

Review

Role of misfolding in rare enzymatic deficits and use of pharmacological chaperones as therapeutic approach

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1. Abstract

Cells have evolved sophisticated molecular control systems to maximize the efficiency of the folding process. However, any subtle alteration of the environment or the protein can lead to misfolding or affect the conformational plasticity of the native states. It has been widely demonstrated that misfolding and/or conformational instability are the underlying mechanisms of several rare disorders caused by enzymatic deficits. In fact, disease-causing mutations often lead to the substitution of amino acids that are crucial for the achievement of a folded conformation, or play a role on the equilibrium between native-state conformers. One of the promising approaches to treat conformational disorders is the use of pharmacological chaperones (PCs), small molecules that specifically bind a target protein and stabilize a functional fold, thus increasing the amount of functionally active enzyme. Molecules acting as PCs are usually coenzymes, substrate analogues behaving as competitive inhibitors, or allosteric modulators. In this review, the general features of PCs are described, along with three examples of diseases (Gaucher disease, Phenylketonuria, and Primary Hyperoxaluria) in which this approach is currently under study at preclinical and/or clinical level.

2. Introduction

The comprehension of the process that allows an unfolded polypeptide chain to acquire the native state, as well as of the role of intracellular systems that assist proteins during the folding process, is a difficult task, as recently reviewed in [1]. The Anfinsen's experiment on the refolding of ribonuclease A demonstrated fifty years ago that the native structure of a protein is determined by amino acids sequence [2], but the efficiency of the folding process is also strongly influenced by the surrounding environment [3]. Protein folding is an exergonic process, because the enthalpic and entropic changes associated with the achievement of a folded structure reduce free energy of the native state with respect to that of the unfolded state [4, 5]. In addition, the process usually occurs very rapidly, in particular in the case of small proteins [6]. Starting from the Levinthal's paradox highlighting that it would be impossible even for a short polypeptide chain to fold in a timeline of seconds by the simple trial of all possible conformations, many studies combining *in silico* and *in vitro* experiments have been focused on the understanding of the thermodynamics and the kinetics of the folding process [1, 7–9].

The current view of protein folding is based on the concept of a free energy landscape represented as a folding funnel (Fig. 1). The wideness of the funnel is the entropy, i.e., the number of possible conformations of the chain, while the height represents the free energy. The polypeptide fluctuates in a conformational landscape where the acquisition of favorable conformations, i.e., those characterized by a lower free energy, drive the chain down in the funnel until the native state [1, 9]. For many proteins, the difference in free energy between the unfolded and the folded state is very low, so that any small alteration of the protein chain and/or of the environment can have a great impact on folding efficiency [1]. In addition, more sophisticated theories and models should be applied when considering the folding of multi-domain protein, which can also fold independently and/or in a sequential way [10]. The presence of folding building blocks, named “foldons” and able to unfold and refold as whole units, has been also advanced [11].

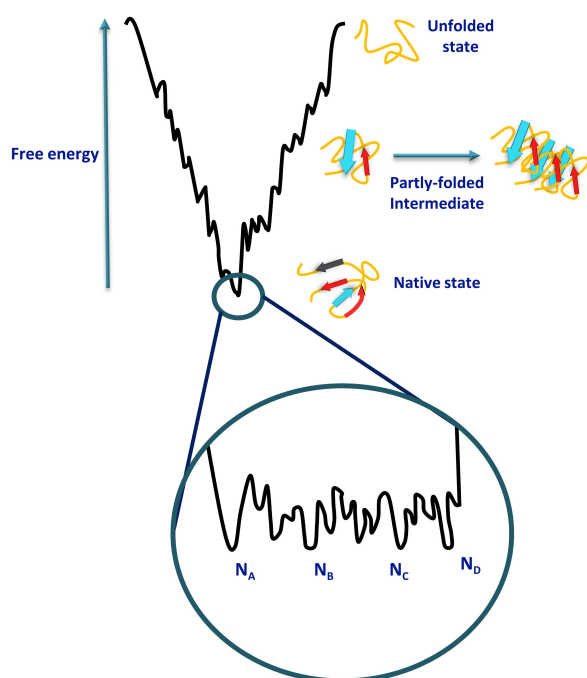


Fig. 1. Schematic representation of the folding funnel. A protein in the unfolded state can reach its native conformation through a process governed by changes in terms of entropy (represented by the wideness of the funnel) and free energy. In the flat bottom different native conformers are displayed ($N_A - D$). The formation of a partly-folded intermediate is also shown.

In the past, most studies on the folding pathway of globular proteins have been guided by two paradigms: (1) the polypeptide chain follows its route in a folding funnel leading to a unique native structure, which represents the most stable conformation under physiological conditions. (2) the major driving force for folding is the “hydrophobic burst”, i.e., the interaction between residues showing hy-

drophobic (aliphatic or aromatic) side chains. Therefore, misfolding mainly arises from intermediates exposing hydrophobic surfaces [12, 13]. Some proteins generate a folding intermediate named molten globule, in which the chain has acquired the secondary structure elements, but most hydrophobic patches are still exposed on the surface, thus explaining why it can be prone to aggregation [14]. In the final steps of the folding process the protein undergoes the complete acquisition of the tertiary and, when present, quaternary structure, thanks to subtle rearrangements also involving amino acid side chains on the surface [15]. Although the formation of a molten globule is often observed, *in vitro* studies have shown that intermediates forming during the folding process of globular proteins can be differently structured, and they can differently populate depending on the environment in which the protein folds [16]. This concept has generated the view that the folding funnel of most proteins is “roughed”. In fact, during the process the chain can be entrapped in local energy minima with a high energy barrier that needs to be overcome to reach the global free energy minimum of the native conformation [17]. The formation of partly-folded intermediates also makes the protein susceptible to non-native interactions that can promote aggregation and/or degradation, thus leading to misfolding [10].

