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Co-administration With the Pharmacological Chaperone AT1001 Increases Recombinant Human α-Galactosidase A Tissue Uptake and Improves Substrate Reduction in Fabry Mice

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Fabry disease is an X-linked lysosomal storage disorder (LSD) caused by mutations in the gene (*GLA*) that encodes the lysosomal hydrolase α-galactosidase A (α-Gal A), and is characterized by pathological accumulation of the substrate, globotriaosylceramide (GL-3). Regular infusion of recombinant human α-Gal A (rhα-Gal A), termed enzyme replacement therapy (ERT), is the primary treatment for Fabry disease. However, rhα-Gal A has low physical stability, a short circulating half-life, and variable uptake into different disease-relevant tissues. We hypothesized that coadministration of the orally available, small molecule pharmacological chaperone AT1001 (GR181413A, 1-deoxygalactonojirimycin, migalastat hydrochloride) may improve the pharmacological properties of rhα-Gal A via binding and stabilization. AT1001 prevented rhα-Gal A denaturation and activity loss *in vitro* at neutral pH and 37 °C. Coincubation of Fabry fibroblasts with rhα-Gal A and AT1001 resulted in up to fourfold higher cellular α-Gal A and ~30% greater GL-3 reduction compared to rhα-Gal A alone. Furthermore, coadministration of AT1001 to rats increased the circulating half-life of rhα-Gal A by >2.5-fold, and in *GLA* knockout mice resulted in up to fivefold higher α-Gal A levels and fourfold greater GL-3 reduction than rhα-Gal A alone. Collectively, these data highlight the potentially beneficial effects of AT1001 on rhα-Gal A, thus warranting clinical investigation.

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INTRODUCTION

Fabry disease (OMIM 301500) is an X-linked lysosomal storage disorder (LSD) caused by inherited mutations in the gene (*GLA*) that encodes α-galactosidase A (α-Gal A; EC 3.2.1.22).[1,2] Deficiency of α-Gal A results in progressive accumulation and deposition of neutral glycosphingolipids with terminal α-galactosyl residues, primarily globotriaosylceramide (GL-3, also known as Gb3 or CTH), in cells of the heart, kidney, skin, brain, eyes, and other tissues, which is believed to contribute to the life-threatening manifestations of Fabry disease.[1–3] The clinical presentation of Fabry disease spans a broad spectrum of severity and roughly correlates with residual α-Gal A activity.[2,4]

Enzyme replacement therapy (ERT) is currently the primary treatment for Fabry disease. ERT is based on the intravenous administration of recombinant human α-Gal A (rhα-Gal A), of which Fabrazyme (agalsidase β; Genzyme, Cambridge, MA) and Replagal (agalsidase alfa; Shire Pharmaceuticals, Cambridge, MA) are the only two approved products. These therapies are generally well-tolerated, and in some patients reduce plasma, urine, and microvascular endothelial GL-3 levels,[5,6] stabilize kidney function,[7] and alleviate neuropathic pain, reverse or improve hypertrophic cardiomyopathy, and increase the ability to sweat.[6,8,9] However, the infused recombinant enzymes tend to be unstable at neutral pH, resulting in a short-circulating half-life of the properly folded active enzyme *in vivo*.[5,10] Furthermore, delivery and uptake of ERT to some cells, tissues, and organs is insufficient in certain cases, as suggested by the inability of infused rhα-Gal A to significantly reduce GL-3 in cardiomyocytes, distal convoluted tubules, and glomerular podocytes, as well as the central nervous system.[5,10] In addition, the infused enzymes can be immunogenic, which may limit efficacy[11] and sometimes adversely affect tolerability.[12,13]

Small molecule pharmacological chaperones (PCs) have been proposed as a potential therapy for Fabry disease.[14] The iminosugar, 1-deoxygalactonojirimycin (AT1001, GR181413A, migalastat hydrochloride) is an analog of the terminal galactose of GL-3 that selectively and reversibly binds and stabilizes wild type and mutant forms of α-Gal A,[15,16] and when used alone as a monotherapy has been shown to reduce the storage of GL-3 *in vitro* and *in vivo*.[17–20] In contrast to ERT, AT1001 is orally available and has broad tissue distribution, including access to the central nervous system.[19] As such, AT1001 is currently in clinical development to evaluate its safety and

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efficacy as a monotherapy for Fabry disease.[21] PCs have also been identified that selectively bind and increase the levels of mutated enzymes associated with several other LSDs, including Gaucher, Tay-Sachs, Sandhoff, GM1-gangliosidosis, and Pompe disease.[22]

Recently, PCs have also shown positive effects on several exogenous recombinant enzymes that are used to treat LSDs, including increased physical stability and improved cellular and tissue uptake. Specifically, the PCs isofagomine and *N*-butyldeoxynojirimycin were shown to increase the *in vitro* cellular uptake and *in vivo* tissue uptake of the recombinant enzymes used to treat Gaucher and Pompe diseases, respectively.[23,24] Similarly, AT1001 was shown to increase the cellular uptake of rhα-Gal A in Fabry patient-derived cells *in vitro*.[23]

In the present study, we demonstrate that AT1001 stabilizes rhα-Gal A (agalsidase β) *in vitro*, minimizing protein denaturation and inactivation at neutral pH and physiological temperature. In addition, incubation of cultured cells derived from Fabry patients with AT1001 results in a concentration-dependent increase in the cellular uptake of rhα-Gal A. Studies in rats and *GLA* knockout (KO) mice indicate that AT1001 coadministration increases the circulating half-life of rhα-Gal A, resulting in significant and dose-dependent increases in rhα-Gal A levels in disease-relevant tissues. Most importantly, we show that AT1001-mediated increases in cellular and tissue levels of rhα-Gal A result in greater reduction of GL-3 compared to rhα-Gal A alone in patient-derived cells and tissues of *GLA* KO mice, indicating a "boost" in the net lysosomal activity from the exogenous recombinant enzyme. Taken together, these data indicate that AT1001 can increase the stability and improve the pharmacokinetic properties of ERT, thereby leading to greater tissue levels and substrate reduction. As such, a therapeutic approach for Fabry disease that combines a small molecule PC with ERT may warrant clinical investigation.

