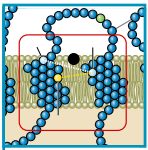# PHARMACOPERONES AS NOVEL THERAPEUTICS FOR DIVERSE PROTEIN CONFORMATIONAL DISEASES

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Tao Y-X, Conn PM. Pharmacoperones as Novel Therapeutics for Diverse Protein Conformational Diseases. *Physiol Rev* 98: 697–725, 2018. Published February 14, 2018; doi:10.1152/physrev.00029.2016.—After synthesis, proteins are folded into their native conformations aided by molecular chaperones. Dysfunction in folding caused by genetic mutations in numerous genes causes protein conformational diseases. Membrane proteins are more prone to misfolding due to their more intricate folding than soluble proteins. Misfolded proteins are detected by the cellular quality control systems, especially in the endoplasmic reticulum, and proteins may be retained there for eventual degradation by the ubiquitin-proteasome system or through autophagy. Some misfolded proteins aggregate, leading to pathologies in numerous neurological diseases. In vitro, modulating mutant protein folding by altering molecular chaperone expression can ameliorate some misfolding. Some small molecules known as chemical chaperones also correct mutant protein misfolding in vitro and in vivo. However, due to their lack of specificity, their potential as therapeutics is limited. Another class of compounds, known as pharmacological chaperones (pharmacoperones), binds with high specificity to misfolded proteins, either as enzyme substrates or receptor ligands, leading to decreased folding energy barriers and correction of the misfolding. Because many of the misfolded proteins are misrouted but do not have defects in function per se, pharmacoperones have promising potential in advancing to the clinic as therapeutics, since correcting routing may ameliorate the underlying mechanism of disease. This review will comprehensively summarize this exciting area of research, surveying the literature from in vitro studies in cell lines to transgenic animal models and clinical trials in several protein misfolding diseases.

I. INTRODUCTION TO PROTEOSTASIS

Homeostasis is the essence of all physiological processes in the animal. In healthy animals, perturbation of any physiological parameter will result in a series of adaptations seeking the return to pre-perturbation level of the particular physiological parameter. If homeostasis cannot be achieved (dyshomeostasis), pathology will ensue, especially after a significant time has passed.

Not only is homeostasis important at the organismal level, but it is also important at the level of individual cells. Proteostasis or protein homeostasis refers to the fact that at the cellular and subcellular (organelle) levels, it is essential to maintain homeostasis of proteins, with protein production, folding, and disposal reaching a balance (20, 216, 319). When stressed, either due to synthesis of misfolded/misassembled protein or other environmental stress such as increased temperature or oxidative stress, heat shock response (230, 299) and unfolded protein response (UPR) (151, 306, 384) are activated and the expression of molecular chaperones is increased, aiding in the folding of misfolded proteins, preventing the accumulation of misfolded proteins, and accelerating the degradation of misfolded proteins. Protein synthesis is also decreased by decreased gene transcription and translation. However, when misfolded proteins do accumulate in the endoplasmic reticulum (ER), therefore leading to persistent ER stress (317, 385), prolonged UPR activation will cause intracellular accumulation of reactive oxygen species (oxidative stress) and consequent cell death (146).

Aging is associated with loss of proteostasis network capacity, reduced activation of the normal protective mecha-

nisms, resulting in increasing difficulty in maintaining proteostasis (25, 140); hence, aging is accompanied by increased incidence of chronic diseases, such as metabolic and neurodegenerative diseases, and some forms of cancer (140, 154) occur. In humans, there are about two missense mutations per gene (318), and ~25–30% of these missense mutations likely affect protein stability or folding (261). With additional mistakes incorporated in gene transcription and splicing, translation, and posttranslational modification and targeting (92), these represent a constant challenge for the cellular proteostasis machinery. In young animals, these challenges are handled ably, but in aging animals, with decreased capacity to respond to these stresses, age-related diseases gradually manifest, especially if there is a genetic component, such as in familial amyotrophic lateral sclerosis (ALS), Parkinson's disease, Huntington's disease, Alzheimer's disease, and other neurodegenerative disease (25, 92, 140, 216). Neurons, due to their structure and inability to regenerate, are the most sensitive to the accumulation of the misfolded proteins (92).

Considering that an average cell contains 10,000–20,000 different proteins ("the proteome"), maintaining the proper balance (concentration), localization, and integrity of these proteins is a daunting challenge for the cell. This is true since many proteins are only marginally stable and are prone to misfolding and aggregation, especially when the cells are faced with exogenous (such as heat shock) or endogenous (such as metabolic) stress conditions (140). To facilitate the maintenance of proteostasis, human cells employ ~1,400 proteins, including 332 molecular chaperones (comprising the chaperome) and their regulators, proteins involved in defending oxidative stress, and protein degradation machinery [ubiquitin-proteasome degradation system (UPS) and autophagy] (40, 140, 205). To maintain proteostasis, cells maintain a delicate balance between protein synthesis, folding, and degradation (132), using a three-pronged strategy of prevention, repair, and damage control (308).

The UPS is known to be critically important in the clearance of soluble monomeric misfolded proteins through ER-associated degradation (ERAD) (249). Misfolded proteins are retro-translocated into the cytosol through the translocon (188, 288), and degraded by the UPS (67, 124, 150, 310). However, UPS is not effective against insoluble protein aggregates (171). Indeed, UPS activity is frequently severely impaired in the presence of aggregated proteins, even in compartments where there are no protein aggregates, suggesting that it is not simply that the aggregates occupy all the proteasomes in the cell (28). The proteasome is also unable to digest proteins with polyglutamine (polyQ) expansion. Autophagy is used to degrade the aggregated proteins (274, 296; for review, see Refs. 86, 215, 265).

When the misfolded proteins overwhelm the cellular machinery for protein folding, proteotoxicity is observed (152, 153). Molecular chaperones antagonize proteotoxicity (18, 21, 39, 40, 71). At the organismal level, proteotoxic stress in one tissue can modulate the proteostasis activity in other tissues through transcellular chaperone signaling (360, 376, 377).

II. QUALITY CONTROL

Proteins of the secretory pathway that are folded in the ER are constantly monitored by the highly redundant ER quality control system (57, 149). Any features indicative of nonnative conformation such as hydrophobic patches exposed on the surface of soluble proteins and mobile loops as well as reduced compactness (globular proteins have a smaller surface area in the native state than in the nonnative state) or increased β-sheet content will result in the protein being retained in the ER and prevented from further forward trafficking to its normal destination. Even in wild-type (WT) proteins, a significant fraction (up to 30%) is misfolded and/or aggregated, despite help of molecular chaperones, and these misfolded proteins are rapidly (within minutes of their synthesis) degraded by the UPS (325). Several G protein-coupled receptors (GPCRs), such as the δ-opioid receptor (282), GnRHR (368), prostaglandin D_2 receptor 1 (275), melanocortin-3 receptor (348), melanocortin-4 receptor (356), CB1 cannabinoid receptor (5), V2R (399), olfactory receptor (239), and D_2 dopamine receptor (104), are inefficiently expressed at the plasma membrane.

For proteins with a linear sequence, the sequon, Asn-X-Ser/Thr (where X is any amino acid except proline), potential N-linked glycosylation can occur on the amide group of asparagine. It is estimated that more than half of all proteins are glycoproteins and more than 90% of the glycoproteins have N-linked glycosylation (11). N-linked glycosylation is a cotranslational modification that is initiated at the luminal face of the ER when the nascent protein is emerging from the ER membrane. Carbohydrates have different compositions in the ER and post-ER locations, and some chaperones such as calnexin and calreticulin recognize the carbohydrates to exert quality control (53, 346, 363, 389).

For proteins in the secretory pathway, nascent proteins are cotranslationally inserted into the ER lumen where the initial folding occurs. There, resident molecular chaperones facilitate the folding of the proteins into their native conformation (120, 141, 143) based on the primary structure of the proteins as elucidated by the elegant studies of Anfinsen (7). Molecular chaperones also prevent the aggregation of folding intermediates with hydrophobic patches exposed by masking these patches and preventing inappropriate liaisons similar to the human chaperones at high school proms. Some molecular chaperones can serve as disaggregases [such as heat shock protein (HSP) 104] (276, 330),

disassociating the aggregated proteins into soluble intermediates, and by cooperating with conventional chaperones such as HSP70 and HSP40, promoting the folding of intermediates (123; reviewed in Ref. 36). It has been suggested that designer protein disaggregase might be developed as a therapeutic for numerous currently incurable neurodegenerative diseases (329).

Proteins that cannot achieve the native conformations have two potential fates: targeting for degradation or undergoing aggregation leading to accumulation of the misfolded protein in the intracellular or extracellular spaces. In the first instance, a loss-of-function disease ensues, whereas the latter causes a gain of function. It is critical to differentiate between these two fates because different strategies are devised for treating loss-of-function versus gain-of-function diseases. Most diseases associated with inactivating GPCRs cause loss of function; few of them cause aggregation, with rhodopsin representing one prominent example. Mutations in α_1-antitrypsin (AAT) cause the mutant proteins to polymerize in the ER of hepatocytes. These intracellular polymers accumulated in the ER are toxic, causing chronic liver diseases, whereas systemic deficiency of AAT also causes pulmonary emphysema.

A. Quality Control at the ER

The ER is the site of synthesis and folding of membrane and secretory proteins (273). On the basis of results obtained in yeast (121), it is estimated that about one-third of all eukaryotic proteins, including all cell-surface and secretory proteins and proteins located in compartments along the exocytic or endocytic pathways, are folded and matured in the ER before exiting the ER and reaching their final destinations (373). Quality control checkpoints within the ER have been known for more than three decades (233, 311; for detailed and excellent reviews, see Refs. 93–95, 110, 148, 149, 211, 346, 409). The ER has a mechanism that prevents the transport of misfolded proteins from exiting the ER and reaching their final destination. Calnexin and calreticulin are involved in the ER retention of glycoproteins of the secretory pathway. They bind to glycoproteins with monoglucosylated glycans (137, 147) so that these glycoproteins can be reglucosylated by the UDP-glucose:glycoprotein glucosyltransferase, an ER sensor for misfolded proteins (339). UDP-glucose:glycoprotein glucosyltransferase only glucosylates misfolded (but not native) glycoproteins by recognizing the innermost N-acetylglucosamine unit of the oligosaccharide and exposed hydrophobic amino acids in the misfolded proteins (339).

