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Introduction

Fabry disease is an X-linked lysosomal storage disorder. It is caused by inherited genetic mutations in α -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. Patients with early-onset (classic) Fabry disease generally have undetectable or very low GLA activity. Later-onset patients usually have low but measurable GLA activity.

Native human GLA is a homodimeric glycoprotein. It is synthesized as a 429 amino acid precursor that is cleaved after the first 31 amino acids to produce the mature lysosomal form. Each mature monomer consists of two domains, a (β)₂-barrel containing the active site pocket, and an anti-parallel β -sheet. Over 400 mutations have been reported in Fabry patients, with ~60% being missense. These missense mutations span all 7 exons and have been identified in 67% of the residues of the full length protein.

We and others have shown that the pharmacological chaperone, AT1001 (migalastat HCl), can increase the levels of GLA in cultured cells and *in vivo*. Here we show that AT1001 increases the levels of mutant GLA in a significant proportion (>60%) of male Fabry patient lymphoblast cell lines representing more than 65 different missense mutations. Mapping of the responsive residues onto the protein structure of human GLA shows that they occur throughout both major structural domains.

To further understand the effect of AT1001 on mutant GLA and its activity *in vivo*, we measured its effects in Fabry R301Q transgenic mice. We show that GLA levels are increased after oral delivery of AT1001, with a concomitant decrease in GL-3 substrate levels in skin, heart and kidney.

Materials and Methods

Cell Lines. Lymphoblast cell lines were cultured in suspension in T25 cm² flasks containing RPMI-1640 medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin at 37°C, 5% CO₂. Three Fabry patient lymphoblast cell lines were provided and attached in T75 cm² tissue culture treated flasks containing DMEM supplemented with 10% fetal calf serum and 1% penicillin-streptomycin at 37°C, 5% CO₂. Three Fabry patient lymphoblast lines (G218E, R301Q, and R301Q) were established at Amicus Therapeutics from blood obtained from clinical investigations. Sixty-eight Fabry patient lymphoblast lines were privately donated. All cell lines were obtained from donors who provided consent for their materials to be used for research purposes.

Animals. Fabry R301Q Tg/KO mice were a gift from Dr. Robert Desnick. Male mice were housed in polysulfone individually ventilated cages with ¹²h light/dark cycle bedding (5:55 a.m. to 5:00 p.m. cage). All studies were conducted at 8 weeks of age under strict adherence to IACUC guidelines.

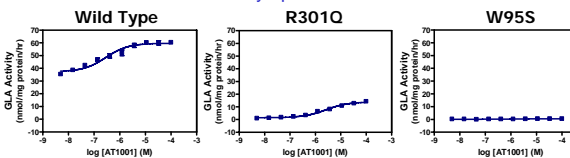
In vitro α -galactosidase A (GLA) enzyme assay. Human lymphoblasts were seeded in sterile clear-bottom 96-well black plates (Costar) at 25,000 cells/well, and incubated at 37°C, 5% CO₂ for 3-6 hrs. Varying concentrations of AT1001, ranging from 1 nM to 5 μ M, were then added, and cells were treated for 5 days at 37°C, 5% CO₂. Cells were washed 3 times with fresh media, then incubated in fresh media for 3 hr at 37°C. After washing 2 times in fresh PBS, lymphoblasts were lysed in 40 μ l of lysis buffer (27 mM sodium citrate, 0.25 M sodium phosphate dibasic, 0.2% Triton X-100, pH 4.6). Ten μ l lysate were added to 50 μ l assay buffer (Lysis Buffer without Triton X-100, but containing 6 mM 4-MU- α -D-galactosylpyruvate (4-MU) and 117 mM NaCl). 4-MU- α -D-galactosylpyruvate (4-MU) was prepared by adding 4-MU-galactosamine (GalNAc), and incubated for 1 hr at 37°C, 70 μ l Stop Solution (0.4 M glycine, pH 10) were then added and fluorescence 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 MicroBCA Protein Assay Kit (Pierce) was used according to manufacturer's instructions to determine protein concentration from 20 μ l of cell lysate. A 4-methylumbelliferone (4-MU) standard curve ranging from 30 μ M to 1.3 nM was run in parallel each day for conversion of fluorescence data to absolute GLA activity expressed as nmol / mg protein / hr or further normalized to % of untreated wild type enzyme activity.

