Glucosylceramide synthase inhibition alleviates aberrations in synucleinopathy models

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Mutations in the glucocerebrosidase gene (GBA) confer a heightened risk of developing Parkinson’s disease (PD) and other synucleinopathies, resulting in a lower age of onset and exacerbating disease progression. However, the precise mechanisms by which mutations in GBA increase PD risk and accelerate its progression remain unclear. Here, we investigated the merits of glucosylceramidase synthase (GCS) inhibition as a potential treatment for synucleinopathies. Two murine models of synucleinopathy (a Gaucher-related synucleinopathy model, GbaA53T/GbaA53T, and an A53T-α-synuclein overexpressing model harboring wild-type alleles of GBA, G3TSNCA mouse model) were exposed to a brain-penetrant GCS inhibitor, G2667161. Treatment of GbaA53T/GbaA53T mice with the GCS inhibitor reduced levels of glucosylceramide and glucosylsphingosine in the central nervous system (CNS), demonstrating target engagement. Remarkably, treatment with G2667161 slowed the accumulation of hippocampal aggregates of α-synuclein, ubiquitin, and tau, and improved the associated memory deficits. Similarly, prolonged treatment of A53T-SNCA mice with G2667161 reduced membrane-associated α-synuclein in the CNS and ameliorated cognitive deficits. The data support the contention that prolonged antagonism of GCS in the CNS can affect α-synuclein processing and improve behavioral outcomes. Hence, inhibition of GCS represents a disease-modifying therapeutic strategy for GBA-related synucleinopathies and conceivably for certain forms of sporadic disease.

Parkinson’s disease | GBA mutations | glucosylceramide synthase | Gaucher disease | Lewy body dementia

Significance

Mutations in the glucocerebrosidase gene (GBA) represent the most common genetic risk factor for Parkinson’s disease (PD), affecting 5–10% of patients and accelerating disease progression. Mutations in GBA and consequent loss of enzymatic activity allow glucocerebrosides to build up in cells. Here, we tested an experimental drug that inhibits the production of glucocerebrosides, hypothesizing that producing fewer lipids may counteract the challenge of clearing them from cells. In mice with mutant Gba, prolonged administration of this inhibitor reduced brain glucocerebroside levels. In addition, this treatment reduced levels of aggregated α-synuclein, a protein that builds up in PD brains, and improved behavioral responses. The findings suggest that reducing glucocerebrosides might represent a viable therapeutic strategy for PD patients carrying GBA mutations.


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Conflict of interest statement: S.P.S., C.V., J.C., C.M.T., A.M.R., H.P., M.A.O., J.C.D., J.M., E.M., B.W., S.H.C., and L.S.S. are employees of Sanofi. Freely available online through the PNAS open access option. 1To whom correspondence may be addressed. Email: richard_sidman@hms.harvard.edu or pablo.sardi@sanofi.com.

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features in vivo, despite the apparent lack of GlcCer accumulation. Sustained administration of an orally available inhibitor of GCS significantly decreased α-synuclein pathology and improved behavioral outcomes in two synucleinopathy models (i.e., Gba<sup>D409V/D409V</sup>, expressing mutant D409V glucocerebrosidase and endogenous α-synuclein; and A53T–SNCA mouse model, over-expressing A53T–α-synuclein and displaying endogenous wild-type murine glucocerebrosidase). The data indicate that brain-penetrant GCS antagonists can modulate α-synuclein homeostasis, thereby reducing the progression of synucleinopathies in mice with and without mutations in Gba.

**Results**

**Brain Penetrant GCS Inhibitor Reduces CNS Glycosphingolipids in Gba<sup>D409V/D409V</sup> Mice.** A potent and orally available inhibitor of GCS (GZ667161, Fig. 1A and B) with good CNS penetration has recently been shown to be effective at reducing the accumulation of glycolipid substrates in the brain and extending life span in a neuropathic murine model of GD (7). Based on these findings, we first tested the effects of GZ667161 in a mouse model of Gaucher-related synucleinopathy (Gba<sup>D409V/D409V</sup>) that presents with progressive accumulation of GlcSph and α-synuclein aggregates in the CNS as well as memory deficits (21). Two Gba<sup>D409V/D409V</sup> cohorts were studied (Fig. S1). In cohort 1 (presymptomatic), drug administration was initiated after Gba<sup>D409V/D409V</sup> pups were weaned at 4 wk of age and continued until killing at 10 mo of age. Gba<sup>D409V/D409V</sup> cohort 2 (postsymptomatic) was administered GZ667161 starting at 6 mo of age until killing at 13 mo of age. Mice were fed GZ667161 compounded in their diet (0.033% wt/wt) for the duration of the study; a control littermate group was fed the same diet lacking the small molecule drug. Similar to previous reports (21), Gba<sup>D409V/D409V</sup> presented no GlcCer accumulation in whole brain lysates compared with wild-type animals, despite displaying 20% residual glucocerebrosidase activity (Fig. 1C and D). Gba<sup>D409V/D409V</sup> cortical glucocerebrosidase activity was not affected by GZ667161 treatment (96 ± 5% of control Gba<sup>D409V/D409V</sup>, n = 11, P = 0.27). Importantly, animals administered the compounded diet exhibited reduced levels of GlcCer in the cerebral cortex (Fig. 1C and D). GZ667161 administration also reduced cortical GlcSph (Fig. 1E and F), another glucocerebrosidase-related lipid known to accumulate in Gba<sup>D409V/D409V</sup> mice (21). These results demonstrate the reduction of glucocerebrosidase substrate glycosphingolipids and confirm the CNS target engagement of the GCS inhibitor.