Hydrophobic amino acids exposed at the surface are detected as misfolded by the ER quality control. Molecular chaperones transiently shield these exposed amino acids that should have been buried inside in the native conforma-

tion (143). The formation of intra- and interchain disulfide bonds in the ER is important for many proteins of the secretory pathway to mature and achieve native conformation (252). Aberrant disulfide bonds are recognized as misfolded and retained for further folding, aided by molecular chaperones and folding catalysts such as protein disulfide isomerase and ERp57 (22, 125, 391).

These and other multiple mechanisms in the ER differentiate properly folded and misfolded proteins. The misfolded proteins are retained in the ER (for example by rebinding of general molecular chaperones or interaction with specific folding factors for some proteins), prevented from trafficking onward, retro-translocated into the cytoplasm through the translocon, and degraded by the UPS system (67, 94, 95, 122, 149, 150, 211, 292, 334). Both integral membrane proteins and other proteins of the secretory pathway undergo this ER quality control. The protein fate is determined by folding, chaperone binding, and tagging for degradation by poly-ubiquitination (132, 211).

Although stringent, quality control at the ER is not fool-proof. Some misfolded proteins can evade the ER quality control monitoring and escape the ER. Some misfolded proteins are transiently diverted to the ER/Golgi intermediate compartment (ERGIC) and cis-Golgi apparatus (48, 85, 238, 255, 269) before being retro-translocated to the ER for ERAD (402) or sorting to lysosome for degradation, or forming toxic aggregates. Indeed, it has been shown that traffic between the ER and the Golgi is required for ERAD (reviewed in Ref. 364). It is also possible that proteins that already exited the ER become misfolded during the trafficking. Therefore, there is additional quality control machinery to monitor and correct misfolding that occurs beyond the ER in the secretory pathway (16, 364).

B. Quality Control at the Golgi Apparatus

First described by Camillo Golgi of the University of Padua as an "internal reticular apparatus" (126), the Golgi apparatus has two functions in the secretory pathway: *1*) sorting proteins into their various final destinations, accomplished by the *trans*-Golgi-network, and 2) modifying the proteins and lipids passing through it, performed in the rest of the Golgi. As the central hub of the secretory pathway, the Golgi cisternae play a critical role in the health of cellular homeostasis. This structure receives the products from the ER and processes them to the mature form for further trafficking to the final destinations in the secretory pathway (411). The majority of the sorting decisions are made in the Golgi apparatus, especially in the *trans*-Golgi network. One well-studied example is the extensive trimming of high-mannose N-glycans derived from the ER in the c*is*-Golgi. These glycoproteins are sensitive to endoglycosidase H (Endo H) in the ER but become insensitive to Endo H at the Golgi and beyond. Rather, because of new sugars added,

glycoproteins become sensitive to neuraminidase. Several conformational features, such as not partitioning into the appropriate lipid microdomains or protein aggregation, are recognized as misfolded and identified by the Golgi quality control, tagged with ubiquitin, and targeted to the endosomal degradation system for disposal (16).

Quality control at the Golgi is demonstrated by early studies showing that some laboratory-generated mutant proteins of the secretory pathway, especially proteins with abnormal transmembrane domains, can escape the ER but are retained in the Golgi (113, 131, 144, 258), suggesting that these proteins can reach conformations that are recognized as native by the ER machinery but are recognized as nonnative by the Golgi quality control machinery. This was subsequently demonstrated in naturally occurring mutations; for example, retention in the Golgi accounts for a portion of the intracellular retention of mutant GPCRs (88, 353, 393).

Mutations in genes for the machinery involved in the trimming, including enzymes, nucleotide sugar transporters, or recycling/reorganization components [such as conserved oligomeric Golgi (COG) complex, SEC, golgins], can all affect glycosylation and cause human illness, currently numbering in several dozens of inherited diseases (108, 109, 369). Common diseases such as diabetes and cancer are also associated with protein glycosylation defect (108, 109, 369). Mutations in COG impair the retrograde vesicular traffic of resident Golgi proteins needed to maintain normal Golgi structure and function (370).

C. Quality Control at the Plasma Membrane

For plasma membrane proteins that have already passed the ER and Golgi quality controls and reached the plasma membrane, they are subject to another round of quality control at the plasma membrane and potentially cleared from the plasma membrane if found to be misfolded. Chaperones, cochaperones, and E2 ubiquitin-conjugating and E3 ubiquitin-ligating enzymes have been identified to promote the lysosomal degradation of defective ΔF508 CFTR proteins at the plasma membrane (266). Similar quality control has been found for other proteins such as CD4, mutant dopamine D4.4 and vasopressin V2 receptors (V2R) (10), and human ether-a-go-go-related gene (hERG) K^+ channel (8). Nonnative membrane proteins that escape the ER quality control monitoring or are generated in post-ER compartments are eliminated from the plasma membrane by this mechanism (9, 266). However, membrane proteins with a misfolded cytoplasmic domain might not be efficiently recognized and degraded by the quality control mechanisms at the plasma membrane and cytoplasm, although the misfolded domain is exposed to the cytoplasm (227).

Quality control at the plasma membrane, like quality control at the other locations, is a double-edged sword. Whereas it protects cells from damaged proteins, it also eliminates proteins that are functional but are mildly misfolded. This presents another hurdle in designing potential therapeutics such as pharmacological chaperones to effectively treat protein conformational diseases.

D. Quality Control at the Mitochondrion

The mitochondrion, representing the cellular machinery for generating the energy currency, ATP, consists of four compartments, including the outer mitochondrial membrane, the intermembrane space, inner mitochondrial membrane, and mitochondrial matrix. Quality control components in the mitochondria consist of molecular chaperones such as HSP60 and HSP70 that aid in the folding, especially in the matrix, and proteases and ubiquitin ligases that target the permanently misfolded proteins for degradation (19, 136). Misfolded proteins in the matrix are retro-translocated into the cytosol for degradation, akin to the retro-translocation of misfolded proteins in the ER for degradation in the ERAD process (231). In addition to sharing some core components, the cytosolic UPS and the mitochondria-associated degradation pathway are also tightly interconnected functionally, and their activities are reciprocally modulated (38). Mitochondrial quality surveillance systems contribute to quality control in extra-mitochondrial compartments, acting on cytosolic or ER substrates; therefore, it is crucial for the maintenance of health at the whole cell level (38, 79). Similarly, several cytosolic pathways, including mitochondrial precursor overaccumulation stress (204), the UPR activated by mistargeting of proteins (396), and the mitochondria to cytosol stress response (386), seek to restore proteostasis and reduce proteotoxicity of mitochondrial insults by affecting cytosolic protein translation, folding, and degradation (79).

The UPR also operates in the mitochondria. It is a protective transcriptional response changing the expression of mitochondrial proteostasis genes (including chaperones and proteases) via mitochondrial-to-nuclear signaling (295, 327), that is, protein misfolding or misassembly in the mitochondrial matrix will result in upregulation of the expression of nuclear genes encoding mitochondrial chaperones and proteases (412). The promoters of these genes also harbor stress elements similar to the genes upregulated with UPR in other organelles such as the ER. In addition, the accumulation of unfolded or misfolded proteins in mitochondria triggers mitophagy, the selective degradation of mitochondria by autophagy (185). The mitochondrial kinase PINK1 and the E3 ubiquitin ligase PARK2/Parkin remove depolarized mitochondria, reducing the unfolded or misfolded protein load. Mutations in *PARK6* (encoding PINK1) or *PARK2* (encoding Parkin) cause familial Parkinson's disease (208, 372), highlighting the importance of

mitochondrial quality control in health and disease (reviewed in Ref. 287).

The UPR likely evolved just to respond to transient disruption of homeostasis (220). Prolonged activation of the mitochondrial UPR can shift a protective mechanism to a potentially harmful one (229). For example, in the presence of deleterious mitochondrial DNA that caused reduced oxidative phosphorylation, the cells seek to recover oxidative phosphorylation activity by constitutive activation of mitochondrial UPR. However, the persistent mitochondrial UPR propagates or maintains the deleterious mitochondrial DNA by promoting mitochondrial biogenesis and dynamics, potentially underlying mitochondrial diseases and aging-associated accumulation of mutated mitochondrial DNA (229).

Reduced activity in degradation (such as due to impaired function of mitochondrial proteases) with consequent proteotoxicity is associated with aging and pathogenesis of many diseases such as neurodegenerative disorders, metabolic syndrome, and cancer (294, 328). Restoring proteostasis by either reducing protein translation (such as by inhibiting the mammalian target of rapamycin pathway) or targeting the UPS represents promising therapeutic options for mitochondrial disease treatment. This has been demonstrated with rapamycin (to inhibit protein translation) in rodent models of Parkinson's disease (184), Leigh syndrome (191, 192), and focal-segmental-glomerulosclerosis (278) or by restoring proteasome function (222, 323). It has been suggested that the benefits of a high dose of rapamycin outweigh the side effects in mitochondrial disease (190).

E. Quality Control at Other Organelles

Another quality control process, called regulation of aberrant protein production (RAPP), is described for mutations in the sequence coding for the signal peptide that results in defective interaction of the nascent peptide with signal recognition particle, leading to increased degradation of the mRNA encoding the mutant protein by Argonaute2, hence decreased translation of the mutant protein (198).

F. Quality Control at the Extracellular Fluid

In addition to the critical importance of proteostasis inside the cells, proteostasis in the extracellular fluid is also critically important. Some of the most debilitating and intractable diseases are caused by misfolding and aggregation of proteins in the extracellular fluid. Alzheimer's disease, type 2 diabetes mellitus, and prion disease are some examples. Recent studies reveal a growing list of abundant chaperones that are constitutively secreted to the extracellular space where they act as sensors and the machinery for disposal of misfolded proteins (400). They bind to the hydrophobic

patches exposed on the misfolded proteins, preventing their aggregation to form insoluble deposits and diminishing their toxicity towards cells. They can also transport the soluble misfolded proteins into the lysosome via receptor-mediated endocytosis for degradation. The extracellular chaperones currently identified are clusterin, haptoglobin, α_2-macroglobulin, apolipoprotein E, caseins, and fibrinogen. Clusterin, haptoglobin, α_2-macroglobulin, and caseins serve as holdases (similar to the intracellular small heat shock proteins) (162), protecting proteins from aggregation and precipitation but directly refolding the misfolded proteins, whereas apolipoprotein E and fibrinogen stabilize proteins in solution (80). Some of the extracellular chaperones are frequently found in extracellular deposits, upregulated in stress or disease conditions, or are genetically associated with some of the diseases mentioned earlier in this paragraph, especially with Alzeheimer's disease (139, 217, 342).

