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Transitioning Pharmacoperones to Therapeutic Use: *In Vivo* Proof-of-Principle and Design of High Throughput Screens

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Abstract

A pharmacoperone (from “pharmacological chaperone”) is a small molecule that enters cells and serves as molecular scaffolding in order to cause otherwise-misfolded mutant proteins to fold and route correctly within the cell. Pharmacoperones have broad therapeutic applicability since a large number of diseases have their genesis in the misfolding of proteins and resultant misrouting within the cell. Misrouting may result in loss-of-function and, potentially, the accumulation of defective mutants in cellular compartments. Most known pharmacoperones were initially derived from receptor antagonist screens and, for this reason, present a complex pharmacology, although these are highly target specific. In this summary, we describe efforts to produce high throughput screens that identify these molecules from chemical libraries as well as a mouse model which provides proof-of-principle for *in vivo* protein rescue using existing pharmacoperones.

Keywords

pharmacoperone; protein trafficking; protein rescue; animal models; high throughput screens; therapeutic approaches

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1. Introduction

For more than 20 years, there has been interest in gene therapy as a means to correct mutational disease. Issues related to the integration of therapeutic DNA into the genome, immune responses, technical problems with vectors (toxicity, immune, inflammatory responses, gene control and targeting issues), chances of inducing tumors, (insertional mutagenesis) and other problems, have made it challenging to reduce this approach to practice, however.

Correcting the folding of misfolded protein mutants and restoring them to function (with pharmacoperone drugs) is a potential alternative to inserting correctly folding proteins by gene therapy (Figure 1). This approach is generally referred to as “rescuing” the protein. It is likely that valuable drugs reside in chemical libraries, yet have been missed, since screening approaches that rely on identification of agonists and antagonists may have failed to identify existing pharmacoperones. There are several advantages to using pharmacoperone drugs; among these advantages are the ability to restore misfolded proteins to function without leaving residual non-functional proteins in other cellular compartments.

Protein rescue with agents that correct folding errors potentially applies to a diverse and vast array of human diseases that result from misfolding or instability of receptors, ion channels, enzymes and other proteins. These include cystic fibrosis (1-5), hypogonadotropic hypogonadism (HH) (6, 7), nephrogenic diabetes insipidus (8-10), retinitis pigmentosa (11), hypercholesterolemia (12), cataracts (13), neurodegenerative diseases (Alzheimer's, Huntington's and Parkinson's, (14-18)), cancer (19), α 1 trypsin deficiency and lysosomal storage disease (20, 21), mucopolysaccharidosis type IIIC (22) and many others. One could envision drugs given in a prophylactic manner (in vitamins, for example) that prevent the misfolding that leads to neurodegenerative disorders (Alzheimer's (misfolded amyloid), (23)) Parkinson's (misfolded α -synuclein) and cataracts (misfolded lens crystalline). In this regard, diseases may be prevented before clinical signs present. In the case of certain proteins (e.g. the GnRHR, V2R and rhodopsin), this approach has succeeded with a striking number of different mutants (24) supporting the view that pharmacoperones will become powerful weapons in our therapeutic arsenal.

A large number of proteins of different classes and structures that have been rescued and restored to function in cell cultures is also suggestive of the broad applicability of this approach (25-39).

Our efforts have focused on G protein coupled receptors (GPCRs), which include the vasopressin type 2 receptor (V2R) and gonadotropin releasing hormone receptor (GnRHR). GPCRs comprise the largest family of validated drug targets — 35-50% of approved drugs derive their benefits by selectively targeting this family.

2. Mechanism of Pharmacoperone Action

GPCRs are subjected to a stringent quality control system (QCS) in the endoplasmic reticulum (ER); this system consists of both, protein chaperones that retain misfolded proteins and enzyme-like proteins that catalyze the folding process. The QCS (consisting of

endogenous chaperone proteins and other factors) assesses structure but not function and insures 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 because they are retained by the QCS even though they may retain (or regain) function.

The functional rescue of misfolded mutant receptors by small non-peptide molecules, originally screened from libraries to serve as receptor antagonists, has now been demonstrated (24). A pharmacoperone is a “molecular scaffold” to promote correct folding of otherwise misfolded mutant proteins within the cell (6, 40). Misfolded proteins are frequently retained by the cellular quality control system (QCS) of the endoplasmic reticulum (ER), do not reach their normal site of function (9, 41) and may result in disease (42). 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 (Figure 1).