**GZ667161 Ameliorates Cognitive Impairment in the Gba-Related Synucleinopathy Mouse Model.** Mutations in GBA are now recognized as an independent risk factor for development of cognitive impairment in patients with PD (24–27). The Gba<sup>D409V/D409V</sup> mice present several distinct features of synucleinopathies, including cognitive impairment and pathogenic accumulation of α-synuclein, ubiquitin, and tau aggregates (21, 22). The assessment of memory function in Gba<sup>D409V/D409V</sup> mice treated with GZ667161 for 2 mo with the novel object recognition test revealed a modest but significant improvement in memory function (cohort 1, Fig. 2C). This improvement in cognitive function was confirmed in the same group of animals with another behavioral paradigm that involved placing the mice in a fear-conditioning chamber, exposing them to a noxious stimulus, and then testing the animals’ context-specific responses (freezing after 24 h. Gba<sup>D409V/D409V</sup> mice treated with GZ667161 for 8 mo showed a greater tendency to display a freezing response in the contextual fear test, indicating greater memory recall than that in the control group (Fig. 2B). Treatment of wild-type mice with GZ667161 had no effect on context-specific responses despite similar GlcCer reduction as in Gba<sup>D409V/D409V</sup> animals (Fig. S2), suggesting that GCS inhibition does not affect memory in animals with normal cognitive function.

To evaluate the therapeutic potential of GCS inhibition, we next tested whether the improvement in cognition could also be realized when administered at a clinically relevant postsymptomatic stage (cohort 2). Testing of 6-mo-old Gba<sup>D409V/D409V</sup> mice before treatment confirmed their contextual memory impairment (contextual freezing response, WT: 59 ± 3%, n = 10; Gba<sup>D409V/D409V</sup>, 24 ± 7%, n = 9, P < 0.01). The remainder of the Gba<sup>D409V/D409V</sup> littermates was randomly assigned to a control group (n = 12) or a GCS inhibitor treatment group (GZ667161, n = 11). Memory function was then evaluated through novel object recognition and contextual fear tests at 12 and 13 mo, respectively. Remarkably, treatment of Gba<sup>D409V/D409V</sup> mice with GZ667161 attenuated memory deficits as assessed with both cognitive tasks, whereas Gba<sup>D409V/D409V</sup> mice treated with the control diet showed no discernible improvement (Fig. 2C and D). Together, the results from these independent cohorts using two different cognitive assessments indicated that GCS inhibition and consequent modulation of lipid homeostasis could not only prevent the development of cognitive deficits but also reverse specific behavioral dysfunction in the mouse model of Gaucher-related synucleinopathy.

**GZ667161 Reduces Pathological Aggregate Accumulation in the Gba-Related Synucleinopathy Mouse Model.** Although the precise pathologies of PD and DLB remain unclear, the findings of progressive accumulation of α-synuclein and other proteins in LBs have implicated protein misfolding as a potential causative mechanism (28, 29). This proteinopathy is replicated in the
Brain Penetrant GCS Inhibitor Reduces CNS Glucosylceramide in a Synucleinopathy Model. To illustrate further the therapeutic potential of GCS inhibition to reduce the accumulation of misfolded α-synuclein aggregates, we next evaluated the efficacy of GZ667161 in A53T–SNCA mice, a well-characterized mouse model of synucleinopathy without mutations in Gba. A53T–SNCA mice express human A53T α-synuclein and carry wild-type alleles of Gba (30). Brains of A53T–SNCA mice exhibit lower glucocerebrosidase activity (~80% residual activity) and no apparent glycosphingolipid accumulation, similar to patients with PD expressing wild-type GBA alleles (11, 16). Transgenic mice were fed GZ667161 (compounded in their diet at 0.033% wt/wt) starting at 6 wk of age. Mice were killed at 8 mo of age, and the levels of glycosphingolipids were quantified by mass spectrometry. A53T–SNCA cortical glucocerebrosidase activity was not affected by GZ667161 treatment (102 ± 4% of control A53T–SNCA, n = 12, P = 0.37). Treatment with GZ667161 reduced the levels of GlcCer in the cerebral cortex, indicating that the GCS inhibitor effectively reduced the synthesis of this glycosphingolipid in the CNS of these animals (Fig. 4A).

