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Palmitoyl:protein thioesterase (PPT1) inhibitors can act as pharmacological chaperones in infantile Batten disease

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ABSTRACT

Competitive inhibitors of lysosomal hydrolases (pharmacological chaperones) have been used to treat some lysosomal storage diseases which result from mis-sense mutations and mis-folded protein but have not been tried in Batten disease, for which there is no current therapy. We synthesized a large number of novel, non-hydrolyzable competitive inhibitors of palmitoyl:protein thioesterase (PPT1) and showed that some could act as chemical chaperones. One inhibitor (CS38: βAGDap(Pal)VKIKK) was taken up by lymphoblasts from patients with mutations leading to the T75P/R151X substitutions and enhanced PPT1 activity 2-fold. A similar 2-fold stimulation with another inhibitor (AcGDap(Palm)GG$(R)_7$) was observed in patients with a G108R amino acid substitution in PPT1. Residual PPT1 activity in both was thermally unstable at pH 7.4 (but not at 4.7) consistent with a mis-folded, unstable PPT1 degraded by the ER stress response. Patients with null mutations did not respond to the pharmacological chaperones.
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1. Introduction

Infantile Batten disease (Infantile Neuronal Ceroid Lipofuscinosis – INCL) is an autosomally inherited neurodegenerative lysosomal storage disease which arises from genetic mutations in the gene (*CLN1*) for palmitoyl–protein thioesterase (PPT1) [1,2]. This results in lysosomal accumulation of autofluorescent material believed to be derived from palmitoylated peptides [3], retinal blindness, ataxia, seizures and early death. Much later onset of the disease is observed in patients with mis-sense mutations and residual enzyme protein up to 2% of control PPT1 activity [4] suggesting that a small increase in activity could delay symptoms into adulthood. It has been assumed that in these mutants, the resultant mis-folded protein is >97% removed by the ER-associated degradation pathway (ERAD) quality control system [5,6].

Competitive small molecule inhibitors of other lysosomal hydrolases, such as α-galactosidase (Fabry disease), β-glucosidase (Gaucher disease), β-galactosidase (GMI-gangliosidosis), and β-hexosaminidase (Sandhoff disease), have been claimed to restore partial activity in non-neural cells by this mechanism and are in clinical trials [7–13]. Success was attributed to the inhibitor acting as a pharmacological chaperone by refolding the enzyme in the ER [10–14]. Other studies have indicated that adding proteostasis regulators (e.g.: Celastrol and MG-132) [13] or heat shock protein [hsp70] [14] can induce a 2-fold increase in lysosomal hydrolase activity. We present data linking specific mutations with protein mis-folding and lack of enzyme activity and show that specific peptide targeting can be used to get these inhibitors to translocate across membranes and restore some PPT1 activity.

2. Materials and methods

2.1. Chemical synthesis of peptides

All peptides were synthesized by the authors using Boc solid phase peptide synthesis involving the protected amino acids Boc-Gly, Boc-Arg(Tos), Boc-Cys(mBz) Boc-Lys(ClZ) and or Boc-Dap(Fmoc). Palmitoyl groups were incorporated (using palmitoyl chloride in CH_2Cl_2) on the side chain of the Dap residue after chain assembly and removal of the Fmoc group [15,16]. The lead compound DAP1 (AcG-palmitoyl diamino proprionate-VKIKK) was based on K-Ras4a (which is physiologically palmitoylated) and structures were confirmed by mass-spectrometry. The corresponding fluorescent-tagged inhibitors (e.g.: CS38; NBD-βAG-Dap(Pal)VKIKK) were synthesized by attaching NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl)- to the N-terminal glycine.

