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Assay strategies for identification of therapeutic leads that target protein trafficking

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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 'rescue' of misfolded and misrouted mutant proteins to restore function, 'shipwrecking' of undesirable proteins by targeting them for destruction, and regulation of levels of partially expressed wild type (WT) proteins at their functional sites of action. Here, we present drug discovery strategies that identify 'pharmacoperones', which are 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 hydrophobic plates in aqueous environments or 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 crystallins). 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 (HTS) of chemical libraries. Such screens are only recently available and provide a means of 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 QCS or otherwise misrouted. When ER retention occurs, the ER becomes stressed and activates pathways that increase the biosynthetic capacity (upregulation of ER-resident chaperones),

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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 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 include: the 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 mellitus, β cells become damaged by an elevated demand for insulin and UPR activation [34].

Strategies such as pharmacoperones, which correct the 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 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 also be used to increase plasma membrane expression of these proteins, and offer a therapeutic means of regulating them. Accordingly, pharmacoperones can be viewed as agents that not only 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, rather than on 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 HTS.

It has certainly not been established that binding at or near the binding site of the natural ligand is a necessary prerequisite 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