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Pharmacological Chaperoning: A Primer on Mechanism and Pharmacology

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Abstract

Approximately forty percent of diseases are attributable to protein misfolding, including those for which genetic mutation produces misfolding mutants. Intriguingly, many of these mutants are not terminally misfolded since native-like folding, and subsequent trafficking to functional locations, can be induced by target-specific, small molecules variably termed pharmacological chaperones, pharmacoperones, or pharmacochaperones (PCs). PC targets include enzymes, receptors, transporters, and ion channels, revealing the breadth of proteins that can be engaged by ligand-assisted folding. The purpose of this review is to provide an integrated primer of the diverse mechanisms and pharmacology of PCs. In this regard, we examine the structural mechanisms that underlie PC rescue of misfolding mutants, including the ability of PCs to act as surrogates for defective intramolecular interactions and, at the intermolecular level, overcome oligomerization deficiencies and dominant negative effects, as well as influence the subunit stoichiometry of heteropentameric receptors. Not surprisingly, PC-mediated structural correction of misfolding mutants normalizes interactions with molecular chaperones that participate in protein quality control and forward-trafficking. A variety of small molecules have proven to be efficacious PCs and the advantages and disadvantages of employing orthostatic antagonists, active-site inhibitors, orthostatic agonists, and allosteric modulator PCs is considered. Also examined is the possibility that several therapeutic agents may have unrecognized activity as PCs, and this chaperoning activity may mediate/contribute to therapeutic action and/or account for adverse effects. Lastly, we explore evidence that pharmacological chaperoning exploits intrinsic ligand-assisted folding mechanisms. Given the widespread applicability of PC rescue of mutants associated with protein

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folding disorders, both *in vitro* and *in vivo*, the therapeutic potential of PCs is vast. This is most evident in the treatment of lysosomal storage disorders, cystic fibrosis, and nephrogenic diabetes insipidus, for which proof of principle in humans has been demonstrated.

1. Introduction

Pharmacological chaperones, pharmacoperones, pharmacochaperones (PCs) are target-specific, small molecules that bind to their target proteins to facilitate biogenesis and/or prevent/correct misfolding [1–4]. Since genetic mutations that produce misfolding mutants account for a wide variety of diseases, the use of PCs holds great promise as a novel therapeutic avenue for the treatment of protein folding disorders that have suboptimum therapeutic options (cystic fibrosis, retinitis pigmentosa, hypogonadotropic hypogonadism) or for which there are effective but expensive/inconvenient therapies (enzyme replacement therapy for lysosomal storage disorders).

The discovery and evolution of PCs has emerged from diverse topical fields, from neuroscience [5] to endocrinology [6], and encompassed multiple levels of biomedical inquiry from genetics, protein structure and folding, protein trafficking, pharmacology and drug discovery and clinical therapeutics. While most PC studies have been performed *in vitro*, demonstrations of PC efficacy in animal models [7–11] and humans [12–16] firmly establish the feasibility of *in vivo* use. The majority of PC targets identified to date are a functionally diverse group of secretory pathway proteins including enzymes, transporters, receptors, and ion channels. Here we provide an integrated resource that reveals the multifaceted nature of PC mechanisms and pharmacology.

2. Structural mechanisms

For integral membrane proteins, mutations that cause misfolding and ER retention may occur in either extracellular or intracellular regions, as well as in transmembrane regions. Such mutations may occur in ligand binding regions or effector activation regions, or in locations distal to these overtly functional sites that are important for native structure and its stabilization [17]. The tertiary structure of proteins is stabilized by a variety of noncovalent interactions, such as hydrogen bonds that together with electrostatic forces form salt bridges, and covalent bonds including disulfide bridges. Exposure of hydrophobic surfaces, unpaired/mispaired cysteines, immature glycans and certain primary amino acid sequence motifs may cause proteins to be terminally misfolded and targeted for degradation [18]. It has long been recognized that ligands have stabilizing effects on their target proteins by increasing folding efficiency, promoting native structure and protecting from proteolytic degradation [19]. It is this phenomenon, applied to misfolding mutants in a cellular context, that has given rise to the emergence of PCs. Although the detailed structural basis for the efficacy of PCs remains to be determined for most protein targets, structural studies of lysosomal enzymes, CFTR and GnRH receptors provide insight into varied mechanisms.