III. PROTEIN CONFORMATIONAL DISEASES

Protein conformational diseases denote the diseases caused by proteins encoded by mutant genes that cannot achieve native conformation resulting in either loss of function or gain of function, leading to human illness. It is estimated that there are hundreds of protein conformational diseases, including many of the diseases not caused by an infectious agent (371). Here we highlight some prominent examples.

A. Familial Hypercholesterolemia

A well-established protein conformation disease is familial hypercholesterolemia caused by mutations in low-density lipoprotein (LDL) receptor (LDLR) gene (44, 156, 225, 362). The LDLR transports the blood cholesterol to the inside of the cells through endocytosis. Plasma LDL-cholesterol levels in patients with heterozygous mutation in *LDLR* (300–500 mg/dl) are twice that of the healthy people. Those in patients who have homozygous or compound heterozygous mutations in *LDLR* (600–1,200 mg/dl) are dramatically higher than those in healthy individuals. The increased levels of LDL-cholesterol lead to tendon xanthoma and atherosclerosis in heterozygous carriers and premature death from heart attack in homozygous or compound heterozygous patients (156). About 53% of the *LDLR* mutations belong to class 2, where the receptor protein is synthesized in the ER as a partially glycosylated form of 120 kDa, but unlike the WT LDLR that quickly exits the ER and becomes the fully glycosylated 160-kDa form, is retained in the ER and degraded. These mutations can be missense or small inframe deletion mutations. These pioneering studies represent some of the earliest examples of protein misfolding as a root cause of human disease.

Other earlier examples include insulin-resistant diabetes due to mutation in the insulin receptor gene (1), sucrase-

isomaltase deficiency due to mutation in the *SI* gene (232), leukocyte adhesion deficiency due to mutations in the common β-subunit (CD18) (207), osteogenesis imperfecta due to mutations in genes encoding pro-α-l collagen and pro-α-2 collagen (*COLIA1* and *COLIA2*) (290), as well as pulmonary emphysema and chronic liver disease due to mutations in α$_1$-antitrypsin gene (*AAT*) (37, 78).

Studies on α$_1$-antitrypsin mutants, especially the major form identified from patients, the so-called Z allele that changes E342 to lysine, showed that due to defective folding (234, 406), E342K does not achieve mature glycosylation (for example containing sialic acid) (97, 183). It is sequestered in the ER and not secreted in similar fashion to the WT α$_1$-antitrypsin (280, 380). This results in low plasma concentrations of functionally active α$_1$-antitrypsin. Subsequently, the ER-retained form is quickly degraded by the proteasome (293). If this degradation is defective (for example, in some patients with severe liver disease associated with α$_1$-antitrypsin deficiency), the mutant α$_1$-antitrypsin that accumulated in the ER is toxic to hepatocytes causing chronic liver injury and hepatocellular carcinoma (398).

B. Cystic Fibrosis

Cystic fibrosis (CF) is another prominent example of a protein conformational disease. Confocal microscopy on primary cultures of airway epithelia in nonpermeabilized cells using antibody directed against an extracellular epitope of CF transmembrane conductance regulator (CFTR) showed that CFTR is located in the apical membrane in non-CF subjects. In contrast, it is either absent or expressed at much lower levels in CF patients, suggesting that at least some CFTR mutants are defective in trafficking to the plasma membrane (84). The misfolded ΔF508 CFTR is retained in the ER by Hsp70 and its co-chaperone CHIP, retro-translocated into the cytosol, tagged with ubiquitin, and degraded by the proteasome (182, 246, 388). When the degradation is blocked by proteasome inhibitor, misfolded CFTR aggregates form aggresomes (114, 195, 390).

The aggresome, a juxtanuclear inclusion body structure, is formed in some cases of expression of misfolded proteins, especially when the proteasome activity is inhibited (114, 195, 390; reviewed in Refs. 209, 213). In transgenic mice expressing mutant superoxide dismutase (SOD) linked to autosomal dominant familial ALS, mutant SOD aggregate into high-molecular-weight insoluble protein complexes (IPCs) resembling aggresomes that are detectable in spinal cord extracts several months before the appearance of motor neuron pathology (194). It has been suggested that the aggresome is cytoprotective, facilitating the degradation of toxic aggregates by recruiting the cytoplasmic misfolded proteins there (359, 367).

C. Prion Disease and Other Neurodegenerative Diseases

Prion proteins exist in normal healthy cells. However, as first suggested by the Nobel Laureate Stanley Prusiner (291), when misfolded, the disease-causing version aggregates and exerts dominant negative effect on the normal prion, leading to the misfolding of the normal prion. Several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, ALS or Lou Gehrig's disease, are also caused by misfolded proteins (amyloid-β peptide and phosphorylated tau, α-synuclein, huntingtin, SOD, respectively), similar to prion diseases. These protein aggregates form toxic inclusions in specific regions of the brain. Aging is an independent risk factor for many of the neurodegenerative diseases. Aging is associated with a decreased capacity for maintaining proteostasis (91) and altered expression of chaperome, which is exacerbated in neurodegenerative diseases (40).

The polyglutamine expansion encoded by CAG trinucleotide repeats in unrelated proteins causes nine neurodegenerative disorders, including spinal and bulbar muscular atrophy (SBMA, also known as Kennedy's disease) caused by mutations in the *androgen receptor*; Huntington's disease caused by mutations in *Huntingtin*; six spinocerebellar ataxias caused by mutations in ataxin1, ataxin2, ataxin3, CACNA1$_A$, and TATA-binding protein, respectively; and dentatorubrol-pallidoluysian atrophy (DRPLA) caused by mutations in the *DRPLA* gene (268). The dynamic nature of these unstable mutations is responsible for the variable phenotype even within a single family.

Many of these misfolded proteins form toxic aggregates; therefore, the mutant protein is usually dominant negative over the WT protein. The accumulation of the toxic aggregates initiates a vicious cycle that feeds on itself causing increasing aggregation and disruption of the proteome, finally manifesting as a pathology. One mechanism of the toxicity is that protein aggregation directly causes an impairment of the UPS function (26). In vitro, transient expression of two unrelated aggregation-prone proteins almost completely inhibits the UPS (26). Dominant negative activity is also the cause of several endocrine diseases, such as neurogenic diabetes insipidus caused by mutations in the *AVP* gene (encoding arginine vasopressin), growth hormone deficiency caused by the *GH1* gene (encoding growth hormone 1), thyroid hormone resistance caused by mutations in the *THRB* gene (encoding thyroid hormone receptor β), and familial hypocalciuric hypercalcemia caused by mutations in the *CASR* gene (encoding the calcium-sensing receptor) (reviewed in Ref. 285). In some genes, both haploinsufficiency and dominant negative activity may cause the disease. For example, although heterodimerization causing dominant negative activity has been observed in many GPCRs (reviewed in Refs. 42, 250, 349, 353), in the MC4R, the vast majority of the heterozygous mutations do

not cause dominant negative activity, even though they may be retained intracellularly (reviewed in Refs. 351, 352).

D. Diseases Caused by Mutations in GPCR Genes

GPCRs comprise the largest family of membrane proteins, regulating almost all physiological processes. In humans, there are 799 GPCRs, with about half encoding olfactory receptors. They mediate the actions of diverse extracellular signals, including photons, ions, odorants, amino acids, fatty acids, nucleotides, peptides, and large glycoproteins (223). They are the leading therapeutic targets in the pharmaceutical market, accounting for >30% of sales, including some of the most commonly prescribed medications (270). Numerous genetic diseases are caused by inactivating or activating mutations in GPCRs (347, 349).

These mutations cause diseases by four major pathways, and we categorize these mutations into four classes (FIGURE 1): class I, decreased mRNA levels with consequent decreased proteins levels, mostly caused by nonsense and frameshift mutations; class II, receptors that are retained intracellularly, primarily in the ER with some mutants retained in the Golgi apparatus; class III, mutant receptors that reach the cell surface but are defective in binding to the ligand; and class IV, mutant receptors that reach the cell surface, bind to the ligand but are unable to transform the ligand binding into intracellular signaling (351, 356), although defective constitutive signaling has also been found. Functional studies, especially in receptors with numerous mutations, show that the most common defect is intracellular retention (class II) (349, 353). In some cases, although the mutations were identified from patients with a particular disease, the mutant receptors have no obvious defect; we classify these mutants as class V.

E. Diseases Caused by Mutations in Polypeptide Hormone Genes

Polypeptide hormones that are secreted and circulating in the blood are also prototypic examples of proteins of the secretory pathway. They are usually produced as prepro-hormones in the ER of the endocrine cells and processed by protease into mature hormones. There are also excellent examples of mutations in these preprohormone genes that cause the encoded protein to be misfolded, and the mature hormone molecules fail to be secreted. Whereas mutations in V2 vasopressin receptor or aquaporin cause nephrogenic diabetes insipidus, neurogenic (familial neurohypophysial) diabetes insipidus is caused by mutations in the *AVP* gene that encodes the arginine vasopressin (AVP) (169, 251). This disease is inherited in an autosomal dominant fashion, suggesting potential neuronal toxicity of mutant proteins. Expression studies in Neuro2A neuroblastoma cells show that mutant AVPs are retained in the ER, not trafficked through the secretory pathway and leads to decreased secretion of AVP (168, 262). ER retention of mutant AVP is confirmed in a transgenic rat model expressing a mutant AVP (331). Dominant AVP mutants form disulfide-linked aggregates in the ER that has a fibrillar substructure, resembling other neurodegenerative diseases that have fibrillar protein aggregates (33). Differentiated Neuro2A cells expressing mutant AVPs have decreased viability, suggesting that the intracellular accumulation of mutant AVPs is toxic, leading to progressive neuronal cell loss (168) that is confirmed in a transgenic mouse model (316). Indeed, the mutant AVPs dimerize with WT AVP and exerts dominant negative effect on WT AVP secretion (170).