2.2. Measurement of PPT1 activity

PPT1 activity was assayed in the soluble fraction of cell lysates at pH 4.7 with a specific fluorescent-based (4-methylum-

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belliferyl-β-gluco-6-thiopalmitate) (MUGSP) substrate in which the generated 4MU-β-glucoside is hydrolyzed by added plant β-glucosidase, as described previously [15]. Inhibitors were added at a final concentration of 1–50 μM and incubations were carried out over the linear range (1–3 h). Mutant cell extracts required longer incubation (18 h) since activity was low. Other lysosomal hydrolase activities were measured as additional controls using similar 4 MU substrates and conditions previously optimized for their hydrolysis [8]. Results are expressed as the mean of duplicate experiments run in triplicate with $p < 0.05$. To determine IC_{50} values, lymphoblast extracts were incubated with increasing concentrations of inhibitor (1–50 μM) for 1 h. For thermal inactivation studies, the lymphoblast cell extracts were pre-incubated at pH 7.4 at temperatures up to 37 °C for 0–90 min, under which conditions there is no loss of PPT activity in normal cell extracts. Aliquots we then incubated at pH 4.7 for the times indicated (1–18 h).

2.3. Cell culture

Immortalized lymphoblasts were grown in suspension culture in RPMI and fibroblasts in DMEM supplemented with 10% fetal calf serum. Small molecule inhibitors were initially dissolved in DMSO and then diluted to 5% DMSO in 5% bovine serum albumin to a final concentration of 1 mg/ml (approximately 2 mM) and added to cells at 5–20 μM final concentration for up to 96 h.

3. Results

3.1. Synthesis of novel in vitro inhibitors of PPT

Inhibitors were synthesized with and without the N-terminal addition of the lipophilic fluorophore NBD and CS38 (NBD-βAGDap(Pal)VKIKK) was a 3-fold better inhibitor than Dap1 (AcG-Dap(Pal)VKIKK) [15] (Table 1). Drugs were tested at a wide range of concentrations and the IC_{50} determined as described previously [15]. Analysis of a large series of substrate-based inhibitors identified the fluorogenic (CS38) as the most potent peptide inhibitor with an IC_{50} of 2 μM (Table 1). The NBD form of the authentic thiol (CS8: GGC(Pal)VKIKK) had comparable inhibitory activity to CS38 (Table 1). Additional peptides with TAT-like polyarginine (R_7) tails (AcG-Dap(Palm)GGR_7) designed to promote cellular uptake, were also strong PPT1 inhibitors, for example AcGDap(Pal)$GG(R)_7$ (Table 1). The addition of an N-terminal Dansyl group did not reduce competitive activity substantially but any truncation of the VKIKK lost inhibitory activity.

Table 1
Relative PPT inhibitory potential of fluorescent labeled peptides with N-terminal VKIKK.

Chemical chaperone		IC_{50} (μM)
CS38	NBD-βAGDap(Pal)VKIKK-OH	2
CS8	NBD-GGC(Pal)VKIKK-NH_2	3
CS63	NBD-βAG(Palmitoleic)VKIKK-OH	5
CS7	NBD-βAGDap(Pal)VKIKK-NH_2	8
CS17	NBD-GDap(Pal)VKIKK-OH	10
	AcGDap(Palm)$GG(R)_7$	16
CS9	NBD-GGS(Pal)VKIKK-NH_2	20
CS4	Dansyl GGC(Pal)AKKKK-OH	25
CS13	NBD-βAGDap(PalKA)VKIKK-NH_2	
CS15	NBD-βAGDap(Pal-OH)VKIKK-NH_2	100

The IC_{50} was determined by assay of PPT1 activity in lymphoblast or fibroblast cell lysates in the presence of a range of concentrations of drug (1–50 μM) as described in the text. Under these conditions the IC_{50} previously published for DAP1(AcG-Dap(Palm)VKIKK) was 10 μM.

3.2. Selection of Batten patient-derived cell lines which might have low PPT1 activity as the result of protein mis-folding

We selected a sub-group of CLN1 patients with residual PPT1 activity that might be helped by therapy based on these studies. From the published PPT1 crystal structure (1EH5.pdb) [17], we determined that a common mutation (C223A), which substitutes Proline for Threonine combined with an exon 5 mutation (C451T) which substitutes a nonsense amino acid (R151X) for Arginine and truncates the protein was far from the active site. BD037, C14522, C7297 and C12488 were all compound heterozygotes of this type and had both low residual levels of PPT1 protein and some detectable PPT1 activity.

