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Assay strategies for identification of therapeutic leads that target protein trafficking

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Abstract

Receptors, enzymes and ion channels are traditional targets of therapeutic development. A common strategy is to target these proteins with agents that either activate or suppress their activity with ligands or substrates that occupy orthosteric sites or have allosteric interactions. An alternative approach involves regulation of protein trafficking. In principle, this approach enables (i) “rescue” of misfolded and misrouted mutant proteins to restore function, (ii) “shipwrecking” of undesirable proteins by targeting them for destruction and (iii) regulation of levels of partially expressed wild-type (WT) proteins at their functional sites of action. Presented here are drug discovery strategies that identify “pharmacoperones,” small molecules that serve as molecular templates and cause otherwise-misfolded mutant proteins to fold and route correctly.

Rescue of mutant proteins

We [1] and others have applied the word “pharmacoperone” (from “pharmacological chaperone”) to reference small molecules that rescue misfolded mutants and restore them to function. These are frequently hydrophobic structures that enter cells by diffusion and serve as a “molecular scaffolding” to promote correct folding [2, 3]. This approach is effective since misfolded proteins frequently retain function, but are identified by the cellular quality control system (QCS) as defective in shape. For that reason alone, they are retained in the endoplasmic reticulum (ER). In other circumstances, mutated trafficking sequences will misroute molecules into the wrong cellular organelle ([4]). In either circumstance, mutants do not reach their normal site of function [5, 6] and result in disease [7].

Retention of functional but imperfect proteins occurs because the chaperone proteins of the QCS are not protein-specific. They recognize general aspects of misfolding (e.g. exposure of

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This article describes a novel approach for drug development by targeting the trafficking of receptor and enzyme proteins. Strategies are described for the development of high throughput assays for the indemnification of this class of drugs.

hydrophobic plates in aqueous environments, unpaired Cys bonds), frequently with relatively low affinity ([8]). Accordingly, G protein-coupled receptors (GPCRs) that retain ligand binding and effector coupling, but are recognized as misfolded by such general criteria, are often retained in the ER. Their rescue with pharmacoperones leads to proper folding, passage through the QCS, restoration to the proper site and restoration of function.

Science writers commenting on this approach [9, 10] 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.

While pharmacoperone drugs are frequently thought of as being useful for rescue of genetic mutants, other diseases of misfolding develop slowly. One could envision drugs given in a prophylactic manner (in vitamins, for example) that prevent the progressive misfolding over time that leads to neurodegenerative disorders such as Alzheimer’s disease (misfolded amyloid), [11, 12]), Parkinson’s disease (misfolded α -synuclein)) and cataracts (misfolded lens crystallines). Such drugs could maintain the stability of proteins and preclude the development of the disease. In this regard, diseases may be prevented before clinical signs present.

Here we summarize the utility of pharmacoperone drugs and describe the underlying means for identifying them by high throughput screening of chemical libraries. Such screens are only recently available and provide a means for identifying novel drugs that would have been missed using previously available screens.

Therapeutic advantages of pharmacoperone drugs

Mutations in enzymes, receptors and ion channels frequently produce misfolded proteins; these are often retained in the ER by the cellular quality control system (QCS) or otherwise misrouted. When ER-retention occurs, the ER becomes stressed and activates pathways that *increase* the biosynthetic capacity (up-regulation of ER-resident chaperones), *decrease* the biosynthetic burden (attenuation of translation) and remove misfolded proteins (by degradation) in the cell [13]. Collectively, this is called the “unfolded protein response” (UPR) [14]. These three pathways address a wide range of dysfunctions that would otherwise be lethal if not for this intervention. The breadth of the functions mitigated by the UPR supports its role as an important mechanism maintaining system robustness. At least three mechanistically distinct components of the UPR (activating transcription factor 6 [ATF6], PKR-like endoplasmic reticulum kinase [PERK] and inositol-requiring element 1 [IRE1]) regulate the expression of numerous genes that both function within the synthetic and secretory pathways and affect cell fate and the metabolism of proteins, amino acids and lipids, responding to the demands imposed by ER stress. When overwhelmed or persistent, the UPR negatively impacts the health of the cell and leads to programmed death. UPR activation, due to ER-retention of proteins [15, 16], causes changes in both therapeutic responses and disease severity that are distinct from the simple loss of function of misfolded proteins [17–21].