Mapping of GLA unrelated residues responsive to AT1001. The X-ray crystal structure of human GLA (PDB: 1R47) determined by Garman and Garbocki (J. Mol. Biol. 337:319-335, 2004) was used to map GLA missense mutations responsive to AT1001 treatment. The structures were generated with SWISS-PDB viewer and rendered with POV-Ray (Persistence of Vision) Render 3.6.6.

In vivo studies and enzyme assays from mouse tissues. Five groups of male R301Q Tg/KO mice were dosed with 0, 10, 30, 100 or 300 mg/kg/day AT1001 in drinking water for 28 days. After dosing, indented tissues were harvested and frozen. Tissue lysates were prepared by homogenizing ~50 mg tissue in Lysis Buffer (see above). 20 μ l lysate were mixed with 50 μ l of substrate (as detailed above). Reaction mixtures were incubated at 37°C for 1 hr. After 70 μ l Stop Solution were added and fluorescence was read on a Victor plate reader as described above. Enzyme activity in the lysates was background subtracted, and normalized for protein concentration using a BCA standard. Assays were run for conversion of fluorescence data to absolute GLA activity expressed as nmol / mg protein / hr.

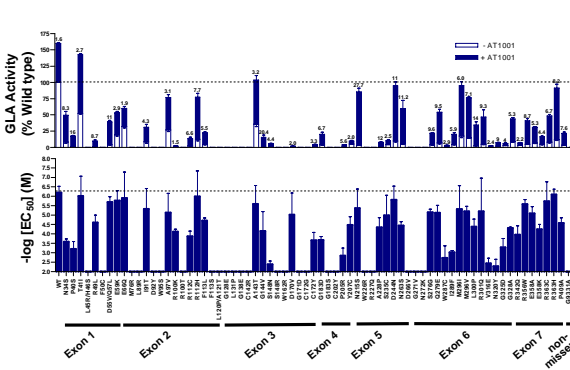
GL-3 analysis from mouse tissues. Tissue samples were washed free of blood, weighed and homogenized with a 200 μ l volume of RNeasy lysis buffer. Homogenate was then extracted using Solid Phase Extraction on a C18 cartridge. The eluent was evaporated and reconstituted prior to injection onto a LCMS system. Nine GL-3 standards were measured using positive ESI-MS/MS. LC separation was achieved on a Zorbax C18 column.

Figure 1. AT1001 Increases GLA Levels in Normal and Fabry Lymphoblasts



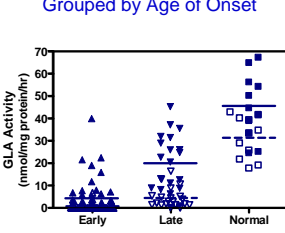
Results: GLA activity (expressed as nmol/mg protein/hr of 4-MU released) was measured in lysates prepared from lymphoblasts incubated with varying concentrations of AT1001. These data show that AT1001 increased the levels of wild type (left panel) and R301Q mutant (middle panel) GLA from normal human and Fabry patient-derived lymphoblasts, but mediated no effect in W95S (right panel) lymphoblasts. Data points are the mean \pm SEM of quadruplicate determinations. In the experiments shown above, GLA levels were increased 1.9-fold and 10.7-fold with EC₅₀ values of 0.3 μ M and 2.6 μ M for the wild type and R301Q, respectively. GLA levels were not increased at any AT1001 concentration in W95S lymphoblasts. The data shown are representative of 3 (wild type), 4 (R301Q) and 3 (W95S) independent experiments, respectively (see Table 1).

