Pharmacoperone identification for therapeutic rescue of misfolded mutant proteins

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INTRODUCTION

Mutations in G protein-coupled receptors (GPCRs) are known to be responsible for over 30 disorders, including cancers, heritable obesity, and endocrine disease. Normally, nascent GPCRs are subjected to the stringent quality control system (QCS) of the endoplasmic reticulum (ER); this system consists of both protein chaperones that promote correct folding by catalyzing the folding process or may retain misfolded proteins. The QCS ensures that only correctly folded proteins enter the pathway leading to the plasma membrane (PM). Because of this, point mutations may result in the production of misfolded and disease-causing proteins that are unable to reach their functional destinations in the cell since they are retained by the QCS even though they may retain (or regain) function when properly trafficked.

The functional rescue of misfolded mutant receptors by small non-peptide molecules, originally screened from chemical libraries to serve as receptor antagonists, has now been demonstrated (Conn et al., 2007); these small molecules (pharmacological chaperones or “pharmacoperones”) serve as molecular templates, promoting correct folding, and allowing the mutants to pass the scrutiny of the QCS and be expressed at the PM (or, in some cases, other cellular loci) where they may function normally. It is important to note that the QCS is not protein-specific; it recognizes general aspects of misfolding (e.g., exposure of hydrophobic plates in aqueous environments or unpaired sulphydryl groups in cysteines), frequently with relatively low affinity. Accordingly, GPCRs that retain ligand binding and effector coupling, but are recognized as misfolded by such general criteria, can be misrouted to the ER. Their rescue with pharmacoperones leads to proper folding, passage through the QCS, restoration to the proper site, and return of function.

In principle, the pharmacoperone rescue approach may apply to a diverse array of human diseases that result from protein misfolding – among these are cystic fibrosis (misfolding of the cystic fibrosis transmembrane conductance regulator, a transporter class ion channel that transports chloride and thiocyanate ions; Dormer et al., 2001; Galiotta et al., 2001; Zhang et al., 2003; Amaral, 2006), hypogonadotropic hypogonadism [HH, The gonadotropin-releasing hormone (GnRH) receptor (GnRHR); Ulloa-Aguirre et al., 2003], nephrogenic diabetes insipidus (Morello and Bichet, 2001; Bernier et al., 2000; Bichet, 2006), retinitis pigmentosa (rhodopsin; Noorzwez et al., 2004), hypercholesterolemia (LDL receptor), cataracts (cystallines; Benedek et al., 1999), neurodegenerative diseases (Huntington’s, Alzheimer’s, Parkinson’s; Heiser et al., 2000; Soto et al., 2000; Forloni et al., 2002; Permanne et al., 2002; Muchowski and Wacker, 2005), and particular cancers (Peng et al., 2003). In the case of certain proteins (e.g., the GnRHR, V2R, and rhodopsin), this approach has succeeded with a striking number of different mutants (Conn et al., 2007), supporting the view that pharmacoperones will become powerful weapons in our therapeutic arsenal (Conn et al., 2007).

It has also become clear that variable (but significant) amounts of even some wild-type (WT) GPCRs are misrouted (i.e., frequently retained in the ER), apparently as a result of misfolding...
3. The physiology of the system is well-characterized in many

1. Gonadotropin-releasing hormone receptor itself is among the smallest GPCRs (328 amino acids in the human). There are technical advantages to small proteins since these require fewer primers for synthesis and for sequencing than do larger GPCRs (typically at least twice the size of the GnRHR), and there is less sequence length that might lead to random mutation during the PCR process. DNA sequencing of mutants is also cheaper than for a larger protein. The size of the GnRHR means that there are fewer domains to consider in identification of important structural motifs and because the size of hydrophobic domains is relatively constant even in different GPCRs (due to the relatively constant size needed to traverse the membrane), the ratio of these to non-hydrophobic regions is relatively high in the GnRHR due to the short NH₂- and COOH-terminals. This promotes the interaction of small hydrophobic molecules that dissolve in the membrane.

2. The ligand (GnRH) itself and analogs are small and have good thermal and chemical stability which has led to the availability of thousands of agonists and antagonists in the literature; these have been used for ¹²⁵I-radioligand assays and coupling of markers (colloidal gold, fluorescein, Texas Red).