2.1 PCs stabilize intramolecular structure

2.1.1 Lysosomal enzymes—Lysosomal storage disorders (LSD) are caused by mutations in a myriad of lysosomal enzymes, including acid-β-glucosidase (Gaucher

disease), α-galactosidase (Fabry disease), and acid-α-glucosidase (Pompe disease). A subset of these mutants possess protein folding defects, resulting in the ER retention of functionally competent enzymes whose native-like state and lysosomal localization can be rescued by PCs [3,20,21]. PCs for lysosomal enzymes are commonly active site inhibitors that create new hydrogen bonding networks and/or van der Waals interactions that stabilize protein structure [3,22,23]. Although distinct in their globular regions, lysosomal enzymes share a common active site that contains a (beta/alpha)8 TIM barrel [24–26]. Structural studies on wild type α-galactosidase reveal that binding of the chaperone deoxygalactonijirimycin yields subtle conformational changes consistent with a lock-and-key mechanism [22]. In contrast, binding of the iminosugar inhibitor isofagomine to acid-β-glucosidase produces a significant rearrangement of loop 1 residues near the active site, causing the extended loop to adopt an alpha helical conformation. This structural rearrangement produces two hydrophobic grooves that extend from the active site into the TIM barrel domain of the enzyme, consistent with an induced-fit mechanism [22,27]. Thus, structural mechanisms of PC chaperoning of lysosomal enzymes can entail either lock-and-key or induced-fit models, dependent on the enzyme and its mutation.

Other enzymes, such as the oxidoreductases tyrosine hydroxylase, tryptophan hydroylase and phenylalanine hydroxylase are also targets for PCs [8,28]. Structural information is largely lacking for PC rescue of these enzymes; however, phenylalanine hydroxylase (PAH) mutants that are causative for phenylketonuria are chaperoned by weak inhibitors that bind the PAH active site, creating hydrophobic stacking interactions in the PAH active site and introducing new hydrogen bonding networks, similar to the interactions observed between the lysosomal enzymes and their respective chaperones [29].

2.1.2 Cystic fibrosis transmembrane conductance regulator (CFTR)—The cystic fibrosis transmembrane conductance regulator (CFTR), a member of the superfamily of ATP-binding cassette transporters, is composed of two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory domain [4]. The most common cystic fibrosis causing mutation (approximately 70% of cystic fibrosis chromosomes [4]), F508del, occurs in the first NBD, causing NBD1 conformational defects which disrupt the interaction of NBD1 with both MSD2 and NBD2 in the carboxy terminal region of the protein, leading to misfolding and ER retention [30,31]. Partial correction of NBD1 folding with “correctors” such as VRT-325, enhances processing of ΔF508 CFTR; however, it does not restore the NBD1/MSD2 interaction [32–34]. For a corrector to be fully efficacious in rescuing ΔF508, it must repair not only the NBD1 conformational defect, but restore contact between NBD1 and MSD2 suggesting that a two-step corrector process may be required [4,35–37]. A second corrector, such as VRT-532, which likely targets the NBD/MSD interface, may be useful in this regard [38]. One of the most efficacious correctors, VX-809, has been tested in clinical trials and is demonstrated to provide a modest improvement in pulmonary function in patients harboring F508del [13,39]. The exact site of action of VX-809 is unclear and multiple sites of action appear likely [4,40–42], perhaps explaining its clinical efficacy. In silico docking experiments with CFTR F508del suggest that the VX-809 aromatic ring(s) occupies a hydrophobic pocket at the NBD1/CL4 (cytoplasmic loop) interface [41,42], as well as interact at the NBD1/NBD2 interface and

NBD1-CL1/MSD1 interface [42]. Thus, it is likely that correctors that bind to distinct sites on CFTR and additively address multiple structural issues will be required to achieve substantial clinical efficacy, and even with NBD1 corrected and the interfaces restored, potentiators may be needed to achieve full functionality [4,34,43–45].

2.1.3 Gonadotropin-releasing hormone (GnRH) receptor—Gonadotropin-releasing hormone (GnRH) receptor is a GPCR whose numerous misfolding mutants lead to hypogonadotropic hypogonadism [2]. While mutations in the “zone of death” (residues Ser168Arg or Ser217Arg inTM4 and 5, respectively) cause terminal misfolding defects [46–48], certain mutations that occur outside this region permit rescue by PCs. These mutations include change-in-charge or gain/loss of a cysteine residue critical to the formation of disulfide bonds. PC rescue of the Asp90Lys change-in-charge mutant proceeds via formation of a surrogate salt bridge between residues Asp98 and Lys121 in TM2 and TM3 domains, respectively [2,49]. This surrogate salt bridge acts as a proxy for the naturally occurring bridge between Glu90 and Lys121 that is eliminated by the Asp90Lys mutation [49]. In the gain-of-cysteine mutant Tyr108Cys, an aberrant disulfide bridge between residues Cys108 and Cys200 distorts GnRH receptor folding, but can be partially rescued with PCs [50]. The loss-of-cysteine mutant Cys200Tyr, prevents disulfide bridge formation between Cys14-Cys200; however, interactions mediated by PCs can restore surface expression and function to near wild type levels [51,52]. Thus, PCs serve as surrogates for disulfide bonds and salt bridges, helping to satisfy kinetic requirements for proper folding of the GnRH receptor mutants [49]. PC rescue of other GPCR mutants, such as vasopressin V2 and leukotriene B4 type-2 receptors, proceeds via stabilization of transmembrane domains, similar to that observed for GnRH receptor mutants possessing mutations in transmembrane regions [53–55].