These are just some of the prominent examples of protein conformational diseases. Many more are described in the literature that are not elaborated herein. Interested readers
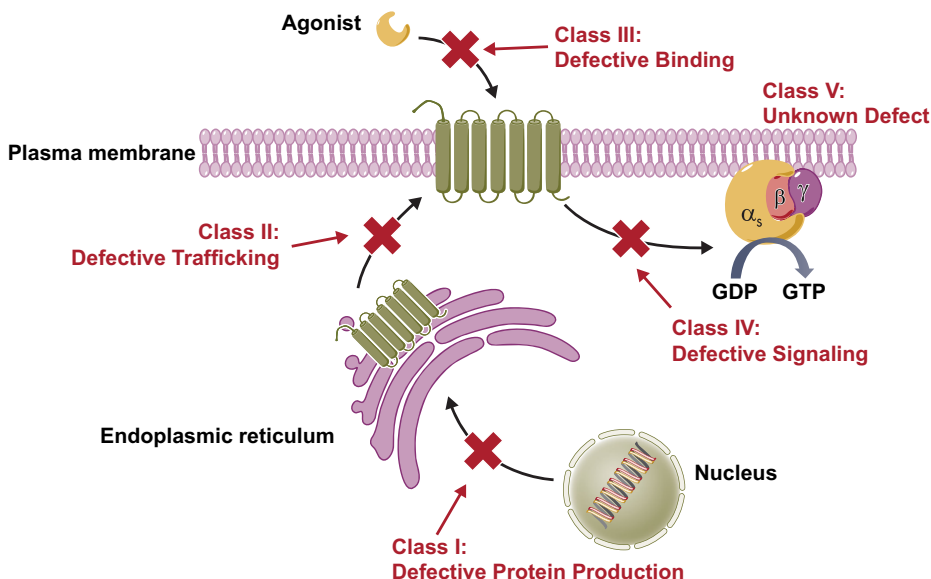

FIGURE 1. Classification of naturally occurring inactivating mutations of GPCRs. [From Tao and Conn (353). Copyright 2014 The Endocrine Society.]

are directed to the following classical as well as current review articles on this topic for further information, with some of these articles focused on loss-of-function mutations and others focused on gain-of-function mutations (14, 15, 41, 56, 64, 65, 70, 129, 142, 212, 279, 312, 338, 344, 371, 392).

IV. MOLECULAR CHAPERONES AS TOOLS AND POTENTIAL THERAPEUTICS FOR CORRECTING MISFOLDED PROTEINS CAUSING PROTEIN CONFORMATIONAL DISEASES

Molecular chaperones are defined as proteins that assist the folding or assembly of other proteins but are not present in the final functional protein (96). They have been traditionally credited with performing two functions: preventing aggregation of folding intermediates or unfolded proteins and facilitating the folding of misfolded proteins. Subsequently, some chaperones are shown to serve as disaggregases, breaking up protein aggregates (276). In mammalian cells, HSP110, HSP70, or Hsc70 and HSP40 work together to slowly dissolve large disordered aggregates, suggesting that HSP110 fulfils a subset of HSP104 activities in mammals (330).

Disulfide bond, covalent interaction between two cysteine residues, is an important co- and posttranslational modification for proteins of the secretory pathway, enhancing structural stability and promoting the assembly of multiprotein complexes. Correct disulfide bond formation is critical for proteins to assume native conformation (103), and aberrant disulfide bonds contribute to protein aggregation, especially in neurological disorders such as Alzheimer's disease, Parkinson's disease, and ALS (6). **FIGURE 2** depicts two disulfide bonds that are essential for proteins to assume native conformation of the GnRHR. Members of the protein disulfide isomerase (PDI) family and ERp57 catalyze the formation of disulfides in the oxidizing environment of

the ER (391). PDI family members are important players in both the formation and reduction of disulfides for correct folding of proteins entering the ER, exhibiting both oxidase activity (introducing disulfides into proteins) and isomerase activity (promoting the rearrangement of incorrect disulfides to correct disulfides) (391). During the normal folding process, cysteine residues that are close to each other can form disulfides in the folding intermediates, although some of these disulfides will not be found in the final native conformation. Some PDI enzymes will reduce non-native disulfides so that native disulfides can form. In misfolded proteins, non-native disulfides are not broken down; rather, they are retained. A tightly controlled redox homeostasis is necessary for the formation of correct disulfide bonding in the ER (206).

Variants in *PDI* are associated with ALS, causing motor dysfunction (394). These PDI variants can impair synaptic protein expression and compromise neuromuscular junction integrity (394). Aberrant disulfide bond formation has been found in mutant rhodopsin (87), MC4R (357), α_1-antitrypsin (309), as well as many other proteins.

Some rhodopsin mutants are misfolded, retained in the ER, and form cytoplasmic ubiquitinated inclusions, aggresome-like structure. The immunoglobulin heavy chain binding protein (BiP), the 78-kDa glucose-regulated protein (259), prevents rhodopsin aggregation, with overexpression of BiP decreasing mutant rhodopsin aggregation and inhibition of BiP resulting in enhanced aggregation (17).

HSP90 has also been shown to be important for the folding of GPCRs. For example, with the MC4R, overexpression of HSC70 increases cell surface expression of WT and mutant receptors and mutant receptor signaling, whereas expression of a cochaperone that accelerates the degradation of HSC70 clients, HSJ1b, decreases MC4R expression (248). Inhibition of endogenous HSP90 by geldanamycin decreases MC4R levels, and expression of the HSP90 cochap-
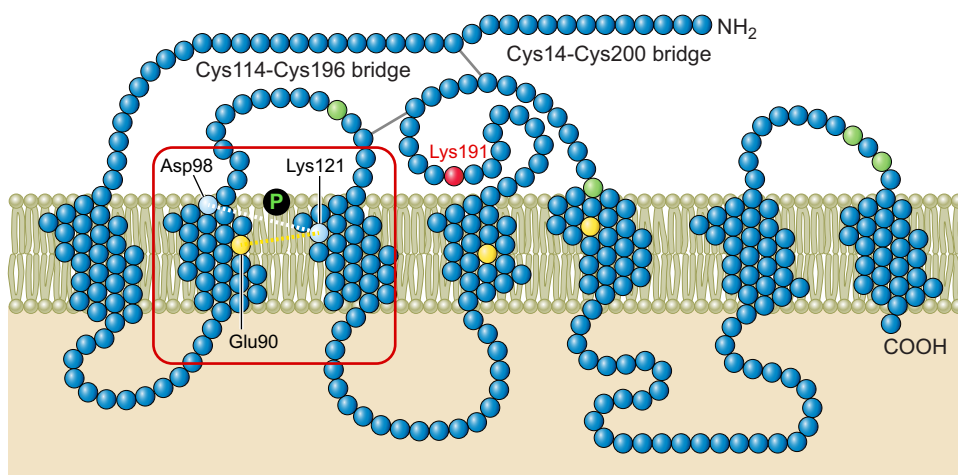

FIGURE 2. Serpentine cartoon of the GnRHR. Highlighted are two disulfide bridges that are required for passage to the plasma membrane and the site of binding of many pharmacoperones of the GnRHR. The position of the 114–196 bridge is common in GPCRs while the 14–200 bridge is uncommon, but its formation is required for the human (but not rat or mouse) GnRHR to traffic correctly.

erone Aha1 (activator of HSP90 ATPase) increases MC4R levels (248).

The molecular chaperones produced endogenously can facilitate the folding of all nascent proteins; therefore, there is no specificity (TABLE 1). Although it is possible to modulate the expression of molecular chaperones to partially correct misfolded proteins in vitro in cell models and in vivo in animal models, the therapeutic potential in humans is limited due to the lack of specificity.

V. CHEMICAL CHAPERONES AS TOOLS AND POTENTIAL THERAPEUTICS FOR CORRECTING MISFOLDED PROTEINS CAUSING PROTEIN CONFORMATIONAL DISEASES

High osmotic pressure conditions cause some organisms, including bacteria, plants, and invertebrate and vertebrate animals, to accumulate low-molecular-weight components, neutral substances such as polyhydric alcohols, sugars, amino acids, and related compounds at moderate to high concentrations (up to several molar) to raise the osmotic pressure in the cytoplasm (405). These organic osmolytes, together with the inorganic ions, account for the majority of osmotic force, contributing to the internal environment where biochemical reactions occur. The major organic osmolytes include polyhydric alcohols (polyols), such as glycerol and sucrose; some free amino acids and derivatives such as taurine and β-alanine; as well as urea and methylamines such as trimethylamine-N-oxide (TMAO), betaine, and sarcosine (405). The organic osmolytes have been shown to be able to stabilize proteins by minimizing the water-protein interface area and shifting the balance towards the native state rather than the denatured state (13). Among the organic methylamines, the fully methylated TMAO is the most potent in stabilizing macromolecules (405).

Glycerol changes the solvent properties of water. It was shown that glycerol is largely excluded from the ordered vicinal water around proteins (115, 116). Therefore, addition of glycerol to a protein solution (or intracellularly) changes the thermodynamics, favoring the native conformation (405). With ΔF508 CFTR, incubation with 10% glycerol induces the EndoH-resistant fraction that bears complex N-linked oligosaccharides, with increased cAMP-stimulated channel activity, suggesting that the glycerol-rescued fraction not only exits the ER but also reaches the plasma membrane where it forms functional channels (321). The effects of glycerol are evident only in temperature-sensitive mutants (83, 321). In addition to glycerol, the other cellular osmolyte TMAO, as well as deuterated water, has similar chaperone effects (43).

Mutations in *aquaporin-2* (*AQP2*) cause non-*X*-linked nephrogenic diabetes insipidus. Some mutant AQP2 are primarily retained in the ER, with reticular and perinuclear vesicular localization (345). Glycerol, TMAO, and DMSO induce an almost complete redistribution of some AQP2 mutants from the ER to the membrane/endosome fractions, where they function as water channels (345).

Sodium 4-phenylbutyrate (PBA) is a new drug recently approved by the United States Food and Drug Administration (FDA) for treating urea cycle disorders. With ΔF508 CFTR, in vitro studies showed that PBA, by downregulating HSC70, induces the trafficking of the mutant CFTR to the plasma membrane with functional restoration of chloride channel activity (313, 315). Further genomic and proteomic analyses identified the proteins modulated by PBA, especially chaperones associated with ERAD, highlighting the importance of members of HSP70 family (332, 333, 395). Randomized, double-blind, placebo-controlled clinical trials show that Buphenyl, the FDA-approved PBA for treating urea cycle disorders by scavenging ammonia, causes statistically significant partial induction of chloride transport in patients homozygous for ΔF508 CFTR, hence a viable therapeutic approach for CF (314, 407).