3.3. Residual activity in T75P mutants is more heat sensitive

Cell lysates of BD037, C14522, C7297 and C12488 were heated at increasing temperatures for 90 min and PPT1 activity compared to that of control lymphoblasts ($n = 3$). There was a rapid loss of PPT1 activity in all four patient samples beginning at 20 °C and typical results (patient C14522) are shown in Fig. 1A. Control extracts showed no loss of activity below 40 °C. We then compared thermolability at pH 7.4, with thermostability at pH 4.7 (data shown for C12488 (Fig. 1B)). All four T75P/R151X mutants were unstable when pre-heated at 37 °C pH 7.4 but were stable at pH 4.7. In contrast, other lysosomal hydrolases, for example α-mannosidase, α-fucosidase and acid sphingomyelinase in the same control or CLN1 cell extract were stable at pH 7.4 and 37 °C (Fig. 1C). The loss of activity is unlikely to result from proteolysis since a cocktail of leupeptin, EDTA, aprotinin and PMSF did not protect against inactivation. Similar thermal instability results were obtained in fibroblasts from a patient (RD55) with a G108R amino acid substitution (Fig. 1D).

3.4. Fluorescently labeled chaperones are taken up by cells and restore PPT activity in intact T75P lymphoblasts

Control (BD116 and C17381) and T75P/R151X mutant (BD037 and C14522) lymphoblastoid cell lines were treated with CS38 (10 μM) for 24–96 h. Uptake by cells was uniform (Fig. 2A) and PPT1 activity increased in the mutants (Fig. 2B) whereas controls were unaffected, possibly because the cells are diluted 15-fold in the PPT1 assay. The 2-fold increase in PPT1 activity in the mutants BD037 and C14522 (Fig 2B and Table 2), suggested that the inhibitor was acting as a chemical chaperone. Higher concentrations of CS38 had no greater effect but we interpret the data in Fig. 2B to suggest that enzyme protein is increased rather than any kinetic effect. The Western blot was not sufficiently sensitive to convincingly support a 2-fold increase in protein. This 80–100% increase compares well with results from chaperone treatments in other lysosomal storage diseases and with a recent study in which heat shock protein hsp70 was shown to produce a 2-fold increase in mutant acid sphingomyelinase activity [7–14]. Our study therefore constitutes an initial proof-of-principle for Batten disease. Since in late-juvenile-onset patients 2% of normal enzyme activity supports normal brain function for at least 10–15 years it could also have clinical significance. The 2-fold increase is all the more impressive since we are working on the assumption that only 50% of the gene product is T75P in these compound heterozygotes, and that R151X did not contribute.

Similar results were observed with two additional late-onset CLN1 patients, C7297, and C12488 (both T75P/R151X amino acid substitutions) using NBD-βAGDap(Pal)VKIKK (CS38), and in fibroblasts from a late-onset patient RD55 with a G108R substitution using AcGDap(Palm)GGR_7 (Table 2). In both cases, increased PPT1