2.2 PCs normalize quaternary structures of multimeric proteins

2.2.1 Rescue of oligomerization-compromised receptors—Many GPCRs and SLC transporters undergo constitutive homo or hetero dimerization as an obligate step in biogenesis [56,57]. Although oligomerization defective mutant GPCRs are retained in the ER, they are not necessarily terminally misfolded since oligomerization competency can be restored by PCs. For example, ER-retained $\alpha_{(1b)}$-adrenoceptor oligomerization-defective transmembrane domain (TMD) mutants can be rescued by $\alpha_{(1b)}$-adrenoceptor antagonists, allowing homo-oligomerization and subsequent maturation to a terminally N-glycosylated $\alpha_{(1b)}$-adrenoceptor that is functionally expressed at the plasma membrane [58]. Similarly, lipophilic β1-adrenergic receptor ligands can restore dimerization of an ER-retained, homodimerization-compromised β1-adrenergic receptor TMD mutant, rescuing surface trafficking and signaling activity [59]. Whether PCs bind to monomers and promote a dimerization-permissive structure or bind to immature dimers, thus stabilizing dimeric structures, remains to be determined.

2.2.2 Rescue of dominate negative effects—Unlike the oligomerization-compromised receptors discussed above, many misfolding mutants retain oligomerization competency and exert a “dominant negative” effect on their wild type counterparts, resulting in ER retention of mutant/wild type oligomers. Such dominant negative effects underlie a

vast number of diseases in which the gene product from a mutant heterozygous allele exerts a dominant negative effect on the gene product of the wild type allele, giving rise to phenotypes ranging from partial function to null-like [60–62]. For example, the *in vitro* dominant negative effects of the retinitis pigmentosa causative rhodopsin mutant P23H, is suppressed by retinoids acting as PCs [63]. Furthermore, the human δ opioid receptor variant Cys-27 exerts a dominant negative effect on the Phe-27 variant, impairing its maturation and targeting it for degradation, an effect that can be overcome by the opioid receptor antagonist naltrexone [64]. The dominant negative effect of a $\alpha_{(1b)}$-adrenoceptor transmembrane mutant on wild type $\alpha_{(1b)}$-adrenoceptor can be rescued by $\alpha_{(1b)}$-adrenoceptor antagonists, requiring only PC binding to the mutant receptor and not the wild type receptor [58]. Interestingly, coexpression of compound mutant heterozygous alleles that recapitulate hypogonadotropic hypogonadism phenotypes *in vitro* yields a promising picture for PC rescue of dominant negative effects associated with a certain hypogonadotropic hypogonadism genotype [17,65,66]. Recently, *in vivo* PC rescue of disease-causative mutant GnRH receptors has been most elegantly demonstrated in a GnRHR[E_{90}K] mouse model of hypogonadotropic hypogonadism [11] and see Conn et al., this issue. While E90K does not exert dominant negative effects in mice, it does so in humans and therefore, this recent finding may be particularly relevant to human disease. Given the many diseases that result from dominant negative effects and the ability of PCs to attenuate these effects, PC therapy may be an efficacious, convenient, and cost-effective approach to treating disease resulting from dominant negative mechanisms.