A few examples of disease and physiological correlates associated with changes in the UPR are relief of UPR results in both improved glucose tolerance and insulin action [19, 22], UPR activation evokes leptin resistance [23], the state of the UPR changes the sensitivity of tumor cells to chemotherapy [24], signal transduction pathways originating in the ER induce apoptosis when protein folding is inhibited [15], disruption of the cellular QCS results in gross immune dysfunction [25] and cytokinesis is related to the state of the UPR [26]. Manipulation of the UPR is also exploited by disease states to increase their severity. For example, enveloped virus infections can increase ER capacity to serve in viral replication [27] and, in some cancers (especially those arising in secretory tissues), the UPR is utilized to sustain active growth [28, 29].

The UPR, is sufficiently sensitive that it can be activated by retention of proteins resulting from a single point mutation in a single protein [30–32]. Left unchecked, the UPR leads to cell death, an event believed to have evolved to remove unregulated cells from organisms [14]. For example, in patients with retinitis pigmentosa, retinal cells undergo apoptosis due to retention of the causative mutant of rhodopsin [33], a GPCR. In type 2 diabetes, β cells become damaged by elevated demand for insulin and UPR activation [34].

Strategies like pharmacoperones that correct misfolding of mutants often successfully restore function to them, remove misfolded proteins from the ER and relieve the UPR. This contrasts with most gene therapy approaches which replace functional loss with a WT gene product but do not remove the mutant, allowing the continued impact on the UPR due to the mutant and the viral vector used to insert the replacement gene, even though function may be restored [35].

The therapeutic reach of pharmacoperone drugs

Two other observations are important, since these extend the therapeutic potential of this class of drugs. First, pharmacoperone drugs need not be present at the time of protein synthesis, but can rescue ER-retained proteins that have already accumulated [36]. This observation is important, since misfolded and mistrafficked mutants need not be (first) degraded and then replaced by newly synthesized protein (i.e. the portion synthesized in the presence of pharmacoperone). If it were necessary for the pharmacoperone drug to be present exactly when synthesis occurred, then the timing of the administration would be extremely complex. Fortunately, this is not the case.

Second, while pharmacoperones are specific for individual proteins, those that rescue one mutant of an individual protein typically rescue most other mutants of the same protein, likely by stabilizing a core region that makes the protein acceptable to the quality control system of the cell. This observation improves the therapeutic reach of these drugs [36–38] since each mutant of an individual protein will not require a separate drug.

In addition to controlling the trafficking of mutants, pharmacoperones may be useful for regulation of the plasma membrane expression of WT proteins since, for some plasma membrane receptors, a percentage of the nascent WT proteins also undergo misfolding (examples in [39–41]) and are retained; this event may serve a cellular regulatory role [42]. Pharmacoperones can be used to increase plasma membrane expression of these, as well,

and offer a therapeutic means of regulating them. Accordingly, pharmacoperones can be viewed as agents that rescue misfolded mutants, but are also potentially able to increase the plasma membrane expression of many WT proteins.

The challenge of using peptidomimetic antagonists as pharmacoperones

In most systems, the initial demonstration of pharmacoperone activity for GPCRs required molecules that interacted with the receptor with high specificity, but did not activate it. Accordingly, the seemingly logical choices were peptidomimetic antagonists. These had the advantage of being small and hydrophobic, enabling diffusion into cells. For this reason, almost all existing pharmacoperones for GPCRs are peptidomimetic antagonists for the receptor. There are a few exceptions: one receptor agonist has been used for this purpose [40] and one molecule has been used that does not appear to compete for the agonist or antagonist binding site [43]. The latter was identified by our group, based on a fortuitous observation, not a rational approach or screening process.