Heterozygous mutations in bone morphogenetic protein (BMP) type II receptor (*BMPR-II*) cause familial pulmonary arterial hypertension. Some mutations in *BMPR-II* cause the mutant receptors to be unable to traffic to the plasma membrane. In vitro studies demonstrated that several chemical chaperones, including thapsigargin, glycerol, or PBA, cause a marked increase in cell surface expression of a mutant BMPR-II, C118W, that is usually retained in the ER (337). The rescued mutant receptor is competent in responding to BMP stimulation with increased phosphorylation of Smad1/5. For kinase-deficient BMPR-II mutants, although BPA can rescue it to the cell surface, there is no

Table 1. *Summary of molecular, chemical, and pharmacological chaperones*

Chaperone	Source	Specificity	Examples
Molecular chaperone	Endogenous	Nonspecific	Calnexin, calreticulin, PDI, ERp57, HSP
Chemical chaperone	Exogenous	Nonspecific	Glycerol, DMSO, TMAO, PBA
Pharmacological chaperone	Exogenous	Specific	Enzyme substrate, receptor agonist, antagonist, and allosteric modulator

DMSO, dimethyl sulfoxide; HSP, heat shock protein; PBA, 4-phenylbutyric acid; PDI, protein disulfide isomerase; TMAO, trimethylamine-N-oxide.

response to BMP stimulation with increased Smad1/5 phosphorylation. In addition, chemical chaperones also increase the cell surface expression of WT BMPR-II (337), suggesting that WT BMPR-II folding and trafficking are not optimal, a theme prevalent in many proteins of the secretory pathway.

In cell and mouse models, PBA was shown to result in partial correction of Z α_1-antitrypsin (47). However, in a preliminary open label study on 10 patients, oral administration of 4-PBA for 14 days does not result in significant increase in blood α_1-antitrypsin levels, despite significant symptomatic and metabolic side effects (361).

In studies with mutant V2Rs, only one of the nine mutants studied has increased cell surface expression after treatment with chemical chaperones including glycerol and DMSO (302). Growing the cells at 27°C or treatment with other osmolytes has no effect on mutant receptor maturation and cell surface density (302). Treatment of four mutant MC4Rs with PBA showed that only one is rescued to the cell surface, whereas it has no effect on the other three mutant receptors (128). Compared with data generated with pharmacoperones (see below), these results suggest that chemical chaperones are effective in only limited mutants and the efficacy is less dramatic compared with pharmacoperones.

Mutation in preproparathyroid hormone (*PPTH*) causes autosomal dominant familial isolated hypoparathyroidism (AD-FIH). The mutant hormone is trapped intracellularly, primarily in the ER, causing ER stress and inducing apoptosis (82). PBA decreases intracellular accumulation and restores normal secretion of the mutant hormone, together with attenuated ER stress and apoptosis, suggesting that chemical chaperones might serve as a therapeutic option for treating this rare disease (82). PBA also increases mutant Factor H secretion, the deficiency of which is associated with higher risk for infections and kidney diseases (4).

In addition to serving as a chemical chaperone, PBA also attenuates the UPR activation (23). It ameliorates ER stress that is critically involved in the pathogenesis of obesity and type 2 diabetes mellitus (271); hence, PBA restores glucose homeostasis in mouse diabetes models and inhibits adipogenesis (23, 272).

In summary, chemical chaperones such as PBA are safe and effective in human therapeutics. When used at the proper concentration, they can potentially ameliorate a number of diseases. For example, it was recently shown that in adipocytes, very low concentrations of PBA inhibit only pathologic ER stress and UPR induced by glucotoxic insults, leaving the physiologically activated response during differentiation unaltered (235). An important question is whether systemic administration of chemical chaperones will shift the set point of proteostasis in the whole organism resulting in undesired side effects. Similar to the molecular chaperones, the lack of specificity limits the therapeutic potential of chemical chaperones (TABLE 1).

VI. PHARMACOLOGICAL CHAPERONES AS POTENTIAL THERAPEUTICS FOR CORRECTING MISFOLDED PROTEINS CAUSING PROTEIN CONFORMATIONAL DISEASES

Pharmacological chaperones, or pharmacoperones (pharmacochaperones), are small molecules that specifically bind to the target protein, stabilizing the native conformation or facilitating the folding of non-native intermediates into native conformation (12, 70, 77, 99, 193, 237, 353, 368). Although detected by the ER quality control system as misfolded, the mutant proteins sometimes retain function. ER quality control does not detect newly folded proteins for function; some mutant proteins can fold normally, hence not defective in trafficking but defective in function. For example, class III and IV GPCR mutants are localized at the cell surface but either cannot bind the ligand (class III) or can bind the ligand but cannot activate downstream signaling (349, 351, 353). P556L TSHR and $\Delta88-92$ MC4R are examples of class III mutations (89, 130), whereas I183N MC3R is a class IV mutation (355). Pharmacoperones cannot correct these class III and class IV mutants.

Pharmacoperones need to be able to cross the plasma and ER membranes, specifically bind to the misfolded proteins in the ER, and escort them out of the ER by promoting the folding of the mutant protein. Usually, there is a positive correlation between the binding affinity and chaperoning potency, with the compounds exhibiting higher affinity showing higher chaperoning activity (175). FIGURE 3 shows several pharmacoperones for the GnRHR. Although they come from diverse chemical groups, the GnRHR recognizes them as specific agonists.

However, there is a trade-off between affinity and in vivo utility, especially with those inhibitors (for enzymes) or antagonists (for receptors) serving as pharmacoperones, because in vivo, these pharmacoperones will need to be displaced from the clients once reaching the final destination. When the affinity is too high, the displacement will be difficult, diminishing potential therapeutic utility. Pharmacoperones do not have to be antagonists; agonists and allosteric modulators can also serve as pharmacoperones (158, 180, 353).

Pharmacoperones can be identified in at least two ways. One is through optimization of natural or synthetic substrates, inhibitors, or ligands, and the other approach is through screening of libraries.
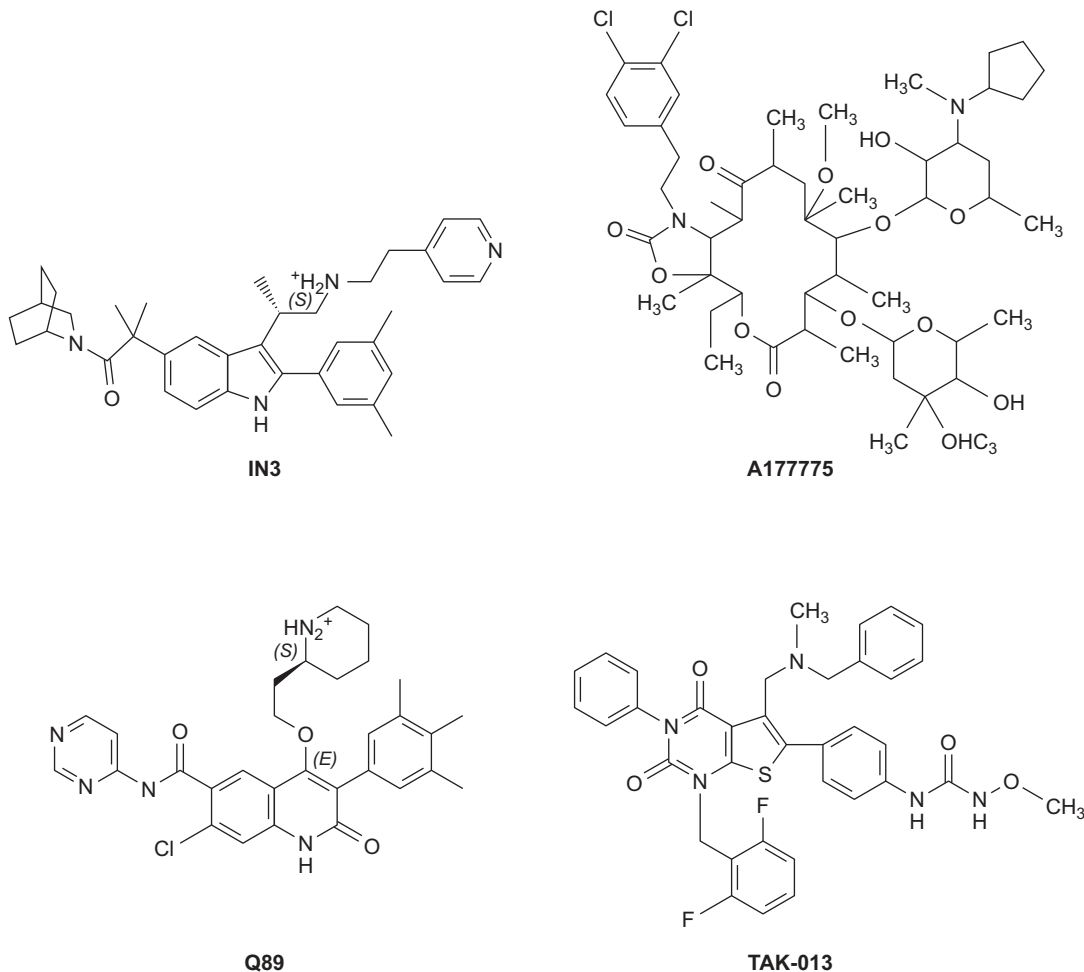
IN3

A177775

Q89

TAK-013

FIGURE 3. Target-specific peptidomimetic antagonists that rescue GnRHR mutants. Target specific pharmacoperones of the GnRHR. These are small hydrophobic drugs that diffuse into the cell and serve as templates to fold or refold misfolded mutants.

A. Cystic Fibrosis

CF is an autosomal recessive fatal genetic disease, affecting multiple organs, including the lungs, intestine, liver, pancreas, and other exocrine glands as well as the male reproductive tract (resulting in infertility in the homozygous males). It is the most common genetic disease in Caucasians, affecting 1 in every 2,000 live births. Recurrent bacterial infection of the lungs due to disruption of the bronchial mucociliary escalator, leading to progressive bronchiectasis and respiratory failure, is usually the cause of death in CF patients. The genetic cause of CF was first revealed in 1989 with the cloning of the *CFTR* gene and identification of mutations in *CFTR* (199, 300). *CFTR* is a large gene, spanning almost 250 kb, with 24 exons, encoding a protein of 1,480 amino acids. The CFTR is a transmembrane protein that serves as a chloride channel, transporting chloride ion across the cell membrane (reviewed in Ref. 210). So far more than 1,900 *CFTR* mutations have been identified (updated at www.genet.sickkids.on.ca/cftr).