The most recent advances in protein chemistry, along with the use of sophisticated structural analyses, have allowed not only to expand the picture of the general principles underlying protein folding, but also to implement our knowledge of the properties of the native state. In fact, the native structure can no longer be considered an arrival point because native proteins show a significant structural plasticity, and fluctuate among various conformers [18, 19]. The latter finding has converted the idea of a sharp folding funnel leading to a unique shape, to that of a flat funnel where an ensemble of native conformers coexists (Fig. 1). Conformational plasticity can promote protein dynamics and adaptability to changes in external conditions, or it can be crucial for the interaction with a particular ligand or binding partner. In small single-domain proteins, the population of rapidly interconverting native conformers has been observed and recently related to the intrinsic fuzziness of some proteins that do not acquire a fully optimized structure in order to expand the ensemble of possible structures and therefore functions that a protein can perform [20]. In contrast to partly-folded intermediates, which usually expose hydrophobic surfaces, native conformers of small proteins can differ from each other for changes mainly involving residues exposed on the surface of the protein. Thus, their association to form aggregates and/or interaction with binding partners is expected to be mainly mediated by electrostatic forces [21]. The consequence is that the surface likely influences the stability of a protein by possibly shifting the equilibrium toward potentially noxious conformers prone to local unfolding [22].

An extreme side of protein conformational plasticity is exemplified by intrinsically disordered proteins, which acquire a folded conformation only in the presence of a binding partner, or by globular proteins carrying intrinsically disordered regions (IDRs) [13]. It has been estimated that about 22% of the human proteome is constituted by proteins showing IDRs [23]. The presence of IDRs is more common in multi-domain proteins of eukaryotes as compared with prokaryotes [24]. Although the role of IDRs of soluble globular proteins is poorly understood, many evidences suggest that they could be able to integrate and transmit a wide range of intracellular signals, mediate protein-protein interactions or post-translational modifications [19].

In the case of multi-domain proteins, the folding process and the stability of the native state can be even more complex. Indeed, besides the moonlighting properties of a growing number of proteins including metabolic enzymes [25], which highlight a functional plasticity, new categories in the field of conformers of the native state have been introduced taking into account the possibility to interconvert among conformers generated by large motions of domains or entire subunits. They include (i) proteins showing short chameleonic sequences that can fold into an α -helix or a β -sheet. (ii) morphoeins, which in solution interconvert among various oligomeric forms possibly resulting in different functions. (iii) metamorphic proteins, which can adopt different folded states that reversibly interconvert [26, 27]. The latter group include proteins involved in important processes such as cell cycle regulation, solute transport, gene expression, as exhaustively reviewed [28–30].

Finally, the global picture of the protein folding process should consider that the events occurring in a cell may differ from those occurring *in vitro* in many respects including: (i) the vectorial protein synthesis, which allows co-translational folding to occur. (ii) the presence of a crowded environment, which can promote aggregation. (iii) the influence of subcellular import, which can occur on the unfolded or the folded state depending on the organelle, and of binding partners, that can promote correct folding. (iv) the action of various classes of molecular chaperones, proteins that use the free energy of ATP hydrolysis to help the folding process by inhibiting or reverting aggregation, promoting the achievement of the native conformation, and/or allowing the degradation of misfolded intermediates [31–35].

3. Protein misfolding and human disease

The global processes of synthesis, recycling, modification, or degradation of the proteome of a cell are governed by molecular complexes that are involved into protein homeostasis regulation (proteostasis) [36]. Proteostasis is crucial for cell survival and for cell response to any

change occurring in the surrounding environment and represent a pivotal mechanism against any kind of external stimuli, including oxidative stress [37]. The proteostasis network controls critical phases in proteins building, such as correct folding and localization, allowing to produce proteins when and where they are needed. Through its crucial role in cellular proteome organization, the proteostasis network also exerts effects that influence the organization at tissue and organism level [38]. Despite the presence of numerous and effective control systems, the accumulation of incorrectly folded or misfolded proteins is not uncommon. Although cells have evolved sophisticated systems to promote folding, any subtle alteration of the environment and/or the polypeptide chain can shift the equilibrium from the native state to an unfolded or partly-folded state, thus leading to misfolding [35]. Protein misfolding in most cases arises from the population of partly-folded intermediates that can be prone to self-associate, are more susceptible to be degraded by the intracellular quality control systems or can be mistargeted to a wrong compartment [22, 39]. However, numerous evidences suggest that also native or native-like intermediates can trigger misfolding. This can occur in the presence of an environmental changes such as increased oxidative stress, altered pH, viscosity or osmolarity of the medium, as well as of a protein change involving surface residues, such as post-translational modifications, or inherited/acquired mutations [22, 40, 41]. In the case of multi-domain proteins, a dysregulation of proteostasis can also arise from interferences within large conformational changes accompanying the interconversion of the different native states conformers or from the appearance of moonlighting properties [42]. An example of this mechanism has been provided by mutations associated with ALAD porphyria, which changed the equilibrium between distinct morphoein forms endowed with different activity. Thus, the classical view of the pathogenesis of misfolding disorders should be expanded to include alteration influencing not the folding process itself, but the conformational plasticity of the native state [43].

Increasing evidences indicate that protein misfolding or altered native state stability is the underlying mechanism of many human disorders, comprising neurological, metabolic, and also infectious diseases [44, 45]. Examples of human diseases associated with protein misfolding are reported in Table 1. In general, two different scenarios can describe the pathogenesis of conformational diseases: (1) loss-of-function, when the effects of misfolding are related to the loss of the functionality specific of the misfolded protein. A typical example is represented by enzyme deficits, where the loss of the catalytic activity of a misfolded enzyme can lead to substrate accumulation, product deficiency, or generation of secondary metabolites [34, 46]. (2) gain-of-function, when misfolded proteins generate species, often ordered aggregates, that become toxic for the cell. The latter possibility is typically ob-

Table 1. Example of misfolding diseases displaying a Loss-of-Function (LoF) or Gain-of-function (GoF) pathogenesis.

