**Introduction**

Gaucher disease (GD) is a lysosomal storage disease caused by a deficiency in acid β-glucosidase (GCase) activity, and subsequent pathological accumulation of the substrate glucosylceramide (GlcCer) in tissue macrophages. Regular infusion of recombinant human GCase (rhGCase; imiglucerase, Cerezyme®, Genzyme Corp.), termed enzyme replacement therapy (ERT), is the primary treatment for Gaucher disease. However, rhGCase has low physical stability (especially at neutral pH and body temperature), a short circulating half-life, and variable access and uptake into different disease-relevant tissues. We have shown previously that the small molecule pharmacological chaperone PC (AT2101; isofagmine bitrate) binds and stabilizes wild-type (WT) and GCase mutants N370S and L444P, resulting in more efficient cellular trafficking and increased cellular GCase levels, AT2101 also binds and stabilizes endogenous mcGCase (i.e., ERT), and may improve its pharmaceutical and pharmacological properties. Therefore, AT2101 may be useful alone (monotherapy) or in combination with rhGCase to treat GD. In this study, we investigated the effects of a second-generation PC, AT3375, on endogenous and exogenous GCase in vitro and in vivo. The data show that AT3375 increases the cellular levels of endogenous WT and mutant GCase with greater potency (EC50, EC50) than AT2101. The magnitude of the increase in GCase activity is the greatest in brain as a result of increased AT3375 potency combined with its improved BBB penetration and PK properties. Importantly, co-administration of AT3375 and AT2101 may improve the properties of rhGCase upon co-administration, thus warranting further investigations for its potential in the treatment of GD.

**1. AT3375 Increases GCase Activity ex vivo with Greater Potency compared to AT2101**

WT and Gaucher patient-derived lymphoblastoid cell lines (LCLs) were incubated with increasing concentrations of AT3375 or AT2101 for 6 days. Day 6, cells were washed twice with DPBS, and GCase activity was measured using a 4-MU assay (expressed as nanomoles of 4-MU liberated per mg protein per hour (nmol/mg protein/hr)). Overall, the EC50 values for AT3375 are consistently lower than those measured for AT2101 in WT and mutant cell lines, indicating that the potency of AT3375 was 2.5- to 10-fold greater than AT2101 in these LCLs.

**2. AT3375 Shows Improved BBB/PK Properties compared to AT2101**

Eight-week old C57BL/6 mice were orally administered AT2101 or AT3375 (30 mg/kg). Plasma and brains were collected as a function of time and PC levels were measured by LC-MS/MS. AT3375 shows higher plasma exposures and brain levels compared to AT2101, resulting in greater brain-plasma ratio.

**3. AT3375 Increases WT and L444P Brain GCase Activity with Greater Potency compared to AT2101**

Eight-week old C57BL/6 mice were orally administered AT2101 or AT3375 (0.1 - 1000 mg/kg) daily for 7 days. Brain tissues were collected 24 hours after the last dose for measurement of GCase activity. AT3375 shows 10-fold greater potency (ED50) than AT2101. In another experiment, eight-week old L444P mice were administered AT3375 (0.1 - 15 mg/kg per day) via drinking water for 28 days followed by a 24-hour washout. Brain samples (n=7) were analyzed for GCase activity using 4-MU and protein levels using Western blotting and IHC (with anti-human GCase). AT3375 increases L444P GCase activity and protein levels in brain (arrows) in a dose-dependent manner, and with greater potency (5-fold increase at 0.1 mg/kg) compared to AT2101 (2-fold increase at 10 mg/kg, inset), *p<0.05 compared to untreated L444P brain.

**4. AT3375 Reduces Epidermal Thickness of L444P Skin**

Eight-week old L444P mice were administered AT3375 via drinking water for 28 days followed by a 24-hour washout. Skin samples (n=7) were collected from the ventral side and stained with H&E. Epidermal thickness was measured using NIH-Elements software. AT3375 leads to significant reduction in epidermal thickening a hallmark of the L444P mouse model (*p<0.05 compared to untreated L444P skin).

**5. AT3375 Increases Visceral Tissue L444P GCase Activity in vivo**

Eight-week old L444P mice were administered AT3375 (0 or 0.1 mg/kg per day) or AT2101 (0 or 10 mg/kg per day) via drinking water for 28 days, followed by a 24-hour washout. Disease-relevant visceral tissues — liver, spleen and lung — were collected and assayed for GCase activity. AT3375 leads to greater tissue L444P GCase levels at a 100-fold lower dose than AT2101. IHC shows a robust increase in GCase signals in L444P liver (arrows) by AT3375; *p<0.05 compared to untreated L444P tissues.

**Conclusions**

- AT3375 increases WT GCase activity in LCLs with a 10-fold greater potency compared to AT2101.
- AT3375 increases mutant GCase activity in N370S and L444P LCLs with 2.5- and 3.8-fold greater potency, respectively, compared to AT2101.
- AT3375 has improved BBB penetration and PK properties leading to a greater increase in WT brain GCase levels compared to AT2101.
- In L444P mice, AT3375 increases mutant GCase levels in disease-relevant tissues including brain, skin, liver, spleen, and lung, with a potency 100-fold greater than AT2101. Additionally, AT3375 is able to reverse epidermal thickening in the skin of L444P mice.
- AT3375 and AT2101 increase the thermostability of GCase at neutral pH in vitro and prevent denaturation of GCase in whole blood, extending the enzyme half-life from <2 hours to greater than 24 hours.
- AT3375 and AT2101 increase the plasma half-life of GCase in vivo resulting in improved rhGCase tissue uptake compared to rhGCase alone.
- Overall, AT3375 is more potent than AT2101 in increasing WT and mutant endogenous GCase in cells and tissues, and improving the skin phenotype in L444P mice. AT3375 also increases the stability and cellular uptake of rhGCase when co-administered to rats. Collectively, these data suggest that AT3375 may be a more effective second-generation PC with improved properties compared to AT2101 and thus warrants further clinical investigation alone or in combination with rhGCase for Gaucher disease.

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**Exploring the Use of the Pharmacological Chaperone AT3375 Alone and in Combination with Recombinant Human Acid β-Glucosidase for Gaucher Disease**


The stability of mcGCase was measured as a function of temperature in an in vitro thermostability assay (TSA) that uses the environment-sensitive dye SYPRO Orange, which binds to exposed hydrophobic residues when proteins unfold. AT3375 and AT2101 significantly increase the stability of mcGCase at neutral pH, as evidenced by the shifting of Tr, from 50 °C to over 56 °C. The stabilizing effect is concentration-dependent. Administration of AT3375 and ERT to rats increases the circulating half-life tested at 37 °C in whole blood. AT3375 and AT2101 extend the Tr of mcGCase from less than 2 hours to well beyond 24 hours, indicating that both PCs could prevent rhGCase inactivation in blood.

**Sprague-Dawley rats (n=3/group) were orally administered AT2101 or AT3375 (3 mg/kg). rhGCase (30 U/kg) was delivered via bolus tail vein injection 30 minutes after PC. Blood was collected as a function of time for measurement of GCase activity and protein levels in plasma. Visceral tissues were collected 24 hours post-rhGCase for the measurement of enzyme uptake. Both AT3375 and AT2101 increase the rhGCase half-life in plasma by 2-fold and increase rhGCase uptake in disease-relevant tissues up to 2-fold (*p<0.05 compared to untreated tissues; #p<0.05 compared to tissues treated with rhGCase alone).**