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## Introduction

Fabry disease is an X-linked lysosomal storage disorder caused by inherited genetic mutations in  $\alpha$ -galactosidase A (GLA) which lead to reduced cellular enzyme activity. Consequently, lysosomal accumulation of the natural GLA substrate, globotriosylceramide (GL-3), occurs and contributes to disease pathology. Accumulation of misfolded GLA enzyme in the ER may lead to cellular stress, which may also contribute to cellular dysfunction and disease.

Male patients with classic Fabry disease have undetectable or very low GLA activity and relatively early disease onset. Later onset patients have low but measurable GLA activity and symptoms are generally milder. Heterozygous females who have inherited one mutant and one wild type allele have traditionally been thought of as carriers; however due to X-inactivation, females may also present with Fabry disease.

We and others have previously shown that the pharmacological chaperone AT1001 (migalastat hydrochloride) can increase the levels of mutant GLA in cultured cells and *in vivo*. Here we show that AT1001 also mediates a concentration-dependent and selective increase in wild type GLA levels in normal human cells. In addition, orally administered AT1001 mediates a dose-dependent and selective increase in GLA levels in tissues of wild type mice. Similarly, in a Phase 1 clinical study in healthy male volunteers, oral administration of AT1001 mediated an increase in GLA levels in white blood cells that persisted for 7 days after drug withdrawal.

To further explore the effect on mutant GLA, we measured the effects of AT1001 in Fabry R301Q transgenic mice. We show that enzyme levels are increased after oral delivery of AT1001 to these mice with a concomitant decrease in GL-3 substrate levels.

## Materials and Methods

**Cell Lines** Lymphoblast cell lines were cultured in suspension in T25 cm<sup>2</sup> flasks containing RPMI-1640 medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin at 37°C, 5% CO<sub>2</sub>. Fibroblast cell lines were cultured at 175 cm<sup>2</sup> tissue culture treated flasks containing DMEM medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin at 37°C, 8% CO<sub>2</sub>. Cell line names, genotypes, and sources are indicated in each of the Figure legends.

**Animals** Fabry R301Q Tg/KO mice were a gift from Dr. Robert Desnick. Male C57BL/6 mice were purchased from Taconic Farms (Germantown, NY) and housed in wire cages at 4 mice per cage. All studies were conducted at 8 weeks of age and conducted under strict adherence to IACUC guidelines.

***In vitro*  $\alpha$ -galactosidase A (GLA) enzyme assay** Human lymphoblasts and fibroblasts were seeded in sterile 96-well black plates (Costar) and incubated at 37°C, 5% or 8% CO<sub>2</sub>, respectively, for 3-6 hrs. Afterward, varying concentrations of AT1001, ranging from 1 nM to 5 nM were added, and cells were incubated for 5 days at 37°C, 5% or 8% CO<sub>2</sub>, respectively. Cells were then washed in fresh media and PBS. After washing, lymphoblasts of fibroblasts were lysed in 27 nM sodium citrate / 46 mM sodium phosphate dibasic, 0.2% Triton X-100, pH 6.8. Lysates were incubated with (4-MU)-D-galactopyranoside, 117 nM N-acetyl-D-galactosamine, GalNAc at 37°C for 1 hr. Afterward, 0.4 M glycine, pH 10.8, was added and fluorescence was read on a Victor plate reader (Perkin Elmer) at 355 nm excitation and 460 nm emission. Raw fluorescence counts were background subtracted, as defined by counts from substrate solution only. A Micro BCA Protein Assay Kit (Pierce) was used according to manufacturer's instructions to determine protein concentration from 20  $\mu$ L of cell lysate. 4-methylumbelliferone (4-MU) standard curve ranging from 30  $\mu$ M to 1.3 mM was run in parallel each day for conversion of fluorescence data to absolute enzyme activity expressed as nmoles / mg protein / hr.