Rare disease	Genetics	Molecular pathogenesis	Compartment	Protein involved	Main symptoms
Huntington disease	Autosomal dominant	LoF and GoF	Nucleus and Cytosol	Huntingtin	Neurodegeneration, incoordination, and behavioural difficulties
Cystic fibrosis	Autosomal recessive	LoF	ER	CFTR	Degeneration of lung function, Sinusitis, Inability to exercise, Male infertility, and repeated infections
Gaucher disease	Autosomal recessive	LoF and GoF	ER	β -Glucocerebrosidase	Anemia, thrombocytopenia, hepatosplenomegaly, aseptic necrosis of bone, and osteoporosis
Phenylketonuria	Autosomal recessive	LoF	Cytosol	PAH	Mental retard, tremors, hyperactivity, and skin conditions (eczema)
Parkinson disease	Autosomal dominant/recessive and acquired	LoF and GoF	Cytosol	α -Synuclein	Tremor, rigidity, slowness of movement, postural instability, difficulty with walking, and nausea
Primary hyperoxaluria type I	Autosomal recessive	LoF	Peroxisome	AGT	Nephrolithiasis, nephrocalcinosis, and progressive renal failure
Fabry disease	X-linked recessive	LoF	Lysosome	α -Galactosidase A	Hypohidrosis, heat intolerance, corneal opacities, arrhythmias, and renal insufficiency

AGT, Alanine: glyoxylate aminotransferase; CFTR, Cystic fibrosis transmembrane conductance regulator; PAH, Phenylalanine-4-hydroxylase.

served in neurodegenerative diseases, where tissue damage is caused by fibrillary aggregates rich in β -structure of proteins or small peptides [22, 47]. A third possibility exists, in which cellular damage is triggered by the molecular response of the cell aiming at restoring proteostasis. Indeed, when the alterations cannot be rescued, signaling pathways are activated that cause cell death by usually inducing apoptosis [48].

4. Use of pharmacological chaperones as therapeutic approach in rare enzymatic deficits

The increasing access to genetic screenings and family segregation studies has allowed the identification of many mutations that have been validated as pathogenic and associated with rare diseases. In the United States, a rare disease is a condition that affects less than 1 in 200,000 people, while in the European Union a disease is classified as rare if it affects less than that 1 in 2000 people [49]. A large subgroup of rare diseases is represented by enzymatic deficits, which are usually characterized by a loss-of-function pathogenesis. Disease-causing mutations can lead to various effects at phenotypic level, spanning from the complete loss of a protein product, the generation of truncated or defective variants, or alterations at sites of post-translational modifications, or protein–protein interactions [50, 51]. In the frequent case in which mutations lead to a missense change, a pathogenic variant is produced that can display a wide spectrum of structural and/or functional defects [52]. Studies performed in the last years

agree upon the finding that misfolding is a common mechanism underlying the loss-of-function of enzymes involved in rare diseases [53, 54]. As such, the loss-of-function is caused by the absence of an adequate amount of an otherwise catalytically-active variant, due to a conformational defect that can promote intracellular aggregation, increased degradation, aberrant subcellular localization, or an aberrant regulation hampering the conversion into active states [34, 45]. Upon this discovery, many efforts have been dedicated to the identification of strategies able to rescue for the effects of destabilizing mutations leading to misfolding. One of those is based on the use of pharmacological chaperones (PCs) [55].

The use of inhibitors to increase the levels of misfolded proteins has been first demonstrated by the pioneering work of Fan JQ and coworkers in 1999, who discovered that a competitive inhibitor of α -galactosidase A at sub-inhibitory concentrations was able to rescue for the Q279E and R301Q mutations in lymphoblasts of Fabry disease patients [56]. The term PC has been then introduced by Morello and co-workers, who discovered small-molecules antagonists of the vasopressin receptor able to rescue folding-defective variants [57]. Since then, several studies have been performed to identify PCs for many proteins, including enzymes associated with rare diseases, as well as to better understand their mechanism of action [54, 58, 59]. PCs should not be confused with molecular chaperones or chemical chaperons [60]. Molecular chaperones include a large group of endogenous proteins that assist protein folding in a non-specific way and prevent aggregation, thus guaranteeing proteostasis [36], while chem-

ical chaperones are small osmolytes that help the folding process in a non-selective manner by a still debated mechanism probably related to a change in the structure of water that can force productive folding [44, 61]. Notwithstanding the similar final effect of promoting the acquirement of the native structures, PCs differ from chemical chaperones because they specifically target a protein prone to misfolding [59]. PCs cause a thermodynamic stabilization of the protein, thus shifting the equilibrium toward the folded and functional conformation [62]. They usually bind to specific domains or inter-domain regions and stabilize the overall structure by providing a molecular scaffold around which mutant or misfolded proteins can assume their three-dimensional structures [34, 63]. From a thermodynamic point-of view, the mechanism of action of PCs can be understood in the light of the thermodynamics of protein folding. Indeed, the energy variation of the folding process can be so small that even subtle amino acid changes affecting the overall stability of an enzyme can narrow the ΔG unfolded-folded state to a degree that phenotypically impairs the production of sufficient amounts of functional enzyme. The interaction of the protein with a PC shifts the equilibrium toward the native states by generating a thermodynamically stable complex, thus providing a positive energetic contribution to the folding process. The latter effect can arise from the binding itself, or from an indirect action on the stabilization of the quaternary structure [64]. In some cases, the increase in the concentration of the folded protein also promotes its correct intracellular targeting, because the protein can successfully be recruited to the proper subcellular localization [65]. This is especially important for proteins that are imported in the ER, destined to the plasma membrane, or secreted in the extracellular space [66]. In proteins showing significant structural plasticity in the native state, PCs can also influence the equilibrium among the different possible conformers, e.g., stabilizing the more active and/or stable one under physiological conditions, possibly through a mechanism of conformational selection as proposed in the case of molecular chaperones [67].