We have summarized the literature for the gonadotropin releasing hormone (GnRH) and vasopressin type 2 (V2) receptor's pharmacoperones with a view toward moving these compounds *in vivo* (24). Science writers commenting on this approach (43, 44) 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. The QCS is not protein-specific; it recognizes *general* aspects of misfolding (e.g. exposure of hydrophobic plates in aqueous environments), frequently with relatively low affinity. Accordingly, GPCRs that retain ligand binding and effector coupling, but are recognized as misfolded by such general criteria, are often retained in the ER and degraded. Their rescue with pharmacoperones leads to proper folding, passage through the QCS, restoration to the proper site and return of function.

2.1 There are a number of principles that have guided our efforts

2.1.1 Most pharmacoperone drugs, used to date, were identified from hits in high throughput screens (HTS) for competitive receptor antagonists and then repurposed. These must be removed after rescue to preclude competition with agonists to avoid the complex pharmacology of trying to rescue function in the presence of its antagonist. This potential therapeutic problem can be addressed by identification of drug candidates in the proposed assays that are pharmacoperones but lack antagonistic activity. We believe that valuable drugs, which affect the trafficking of GPCRs, may have been overlooked because of this limitation (45, 46).

2.1.2 It has 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 information to suggest otherwise (47-49). 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 (or agonism). Accordingly, identification of

non-antagonistic (i.e. allosteric) pharmacoperones is a reasonable and therapeutically important goal.

2.1.3 Pharmacoperone drugs need *not* be present at the time of protein synthesis, but can rescue ER-retained proteins that have already accumulated (Figure 1) (50). This observation increases the therapeutic usefulness, since misfolded mutants need not be (first) degraded and then replaced by newly synthesized protein (i.e. the portion synthesized in the presence of pharmacoperone).

2.1.4 While pharmacoperones are specific for individual protein mutants, those that rescue one mutant of an individual protein typically rescue most mutants of the same protein, likely by stabilizing a core microdomain that makes the protein acceptable to the quality control system of the cell. This observation also improves the therapeutic reach of these drugs (24, 28, 51) since each mutant of an individual protein will not require a different drug.

3. Physiological Significance of the Targets Selected

3.1 GnRHR

The GnRHR resides in the gonadotrope cells of the pituitary and is responsible for producing responses to hypothalamic GnRH, such as the releasing of the gonadotropins, luteinizing hormone and follicle stimulating hormone. The hGnRHR has been a central focus of drug development and understanding the mechanism of GnRH action has already led to useful drugs (agonists and antagonists) for the treatment of disorders of reproduction and for cancer. When the function of this receptor is lost due to mutation, the disease hypogonadotropic hypogonadism results. Because this target is causally and mechanistically associated with pathophysiological responses, the proposed intervention with pharmacoperone drugs results in valid therapeutic approaches.

3.2 V2R

The V2 receptor is expressed in the distal convoluted tubule and the collecting ducts of the kidney. V2R responds to vasopressin by stimulating mechanisms that concentrate the urine and maintain water homeostasis in the organism. When the function of V2R is lost due to mutation, the disease nephrogenic diabetes insipidus results (8, 24). The current goal of treatment is to control the body's fluid levels and problems with electrolyte imbalances. Patients must drink large quantities of water to offset the loss. If the affected person does not drink enough fluids, high urine output may cause dehydration and high serum osmolality. NDI is caused by an inborn error of metabolism that is present at birth; it is a chronic condition requiring lifelong treatment. Complications can include dilation of the ureters and bladder, hypernatremia, severe dehydration and shock. The importance of such non-antagonistic pharmacoperones was shown in a study of patients with X-linked NDI. Mutant vasopressin 2 receptors in NDI resulted in misrouted proteins that were trapped in the ER, degraded and did not reach the plasma membrane in the collecting ducts of the kidney where they would normally reabsorb water. *In vitro* studies indicated that a non-peptide V1a receptor antagonist rescued cell surface expression and function of mutant V2 receptors. When applied *in vivo*, a short-term treatment with a V1a receptor antagonist showed that

patients given this molecule decreased both 24-h urine volume and water intake. Maximum increase in urine osmolality was observed on day 3 and sodium, potassium, creatinine excretions and plasma sodium were constant throughout the study (10). Unfortunately, the trade-off between antagonism and pharmacoperone activity resulted in complex pharmacology that did not allow full exploitation of pharmacoperone activity.

We have focused on two elements which we believe would be valuable for moving this field forward:

- Reliable high throughput screens for identification of pharmacoperone drugs.
- Animal models that enable optimization of rescue conditions with a view toward human use.

