Neuroprotection and lifespan extension in Ppt1−/− mice by NtBuHA: therapeutic implications for INCL

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Infantile neuronal ceroid lipofuscinosis (INCL) is a devastating childhood neurodegenerative lysosomal storage disease (LSD) that has no effective treatment. It is caused by inactivating mutations in the palmitoyl-protein thioesterase-1 (PPT1) gene. PPT1 deficiency impairs the cleavage of thioester linkage in palmitoylated proteins (constituents of ceroid), preventing degradation by lysosomal hydrolases. Consequently, accumulation of lysosomal ceroid leads to INCL. Thioester linkage is cleaved by nucleophilic attack. Hydroxylamine, a potent nucleophilic cellular metabolite, may have therapeutic potential for INCL, but its toxicity precludes clinical application. We found that a hydroxylamine derivative, N-(tert-Butyl) hydroxylamine (NtBuHA), was non-toxic, cleaved thioester linkage in palmitoylated proteins and mediated lysosomal ceroid depletion in cultured cells from INCL patients. In Ppt1−/− mice, which mimic INCL, NtBuHA crossed the blood-brain barrier, depleted lysosomal ceroid, suppressed neuronal apoptosis, slowed neurological deterioration and extended lifespan. Our findings provide a proof of concept that thioesterase-mimetic and antioxidant small molecules such as NtBuHA are potential drug targets for thioesterase deficiency diseases such as INCL.

Although lysosomal storage diseases (LSDs) are individually rare, cumulatively they affect 1 in 5,000–7,500 live births1,2. Neurodegeneration is a devastating manifestation in the majority of the more than 50 LSDs. Neuronal ceroid-lipofuscinoses (NCLs), commonly known as Batten disease3, constitute a group of the most common (1 in 12,500 births) genetically distinct, neurodegenerative storage disorders, characterized by progressive psychomotor deterioration with onset around early childhood to adolescence. Mutations in eight different genes underlie various types of NCLs and several more NCL genes have been predicted to exist4. Autofluorescent cytoplasmic inclusion granules in neurons and in other cell types are associated with selective destruction of neurons in the brain and in the retina5. The NCLs as a group are broadly divided into two categories: one in which the gene mutations underlie a defect in a transmembrane protein and the other in which activities of soluble lysosomal enzymes are impaired6. Notably, the deficiency of two soluble lysosomal enzymes, cathepsin D and PPT1, underlies two of the most lethal NCLs: congenital NCL and INCL, respectively.

More than a decade ago, the purification and characterization of PPT1 from bovine brain substantially advanced our understanding of protein de-acylation7. PPT1 catalyzes the hydrolysis of the thioester linkage that attaches a 16-carbon fatty acid (predominantly palmitate) to cysteine residues in polypeptides. Subsequent molecular cloning and expression of bovine and rat PPT1 (ref. 8) facilitated further characterization and the demonstration that PPT1 is a lysosomal enzyme9,10. Previous linkage analysis localized the candidate locus for INCL to chromosome 1p32 (ref. 11), a region of chromosome 1 in which the PPT1 gene is colocalized. Finally, the identification of inactivating mutations in the PPT1 gene12 in INCL patients clearly established the molecular basis of this autosomal recessive neurodegenerative LSD. Children afflicted with INCL are normal at birth, but they develop psychomotor retardation by 11–18 months of age and are completely blind as a result of retinal degeneration by 2 years of age. By 4 years of age, these children manifest virtually no brain activity and they remain in a vegetative state for several more years before eventual death13. These grim facts underscore an urgent need for the development of rational and effective therapeutic strategies for this devastating childhood neurodegenerative LSD.

Palmitoylation (S-acylation) has emerged as an important, and the only reversible, post-translational lipid modification of proteins14,15. Thus, dynamic palmitoylation (palmitoylation-depalmitoylation) provides an important regulatory mechanism for the function of many proteins16,17. Although palmitoylation is critical for protein function, depalmitoylation is equally important for these proteins to recycle or undergo degradation by lysosomal hydrolases. PPT1 deficiency impairs degradation of palmitoylated proteins (constituents of ceroid) by lysosomal hydrolases and, consequently, abnormal ceroid accumulation in lysosomes is suggested to cause INCL pathogenesis18 (Supplementary Fig. 1). Because the thioester linkage is labile and nucleophilic attack cleaves this linkage19, we hypothesized that nucleophilic small molecules may have therapeutic potential for INCL. Hydroxylamine20, a potent nucleophilic metabolite present in all plants and animals21, cleaves thioester linkage with high potency and specificity14,15. Thus, hydroxylamine functionally mimics all thioesterases, including PPT1. However, hydroxylamine at a high concentration stimulates the production of methemoglobin, which, unlike hemoglobin, does not transport oxygen to the tissues. This toxicity precludes its clinical application.

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We rationalized that derivatives of hydroxylamine may be non-toxic while retaining the ability to cleave thioester linkage. We first screened a panel of 12 hydroxylamine derivatives and identified NtBuHA, which was non-toxic, cleaved thioester linkage in palmitoylated proteins, mediated depletion of lysosomal ceroid deposits and suppressed apoptosis in both cultured cells from INCL patients and in the brains of Ppt1−/− mice, which recapitulate virtually all clinical and pathological features of INCL. Moreover, we found that NtBuHA was neuroprotective and modestly, albeit substantially, extended lifespan in these mice without elevating methemoglobin levels. Our findings provide a proof of principle that non-toxic nucleophilic small molecules with antioxidant property, such as NtBuHA, are potential drug-targets for diseases caused by thioesterase deficiency, such as INCL.

RESULTS

Screening of hydroxylamine derivatives that mimic thioesterases

Thioester linkage in palmitoyl-CoA, as well as in palmitoylated proteins (constituents of ceroid) 18, is cleaved by hydroxylamine with high specificity 14,15,22. To determine whether hydroxylamine derivatives retain this property, we first screened a panel of 12 hydroxylamine derivatives (Supplementary Table 1) for their ability to cleave thioester linkage in [14C]palmitoyl-CoA. We used [14C]palmitoyl-CoA because it is a model substrate of thioesterases including PPT1 and mediates the release of free [14C]palmitic acid (Fig. 1a). As a positive control, we used the parent compound, hydroxylamine. We found that, like hydroxylamine, both water-soluble (Fig. 1b) and DMSO-soluble (Fig. 1c) hydroxylamine derivatives cleaved thioester linkage in [14C]palmitoyl-CoA, releasing free [14C]palmitate, albeit at varying degrees of efficiency. The densitometric quantitation of the released free [14C]palmitate bands in thin-layer chromatography (TLC) (Fig. 1b,c) confirmed these results.