Although their action is protein and mutation-specific, different studies demonstrate that they can be efficacious for more than 50% of missense mutations [68]. For this reason, PCs therapy offers a promising alternative approach for rare diseases for which only organ transplant or enzyme replacement are available [69]. Compounds acting as PCs identified so far have different protein targets including secreted proteins, transcriptions factors, ion channels, G protein-coupled receptors, and disease-causing enzymes such as in particular lysosomal hydrolases [54, 59, 70–73]. The general rules that defined first generation PCs are: (i) they target proteins bearing mutations that make the overall structure unstable but do not affect the functional properties. (ii) they must be specific for the target protein, e.g., used at very low concentrations (in the nanomolar range), and in the case of enzymes they must bind at the active

site [74]. Enzyme ligands effective as PCs are either vitamin derivatives functioning as coenzymes, or substrate analogues acting as competitive inhibitors [65]. The rationale at the basis of the action of these two classes of PCs is shown in Fig. 2A,B. It is known that when a coenzyme binds to the apoprotein not only has a prosthetic role, by participating to the catalytic pathway, but can also exert a chaperone role, by inducing an overall stabilization of the native structure or by promoting the folding of partly-folded intermediates [75–77]. As for substrate analogues, the use of PCs can be considered a counterintuitive approach [62]. They behave as competitive inhibitors of the target enzyme, bind the active site with high specificity and promote folding, then dissociate from the target protein in the presence of the physiological substrate, thus restoring activity. Recently, the term “second-generation PCs” has been introduced to indicate non-inhibitor molecules, as allosteric modulators able to promote folding. The main difference with respect to “first-generation PCs” is their binding to an enzyme pocket far from the active site [72, 73] (Fig. 2C). In some cases, such as for G-protein coupled receptors, PCs working as allosteric modulators can represent a more promising approach, as compared with classical active site ligands, because they do not compete with the physiological substrate, thus minimizing untoward inhibition effects [73].

We focused this review on three examples of rare diseases due to enzymatic deficits for which a therapy with molecules acting as PCs has been investigated. Gaucher disease has been one of the first disorders for which the use of substrate analogues as PCs has been proposed and tested at clinical level. Phenylketonuria represents a paradigmatic example of the role of coenzymes as PCs, as well as of the development of second-generation PCs. Primary Hyperoxaluria Type I is a disease in which both coenzymatic forms and substrate analogues have been tested for their chaperone activity.

4.1 Gaucher disease

Gaucher disease (GD, OMIM 230800) is classified as lysosomal storage disorder (LSD). Lysosomes are organelles involved in the catabolism of different class of molecules such as cholesterol, glycoproteins, glycosphingolipids, peptides, and glycogen, thanks to the presence of more than 50 hydrolases in their matrix [78, 79]. If one of these metabolic pathways is blocked by the loss of a specific enzyme activity, the substrate accumulates in the lysosome, resulting in enzyme-specific clinical manifestations [80]. Most LSDs show autosomal recessive inheritance, with a small percentage inherited as X-linked trait [78]. Although each LSD is rare, their prevalence as a group can be as high as 1/1000, in particular in some populations such as Ashkenazi jewish [81, 82]. The use of PCs has emerged as promising approach for the treatment of various LSDs, including in particular GD, Fabry disease, and Krabbe disease [83].

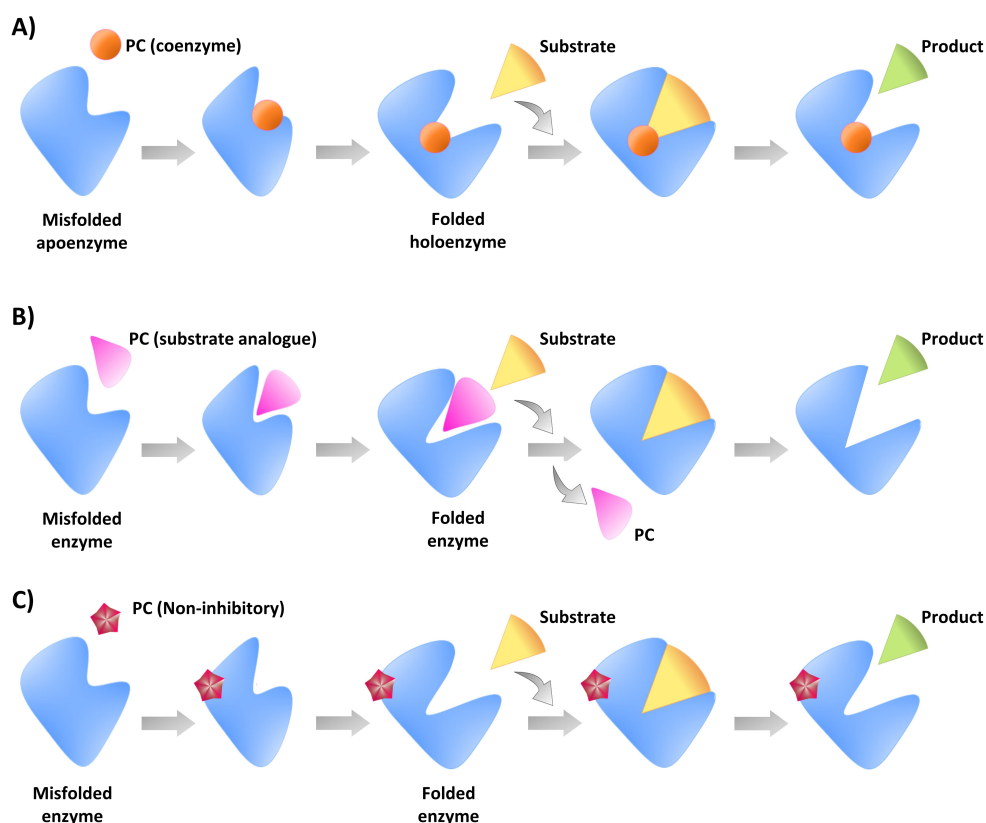


Fig. 2. Schematic representation of the PCs mechanism of action. (A) Coenzymes act on misfolded apoenzymes, inducing the proper folding of the holoenzyme and restoring its catalytic activity. (B) Substrate analogues can behave as competitive inhibitors and bind the active site of misfolded enzymes, promoting the achievement of the folded state. In the presence of the physiological substrate the PC dissociates, and the enzyme activity is restored. (C) Second-generation PCs bind as allosteric ligands far from the active site and promote correct folding, or stabilize an active conformation of a protein through conformational selection.