4. High throughput screening assays

4.1 Principle of the HTS

We first established an assay for pharmacoperones of the V2R. The primary assay uses a HeLa cell line constitutively expressing hV2R[L⁸³Q] (the V2 mutant assay) expressed under the control of a tetracycline-regulated (TET-off) transactivator (52-54). In the absence of doxycycline (“Dox”) (stable analog of tetracycline), the mutant is expressed; it is then misrouted and retained in the ER. Following pretreatment with pharmacoperone, the mutant is rescued and trafficked to the plasma membrane. The rescued mutant is then responsive to native vasopressin and coupled to the production of cAMP for the mutant assay. In the HTS, the level of functional hV2R (mutant) present in each test well is quantitated using a luminescent-based cyclic adenosine monophosphate (cAMP) assay (cAMP-Glo, Promega). This allows the screen to identify compounds which increase the trafficking of mutant in our model system. To triage assay artifacts and compounds with intrinsic off-target activity, compounds are then counter screened with the same cell line as the primary assay in the presence of doxycycline, which shuts off the mutant expression. To eliminate artifacts resulting from the luminescence assay, an orthogonal radiolabeled cAMP RIA or IP assay is used. Compounds are profiled for nonspecific cytotoxicity and specificity for hV2R over other GPCR systems. Increase of total hV2R at the cell surface are confirmed using radioligand binding and fluorescently tagged receptor localization studies. All validated hits are screened for any direct hV2R agonist or antagonist activity and profiled for specificity using a panel of other cellular receptors.

In the hV2R[L⁸³Q] assay, cells are challenged with an EC₁₀₀ dose of vasopressin, then lysed and cAMP levels determined. The counter screen protocol is identical to the primary HTS assay protocol with the exception that the hV2R[L⁸³Q] cells are incubated in the presence of doxycycline for 36 hours prior to assay. During this time the gene for the mutant is off and no measurable mutant remains in the cell. Accordingly the hV2R[L⁸³Q] primary assay and counter screen protocols confirm the expected pharmacology of positive and negative controls (Figure 2).

The SR121463 (originally developed as a receptor antagonist by Sanofi-Aventis Recherche & Développement, obtained with thanks to Dr. Claudine Serradeil) can be used as a

pharmacoperone for the V2R mutant. It yields an EC_{50} of 1.97 ± 0.25 nM in the “-Dox” assay, and signal-to-background ratio (S/B) of ~ 7 ($n=4$). Both protocols are high throughput compatible in both 384 and 1,536 well formats.

In the case of the hGnRHR mutants, the Merck compound IN3 (6, 28, 50) is an effective and target specific pharmacoperone; it was also initially developed as a GhRHR antagonist.

4.2 LOPAC (Library of Pharmacologically Active Compounds) pilot screen

To determine the performance of our optimized hV2R[L⁸³Q] pharmacoperone assay in terms of robustness (Z') under HTS conditions, we used the assay to screen the Sigma LOPAC (Library of Pharmacologically Active Compounds). Briefly, compounds were analyzed at a single concentration of 6 μ M (0.4% DMSO) using SR121463 as a positive control. Each plate contained high and low signal control wells, which were used in Z' factor calculations. An activity scatterplot of all compounds tested, as well as positive and negative controls, is shown in Figure 3.

As indicated from the positive and negative control scatterplots (Figure 3a), the assay demonstrated a high Z' factor (0.85 ± 0.05) for the entire LOPAC screen, indicative of an excellent assay window. Day-to-day reproducibility of the assay is also excellent. As indicated in Figure 3b, a scatterplot of replicate measurements yields an $r^2 = 0.86$, indicating high reproducibility in hit identification. It is important to note that a high number of hits are expected from LOPAC screens due to the high proportion of pharmacologically active compounds present in the LOPAC itself. Using nominal hit-cutoffs (55) a hit rate of 0.78% was calculated. Based on results from previous cell-based LOPAC pilot screens, we anticipate an actual HTS hit rate $< 1\%$. The results presented above show a robust and reproducible HTS compatible assay, ($S/B \sim 6$ and $Z' \sim 0.8$).

Another important parameter to assess for HTS readiness is the susceptibility of an assay to artifact. For example, in the case of the hV2R[L⁸³Q] pharmacoperone assay, compounds that non-specifically activate cyclase activity, agonists of endogenous GPCRs, cytotoxic compounds, and compounds that interfere with the luciferase-based detection reagents (via biochemical or optical means) may be falsely identified as hits. To assess the pharmacology of hits, as well as the contribution of artifact to the primary assay readout, we cherry-picked and titrated all hits from the LOPAC screen. Compounds were tested starting from 25 μ M with 3-fold series dilution steps for 10 points ($n=3$) in both the primary hV2R[L⁸³Q] pharmacoperone assay as well as its counter screen (i.e. pretreatment of the same cells with doxycycline 36 hours prior to assay). All hits confirmed activity in the titration assay, albeit with varying degrees of potency; a subset of the selected hits are shown in Table 1.