Figure 2. Responses to AT1001 in Male Patient Lymphoblasts



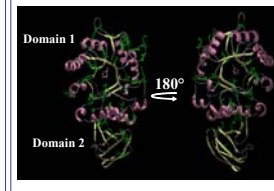
Results: Male Fabry patient-derived lymphoblasts representing 71 different genotypes (69 missense, 1 splice site, and 1 frameshift) were profiled for magnitude and potency of GLA increase in response to treatment with varying concentrations of AT1001. **Top panel.** The average baseline (empty bars) and maximally increased (filled bars) GLA activity in the absence or presence of AT1001 are shown. Data are normalized to percentage of GLA activity in untreated wild type lymphoblasts run in parallel. The dotted line represents the wild type (WT) GLA activity level. The number at the top of each bar represents the average relative increase in GLA activity over baseline for each cell line. Cell lines with no bar associated showed no detectable baseline GLA activity, nor any response to AT1001 at the concentrations tested. **Bottom panel.** The average potency of AT1001 for mediating an increase in GLA activity for every cell line is shown. Potency is expressed as the negative log of the concentration at which 50% of the maximum effect is achieved (EC₅₀). The dotted line represents the potency of the wild type control (log[EC₅₀] = 6.1 or 760 nM). **Log[EC₅₀]** is represented by bars with lower -log[EC₅₀] and higher potency to AT1001 was represented by bars with higher -log[EC₅₀]. Cell lines with no bar associated indicated that no response to AT1001 was measured. For both panels, bars represent the mean \pm SEM of at least 3 independent determinations (see Table 1 for the exact values and number of independent experiments determined for each cell line). **Broken horizontal line.** The exons where the mutations occur in the gene are indicated. Missense genotypes are shown in order from N-terminus to C-terminus of the protein. Wild type (WT) is shown at the far left and non-missense mutations are shown at the far right.

Figure 3. Responses to AT1001 Grouped by Age of Onset



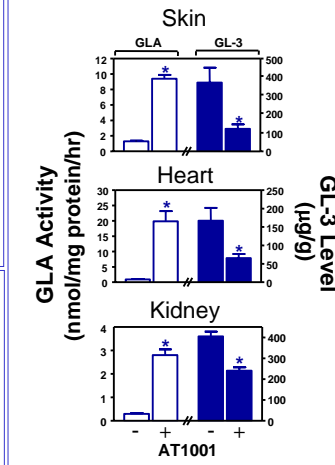
Results: GLA activity was measured in lysates prepared from male Fabry patient-derived lymphoblasts of those of normal males or females incubated in the absence or presence of AT1001 and grouped based on approximate age of disease onset. Open symbols represent baseline GLA activity, with the average for each group denoted by dotted lines. Filled symbols represent maximal GLA activity after AT1001 treatment, with the average for each group denoted by solid lines.

Figure 4. Responsive Mutations Mapped onto the GLA Protein Structure



Results: Residues mutated in Fabry disease and responsive to AT1001 treatment were mapped onto the structure of the GLA monomer (PDB:1R47, Garman and Garbocki, J. Mol. Biol. 337:319-335, 2004). The residues depicted in gray, when mutated, were responsive to AT1001 treatment (see Figure 2 and Table 1). The galactose ligand is shown bound in the active site.

Figure 5. AT1001 Reduces GL-3 Levels in R301Q GLA Tg/KO Mice



Results: Administration of AT1001 *ad libitum* in drinking water (100 mg/kg/day; 4 weeks) to male R301Q GLA Tg/KO mice increases mutant GLA levels, as measured by activity in tissue lysates, and reduces GL-3 substrate levels (measured by LC-MS/MS) significantly in skin, heart, and kidney (***p<0.05, t-test, vs. untreated, n=7 mice per group). The relative increases in GLA levels were 7.3-, 21.5-, and 9.3-fold in skin, heart, and kidney, respectively. GL-3 levels were decreased by 67%, 61%, and 41% in skin, heart, and kidney, respectively. In this study, four doses of AT1001 were tested (10, 30, 100, and 300 mg/kg/day), and all showed significant increases in GLA levels and significant decreases in GL-3 levels.

Summary & Conclusions

- The small molecule pharmacological chaperone AT1001 (migalastat HCl) increases the levels of mutant GLA *in vitro* in a significant proportion of cells derived from Fabry patients with different genotypes.
 - 45 of 69 different missense genotypes responded to AT1001.
 - Magnitude and potency of response varied with respect to genotype.
 - A majority of cell lines associated with an early-onset phenotype responded.
 - A high majority of cell lines associated with a later-onset phenotype responded.
- Mapping of the responsive residues onto the protein structure of human GLA shows that they occur throughout both major structural domains.
- Oral delivery of AT1001 to R301Q GLA Tg/KO mice, an *in vivo* model of Fabry disease, increases GLA levels and reduces GL-3 substrate levels in Fabry disease-relevant tissues.
 - Significant increases in GLA levels were observed in skin, heart, and kidney.
 - Significant decreases in GL-3 substrate levels were observed in skin, heart, and kidney.
- These data indicate that AT1001 merits further evaluation as a treatment for Fabry disease.
- Currently, AT1001 is being evaluated in Phase 2 clinical trials for Fabry disease.