The most common mutation in *CFTR*, ΔF508, resulting from an inframe deletion of three nucleotides, is found in ~70% of the CF chromosomes (199). Subsequent studies quickly identified the defect of this common CFTR mutant: defective intracellular transport and processing of CFTR is the molecular basis for most cystic fibrosis (63). Unlike the WT CFTR, ΔF508 is expressed as an incompletely glycosylated protein that is sensitive to EndoH digestion, suggesting that it is retained in the ER, whereas the mature form of the WT CFTR is resistant to EndoH treatment but sensitive to neuraminidase digestion, suggesting that it has already exited the ER. The ΔF508 CFTR is temperature sensitive: incubation of the cells expressing ΔF508 CFTR at reduced temperatures enhances the maturation of the mutant protein (83), highlighting that this mutant CFTR can be corrected. Of special relevance for clinical care is that restoration of partial (6–25%) membrane expression can lead to dramatic improvement in patients' health (189, 410).

After successful correction by decreased temperature and chemical chaperones was demonstrated, extensive studies were performed to identify small molecule compounds that can serve as pharmacoperones, correcting ΔF508 CFTR trafficking defect (54, 55, 90, 236, 277, 304, 305, 320, 375, 387, 408; reviewed in Refs. 138, 240). These studies led to the identification of chemically diverse compounds, including a promising drug candidate, VX-809/Lumacaftor, that

was tested in clinical trials. Unfortunately, when administered alone, VX-809 leads to no significant clinical improvement in ΔF508-CFTR patients (68), although combination with a CFTR potentiator VX-770 results in a small improvement in lung function in these patients. One drawback is that VX-770 has been shown to decrease the rescue effect of VX-809 on the functional expression of ΔF508-CFTR at the plasma membrane (66, 379). More potent pharmacoperones and other combination protocols targeting different mechanisms are needed (286, 366, 378).

B. Lysosomal Storage Disorders

Lysosomal storage disorders (LSDs) are a group of more than 50 disorders, occurring in from 1:1,500 to 7,000 live births (340). LSDs are generally characterized by the deficiency in a lysosomal enzyme, resulting in the accumulation of the macromolecular substrates and progressive deterioration in the patients, leading to organ dysfunctions and premature death (247). The deficiency of different lysosomal enzymes results in the accumulation of various glycosphingolipids, causing distinct diseases (such as Fabry disease or Gaucher disease). These rare inborn errors of metabolism disorders exhibit a broad spectrum of phenotypes. In severe cases, the lysosomal build-up of undigested substrate starts prenatally. In many cases, onset happens in early infancy in patients with mutations resulting in very low residual enzyme activity. On the other end of the spectrum, some patients have late-onset disease due to less severe mutation with higher residual enzyme activity. Analysis of the correlation between residual enzyme activity and disease severity suggested that a modest restoration of 10–15% of enzyme activity may be sufficient to prevent the pathogenesis of LSD (100, 226, 326); it is not necessary to restore enzyme activity to the normal level for a significant clinical benefit. This also explains that the penetrance is not complete in many cases of mutations with residual enzyme activity.

Current treatment strategies include enzyme replacement therapy, substrate reduction therapy, bone marrow transplantation, with gene and stem cell therapies actively investigated. Another strategy is using small molecule inhibitors at subinhibitory concentration, a paradoxical approach (99). This approach was first studied in Fabry disease, a disorder of glycosphingolipid metabolism caused by deficiency of lysosomal α-galactosidase A (α-Gal A) encoded by the *GLA* gene, resulting in renal failure, myocardial infarction and stroke, and premature death (118). More than 400 mutations in *GLA*, primarily missense mutations with some nonsense mutations and indels of single amino acids, have been identified (247). Fabry disease is one of the few X-linked LSDs, although female heterozygous patients are also affected, likely due to random X chromosome inactivation. Currently, the main lines of treatment are enzyme replacement or substrate reduction therapy (247). Some of the mutant enzymes in Fabry disease have kinetic properties similar to those for WT α-Gal A, but they are less stable and form aggregates in the ER and have accelerated degradation, resulting in enzyme deficiency.

Fan et al. (101) showed that in Fabry lymphoblasts, a potent competitive inhibitor of α-Gal A, 1-deoxy-galactonojirimycin (DGJ), enhances α-Gal A activity when used at subinhibitory intracellular concentrations by enhancing transport and maturation of the WT and mutant enzymes [later it was shown that the pharmacoperone releases the mutants α-Gal A from BiP (401)]. In transgenic mice overexpressing a mutant α-Gal A, DGJ is able to substantially increase the enzyme activity in some organs when administered orally (101). This pioneering study demonstrated that paradoxically, competitive inhibitors can treat genetic metabolic diseases caused by enzyme deficiency (reviewed in Ref. 99). Subsequent studies with a large panel of mutants showed that DGJ can substantially increase the enzyme activity of up to 60% of mutations from both classic and cardiac forms of Fabry disease patients, suggesting that it may be used to treat many Fabry disease patients with missense mutations (165, 397). In vivo experiments in transgenic mice expressing mutant human α-Gal A showed that DGJ is safe and effective in restoring enzyme activity in multiple tissues and decreasing substrate storage (166, 202).

Clinical proof of concept for the pharmacoperone approach has been reported in a patient with the cardiac variant of Fabry disease, harboring G328R mutation that retains residual α-Gal A activity (112). Infusion of the weak inhibitor of α-Gal A, galactose, is safe and effective therapeutically, with marked improvement in cardiac contractility (112). The cardiac variant of Fabry disease, in contrast to the classic form of the disease, has later onset and milder phenotype, without the severe vascular phenotype that usually results in death in early adulthood. Previous in vitro studies showed that galactose increases the activities of these variants (167, 267). However, in a clinical trial, pharmacoperone treatment in patients with mutant α-Gal A does not result in significant decrease in the number of globotriaosylceremide inclusions per kidney interstitial capillary, although there may be improvements in gastrointestinal symptoms (119).

Since the original studies of Fan et al. (101), similar findings have been reported for Gaucher disease (200, 228, 322), Tay-Sachs and Sandhoff diseases (365), GM$_1$-gangliosidosis (244), Pompe disease (201), and Schindler/Kanzaki disease (69). For example, Gaucher disease, the most prevalent lysosomal storage disease, is caused by mutations in the gene encoding β-glucocerebrosidase, *GBA1*, leading to accumulation of glucosylceramide in the macrophages. Mutant enzymes are retained in the ER and degraded by the proteasome (307, 324). Both inhibitors and noninhibitors

serving as pharmacoperones have been identified, rescuing some of the mutant enzymes to the lysosome (3, 61, 200, 228, 322, 341; reviewed in Ref. 196), although some other effects such as shifting the pH optimum have also been observed (341). Because of the inverse relationship between β-glucocerebrosidase activity and α-synuclein aggregation (linked to Parkinson's disease) (245), enhancement of β-glucocerebrosidase activity, especially by noninhibitory pharmacoperones, might have implications for the treatment of Parkinson's disease, as recently demonstrated in induced pluripotent dopaminergic stem cells from Gaucher disease patients (2). Treatment of these cells with noninhibitory pharmacoperone NCGC607 leads to decreased α-synuclein levels in these cells, with potential utility for treating Parkinson's disease and other synucleinopathies (2). In vivo, an earlier study showed that an inhibitory pharmacoperone, isofagomine, when administered orally, can improve motor function, abolish microglial inflammation in the substantia nigra, and decrease the number of small α-synuclein aggregates (298). Since the transgenic mice overexpress WT human β-glucocerebrosidase, this represents another example of the imperfect maturation of WT protein.

Schindler/Kanzaki disease is an inherited neurological disease caused by mutation in a gene encoding a lysosomal enzyme, α-N-acetylgalactosaminidase (α-NAGAL), *NAGA*. Both DGJ and DGJNAc (2-acetamido-1,2-dideoxy-d-galactonojirimycin, a specific inhibitor for α-NAGAL) can bind to the mutant human α-NAGAL, stabilizing and chaperoning it to the lysosome (69).

A distinct advantage of pharmacoperones over enzyme replacement is the ability to cross the blood-brain barrier compared with the inability of enzymes to do so, therefore better suited for LSDs that have neurological dysfunctions. However, pharmacoperones cannot correct all mutant enzymes (27, 69), correcting ~50% of mutant enzymes causing Fabry disease. Another interesting point raised is the specificity of the pharmacoperones. DGJ is a pharmacoperone in clinical trial for Fabry disease; its actions on α-NAGAL therefore represent an off-target effect, a potential source for side effects (69).

Interestingly, the WT enzymes are not folded optimally which can be improved with pharmacoperones (69, 101, 289). For example, pharmacoperones increase the maturation of recombinant human α-glucosidase and α-galactosidase A when co-administered (289). Therapeutically, this is important because although pharmacoperones can be used in patients with misfolded enzymes, in patients that have almost no residual enzyme activities, pharmacoperones alone are likely not effective in treating the patients. They will need enzyme replacement. However, even in these patients, pharmacoperones co-administration will likely increase the efficacy of the enzymes administered, significantly reducing the cost of treatment.

C. p53 and Cancer

The tumor suppressor p53, a DNA-binding transcription factor, is a key regulator of the cell's defense against cancer pathogenesis. p53 and associated cell-cycle pathways arrest the cell cycle and induce senescence and apoptosis in an emerging cancerous cell and upon cellular stress by controlling gene transcription as well as a variety of other mechanisms (381, 382). For cancer pathogenesis, p53 or its pathways must be inactivated by mutation. Indeed, loss of p53 function is found in almost all human tumors, with mutation in *TP53* (that encodes p53) observed in ~50% of human cancers, almost exclusively in the core DNA-binding domain (35, 186, 187), whereas in the remaining tumors, p53-associated pathways are deficient. Many oncogenic p53 missense mutants have decreased stability, with increased elimination by denaturation and aggregation (46). Methods to stabilize p53 mutants can potentially reactivate apoptotic signaling pathways in tumor cells, hence being used to treat cancer with these destabilizing p53 mutations.