As expected, the counter screen was effective at identifying false positives such as forskolin, as well as cytotoxic compounds (such as Mitoxantrone). Some compounds showed activity only in the primary screen, such as L-368,899, an oxytocin receptor agonist. Given that the oxytocin receptor shares homology with the vasopressin receptor, this compound may be useful for further characterization as a V2R pharmacoperone. Combined, these results demonstrate the ability of the hV2R[L⁸³Q] pharmacoperone assay to identify potent hits, and the usefulness of the counter screen to triage off-target hits.

5. SDDL (Scripps Drug Discovery Library) compounds

SDDL compounds are selected based on scaffold novelty, physical properties and spatial connectivity. A summary of select SDDL properties is shown in Figure 4.

By design, the diversity of the SDDL mimics that of much larger collections found at major pharmaceutical companies, yet is responsive to lessons learned from successful drug discovery efforts and emerging trends in HTS library construction (56-60). The SDDL continues to be augmented with diverse small molecule scaffolds, as well as focused sub-libraries targeted to popular drug-discovery targets and compound collections provided by Scripps' distinguished chemistry faculty.

In its current state, the SDDL has several focused sub-libraries for screening popular drug-discovery target classes (e.g. kinases/transferases, GPCRs, ion channels, nuclear receptors, hydrolases, transporters), as well as diverse chemistries (e.g. click-chemistry, PAINS-free, Fsp³ enriched, and natural product collections) and physical properties ("rule-of-five," "rule-of-three," polar surface area, etc.) (60-65).

The novel screening approach presented here will provide a basis for other researchers interested in identifying small molecules that regulate the trafficking and folding of proteins. Regulation of these processes by pharmacoperones is not unique to GPCRs but pharmacoperones can be used to restore the functional expression of enzymes and ion channels as well (41, 42). Therapeutics based on this approach will become increasingly important in the coming years.

An analogous assay for pharmacoperones of the GnRHR is in development.

6. An *In Vivo* Proof-of-Principle for Pharmacoperone Action in Mice

We recently reported (66) the efficacy of pharmacoperone drugs in a newly developed mouse model expressing mutant GnRHR[E⁹⁰K] in the pituitary and showing the hallmarks of HH. This disease is associated with reproductive failure in both males and females.

Because we are using receptor antagonist drugs that are repurposed as pharmacoperones, the rescue of all parameters is not 100% complete. Also, the drug must be given in a pulsatile fashion so that, after rescue of the receptor, it is washed out to enable occupancy by the endogenous ligand.

We use these drugs because non-antagonistic pharmacoperones are currently unavailable for HH (or other diseases, with modest exceptions, (47)). Our data show that available drugs will likely be useful to alleviate considerable suffering associated with diseases of misfolding. Pharmacoperone "IN3" (and other pharmacoperones that we use) have already been subjected to off-target and toxicology studies in humans and we are not assessing their toxicity in the mouse.

6.1 We chose GnRHR[E⁹⁰K] as a model mutant since

6.1.1 The physiology of the GnRHR is well-characterized in humans, mice and many other animal models (67, 68); the mutant GnRHR[E⁹⁰K] acts similarly in mice and humans (28, 69).

6.1.2 A great deal of information is also available regarding the cellular mechanism of action of the GnRHR (70). In addition, we have available, substantive information on the mechanism of misfolding (67, 68), mutant interactions with pharmacoperones (24, 71) and the molecular basis of the dominant-negative effect (50). This effect occurs when a misfolded mutant is retained by the ER and, because this receptor (normally) oligomerizes, it also retains WT receptor. This enhances the deleterious impact of the mutant and likely increases the severity in patients.

6.1.3 The mutant GnRHR[E⁹⁰K] has, in cell cultures, already established general principles that are applicable to other diseases and misfolded molecules. Some examples are (a) The rescue of misfolded mutants by pharmacoperones, discovered in this system, appears to be generalizable to many diseases of misfolding (42). (b) The dominant-negative effect, which we first reported (72) to be based on ER-retention of WT protein by misrouted mutants, has emerged as an important feature of the regulation of other WT-mutant hetero-oligomers to the plasma membrane. For many GPCRs that oligomerize in order to traffic to the plasma membrane, a misfolded mutant also retains the WT molecule. Some examples are the delta-opiate receptor (73), histamine receptor (74), GHRH receptor (75), MC1 receptor, (76-78), TSH receptor (79), gonadotropin receptors (80-82) rhodopsin (83) and others (84, 85). (c) Constitutive oligomerization in the ER, first shown in this system (86), has also been demonstrated for receptors of melatonin (87), dopamine D2 (88), vasopressin (89), serotonin (90), δ -opioids (91), β_2 -adrenergics (92-94), GABA_B (95-97), and follicle-stimulating hormone (98). (d) The observation that individual pharmacoperones rescue most mutants, noted above, (28) is generalizable, also applying to the V2R (99) and other receptors. (e) The observation that pharmacoperones rescue previously retained mutants (50) as well as newly synthesized ones appears generalizable to the V1 and V3 receptors (100). (f) We have shown that GFP and HA-tagging have a problematic impact on data interpretation (86), was subsequently observed by others as a concern (101, 102). It is reasonable and likely that we will continue to learn generalizable aspects applicable to rescue of other molecules from examination of this system.