GD is the most common and the most studied LSDs. The disease shows an autosomal recessive inheritance and is due to mutations in the *GBA1* gene encoding β -Glucocerebrosidase (GBA) [80]. This enzyme is activated by saposin C and hydrolyzes the sphingolipid glucosylceramide into glucose and ceramide. Pathogenic mutations cause a functional deficiency of GBA leading to the accumulation of undigested glucosylceramide in lysosomes that in turn causes impairment of autophagy, mitochondrial dysfunction, and inflammation [80, 84]. In particular, substrate accumulation is observed in macrophages and they become the so-called Gaucher cells, in which the high concentration of fibrillar or aggregated structures gives rise to a wizened aspect [85, 86]. Three types of GD are known, based on their clinical features and in particular the involvement of central nervous system manifestations. GD Type I accounts for most cases and is considered as non-neuronopathic, although the development of neurological symptoms can develop later in life. Type II is the most severe form, also defined as neuronopathic infantile, cerebral, or perinatal lethal. The estimated life expectancy is about 3 years, because of severe neurological complications. Type III is of-

ten confused with Type-I, because of the similarity in symptoms at the beginning, but then progresses with neurological involvement within the second decade of life [86]. It has been reported that the heterozygosis for mutations in the *GBA1* gene typical of GD are also associated with an increased risk of Parkinson's disease [87] or with the possibility to develop different forms of cancer [84]. Indeed, Parkinson-like symptoms have been also found as a possible complication in GD Type I, probably dependent on an inverse relationship between GBA and α -synuclein levels [88, 89].

Two specific therapeutic approaches are currently available for the treatment of GD: (1) Enzyme Replacement Therapy (ERT), in which recombinant GBA is administered intravenously to rescue the enzyme deficit. Approved pharmaceutical formulations are imiglucerase (Cerezyme®, Sanofi-Genzyme); velaglucerase (Vpriv®, Shire) and taliglucerase (Elelyso®, Pfizer). (2) Substrate Reduction Therapy (SRT), in which a small molecule is employed as a weak inhibitor of glucosylceramide synthase to reduce the burden of gkycosylceramide. Available drugs are N-butyl- α -deoxynojirimycin (Miglustat, Zavesca®, Actelion)

and Eliglustat (Cerdelga®, Sanofi-Genzyme) [86, 90]. Although all these treatments have proven useful and quite well tolerated by GD patients, they show high costs and are not effective against the neuropathic forms of the disease, thus prompting for the search of new approaches. In this regard, the analysis of disease pathogenesis has revealed that among the hundreds of GD-causing mutations, many are missense changes leading to the loss of GBA function by interfering with the folding process of the protein and lead to various effects including premature degradation, aggregation, or retainment in the ER [91]. This is true particularly for the L444P mutation, one of the two most common and associated with neurological impairment [92]. Therefore, the possibility to identify PCs specifically designed to facilitate folding, trafficking to lysosomes and function of mutant GBA has been attractive. PCs also have the potential to prevent or attenuate other cellular responses, including ER stress and the unfolded protein response that can lead to apoptosis and other inflammatory consequences [93].

Many *in vitro* studies also including high-throughput screening campaigns have been performed to identify small molecules as PCs in GD. Most are active-site specific ligands and reversible competitive inhibitors of GBA. Good candidates belong to the iminosugars family, inhibitors of the enzyme that also display chaperone activity when used at sub-inhibitory concentrations [94, 95]. *N*-nonyldeoxynojirimycin has been one of the first molecules identified, but showed poor selectivity, being not able to distinguish between α - and β -glucosidases [78]. Therefore, derivatives with a sp^2 -iminosugar structure were synthesized and they were found to be endowed with a higher selectivity against GBA [96, 97]. Isfagomine was also considered a promising candidate based on *in vitro* and *in vivo* studies [98, 99]. Tested in patients with the commercial name of Plicera® (isfagomine D-tartrate), isfagomine showed efficacy in phase I trials, but then failed in phase II trials, which did not reveal significant clinical improvements in patients without previous history of ERT (Trial ID NCT00433147, NCT00446550). Since the failure in clinics was mainly attributed to the high hydrophilicity of iminosugars, attempts have been made to generate substituted forms that showed effectiveness in fibroblasts of patients [100]. An attempt in this direction involved the synthesis of alkylated trihydroxypiperidine compounds. Experiments performed in patient fibroblasts have shown that one hit compound is effective in rescuing for the N370/RecNcil but also the L444P mutation in fibroblasts derived from Gaucher patients compound heterozygous for the N370/RecNcil mutations and homozygous for the L444P mutation, the latter associated with neurologic impairment [101]. Among non-iminosugar molecules, Ambroxol, commercially available as an expectorant drug for newborns, is a mixed-type pH-dependent inhibitor of GBA able to cross the blood-brain barrier, thus possibly useful also against the neuropathic forms of the disease. Am-

broxol enhanced GBA activity in patients-derived fibroblasts and lymphoblasts [102]. Its effectiveness in non-human primates and patient-derived macrophages has been also reported [103, 104]. After some encouraging results in a pilot study no large clinical trials have been undertaken, and the drug has been used as off-label treatment [105]. An investigator-based registry recently published suggested a re-evaluation of the use of Ambroxol as a safe and cheap option for GD patients, although a wider and placebo-controlled study would be necessary to obtain approval and overcome the current limitation to prescriptions by physicians [106].

Recently, researchers have been focused on the possibility to identify second generation PCs able to stabilize GBA without interfering with the hydrolytic activity. This possibility has been raised after the identification of an allosteric binding site on the protein [107]. Several molecules have been identified [108, 109]. Under this perspective, the L-iminosugar series have also received attention because while D-iminosugars act as competitive inhibitors, the L-enantiomers are often found to be non-competitive inhibitors of GBA. L-iminosugars have proven effective in fibroblasts derived from N370S homozygous GD patients [110]. Non-inhibitory PCs represent a promising option in GD, because many active-site directed molecules failed in clinical trials probably as a consequence of an unfavorable balance between inhibitory versus chaperone behavior. New cellular models for pre-clinical testing and appropriate clinical trials will be necessary for a rapid development of effective drugs [111].