Table 1. Fabry Lymphoblast Cell Line Screening Summary

Genotype	Onset	Baseline Value \pm SEM; (n, WT)	Relative Increase \pm SEM	EC ₅₀ \pm SEM (n) μ M	n	Genotype	Onset	Baseline Value \pm SEM; (n, WT)	Relative Increase \pm SEM	EC ₅₀ \pm SEM (n) μ M	n	Genotype	Onset	Baseline Value \pm SEM; (n, WT)	Relative Increase \pm SEM	EC ₅₀ \pm SEM (n) μ M	n	
Wildtype	Unaffected	35 \pm 2 (100/0)	1.6 \pm 0.01**	0.8 \pm 0.1	30	L311P	Early	0 \pm 0 (0/0)	-	-	2	D266V	Early	0 \pm 0 (0/0)	-	-	2	
R301Q	Early	18 \pm 0.8 (81)	0.8 \pm 0.2**	369 \pm 22	2	G218E	Early	0 \pm 0 (0/0)	-	-	2	G271R	Early	0 \pm 0 (0/0)	-	-	2	
F40S	Early	0.5 \pm 0.1 (5)	16.7**	648 \pm 140	1	N272R	Early	0 \pm 0 (0/0)	-	-	6	S275R	Early	0 \pm 0 (0/0)	-	-	4	
T41I	Late	16.2 \pm (52)	2.7 \pm 0.04**	9.8 \pm 0.0	3	A414T	Late	11 \pm (2)	3.2 \pm 0.3**	2.6 \pm 1	6	S276G	Early	18.0 \pm (40)	6.7 \pm 2.0**	7.2 \pm 0.5	4	
L48R/H48E	Early	0 \pm 0 (0/0)	-	-	3	G144V	Early	0.1 \pm 0.1 (3)	20.9**	71 \pm 31	1	G278E	Late	27.1 \pm (6)	9.5 \pm 2.2**	7.5 \pm 1.0	1	
R49L	NA	0.4 \pm 0.1 (2)	8.7 \pm 2.3*	25 \pm 4	3	S148H	Early	0.8 \pm 0.3 (8)	4.4 \pm 1.7**	4100 \pm 60	4	W292C	Early	0.2 \pm 0.1 (6)	3.5 \pm 0.6**	1900 \pm 900	5	
E26K	Early	0 \pm 0 (0/0)	-	-	1	S168H	Early	0 \pm 0 (0/0)	-	-	2	D296E	Early	14.0 \pm (14)	37.0 \pm 6.0**	5200 \pm 700	1	
D55V/Q57L	NA	1.4 \pm 0.2 (5)	11 \pm ***	2 \pm 0.2	5	W162G	Early	0 \pm 0 (0/0)	-	-	6	M294L	Late	5.4 \pm (27)	5.9 \pm 0.7**	4.9 \pm 2.3	4	
E59K	Early	6.8 \pm 0.5 (19)	2.9 \pm 0.4**	17 \pm 0.3	3	D179V	Early	0.4 \pm 0.1 (2)	2.0 \pm 0.7*	9.5 \pm 4.4	4	M295V	Late	5.8 \pm 0.5 (17)	5.6 \pm 0.4**	5.7 \pm 0.8	30	
I66Q	Late	11 \pm (2)	19.9 \pm 1.1*	12 \pm 0.5	3	G171D	Early	0 \pm 0 (0/0)	-	-	2	L300P	Early	19.0 \pm (25)	14.3 \pm 3*	41 \pm 7	3	
M78R	Early	0 \pm 0 (0/0)	-	-	3	C172I	Early	0 \pm 0 (0/0)	-	-	2	R301Q	Late	19 \pm 0.1 (45)	102 \pm 18**	9.0 \pm 2.2	4	
L30R	Early	0 \pm 0 (0/0)	-	-	3	C172Y	Early	0.6 \pm 0.3 (26)	3.3 \pm 0.9**	220 \pm 70	3	V164L	Late	0 \pm 0 (0/0)	-	-	300 \pm 180	4
D17V	Late	3.1 \pm (4)	4.3 \pm 0.8*	4.8 \pm 1.7	3	G193D	Early	1.5 \pm 0.6 (13)	6.7 \pm 1.4**	210 \pm 20	3	N291Y	Early	68.0 \pm (56)	37.0 \pm 6.0**	5200 \pm 700	1	
D29Y	Early	0 \pm 0 (0/0)	-	-	4	G193S	Early	0 \pm 0 (0/0)	-	-	6	G193D	Late	0.7 \pm (25)	2.4 \pm 0.8**	520 \pm 150	4	
