mutants). Because they were designed as GnRHR peptidomimetics, however, all of the drugs examined would be expected to compete with the natural ligand for these receptors, even though the precise locus of binding may...
differ slightly. Accordingly, it is conceivable that they serve as similarly located nuclei, stabilizing the ligand binding site of the mutants. This observation also leads to the consideration that the ability of the mutants to escape degradation by the quality control apparatus of processing may be, as an evolutionary matter, related to stability of the natural ligand binding site.

Alternatively, pharmacoperones that do not compete for the natural ligand binding site can certainly be envisioned since the existence of molecule-stabilizing allosteric interactions suggests that such compounds would exist (Conn et al., 2002). The ability to bind outside the natural ligand binding site may make such molecules advantageous as therapeutic agents because it might not be necessary to remove these before activating the receptor with an agonist. It is conceivable that such agents might show a more heterogeneous pattern of mutant rescue by interacting at diverse sites than the heterogeneous pattern measured for the peptidomimetics in the present study. Likewise, shipwrecking (Conn et al., 2002) compounds that interfere with the structure of wild type receptors may be highly heterogeneous for the same reason.

It is notable that the normal expression of the human GnRHR, but not the rat GnRHR, can be increased by the agents that were successful in rescuing receptors. It has become clear that the presence of Lys191 in the primate sequence (Arora et al., 1999; Maya-Núñez et al., 2000, 2002) limits the percentage of the synthesized receptor that reaches the plasma membrane to about 50% (Conn et al., 2002). The pharmacoperones function to override this inhibition, presumably due to structural stabilization, but are unable to do so in the rodent sequence, which lacks this “extra” amino acid and is already more efficiently routed to the membrane. The ability of all the rescue agents with high

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**Fig. 5.** Quinolones show the effect of the 1 μg/ml concentration of each member of the specified class of compounds in rescuing each component of the receptor palette. This figure shows IP production in the presence of 10^{-7} M buserelin. The transfection of cells and measurement of IP production occurred as described under Materials and Methods.

**Fig. 6.** Erythromycin macrolides show the effect of the 1 μg/ml concentration of each member of the specified class of compounds in rescuing each component of the receptor palette. This figure shows IP production in the presence of 10^{-7} M buserelin. The transfection of cells and measurement of IP production occurred as described under Materials and Methods.
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Cystic fibrosis, nephrogenic diabetes insipidus, hypercholesterolemia, retinitis pigmentosa, cataracts, Alzheimer’s, and other diseases (Conn et al., 2002). The data in this study provide the basis for structural design of pharmacoperones, as well as for “shipwrecking” agents (Conn et al., 2002), that cause the misrouting of proteins that would, in their absence, route correctly.

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References
Maya-Núñez G, Janovick J, Ultos-Aguirre A, Soderlund D, Conn PM, and Mendez JP

Fig. 7. This figure shows the unrescued (no drug) coupling of receptor in the absence (upper image) or presence (lower image) of 10−7 M buserelin.


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