6.1.4 Biochemical, Evolutionary and other Pragmatic Reasons for Mutation E⁹⁰K selection: At the time we began design of this animal, sixteen mutations (N¹⁰K, T³²I, E⁹⁰K, Q¹⁰⁶R, A¹²⁹D, R¹³⁹H, S¹⁶⁸R, A¹⁷¹T, C²⁰⁰Y, S²¹⁷R, R²⁶²Q, L²⁶⁶R, C²⁷⁹Y, Y²⁸⁴C, L³¹⁴X and a splice junction mutation at the intron 1-exon 2 boundary) had been identified from patients with HH. Our selection was based on the following considerations:

6.1.4.1 In cell cultures mutant mouse GnRHR[E⁹⁰K] is fully retained in the ER (72), identically as in the case of its human counterpart. This is important since the mouse orthologs of many misrouted human GnRHR mutants are correctly routed because trafficking of the mouse GnRHR (and its mutants) is less stringently

controlled (69). S¹⁶⁸R and S²¹⁷R did not rescue with any class of pharmacoperone (46, 49, 103) so these were eliminated. We now know that this failure to rescue results from a substantial thermodynamic change in the transmembrane component of these mutants (68). We participated in the development of an animal expressing L¹¹⁷P by mutagenesis. This mutation has not been reported in human disease; subsequent to development of this animal, we learned (103) that it also cannot be rescued by pharmacoperones (due to the (covalent) right angle bend caused by the presence of proline) and it would have been excluded for this reason as well. Accordingly, this mutation could not be used in these studies.

6.1.4.2 We wanted mutants that rescued well with pharmacoperones. R¹³⁹H, E⁹⁰K, C²⁷⁹Y rescue best (i.e. high ratio of rescue (using pharmacoperone IN3, chemical structure below, section 6) to basal expression in cell culture models (28)).

6.1.4.3 We also wanted a mutation at a highly conserved site so that the mouse would have a high chance of being an effective model for the human.

E⁹⁰K met all our criteria and is found in human HH disease and we know a great deal about its behavior in COS cells (104).

6.1.5 E⁹⁰K has a known biochemical mechanism of disruption of the receptor. The E⁹⁰-K¹²¹ bridge is a highly *conserved feature essential for trafficking of the GnRHR to the PM*. This bridge establishes a physical interaction between transmembrane segment (TMS) 2 and TMS3. *Because this salt bridge appears to be a fundamental requirement for correct routing, E⁹⁰K results in a routing defect in both mouse and human GnRHR* (6, 69, 105). In this mutant the E⁹⁰-K¹²¹ salt bridge is broken (i.e. the mutant, K⁹⁰-K¹²¹ opposes two positive charges and the bridge cannot form) (6, 106, 107). The strength of this model is increased by knowledge of the biochemical mechanism of action of the mutation (71, 107).

6.2 Development and genetic characterization of the model animal

We have published the technique for creating these animals (108). Briefly, *Gnrhr*^{E90K} “knock-in” mice (K⁹⁰neo) were generated by homologous recombination in C57BL/6; 129SvEvTac hybrid RJ2.2 mouse embryonic stem (ES) cells (109). Previously, we removed *neo* and characterized the E⁹⁰K phenotype (108). E⁹⁰K/E⁹⁰K males have slightly smaller testes compared to controls but are fertile. E⁹⁰K/E⁹⁰K females generate antral follicles but do not ovulate (108). Interestingly, when *neo* was left in the locus, the HH phenotype was more severe, making it a better mouse model for pharmacoperone trials.

6.3 Phenotypic characterization of the model animal

Circulating levels of gonadotropin and sex steroids were decreased in the homozygous mutant, compared with WT. Histological analysis (Fig. 5) of the ovaries of adult homozygous females showed absence of large antral follicles and corpora lutea. Mutant follicles were arrested at the early antral stage with only a few layers of granulosa cells and early antrum formation visible in about 30% of the follicles. Affected males showed small testes (Fig. 6).

6.4 Surgical methods

A catheter was surgically inserted in the left carotid of the animal and the animal was attached to an infusion system in a specially designed cage, as shown (Fig. 7).