The use of peptidomimetic antagonists as pharmacoperones presents a complex pharmacology due to competition with endogenous agonists following receptor rescue. *In vivo*, this competition necessitates episodic administration and the requirement for washout. Oral dosing with pharmacoperones that do not compete with the naturally occurring ligand binding site would be more facile. For this reason, pharmacoperone drugs that stabilize the receptor or mutant but are not agonists or antagonists are a desirable target of high-throughput screening (HTS).

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 existing information to suggest otherwise [43]. 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 desirable goal of screening campaigns.

Selecting model GPCR targets

Mutations in GPCRs are known to be responsible for over 30 disorders, including cancers, heritable obesity and endocrine disease; two that are addressed in the proposed project are hypogonadotropic hypogonadism and nephrogenic diabetes insipidus, caused by mutations in the gonadotropin releasing hormone receptor and the vasopressin type 2 receptor.

Gonadotropin Releasing Hormone Receptor (GnRHR)

The human GnRHR 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. The GnRHR itself is one of the smallest GPCRs (328 amino acids in the human and most non-rodent mammals; 327 in rat and mouse sequences); it may be a “limit” size containing only the bare essentials required for ligand binding and signal transduction. There are technical advantages to working with 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. The ligand (GnRH) itself and analogs are small (decapeptides) and have good thermal and high chemical stability (i.e. no oxidizable Met and no internal Cys bridges). Its size and stability has led to the availability of thousands of analogs and peptidomimetics.

The physiology of the system mediated by the GnRHR is well-characterized in many animal models and we have a broad basis for understanding processing differences now known to occur in different species [44].

Finally, a great deal of information is also available regarding the cellular mechanism of action and trafficking of the GnRHR [45]. We have available substantive information on the mechanism of misfolding [39, 42], mutant interactions with pharmacoperones *in vitro* [46] and the molecular basis of the dominant-negative effect.

Our laboratory showed the successful rescue and restoration to function of a mutant of the GnRHR receptor in a mouse model [47]. Because this was a catheterized animal knock-in, it was possible to optimize dose and washout conditions, as well as characterize the physiological, anatomical and cellular responses to rescue.

Vasopressin Type 2 Receptor (V2 Receptor)

As a second model to study, we selected the V2 receptor as it shares many of the desirable characteristics of the GnRHR (small size, relevance to human and animal well-being, availability and chemical stability of ligands that serve as “positive control” pharmacoperones). The V2R also offers the advantage of a very large palette of mutants (hundreds, compared to just 25 or so for the hGnRHR) and chemical agents are available that have pharmacoperone properties. The majority of these mutants are rescuable in cell cultures [6, 48], and the control rescue compounds, as in the case of the hGnRHR, are available [49].

Design of Screens for HTS for pharmacoperones of the GnRH and V2 receptors

Assays amenable for HTS are typically homogeneous in design, involving relatively few reagent additions, are devoid of wash steps and are compatible with multi-modal detection readers.

HeLa cell lines stably expressing the rescuable mutants, hGnRHR[E⁹⁰K] [50] or hV2R[Y¹²⁸S] [51], under control of the tetracycline-controlled transactivator were prepared. Use of this transactivator means that when tetracycline or its analog, doxycycline is present in the media with the HeLa cells that the gene is shut off and the receptor protein does not accumulate [52]. Previously it was demonstrated that there is literally no measurable expression, as assessed by either protein expression or real-time PCR [53, 54] in the presence of tetracycline because the mutant GPCRs are under the control of this transactivator. Removing doxycycline effectively turns on gene expression of these mutants and affords us the ability to screen for pharmacoperone. 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) serve as an excellent negative control line. In both cases, coupling to second messenger is the measured endpoint.