***In vitro* glucocerebrosidase (GCase) enzyme assay** Gaucher patient fibroblasts were seeded in sterile 96-well black plates and incubated at 37°C, 8% CO<sub>2</sub> for 3-6 hrs. Varying concentrations of AT1001 (Tartaric or AT1001), ranging from 60 nM to 1.3 nM or 1 mM to 51 nM, respectively, were added and incubated for 5 days at 37°C, 8% CO<sub>2</sub>. Cells were then washed in complete growth medium followed by PBS. After washing, half of the samples were treated with 1M buffer alone (100 mM sodium citrate / 100 mM sodium phosphate dibasic, 0.25% Triton laurocholate, 0.1% Triton X-100, pH 5.2) and the other half were treated with 2.5 mM carbonyl beta epoxide (CBE) in 1M buffer. Plates were incubated for 45 min at 37°C. Subsequently, 4-methylumbelliferone (4-MU) standard curve ranging from 30  $\mu$ M to 1.3 mM was run in parallel each day for conversion of fluorescence data to absolute enzyme activity expressed as nmoles / mg protein / hr.

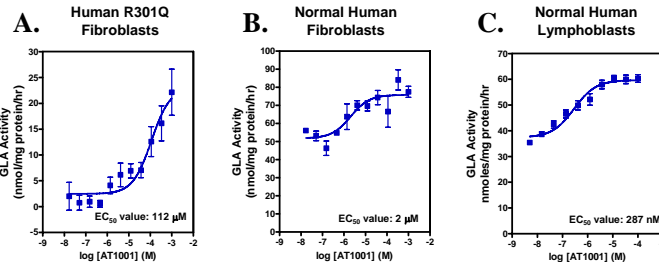
***In vitro* acid  $\alpha$ -glucosidase (GAA) enzyme assay** Pompe patient fibroblasts were seeded in sterile 96-well black plates and incubated at 37°C, 8% CO<sub>2</sub> for 3-6 hrs. Varying concentrations of AT2220 HCl or AT1001, ranging from 1 mM to 51 nM, were added and incubated for 5 days at 37°C, 8% CO<sub>2</sub>. After washing, 30  $\mu$ M citric acid, 40 mM sodium phosphate dibasic, pH 4.0, containing 4-MU-D-glucoside was added and plates incubated at 37°C for 3 hrs. Afterward, 0.4 M glycine, pH 10.8, was added and fluorescence was read on a Victor plate reader at 355 nm excitation and 460 nm emission. Raw fluorescence data were background subtracted and normalized to percent of basal fluorescence, and used to determine fold increase and EC<sub>50</sub>.

***In vivo* studies and enzyme assays from mouse tissues** For the study corresponding to Figures 3 and 4, four groups of 10 male C57BL/6 mice were dosed with 0, 1, 10 or 100 mg/kg/day AT1001 for 28 days. For the study corresponding to Figure 5, two groups of 6-7 male R301Q Tg/KO mice were dosed daily with 0 or 30 mg/kg AT1001 by oral gavage for 28 days. After dosing, indicated tissues were harvested and frozen. Tissue lysates were prepared by homogenizing tissue in GLA, GCase, or GAA assay buffer (same as detailed in sections above). Lysate was combined with the respective assay buffer and substrate solution. For the GCase assay, lysates were treated with and without CBE prior to addition of substrate. Reaction mixtures were then incubated at 37°C for 1 hr. Afterward, 0.4 M glycine, pH 10.8, was added and fluorescence was read on a Victor plate reader at 355 nm excitation and 460 nm emission. Enzyme activity in the lysates was background subtracted, and normalized for protein concentration using the MicroBCA Protein Assay Kit. 4-MU standard curve was run for conversion of fluorescence data to absolute enzyme activity expressed as nmoles / mg protein / hr or further normalized to % of untreated activity.