6.5 Infusion and the pharmacology of drug administration

Currently available pharmacoperones for this system are repurposed (peptidomimetic) GnRHR antagonists. The molecules we used were developed by pharmaceutical companies as specific and orally bioavailable GnRHR antagonists and subsequently abandoned. In our search for structures that might bind and stabilize mutants of the GnRHR, we recognized that these molecules were candidates to be pharmacoperones since they bound the mutants with high affinity and selectivity yet did not activate it. Because these compounds are antagonists, they must be washed out of the system before (endogenous) agonist can occupy/activate the receptor.

Our proof of principle studies have relied on the Merck compound, “IN3”, ((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) at a concentration of 5 µg/ml, infused in heparin-saline at 25 µl/h. We have studied this compound extensively in cell cultures and pharmacological data is available with bioavailability of about 25% by i.m. or s.c. administration and a half-life of about 3 h (in humans, dogs and rats) (6, 28, 50, 71, 107, 110-112). We selected this drug since a half-life of this order is desirable as it allows sufficient time for the drug to access the vasculature of the pituitary gonadotrope without creating the need for a high concentration. This amount of time also allows for adequate washout in a 1-2 day pulse protocol.

6.6 Demonstration of In Vivo Rescue

We used an infusion system which relies on i.v. administration of the drug IN3, to demonstrate *in vivo* rescue of the mutant. Endogenous GnRH binds the GnRHR and results in LH release to the peripheral circulation. Because testis growth is closely related to circulating LH levels, testicular weight is a good and convenient indicator of its average level over time. The primary indication of human HH is the inability to elaborate pulsatile LH and testes remain small. The data (Fig. 6) shows the 90-day WT control (+/+) and K⁹⁰neo/K⁹⁰neo homozygous animals. A 60-day-old homozygous mutant was exposed to infusion of 5 µg/ml pharmacoperone IN3 for 30 days at a rate of 25 µl/h. The animal (Fig. 6) received pulses of 6 h every three days. Male K⁹⁰neo/K⁹⁰neo animals displayed decreased gonadal size compared to wild-type animals or heterozygotes at 60 days of age. Males were infused at a constant rate with saline-heparin (SH) or 5 µg/ml pharmacoperone IN3, were varied. The frequency (pulses/day) and pulse duration (hours) of pharmacoperone administration in the model animal to identify conditions which rescued the mutant. A selection of these conditions is shown (Fig 8). The optimum drug infusion conditions produced, in 30 days, an animal with a testis weight that was 72 fold the mean aged-matched untreated animals (0.045; N=5 g) at 90 days of age. We created a pool of “rescued” testes from these animals (0.09 ± 0.005/2 testes; N=14) to compare with untreated mutants, heterozygotes, or wild-type animals at 90 days of age. After 30 days of IN3 infusion, testis

weight was markedly increased and spermatogenesis was restored to WT levels in all animals.

Because the mouse tether and jacket interfere with mating studies, we isolated sperm from the epididymis of a *single* IN3-rescued K⁹⁰neo/K⁹⁰neo male and performed sperm analysis and *in vitro* fertilization (IVF). Sperm from K⁹⁰neo homozygotes possessed abnormal morphologies including loops in the sperm tails (Fig. 9), likely to be the cause of low sperm progression. In a single animal, IN3-rescue produced an animal with virtually normal sperm counts and good progression. Sperm from this *single* animal were used to produce blastocysts that were implanted into a surrogate female and resulted in a mouse pup with the expected (heterozygous) genotype.

Steroidogenic acute regulatory protein (StAR) mediates cholesterol transfer within the mitochondria, the rate-limiting step in the production of steroid hormones; its production is mediated by the luteinizing hormone levels in the testes. We examined StAR protein levels in 3 testes each of wild-type, K⁹⁰neo homozygotes and IN3-treated K⁹⁰neo homozygotes by Western blotting. Compared to the wild-type (arbitrarily 100% by densitometry), StAR protein was markedly decreased to 28% in K⁹⁰neo homozygotes. The loss was substantially, but not totally, reversed by IN3 treatment (54%) (Fig. 10). Side chain cleavage cytochrome (P450_{scc} or CYP11A1) is a mitochondrial enzyme that catalyzes conversion of cholesterol to pregnenolone. This is the first reaction in the process of steroidogenesis in all mammalian tissues that specialize in the production of various steroid hormones. Similar to that of StAR, there was a modest decrease in this enzyme in the K⁹⁰neo homozygotes as assessed by Western blotting (34%) (Fig. 10). CYP11A1 protein expression was increased by IN3 treatment (50%), but not to wild-type levels (100%). The more modest loss of P450_{scc} compared with StAR proteins may reflect the observation that this enzyme is always active, but its activity is limited by the supply of cholesterol in the inner mitochondrial membrane.