In both the GnRHR and the V2R assays, the mutant receptor is expressed as misfolded protein which prevents it from trafficking to the cell membrane to function properly as a GPCR. Both assays also seek to identify molecules that product a gain of activity and restore proper folding of the receptors thus allowing for correct trafficking and subsequent regulation by their natural agonist.

The coupling to G proteins is different for GnRH and V2R and because of this the end-point is different for each target. For V2R, which is Gs-coupled, we measure the amount of cyclic AMP produced when the cells are in the presence of vasopressin. In this scenario, compounds that correct the misfolding, result in vasopressin activation of the Gs coupled system thereby increasing production of cAMP (Figure 1).

For GnRH, which couples through Gq, we measure the amount of calcium released into the cell from the ER. When the cells are in the presence of GnRH and a compound that corrects the misfolding, this results in phospholipase C activation which ultimately results in the accumulation of inositol phosphatase 3 (IP3) with in the cells which then triggers the release of the Ca^{2+} from the stores (Figure 2).

Both assays were miniaturized to 1,536 well assays and optimized [50, 51]. The optimization included determining the best conditions for cell number per well, incubation times in the presence of compound, agonist concentration necessary for receptor signaling, DMSO tolerance and other indications of assay robustness. Selection of the optimal condition for each of these parameters is always based on Z' value being greater than 0.5 [55]. This statistic, a pure number, assesses whether the response in a particular assay is adequate to warrant further attention [55, 56]. The optimized conditions and protocols are summarized in figure 3.

Once optimal conditions are achieved, online robotic validation is to done to determine the performance of the optimized assays over time. Fortunately good control compounds, pharmacoperones, exist for both assays and these are used to monitor pharmacologic response as well as to determine Z' [3, 51] (see Figure 4).

The counter screen assays for both GnRHR and V2R, as discussed earlier, utilize the same cells which have been treated with doxycycline to repress the Tet responsive promoter effectively rendering them receptor expression free. This gives us an exceptional opportunity to identify artifacts affecting the cells and or readout in a manner independent of the V2 or GnRH receptors themselves. The hit cutoff is determined in manner similar to the primary assay and applied accordingly. Here an active in the counter screen, if also active in the confirmation assay, would be ruled out from further progression.

All compounds that are progressed forward at this stage are subjected to *in silico* chemical triage to identify “PAINs,” highly promiscuous compounds (frequently appear as positives

in unrelated assays) [57] and preliminary molecular structure clustering prior to assembly of a finalized list for concentration response curves (CRC) generation. Compounds of interest are picked for CRCs and are reformatted in 10 point three fold serial dilution format and tested in triplicate.

Typical 640K library HTS campaigns such as those described here complete in about 6 months from the point of assay transfer through delivery of the final report to all investigators.

Selecting a soluble enzyme model target

Disease due to misrouting of enzymes that metabolize oxalic acid

Hyperoxalosis is a deadly disease with poor prognosis and presently with limited therapy options. Primary hyperoxaluria Type I (PH1) is due to an autosomal recessive disorder of glyoxylate metabolism caused by the deficiency or misrouting of liver peroxisomal alanine:glyoxylate aminotransferase (AGT). This is a rare disease. AGT is a pyridoxal 5'-phosphate-dependent enzyme normally present in the peroxisomes that catalyzes the conversion of Ala and glyoxylate to pyruvate and Gly, respectively. When active AGT is absent in the peroxisome, glyoxylate is oxidized to oxalate in the cytosol and is excreted by the kidneys. In the disease state, the increased oxalate concentration in the urine leads to the formation and deposition of insoluble calcium oxalate crystals and the disease states, nephrocalcinosis and urolithiasis, and frequently, renal failure and calcium oxalate deposition in other tissues. This systemic oxalosis is potentially lethal [58]. Common treatments are directed at decreasing oxalate in the body, often by dialysis or by kidney transplantation [58]. Available treatments also include vitamin B6 therapy, but only a small percentage of patients respond to B6 therapy [59, 60].