To determine whether the hydroxylamine derivatives are non-toxic, we treated cultured fibroblasts from INCL patients with each of the 10 of 12 derivatives and evaluated their viability. We excluded N,N-dibenzylhydroxylamine and N-tert-butyl-O-[1-(4-(chloromethyl)phenyl)ethyl]-N-(2-methyl-1-phenylpropyl) hydroxylamine, as these derivatives were insoluble in tissue culture medium. We found that all of the tested compounds, except for N-benzoyl-N-phenyl hydroxylamine, were non-toxic at concentrations up to 1 mM (Supplementary Fig. 2).

NtBuHA is non-toxic to cultured cells from INCL patients

To evaluate whether NtBuHA is non-toxic, we cultured lymphoblasts from INCL patients in the absence and presence of varying concentrations of NtBuHA (0–5 mM) and determined the viability of the cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method 15. It should be noted that, although 1 mM NtBuHA was non-toxic to cultured INCL lymphoblasts, the viability of the cells gradually declined at higher concentrations (Fig. 1d). However, when these cells were cultured in medium containing 500 µM NtBuHA for varying lengths of time (12–60 h), cell viability was not altered (Supplementary Fig. 3a).

To further confirm these results, we tested the plating efficiency of the cells after treatment with NtBuHA at varying concentrations ranging from 0.1 to 2.5 mM. We found that the plating efficiency of the cells remained unaffected after treatment with NtBuHA at concentrations of up to 1 mM, and the plating efficiency slightly declined when the NtBuHA concentration was raised to 2.5 mM (Fig. 1e and Supplementary Fig. 3b). Taken together, these results indicate that NtBuHA is non-toxic to cultured cells from INCL patients at a wide range of concentrations.

NtBuHA cleaves thioester linkage in [14C]palmitoyl-CoA

We chose to study NtBuHA in further detail because a previous report indicated that this compound manifested potent mitochondrial antioxidant activity and prolonged longevity in normal mice and rats 24. These properties of NtBuHA are especially relevant because high levels of endoplasmic reticulum (ER) and oxidative stress have been reported to cause apoptosis in cultured cells from INCL patients, as well as in the neurons of Ppt1−/− mice 25, contributing to neurodegeneration 26–28 and shortened lifespan 25. Consistent with these findings, high levels of apoptosis have been reported in brain tissues of INCL patients 29. Thus, we sought to determine whether NtBuHA-mediated cleavage of thioester linkage in INCL cells is dose and time dependent. We incubated 0.1 µCi of [14C]palmitoyl-CoA with varying concentrations of NtBuHA (0–500 µM) and measured the levels of free [14C]palmitate. NtBuHA efficiently cleaved thioester linkage and released free [14C]palmitate in a dose-dependent manner (Supplementary Fig. 4a).

We then incubated 0.1 µCi of [14C]palmitoyl-CoA with 500 µM NtBuHA, which yielded the highest amount of free [14C]palmitate, and stopped the reaction at varying incubation times. NtBuHA mediated the cleavage of thioester linkage in [14C]palmitoyl-CoA and the release of free [14C]palmitate occurred in a time-dependent manner (Supplementary Fig. 4b). Taken together, these results suggest that NtBuHA efficiently cleaves thioester linkage in a dose- and time-dependent manner.

NtBuHA cleaves thioester linkage in palmitoylated proteins

To determine whether NtBuHA cleaves thioester linkage in palmitoylated proteins, we used cultured lymphoblasts from INCL patients labeled with [35S]cysteine, as previously reported 18. We found that, compared with the cells grown in medium without NtBuHA, cells grown in NtBuHA-containing medium for 24 and 48 h yielded appreciably less intense [35S]cysteine-labeled lipid thioester bands (Fig. 1f and Supplementary Fig. 5a). To confirm these results, we performed identical experiments using cultured lymphoblasts from nine different INCL patients who carried varying inactivating mutations in the PPT1 gene (Supplementary Table 2). Compared with the density of the [35S]cysteine-labeled lipid thioester bands from untreated INCL cells, those of the NtBuHA-treated counterparts yielded substantially less dense bands (Fig. 1g and Supplementary Fig. 5b).

The loss of intensity of [35S]cysteine-labeled lipid thioester bands in NtBuHA-treated cells also indicates that NtBuHA cleaved the thioester linkage in S-acylated proteins, much like its parent compound, hydroxylamine. As expected, the normal control cells (n = 4) exhibited extremely low levels of [35S]cysteine-labeled lipid thioester accumulation, unlike the more dense bands characteristically found in INCL cells (Fig. 1g). Collectively, these results indicate that NtBuHA cleaves thioester linkage in palmitoylated proteins in cultured cells from INCL patients in a dose- and time-dependent manner and mediates depletion of lipid thioesterases in these cells.

NtBuHA depletes granular osmiophilic deposits in INCL cells

A physical correlate of ceroid deposition in the cells of INCL patients is the presence of granular osmiophilic deposits (GRODs) 13. We sought to determine whether NtBuHA mediates the depletion of GRODs in INCL cells, which are detectable by transmission electron microscopy (TEM). Accordingly, we analyzed the levels of GRODs by TEM of INCL cells (Fig. 1g). Collectively, these results indicate that NtBuHA cleaves thioester linkage in palmitoylated proteins in cultured cells from INCL patients in a dose- and time-dependent manner and mediates depletion of lipid thioesterases in these cells.
decreased ($t_{77} = 7.61, P < 0.001$, permutation $t$ test) number of GRODs in NtBuHA-treated cells (Fig. 2b). The morphometric analysis of the GRODs revealed that the size of the GRODs, when present in NtBuHA-treated cells, was also significantly smaller ($t_{66} = 4.68, P < 0.001$ permutation $t$ test) than those of the untreated cells (Fig. 2c).

We also conducted similar experiments using cultured fibroblasts from INCL patients. We found that, compared with the untreated INCL fibroblasts, which contained numerous highly dense GRODs (Supplementary Fig. 6a), the NtBuHA-treated cells contained a markedly lower number of GRODs (Supplementary Fig. 6b,c). These results indicate that NtBuHA is effective at mediating the depletion of GRODs in both cultured lymphoblasts and fibroblasts from INCL patients.