RESULTS

AT1001 stabilizes rhα-Gal A, preventing denaturation and loss of activity

The effect of AT1001 binding on the stability of rhα-Gal A was assessed using a fluorescence-based thermal denaturation assay.[25] At neutral pH, rhα-Gal A was significantly less stable [melting temperature (T_m) 47 °C] than at acidic pH (T_m 58 °C) (**Figure 1a**). Importantly, coincubation with AT1001 at neutral pH resulted in a concentration-dependent stabilization of rhα-Gal A, with 100 µmol/l AT1001 shifting the T_m to 58 °C, similar to that observed for the enzyme alone at acidic pH (**Figure 1a**). A concentration-dependent stabilization of rhα-Gal A with AT1001 at acidic pH was also observed. The T_m for rhα-Gal A shifts from 58 °C to 68 °C and 72 °C with 10 and 100 µmol/l AT1001, respectively (data not shown). Furthermore, incubation at neutral pH/37 °C resulted in time-dependent denaturation of rhα-Gal A, with a half-life of ~9 hours (**Figure 1b**). Incubation in the presence of AT1001 (10 µmol/l; **Figure 1b**), or in acidic buffer (data not shown), prevented rhα-Gal A denaturation for up to 24 hours. Additionally, incubation of rhα-Gal A in human whole blood resulted in a time-dependent loss of activity, with a half-life of ~2 hours (**Figure 1c**). Again, coincubation with AT1001 (1 µmol/l) increased the half-life of rhα-Gal A in blood to ~8 hours. Taken together, these results demonstrate that AT1001 stabilizes rhα-Gal A, preventing pH-, time-, and temperature-dependent denaturation and inactivation.

AT1001/rhα-Gal A coincubation results in greater α-Gal A levels and enzyme activity in Fabry fibroblasts compared to incubation with rhα-Gal A alone

The effect of AT1001 coincubation on rhα-Gal A was assessed in Fabry patient-derived fibroblast cell lines that show rhα-Gal A-dependent reduction of elevated GL-3 (**Supplementary Figure S1**). In these cell lines, 5-hour incubation with rhα-Gal A (0.4 nmol/l–0.5 nmol/l) led to a partial (≤50% of maximum) increase in α-Gal A activity and decrease in GL-3 (**Supplementary**

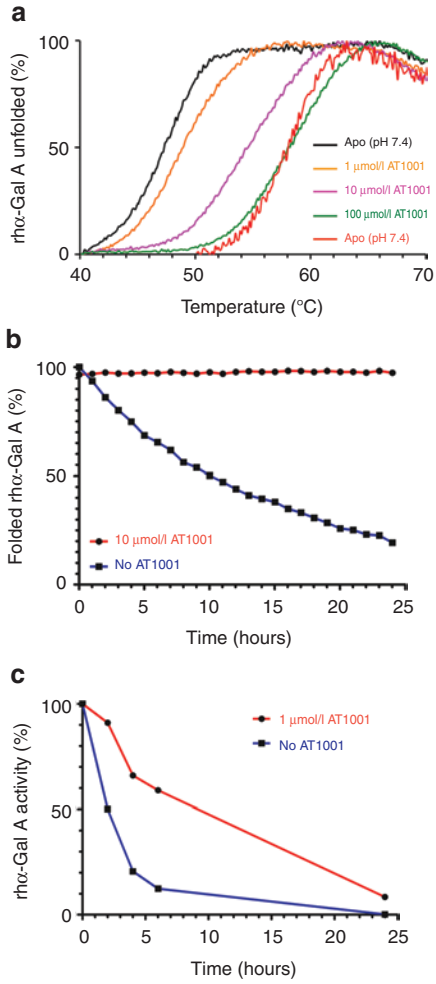

Figure 1 AT1001 increases the physical stability of recombinant human α-Gal A (rhα-Gal A) *in vitro*. (**a**) Thermal stability scans of rhα-Gal A in the absence and presence of various AT1001 concentrations. All experiments were performed at pH 7.4, with the exception of Apo (pH 5.2; red). Unfolding of rhα-Gal A was monitored by changes in the fluorescence of SYPRO Orange as a function of temperature. (**b**) Time course of rhα-Gal A unfolding in neutral buffer (pH 7.4) at 37 °C in the absence and presence of 10 µmol/l AT1001. Unfolding of rhα-Gal A was monitored by changes in the fluorescence of SYPRO Orange as a function of time. (**c**) Time course of rhα-Gal A inactivation in human whole blood at 37 °C in the absence and presence of 1 µmol/l AT1001. α-Galactosidase A (α-Gal A) enzyme activity was determined at the indicated time points using 4-methylumbelliferyl-α-D-galactopyranoside (4-MUG). To obtain relative enzyme activity levels, measurements at the various time points were compared to the activity at time zero.

Figure S2a–c). In C52S Fabry fibroblasts that have no endogenous α-Gal A activity and show no increase in α-Gal A levels in response to AT1001 alone[17], 5-hour coincubation with rhα-Gal A (0.5 nmol/l) and increasing concentrations of AT1001 (10–1,000 μmol/l) resulted in 4.4–5.6-fold more cellular α-Gal A protein 2 days later compared to rhα-Gal A incubation alone (**Figure 2a** and **Table 1**). As expected, 5-hour incubation with AT1001 alone (10–1,000 μmol/l) showed no increase in α-Gal A protein levels (**Figure 2a**) or activity (data not shown). However, after 5-hour rhα-Gal A and AT1001 coincubation, approximately threefold higher α-Gal A activity was seen 2 days later as compared to rhα-Gal A incubation alone [**Figure 2b** (blue bars) and **Table 1**]. The concentration of rhα-Gal A that yielded 50% of the maximal effect (EC_{50} value) was reduced approximately tenfold following AT1001 coincubation compared to incubation with rhα-Gal A alone [4.6 ± 1.3 nmol/l (n = 4) versus 48 ± 14 nmol/l (n = 4), respectively; P<0.05 by t-test; **Figure 2c** (blue lines)], indicating that the potency of rhα-Gal A uptake was greater when coincubated with AT1001. In Fabry fibroblasts that endogenously express AT1001-responsive mutant forms (R301Q and L300P),[17] rhα-Gal A and AT1001 coincubation similarly resulted in 3.0–3.3-fold higher α-Gal A activity compared to rhα-Gal A alone (**Table 1**). Incubation with 1,000 μmol/l AT1001 alone minimally increased α-Gal A activity in these cells 2 days later [1.5 ± 0.3-fold and 1.8 ± 0.1-fold above baseline levels in R301Q and L300P, respectively; n = 4 each], but the magnitudes of increase did not reach those seen with either rhα-Gal A incubation alone or with rhα-Gal A and AT1001 coincubation (**Table 1**).