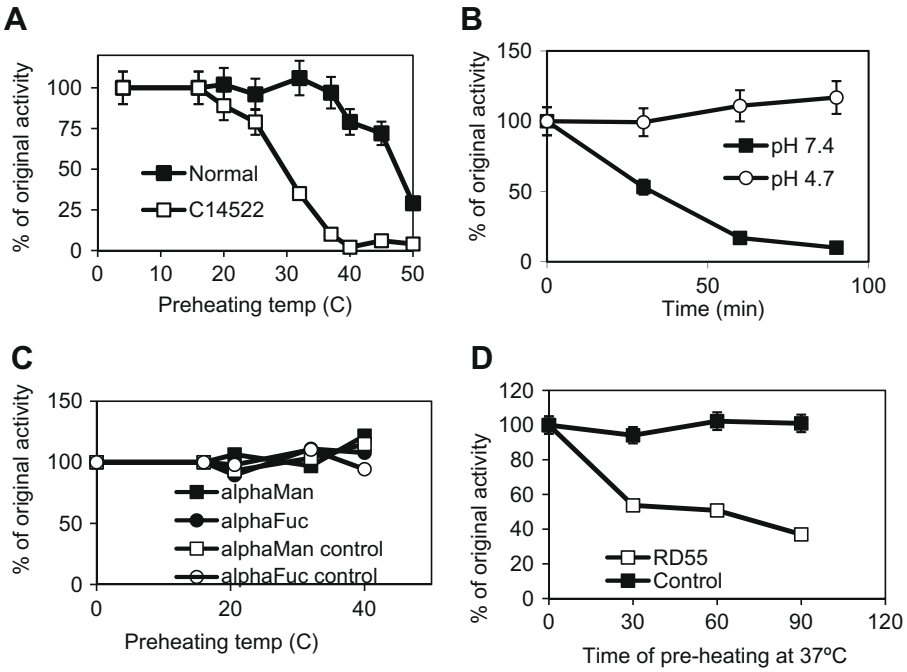

Fig. 1. Increased thermolability of mutant cell extracts at pH 7.4. PPT1 activity in mutants was less than 2% of that in controls, so results are expressed as percent of original activity. Panel A: Lymphoblasts from patient C14522 (T75P) showed increased loss of PPT1 activity after 90 min pre-treatment at the temperatures indicated at pH 7.4. Panel B: Patient lymphoblast C12488 (T75P) extracts were stable when pre-heated at pH 4.7 but showed thermolability similar to C14522 when pretreated at 37 for 90 min at pH 7.4. Panel C: Representative lysosomal hydrolases (α-mannosidase and α-fucosidase) were unaffected by pre-treatment of BD037 (T75P) at pH 7.4 and 37°. Panel D: Patient RD55 (G108R) fibroblast extracts were stable when pre-heated at pH 4.7 but showed increased thermolability when pretreated at 37 for 90 min at pH 7.4. Results are the mean of duplicate experiments run in triplicate with $p < 0.05$.

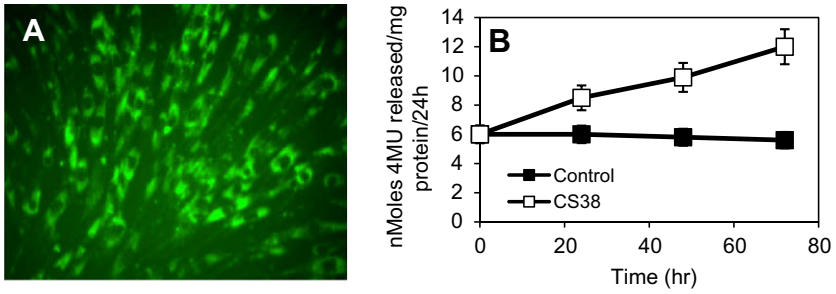

Fig. 2. Panel A: Cells were cultured as described in the text and fibroblasts treated with CS38 for 24 h. Cells were fixed and examined by fluorescence microscopy as described in the text. A similar uptake pattern was observed by lymphoblasts (data not shown). Panel B: BD037 lymphoblasts were cultured as described in the text and treated with CS38 at 20 μM (the limit of drug solubility) for up to 96 h. Harvested cells were lysed and assayed for PPT1 activity as described in the text. Results are the mean of triplicate assays run in duplicate. Similar results were obtained for C14522 as shown in Table 2. Normal lymphoblasts were unaffected by such treatment since the inhibitor is diluted during preparation of the cell extract.

Table 2
Differential effect of chaperone CS38 on lymphoblastoid cell lines and fibroblasts (RD55) from patients with CLN1 mutations.

Patient	Mutant PPT	Age of onset of symptoms	Effect on PPT1 activity
BD445	T29A	6 months	None
BD329	A364T/ R122W	3 months	None
BD037	T75P/R151X	7 years	2-fold increase
C14522	T75P/T75P	7 years	2- to 3-fold increase
RD55	G108R	20 years	2-fold increase

All results in this summary were the average of three experiments run in duplicate in which the chemical chaperone (CS38) was added to lymphoblast cultures from patients (as indicated) with defined mutations for 72 h and the PPT1 activity determined on cell lysates as described in the text. PPT activity was expressed as units per mg total cell protein. In the case of RD55 the cells were fibroblasts and the PPT1 inhibitor used (20 μM) was AcGDap(Pal)G_7.

activity was in the range of 1.5- to 2-fold ($p < 0.05$) suggesting that some enzyme stabilization was occurring.