**Normal serum methemoglobin in NtBuHA-treated Ppt1−/− mice**

Given that hydroxylamine stimulates methemoglobin production, we sought to determine whether the hydroxylamine derivative (NtBuHA) elevated serum methemoglobin levels in mice. Accordingly,
Figure 2  NtBuHA mediates the depletion of ceroid and GRODs in cultured INCL cells. (a) Electron micrographs of cultured lymphoblasts from INCL patients showing the intracellular accumulation of GRODs (inset) and the effect of NtBuHA treatment (250 μM, 3 weeks) on the accumulation of GRODs. Note that GRODs were completely depleted in most NtBuHA-treated cells (NtBuHA (1)), but a few cells contained GRODs (2). Scale bar represents 500 nm. (b) Box plot analysis of GRODs counted from the TEM in untreated (n = 23) and NtBuHA-treated (n = 56) cells (t_{22} = 7.61, P < 0.001, permutation t test). (c) Morphometric analysis of the GRODs in untreated (n = 47) and NtBuHA-treated (n = 21) cells (t_{66} = 4.68, P < 0.001, permutation t test). (d) Electron micrographs of cerebral cortices of 6-month-old wild-type mice (WT, left) and untreated (middle) and NtBuHA-treated Ppt1−/− mice (right). We found no GRODs in wild-type mouse cortices, whereas those of the untreated Ppt1−/− mice showed a very high level of GRODs. Cortices from NtBuHA-treated mice showed a significantly reduced number of GRODs compared with those of untreated mice (t_{66} = 2.89, P = 0.012, permutation t test). Scale bar represents 2 μm. (e) Quantitation of GRODs per cell in the brain sections from wild-type mice and untreated and NtBuHA-treated Ppt1−/− mice. Representative electron micrographs from 3–6 mice from each group were analyzed. (f) Autofluorescence visualized by dark field microscopic analysis of cortical tissue sections of 6-month-old wild-type mice (left) and untreated (middle) and NtBuHA-treated (right) Ppt1−/− mice. Densitometric analyses of autofluorescence in the brain tissues of untreated and NtBuHA-treated Ppt1−/− mice (g) revealed a significant decline in autofluorescence in treated mice (t_{28} = 11.78, P < 0.001, permutation t test). Scale bar represents 50 μm. Box-whisker plots represent the first three quartiles (25%, median and 75%) and values 1.5x the interquartile range below the first quartile (lower horizontal line) and above the third quartile (upper horizontal line).

we measured the blood methemoglobin levels in wild-type, untreated- and NtBuHA-treated Ppt1−/− mice. We found that treatment of Ppt1−/− mice with NtBuHA for up to 3 months did not lead to elevation of the methemoglobin levels (Table 1). We conclude that this derivative of hydroxylamine, at the dosages used in our experiments, is unlikely to have toxicity from the standpoint of increased methemoglobin levels.

We next sought to determine whether NtBuHA crosses the blood-brain barrier. Accordingly, we provided wild-type and Ppt1−/− mice with NtBuHA in their drinking water and determined the levels of NtBuHA in perfused brain tissues by multiple reaction-monitoring mass spectrometry, as previously reported. We found that NtBuHA, administered to 3- and 6-month-old Ppt1−/− and wild-type mice, was readily detectable in the brain (Table 2). These results suggest that orally administered NtBuHA is readily absorbed from the gastrointestinal tract and crosses the blood-brain barrier.

NtBuHA treatment depletes GRODs in Ppt1−/− mouse brain

To determine whether the beneficial effects of NtBuHA observed in cellulo are reproducible in vivo, we treated 3-month-old Ppt1−/− mice (n = 6) with NtBuHA continuously for 3 months. We tested these mice at 6 months of age, as this is the age at which they begin to manifest signs of neurological impairment and characteristic pathological features of INCL. Untreated age- and sex-matched Ppt1−/− mice (n = 6) served as controls. At the end of the treatment period, the mice were killed and the cortical tissues were examined by TEM. We found that, although we detected no GRODs in the wild-type mice, high levels of GRODs were readily detectable in the cortical tissues of untreated Ppt1−/− mice (Fig. 2d). Notably, the NtBuHA-treated Ppt1−/− mice showed a markedly reduced number of GRODs (Fig. 2d). Quantitation of the GRODs in the brain cells of untreated and NtBuHA-treated Ppt1−/− mice showed a significant reduction (t_{16} = 2.89, P = 0.012, permutation t test) in the number of the GRODs (Fig. 2e). These results indicate that NtBuHA-treatment mediates depletion of GRODs in the brains of Ppt1−/− mice.

Table 1  Serum methemoglobin levels in wild-type, untreated- and NtBuHA-treated Ppt1−/− mice

| Mice          | Treatment | Methemoglobin (% ± s.d.) | Number of mice |
|---------------|-----------|--------------------------|----------------|
| Wild type     | Untreated | 1.60 ± 0.69              | 8              |
|  Ppt1−/−      | Untreated | 1.50 ± 0.66              | 8              |
|  Ppt1−/−      | NtBuHA    | 1.59 ± 0.58              | 8              |
that the intensity of autofluorescence in NtBuHA-treated pretreated and co-treated INCL lymphoblasts were either untreated and INCL lymphoblasts. Top middle, NtBuHA pretreated. Top right, NtBuHA necrotic cells using propidium iodide (PI). Top left, untreated control

Table 2 NtBuHA levels in brain tissues of mice receiving NtBuHA in their drinking water

| Genotype of mice | Age     | NtBuHA in brain tissue (ng per mg of brain tissue ± s.d.) |
|------------------|---------|---------------------------------------------------------|
| Wild type        | 3 months| 4.44 ± 0.50 (n = 3)                                      |
| Ppt1−/−          | 3 months| 4.87 ± 0.25 (n = 3)                                      |
| Wild type        | 6 months| 4.73 ± 0.48 (n = 3)                                      |
| Ppt1−/−          | 6 months| 5.65 ± 0.51 (n = 3)                                      |

n represents the number of mice tested.

do the brain tissues of NtBuHA-treated Ppt1−/− mice for autofluorescence and compared them with untreated Ppt1−/− and wild-type mice. We found that, although the brain tissues from wild-type mice had no autofluorescence, those of the untreated Ppt1−/− mice manifested intense autofluorescence (Fig. 2f). Notably, NtBuHA-treated Ppt1−/− mice showed significantly reduced (t = 17.78, P < 0.001, permutation t test) autofluorescence (Fig. 2f). Quantitative analysis revealed that the intensity of autofluorescence in NtBuHA-treated Ppt1−/− mice was substantially lower (than that in untreated Ppt1−/− mice (Fig. 2g). Together, these results suggest that NtBuHA mediates the depletion of intracellular ceroid and decreases the number of GRODs per cell and the intensity of autofluorescence.