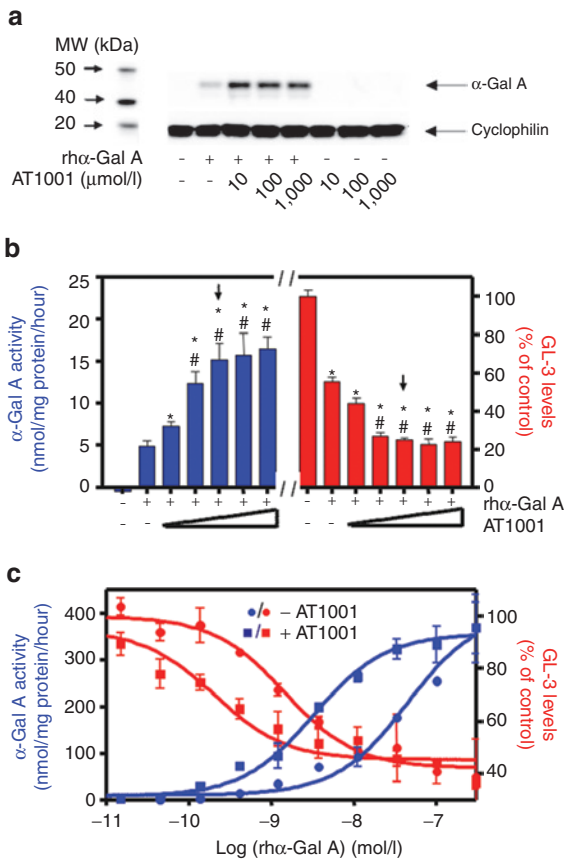

Figure 2 AT1001/recombinant human α-Gal A (rhα-Gal A) coincubation leads to greater α-Gal A levels and activity in Fabry fibroblasts. (**a**) Fabry fibroblasts expressing C52S α-Gal A were incubated with rhα-Gal A (0.5 nmol/l) alone or in the presence of increasing concentrations of AT1001 for 5 hours. α-Gal A protein levels in cell lysates were measured by western blot 2 days later. The data shown are representative of four independent experiments (**Table 1**). (**b**) C52S Fabry fibroblasts were incubated with rhα-Gal A (0.5 nmol/l) alone or in the presence of increasing concentrations of AT1001 (0.1, 1, 10, 100, or 1,000 μmol/l) for 5 hours. α-Galactosidase A (α-Gal A) activity (blue bars) in cell lysates was then measured 2 days later. The data points shown are the mean ± SEM of four wells tested in parallel from one representative of three independent experiments (**Table 1**). Globotriaosylceramide (GL-3) levels (red bars) were measured 10 days later. The data points represent mean ± SEM of three wells tested in parallel from one representative of three independent experiments (**Table 1**). The arrows indicate 10 μmol/l AT1001. (**c**) C52S Fabry fibroblasts were incubated with increasing concentrations of rhα-Gal A alone or in the presence of 1 mmol/l AT1001 for 5 hours. α-Gal A activity (blue lines) and GL-3 levels (red lines) in cell lysates were measured 2 and 10 days later, respectively. The data points shown are the mean ± SEM of four wells tested in parallel from one representative experiment. Similar effects of coincubation described in panels **a** and **b** were seen in R301Q and L300P fibroblasts (**Table 1**). *P < 0.05 compared to baseline; #P < 0.05 compared to rhα-Gal A alone, as determined by one-way ANOVA with Bonferroni *post-hoc* analysis.

AT1001/rhα-Gal A coincubation leads to greater GL-3 reduction in Fabry fibroblasts compared to incubation with rhα-Gal A alone

Coincubation of C52S fibroblasts with rhα-Gal A (0.5 nmol/l) and AT1001 (1,000 μmol/l) for 5 hours resulted in 2.3- and 2.2-fold decreases in cellular GL-3 levels when measured 10 and 14 days postincubation, respectively, as compared to 1.4- and 1.5-fold reductions following incubation with rhα-Gal A alone (**Supplementary Figure S2d**); this effect was dependent on AT1001 concentration [**Figure 2b** (red bars) and **Table 1**]. At a clinically achievable concentration of AT1001 (10 μmol/l, the approximate maximal plasma concentration in humans following oral administration of 150 mg AT1001),[26] the magnitude of the decrease in cellular GL-3 levels that was seen 10 days after coincubation was nearly maximal [**Figure 2b** (red bars) and **Table 1**]. The EC_{50} value for decreasing GL-3 levels in C52S fibroblasts was approximately sixfold lower following AT1001 coincubation compared to incubation with rhα-Gal A alone [0.12 ± 0.03 nmol/l (n = 5) versus 0.7 ± 0.3 nmol/l (n = 5), respectively; P < 0.05 by t-test; **Figure 2c** (red lines)], indicating that AT1001 also increased the potency of rhα-Gal A-mediated GL-3 reduction. The effect of coincubation on both α-Gal A and GL-3 levels was dependent upon simultaneous addition of rhα-Gal A and AT1001 in the growth media (**Supplementary Figure S3**). In R301Q and L300P fibroblasts, rhα-Gal A and AT1001 coincubation similarly resulted in decreases in cellular GL-3 levels (2.7- and 1.7-fold reductions, respectively) that were greater than those seen with rhα-Gal A alone (1.5- and 1.2-fold reductions, respectively) (**Table 1**).