3. The physiology of the system is well-characterized in many animal models and there is a broad basis for understanding processing differences now known to occur in closely related species (Knollman et al., 2005). We have taken advantage of the large number of GnRHR sequences that are available and been able to determine how changes in routing have been impacted by sequence changes (i.e., natural mutation; Janovick et al., 2006). Among primate receptors, for example, there are specializations that make these receptors able to decode frequency-modulated, as well as amplitude-modulated, signals (Janovick et al., 2007b), and note structural changes among particular animals that appear to be explained by reproductive specializations (Knollman et al., 2005; Janovick et al., 2006, 2007b).

The human GnRHR, unlike the rat and mouse counterparts, appears “balanced” in its distribution between the PM and ER (Conn et al., 2006a,b), so much that about 50% of the human WT GnRHR (in cells transfected with the corresponding sequence) is retained in the ER and can be “rescued” by the approach described above. At first consideration this appears to be an inefficient use of newly synthesized protein; however, the strong and convergent evolutionary pressure for this suggests a regulatory advantage (Conn et al., 2006a,b; Ulloa-Aguirre et al., 2006). This system offers the ability to examine the evolution of the QCS system since these receptors have been cloned from a wide range of animals (fish, birds, reptiles, many mammals, and multiple primates; Janovick et al., 2003b).

4. A great deal of information is also available regarding the cellular mechanism of action and trafficking of the GnRHR (Jennes et al., 2009) as is substantive information on the mechanism of misfolding (Conn et al., 2006a,b), mutant interactions with pharmacoperones (Ulloa-Aguirre et al., 2003) and the molecular basis of the dominant-negative effect (Conn et al., 2006a,b). Recent studies (Janovick et al., 2007a) indicate that the mutant receptor that is already trapped in the ER can be freed by pharmacoperones — a surprising result that increases the potential therapeutic reach of this approach since pharmacoperones do not need to be present at the moment of receptor synthesis.

**PHARMACOPERONES**

A pharmacoperone (from “pharmacological chaperone”) is a small molecule that enters cells and serves as a “molecular scaffold” to promote correct folding of otherwise-misfolded mutant proteins within the cell (Conn et al., 2002; Janovick et al., 2002). Misfolded proteins are often retained by the cellular QCS of the ER. For this reason, they do not reach their normal site of function (Bernier et al., 2004; Ulloa-Aguirre et al., 2004). This event may result in disease (Castro-Fernandez et al., 2005). Pharmacoperones can rescue misfolded receptor mutants and restore them to function, which is a potentially useful therapeutic approach when the target is a misfolded/misrouted protein. The literature was summarized for the GnRH and vasopressin type 2 (V2) receptors pharmacoperones with a view toward moving these compounds in vivo (Conn et al., 2007). Science writers commenting on this approach (Hunter, 2004; Kresge, 2004) have observed that rescue with pharmacoperones is a viable “alternative (to gene therapy)” since it serves as a means of “skirting gene therapy to correct genetic defects.” This view is supported by the consideration that correction of defective protein folding appears significantly less challenging than replacement of a defective gene (or gene product) by a perfect one. One could, in fact, envision drugs given in a prophylactic manner (in vitamins, for example) that prevent the misfolding that leads to neurodegenerative disorders (Bellotti et al., 2007; Sun et al., 2007) and cataracts. In this regard, diseases may be prevented before clinical signs present themselves.

In addition to controlling the trafficking of mutants, this class of drugs may be useful for regulation of the PM expression of WT proteins since, for some PM receptors, a percentage of the nascent WT proteins also undergo misfolding (examples in: Conn et al., 2006a) and are retained; this event may serve a cellular regulatory...
role (Conn et al., 2006a,b). Pharmacoperones can be used to increase PM expression of these, as well, and offer a therapeutic means of regulating these GPCRs. Accordingly, pharmacoperones can be viewed as agents that rescue misfolded mutants, but are also potentially able to increase the PM expression of WT proteins.