NtBuHA ameliorates ER and oxidative stress

Previously, we reported that ER and oxidative stress contribute to neuropathology in INCL26–28. Thus, we sought to determine whether NtBuHA treatment of Ppt1−/− mice ameliorates these stresses. We performed western blot analysis of proteins in brain lysates from untreated and NtBuHA-treated Ppt1−/− mice and determined the levels of the ER stress markers Grp-78, Grp-94 and ATF6. We found that the levels of all three of the ER stress markers were markedly reduced in the brain tissues of NtBuHA-treated Ppt1−/− mice compared with untreated mice (Supplementary Fig. 7).

We then performed western blot analysis of brain lysates from NtBuHA-treated and untreated Ppt1−/− mice to determine the levels of the oxidative stress markers superoxide dismutase-2 (SOD2) and catalase. The levels of these two enzymes increase in response to elevated oxidative stress to maintain cellular homeostasis, and a decrease in the levels of SOD2 and catalase can occur if a treatment is effective at reducing oxidative stress. We found that NtBuHA treatment substantially reduced the levels of both SOD2 and catalase (Supplementary Fig. 7). Taken together, these results indicate that NtBuHA treatment is effective at reducing both ER and oxidative stress in the brains of Ppt1−/− mice.

NtBuHA suppresses apoptosis in cultured INCL cells

Next, we sought to determine whether NtBuHA treatment protects cultured INCL lymphoblasts from apoptosis, which has been reported to be one of the major contributors of neuronal death in INCL patients29. Because PPT1 is ubiquitously expressed in all mammalian tissues and organs32,33, we used cultured cells from INCL patients in our in vitro experiments. Moreover, these cultured cells manifest pathological changes (that is, accumulation of GRODs and increased apoptosis) that are virtually identical to those found in postmortem brain tissues from INCL patients26–28. Accordingly, we pretreated cultured lymphoblasts from INCL patients with 1 mM NtBuHA for 12 h and then treated them with H2O2 (500 µM) in the presence or absence of NtBuHA (1 mM) for 3 h. As a control, we treated INCL lymphoblasts without NtBuHA pretreatment with the same concentration of hydrogen peroxide for 3 h.

Apoptosis was detected by annexin V staining and quantified by fluorescence-activated cell sorting (FACS) analysis. We found that H2O2 treatment elevated the levels of apoptosis in cells that were not pretreated with NtBuHA (Fig. 3a,b). However, in NtBuHA-pretreated lymphoblasts, H2O2 failed to induce apoptosis above the basal level (Fig. 3a,b). Similarly, cells that were first pretreated with NtBuHA and then incubated with H2O2 in presence of NtBuHA (co-treated) also showed no alteration in apoptosis above the basal level in these cells (Fig. 3a,b). Together, these results suggest that NtBuHA protects cultured INCL lymphoblasts from oxidative stress–mediated apoptosis.

Reduced apoptosis in NtBuHA-treated Ppt1−/− mouse brain

to determine whether NtBuHA treatment protects against neuronal apoptosis in vivo, we performed TUNEL assay using cortical tissue sections from 6-month-old wild-type mice and untreated and NtBuHA-treated Ppt1−/− mice. Although we found virtually no TUNEL-positive apoptotic cells in the brains of wild-type mice (Fig. 4a), there were numerous apoptotic cells in the brains of the untreated Ppt1−/− mice (Fig. 4b). In contrast, the brain tissues of

| Genotype of mice | Age     | NtBuHA in brain tissue (ng per mg of brain tissue ± s.d.) |
|------------------|---------|---------------------------------------------------------|
| Wild type        | 3 months| 4.44 ± 0.50 (n = 3)                                      |
| Ppt1−/−          | 3 months| 4.87 ± 0.25 (n = 3)                                      |
| Wild type        | 6 months| 4.73 ± 0.48 (n = 3)                                      |
| Ppt1−/−          | 6 months| 5.65 ± 0.51 (n = 3)                                      |
NtBuHA-treated Ppt1−/− mice showed significantly lower levels (t10 = 15.74, P < 0.001, permutation t test) of TUNEL-positive cells (Fig. 4c,d).

To further confirm these results, we performed western blot analysis of brain tissue lysates for two biochemical correlates of the oxidative stress–mediated mitochondrial pathway of apoptosis: cleaved caspase-9 and cleaved PARP-1. Compared with the brain tissues of wild-type mice, those of untreated Ppt1−/− mice showed significantly higher levels of both cleaved caspase-9 and cleaved PARP-1 (Fig. 4e–g). This suggests that NtBuHA protects brain neurons from apoptosis in an INCL mouse model.

NtBuHA retards progression of brain atrophy in Ppt1−/− mice

Elevated levels of apoptosis in the brain of Ppt1−/− mice26–28 and in INCL patients29 are suggested to cause brain atrophy. Thus, we determined the brain size and weight of untreated and NtBuHA-treated Ppt1−/− mice and wild-type mice. We found that the size and weight of the brains of untreated Ppt1−/− mice were markedly decreased compared with those of wild-type mice (Fig. 5a,b). In contrast, both of these parameters were markedly higher in NtBuHA-treated Ppt1−/− mice compared with those of the untreated Ppt1−/− mice (Fig. 5a,b).

To determine whether the brain size and weight correlated with neuron density, we performed immunohistochemical analyses of cortical tissue sections from wild-type mice and untreated and NtBuHA-treated Ppt1−/− mice using antibody to NeuN, a neuronal marker protein. Compared with the brains of wild-type mice, those of the untreated Ppt1−/− mice had a significantly reduced number (t6 = 21.8, P = 0.008, permutation t test) of NeuN-positive cells (Fig. 5c,d). In contrast, the brains of NtBuHA-treated Ppt1−/− mice showed a modestly, but significantly, higher (t6 = 8.17, P = 0.002, permutation t test) number of NeuN-positive cells than those of untreated Ppt1−/− mice (Fig. 5c,d).

