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Functional Screening of Pharmacological Chaperones via Restoration of Enzyme Activity upon Denaturation

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Supporting Information

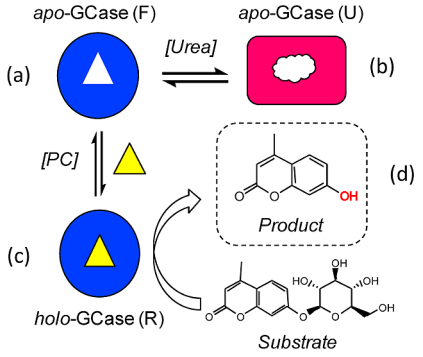
ABSTRACT: Pharmacological chaperones (PCs) are small molecules that stabilize and promote protein folding. Enzyme inhibition is widely used for PC selection; however, it does not accurately reflect chaperone activity. We introduce a functional assay for characterization of PCs based on their capacity to restore enzyme activity that is abolished upon chemical denaturation. Dose-dependent activity curves were performed as a function of urea to assess the chaperone potency of various ligands to β-glucocerebrosidase as a model system. Restoration of enzyme activity upon denaturation allows direct screening of PCs for treatment of genetic disorders associated with protein deficiency, such as Gaucher disease.

Gaucher disease (GD) is the most prevalent lysosomal storage disorder caused by a deficiency in β-glucocerebrosidase (GCase) that results in deleterious cellular accumulation of glucosylceramide (GlcCer). GCase catalyzes β-glycosidic bond cleavage of GlcCer to produce glucose and ceramide, a bioactive sphingolipid involved in heat stress response and apoptosis. More than 200 mutations in the GCase gene can lead to aberrant folding during protein assembly, which triggers endoplasmic recticulum (ER)-associated degradation pathways.[1] Defective GCases form non-native conformers with lower thermal stability under neutral pH conditions of the ER that are susceptible to proteolysis with a reduced level of trafficking.[2] However, most clinically relevant GCase mutants retain partial catalytic activity (<30%) under acidic conditions in the lysosome.[3] To date, enzyme replacement therapy using recombinant enzyme is the most widely used treatment for type I GD.[4] Alternatively, enzyme enhancement therapy based on small molecules that function as pharmacological chaperones (PCs)[5] offers a promising approach for salvaging endogenous GCase via stabilization of the native conformer for treatment of neuronopathic forms of GD (type II/III).

Since the first evidence of PCs as therapeutic agents for GD,[6] drug candidates have largely consisted of reversible active site inhibitors of GCase based on substrate mimics, such as natural and alkylated iminosugar analogues.[7] This has led to a counterintuitive principle that inhibitors enhance the activity of mutant enzymes by manipulating the ER folding environment. High-throughput screening of chemical libraries thus relies on enzyme inhibition,[8] where the inhibitor potency for GCase is used as a major criterion for PC selection. However, primary screening by inhibition is biased toward the detection of ligands that influence the interaction of wild-type, folded GCase with a single fluorogenic substrate probe.[9]

Indeed, not all inhibitors act as protein stabilizers as exemplified by false positives that lack relevant chaperone activity.[10] For this reason, thermal stability assays are required as a secondary screen to confirm chaperone activity prior to testing with GD patient-derived cell cultures.[11] Herein, we introduce a new assay for characterization of PCs based on their capacity to restore enzyme activity that is abolished in urea (Scheme 1) to address the high rate of attrition of conventional screens. Urea serves as a convenient perturbant to modulate the equilibrium distribution of protein conformers in solution without precipitation unlike thermal denaturation.[12] Seven model PCs were examined in this study, including competitive, mixed-type, and covalent inhibitors of GCase together with GlcCer (natural substrate) and mannitol (MNT, nonspecific osmolyte) as depicted in Figure S1 of the Supporting Information. A fluorogenic substrate is then used to evaluate the residual activity of the stabilized, refolded enzyme in urea relative to native holo-GCase (i.e., 0 M urea with PC) by measuring the extent of product formation. Similar experiments

Scheme 1. Restoration of Enzyme Activity upon Denaturation (READ) for Functional Screening of PCs[a]



[a](a) Recombinant GCase (folded apoenzyme, F) is first equilibrated under neutral pH conditions. (b) GCase is then partially or fully denatured (unfolded apoenzyme, U) in urea. (c) PC is introduced to shift the equilibrium distribution of the native, active enzyme state (refolded holoenzyme, R). (d) Normalized enzyme activity is measured by extent of product formation after thermal quenching. PCs can function as enzyme enhancers via reversible orthosteric and/or allosteric binding.