PROBLEMS WITH EXISTING PHARMACOPERONES AND SCREENING TECHNIQUES

One problem with in vivo use of known pharmacoperones is that almost all existing ones for GPCRs are peptidomimetic antagonists directed against a site near that for the native ligand. This is because existing pharmacoperones, including In3 described below, were selected from assays that identified antagonists with target specificity. In3 is highly specific for the GnRHR and was screened for more than 100 other structures, including α-adrenergics, β-adrenergics, adenosine receptor, bradykinin, CB1 and CB2, dopamine receptors, neurokinins, prostanoid receptors, serotonin receptors, somatostatin, calcium, sodium, potassium channels, monoamine oxidases, and several phosphatases. There are a few exceptions to the use of antagonists-only as pharmacoperones: one agonist has been used for this purpose (Petaja-Repo et al., 2002) and one molecule has been used that does not appear to compete for the agonist or antagonist binding site (Janovick et al., 2009b). The latter was identified by our group, based on a fortuitous observation, not a screening process. The “overlap” of the majority of the pharmacoperones with the binding site means that there will likely be competition in vivo that will necessitate episodic administration and washout when used in vivo. From a therapeutic point of view, this presents a hurdle since oral dosing, while desirable (and possible with small molecules), is more difficult (there must be a washout period to prevent the pharmacoperone from competing with the endogenous ligand). It has certainly not been established that binding at or near the binding site of the natural ligand is a necessary pre-requisite for pharmacoperone activity and there is extant information to suggest otherwise (Conn and Janovick, 2009; Janovick et al., 2009a,b). This would, in fact, be an unexpected requirement since one could imagine pharmacoperones that might stabilize the correctly routed form of the receptor and not show any antagonism. Accordingly, identification of non-antagonistic pharmacoperones is a reasonable goal.

Since most high throughput screens (HTS) approaches in the pharmaceutical industry revolved around assay models that identified agonists and antagonists, pharmacoperones that were not also agonists or antagonists would have been missed. Accordingly, rethinking the nature of the assay is important both to identify such overlooked structures, but also because they may prove to be more useful and therapeutically facile to use.

It is also worth noting that use of pharmacoperones drugs may unexpectedly alter physiological regulation. As an example, while correction of decreased LH in the male due to the E90K mutations in the GnRHR is likely to be straightforward, such a correction in females may be quite difficult, due to physiological cyclicity and elaborate feedback mechanisms.

NEW SCREENING TECHNIQUES

We have recently developed a new approach for the identification of pharmacoperones for hGnRHR mutants based on increase of activity of model mutants.

Primary screen

The primary screen for pharmacoperones consists of measuring gain of activity in stable HeLa cells expressing the indicated mutant (hGnRHR[E90K]) under control of the tetracycline-controlled transactivator; the parent line was kindly supplied by Dr. Peter Seeburg (Max-Planck-Institut für Medizinische Forschung). Preparation of this mutant has been described (Janovick et al., 2002). The DNA was inserted in the host cell according to published methods (Krestel et al., 2001).

Negative screen

This assay is important in order to preclude moving “false” positives through the process. Stable cell lines which contain the tetracycline-controlled transactivator, which shuts the gene off in the presence of this antibiotic are used (Krestel et al., 2001). There is literally no measurable expression, as assessed by protein expression or real-time PCR in the presence of tetracycline because the mutant GPCRs are under the control of this transactivator. Accordingly, cells cultured in the presence of tetracycline are substantially identical to the primary screen, but lack expression of the target gene and gene product. These cells (in the presence of tetracycline) will serve as an excellent negative control line. In both cases, coupling to second messenger is the measured endpoint.

Candidate compounds could also be screened in a radioligand assay (Janovick et al., 2007b) to determine if they compete with receptor agonists and antagonists. Other routine necessary negative controls, secondary screens, counter-screens and tests for specificity, range, linearity, accuracy, precision, limit of detection and limit of quantitation, robustness, reagent stability, and otherwise are not described in this manuscript due to space limitations.

We expect this assay to identify as “specific” other proteins that alter cellular machinery (e.g., ubiquitin ligases, heat shock proteins). It will be necessary to subject hits to a qualifying assay with a non-GnRHR GPCR to exclude such false positives.

SELECTION OF THE MUTANTS

GnRHR mutant

The naturally occurring E90K mutation was selected. Modeling studies (Janovick et al., 2003a) for the hGnRHR and the data (Janovick et al., 2002, 2003a; Knollman et al., 2005) support the view that the E90–K121 salt bridge is a fundamental and evolutionarily conserved determinant required for correct protein trafficking to the PM in all mammals examined. This bridge, linking TMS2 to TMS3 is highly conserved in the GnRHR. E90 is conserved in mammals. Even in non-mammals, the TMS2–TMS3 salt bridge is needed and may have been formed by a D98–K121 bridge. D98 is absolutely conserved in all mammalian, reptilian, avian, and piscine GnRHRs sequenced to date. K121 is conserved in the same groups; in fruit flies, the residue is also a conservative change R121. A negative amino acid in TMS2 is also found in all other GPCRs (Ni and Lee, 2007) of the B-class, except the glucagon receptor. Because this salt bridge is a basic requirement for correct routing, E90K results in a routing defect in both mouse and human GnRHR (Brothers et al., 2004; Knollman et al., 2005; Janovick et al., 2007a). This leads to full, but resuable, ER-retention (Brothers et al., 2004) and the predicted phenotype. While it is true that the orthologs of many misrouted human mutants are correctly routed in the mouse.
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Advantages and use of the stably transfected “tta” cells as an assay for pharmacoperones