**GL-3 analysis from mouse tissues** Tissue samples were washed free of blood, weighed and homogenized with a solvent system in a FastPrep<sup>®</sup> system. Homogenate was then extracted using Solid Phase Extraction on a C18 cartridge. The eluent was evaporated and reconstituted prior to injection onto a LCMS system. Nine GL-3 isoforms were measured using positive ESI-MS/MS. LC separation was achieved on a Zorbax C18 column.

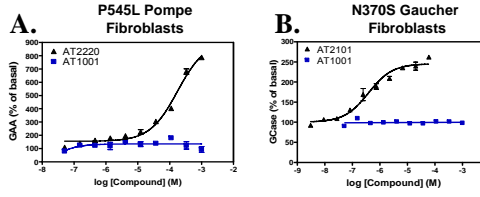
**Phase 1 clinical study and human white blood cell (WBC) GLA enzyme assay** This was a Phase 1, single-center, parallel, double-blind, placebo controlled, safety, tolerability, and pharmacokinetics of AT1001. The study consisted of 2 dose levels (50 mg and 150 mg) for 7 days. 6 clinically healthy volunteer subjects were allocated to each of the active treatment groups and 2 subjects to the placebo, with a 2-week safety evaluation period (from the first dose). For isolation of WBCs and enzyme assay, blood was drawn into an 8 mL Vacutainer CPT tube (Becton Dickinson) from each individual just prior to the first dose, and at 4, 6, and 14 days afterward. WBCs were immediately harvested according to the manufacturer's protocol, pelleted, and frozen at -80°C. Cell pellets were washed and lysed by sonication at room temperature in WBC lysis buffer (27 mM citric acid, 46 mM sodium phosphate dibasic, 0.5% Triton X-100, pH 6.8). Lysates were frozen on a dry ice/acetone bath, then stored at -80°C until use. To assay, 50  $\mu$ L of lysate was combined with 50  $\mu$ L of 117 nM sodium citrate, and 50  $\mu$ L of 5 mM 4-MU-D-galactopyranoside. Reaction mixtures were then incubated at 37°C for 1 hr. Afterward, 100  $\mu$ L of stop solution (0.2 M NaOH-glycine, pH 10.7) was added, and fluorescence was read on a Victor plate reader at 355 nm excitation and 460 nm emission. Enzyme activity in the lysates was background subtracted, and normalized for protein concentration using the MicroBCA Protein Assay Kit. 4-MU standard curve was run for conversion of fluorescence data to absolute enzyme activity expressed as nmoles / mg protein / hr. Data were normalized to the fraction of pre-dose enzyme activity.

**Figure 1. AT1001 Increases GLA Levels in Fabry (R301Q) Patient Fibroblasts and Normal Human Fibroblasts & Lymphoblasts**



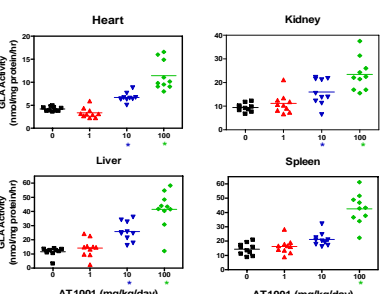
**Results:** Panel A. AT1001 treatment in culture medium for 5 days mediates a concentration-dependent increase in GLA levels of 8.2  $\pm$  2.8-fold (n=4), with an EC<sub>50</sub> value of 41  $\pm$  23  $\mu$ M (n=4) in R301Q patient-derived fibroblasts (DMN94.29; cells were a gift from Dr. Raphael Schiffmann). The data shown are representative of four separate experiments. Panel B. AT1001 treatment in culture medium for 5 days mediates a concentration-dependent increase in GLA levels of 1.5  $\pm$  0.14-fold (n=5), with an EC<sub>50</sub> value of 8.3  $\pm$  2.5  $\mu$ M (n=5) in normal human fibroblasts (CRL2003; Coriell). The data shown are representative of five separate experiments. Panel C. AT1001 treatment in culture medium for 5 days mediates a concentration-dependent increase in GLA levels of 1.9  $\pm$  0.3-fold (n=34), with an EC<sub>50</sub> value of 0.59  $\pm$  0.11  $\mu$ M (n=34) in normal human lymphoblasts (GM03201; Coriell). The data shown are representative of 34 separate experiments. Additionally, 10 other normal human lymphoblast lines were tested, and preliminary data yield comparable results.