To evaluate the degree of protection provided by NtBuHA in neurons from various cortical layers, we immunostained brain sections from wild-type mice and untreated and NtBuHA-treated Ppt1−/− mice using antibodies to Cux1 (cortical layers II-IV) and Ctip2 (cortical layers IV-VI). We then quantitated the Cux1- and Ctip2-positive neurons in these sections. Compared with wild-type mice, the numbers of Cux1- and Ctip2-positive neurons in the brains of Ppt1−/− mice were reduced to 53% and 35%, respectively. In contrast, the brains of the NtBuHA-treated Ppt1−/− mice showed a markedly increased number of both Cux1- and Ctip2-positive neurons in the mediolateral secondary visual cortex compared with those of the untreated Ppt1−/− mice (Supplementary Fig. 8). Given that one of the prominent neuropathological features of INCL is cortical atrophy, we also measured the cortical thickness in the mediolateral secondary visual cortex region (Supplementary Fig. 9a) of cresyl violet–stained brain sections from wild-type, untreated Ppt1−/− and NtBuHA-treated Ppt1−/− mice (Supplementary Fig. 9b). Quantitative analysis revealed a marked decline in cortical thickness in untreated Ppt1−/− mice compared with wild-type mice, whereas this decline was less pronounced in the NtBuHA-treated Ppt1−/− mice (Supplementary Fig. 9c). Cumulatively, these results provide supporting evidence that NtBuHA treatment has neuroprotective effects in the brains of Ppt1−/− mice.

Next, we sought to determine whether NtBuHA treatment of Ppt1−/− mice manifests any beneficial effect on astroglial activation, as assessed by higher levels of expression of glial fibrillary acidic protein (GFAP). Previous studies have found that increased GFAP expression precedes neuronal death in these mice34. We first performed western blot analysis of cortical lysates from wild-type mice and those of the untreated- and NtBuHA-treated Ppt1−/− mice using GFAP and NeuN antibodies, respectively. We found markedly higher levels of GFAP expression in the brain tissue lysates of the untreated Ppt1−/− mice compared with those of wild-type mice (Fig. 5e,f). However, the levels of NeuN were inversely proportional to those of the GFAP (Fig. 5e,g). Immunohistochemical analysis of cortical tissues corroborated the results of the western blot analyses (Fig. 5h,i). Compared with the untreated Ppt1−/− mice, the NtBuHA-treated Ppt1−/− mice showed significantly decreased GFAP levels (t6 = 5.19, P = 0.03, permutation t test), whereas NeuN levels were moderately elevated (Fig. 5e–i). These findings indicate that NtBuHA has beneficial effects in reducing the activation of astroglia and may be involved in preventing neurodegeneration in this mouse model of INCL.
NtBuHA preserves motor function and extends lifespan

Impaired motor coordination is one of the first signs of neurological deterioration in NiCl1 patients. Given that NtBuHA functionally mimics PPT1, we tested wild-type mice and untreated and NtBuHA-treated Ppt1−/− mice at 6 and 8 months of age for motor coordination using the rotarod performance test. We first performed the rotarod test using 3-month-old wild-type and Ppt1−/− mice because they do not manifest signs of motor deficit at this age. As expected, there were virtually no differences in performance between the wild-type and Ppt1−/− mice at all three speeds (4, 8 and 12 r.p.m. for 60 s; Supplementary Fig. 9d). We then repeated the same test using 6-month-old wild-type, untreated Ppt1−/− and NtBuHA-treated Ppt1−/− mice. Untreated 6-month-old Ppt1−/− mice manifested a clear motor deficit, whereas NtBuHA-treated mice had near-normal motor coordination (Fig. 6a–c and Supplementary Video 1). The rotarod test on 8-month-old wild-type mice and untreated and NtBuHA-treated Ppt1−/− mice yielded similar results (Fig. 6d).

To determine whether NtBuHA treatment of the Ppt1−/− mice improves their endurance, we repeated the rotarod test at the highest speed (12 r.p.m.) for 300 s instead of 60 s using wild-type mice and untreated and NtBuHA-treated Ppt1−/− mice. The endurance time of the wild-type mice was markedly higher than that of the untreated Ppt1−/− mice (wild type, 224 ± 84 s; untreated Ppt1−/−, 12 ± 6 s; Supplementary Fig. 9e). In contrast, the endurance time of the NtBuHA-treated Ppt1−/− mice was modestly longer than that of the untreated Ppt1−/− mice (untreated Ppt1−/−, 12 ± 6 s; NtBuHA-treated Ppt1−/−, 53 ± 24 s).

We also tested the Ppt1−/− mice for their exploratory behavior using the open field test. Compared with 6- and 8-month-old untreated Ppt1−/− mice, those treated with NtBuHA showed improved exploratory behavior (Fig. 6f). Taken together, these results suggest that NtBuHA treatment of Ppt1−/− mice not only cleaves thioester linkage in palmitoylated proteins, mediates depletion of ceroid, reduces ER and oxidative stress, and suppresses neuronal apoptosis, but also considerably slows the deterioration of motor coordination and exploratory behavior.

To determine whether the observed beneficial effects of NtBuHA treatment extended the lifespan of the mice, we divided 190 3-month-old Ppt1−/− mice into two groups: one group (n = 110) received no treatment (control) and the other group (n = 80) was treated with NtBuHA. The status of all mice was monitored on a daily basis and the mice were killed when they could not reach for food and water. The lifespan of NtBuHA-treated mice was extended compared with that of the untreated mice (Fig. 6g). The quantitative analysis confirmed...
that NiBuHA treatment conferred a significant extension of median survival time (untreated, 242 d; treated, 277 d; \( \chi^2(161, df = 1), P < 0.001 \)) and lengthened the maximum lifespan (untreated, 311 d; treated, 311 d). These results strongly suggest that NiBuHA treatment of Ppt1\(^{-/-}\) mice not only slows down the progression of neuropathology, but also modestly extends their lifespan.

**DISCUSSION**

We found that thioesterase-mimetic, nucleophilic small molecules with antioxidant properties, such as NiBuHA, cleaved thioester linkage in palmitoylated proteins (constituents of ceroid) with high specificity. We also found that this small molecule was non-toxic, mediated depletion of ceroid deposits and suppressed apoptosis in cultured cells from INCL patients. Furthermore, results obtained in cellulo were reproduced in vivo when Ppt1\(^{-/-}\) mice were treated with NiBuHA. Notably, in Ppt1\(^{-/-}\) mice, orally administered NiBuHA crossed the blood-brain barrier, did not stimulate methemoglobin production, mediated ceroid depletion, suppressed neuronal apoptosis, slowed neurological deterioration and caused a modest, albeit significant, extension of lifespan.