Testes were assessed by quantitative real-time PCR for mRNA levels of hydroxyl steroid dehydrogenase 17 β (Fig. 11). This enzyme catalyzes conversion of the poorly bioactive 17-keto steroids to the highly bioactive 17 β -hydroxysteroids (113). Levels were similar comparing WT and heterozygotes mRNA levels in E⁹⁰Kneo homozygotes were markedly reduced compared with either group. Treatment with IN3 increased *Hsd17b* expression in homozygotes, but not the same levels as observed for WT. Despite the “sub-normal” level of this rate-limiting enzyme, circulating levels of testosterone were improved in IN3-treated homozygous mutants (Fig. 12).

These studies show that mutant GnRHR[E⁹⁰K] can be rescued *in vivo* and its translocation to the plasma membrane also restores other functions associated with the normal functioning of the GnRHR.

7. Conclusions

The studies that we summarize here describe the basis of development of high throughput screens for pharmacoperones of GPCRs, among the most common drug targets. In principle, these assays can be extended to other proteins. Of note, this approach is designed to identify drug leads that are specifically pharmacoperones and not simply antagonists that also show

pharmacological activity. Such (non-antagonists) are valuable as they will show a more simple pharmacology that is afforded by pharmacoperones that are also antagonists. While such combination drugs can be shown to be useful in the mouse mutant model described, their use requires pulsatile administration so that the misrouted protein can first be rescued and then the drug removed to enable occupancy by endogenous agonist. We predict that, a drugs are identified that lack antagonistic activity, pharmacoperone rescue will provide a new approach for therapeutic development, useful for a range of diseases.

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Abbreviations

GPCRs	G protein coupled receptors
V2R	vasopressin type 2 receptor
GnRHR	gonadotropin releasing hormone receptor
QCS	quality control system
ER	endoplasmic reticulum
PM	plasma membrane
GnRH	gonadotropin releasing hormone
LH	luteinizing hormone
FSH	follicle stimulating hormone
HH	hypogonadotropic hypogonadism
NDI	nephrogenic diabetes insipidus

cAMP	adenosine monophosphate
“Dox”	doxycycline
S/B	signal-to-background ratio
TMS	transmembrane segment
IVF	<i>in vitro fertilization</i>
StAR	Steroidogenic acute regulatory protein

Fig. 1. The canonical pathway of protein translation from mRNA

emphasizing that misfolded proteins are retained in the cell (ER and elsewhere) and can be rescued by target specific pharmacoperone drugs (red arrow) that correct misfolding and restore function. Modified from (24) and reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics.

Fig. 2. Responses of controls in the 384-well formatted hV2R[L⁸³Q] Pharmacoperone Assay
(a) Vasopressin titrations. In the absence of doxycycline (“Dox”), hV2R[L⁸³Q] is synthesized and retained in the ER; a vasopressin challenge yields no cAMP response (triangles). Following pre-treatment with 100 nM of the pharmacoperone SR121463 (“SR”), hV2R[L⁸³Q] is rescued and trafficked to the plasma membrane, and a robust cAMP response to vasopressin challenge is observed (circles). Also included are the results of challenging the cells with vasopressin after preincubation with SR and doxycycline (squares). **(b) Pharmacoperone titrations.** In the absence of Dox, increasing concentrations of pharmacoperone SR121463 increases the amount of hV2R[L⁸³Q] on the surface of the cells, with the result of increasing cAMP responses after challenge with an EC₁₀₀ of vasopressin (circles). An EC₁₀₀ challenge of vasopressin to cells in the absence of doxycycline (squares) or pretreated with doxycycline (triangles) results in no appreciable cAMP response. Each dataset is normalized to either the 100% response of either VP (left) or SR (right) dose-response curve. Reprinted from (54) with permission of the publisher.

Fig. 3. Scatter plot analysis of the high-throughput LOPAC pilot screen

(a) All data from all assay plates ($n=17$, triplicate results), including controls, are displayed. Large separation in activity ($Z' = 0.85 \pm 0.05$, $S/B = 7.3 \pm 0.6$) between wells dosed with SR121463 (“high control”) and DMSO (“low control”), as well as a broad distribution of hits indicate an excellent HTS assay. (b) Representative graph of compound activity correlating plate replicas. The best fit line has an $r^2 = 0.86$, indicative of the high fidelity of the assay's hit identification. Reprinted from (54) with permission of the publisher.

Fig. 4. Distribution plots of SDDL properties
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Fig. 5. Phenotype of $K^{90}\text{neo}$ heterozygous and homozygous female mice

Ovaries from mice of the indicated genotypes were collected at 90 days of age, imaged with a stereomicroscope and processed for histology (H&E staining). Wild-type (a) and heterozygotes ($K^{90}\text{neo}/+$) (b) ovaries were indistinguishable. Both exhibited similar size, presence of follicles at all stages of development and presence of corpora lutea. The ovaries of $K^{90}\text{neo}/K^{90}\text{neo}$ females (c) were smaller and exhibited no follicular development past the secondary follicle stage. The stereomicroscope image is shown on the left (scale bars= 0.5 mm) and the H&E stained sections are on the right (scale bars = 0.2 mm). Reprinted from (66) with permission of the publisher.