**Figure 2: AT1001 is Selective for GLA *In Vitro***



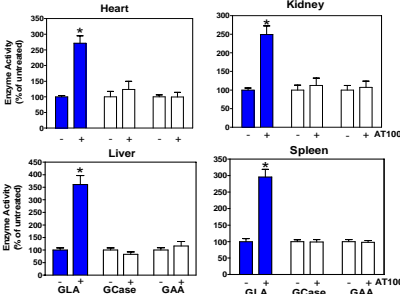
**Results:** Panel A. AT1001 treatment in culture medium for 5 days does not mediate a significant increase in P545L mutant acid  $\alpha$ -glucosidase (GAA) levels in Pompe patient-derived fibroblasts at any concentration tested (n=3; 84R9390; cells were a gift from Dr. W. J. Kleijer). In contrast, the positive control chaperone for this enzyme, AT2220 (1-deoxyxojymycin hydrochloride) significantly increases mutant GAA levels with a maximal magnitude of 6.4  $\pm$  0.3-fold (n=15), and an EC<sub>50</sub> value of 71  $\pm$  8  $\mu$ M (n=15) in these cells. The data shown are normalized to basal GAA activity (100%) and are representative of 3 separate experiments. Panel B. AT1001 treatment in culture medium for 5 days does not mediate a significant increase in N370S mutant glucocerebrosidase (GCase) levels in Gaucher patient-derived fibroblasts at any concentration tested (n=3; DMN89.45; cells were a gift from Dr. Raphael Schiffmann). However, the positive control chaperone for this enzyme, AT2101 (isofagomine tartrate) significantly increases mutant GCase levels with a maximal magnitude of 2.8  $\pm$  0.1-fold (n=3), and an EC<sub>50</sub> value of 0.6  $\pm$  0.1  $\mu$ M (n=3) in these cells. The data shown are normalized to basal GCase activity (100%) and are representative of 3 separate experiments.

**Figure 3. Orally-administered AT1001 Increases GLA Levels in Wild Type Mouse Tissues**



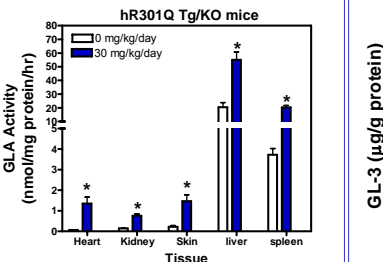
**Results:** Oral administration of AT1001 (0, 1, 10, or 100 mg/kg/day in drinking water; 28 days) to male C57BL/6 wild type mice increases GLA levels in heart, kidney, liver, and spleen with a maximum increase of 3.5  $\pm$  0.05, 3.3  $\pm$  0.6, 3.5  $\pm$  0.7, and 2.9  $\pm$  0.2-fold, respectively at 100 mg/kg/day. The levels were increased significantly, as determined by ANOVA analysis followed by Tukey's post-hoc analysis (p<0.05). The data shown are representative of two separate measurements.

**Figure 4. AT1001 is Selective for GLA *In Vivo***



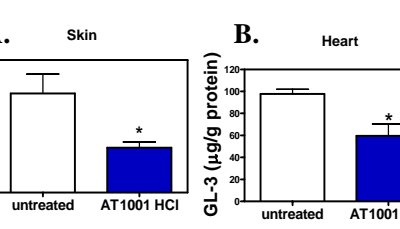
**Results:** Oral administration of AT1001 (100 mg/kg/day in drinking water; 28 days) to male C57BL/6 wild type mice significantly increases GLA levels in heart, kidney, liver, and spleen (3.5  $\pm$  0.05, 3.3  $\pm$  0.6, 3.5  $\pm$  0.7, and 2.9  $\pm$  0.2-fold, respectively; \*p<0.05), but does not affect GCase or GAA activity.