We use HeLa cells for the basis of our screen; such cells are human in origin and contain the same major (endogenous) chaperone proteins that are found in most human cells (Lievremont et al., 1997; Feder and Hofmann, 1999; Ravindran et al., 2005); hGnRHR is processed, expressed, and coupled properly in these cells (Knollman and Conn, 2008) and they are adherent and well-adapted to cell culture. A special feature of the cell lines used (Krestel et al., 2001) is that the GPCRs or GPCR mutants are expressed under the control of the tTA (tetracycline-controlled transactivator). In the absence of tetracycline, the GPCR is expressed. In the presence of tetracycline, the GPCR is not measurably expressed. This model allows use of the GPCR to measure signal in the HTS (i.e., no tetracycline).

Cell culture and RNA extraction for real-time PCR

Stably transfected HeLa (tTA) cells were stably transfected with hE90K and then plated at 200,000 cells per well in a 6-well culture plate for extracting RNA. The cells were grown in DMEM/10% fetal calf serum, containing gentamicin, in a humidified atmosphere at 37°C. To measure the time needed to “turn off” the gene, 1 μg/ml Tetracycline was added to each well and remained on the indicated cells for a maximum of 12 h. Cells were examined at differing times by dumping off medium, blotting, placing on ice, and washing two times with 2.5 ml 1× PBS (ice cold). Then 1 ml TRIZOL reagent is added per well and followed according to the manufacturer’s protocol. The cells were pipetted seven times through an RNase free pipet tip to lyse and transferred to a 1.5-ml RNase free centrifuge tube and frozen at −80°C until the total RNA was extracted. After extracting the RNA, it was dissolved in 25 μl diethylpyrocarbonate-treated water.

Real-time PCR

The TaqMan assay was used with the TaqMan PCR Core Reagent Kit with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The full length sequence of the human GnRH receptor was used to design the primers and probe for the real-time PCR assays for the mutant E90K. The TaqMan probe for the GnRHR mutant E90K was synthesized by Applied Biosystems; 5′-FAM-CTCCCTGGCTATCACGAGGCCCCTAG-MGB-3′. For the internal control, 18S RNA, ABI’s MGB probe was used in the TaqMan assay.

The following evidence suggests that this screen will be extremely valuable:

- RNA for the GPCR mutant is expressed under the control of tetracycline. Stable cells were cultured in the presence of tetracycline for varying amounts of time in order to show a time course for complete extinction of expression of the E90K mutant. Total RNA was extracted from the cells followed by reverse transcription (RT) for obtaining single strand DNA for use in real-time PCR. One microgram of total RNA was used for RT to generate single strand DNA. Relative quantitative standard curves for E90K and 18S RNA (the internal housekeeping gene product used to normalize the data or calculate a ratio between the gene of interest and the endogenous and unchanged standard) were generated by serial dilutions of 1:10 of the RT product ranging from 1 to 10 ng. Ct values (Ct, the cycle threshold, is the cycle at which the fluorescence from a sample crosses the set threshold) were obtained, averaged, and converted to (logarithm) nanograms using the straight line extrapolation equation: \( (\text{ave Ct value} - b) / m \); \( b \) = y-intercept of standard curve line, \( m \) = slope of standard curve line.

The ratio of E90K to 18S RNA was determined. The data (Figure 1) show that by 12 h in the presence of tetracycline, the RNA for hGnRHR[E90K] is no longer detectable.

- The rescuable GPCR mutant is under the control of tetracycline and rescuable by pharmacoperone, In3. In this study (Figure 2), 20,000 stable cells/250 μl were cultured (or rat; Knollman et al., 2005), E90K behaves similarly in mouse and human sequences (Knollman et al., 2005); the absence of K191 is without impact (Knollman et al., 2005). Further, in cell expression studies, mouse E90K, like the human mutant can be rescued by pharmacoperones (Knollman et al., 2005).