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are also performed for apo-GCase without ligand as a reference state to compare the dose response of PCs as a measure of their chaperone activity. Various methods can be used for READ, including fluorescence microarrays and multiplexed separation techniques, such as capillary electrophoresis (CE). CE with UV detection offers several advantages in terms of high selectivity, small sample requirements, and short analysis times that prevent spectral interference by resolving excess urea and free ligand, where both substrate and product are monitored simultaneously, including their stereoisomers.[13]

Figure 1a depicts a series of electropherograms showing the impact of PC binding on the modulation of GCase activity

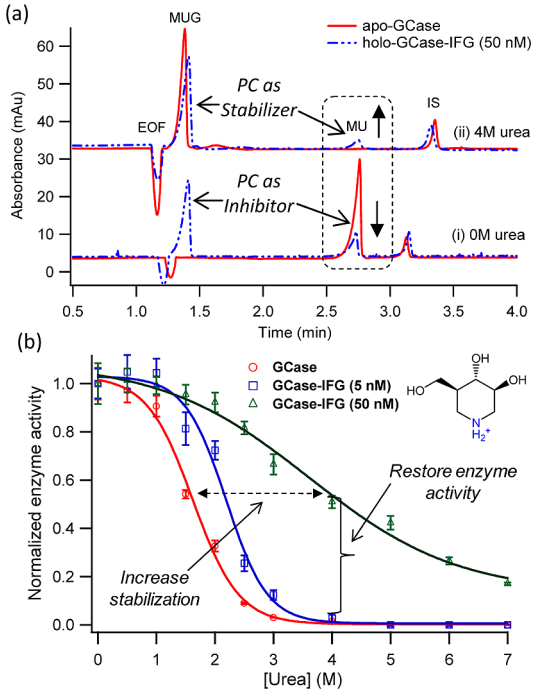

Figure 1. (a) Electropherogram overlay depicting the contradictory properties of PCs, such as the fact that IFG is a potent active site inhibitor of GCase that attenuates substrate turnover for the native enzyme at 0 M urea, whereas it enhances catalytic activity at 4 M urea, where apo-GCase is inactive. (b) Dose-dependent activity curves for GCase for characterization of PCs as a function of ligand type and dosage when using READ, where the error represents $\pm 1\sigma$ with a precision (n = 3) of <5%.

under neutral pH conditions at 0 M (folded and active) and 4 M urea (unfolded and inactive). Under alkaline buffer conditions in CE, excess urea and ligand are neutral and comigrate with the electroosmotic flow (EOF), which is then followed by the substrate (MUG), product (MU), and internal standard (IS) that migrate as anions with increasing charge density. The potent competitive inhibitor of GCase, isofagamine (IFG) (IC_{50} = 4.6 nM at pH 7.2),[12] significantly attenuates product turnover at 50 nM under native conditions, whereas it restores ~50% of normalized activity at 4 M urea that is nearly fully abolished (≈1.5%) for ligand-free apo-GCase. Thus, READ directly measures the chaperone capacity of a ligand to restore GCase activity while also revealing the potency of inhibition (or activation). Noteworthy is the fact that this approach avoids false negatives when using classical inhibitor screens because ligand interactions that do not inhibit GCase can still facilitate folding to enhance enzyme activity under denaturing conditions.

Figure 1b depicts normalized GCase activity curves for IFG as a function of urea at two dosage levels relative to apo-GCase. Each activity curve reflects the extent of ligand-induced stabilization of GCase that increases the population of the refolded yet catalytically active enzyme in urea. Two parameters can be derived from these activity curves that are related to chaperone activity of a ligand similar to equilibrium protein unfolding,[14] namely, the midpoint for urea inactivation (C_M) and cooperativity for inactivation (m). The former parameter reflects the intrinsic thermodynamic stability of apo-GCase and/or specific enzyme−ligand complex (holo-GCase), whereas the latter term indicates the sensitivity of enzyme conformers to urea denaturation. Figure 1b highlights that addition of only 5 nM IFG (using 25 nM GCase) results in a shift in C_M relative to that of apo-GCase (ΔC_M) of 0.57 M that is further increased to ~2.0 and ~4.4 M (data not shown) with 50 and 500 nM IFG, respectively, in a dose-responsive manner as shown in Figure S2 of the Supporting Information. Similarly, a dose-dependent decrease in cooperativity relative to that of apo-GCase (Δm) was measured with IFG as indicated by a more shallow linear transition between active and inactive enzyme conformers. The lower cooperativity for holo-GCase is likely associated with independent unfolding of the three non-contiguous domains of GCase that is enhanced upon localized stabilization of the active site (in domain III) by competitive inhibitors, such as IFG.[15] This is in contrast to greater unfolding cooperativity upon ligand binding of single-domain proteins.[14] In general, the chaperone potency of a ligand for GCase is associated with greater holoenzyme stabilization (ΔC_M), higher catalytic activity in urea, and reduced cooperativity (Δm) that is achieved at low dosage levels. However, because of the 10-fold weaker potency of IFG at pH 5.2,[12] ligand binding imparts a smaller increase in holo-GCase stability under acidic conditions as shown in Figure S3 of the Supporting Information. Dynamic protein unfolding studies were also performed (Figure S4 of the Supporting Information) to relate GCase activity curves with urea-induced structural changes to enzyme that are dependent on ligand type, dosage, and/or buffer pH.