A phenotypic approach to drug discovery can also be leveraged to identify pharmacoperone drugs that can correct trafficking errors of the enzyme that is the underlying cause of hyperoxalosis; a potential research opportunity of interest to us that is responsive to our current capabilities.

To address this we developed a HTS amenable high-content assay to identify molecular pharmacoperones for the treatment of primary hyperoxaluria type 1 [4]. This miniaturized 384 well plate-formatted cell-based approach employs the CHO-GO cells expressing the AGT-170 genetic mutation which causes the oxalate to be misrouted to the mitochondria [4]. We can readily detect the co-localization and routing of the mutant AGT via immunostaining fixed cells with anti-AGT antibody [61] and anti-PMP70 antibody followed by secondary antibody labelling along with Hoescht nuclear staining.

Using these techniques we were able to readily distinguish a difference between AGTmi cells which do not elicit a PH1 phenotype versus those that do; for example the AGT- 170 mutant containing cells. This assay is compatible with fully automated robotic screening.

Concluding remarks

In addition to the three assays described herein, there are recent reports (both in 2014) of assays that detect pharmacoperones by *in silico* screening [62] and by affinity-based screening methods [63], suggesting the growing interest in identifying this class of drugs from chemical libraries. Collectively, these new tools are likely to result in useful structures that produce novel therapeutics.

In principle, the pharmacoperone rescue approach might apply to a diverse array of human diseases that result from protein misfolding—among these are cystic fibrosis [64–67] hypogonadotropic hypogonadism (HH, [46]), nephrogenic diabetes insipidus [6, 68, 69], retinitis pigmentosa [70], hypercholesterolemia, cataracts [71], neurodegenerative diseases (Huntington's, Alzheimer's, Parkinson's [72–76]) and particular cancers [77]. In the case of certain proteins (e.g. the GnRHR, V2R and rhodopsin), this approach has succeeded with a striking number of different mutants [49], supporting the view that pharmacoperones will become powerful weapons in our therapeutic arsenal [49]. It has also become clear that variable (but significant) amounts of even some WT GPCRs are misrouted (i.e. retained in the ER), apparently as a result of misfolding [41, 78–83]. This suggests a level of post-translational control that may provide yet another level of potential therapeutic intervention [84]: increasing the routing of WT GPCRs. In principle the (endogenous) chaperoning system could mediate the fraction of the newly synthesized receptor that traverses to the PM and may contribute to the physiological changes in levels of GnRHR that are observed in different physiological settings [85]. We anticipate that this will become an interest area of interest that will yield novel therapeutics.

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Figure 1.

Representation of a cell based assay to monitor for pharmacoperone rescue of the vasopressin receptor function. Untreated cells, “Doxycycline minus“, have an active tet transactivator resulting in expression of misfolded mutant protein. Upon treatment of the cells with vasopressin and a pharmacoperone (SR121463) receptor folding is corrected and function is rescued resulting in a Gs coupled response which is readily monitored using cAMP-Glo from Promega Corp. Here, a diminution in luminescence is indicative of receptor activation.

Figure 2.

Gonadotropin releasing hormone (GnRH) receptor (GnRHR) pharmacoperone assay principle. The mutant GnRHR is retained in the lumen of untreated cells. Addition of pharmacoperone rescues GnRHR, and the receptors are trafficked to the plasma membrane. GnRHR on the plasma membrane is now responsive to agonism by GnRH, which is quantitated using Fluo-2 detection reagents. The level of functional GnRHR (mutant) is proportional to the magnitude of Gq modulated signaling, IP₃ induction, and subsequent Ca²⁺ released and detected in the cells using Fluo-2 and reading on a FLIPR.

HTS Protocols

Figure 3.

Flow diagrams of the HTS protocols used during the fully automated 1536 well format HTS.

Figure 4.

A. V2R control and B. GnRH control concentration response curves including EC50 and Hill Slope data generated using GraphPad Prism along with structures C. and D., respectively