**Figure 6. Orally-administered AT1001 Increases GLA Levels in hR301Q Tg/KO Mouse Tissues**



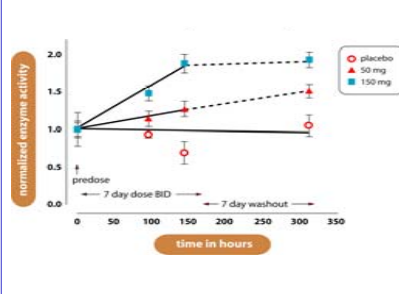
**Results:** Administration of AT1001 by daily oral gavage (30 mg/kg; 4 weeks) to male R301Q Tg/KO mice increases mutant GLA levels significantly (p<0.05, t-test, vs. untreated) in heart, kidney, skin, liver, and spleen tissues (11  $\pm$  0.1, 5.5  $\pm$  0.3, 6.2  $\pm$  0.7, 3  $\pm$  0.02, and 5  $\pm$  0.1-fold, respectively; n=6-7 mice for each tissue). The data shown are representative of two separate measurements. Two similar experiments were conducted in male and female R301Q Tg/KO mice orally administered AT1001 (30 mg/kg/day in drinking water; 4 weeks) and comparable increases in mutant GLA levels were observed in all treated tissues.

**Figure 7. AT1001 Reduces GL-3 Substrate Levels in hR301Q Tg/KO mice *In Vivo***



**Results:** Administration of AT1001 by daily oral gavage (30 mg/kg; 4 weeks) to male R301Q Tg/KO mice reduces GL-3 substrate levels (measured by LC-MS/MS) significantly in skin and heart. Panel A. AT1001 significantly reduced GL-3 levels after daily po dosing in skin tissue of treated R301Q Tg/KO mice (p<0.05; n=6-7 mice per group). Panel B. AT1001 significantly reduced GL-3 levels 1.6-fold after daily po dosing in heart tissue of treated R301Q Tg/KO mice (p<0.01; n=6-7 mice per group). GL-3 levels were also analyzed in kidney tissue and showed a trend towards reduction, but did not reach statistical significance.

**Figure 5. Orally-administered AT1001 Increases GLA Levels in Healthy Human Volunteer White Blood Cells**



**Results:** Oral administration of AT1001 to healthy human volunteers (50 and 150 mg twice daily for 7 days; n=6 for treatment groups; n=2 for placebo) resulted in a dose-related increase in GLA levels in white blood cells by up to 2-fold. No increase in GLA levels were observed from the placebo group. Elevated GLA levels persisted for 7 days after drug withdrawal. AT1001 was orally available and was generally well tolerated at all doses, with no serious adverse events occurring in any treatment group (data not shown).

## Summary & Conclusions

We have identified a small molecule pharmacological chaperone, AT1001 (migalastat hydrochloride), that increases the levels of wild type and mutant GLA *in vitro*, *in vivo*, and in healthy human volunteers.

- AT1001 is a potent inhibitor of wild type GLA (K<sub>i</sub> value: 30±2 nM, n=5; data not shown).
- AT1001 selectively increases GLA levels in wild type & R301Q Fabry patient cell lines *In vitro*.
- Oral delivery of AT1001 selectively increases GLA levels in tissues of wild type and R301Q Tg/KO mice *In vivo*.
- Oral delivery of AT1001 increases GLA levels in white blood cells of normal healthy human males. Elevated GLA levels persist for 7 days after drug withdrawal.

Oral delivery of AT1001 leads to significant reduction of GL-3 levels in skin and heart tissue of R301Q Tg/KO mice *In vivo* and shows a trend towards reduction in the kidney.

These data suggest that AT1001 merits further evaluation as a treatment for Fabry disease.

Currently, AT1001 is being evaluated in Phase 2 clinical trials for Fabry disease.