4.2 Phenylketonuria

Phenylketonuria (PKU, OMIM 264600) is an autosomal recessive disease caused by an error in amino acid metabolism. The prevalence is different around the world, from 1:10000 in Europe, to 1:15000 in USA. In some countries like Turkey the prevalence is higher (1:4000), because of consanguineous marriages within the population [112]. This disease is mostly diagnosed in the first days of life, through a blood test followed by genetic analysis indicating the presence of a pathogenic mutation in the gene encoding phenylalanine hydroxylase (PAH). PAH is a hepatic enzyme that catalyzes the rate-limiting step of dietary phenylalanine (Phe) catabolism, leading to the production of tyrosine [77, 113]. In the absence of PAH, Phe accumulates in the bloodstream mainly leading to neurological symptoms, such as mental retardation, motor disorders, and depression, as well as other symptoms, such as eczematous rash, seizures, and autism [114]. The classification of PKU severity is based on the blood Phe concentration. The normal range of concentration is 50–110 $\mu\text{mol/L}$. Concentrations of 120–600 $\mu\text{mol/L}$ are classified as hyperphenylalaninemia, while levels of 600–1200 $\mu\text{mol/L}$ are classified as mild phenylketonuria. Classic PKU is associated with levels of blood Phe above 1200 $\mu\text{mol/L}$ [112, 115].

PAH is a homotetrameric cytosolic enzyme of 50 kDa subunits that catalyzes a monooxygenase reaction requiring molecular oxygen, iron, and the cofactor tetrahydrobiopterin (BH4). Each subunit is made up of a regulatory domain comprising an ACT domain and an N-terminal unstructured tail, a catalytic domain, and a tetramerization domain [77]. PAH is regulated by three mechanisms: (i) activation at high concentrations of the substrate Phe; (ii) inhibition by the cofactor at physiological Phe concentrations; (iii) phosphorylation at a specific serine residue (Ser16) by a cAMP-dependent kinase that activates the enzyme synergically with Phe [77]. The recently solved structure of full-length human PAH in the Phe-free form and complexed with BH4 has revealed the structural bases of the complex regulation of the enzyme by the cofactor and suggested a certain degree of conformational plasticity of the enzyme [116]. Moreover, it is known that PAH exists as an equilibrium between a “resting” and an “activated” state, being the first favored at basal Phe concentration ($<50 \mu\text{M}$), and the second favored in the presence of higher Phe concentrations ($\gg 50 \mu\text{M}$). This equilibrium is essential to allow Phe degradation at potential neurotoxic concentrations, while maintaining adequate availability of Phe and tyrosine for anabolic processes at basal levels [77, 117]. Phe binds to an allosteric site formed at the interface between two ACT domains at the N-terminus of the protein only in the activated state [118]. Structural and biophysical studies have indicated that binding of Phe to the allosteric site stabilizes a large repositioning of regulatory domains, exposing the active site [119, 120]. The latter movement also stabilizes the protein against denaturation and proteolytic cleavage [121, 122].

In PKU patients, accumulating Phe is transaminated to phenylpyruvate that not only accumulates at toxic concentrations, but also generates secondary metabolites such as phenyllactate and phenylacetate. The neurological symptoms of PKU are probably due to accumulating Phe that saturates the LAT-1 transporter of neutral amino acids in the central nervous system. In addition, decreased levels of tyrosine may also play a role on disease pathogenesis because this amino acid is an important precursor of dopamine, norepinephrine, and adrenaline. PKU treatment must be initiated within the first 10 days of life to prevent any irreversible tissue damage [123]. However, no definitive treatments are currently available. A Phe-restricted diet allows to prevent neurological symptoms, but strict adherence to treatment is difficult to obtain from patients [124]. Other approaches include the administration of large-neutral amino acids, to prevent excessive increase in the brain, and ERT based on the administration of the enzyme Phe ammonia lyase from *Anabaena variabilis*, which catabolizes Phe to transcinnamic acid and ammonia [112]. The enzyme is usually intraperitoneally administered in a PEGylated form, but the encapsulation in erythrocytes to protect from degradation and immunological reaction of pa-

tients has been tested at preclinical level [125].

In the last years, numerous studies have been performed to better understand PKU pathogenesis. Among the 1000 + of different mutations identified by genetic analysis of the patients, it has been observed that in many cases the loss of PAH enzymatic function results from single amino acid replacements that increase the tendency to misfolding, conformational destabilization and rapid degradation [68, 126, 127]. In addition, the data on the complex regulation of the enzyme, and the associated conformational changes, in particular those occurring upon the shift between the “resting” and the “activated” state, have led to a new view of PKU pathogenesis in which pathogenic mutations can affect the equilibrium between the conformers, so that the enzyme is not able to sense and respond to increased Phe concentration. The latter view point more to an inability to shift between native conformers rather than to misfolding itself as the cause of PAH functional deficit in PKU [128]. Overall, the latter results have prompted researchers to implement approaches based on small-molecules acting as PCs for PAH that could address the loss-of-function molecular phenotype and restore the Phe degradation ability.

A first approach has been the pharmacological administration of the natural cofactor BH4, approved by FDA and EMA and commercialized as sapropterin dihydrochloride [129, 130]. In responsive patients, BH4 decreases blood Phe concentration and/or increases dietary Phe tolerance [131–133]. It has been reported that a minority of PKU patients are responsive to BH4, and that responsiveness is usually associated with mutations giving rise to mild effects, although clear genotype/phenotype correlations have not been established. Attempts to implement personalized medicine in PKU have been made using an approach called activity landscape, which provides a link between mutations, their functional consequences on PAH activity, and the responsiveness to BH4. This approach allowed to analyze the enzyme kinetics in a wide range of substrate and cofactor concentrations, thus better dissecting the effects of each mutation, as well as predict disease severity and possible responsiveness based on the genotype [134, 135].

The understanding of structural bases of BH4 responsiveness is not straightforward. It is known that BH4 and PAH concentrations are similar in liver hepatocytes, and that the cofactor forms a dead-end complex with the enzyme at physiological Phe concentrations [136]. When blood Phe concentration increases, PAH is activated by allosteric Phe binding. A first hypothesis to explain responsiveness was linked to the mere kinetic role of BH4. However, only few PKU-linked variants display a low affinity for the coenzyme. Further investigations demonstrated that BH4 is effective in counteracting the effects of misfolding-inducing mutations, thus behaving as a PC [137]. The chaperone role of BH4 has been evidenced on both purified variants, where BH4 is able to protect PAH unsta-

ble variants from thermal denaturation and their stabilizing effects propagated throughout all the protein structure, and cellular model systems, where a protection against degradation by the ubiquitin proteasome system has been shown [138, 139]. The structure of full-length PAH has revealed that BH4 binding to the apoenzyme causes conformational changes that propagate from the regulatory domain to the oligomerization domain and also involve the active site [116]. These changes have also explained the thermodynamic stabilization exerted by BH4, which results in an increased resistance to thermal stress. In addition, the changes in one monomer also influence dimerization and tetramerization.