3.5. Chemical chaperones do not increase the activity levels of inactive enzymes

The lymphoblast cell line BD445 was derived from a Greek/Irish ancestry child with onset at 6 months and death at 7.5 years. The nonsense mutation results in T29A and premature chain termination. These lymphoblasts are therefore a classical infantile CLN1 with no residual enzyme. Treatment with DAP1 or CS38 for 3 days did not restore any PPT1 activity. We also tested BD329 in which the mutation was the Finnish R122W but also saw no increase in PPT1 activity (summarized in Table 2) probably because it is very near the active site [17]. Although single examples, such mutations (which are non-correctable by chemical chaperones), serve as a

control for non-PPT1-mediated DAP1 phenomena, such as detergent effects.

4. Discussion

Our results suggest that NBD-GDap(Pal)VKIKK is a potent non-hydrolyzable competitive inhibitor of PPT1 (IC_{50} of 2 μM) which is taken up by cultured fibroblasts and transformed lymphoblastoid cell lines. It was then able to promote some additional hydrolysis of thioester peptides in four CLN1 patients with T75P and one with G108R amino acid substitutions. We propose that the inhibitor is acting as a pharmacological chaperone and that our results compare well with similar pharmacological chaperone studies on fibroblasts from patients with other lysosomal storage disease. We demonstrate that T75P and G108R amino acid substitutions result in a much more thermally labile enzyme at pH 7.4 than wild-type PPT1 but that both normal and mutant enzymes are stable at lysosomal pH 4.7. Therefore we propose that Batten patients who are homozygous for T75P and G108R or compound heterozygotes, could respond to small molecule chaperones (competitive inhibitors) which fit the criterion of "active-site-specific chaperones". The 2-fold increase in PPT1 activity may seem modest but there is currently no therapy available to reverse the neurological decline in INCL and 5% of residual activity of lysosomal hydrolases is often sufficient to delay onset of symptoms into the 3rd or 4th decade of life. Combination therapy with hsp70 [14] could produce an additive effect since the mechanisms of action are quite different, although it seems unlikely that this 70-kDa protein could ever cross the blood brain barrier.

A likely explanation for the action of these inhibitors as pharmacological chaperones is that residue Threonine 75 in palmitoyl–protein thioesterase (PPT1) is located at the N-terminus of a solvent exposed helix leading into an extended turn region [17] and substitution by Proline leads to loss of hydrogen bonding because of the cyclic Proline structure. The backbone dihedral angles of Thr75 are (154°, −140°) (Phi, Psi) and are located in the β sheet region of the Ramachandran map while substitution with Proline would favor Psi angles closer to 90°. Residue 75 is remote from the active site and the chaperone must work by binding to the active site and stabilizing the enzyme. However, the backbone reorganization of the turn to accommodate the cyclic structure of Proline makes the enzyme thermally labile, as observed experimentally. The other potential mis-fold-inducing substitution we studied, G108R is also theoretically likely to be thermally destabilized by mutation to a conformationally more restricted amino acid and this is what we observe experimentally.

5. Conclusion

In conclusion, we have shown that point mutations in the CLN1 gene, result in a mis-folded protein which is prematurely degraded but which can be partially protected by pharmacological chaperones based on a palmitoylated basic peptide structure. Future studies will be directed towards understanding how these novel PPT1 inhibitors might gain access to the endoplasmic reticulum, bind to the active site, help it fold properly, target it to the lysosome (where excess of substrate (the storage material) will compete with and dissociate the inhibitor) and lead to degradation of the storage material. In this regard it is encouraging that of all the many types of quantum dot coating tested, only a peptide containing the GDap(Pal)GVIKK sequence was able to be taken up by cells into compartments other than endosomes [18].

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