2.2.3 PC treatment influences stoichiometry of heteropentameric receptors—

Neuronal nicotinic acetylcholine (nACh) receptors are pentameric ligand-gated cation channels composed of α and β subunits, the interfaces of which form two orthostatic nicotine binding sites [67]. Multiple α(2-10) and β(2-4) subunit isoforms exist allowing the formation of nACh receptor subtypes composed of various α and β subunit stoichiometries. It has long been recognized that nicotine upregulates the number of β2 subunit-containing receptors, such as α4/β2 receptors, by facilitating an inefficient assembly process [68–73]. In contrast, nicotine does not upregulate the number of β4 subunit-containing receptors, presumably owing to their already efficiently assembly [68,70,74]. More subtle than upregulation of receptor number, nicotine treatment can alter nACh receptor stoichiometry of either β2 or β4 subunit-containing receptors. nACh receptors composed of α and β subunits can exist in one of two pentameric stoichiometries: 2(α)/3(β) or 3(α)/2(β) [72,75]. It is believed that nicotine acts on tetrameric 2(α)/2(β) intermediates to bias the incorporation of a β subunit into the fifth position, thus favoring the production of 2(α)/3(β) receptors [72,74,76], a phenomenon termed “SePhaChARNS” selective pharmacological chaperoning of acetylcholine receptor number and stoichiometry [5]. The ability of nicotine to upregulate the number of certain nACh receptor subtypes, as well as to favor 2(α)/3(β) stoichiometries, may have clinical ramifications for patients with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), a form of epilepsy linked to nACh receptor mutants. Recombinant receptors incorporating ADNFLE mutants display increased α:β ratio stoichiometry relative to wild type receptors [77] and treatment with nicotine normalizes the stoichiometry of these mutant receptors to wild type α:β ratios, an effect suggested to

underlie the decreased number of seizures in patients with ADNFLE who use nicotine-containing products.

3. Cotranslational vs. post translational modification

While pharmacological chaperoning may occur cotranslationally, as for calcium-sensing receptor mutants [78], most PCs act posttranslationally in various ER compartments, including in the transitional ER where PC correction of long QT-linked HERG (Kv11.) mutants occurs [79]. Because it is not required that most PCs be present during protein synthesis, previously synthesized pools of target proteins can be rescued [53,80–82]. Once PCs have induced native-like structures that permit their target proteins to pass the ER quality control system, these proteins are trafficked to their functional destination and the presence of the PC appears to no longer be required [83]. Thus, the duration of PC exposure that is necessary to rescue a protein is usually far shorter than the half-life of its target protein [79,83], a phenomenon of clinical importance (see section 5.2).

4. PCs affect the interaction of target proteins with molecular chaperones

4.1 ER resident molecular chaperone

Calnexin is an ER resident lectin that interacts with monoglucosylated N-linked glycans within nascent glycoproteins to aid in their folding [18]. Calnexin binding/unbinding is a dynamic process whereby immature precursors undergo multiple binding/unbinding cycles in which glucosidase II removes glucoses, while UDP-glucose:glycoprotein glucosyltransferase, a folding sensor, reglucosylates folding intermediates for additional calnexin binding cycles. For known PC targets, calnexin binds to both immature wild type proteins and their misfolding mutants, the latter of which show prolonged association with calnexin as demonstrated for mutant vasopressin V2 [84] and V1B/V3 receptors [85], human melanin concentration hormone receptor 1 [86], and the potassium channel human ether-a-go-go HERG [87]. PC binding promotes native-like folding of not only these misfolding mutants, but also of their inefficiently processed wild type receptors, thus facilitating dissociation from calnexin. In contrast, for efficiently processed wild type receptors, such as rhodopsin, PCs facilitate the release of misfolding mutants from calnexin, but do not affect the interaction of calnexin with wild type receptors [88]. For proteins that undergo calnexin binding, the efficacy of a PC appears to be greatest when the amount of protein folding intermediate bound to calnexin is highest. Thus, calnexin binding appears to protect both inefficiently processed wild type receptors, and their corresponding misfolding mutants, in an intermediate folding state until PCs can facilitate folding [88]. Such mechanisms may be particularly important for wild type and mutant human GnRH receptors, which exhibit a complex, species-specific relationship between calnexin binding and PC rescue [89]. While calnexin binding is critical for PC rescue of several proteins, not all PC-mediated rescue of disease linked mutants is calnexin-dependent as noted for benzoquinolizinium rescue of the CFTR F508del mutant [90].

4.2 Cytoplasmic molecular chaperones

In addition to affecting the interaction of target proteins with ER resident molecular chaperones, PCs can also affect the interaction of integral membrane proteins with

cytoplasmic molecular chaperones. In this regard, PC stabilization of A1 adenosine receptor folding intermediates promotes the dissociation of the receptor from HSP 40 protein D1 receptor interacting protein 78 (DRiP78) [91]. Intriguingly, PCs promote the interaction of the prostanoid DP1 receptor and ANKRD13C [92], a cytoplasmically localized molecular chaperone for GPCRs that aids in forward trafficking [93].

5. Pharmacology of PCs

5.1 PC EC_{50}s

Although a drug's EC_{50} value for chaperoning is strongly correlated with its ligand binding affinity (Kd) and/or its ability to activate/inhibit its protein target [53,94–96] chaperoning EC_{50s} are generally, but not always [97], 100 fold higher (i.e., less potent) than those required for functional activation/inhibition [80,81,95]. The lower potency of chaperoning presumably derives from lower affinity interactions with immature intermediates, relative to high-affinity interactions with native, mature conformations [71]. For secretory pathway proteins, additional factors affecting the efficacy and potency of PCs include the ability of PCs to access their targets in the ER, as well as the unique environment of the ER lumen which is distinct in protein concentration, pH, ionic environment, membrane potential, and the presence of ER resident proteins [95].