AT1001 coadministration increases the circulating half-life of rhα-Gal A in rats and mice

To investigate the effects of increased rhα-Gal A stability *in vivo*, AT1001 was coadministered with rhα-Gal A in rats. Oral administration of AT1001 (3 mg/kg) 30 minutes before bolus tail vein injection of rhα-Gal A (10 mg/kg) (*i.e.*, at AT1001 T_{max}, to maximize the physical interaction between the two in the circulation),

Table 1 Effect of rhα-Gal A and AT1001 coincubation on cellular α-Gal A and GL-3 levels in Fabry patient-derived fibroblasts

Fibroblast genotype	rhα-Gal A (nmol/l)	AT1001 (μmol/l)	α-Gal A protein (increase relative to rhα-Gal A alone)	α-Gal A activity (nmol/mg/hour)	GL-3 levels (% of control)
C52S (c.155 G>C)	0	0	nd	0 ± 1	100 ± 6
	0.5	0	1	8 ± 3	67 ± 7*
	0.5	0.1	nt	14 ± 6*	56 ± 8*
	0.5	1.0	nt	20 ± 6*	44 ± 9*,**
	0.5	10	4.4 ± 1.1*,**	20 ± 5*	40 ± 8*,**
	0.5	100	4.7 ± 1.5*,**	22 ± 8*,**	37 ± 10*,**
	0.5	1,000	5.6 ± 2.0*,**	25 ± 11*,**	39 ± 8*,**
R301Q (c.902 G>A)	0	0	nd	1 ± 1	100 ± 4
	0.5	0	1*	7 ± 3	67 ± 10*
	0.5	0.1	nt	8 ± 3	67 ± 9*
	0.5	1.0	nt	14 ± 3*	51 ± 9*
	0.5	10	4.8 ± 1.5*,**	14 ± 4*	39 ± 9*,**
	0.5	100	5.0 ± 1.6*,**	22 ± 4*,**	38 ± 8*,**
	0.5	1,000	4.7 ± 1.5*,**	23 ± 9*,**	36 ± 7*,**
L300P (c.899 T>C)	0	0	nd	0.4 ± 0.5	100 ± 4
	0.5	0	1	6 ± 2	84 ± 8
	0.5	0.1	nt	8 ± 3*	79 ± 9*
	0.5	1.0	nt	14 ± 5*,**	67 ± 9*
	0.5	10	4.1 ± 0.9*	16 ± 4*,**	62 ± 10*,**
	0.5	100	4.5 ± 1.3*,**	18 ± 4*,**	58 ± 8*,**
	0.5	1,000	4.2 ± 1.3*,**	18 ± 5*,**	60 ± 7*,**

Abbreviations: GL-3, globotriaosylceramide; nd, not detectable; nt, not tested; rhα-Gal A, recombinant human α-Gal A; α-Gal A, α-galactosidase A.
Three different male Fabry patient-derived fibroblast cell lines (with the indicated genotypes) were incubated with rhα-Gal A (0.5 nmol/l) alone or in the presence of increasing concentrations of AT1001 for 5 hours. α-Gal A protein levels and α-Gal A activity were measured in cell lysates 2 days later. Cellular GL-3 levels were measured 10 days later. Data represent the mean ± SEM of three or more independent experiments.
*$P < 0.05$ compared to baseline; **$P < 0.05$ compared to rhα-Gal A incubation alone; one-way ANOVA with Bonferroni *post-hoc* analysis.

resulted in an approximate 2.6-fold increase in the circulating half-life of the enzyme (**Figure 3a**, upper panel). In the absence of AT1001, rhα-Gal A activity declined rapidly, with a half-life of 24 ± 0.5 minutes; AT1001 coadministration increased the half-life to 63 ± 4 minutes. Western blotting indicated that coadministration led to 2.5- and 1.5-fold higher rhα-Gal A protein levels in plasma 60 and 240 minutes postinjection, respectively (**Figure 3a**, lower panel). Similarly, studies conducted in *GLA* KO mice demonstrated that oral administration of AT1001 (3 or 10 mg/kg) 30 minutes before injection of rhα-Gal A (1 mg/kg) also significantly increased the circulating levels of recombinant enzyme. In the absence of AT1001, plasma α-Gal A levels were nearly undetectable 240 minutes postinjection. In contrast, AT1001 coadministration led to increases in α-Gal A activity 60-, 120-, and 240-minutes postinjection that were up to 12-, 40-, and 90-fold greater, respectively, than those seen following administration of rhα-Gal A alone (**Figure 3b**, upper panel). Western blotting confirmed the higher plasma α-Gal A protein levels in *GLA* KO mice (**Figure 3b**, lower panel).

Coadministration of AT1001 increases tissue α-Gal A uptake and GL-3 reduction in *GLA* KO mice

Preliminary studies demonstrated that a single tail vein injection of rhα-Gal A dose-dependently increased α-Gal A activity and reduced GL-3 levels in the skin, heart, and kidney of *GLA* KO mice 7 days postadministration, with 3 mg/kg showing the greatest effects (**Supplementary Figure S4**). Three days postadministration of 1 mg/kg rhα-Gal A, significant increases in α-Gal A activity were seen that were up to 2.0-, 8.5-, and 5.4-fold above baseline in skin, heart, and kidney, respectively [**Figure 4a** (red bars) and **Table 2**]. Importantly, oral administration of AT1001 30 minutes before, and 2 hours after, rhα-Gal A injection resulted in even greater α-Gal A levels that were up to 2.7-, 4.1-, and 5.2-fold higher, respectively, than those seen with rhα-Gal A alone [**Figure 4a** (blue bars) and **Table 2**]. In contrast, administration of a tenfold lower dose of rhα-Gal A (0.1 mg/kg) did not result in increased α-Gal A activity above baseline [**Figure 4a** (pink bars) and **Table 2**]; however, α-Gal A levels were significantly increased up to 1.6-, 2.5-, and 3.5-fold above baseline in skin, heart, and kidney, respectively, following coadministration with AT1001 [**Figure 4a** (green bars) and **Table 2**]. These effects on α-Gal A tissue levels were also seen 7 days after coadministration (**Table 2**). Notably, AT1001 coadministration with 1 mg/kg rhα-Gal A resulted in significantly greater enzyme levels in heart and kidney compared to those seen following 3 mg/kg α-Gal A alone (**Table 2**).