Binding of a specific double-stranded DNA (45), heparin, or a peptide derived from p53-binding protein (111) can stabilize mutant p53. The peptide, acting as a chaperone, even restores sequence-specific DNA binding activity of a highly destabilized mutant I195T to almost that of the WT p53 level (111). Importantly, small molecule compounds have been identified, either by chemical library screening or virtual screening and rational drug design, that can act as pharmacoperones, specifically stabilizing p53 mutants, including some frequently found in human cancers (35, 49, 50, 106, 218, 219, 301; reviewed in Ref. 51). These compounds inhibit tumor cell growth both in vitro and in vivo; they also work synergistically with traditional chemotherapeutic drugs (52). One pharmacoperone, APR-246, shows promising results in a phase I/II clinical trial, being safe and effective in inducing cell cycle arrest, apoptosis, and target gene expression (224). Precision medicine targeting the specific mutation in *TP53* is feasible.

D. Diseases Caused by Mutations in GPCR Genes

Morello et al. (256) reported the utility of pharmacoperones for *AVPR2* mutations associated with nephrogenic diabetes insipidus. In this study, selective nonpeptidic V2R antagonists SR121463A and VPA-985 were shown to be able to correct eight naturally occurring *AVPR2* mutations, and this effect is not blocked by peptide antagonists, suggesting that the site of action is intracellular. It was suggested that because of their hydrophobic nature, SR121463A and VPA-985 can pass through the plasma and ER membranes and bind to the misfolded mutants in the ER and rescue them onto the cell surface. Once they reach the cell surface, at least some of them are functional in responding to agonist stimulation. Since this study, pharmacoper-

Table 2. *Pharmacoperones for misfolded GPCRs*

Receptor	Diseases or Function	Reference Nos.
Rhodopsin	Retinitis pigmentosa	263, 264
V2R	Nephrogenic diabetes insipidus	256
GnRHR	Hypogonadotropic hypogonadism	177, 221
LHCGR	Hypergonadotropic hypogonadism	260
CaSR	Familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism	160
FSHR	Hypergonadotropic hypogonadism	178
MC4R	Obesity	102, 297
δ-Opioid receptor	Pain perception	281
μ-Opioid receptor	Pain perception	60
κ-Opioid receptor	Pain perception	62
D_4 dopamine receptor	Idiopathic Parkinson's disease	374
MCHR	Energy homeostasis	98
Kinin B1 receptor	Allergic airway disease	105
V_{1a} receptor	Arterial hypertension, congestive heart failure, and peripheral vascular disease	145
V_{1b}/V_3 receptor	Stress response	303
A_1 adenosine receptor	Type 2 diabetes and cardio- and neuroprotection	243

ones have been identified for both naturally occurring and laboratory-generated mutant GPCRs **(TABLE 2)** (reviewed in Refs. 75, 76, 353). **FIGURE 4** demonstrates the cellular mechanism of pharmacoperone action.

In vitro studies on multiple mutations revealed that some mutants do not signal even after being rescued to the cell surface, suggesting additional defects in ligand binding and/or signaling. For example, in the MC4R, we show that G98R can be efficiently rescued to the cell surface with two pharmacoperones, ML00253764 and Ipsen 5i, but no sig-

naling can be obtained (350, 354). In the GnRHR, one mutant cannot be rescued. Therefore in vitro experiments can be used to predict which patients will benefit from the pharmacoperone treatment. Even in previously undruggable GPCR, the Frizzled4 (Fz4) receptor, pharmacoperones were identified for a misfolded mutant (117).

The first clinical trial of this strategy was again reported in nephrogenic diabetes insipidus patients (31). Short-term treatment with a nonpeptidic V1a receptor antagonist (SR49059) in X-linked nephrogenic diabetes insipidus pa-
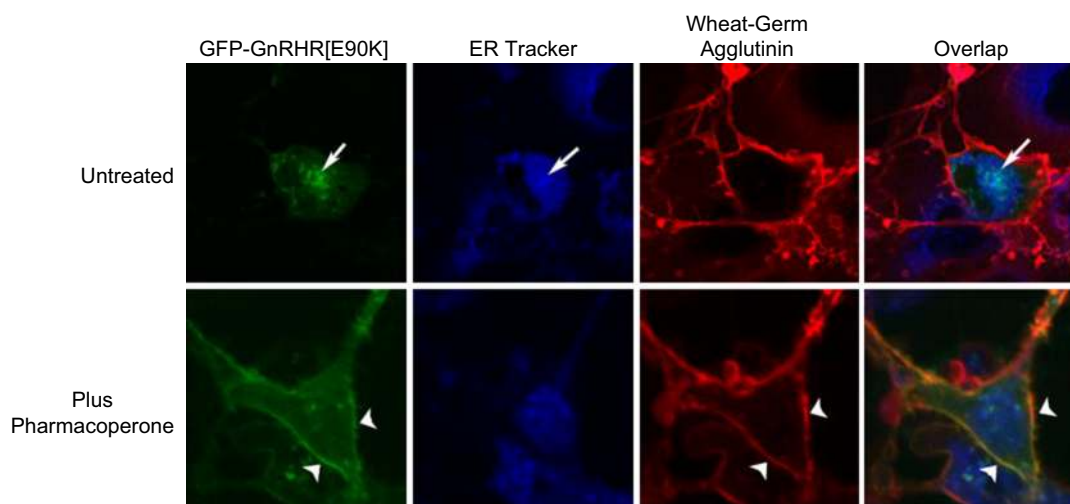

FIGURE 4. Cellular demonstration of the underlying mechanism of pharmacoperone rescue. Confocal images of Cos-7 cells transiently cotransfected with the GFP-tagged human GnRHR in green and the dominant-negative human GnRHR (E90K) and labeled with ER tracker (blue) and wheat-germ agglutinin Alexa-633 (red). The yellow color shows the overlap between the plasma membrane marker and the mutant receptor. Treatment with 1 μg/ml IN3 for 4 h allows the mutant receptor to leave the ER and move to the plasma membrane (arrowheads). [Modified from Janovick et al. (172). Copyright 2007 Elsevier.]

tients results in decreased urine volume and water intake and increased urine osmolality (31). Although the clinical trial was ended due to hepatic toxicity (31), it showed that indeed this approach is clinically feasible. Similarly, in vivo studies in mice with a knockin mutation of GnRHR, pulsatile administration of an antagonist that serves as a pharmacoperone rescues the mutant GnRHR, E90K, from ER to the plasma membrane, which responds to the stimulation to the endogenous agonist, resulting in restored steroidogenesis and spermatogenesis, at least partially, and serum androgen completely (181). Similar in vivo experiments in other GPCRs where pharmacoperones have been identified are eagerly awaited.

E. Miscellaneous Diseases

Tyrosinase is the key enzyme in melanin synthesis; mutations in tyrosinase (*TYR*) gene are associated with oculocutaneous albinism type 1. The mutant tyrosinases are retained in the ER and have prolonged association with calnexin and calreticulin (32, 135). Interestingly, in advanced and metastatic melanoma, tyrosinase activity is also diminished due to retention of tyrosinase in the ER (134). Tyrosinase catalyzes the oxidation of tyrosine and L-3,4-dihydroxyphenylalanine (DOPA) to dopaquinone (335). Both the substrate tyrosine and the cofactor DOPA are required for the promotion of folding, ER exit, and transport to Golgi apparatus of tyrosinase, serving as pharmacoperones (133). The effect of DOPA is evident both co- and posttranslationally, promoting the folding of both nascent and preexistent tyrosinase (133). It is suggested that these compounds stabilize the tyrosinase so that it cannot serve as the substrate for UDP-glucose:glycoprotein glucosyltransferase to be reglucosylated, hence released from the ER (133).

The sulfonylurea receptor 1 (SUR1), a member of the multispanning transmembrane proteins of the ATP-binding cassette (ABC) family (like CFTR), oligomerizes with inwardly rectifying K^+ (Kir) channel Kir6.2 to form ATP-sensitive potassium channel (K_{ATP}) to regulate insulin secretion in pancreatic β cells in response to blood glucose concentration. Loss-of-function mutations in genes encoding either the SUR1 (*ABCC8*) or Kir6.2 (*KCNJ11*) channel subunit of the K_{ATP} cause congenital hyperinsulinemia or persistent hyperinsulinemic hypoglycemia of infancy (164). Some of the causative mutations in *SUR1* result in the mutant channel proteins failing to reach the cell surface, although they retain at least partial channel activity if coaxed to the cell surface (for example, by masking the ER retention motif through site-directed mutagenesis) (57, 358). This is because the structural features required for trafficking are different from those required for proper channel function (58). Interestingly, sulfonylureas, K_{ATP} channel antagonists that have long been used to treat type 2 diabetes, can correct the trafficking defects in some of these mutations (403, 404).

Mutations in phenylalanine hydroxylase (*PAH*) cause the inborn error of metabolism, phenylketonuria (OMIM no. 261600), with L-Phe accumulating in the blood that is toxic to the brain. Of the more than 500 disease-causing mutations (see PAH Mutation Analysis Consortium database at http://www.pahdb.mcgill.edu/; Ref. 155), the most common defect is misfolding and consequent accelerated turnover, leading to loss of enzymatic activity (283). High-throughput screening identified compounds that could serve as pharmacoperones for the mutant PAH, hence promising alternative treatments for phenylketonuria (284).

Menkes disease is a neurodegenerative disorder with severe neurological symptoms such as seizures, lethargy, and hypotonia, resulting in death in early childhood. It is caused by copper deficiency due to mutations in *ATP7A* that codes for the copper ATPase, copper-transporting ATPase 1 (383). Copper-transporting ATPase 1 that is localized in the *trans*-Golgi network functions as an intracellular pump to transport copper into the *trans*-Golgi network for incorporation into copper-requiring enzymes; those localized on the plasma membrane are involved in copper transport out of the cells (241). Some of the mutant copper-transporting ATPase 1s are misfolded and retained in the ER (203). The mutants are temperature sensitive and can be corrected by chemical chaperones, further supporting the suggestion that the ER-retained mutant is misfolded (203). In vitro, copper supplementation in the culture media results in the correction of the mislocalization (203). Therefore, the substrate for the transporter serves as a pharmacoperone, facilitating the folding of the mutant protein. In Menkes patients with residual copper-transport activity, early treatment with copper injections can normalize clinical outcomes and increase survival (197).

In summary, compared with the nonspecific molecular chaperones and chemical chaperones, pharmacoperones have significant therapeutic potential due to their specificity **(TABLE 1)**. Pharmacoperones only affect the folding of the target protein, be it enzyme or receptor, without affecting the folding of other proteins. Therefore, they are expected to have significantly less rates of deleterious side effects.