5.2 Competitive antagonists/inhibitors

While high-affinity antagonist/inhibitor PCs are highly efficacious in rescuing a variety of disease-causative ER-retained misfolding mutants, proteins rescued in this way can arrive at their functional destination in an antagonist/inhibitor-bound state, potentially preventing agonist activation or substrate access, the latter of which can be circumvented by using subinhibitory concentrations [81,98,99]. In cell culture systems, PCs can be dissociated from their targets by either removal/washout from the culture medium or via competition with high concentrations of agonist/substrate [100], provided that PC binding is not irreversible or PCs are not ineffectively removed [81]. Therefore, "functional rescue" includes not only the ability of the PC to promote the proper folding and trafficking of its target protein, but the ability of agonists/substrates to displace PC antagonists/inhibitors. The displacement of antagonist PCs is particularly difficult for receptors and ion channels for which the "rescue EC_{50}" for most antagonist PCs is higher than their IC_{50}s for inhibiting activity. While high-affinity sulfonylurea drugs effectively rescue ATP-sensitive potassium channels (K_{ATP}) misfolding mutants, these drugs do not easily dissociate from the channel upon its surface expression, thus yielding nonfunctional channels [101]. Conversely, high concentrations of low-affinity sulfonylureas are effective PCs that readily dissociate, allowing functional recovery; however, in general the need for high drug concentrations is not optimum for the development of clinically favorable PCs. In general, low concentrations of high-affinity PC antagonists provide the most efficacious rescue while still allowing antagonist dissociation [96,100,102]. While it remains unclear whether receptor antagonist PCs will possess broad therapeutic application, the efficacy of a vasopressin V2 receptor antagonist PC in treating nephrogenic diabetes insipidus patients has been demonstrated in humans [12] and a peptidomimetic GnRH receptor antagonist has recently been demonstrated to rescue a GnRH receptor mutant in an *in vivo* mouse model [11].

Because most PCs are effective chaperones of accumulated pools of non-natively folded proteins and because the PC exposure time required for chaperoning is far shorter than the half-life of the chaperoned protein [79,83], intermittent (pulsatile) PC administration may provide PC-free periods during which chaperoned protein can be active, provided PC dissociation occurs. Such an approach may be particularly useful in the case of lysosomal storage disorders where substrate reduction is the objective. In fibroblasts from Fabry disease patients, the iminosugar migalastat DGJ, a reversible active-site inhibitor, structurally stabilizes α-galactosidase A, allowing lysosomal targeting, improved enzymatic activity, and substrate reduction [103]. Dissociation of DGJ from α-galactosidase A is likely aided by a lower binding affinity in acidic environment lysosome [104], as well as high levels of competitive substrate [105], thus allowing active site inhibitors to be used at subinhibitory concentrations for chaperoning [106]. Excitingly, phase II clinical trials have demonstrated that every-other-day, oral administration of migalastat HCl increases α-galactosidase A activity and this is accompanied by successful substrate reduction in patients carrying PC-responsive genotypes. Given the short plasma half-life of migalastat (3–4 hrs), the rapid action of chaperones on protein folding, and the long protein half-life of α-galactosidase A (> 100 hrs, depending on stability of the phenotype [103]), alternate day administration permits chaperoning, while providing periods of inhibitor-free enzyme activity. Such an approach may have a significant impact on the treatment of lysosomal storage disorders which are currently treated by enzyme replacement therapy. Very recently, optimization of a pulsatile drug administration protocol has proved to be highly effective in rescuing mutant GnRH receptors *in vivo* [11] and see Conn this issue.

5.3 Agonists/inverse agonists/protean agonists

For many receptors, PC rescue of misfolding mutants is achievable using either an antagonist or agonist, indicating that chaperoning is independent of signaling and is likely due to binding site occupancy [94,107–109]. Unlike antagonist PCs, most agonist PCs do not need to be displaced from their targets for receptor activation [109], unless the target receptor arrives at the cell surface in an agonist-induced desensitized state as observed for chaperoned growth hormone receptors [110]. While many GPCR agonists promote receptor internalization and desensitization via arrestins, vasopressin V2 receptor agonist PCs have been identified that do not trigger arrestin recruitment, thus exhibiting “biased agonism” that can prevent AVP-induced arrestin recruitment [108]. Although biased agonist PCs have the advantage of not inducing internalization, their ability to rescue some mutants may be more restricted than rescue by antagonist PCs [108]. For nACh receptors, agonists are more potent PCs than antagonists, possibly because heteromeric assembly may occur more efficiently if the receptor is in an activated or desensitized state [72]. In addition to agonist PCs, inverse agonists PC have been identified for the prostanoid DP1 receptor [92]. Moreover, V2R antagonists are “protean agonists” serving as PCs for certain mutants while acting as inverse agonists at wild type receptors [111] further revealing the complexities of pharmacological chaperoning.