Concomitant with the effects on α-Gal A tissue levels, AT1001 coadministration also resulted in greater tissue GL-3 reduction in

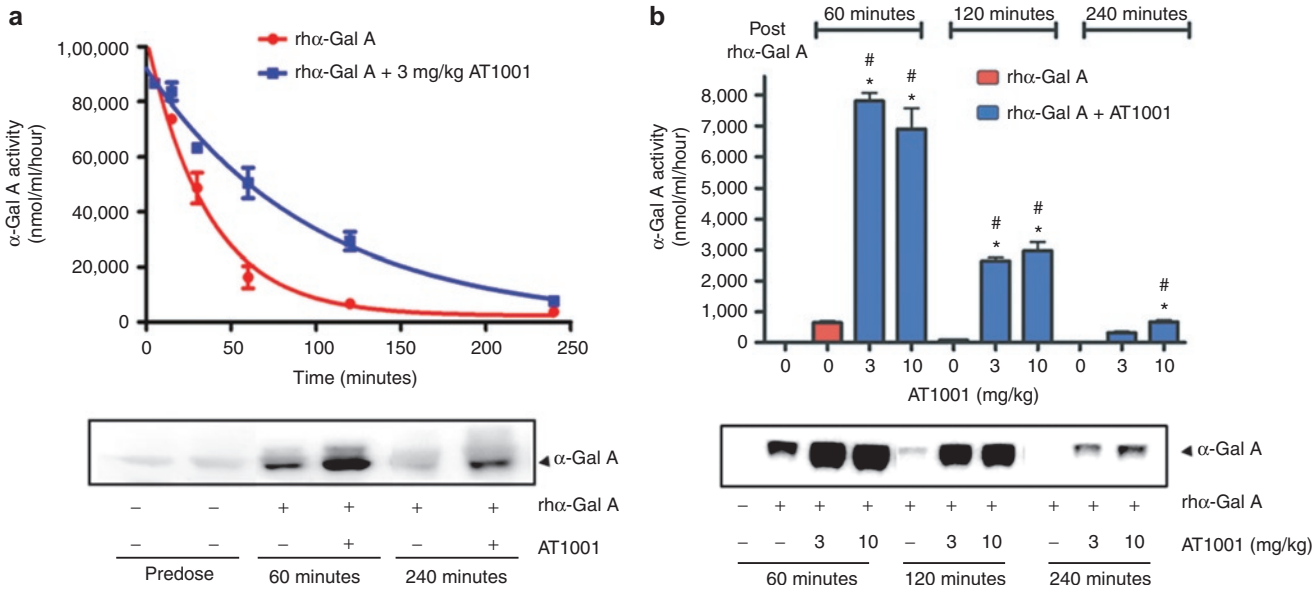

Figure 3 AT1001 increases the circulating half-life of recombinant human α-Gal A (rhα-Gal A). (**a**) Eight-week-old male Sprague-Dawley rats were administered either vehicle (water) or AT1001 (3 mg/kg) via oral gavage. Thirty minutes later, vehicle (saline) or rhα-Gal A (10 mg/kg) was administered via bolus tail vein injection. Blood was collected at the indicated time points, and α-galactosidase A (α-Gal A) activity (upper panel) and protein levels (lower panel) were measured in plasma as described in Materials and Methods section. The data shown are an average of three independent studies. Each time point represents the mean ± SEM of the activity measured from nine rats. Each lane on the western blot contains plasma from a single rat, and is representative of two rats in each group. (**b**) Twelve-week old male *GLA* knockout (KO) mice were administered either vehicle (water) or AT1001 (3 or 10 mg/kg) via oral gavage. Thirty minutes later, vehicle (saline) or rhα-Gal A (1 mg/kg) was administered via bolus tail vein injection. Blood was collected at the indicated time points, and α-Gal A activity (upper panel) and protein levels (lower panel) were measured in plasma as described in Materials and Methods section. Each bar represents the mean ± SEM of the activity measured from 5 mice/group. Each lane on the western blot contains plasma from a single mouse, and is representative of two mice in each group. *$P < 0.05$ compared to baseline; #$P < 0.05$ compared to rhα-Gal A administration alone; one-way ANOVA with Bonferroni *post-hoc* analysis.

GLA KO mice. Seven days after a single administration of 1 mg/kg rhα-Gal A, skin, heart, and kidney GL-3 levels were reduced by 69 ± 4%, 71 ± 3%, and 58 ± 5%, respectively [**Figure 4b** (red bars) and **Table 2**]. Oral administration of AT1001 (30, 100, or 300 mg/kg) 30 minutes before, and 2 hours after, rhα-Gal A administration reduced GL-3 levels by 93 ± 2%, 94 ± 2%, and 93 ± 3%, respectively [**Figure 4b** (blue bars) and **Table 2**]. Notably, coadministration of AT1001 with 1 mg/kg rhα-Gal A reduced tissue GL-3 levels more than administration of the higher dose of 3 mg/kg rhα-Gal A alone (**Table 2**). Importantly, administration of the lower dose of 0.1 mg/kg rhα-Gal A reduced GL-3 levels approximately twofold in heart only [*i.e.,* there was no significant effect on skin or kidney GL-3 levels; **Figure 4b** (pink bars) and **Table 2**]; however, GL-3 was significantly reduced in all three tissues following coadministration of AT1001 with the low dose of rhα-Gal A [**Figure 4b** (green bars) and **Table 2**]. Similar effects of coadministration were seen with 0.3 mg/kg rhα-Gal A (**Table 2**). Furthermore, immunohistochemistry demonstrated that coadministration results in greater GL-3 reduction in cardiac smooth muscle cells of blood vessel walls and in renal distal tubular epithelial cells in the *GLA* KO mice (**Figure 4c**).

Lastly, the effects of coadministration were investigated using a single administration of AT1001 at doses (3 and 10 mg/kg) that lead to plasma exposures in mice that are comparable to those seen in humans following oral administration of 150 mg and 450 mg, respectively.[21,26] Again, administration of AT1001 30 minutes before rhα-Gal A (1 mg/kg) resulted in sustained circulating plasma levels of α-Gal A (**Figure 3b**), and up to 2.3-, 2.6-, and 2.5-fold greater enzyme levels 1 day postadministration in skin, heart, and kidney, respectively, compared to those seen following rhα-Gal A administration alone (**Figure 5a** and **Supplementary Table S1**); greater α-Gal A levels were also seen 3 and 7 days postinjection (**Supplementary Table S1**). Importantly, coadministration also led to significantly greater reduction in tissue GL-3 levels compared to administration of rhα-Gal A alone, with ~4.0-, 2.5-, and 2.0-fold greater reductions seen in skin, heart, and kidney, respectively (**Figure 5b**). Collectively, these data indicate that coadministration of AT1001 to *GLA* KO mice at doses that yield clinically achievable exposures can significantly increase α-Gal A activity and reduce GL-3 levels in disease-relevant tissues to a greater extent than seen following administration of rhα-Gal A alone.