In the CNS, a large number of protein functions require dynamic palmitoylation.\(^{16,37}\) Although post-translational lipid modifications of proteins, such as palmitoylation, are critical in many biological processes, removal of the lipid moieties (depalmitoylation) from these lipid-modified proteins is also important for their recycling or degradation in lysosomes. Thus, genetic deficiency of the lysosomal thioesterase PPT1 leads to a devastating neurodegenerative LSD, INCL. Because children afflicted with INCL are normal at birth and disease manifestation does not occur before 11–18 months of age, there is a window of opportunity to develop and implement therapeutic interventions that arrest disease pathology.

To date, INCL is the only well-characterized neurodegenerative LSD caused by the deficiency of PPT1. Mammalian thioesterases are both cytosolic (APT1 and APT2)\(^{38,39}\) and lysosomal (PPT1 and PPT2)\(^{40,41}\), which catalyze the cleavage of thioester linkage in S-acylated (S-palmitoylated) proteins. Hydroxylamine cleaves thioester linkage with high specificity\(^{14,15}\), although it was not known whether its derivatives can cleave thioester linkage. We found that its derivatives, including NiBuHA, also cleave this linkage in S-acylated proteins and mediate ceroid depletion. However, although NiBuHA depalmitoylates S-acylated proteins, it is unlikely that it would be effective at depalmitoylating proteins in which the palmitate is linked to cysteine residues via amide linkage (N-palmitoylation).

In addition to its role in enhancing membrane affinity, palmitoylation promotes protein–protein interactions.\(^{14,15}\) A recent report suggested that palmitoylation-induced aggregation of mutant cysteine-string protein-\(\alpha\) (CSP\(\alpha\)), and possibly other proteins that undergo palmitoylation, may underlie adult-onset NCL.\(^{40}\) Thus, thioesterase-mimetic small molecules such as NiBuHA may ameliorate the pathogenesis of diseases caused not only by thioesterase
deficiency, but also those caused by the accumulation of aggregate-prone palmitoylated proteins. We previously reported that high levels of ER and oxidative stress lead to neuronal apoptosis in INCL cells and in Ppt1−/− mice26–28. It has been reported that NtBuHA has a potent antioxidant property24. We found that NtBuHA not only mediates depletion of intracellular ceroid, but also protects Ppt1-deficient cells from oxidative-stress-mediated apoptosis as a result of its anti-oxidant property. This may suggest that the neuroprotective effects of NtBuHA may stem not only from its ability to cleave thioester linkage in S-acylated proteins, but also from its anti-oxidant property. Given that NtBuHA functionally mimics all thioesterases, we propose that nucleophilic small molecules with antioxidant properties similar to that of NtBuHA may have therapeutic potential not only for INCL resulting from PPT1 deficiency, but also for as yet unrecognized diseases resulting from deficiencies of other thioesterases.

Although several therapeutic approaches are currently being used for the LSDs with systemic (visceral) involvement41, the development of effective treatment strategies for LSDs affecting the CNS remains challenging42. For INCL, several approaches are currently being used to develop an effective treatment strategy for this uniformly fatal disease43–48. However, delivering macromolecules to the brain as therapeutics for neurodegenerative diseases remains challenging. Consequently, most of the potential therapies face this challenge, although new strategies to overcome the obstacles are being tested49. We used a mechanistic approach to identify non-toxic, small molecules with antioxidant and thioesterase-mimetic properties that readily cross the blood-brain barrier. Our results indicate that NtBuHA fulfills at least some of the criteria required to be considered a potential drug target for INCL.

Despite the positive beneficial effects of NtBuHA, Ppt1−/− mice ultimately succumbed to INCL. Although we do not have a clear understanding of the mechanism of death of the NtBuHA-treated mice, there are several possibilities that may account for this outcome. One of these possibilities is that the time of initiation of NtBuHA treatment may be critical. The other possibility is that our NtBuHA dosage may not have been adequate to maintain the full therapeutic level in the brain. To the best of our knowledge, no off-target adverse effects of NtBuHA have been reported, but, although we uncovered no adverse effects of this small molecule, we are nonetheless mindful that, as with most drugs, NtBuHA may also have such adverse effects. Nevertheless, our findings provide a proof of concept that thioesterase-mimetic small molecules with antioxidant properties, such as NtBuHA, may offer a mechanism-based potential therapeutic option not only for INCL, but also for as yet unrecognized diseases that are caused by the deficiency of either cytosolic or lysosomal thioesterases.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.S. and G.C.C. designed and performed the majority of the experiments. S.P. performed genotyping of the mice, densitometric analyses, motor function and behavior testing, and analyzed the data. Z.Z. designed some of the experiments, performed data analysis and prepared the illustrations. A.L. provided statistical analyses of the data. A.B.M. conceived the project, designed some of the experiments and wrote major portions of the manuscript. All of the authors participated in the writing and editing of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents. All hydroxylamine derivatives and other reagents were purchased from Sigma. [14C]palmitoyl-CoA and [14C]palmitic acid were from Perkin Elmer and [35S]cysteine was purchased from MP Biomedicals.

Cell culture. Immortalized lymphoblasts from normal subjects and INCL patients were obtained from the laboratory of the late K.E. Wisniewski (Institute for Basic Research in Developmental Disabilities). PPT1-deficient fibroblasts were derived from skin biopsy samples from an INCL patient admitted to an ongoing clinical protocol (www.clinicaltrials.gov; NCT00028262, protocol #01-CH-0086), approved by the Institutional Review Board of the National Institute of Child Health and Human Development. This patient was homozygous for one of the most lethal PPT1 mutations (R122W). A list of the cell lines used and the PPT1 mutations they carried is provided in Supplementary Table 2. Fibroblasts were cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS, vol/vol), 2 mM glutamine, 100 U ml−1 penicillin and streptomycin at 37 °C in humidified atmosphere with 5% CO2. Lymphoblasts were cultured in RPMI supplemented with 16% FBS at 37 °C in humidified atmosphere with 5% CO2.

Mice. Ppt1−− mice (a generous gift from S.L. Hofmann, University of Texas Southwestern Medical Center) were generated by targeted disruption of the last exon in the Ppt1 gene in embryonic stem cells as previously reported25. These mice were subsequently backcrossed for ten generations with wild-type C57BL/6 mice to obtain congenic C57 background and a breeding pair was kindly given to us by M.S. Sands (Washington University School of Medicine) to start our Ppt1−− mouse colony at the National Institutes of Health. Both male and female mice were used in this study. Mice were housed and maintained in a pathogen-free facility under a 12-h light and 12-h dark cycle. Mice were provided with food and water ad libitum. All procedures were carried out under an animal protocol approved by the Animal Care and Use Committee of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. 3-month-old Ppt1−− mice were given NtBuHA in their drinking water (1 mM NtBuHA and 1 mM NaCl) as previously reported26.