GZ667161 Ameliorates Cognitive Impairment in a Synucleinopathy Mouse Model. Next, we examined the ability of GCS inhibition to influence cognitive function in A53T–SNCA mice. Animals were tested for novel object recognition initially at 3 mo post-treatment with GZ667161. A53T–SNCA mice treated with GZ667161 showed a trend toward an improved response in this test (Fig. 4B). This trend was confirmed when the mice were retested at a later time point using conditioned fear testing. A53T–SNCA mice treated with GZ667161 for 6.5 mo showed a significantly improved contextual response (Fig. 4C), which indicated that inhibition of GCS alleviated the aberrant cognitive response in a murine model of synucleinopathy.

GZ667161 Affects α-Synuclein Proteostasis in a Synucleinopathy Mouse Model. We then evaluated whether GCS inhibition would affect the subcellular localization of α-synuclein in A53T–SNCA mice. Previously, our group has shown that glucocerebrosidase augmentation can affect α-synuclein cellular distribution in A53T–SNCA mice. Cortical tissue homogenates from A53T–SNCA mice were subjected to serial fractionation to separate the cytosolic-soluble, membrane-associated, and cytosolic-insoluble forms of α-synuclein (18). No changes in the levels of cytosolic soluble α-synuclein were observed in the CNS of GZ667161-treated mice (Trion sol: 114 ± 8% of control, n = 14, P = 0.17, Fig. 5A). However, the levels of the membrane-associated and insoluble α-synuclein species were significantly decreased in response to GZ667161 treatment (Trion sol: 75 ± 8% of control, n = 14, P < 0.05; SDS soluble: 81 ± 3% of control, n = 14, P < 0.01, Fig. 5A), suggesting that the GZ667161-mediated reduction in glycosphingolipids affected the subcellular distribution of α-synuclein. Recent studies have demonstrated that alterations in lipid membrane composition can greatly affect the kinetics of α-synuclein membrane-induced aggregation (31, 32).

GZ667161 Reduces Pathological Aggregate Accumulation in a Synucleinopathy Mouse Model. Concordance of tau-, ubiquitin- and α-synuclein-associated pathology frequently occurs in patients with PD and DLB (33-35). We therefore evaluated the effect of GZ667161 on the accumulation of pathological aggregates in the A53T–SNCA mouse model by immunohistochemical morphometric analyses (21). Quantification of ubiquitin and tau aggregates in the mouse hippocampus confirmed the progressive accumulation of pathological aggregates during the 7-mo study (A53T–SNCA baseline vs. control, Fig. 5B and C). Also, immunohistochemical staining of hippocampal sections of A53T–SNCA animals for pathological and behavioral aberrations associated with GBA-mediated synucleinopathies.

**Fig. 2.** GCS inhibition ameliorates memory deficits in a mouse model of Gaucher-related synucleinopathy. Two independent randomized cohorts of Gba<sup>D409V/D409V</sup> mice received GZ667161 or control diet. Age-matched WT mice served as positive controls. (A) Cohort 1 mice were subjected to the novel object recognition test 2 mo after treatment initiation. None of the groups showed an object preference after exposure to two identical objects during training (clear white, blue, and red bars). After 24 h, the mice were presented with a novel object. In the testing trial (hatched bars), WT mice investigated the novel object significantly more (P < 0.01). Gba<sup>D409V/D409V</sup> mice (blue hatched bar) showed no preference for the novel object (cognitive impairment), and GZ667161-treated Gba<sup>D409V/D409V</sup> mice showed a modest but significant increase in investigation of the novel target (red hatched bars, P < 0.05). (B) The memory impairment of cohort 1 Gba<sup>D409V/D409V</sup> mice was corroborated by decreased freezing responses in the contextual fear conditioning test at 9 mo of age (blue bar, P < 0.05), whereas littermate mice treated with GZ667161 showed a better contextual memory response (red bar). (C and D) Cognitively impaired Gba<sup>D409V/D409V</sup> mice were randomized to receive GZ667161 (n = 15) or control diet (n = 14) from 6 to 13 mo of age (cohort 2, symtomatic). GZ667161 treatment attenuated the memory impairment in the novel object recognition and contextual fear tasks. The horizontal line demarcates 50% target investigations, which represents no preference for either object. The results are represented as means ± SEM, with n ≥ 10 per group (*P < 0.05; **P < 0.01).
ubiquitin and tau revealed a marked reduction in aggregate pathology in animals exposed to the GCS inhibitor GZ667161 (Fig. 5B and C). Immunostaining for proteinase K-resistant α-synuclein in A53T–SNCA mice failed to reveal a distinct signal, presumably due to the large amounts of exogenous human α-synuclein. Importantly, treatment with the GCS inhibitor reduced human α-synuclein levels in membrane-associated fractions (Fig. 5A), analogous to the effects on accumulation of mouse endogenous tau and ubiquitin aggregates (Fig. 5B and C). These results affirmed that a reduction in glycosphingolipids in the CNS limited the development of abnormal pathological inclusions in a synucleinopathy animal model expressing wild-type glucocerebrosidase.