5.4 Allosteric ligands

Issues encountered with orthosteric agonists and antagonist PCs may be circumvented by developing PCs that act allosterically. Allosteric agonists that act as cotranslational

stabilizers at a conformational checkpoint during calcium-sensing receptor biosynthesis are a promising intervention for recovering loss-of-function mutations associated with hypocalciuric hypercalcemia [112,113]. Furthermore, ER-retained mutant luteinizing hormone [114] and follicle-stimulating hormone [115] receptors associated with reproductive disorders are rescued by allosteric activators such as thienopyrimidine. These allosteric PCs may be particularly important for avoiding receptor desensitization observed with orthosteric agonist PCs. For lysosomal storage disorders, the ability of the allosteric ligand N-acetylcysteine to chaperone α-glucosidase A mutants offers an advantage over active-site directed ligands that may potentially inhibit catalytic activity [116]. Lastly, a newly proposed model of phenylalanine hydroxylase allosteric regulation suggests that compounds, such as phenylalanine, that stabilize the ACT:ACT interface, may allow identification of allosteric PCs that will be useful in treating the autosomal recessive disorder phenylketonuria [117].

6. Considerations of functional rescue

The degree of functional rescue depends on the absolute amount of protein rescued, the intrinsic activity of the rescued protein and its stability at the appropriate functional location. Protein misfolding mutants that are rescued by PCs can be functionally indistinguishable from their wild type counterparts [17,59], display partial activity [118], or be nonfunctional as is the case for rescued G protein-coupling deficient μ-opioid receptor mutants [107]. For rescued proteins that display compromised functionality, potentiators in combination with PCs may provide additive or even synergistic effects on function as noted above for CFTR F508del [44,45]. Such a cocktail approach may also be applicable to the rescue of mutant K_{ATP} channels associated with congenital hyperinsulinism in which case carbamazepine, working as a PC, may produce synergist effects with the channel opener diazoxide [119].

In addition to the intrinsic functional properties of rescued mutants, an important factor in considering the degree of functional rescue is the stability of the protein at its functional location. Subsequent to the rescue of misfolding mutants and their delivery to functional destinations, some chaperoned proteins, such as vasopression V1a receptor mutants, undergo agonist-mediated endocytosis similar to wild type receptors [81]. Similarly, the cell surface residency time of mutant μ-opioid receptors is unaffected following PC rescue [107]. In contrast, following PC treatment, some nicotinic receptor subtypes display an increased surface residency time [72] potentially augmenting the potential therapeutic effects of nACh receptor PCs. Similarly, vasopressin V2 receptor mutants rescued by “biased” agonists, stabilize receptor surface expression since, unlike full agonists, they do not activate pathways that trigger receptor internalization [108]. At the other extreme, rescued vasopressin V2 R137H receptors undergo accelerated rates of β arrestin-dependent internalization [120,121], similar to PC-rescued δ-opioid receptor variant-Cys-27 which is unstable at cell surface and rapidly undergoes constitutive endocytosis [122]. Interestingly, in the absence of PCs, the CFTR F508del mutant displays a six fold increase in its endocytic rate relative to wild type CFTR, however, CFTR F508del rescued by correctors display wild type rates of endocytosis [123]. Not only can correctors affect the kinetics of endocytosis, but recent evidence indicates that correctors redirect CFTR F508del from a degradative pathway following endocytosis to a recycling pathway [124].

Another factor in considering the therapeutic potential of a chaperone is whether a rescued mutant needs to be restored to the same functional extent as wild type. Since heterozygous individuals carrying CFRT F508del mutation are asymptomatic, and based on inferences from data obtained in cultured human bronchial epithelial cells [39] and human cystic fibrosis ciliated surface airway epithelium [125], it has been suggested that individuals homozygous for the CFTR F508del mutation may achieve therapeutic benefit from the rescue of approximately 20–25% of CFTR F508del mutants, provided full functionality of the rescued CFTR F508del is achieved. Similarly, for lysosomal storage disorders, functional rescue of mutant lysosomal enzyme activity of 5–15% is thought to be the critical threshold for achieving therapeutic levels of substrate reduction [3,126].