Fig. 6. Male phenotype

Testes from the indicated genotypes were collected at 90 days of age, imaged with a stereomicroscope and stained. Wild-type (a) and $K^{90\text{neo}}/+$ (b) testes were indistinguishable in size, appearance, abundance of Leydig cells and presence of spermatozoa in the seminiferous tubules. The testes of $K^{90\text{neo}}/K^{90\text{neo}}$ males were smaller and exhibited varying degrees of hypogonadism. There were few to no Leydig cells or elongated spermatids (c). Treatment of $K^{90\text{neo}}/K^{90\text{neo}}$ males with pharmacoperone IN3 (d) resulted in increased testis size and restored spermatogenesis. The stereomicroscopic image is shown in the left column (bar = 2 mm) and the H&E stained sections are shown on the middle and right (scale bars = 0.1 mm (testis)). Reprinted from (66) with permission of the publisher.

Fig. 7. Mouse in infusion tether

A catheter was placed in the left carotid of a 60 day old male animal that was outfitted with a jacket and placed in a cage that allowed free-ranging while drug or saline containing heparin was infused. Pencil-drawn image made available by Instech and reprinted with permission.

Fig. 8. Testicular weight
was determined in 60-day-old animals after 30 days infusion with pharmacoperone IN3 (each 1, 2 or 3 days with pulse duration of 2, 4, 6 or 8 hours). Reprinted from (66) with permission of the publisher.

Fig. 9. Morphology of Sperm

Images are shown of normal sperm, as well as those from untreated $K^{90\text{neo}}/K^{90\text{neo}}$ mutants. These lack heads, have thin heads or tail loops. Images by Drs. Eunju Kang and Shoukhrat Mitalipov, the Oregon National Primate Research Center.

Fig. 10. Expression of StAR (4) and CYP11A1 (5) protein in wild-type, $K^{90}neo/K^{90}neo$ and IN3-rescued $K^{90}neo/K^{90}neo$ males. 50 μ g of protein of testicular protein from wild-type, $K^{90}neo/K^{90}neo$ and IN3-rescued $K^{90}neo/K^{90}neo$ groups were analyzed by immunoblotting. Immunoblots show relative expression levels of StAR (A) and CYP11A1 (B) in testes from three different mice with β -actin expression as a loading control. Modified from (66) and reprinted with permission of the publisher.

Fig. 11. Hsd (hydroxysteroid dehydrogenase)17b3 qPCR

Real-time PCR was performed using total RNA extracted from the testis. Data were analyzed by the CT method and reported as copy number relative to that of *Gapdh*. Wild-type and K⁹⁰neo heterozygotes, which are both phenotypically normal, exhibited similar levels of *Hsd17b3* mRNA. K⁹⁰neo/K⁹⁰neo males, which exhibit hypogonadism, had reduced *Hsd17b3* mRNA ($P < 0.05$). *Hsd17b3* expression was increased in K⁹⁰neo/K⁹⁰neo males following pharmacoperone treatment ($P < 0.05$). Error bars show SEM. Reprinted from (66) with permission of the publisher.

Fig. 12. IN3 treatment restores testosterone levels in $K^{90}neo/K^{90}neo$ males

Serum testosterone was measured by RIA. $K^{90}neo/K^{90}neo$ males exhibited less serum testosterone than heterozygotes. Testosterone levels were restored in $K^{90}neo/K^{90}neo$ males after pharmacoperone treatment. Error bars show SEMs. IN3-treated animals were 60 days old, then treated for 30 additional days (90 days at euthanizing). Reprinted from (66) with permission of the publisher.

Table 1

Primary assay and counter screen results for three hits from the LOPAC screen. For EC₅₀ determination compounds were titrated starting from 25 μ M with 3 fold series dilution steps for 10 points (n=3).

Structure	Vendor information	LOPAC Screen Average % Response $\pm$ Std Dev	EC50 (μ M) -Dox	EC50 (μ M) +Dox	Name	Selectivity	Dose Response Curves Triangles = -Dox Circles = +Dox
Sigma Aldrich F6886	104 $\pm$ 0	0.43	0.59	Forskolin	Activates adenylate cyclase

Sigma Aldrich L2540	74 $\pm$ 2	8.30	>25	L-368,899	Non-peptide oxytocin receptor antagonist

Sigma Aldrich M6545	51 $\pm$ 1	>25	>25	Mitoxantrone	DNA synthesis inhibitor (cytotoxic)