It should be mentioned that pharmacoperones can be a double-edged sword. They might account for some of the unexplained side effects of current drugs by increasing the expression levels of drug targets (364). In these cases, selectively interfering with the biosynthesis of drug targets in the ER and inducing the quality control system to block their transport or accelerate their degradation might alleviate these side effects, complementing the current pharmacotherapy (364).

VII. HIGH-THROUGHPUT SCREENS TO IDENTIFY DRUGS FOR PROTEIN CONFORMATIONAL DISEASES

Many of the current studies on pharmacoperones are the result of investigations on known compounds, be they substrates or ligands. Many of these compounds are also antagonists that block the rescued protein function, although a few agonists have also been found to act as pharmacoperones. High-throughput screening to identify additional small molecules that correct the folding defect without blocking protein function has been employed in a few cases.

The word *pharmacoperone* refers to small molecules that rescue misfolded mutants and restore them to function (77). These are usually hydrophobic structures that enter cells by diffusion and then function as "molecular templates" to promote correct folding of proteins (73, 176, 177). Such putative drugs can correct the trafficking of mistrafficked proteins that do not ordinarily reach their normal site of function (29, 30, 368), and in principle, these drugs can treat disease (59). Science writers have welcomed this approach (24), noting that it is a practical "alternative (to gene therapy)," one that serves as a means of "skirting gene therapy to correct genetic defects." It is also worth noting that using pharmacoperones to correct defective protein folding is less of a challenge than it is to totally replace a defective gene by genetic engineering or to use enzyme replacement therapy. Moreover, remediation of the defective proteins (from the ER or otherwise) removes retained material that can be toxic or that can activate the UPR and result in other undesirable actions. An in vivo proof of principle was recently published (163).

One could envision "lifestyle drugs" given in vitamin supplements that might preclude misfolding that leads to diseases such as Parkinson's disease (caused by misfolded α-synuclein), cataracts (misfolded lens crystallins) (107), or Alzheimer's disease (misfolded amyloid) (343)). For these reasons, identification of such agents by high-throughput screening can be a potentially fruitful effort.

A. Design of Screens for Pharmacoperones for GPCRs

In the case of screens for two GPCRs, GnRHR (74) and V2R (180, 336), the screens employ stable HeLa cell lines expressing the misfolded mutants of these receptors, human GnRHR $E^{90}K$ or human V2R $Y^{128}S$, respectively. These mutants are expressed under the control of the tet (tetracycline) (OFF) transactivator. Accordingly, when tetracycline or doxycycline is present in the medium, the receptor gene is not expressed and the mutant protein does not accumulate in the cell (214). There is literally no detectable receptor gene or protein expression, as measured by either real-time polymerase chain reaction or by measuring protein expres-

sion (72, 179) when these antibiotics are included in the medium. Removal of antibiotic supplements rapidly restores gene expression of these genes. Not surprisingly, antibiotic coculture results in a cell that is identical to those used in the primary screen, but one that lacks mutant expression. Accordingly, cells cultured with tetracycline or doxycycline may be used as negative control lines. For both of these GPCRs, the activation of second messengers can be assessed and used as an indicator of cellular rescue.

It was clear from the earlier studies that misfolded GnRHR and the V2R mutants are retained in the ER and do not traffic to the plasma membrane. As such, second messengers are not generated upon agonist treatment. Both assays show gain of activity in the presence of pharmacoperone drugs which restore proper folding of the receptors and enable correct trafficking and activation by their natural agonists and second messenger coupling. For V2R, which is $G\alpha_s$ coupled (34), we measure the amount of cAMP produced when the cells are in the presence of vasopressin. Compounds that correct the misfolding can potentially result in vasopressin activation of the G_s-coupled system, thereby increasing the adenylate cyclase activity and production of cAMP. For GnRHR, which couples through $G\alpha_q$ (161), we measure the amount of calcium released into the cell from the ER. Compounds that correct the misfolded GnRHR mutants can potentially result in GnRH activation of phospholipase C and accumulation of inositol trisphosphate 3 within the cells which then triggers the release of Ca^{2+} from the ER stores.

B. Design of Screens for Pharmacoperones for Soluble Enzymes

Hyperoxalosis is a deadly disease with limited therapeutic options, such as lifetime dialysis or dual-organ transplantation. Treatments for hyperoxalosis also include vitamin B_6 therapy, but only a small percentage of patients respond to this approach (253, 254). Primary hyperoxaluria type I (PH1) is an autosomal recessive disorder of glyoxylate metabolism caused by misrouting of liver peroxisomal alanine: glyoxylate aminotransferase (AGT). When active AGT is absent in the peroxisome, glyoxylate is oxidized to oxalate in the cytosol and is excreted by the kidneys, resulting in increased oxalate concentration in the urine and deposition of insoluble calcium oxalate crystals. Systemic oxalosis is potentially lethal (81). Common treatments are directed at decreasing oxalate in the body, often by dialysis or by kidney transplantation.

Looking for drugs that promote the distribution of soluble enzymes to a cellular compartment required a fundamentally different approach than redistribution of a GPCR from ER retention to the plasma membrane. A cell-based high content assay (242) was miniaturized into a 1,536-well plate format and used to screen ~4,000 pharmacologically

relevant compounds including FDA-, European Union-, and Japanese-approved drugs (157). This assay employs CHO cells stably expressing AGT-170, a mutant that predominantly resides in the mitochondria instead of peroxisome as the WT AGT. The high content assay optically monitors the relocation of the mutant AGT to the peroxisomes through automated image acquisition and analysis. Two analogs were identified by this approach, demonstrating the applicability of this assay for large-scale high-throughput screens. One of these drugs, monensin, was further characterized in Western blot assays to confirm that partial proper relocation of AGT in cells occurred (157).

In summary, traditionally pharmacoperones were discovered based on known properties of chemicals such as enzyme substrates (for enzymes) or receptor ligands (for receptors). However, with the advances in high-throughput and ultra-high-throughput screening, screening libraries for novel pharmacoperones with more ideal profiles (e.g., not acting as enzyme inhibitors for enzymes or antagonists for receptors) will be increasingly important to move this field forward towards translational research from the bench into the bedside. A multidisciplinary team including medicinal chemists will be needed to exploit this opportunity.

C. Potential Therapeutic Problems With Hits

Almost all existing pharmacoperones for GPCRs were selected from antagonist screens for receptors, with only a few examples of agonists or allosteric modulators serving as pharmacoperones (158, 159, 180; reviewed in Ref. 353). This occurred because investigators selected agents that interacted with the protein of interest, but did not activate it. Dual antagonist-pharmacoperone activity is problematic since these drugs present a complex pharmacology, with the antagonists competing with endogenous agonists following receptor rescue. This competition presents the requirement for episodic administration and washout to enable the receptor to be activated by endogenous agonists (181). The availability of non-antagonists will facilitate oral dosing. Agonists can also serve as pharmacoperones. However, agonists stimulate the signaling, and consequent internalization and downregulation of the receptor therefore can potentially decrease the expression of the normal WT receptor (158). For misfolded receptors, the net expression on the cell surface is also the balance of the chaperoning activity promoting the forward trafficking and the post-signaling downregulation. For these reasons, pharmacoperone drugs that stabilize the receptor or mutant but are not agonists or antagonists are a desirable goal in high-throughput screening work. We have recently shown that in a large screen for pharmacoperone activity, there is no correlation between pharmacoperone and antagonist activities (180). Accordingly, identification of non-antagonist pharmacoperones should be viewed as an achievable goal.

There are two remaining observations that are significant to mention. First, it is clear that pharmacoperone drugs need not be present at the precise time of protein synthesis (172, 173). This means that ER-accumulated misfolded and mistrafficked mutants need not be (first) degraded and subsequently replaced by recently synthesized proteins in the presence of pharmacoperone. Second, while pharmacoperones are specific for individual proteins, those that rescue one mutant of an individual protein frequently rescue multiple 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 (172–175, 256, 257), since each mutant of an individual protein will not require a separate drug. However, there are also cases where some mutants cannot be rescued to the correct location, likely due to the severe defect in folding, or are not functional despite being corrected to the proper location. For example, G98R MC4R can be efficiently escorted to the cell surface using pharmacoperones but cannot initiate signaling upon agonist stimulation (159, 350, 354).

For lysosomal enzymes, mutations of which cause lysosomal storage diseases, pharmacoperones that are not enzyme inhibitors also have therapeutic advantage over pharmacoperones that are also enzyme inhibitors (196). For pharmacoperones that are also enzyme inhibitors, after the mutant enzyme reaches the lysosome, the bound pharmacoperone has to be outcompeted by the substrate because the pharmacoperone is bound to the active site of the mutant enzyme. For pharmacoperones that are not enzyme inhibitors and are not bound to the active site of the mutant enzyme, the mutant enzyme can be functional without the need for the pharmacoperone to be displaced by the substrate because the active site is free for the substrate to bind. This has been demonstrated in a cellular model of Gaucher disease with compounds derived from high-throughput screening (3, 127).

The arrival of validated screens for non-antagonist pharmacoperones will likely herald new advances in this novel approach to the rescue of mistrafficked proteins.

VIII. CONCLUSIONS

The average human cell has 10,000–20,000 different proteins at their typical concentrations and locales. To maintain proteostasis, cells have stringent quality control mechanisms in different locations, ensuring balance in protein synthesis, folding, trafficking, and degradation. Although essential for homeostasis, this stringent monitoring results in many mutant proteins being detected as misfolded, therefore retained in the ER and some other locales, although the mutant proteins might be perfectly functional, be it as enzyme, or receptor, or as an ion channel, if they could reach their normal destination. Although molecular or chemical

chaperones can help the mutant proteins to fold and are useful tools for studying mutant protein folding, they are of limited value as a therapeutic in patients due to the nonspecific action. Pharmacoperones have great potential because different from molecular and chemical chaperones, they can act as specific folding templates for the mutant proteins, helping them reach their native conformation, passing the quality control monitoring, and reaching their normal destination. Some of the rescued mutants might be functional. Because the pharmacoperones are small molecules, they can be administered orally, reaching all parts of the body including the central nervous system at much lower costs than alternative treatments such as enzyme replacement therapy and are much safer than gene therapy. Pharmacoperones have great potential for all protein conformational diseases, especially for some of the orphan diseases where there are no current therapeutics.

ACKNOWLEDGMENTS

GRANTS

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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