Screening for hydroxylamine derivatives that cleave thioester linkage. To determine whether hydroxylamine derivatives, like their parent compound hydroxylamine, cleave thioester linkage, we used the model thioester substrate of PPT1, [14C]palmitoyl-CoA. We screened 12 hydroxylamine derivatives (Supplementary Table 1) using hydroxylamine (1 M) and recombinant human PPT1 (a generous gift from S.L. Hofmann) as controls. Briefly, we incubated 0.1 μM of [14C]palmitoyl-CoA with each of the 12 hydroxylamine derivatives (1 mM, pH 7.4) at 23–25 °C for 1 h. [14C]palmitate released was then extracted using a mixture of chloroform:methanol (1:1) and resolved by thin layer chromatography using a mixture of chloroform:methanol:water (65:25:4). As a control, [14C]palmitoyl-CoA was either treated with hydroxylamine or recombinant human PPT1.

MTT assay. Cell viability was determined according to a previously reported method21. Briefly, following treatment with hydroxylamine derivatives, INCL cells were incubated with MTT (Sigma) for 4 h at 37 °C. Formazan crystals formed this way were dissolved in acidiﬁed isopropanol and absorption was measured at 570 nm. Viability was expressed as percent of untreated control.

Plating efficiency assay. Plating efficiency was evaluated as described previously50. Briefly, after treatment with varying concentrations of NtBuHA (0–2.5 mM), INCL fibroblast cells were harvested and plated at a density of 100 cells per 50-mm tissue culture dish. 10 d later cells were fixed with 2.5% glutaraldehyde (vol/vol) and stained with 0.5% crystal violet (wt/vol). Plates were air dried. Crystal violet–positive cell colonies were then counted manually and plating efficiency was expressed as number of colonies per 100 cells plated.

Labeling cells with [35S]cysteine. Lymphoblasts were labeled with [35S]cysteine as previously described18 with minor modifications. Briefly, labeled cells were treated with 500 μM of NtBuHA at every 12 h for 48 h at 37 °C. Cells were then washed twice with ice-cold PBS and centrifuged at 2,250 g at 4 °C for 5 min. The pellet thus obtained was resuspended in PBS and lipid thioesters were extracted as described previously18.

Densitometric analyses of [35S]cysteine-labeled lipid thioester bands in TLCs. Intensities of the identiﬁable [35S]cysteine-labeled lipid thioester bands in autoradiographs of TLCs were quantified using QuantityOne software (Biorad). Band intensities from at least three different experiments were normalized and expressed as the intensity per mm2.

TEM. TEM of INCL lymphoblasts and fibroblasts was performed as previously described26. Briefly, cells were first treated with 250 μM NtBuHA for 3 weeks. Medium was replaced with fresh 250 μM NtBuHA at every 72 h. After treatment, cells were fixed with 2.5% glutaraldehyde in sodium phosphate buffer, washed three times with Millonig’s phosphate buffer and stained with 2.5% uranyl acetate (wt/vol). Ultra-thin sections were then prepared using AO Reichert ultracut ultramicrotome, stained with lead citrate and examined by using Zeiss EM10 CA. The number and size of the GRODs were determined by using Imagel (US National Institutes of Health).

For brain TEM, cortical tissue sections were prepared from wild-type mice and untreated and NtBuHA-treated Ppt1−− mice. Cortical tissues (approximately 1 mm3) were dissected from the mice, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and washed three times in 0.1 M sodium cacodylate buffer at 23–25 °C. Tissues were then post-fixed with 1% osmium tetroxide (vol/vol), dehydrated by sequential treatment of 50%, 70%, 90% and 100% ethanol, and treated with Spurr’s resin/ethanol using variable wattage Pelco BioWave Pro microwave oven (Ted Pella). Cortical tissues were embedded and polymerized in 100% resin for 18 h at 70 °C. Tissue sections (50 nm thick) were prepared using Reichert-Jung Ultracut-E ultramicrotome and collected on LuxFilm grids (Ted Pella) of 30-nm film thickness. The grids were then post-stained with uranyl acetate and lead citrate and examined in a FEI Tecnai G2 transmission electron microscope operating at 80 kV.

Detection of apoptosis. To evaluate whether NtBuHA protects against oxidative stress–induced apoptosis in INCL lymphoblasts, we first treated the cells with 1 mM NtBuHA for 12 h and then incubated with 500 μM H2O2 for 3 h in the presence or absence of NtBuHA (1 mM). Untreated INCL lymphoblasts (control) were also treated with H2O2 for 3 h. For the detection of apoptosis, the cells were stained with annexin V using Apoptosis detection kit (Biovision) and analyzed by FACS (Guava EasyCyte Mini System, Millipore).

TUNEL assay. To determine the level of apoptosis in the brain sections of mice, TUNEL assay was performed using TUNEL assay kit (Trevigen) as per manufacturer’s instruction.

Western blot analysis. Western blot analysis was performed as previously described26. Briefly 30 μg of protein were resolved in 4–12% Bis Tris gel (Invitrogen) and electro-transferred to PVDF membrane. The membranes were blocked with 5% non-fat dried milk (wt/vol) in Tris-buffered saline with Tween-20 (TBS-T) for 1 h at 23–25 °C and were then incubated overnight with primary antibodies at 4 °C. After washing three times, the blots were incubated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at 23–25 °C and developed using enhanced chemiluminescence detection reagents (Pierce). We used antibodies to ATF6 (Imagex, IMG-273, 1:200), SOD2 (Upstate, 06-984, 1:500), caspase-9 (Upstate, 05-572, 1:1,000), cleaved PARP-1 (BD Pharmingen, 51-9000017, 1:1,000), catalase (Abcam, ab16731, 1:2,000), GFAP (Upstate, 06-984, 1:500), caspase-9 (Upstate, 05-572, 1:1,000), cleaved PARP-1 (BD Pharmingen, 51-9000017, 1:1,000), catalase (Abcam, ab16731, 1:2,000), GFAP (Cell Signaling, 3670, 1:1,000), Grp78/Grp94 (Cell Signaling, 2104, 1:1,000), NeuN (Chemicon, MAB377, 1:1,000) and β-actin (Abcam, IMG-273, 1:200), SOD2 (Upstate, 06-984, 1:500), caspase-9 (Upstate, 05-572, 1:1,000), cleaved PARP-1 (BD Pharmingen, 51-9000017, 1:1,000), catalase (Abcam, ab16731, 1:2,000), GFAP (Cell Signaling, 3670, 1:1,000), Neun (Chemicon, MAB377, 1:1,000) and β-actin (US Biological, CA0760-40, 1:4,000). Densitometric quantitations were performed using QuantityOne software (Biorad).