7. Unrecognized pharmacological chaperoning

7.1 On-target activity of PCs

Given the number and diversity of proteins that undergo pharmacological chaperoning *in vitro*, and the demonstration that pharmacological chaperoning occurs *in vivo* [9,11,12,14–16,127], it is possible that the pharmacological effects of some clinically relevant drugs may be due, in part, to their unrecognized activity as pharmacological chaperones. This is most evident with drugs acting on the central nervous system. One of the best studied compounds in this regard is the nACh receptor agonist nicotine, the chaperoning activity of which is thought to contribute to the addictive properties of nicotine, as well as to its therapeutic effect on Parkinson's disease and potential to treat epilepsy associated with nACh receptor mutants [5]. Other central nervous system proteins that likely undergo *in vivo* chaperoning include multiple types of opioid receptors which are chaperoned *in vitro* by a variety of clinically used opioids [64,122,128,129]. Furthermore, antipsychotics that bind to dopamine $D_{2–4}$ receptors are potent chaperones of dopamine D4 receptor folding mutants, as well as wild type D4 receptors [130,131]. Such chaperoning activity may explain the paradoxical upregulation of D2 receptors by both receptor agonists and antagonists. Lastly, folding-impaired human serotonin transporter (SERT) mutants are effectively rescued by the noncompetitive SERT inhibitor ibogaine [132], a drug of abuse that has been used to treat addiction. The notion that the effects of psychiatric drugs may be partially attributable to the binding of their targets in the early secretory pathway has been well-considered by Lester et al. [133].

It is possible that clinically-used drugs that block their targets may exert chaperoning activity *in vivo* with little functional consequences. Sulfonylureas are K_{ATP} channel inhibitors that are commonly prescribed to treat type II diabetes. *In vitro* these drugs rescue K_{ATP} channel mutants responsible for congential hyperinsulinism [101] and upregulate surfaces levels of wild type K_{ATP} [101,134]. It is conceivable that sulfonylureas may act as PCs on wild type K_{ATP} in patient populations, however, such action may be clinically inconsequential since sulfonylureas block the K_{ATP} channels they chaperone. Similarly, the sodium channel blocker and local anesthetic lidocaine rescues inefficiently processed wild type $NaV_{1.8}$ sodium channels [135] but would also inactivate surface $NaV_{1.8}$ channels likely rendering such rescue functionally undetectable. Thus, it is possible that some clinically-

used drugs may exert *in vivo* chaperoning activity on their know targets with little functional consequence.

7.2 Off-target chaperoning

A variety of clinically-used drugs have been noted to have "off-target" chaperoning activity *in vitro*, facilitating the biogenesis of unintended targets. In this context, it has been suggested that calcium-sensing receptor upregulation observed in patients chronically treated with dihydropyridine calcium channel blockers may be partially due to the ability of dihydropyridines to act as "unintended" calcium-sensing receptor PCs (see Breitwieser this issue). Likewise, carbamazepine, a sodium channel blocker and clinically used anticonvulsant, rescues K_{ATP} potassium channel mutants at concentrations relevant to sodium channel blockade, suggesting that clinical doses may promote the biogenesis of K_{ATP} potassium channels [119,136]. Other clinically used drugs observed to act as PCs *in vitro* include cyclosporine which rescues defective ABCB1 transporters [137].

Intriguingly, the adverse effects of some widely-used therapeutics agents may result from their ability to act as anti-chaperones, causing misfolding of off-target proteins, a phenomenon termed shipwrecking [46]. The best characterized of these anti-chaperone targets is the cardiac potassium channel human ether-a-go-go (HERG) which mediates adverse cardiac effect of several drugs. In this regard, the serotonin selective reuptake inhibitor fluoxetine, its metabolite norfluoxetine, and the tricyclic antidepressant desipramine disrupt ER processing of HERG, an effect thought to underlie drug-induced (acquired) long QT syndrome [138,139]. More recently, the antiprotazoal pentamidine has been proposed to act as an anti-chaperone by binding to HERG folding intermediates and preventing their maturation and export [97]. In addition to these direct anti-chaperone effects on HERG processing, the Na^+/K^+ ATPase inhibitor digoxin indirectly disrupts HERG biogeneisis via a $[K+]_i$ –dependent conformational defect in HERG folding [140], underscoring the intricacies of studying pharmacological chaperoning. Adding to this complexity, astemizole, an antihistamine known to cause fatal arrhythmias by blocking HERG channel activity, can rescue digoxin-induced, $[K+]_i$ –dependent HERG conformational impairments [140] consistent with astemizole's activity as a HERG pharmacological chaperone [95].