Notwithstanding the satisfactory results obtained by BH4 administration, responsiveness is limited to a minority of patients, and a variable clinical outcome is observed even among responders [140]. Therefore, a search for other molecules acting as PCs has been undertaken. One of the tested possibilities has been the search for coenzyme analogues performed by virtual screening followed by *in vitro* and *in vivo* assays. This strategy allowed to identify two compounds that bind at the BH4 site and induce a significant stabilization of PAH against misfolding and degradation, one of which is more effective than the cofactor itself [141]. A strategy based on the identification of substrate analogs has been also undertaken. In 2008, high-throughput screening of molecules able to induce a thermal stabilization of PAH allowed to identify two hit compounds effective both *in vitro* and *in vivo* [117]. The binding at the PAH active site was also determined by crystallography, thus providing insights into the molecular mechanism explaining the chaperone effect [139]. Recently, Lopes R.R. *et al.* [142] applied a different strategy to target the whole PAH active site, including the binding sites for the substrate, the cofactor, and the non-heme ferric center. Through a combination of studies using purified PAH and cellular models, aiming at investigating both the direct binding of the molecules and their protective effect against enzyme denaturation/degradation, the authors were able to identify putative PCs that stabilize PAH in the active conformation. Interestingly, one of them binds a region remote from the catalytic and the allosteric sites, thus possibly representing a “second-generation” non-inhibitory PC [142]. In this regard, a strategy based on the use of small-molecules that bind PAH in a site remote from the active site has been also considered promising for mutations affecting the equilibrium among alternative PAH structures. This approach is based on the idea that any molecule able to specifically bind the “activated” conformation would rescue for the mutations effects while avoiding the risk of a constitutive activation of the enzyme which could lead to unwanted dangerous reduction in the plasmatic Phe concentration [128].

4.3 Primary hyperoxaluria type I

The term hyperoxaluria refers to a pathologic increase in urinary excretion of oxalate. In humans, oxalate is an end-product of metabolism, mainly produced in the liver from the catabolism of hydroxyproline and glycolate [143, 144]. Any defect in the production or excretion of oxalate leads to its progressive accumulation in the kidneys, thus allowing the formation of the poorly soluble calcium oxalate (CaOx) that precipitates generating kidney stones. Hyperoxalurias can be distinguished into two categories: (i) primary hyperoxalurias, rare inborn errors of glyoxylate metabolism that result in a high endogenous oxalate production. (ii) secondary hyperoxalurias, non-genetic forms of the disease caused by an increased exogenous oxalate absorption, as consequence of various alterations including intestinal inflammation, bariatric surgery, or excessive intake of oxalate precursors [145]. The three types of PH are distinguished based on the mutated gene. Among them, Primary Hyperoxaluria Type 1 (PH1, OMIM 259900) is the most frequent (about 80% of PH patients) and severe form [146, 147]. PH1 is due to mutations in the *AGXT* gene encoding liver peroxisomal alanine: glyoxylate aminotransferase (AGT), and is characterized by an autosomal recessive inheritance. The global prevalence is about 1 to 3 per million people and the incidence is approximately 1:120000 live births in Europe and North America [148, 149]. Patients affected by PH1 show a deposition of CaOx and its progressive accumulation in the kidneys eventually leading to renal failure. Upon renal failure, the excessive oxalate synthesis by the liver is compounded by the failure to remove it from the body. This results in a potentially fatal condition named systemic oxalosis, with CaOx to deposit into many organs and tissues [150, 151].

AGT is liver peroxisomal homodimeric enzyme belonging to the Fold Type I family of pyridoxal 5'-phosphate (PLP)-dependent enzymes [152]. Each subunit is made up of an N-terminal arm wrapping over the surface of the neighboring subunit, a large domain comprising the active site, and a small C-terminal domain where the regions mediating peroxisomal targeting are located [153]. PLP is covalently bound through a Schiff base with Lys209 and interacts with the apoprotein through residues belonging to both subunits [152]. AGT is functionally involved in the metabolism of glyoxylate, and it catalyzes the transamination of L-alanine and glyoxylate into pyruvate and glycine. Although the catalytic pathway of the reaction is typical of PLP-dependent transaminases, AGT is peculiar because the equilibrium of the reaction is largely shifted toward glyoxylate-to-glycine conversion, in line with the role of the enzyme in glyoxylate detoxification [154]. This explains why in PH1 patients the deficit of AGT causes glyoxylate accumulation, thus allowing its conversion to oxalate by lactate dehydrogenase in hepatocyte cytosol [146, 155].

Classical treatments for PH1 aim at preventing kidney failure or restoring kidney function. They include a high fluid intake, the administration of crystallization inhibitors, lithotripsy, dialysis, and/or kidney transplantation procedures [147]. Only two therapeutic approaches directed to the causes of the disease are available so far: the administration of pharmacological doses of Vitamin B6 in form of pyridoxine (PN), and liver transplantation [145, 156]. Liver transplantation is the most resolutive action to restore the total activity of AGT, but it is a very invasive procedure associated with significant potential side-effects. On the other hand, the administration of PN, that is converted to the natural cofactor of AGT, PLP, is effective only in a small percentage (less than 30%) of patients [147, 157]. Very recently, a drug based on the RNA silencing technology named Lumasiran (Oxlumo™) has been approved for the treatment of PH1 [158]. It is directed against the *HAO1* gene encoding glycolate oxidase, and it represents a SRT approach because it counteracts the synthesis of glyoxylate. An analogous technology having as target lactate dehydrogenase (Nedosiran) is currently under clinical testing (NCT04580420 NCT03392896 NCT02795325). Although these new approaches hold promise for PH1 patients, they represent very expensive long-life treatments, whose risk/benefit ratios should be carefully evaluated by physicians. Thus, alternative approaches based on small molecules or biologic drugs are currently under investigation [159].