DISCUSSION

Regular infusion of rhα-Gal A is the primary treatment for Fabry disease, and is associated with clinical efficacy. However, rhα-Gal A has some limitations, including instability at neutral pH,[27] a short-circulating half-life,[5] and variable and inefficient uptake into some cell types and tissues.[28–33] AT1001 is a PC that selectively binds to endogenous α-Gal A, increasing physical stability, lysosomal trafficking, and activity in cultured cells[14,16,17,20,34] and in Fabry disease-relevant tissues *in vivo*.[18,19] Recently, PCs have been shown to increase the stability[23,24,35,36] and to improve the cellular and tissue uptake of several exogenous recombinant enzymes that are used to treat LSDs, including rhα-Gal A.[23,24,35,36]

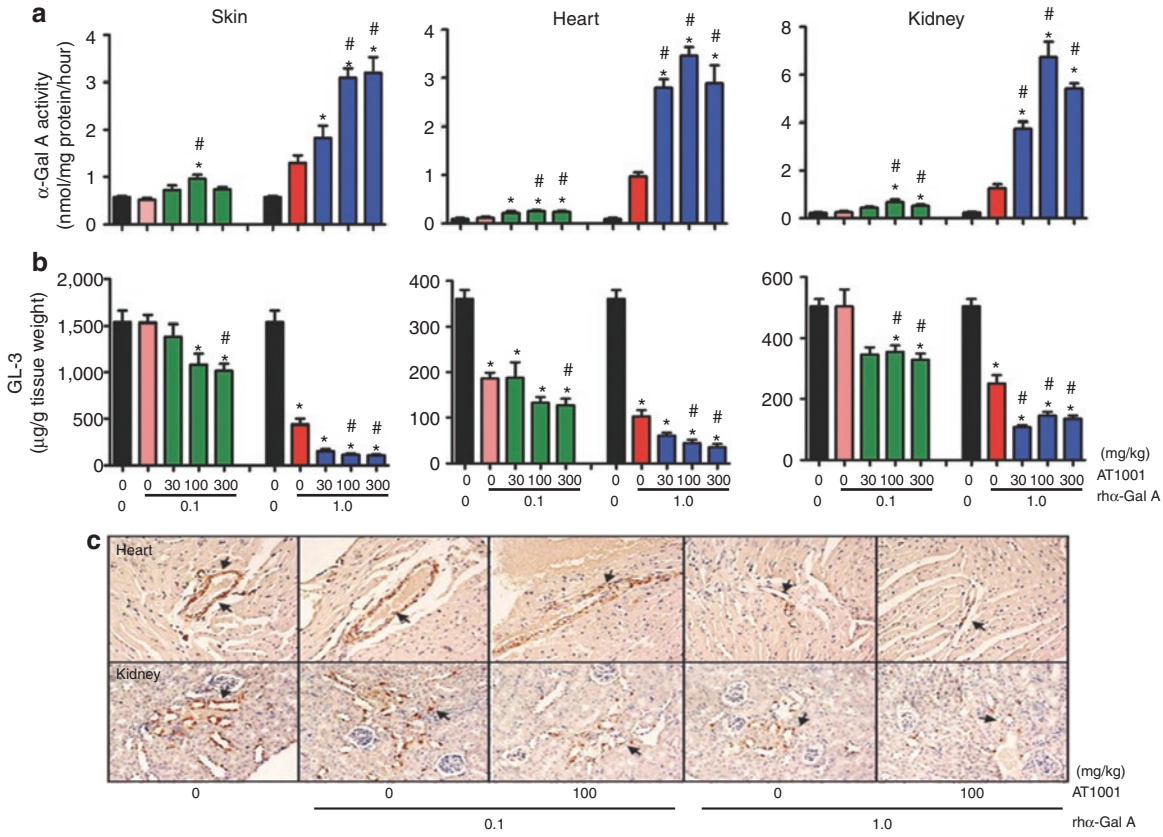

Figure 4 Coadministration of AT1001 promotes greater α-galactosidase A (α-Gal A) uptake and globotriaosylceramide (GL-3) reduction in tissues of *GLA* knockout (KO) mice. Twelve-week-old male *GLA* KO mice were administered either vehicle (water) or AT1001 (30, 100, or 300 mg/kg) via oral gavage. Thirty minutes later, vehicle (saline) or recombinant human α-Gal A (rhα-Gal A) (0.1 or 1 mg/kg) was administered via bolus tail vein injection, which was followed 2 hours later by a second oral administration of AT1001 (using an equivalent dose to the first administration). Skin, heart, and kidney were collected (**a**) 3 or (**b**) 7 days postadministration for measurement of α-Gal A activity and GL-3 levels, respectively, as described previously.[19] The α-Gal A and GL-3 data were pooled from two independent studies, and represent the mean ± SEM for 14–16 mice/group, with the exceptions of all 30 mg/kg AT1001 dose groups and the 0.3 mg/kg rhα-Gal A ± AT1001 dose groups (mean ± SEM for 7–8 mice per group). **P* < 0.05 compared to baseline; #*P* < 0.05 compared to rhα-Gal A administration alone; one-way ANOVA with Bonferroni *post-hoc* analysis. (**c**) Cell type-specific reduction of GL-3 in *GLA* KO mice was assessed by immunohistochemistry using an anti-GL-3 antibody as described previously.[19] The GL-3 signal is represented as brown spots (denoted with arrows). The data shown are representative photomicrographs from 7 to 8 mice/group (magnification: ×20).

Our studies indicate that the binding of AT1001 to rhα-Gal A significantly increases the physical stability of the enzyme in neutral pH buffer, as well as in human blood *ex vivo*. In addition, coincubation of rhα-Gal A with AT1001 results in greater α-Gal A uptake and activity in Fabry patient-derived fibroblasts. An earlier study revealed similar findings in a cell-based assay.[23] Several mechanisms could explain these effects of AT1001, including: (i) extracellular stabilization of rhα-Gal A, leading to higher concentrations of properly folded, functional enzyme that can bind to and be internalized by mannose 6-phosphate receptors on the cell surface, (ii) stabilization of rhα-Gal A in endocytic vesicles and other nonlysosomal compartments, leading to less intracellular degradation, and/or (iii) stabilization and prolonged half-life of functional rhα-Gal A in lysosomes. As the effects of coincubation on cellular α-Gal A levels require the simultaneous presence of rhα-Gal A and AT1001 in the growth medium for only a few (3–5) hours, we speculate that the increased uptake results from extracellular stabilization of the properly folded protein, consistent with previously proposed mechanisms.[23,24] However, it is possible that dissociation of AT1001 from rhα-Gal A is sufficiently slow, such that the internalized enzyme remains stable subsequent to endocytosis and/or lysosomal delivery. To gain more insight into the mechanisms that mediate the effects of AT1001, future studies should directly measure the rate of AT1001 entry and exit from lysosomes, the binding affinities of rhα-Gal A for the mannose 6-phosphate receptors in the absence and presence of AT1001, and the dissociation rates of AT1001 from rhα-Gal A at both neutral and lysosomal pH.