W95S	Early	0 \pm 0 (0/0)	-	-	3	P208R	Early	0.3 \pm 0.1 (8)	5.6 \pm 1.5**	1400 \pm 300	3	G124A	Early	3 \pm 0.3 (8)	5.3 \pm 0.6**	49 \pm 1	3	
A97V	Early	9 \pm 2 (27)	3.1 \pm 0.5*	7.4 \pm 2.9	4	C260Y	Early	0 \pm 0 (0/0)	-	-	6	R434Q	Early	11 \pm 0.2 (23)	2.2 \pm 0.5**	110 \pm 27	4	
R100K	Early	0.2 \pm 0.0 (5)	1.5 \pm 0.2**	77 \pm 4.5	3	Y267C	Early	1.9 \pm 0.6 (2)	2.0 \pm 0.2*	34 \pm 7	4	R356W	Late	13 \pm 0.1 (5)	11 \pm 1*	2.6 \pm 0.2	3	
R107T	Early	0 \pm 0 (0/0)	-	-	6	N218E	Early	13 \pm 0.2 (1)	28 \pm 2*	43 \pm 14	3	E358K	Early	10.6 \pm (6)	5.5 \pm 2.2*	83 \pm 12	4	
R112I	Early	0.3 \pm 0.1 (2)	6.6 \pm 0.6*	140 \pm 17	3	W234L	Early	0 \pm 0 (0/0)	-	-	2	E158R	Early	16 \pm 0.4 (2)	4.4 \pm 1.7**	57 \pm 2	4	
R112L	Late	5.3 \pm 1.0 (3)	4.1 \pm 1.0**	1.04 \pm 0.4	3	R270Y	Early	0 \pm 0 (0/0)	-	-	2	R363C	Late	38 \pm 1.9 (13)	6.7 \pm 1.4**	1.8 \pm 0.1	3	
F113L	Late	1.8 \pm 0.4 (4)	5.5 \pm 1.1*	20 \pm 1.3	4	A238P	Early	0.1 \pm 0.1 (5)	12.3**	45 \pm 9	3	R380K	Late	4.5 \pm 0.2 (3)	6.2 \pm 0.8**	0.8 \pm 0.1	3	
F113T	Late	0 \pm 0 (0/0)	-	-	6	S232C	Late	1.8 \pm 0.5 (2)	2.5 \pm 0.4*	10 \pm 4	3	P490A	Late	13 \pm 0.4 (5)	7.6 \pm 2.2*	27 \pm 3	3	
L126P/A112I	Early	0 \pm 0 (0/0)	-	-	2	D244H	Early	37 \pm 0.5 (1)	11 \pm 3*	1.6 \pm 0.3	3	G931A	NA	0 \pm 0 (0/0)	-	-	2	
G236E	Early	0 \pm 0 (0/0)	-	-	5	N285S	Early	22 \pm 0.7 (5)	11 \pm 2*	39 \pm 3	3	K960G	NA	0 \pm 0 (0/0)	-	-	8	

GLA activity was measured in lysates prepared from cells incubated in the absence or presence of AT1001. Differences between treated and untreated cells were determined using a two-tailed, paired Student's t-test ($p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). ** indicates no significant increase in GLA activity. * indicates that the approximate age of disease onset information is not available. Wherever a significant increase in GLA activity was observed, a corresponding EC₅₀ value for AT1001 was calculated. Baseline values are the absolute GLA activity (nmol/mg protein/hr) measured in lysates from untreated cells. % WT was derived from the absolute GLA activity measured in lysates from untreated cells and is expressed as a percentage of the absolute GLA activity measured in wild type cell lysates assessed in parallel.