Discussion
The link between synucleinopathies (including PD and DLB) and GBA mutations has been consolidated in recent years. It is now widely accepted that disease-segregating mutations in GBA not only increase the risk of PD and DBL but, more importantly, they accelerate the progression of these diseases (3–5, 14, 24–27). Although the precise mechanistic basis of GBA-mediated PD remains unknown, clinical and experimental evidence indicates that glucocerebrosidase haploinsufficiency, as a result of GBA mutations, can interfere with α-synuclein processing and contribute to the pathological accumulation of the protein (10, 15, 16). The present studies support this premise by demonstrating that a reduction in glucocerebrosidase-related lipids (through a brain-penetrant GCS antagonist) can improve behavioral and pathological defects in synucleinopathy mouse models. Patients who harbor GBA mutations present a faster deterioration of cognitive functions (25–27). Similarly, partial reduction in brain glucocerebrosidase activity can exacerbate cognitive deficits in animal models (16, 21). The GbaD409V/D409V mouse model recapitulates many of the aberrant biochemical characteristics noted in brains from patients with PD and DLB and also features measurable changes in GlcCer and cognitive functions.

Fig. 3. GCS inhibition reduces pathological aberrations in a mouse model of Gaucher-related synucleinopathy. Brain sections from WT and GbaD409V/D409V mice were stained for proteinase K-resistant α-synuclein (A), ubiquitin (B), and tau (C) aggregates. GZ667161 reduced aggregated protein levels of GbaD409V/D409V mice. The representative images (Right) show proteinase K-resistant α-synuclein immunoreactivity (A, red), ubiquitin (B, green), and tau (C, green) in the hippocampi of GZ667161-treated and control GbaD409V/D409V mice of cohort 1. DAPI-stained cell nuclei fluoresce blue. All data represent the mean ± SEM, with n ≥ 8 per group (*P < 0.05; **P < 0.01).

Fig. 4. GCS inhibition reduces GlcCer and affects cognition in the A53T–SNCA mouse model of synucleinopathy. A53T–SNCA mice were fed GZ667161 from 6 wk of age to 8 mo. Equivalent littermates were fed a control diet to monitor disease progression, and age-matched untreated WT mice were positive controls. (A) GZ667161 treatment reduced brain GlcCer in A53T–SNCA mice. (B) All mice subjected to the novel object recognition test at 4.5 mo of age showed no object preference after exposure to two identical objects during training (clear white, blue, and red bars). WT mice investigated the novel object significantly more frequently than A53T–SNCA mice (white hatched bars, P < 0.01), but A53T–SNCA mice (blue hatched bar) showed no such preference, indicating cognitive impairment. GZ667161-treated A53T–SNCA mice showed a nonsignificant trend toward cognitive improvement (red hatched bars, P = 0.11). (C) The memory impairment of A53T–SNCA mice was corroborated by a decreased freezing response in the contextual fear-conditioning test at 8 mo of age (blue bar, P < 0.05). Treatment of A53T–SNCA mice with GZ667161 significantly attenuated the contextual memory responses at this later time point (red bars, P < 0.05). Bars with different letters are significantly different from one another (P < 0.05). All data represent the mean ± SEM, with n ≥ 10 per group (*P < 0.05; **P < 0.01).
deficits in memory. Because few patients carrying GBA mutations will develop cognitive impairment, it was relevant to evaluate whether the salutary effects can also be realized in animals with overt disease. GCS inhibition in both early and late symptomatic Gba<sup>[D409V/D409V]</sup> mice was effective in reversing cognitive impairment. This recovery in cognition was associated with reduction in glycosphingolipid levels and a measurable decline in the accumulation of pathological aggregates. Remodeling of the neuronal glycosphingolipid topography may possibly improve lysosomal function, a requirement for correct synaptic activity and proper functioning of pathways that degrade aggregated proteins (31, 36, 37). These results strongly suggest that brain-penetrant GCS inhibitors may impede progression of (and perhaps even reverse) some aspects of Gaucher-related parkinsonism and associated synucleinopathies.