![Figure 1](https://via.placeholder.com/150)

**FIGURE 1** | Tetracycline inhibits accumulation of E90K mRNA (normalized with 18S RNA) and assessed by real-time PCR.

![Figure 2](https://via.placeholder.com/150)

**FIGURE 2** | Loss of IP production (in the presence of tetracycline) in stably transfected cells with or without In3 treatment shows that expression of the (rescued) mutant protein is under control of this antibiotic.
Figure 3 | Well-by-well IP stimulation in response to a GnRH agonist, following rescue of mutant E90K by maximum, EC80, or no pharmacoperone (In3).

Data is graphed by row, then column. The upper graphic shows the absence of tetracycline; the lower graphic shows the presence of tetracycline. Note that the y-axis differs.

In the presence of increasing concentrations of tetracycline, which was continuously present thereafter, for a total of 48 h. Cells (48-well plates) were treated with or without pharmacoperone In3 (“In3” is our lab name for pharmacoperone, [(2S)-2-[[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxoethyl]-2-(3,5-dimethylphenyl)-1H-indol-3-yl]-N-(2-pyridin-4-ylethyl)propan-1-amine], which was originally developed as a specific antagonist of the GnRHR (Ashton et al., 2001). The pharmacoperone is then removed and cells labeled with 3H-inositol for 18 h and then stimulated with 10^-6 M Buserelin and total inositol phosphate (IP) was then measured by ion exchange chromatography (Huckle and Conn, 1987). These studies show that stimulation of IP production in response to the GnRHR agonist, Buserelin, is lost in the presence of tetracycline and that the known pharmacoperone In3 can rescue this mutant with an excellent signal-to-noise ratio (Figure 2). The IP assay, using disposable columns, resin, and a skilled operator can conveniently assay 1,000 samples in a day.

In order to perform (i) a preliminary assessment of the assay performance and (ii) demonstrate the ability to perform the assay in 96-well plates, the study, shown in Figure 3, was performed (all wells in 1% DMSO). This study also (iii) shows the utility of cells treated with tetracycline (which extinguishes expression of the mutant hGnRHR gene) as a negative screen. The upper frame in Figure 3 demonstrates that positives can be identified with a good sensitivity and high signal-to-noise ratio, with minimal wall effects and good reproducibility.

In this study, stable cells were cultured in the absence (upper frame) or presence (lower frame) of tetracycline which was continuously present thereafter, as described above (“b”). Pharmacoperone was added at the indicated concentrations in 1% DMSO final. Total IP was measured. The x-axis was numerically compressed for this image and is labeled 0–32. There are 32 (interspersed) wells of either DMSO only (DMSO control, green), an EC80 dose of pharmacoperone In3 in 1% DMSO (red) or a saturating dose of In3 in 1% DMSO (black); 3 x 32 = 96. Every third well contained DMSO, EC80 dose of pharmacoperone In3, or a saturating dose of pharmacoperone In3 so that the next row of wells contained the same treatment diagonally. In Figure 3, rescue was performed on cells in the absence of tetracycline (which allows the receptor mutant to be expressed). This would serve as the primary screen.

In the lower image of Figure 3, tetracycline was present and, accordingly, the gene for the hGnRHR mutant was shut off. This would serve as a negative screen for agents that affect their action by some means other than through the hGnRHR. In that image, there is no significant or reproducible response difference between cells that have been incubated with pharmacoperone In3 because the gene for the mutant receptor is not expressed. Agents that act through a means other than the hGnRHR would be expected to still respond since, other than lacking the hGnRHR (mutant), the cells are otherwise identical.

Figure 4 shows the same data expressed in a “column then row” format instead of the “row then column” format above. This shows, by inspection, the absence of wall effects. These are aberrant effects sometimes observed for cells plated in a well adjacent to an edge or corner of a culture plate.

CONCLUSION
While pharmacoperones may also be agonists or antagonists and are frequently selected from HTS assays for such drugs, this is not a requirement. Pharmacoperones have the potential to override normal physiological levels of receptors and other proteins. This approach may result in altered regulation and may change the number of receptors present at the PM by rescuing misfolded mutants or increasing PM expression of fractionally expressed WT receptors. Accordingly, the screening approach we describe is useful for treatment of diseases in which the underlying cause is loss of receptors due to mutation and subsequent misrouting (retention in the ER). This is a novel screen that may lead to identification of pharmacoperones that do not compete with the endogenous ligands, since these agents may have a therapeutic advantage. In
principle this approach can form the basis of an HTS for the identification of pharmacoperone drugs for mutant GPCRs, enzymes, or ion channels. Because there are a paucity of such drugs known, the ability to identify them in chemical libraries is one of the keys to moving this approach forward.

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