Immunohistochemistry. The NtBuHA-treated and untreated mouse brain tissues were fixed in 3.7% paraformaldehyde (wt/vol), embedded in paraffin and processed for histological analyses. Briefly, after being treated with xylene and then successively with different concentration of ethanol in water (100% to 0%) tissue sections were incubated overnight in sodium citrate buffer at 95 °C. Sections were then blocked with 5% BSA (wt/vol) in PBS, probed overnight with antibodies to GFAP (Cell Signaling, 3670, 1:200) and NeuN (Chemicon, MAB377, 1:1,000). Sections were then washed three times with 1x phosphate-buffered saline (PBS), incubated with biotinylated antibody to mouse (1:500, BA-2000, Vector Laboratories) for 1 h at 23–25 °C followed by washing for three times with

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PBS and incubation with ABC complex according to the protocol provided by the manufacturer (Vector Laboratories). Cells were counted using SPOT Advanced Plus software, version 4.7 (Diagnostic Instruments).

**Immunofluorescence of neurons in various cortical layers.** 6-month-old wild-type, untreated Ppt1−/− and NtBuHA-treated Ppt1−/− mice were anesthetized and perfused with cold PBS followed by 4% paraformaldehyde. Brains were placed in the same fixative overnight at 4 °C and were frozen in 30% sucrose solution (wt/vol) in PBS. Frozen sections were permeabilized in 0.4% Triton X-100 (wt/vol), blocked in 5% BSA and incubated in primary antibodies overnight at 4 °C. We used antibodies to Cux1 (Santa Cruz Biotechnology, sc-13024, 1:100) and Ctip2 (Abcam, ab18465, 1:500). Sections were washed and incubated with Alexa Fluor–conjugated secondary antibodies (Invitrogen, A11008, 1:500; A21209, 1:2,000) at 23–25 °C for 1 h. Nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) and fluorescence was visualized using an LSM-510 inverted confocal microscope (Zeiss). The captured images were processed using the LSM Image Browser, version 4.2 (Zeiss).

**Cresyl violet staining.** 5-µm-thick brain sagittal sections from 6-month-old wild-type, untreated or NtBuHA-treated Ppt1−/− mice were deparaffinized, hydrated and stained with 0.5% cresyl violet solution. At least four comparable sections from each brain were used for measurement of cortical thickness and a similar area (secondary visual cortex mediolateral) for all the sections was used.

**Motor coordination performance test.** Motor coordination of the untreated and NtBuHA-treated Ppt1−/− mice was assessed using rotarod (UGO Basile) performance test35 at three different speeds (4, 8 and 12 r.p.m.). At all of these speeds, the direction of rotation was reversed every 15 s. To determine replicability, the tests were performed by two independent investigators, each of whom performed at least three separate experiments following identical protocols, and the results were compared to determine if they were replicable.

**Rotarod endurance test.** For testing endurance on rotarod, we used 6-month-old wild-type (n = 6), untreated Ppt1−/− (n = 8) and NtBuHA-treated Ppt1−/− (n = 7) mice. The mice were trained on rotarod at 12 r.p.m. (300 s d−1) for 3 consecutive days before performing the test. The direction of rotation was reversed every 15 s and the mice were allowed to stay on the rotating rod for up to 300 s. After 3 d of training, the mice were designated as group A (wild-type), group B (untreated Ppt1−/−) and group C (NtBuHA-treated Ppt1−/−). The recordings were conducted on day 4 by an operator who was unaware of the identity of the groups. For all experiments, the training and the rotarod test were conducted in the afternoon between 3–5 p.m.

**Open field test.** The exploratory behavior of untreated and NtBuHA-treated Ppt1−/− mice was evaluated by open field test as previously described56. These tests were performed by two independent investigators each of whom carried the identical protocol in at least three separate experiments and the results were compared to determine if they were replicable.

**Detection of NtBuHA in brain tissues by mass spectrometry.** Targeted detection of NtBuHA was performed using multiple reaction-monitoring mass spectrometry. For metabolite extraction, three volumes 50% chilled methanol (vol/vol) containing internal standards was added to the tissue sections in the Magna-Lyser tubes containing ceramic beads (Roche). The tissue samples were homogenized by spinning the tubes three times with a 10–30-s pulse in the Magna Lyser homogenizer (Roche) at 7,000 r.p.m. The supernatant was transferred to a fresh tube and an equal volume of chilled 100% acetonitrile (vol/vol) was added, vortexed and incubated on ice for 15 min. The tubes were centrifuged at 20,000 g at 4 °C for 15 min. The supernatant was transferred to a fresh tube and dried under vacuum and stored at −80 °C until mass spectrometry analysis. The samples were resolved on an Acquity UPLC BEH C18 1.7 µm, 2.1 × 100 mm column online with a triple quadruple mass spectrometer (Xeo-TQ, Waters) operating in the multiple reactions monitoring mode as previously described58.

**Determination of lifespan in untreated and NtBuHA-treated animals.** Both the untreated (n = 110) and NtBuHA-treated (n = 80) Ppt1−/− mice were used to evaluate longevity. When the mice were either dead or were unable to reach for food and water, they were killed according to the protocol. Kaplan-Meier analysis was performed to measure cumulative survival and log rank test was used to determine whether the median lifespan in NtBuHA-treated animals was significantly different from that of untreated counterparts.

**Statistical analysis.** All quantitative results including motor function and exploratory behavioral analyses were performed by investigators who were unaware of the genotype or the treatment of the respective mice. For in vivo experiments, the numbers reported (n) are biological replicates. To confirm the interassay reproducibility of the results, at least three independent experiments were performed. In some of the experiments, due to the relatively small sample sizes, two-sample permutation t tests were conducted to compare the independent groups and P < 0.05 was considered significant. All data are expressed as the mean of at least three independent experiments ± s.d., and are also summarized in the legend of each figure with box-whisker plots, along with P values for group comparisons. The results of lifespan studies were compared by Kaplan-Meier curves and log rank test.

50. Franken, N.A., Rodermond, H.M., Stap, J., Haverman, J. & van Bree, C. Clonogenic assay of cells in vitro. Nat. Protoc. 1, 2315–2319 (2006).