Similar to the stabilizing effects seen *in vitro*, AT1001 coadministration to mice and rats also prolonged the circulating half-life of rhα-Gal A. Importantly, these effects were seen at doses that result in plasma concentrations of ~10 μmol/l and 30 μmol/l, which are comparable to those achieved in humans following oral administration of 150 and 450 mg AT1001, respectively.[21,26] Furthermore, AT1001 coadministration also resulted in up to threefold higher levels of α-Gal A activity in disease-relevant tissues of *GLA* KO mice that were sustained well above baseline for up to 7 days. We hypothesize that binding of AT1001 to rhα-Gal A may sufficiently increase the physical stability of the exogenous enzyme to minimize or prevent

Table 2 Effect of rhα-Gal A and AT1001 coadministration on α-Gal A and GL-3 levels in tissues of *GLA* KO mice

		Skin			Heart			Kidney		
AT1001 (mg/kg)	0	30	100	300	0	30	100	300	0	30	100	300
Necropsy day[a]	rhα-Gal A (mg/kg)						α-Gal A levels (relative to untreated)					
3	0.1	nc	1.2 ± 0.1	1.6 ± 0.1*,**	1.4 ± 0.1	1.2 ± 0.1	2.0 ± 0.1*	2.5 ± 0.1*,**	2.3 ± 0.1*,**	nc	2.0 ± 0.1	3.5 ± 0.1*,**	3.0 ± 0.1*,**
	1	2.0 ± 0.2	3.0 ± 0.3*	5.2 ± 0.2*,**	5.5 ± 0.3*,**	8.5 ± 0.1	28 ± 0.2*,**	35 ± 0.2*,**	29 ± 0.2*,**	5.4 ± 0.2	19 ± 0.3*,**	28 ± 0.6*,**	23 ± 0.2*,**
7	0.1	nc	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.3**	1.1 ± 0.1	1.5 ± 0.1*,**	1.8 ± 0.1*,**	2.2 ± 0.1*,**	nc	1.4 ± 0.1*,**	1.3 ± 0.1*	1.2 ± 0.1
	0.3	nc	nd	3.2 ± 0.2*,**	3.5 ± 0.1*,**	2.0 ± 0.1	nd	4.0 ± 0.1*,**	5.0 ± 0.2*,**	nc	nd	3.0 ± 0.2*,**	2.5 ± 0.1*,**
	1	1.7 ± 0.1	2.6 ± 0.3*	4.4 ± 0.2*,**	4.3 ± 0.2*,**	6.0 ± 0.1	10 ± 0.2*	14 ± 0.2*,**	17 ± 0.2*,**	2.3 ± 0.2	5.0 ± 0.1*	9.0 ± 0.2*,**	8.3 ± 0.2*,**
	3	5.4 ± 0.3*	nd	nd	nd	8.4 ± 1.0*	nd	nd	nd	7.0 ± 2.0*	nd	nd	nd
							GL-3 levels (% reduction)						
7	0.1	nc	10 ± 4	30 ± 7*	34 ± 5*,**	48 ± 3*	55 ± 8*	67 ± 3*	69 ± 2*,**	nc	37 ± 5	40 ± 5*,**	46 ± 4*,**
	0.3	25 ± 8	nd	53 ± 10*,**	45 ± 8*,**	41 ± 10	nd	83 ± 3*,**	74 ± 6*,**	24 ± 2*	nd	42 ± 5*,**	33 ± 2*
	1	69 ± 4*	89 ± 1*	92 ± 2*,**	93 ± 2*,**	71 ± 3*	85 ± 2*	93 ± 2*,**	94 ± 2*,**	58 ± 5*	93 ± 3*,**	85 ± 2*,**	83 ± 2*,**
	3	87 ± 3*	nd	nd	nd	83 ± 6*	nd	nd	nd	71 ± 1*	nd	nd	nd

Abbreviations: GL-3, globotriaosylceramide; KO, knockout; nd, not detectable; nc, no change; rhα-Gal A, recombinant human α-Gal A; α-Gal A, α-galactosidase A.
Twelve-week-old male *GLA* KO mice were administered AT1001 and/or rhα-Gal A as described in Materials and Methods section. Mice were sacrificed 3 or 7 days post-rhα-Gal A injection to measure tissue α-Gal A and GL-3 levels.[19] The α-Gal A data were normalized relative to baseline levels. The GL-3 data were normalized relative to baseline levels of untreated *GLA* KO (100%) and wild-type C57BL/6 mice (0%).
[a]Post-rhα-Gal A administration.
*$P < 0.05$ compared to baseline; **$P < 0.05$ compared to rhα-Gal A alone, as determined by one-way ANOVA with Bonferroni *post-hoc* analysis. The α-Gal A and GL-3 data were pooled from two independent studies and represent the mean ± SEM for 14–16 mice/group, with the exceptions of all 30 mg/kg AT1001 dose groups and the 0.3 mg/kg rhα-Gal A ± AT1001 dose groups (mean ± SEM for 7–8 mice/group).

thermally and neutral pH-mediated denaturation, as well as proteolysis, in the blood. A longer circulating half-life may offer more chances for recognition by mannose 6-phosphate receptors and uptake into disease-relevant cells/tissues. In addition, reduced asialoglycoprotein receptor-mediated clearance of rhα-Gal A by the liver could contribute to the prolonged circulating half-life and higher tissue levels of exogenous enzyme.[37] As elevated levels of rhα-Gal A in target cells and tissues are desirable for the treatment of Fabry disease, with some cell types showing very poor uptake,[5,28–33,38] AT1001 coadministration could have a significant impact on the overall efficacy of rhα-Gal A. To this end, AT1001 coadministration significantly improved uptake of rhα-Gal A into smooth muscle cells of cardiac blood vessels and distal tubular epithelial cells of the kidney. Future studies to more closely investigate the effects of AT1001 on rhα-Gal A uptake into different disease-relevant cell types are warranted.