8. Do PCs exploit intrinsic ligand-assisted folding mechanisms?

8.1 The ER as a storage reservoir of folding competent intermediates

It is well-established that numerous wild type proteins are "inefficiently" processed in the ER including G protein-coupled receptors (GnRH [141,142], calcium-sensing [143], δ and μ opioid [53,94,144], and vasopression V2 and V3 receptors [85]), ion channels (nAChRs [71], $GABA_A$ receptors [145], Na_v sodium channels [146]), and tyrosine kinase receptors such as the growth hormone receptor [110], with up to 70% of these nascent subunits/receptors degraded without use. A significant portion of these unused subunits/receptors, however, are not terminally misfolded since treatment with either PCs or proteasome inhibitors promotes native-like folding and forward-trafficking of functional proteins [53,71,85,94,110,141–146]. Thus, for inefficiently processed secretory pathway proteins, it

has been suggested that the ER serves as a storage reservoir of viable folding intermediates that are subject to post-translational regulatory mechanisms [145,147]. As discussed below, one such post-translational regulatory mechanism may be ligand-facilitated folding executed by a protein's cognate ligand.

8.2 Ligand-assisted folding/stabilization by "cognate ligand chaperones"

Reminiscent of pseudosubstrates and cofactors, which regulate the maturation and folding of their targets [148], evidence is accumulating that endogenous ligands act as "cognate ligand chaperones" to regulate the biogenesis of their target proteins. Coexpression of growth hormone and its receptor shows that growth hormone binds to nascent growth hormone receptors in the ER to facilitate receptor maturation, possibly by assisting dimerization [110]. In this way, the chaperoning of the growth hormone receptor by growth hormone may represent an autocrine regulatory mechanism. In the central nervous system, ionotropic receptors appear to be subject to regulation by cognate ligand chaperones. The neurotransmitter γ-aminobutyric acid (GABA) acts in the early secretory pathway to promote surface expression of functional recombinant $GABA_A$ receptors [145] and treatments that increase levels of endogenous GABA promote $GABA_A$ receptor maturation in cultured neurons (unpublished). Exogenous application of physiologically relevant concentrations of choline promotes the maturation of recombinant α4β2 nAChRs suggesting that ambient choline or acetylcholine in the nervous system may regulate the biogenesis of nAChRs [71]. Based on the observation that glutamate receptor ligand binding domain mutants are retained in the ER, it has long been proposed that the neurotransmitter glutamate is a cognate ligand chaperone for ionotropic glutamate receptors [149] including NMDA [150], AMPA [151,152] and kainate [153–155] receptor subtypes. Lastly, wild type calcium-sensing receptor, which is chaperoned by agonists/antagonist and allosteric agonists, may undergo chaperoning by endogenous modulators such as the orthosteric agonist calcium and the high-affinity allosteric agonist glutathione, each of which is abundant within the ER lumen (see Breitwieser this issue). Thus, PCs may exploit heretofore underappreciated ligand-assisted folding mechanisms that are intrinsic to protein folding processes.

9. Conclusions

It is very clear is that in cell culture models PCs can rescue many ER-retained, disease-causative misfolding mutants; thus, PCs have a vast potential to mitigate a wide range of protein misfolding diseases. The potential therapeutic utility of pharmacological chaperones is supported by proof of concept trials in humans, including those for cystic fibrosis, lysosomal storage disorders, and nephrogenic diabetes insipidus. Given that protein misfolding mutants that respond to PCs *in vitro* are those that show a clinical effect [14], the emergence of PCs as therapeutic tools has enormous potential. PCs work on intra and intermolecular levels to provide structural fidelity to disease-causative protein misfolding mutants, allowing them to pass quality control mechanisms and traffic to their functional destination. Some rescued proteins possess wild type functionality, however, others are functionally compromised or unstable. As *in vivo* application moves to the forefront, practical considerations, such as the need for potentiators (cystic fibrosis) or pulsatile

administration (hypogonadotropic hypogonadism, nephrogenic diabetes insipidus), become evident and optimization of treatment approach will be necessary to realize the full potential of PCs. The identification of efficacious PCs should be greatly accelerated by the use of cell-based high-throughput screening [156–159]. Largely uninvestigated, pharmacological chaperoning activity of currently used therapeutic agents may partially account for their beneficial clinical effects, and in some cases may underlie drug toxicity. Lastly, PCs appear to exploit existing physiological mechanisms that allow cognate ligands to regulate the biogenesis of their wild type target proteins. In this light, PCs may not only be therapeutically useful for rescuing disease-causative protein misfolding mutants, but also for increasing levels of inefficiently expressed wild type protein.

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