The pathological hallmark of PD and DLB is the accumulation of α-synuclein within Lewy bodies and neurites of the nervous system (38). α-Synuclein is an intrinsically disordered protein that has been implicated in vesicle trafficking and in synaptic plasticity of neurons (31). The interaction between α-synuclein and lipid surfaces is key to mediating its normal function, and alterations in the lipid membrane composition can trigger the formation of pathological amyloid fibrils (31, 39). For example, changes in the composition of sphingolipids have been reported to stabilize soluble oligomeric forms of isolated recombinant α-synuclein (13). Endogenously produced α-synuclein can also accumulate in induced pluripotent (iPS) cells from patients with PD who harbor homozygous or heterozygous GBA mutations, presumably because of the presence of increased levels of membrane glycosphingolipids (12, 13, 40). It appears reasonable, then, that persistent reduction in brain glycosphingolipids of the synucleinopathy animal slowed the accumulation of aggregated proteins (ubiquitin, α-synuclein, and tau), indicating the potential for the orally available brain-penetrant GCS inhibitor to modify disease progression in patients with PD carrying GBA mutations.

The precise mechanisms underlying PD pathogenesis are still unknown. Functional studies of PD-associated genes support a central pathogenic role of lysosomal pathways (41). GBA mutations result in reduced lysosomal glucocerebrosidase activity. However, it is unclear whether alternative lysosomal insults might affect glucocerebrosidase-related lipid accumulation and, therefore, be responsive to GCS inhibition. A number of patients with PD without mutations in GBA exhibit lower levels of CNS glucocerebrosidase activity, suggesting a role for this lysosomal enzyme in disease despite the lack of apparent lipid accumulation (9, 11). Similarly, the A53T–SNCA mice present reduced glucocerebrosidase activity and no evident glycosphingolipid accumulation (16, 22). Prolonged treatment with the GCS inhibitor improved behavioral endpoints, modulated α-synuclein homeostasis, and reduced the accumulation of ubiquitin and tau aggregates in this animal model expressing wild-type glucocerebrosidase, suggesting that the therapeutic benefit might also extend to patients displaying reduced glucocerebrosidase activity despite carrying wild-type GBA alleles.

GCS inhibitors have been proven safe and tolerable in the clinical setting (42). Translation of the present findings to the GBA-PD patient population will require identification of a prodromal or early symptomatic patient population (to restore function of impaired neurons before their demise) and potentially lengthy clinical trials (due to the slow and variable disease progression and current lack of disease progression biomarkers).

In summary, the present findings demonstrate that reducing the levels of CNS glycosphingolipids by antagonizing GCS corrected the hallmark pathological and functional phenotypes in two
animal models of synucleinopathy. Glucocerebrosidase deficiency [due to GBA4 mutations (1, 21) or α-synuclein–mediated inhibition (9, 13)] can lead to altered glycosphingolipid homeostasis, consequent α-synuclein misprocessing and intracellular deposition, generalized neuropathy, and the development of behavioral deficits. Reducing the levels of glycosphingolipids through GCS inhibition disrupted the pathogenic cycle of aberrant protein aggregation and functional deficits. Together, the data presented herein provide robust in vivo evidence supporting GCS inhibition as a novel disease-modifying therapeutic approach for GBA-related synucleinopathies, including PD, and support the advancement of GCS antagonists toward clinical testing.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee at Sanofi approved all procedures. Experimental details regarding animal use are provided in "Materials and Methods."

Administration of the Glucosylceramide Synthase Inhibitor GZ667161

A subset of animals received the glucosylceramide synthase inhibitor GZ667161 through their pelleted diet [0.033% (wt/wt)]. In each experimental cohort, sex and sibling were randomly matched for group assignment. GBA<sup>D409V/D409V</sup> cohort 1 drug administration was initiated after pups were weaned at 4 wk of age and was continued until killing at 10 mo of age. GBA<sup>D409V/D409V</sup> cohort 2 were administered GZ667161 starting at 6 mo of age until killing at 13 mo of age. AS3T–SNCA mice were exposed to the drug starting at 6 wk of age until killing at 8 mo.