Figure S5 of the Supporting Information compares activity curves of two distinct noncarbohydrate PCs derived from a FDA-approved drug library that function as mixed-type inhibitors of GCase. Ambroxol (ABX) is an antimucolytic agent recently shown to enhance the activity of mutant GCase in GD patient cells.[8] In contrast, diltiazam (DTZ) is a Ca^{2+} channel blocker that enhances GCase activity in cellulo for several GD variants.[16] Although DTZ and ABX are both mixed-type inhibitors of GCase with similar potencies ($IC_{50} \approx 50-60$ μM at pH 7.2),[12] DTZ has greater chaperone activity than ABX as reflected by its larger ΔC_M at each dosage level. For instance, 60 μM DTZ was able to restore the normalized GCase activity to ~30% in 4 M urea, which is within the critical threshold of activity under which symptoms of GD are observed.[17] In contrast, an equivalent dosage of ABX produced no significant changes in protein stability or GCase activity. Thus, inhibition type and potency alone do not reliably predict chaperone properties. GCase activity curves were also performed when using 5 nM GlcCer and 0.5 mM MNT that generate similar enzyme enhancement despite doses that vary over 5 orders of magnitude (Figure S6 of the Supporting Information). X-ray crystal structures have shown that polyols bind to the active site

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of GCase, however, with much lower affinity than IFG.[18] Table S1 of the Supporting Information summarizes three key parameters derived from READ that highlight the wide disparity in chaperone activity measured among seven model ligands, including ΔC_M, Δm, and percent activity at 4 M urea. Bromhexine (BHX) is a prodrug of ABX with a weaker potency ($IC_{50} \approx 86$ μM), whereas fluphenazine (FLZ; $IC_{50} \approx 140$ μM)[12] is an antipsychotic drug for the treatment of schizophrenia, which is a thermal stabilizer of GCase yet does not enhance activity in cellulo;[8] the latter observation is likely due to the limited aqueous solubility of FLZ.

To clearly rank chaperone potency for ligands over a wide dynamic range, Figure 2 compares two log-based READ indices

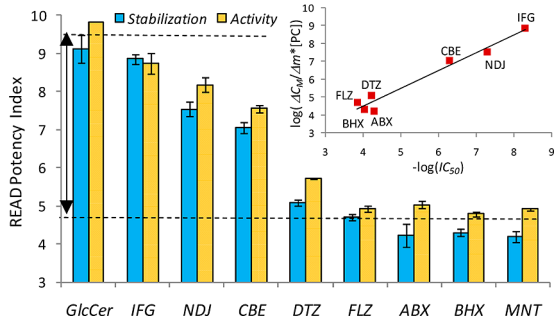

Figure 2. Two log-based READ indices for assessing the chaperone potency of a ligand in enhancing the stability and restoring the activity of denatured GCase relative to GlcCer and MNT as controls. Ligand binding that induces stabilization to GCase also increases activity at 4 M urea (a key transition point where apo-GCase is inactivated). Overall, there was a linear correlation (e.g., slope = 0.996; $R^2 = 0.961$) between inhibitor potency (IC_{50}) and PC-induced stabilization (i.e., ΔC_M) as shown in the inset, with notable exceptions among mixed-type inhibitors.

derived from ΔC_M and percent activity (at 4 M urea) values normalized to dosage level. GlcCer was found to have the highest chaperone potency among all ligands tested, including IFG. In contrast, MNT induced the weakest stabilization to GCase, which served as a lower "cutoff" for exclusion of ligands with marginal chaperone activity, such as BHX, ABX, and FLZ. The latter compounds require excessively large doses (>200 μM) to elicit a measurable response that is similar to that of polyol-based osmolytes; this is undesirable as it contributes to drug toxicity with reduced selectivity in vivo. Both covalent and competitive inhibitors of GCase, conduritol β-epoxide (CBE) and *N*-nonyldeoxynojirimycin (NDJ), possess greater chaperone activity relative to noncarbohydrate ligands; however, they lack GCase specificity.[11] Thus, enzyme controls must be included in primary screens to confirm ligand selectivity with an adequate READ potency index (>6). Overall, the inset of Figure 2 shows that there is a linear correlation between ligand-induced stabilization and IC_{50} values similar to trends for GCase melting temperature transitions.[19] However, there were several exceptions among mixed-type inhibitors that have greater chaperone potency despite their weaker inhibition properties, such as DTZ and FLZ.

In summary, READ directly measures the chaperone capacity of ligands that enhance enzyme activity under denaturing conditions. Although inhibitor potency is an approximate indicator of chaperone activity,[19] lead candidates are prone to false positives that require retesting prior to cell-based assays. Moreover, screening by inhibition excludes the detection of PCs that may stabilize the native enzyme conformer without substrate displacement. Computational studies have revealed the existence of allosteric sites in GCase that may represent targets for non-active site PCs.[20] Future work will adapt READ to enable high-throughput screening of novel classes of PCs that function as stabilizers yet properly refold denatured or mutant enzymes without adverse inhibition.

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of materials and methods, Table S1, and Figures S1–S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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