Among the more than 200 PH1-associated mutations identified so far, missense mutations are well-represented [160]. It has been widely demonstrated that most pathogenic amino acid substitutions do not affect the intrinsic catalytic activity of the protein, but rather alter its folding pathway. Thus, misfolding has emerged as one of the main mechanisms underlying the AGT loss-of-function in PH1. The downstream effects of AGT misfolding are various and include a reduced stability of the dimeric unit, a higher susceptibility to proteolytic degradation, an increased aggregation propensity, or mistargeting to mitochondria where AGT is functionally ineffective [161, 162]. The latter is a pathogenic mechanism peculiar of PH1 in which a polymorphism normally present in 20% of Caucasian population generates a weak mitochondrial targeting sequence, whose effects become evident only if pathogenic mutations interfering with folding are concomitantly present [163–165].

On the basis of these knowledge, the possibility to develop a non-invasive treatment approach for PH1 based on the use of PCs has been considered. First, it must be underlined that a chaperone action of the PLP coenzyme on AGT has been demonstrated for most variants showing folding defects [151, 161, 166]. PLP is bound at the interface between the two subunits of the AGT dimer. The chaperone effect is mediated by a variety of mechanisms including (i) the promotion of dimerization [167, 168]; (ii) an im-

proved efficiency of the folding process, with also includes the correct targeting [163, 166]; (iii) an overall stabilization of AGT native structure against electrostatically-driven aggregation [169]. Based on the chaperone role of PLP, which allows to rationalize the effects of PN administration to PH1 patients, cellular studies on other B6 vitamers have been performed. They showed that other vitamers can be more effective than PN in rescuing folding-defective variants. In fact, in the presence of excess PN, pyridoxine phosphate accumulates in the cytosol and competes with PLP leading to the formation of an inactive complex. This explains why AGT inhibition is observed upon PN administration to cell expressing the wild-type form [170]. Recent data based on a combined molecular/cellular study on variants bearing mutations of interface residues have allowed to establish that (i) the higher is the structural perturbation caused by a mutation, the lower is responsiveness to PN [166], and that (ii) the co-inheritance of the minor allele polymorphism can not only influence the severity of the effect of PH1-associated mutations, but also their responsiveness to PN [165].

Another molecule that acts as PC for AGT is aminooxyacetic acid. It binds the active site of AGT and behaves as competitive tight-binding inhibitor that forms an oxime with the carbonylic group of PLP. Although data in cellular models indicate that aminooxyacetic acid behaves as a PC for AGT pathogenic variants showing folding defects, promoting the achievement of their correctly folded structure, it is not specific, being able to interact with many PLP-enzymes and free PLP. For these reasons, a small screening campaign based on the aminooxyacetic acid scaffold has been undertaken and led to the identification of hit-compounds that are active on some of the most frequent AGT variants as promising candidates to be developed for the treatment of PH1 [171].

Recently, cycloserine enantiomers have been also considered as a possible AGT inhibitors suitable as PC. L-cycloserine is a synthetic compound used as regulator of lipid metabolism *in vitro*, while D-cycloserine, the natural enantiomer, is used as second-line approach in the treatment of tuberculosis [172]. Both compounds behave as irreversible inhibitors of several PLP-dependent transaminases, racemases and decarboxylases [173]. A study on the interaction of cycloserine with AGT has demonstrated that both enantiomers are able to bind the AGT active site with high affinity and inhibit transaminase activity in a reversible competitive fashion although with different mechanisms. In fact, while the L-enantiomer is a classical competitive inhibitor, the D-enantiomer is a time-dependent inhibitor. Interestingly, only D-cycloserine displays a PC activity on a mutant form of AGT prone to misfolding. The latter data have suggested that a new line of intervention against PH1 could be also based on a drug repositioning approach [174].

As already mentioned, one of the downstream effects of AGT misfolding in PH1 is mitochondrial mistargeting. A solid platform based on the use of Chinese Ham-

ster Ovary cells expressing the most frequent mistargeted variant G170R, has been implemented to identify small molecules able to promote folding and reroute the protein to peroxisomes [175]. The application to the screening of libraries of drugs accepted by FDA and/or EMA, led to the selection of three active drugs belonging to the family of ionophores, which showed EC50 in the micromolar range [176].

5. Conclusions

The use of PCs represents a promising pharmacological strategy to treat rare disorders associated with protein misfolding. The administration of coenzymes, such as BH4 in PKU or PN in PH1, represents a safe and cost-effective approach that is usually attempted in all patients bearing missense mutations. However, the efficacy is limited to a minority of patients, and no clear genotype/phenotype correlations have been established, possibly due to the influence of modifier genes and/or environmental factors. Substrate analogues have also represented a cheap solution for some disorders, although their efficacy *in vitro* does not always translate into efficacy in clinical trials. The latter finding can be partly ascribed to the fact that most PCs are also inhibitors of the target enzyme, and it is very difficult to balance the inhibitory and the chaperone effects. Therefore, second generation PCs able to bind enzyme clefts different from the active site are considered a good solution to improve folding efficiency of conformational variants while avoiding the inhibition of catalytic activity.

6. Author contributions

BC conceived the content of the review, supervised the work, participated in the original draft of the manuscript and edited the final version; GP, SG, LG, EML and IL participated in the original draft of the manuscript.

7. Ethics approval and consent to participate

Not applicable.

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10. Conflict of interest

The authors declare no conflict of interest.

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Abbreviations: ID, intrinsically disordered; IDRs, intrinsically disordered regions; PC, pharmacological chaperone; GD, Gaucher disease; LSD, lysosomal storage disorder; GBA, β -glucocerebrosidase; ERT, Enzyme Replacement Therapy; SRT, Substrate Reduction Therapy; PKU, phenylketonuria; PAH, phenylalanine hydroxylase; Phe, phenylalanine; BH4, tetrahydrobiopterin; CaOx, calcium oxalate; PH1, Primary Hyperoxaluria Type I; AGT, alanine:glyoxylate aminotransferase; PLP, pyridoxal 5'-phosphate; PN, pyridoxine.

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