Importantly, the combination of AT1001 and rhα-Gal A leads to a greater effect on the key substrate GL-3 in Fabry fibroblasts and in cells and tissues of *GLA* KO mice. In the cell-based studies, the greatest GL-3 reduction seen after coincubation followed an unexpectedly long time-course, requiring 10–14 days to manifest. This may be due to relatively slow GL-3 turnover in these cells, particularly if the substrate is accumulated in nonlysosomal cellular compartments and requires redistribution to lysosomes for hydrolysis.[3,39] However, the possibility of prolonged binding and stabilization of rhα-Gal A by AT1001 in lysosomes cannot be ruled out. In our studies with *GLA* KO mice, tissue GL-3 levels were only assessed 7 days after coadministration; thus, studies to investigate the time course for GL-3 reduction *in vivo* are warranted.

It should be noted that the maximal effect of coincubation on GL-3 reduction in Fabry fibroblasts was seen with 0.5 nmol/l rhα-Gal A, a concentration that is just below the observed EC_{50} value and that leads to only partial GL-3 reduction (≤50% of maximum). Importantly, coincubation with AT1001 resulted in an approximately sixfold leftward shift in the EC_{50} value, indicating that AT1001 actually increases the potency of α-Gal A. Indeed, AT1001 coincubation with 0.5 nmol/l rhα-Gal A resulted in robust GL-3 clearance that was similar to the maximum reductions seen following incubation with higher concentrations of rhα-Gal A alone (*i.e.,* 5 nmol/l–50 nmol/l). An increased potency for GL-3 reduction was also observed in tissues of *GLA* KO mice following coadministration. This was most evident by the enhanced efficacy seen at the lower dose of rhα-Gal A (0.1 mg/kg), which had minimal effects on skin and kidney GL-3 levels when administered alone.

In humans, the maximum concentration of rhα-Gal A measured in plasma during infusion with 1 mg/kg agalsidase β is approximately 50 nmol/l.[5,40] While our cell-based studies suggest that further stabilization of rhα-Gal A at this concentration may not have a significant added benefit, our *in vivo* studies clearly show improved GL-3 clearance at both therapeutic (1 mg/kg) and subtherapeutic (0.1 and 0.3 mg/kg) dose levels. It is possible that GL-3 reduction in cultured fibroblasts is particularly sensitive to low concentrations of rhα-Gal A, whereas the potency for mediating GL-3 reduction in disease-relevant tissues *in vivo* is lower. Hence, AT1001-mediated increases in the maximal concentration and/or duration of circulating rhα-Gal A may be primarily responsible for the greater tissue uptake and GL-3

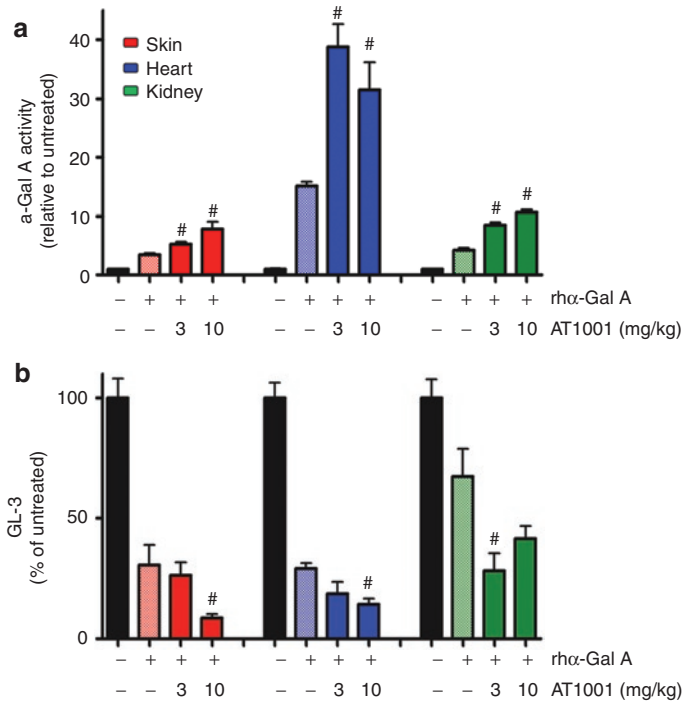

Figure 5 A single oral administration of AT1001 at doses that yield clinically achievable exposures in humans increases the tissue levels of recombinant human α-Gal A (rhα-Gal A) and leads to greater globotriaosylceramide (GL-3) reduction in *GLA* knockout (KO) mice. Twelve-week-old male *GLA* KO mice were given a single oral administration of vehicle (water) or AT1001 (3 or 10 mg/kg, which result in plasma C_{max} values of ~10 and 30 μmol/l that are roughly equivalent to the C_{max} values seen in humans following administration of 150 and 450 mg AT1001, respectively) 30 minutes before bolus tail vein injection of 1 mg/kg rhα-Gal A. Mice were killed 1 or 7 days later for measurement of tissue (**a**) α-galactosidase A (α-Gal A) and (**b**) GL-3 levels (see Materials and Methods). α-Gal A data were normalized relative to baseline levels; GL-3 data were normalized relative to baseline levels of untreated *GLA* KO (100%) and wild-type C57BL/6 mice (0%). The bars on each graph represent the mean ± SEM for 5 mice/group. $^{\#}P < 0.05$, compared to rhα-Gal A administration alone, as determined by one-way ANOVA with Bonferroni *post-hoc* analysis.

reduction seen in mice. Furthermore, the half-life of rhα-Gal A in human plasma after infusion is about 45 minutes,[41] with mean plasma concentrations decreasing to ~0.5 nmol/l within 9 hours postinfusion.[5] Thus, there may be a period of several hours following infusion when AT1001 could have a substantial impact on the low circulating levels of rhα-Gal A levels due to binding and increased stability. Our data suggest that improved efficacy could be derived from the currently recommended dose and regimen of rhα-Gal A when coadministered with AT1001. In addition, the sustained plasma and tissue α-Gal A levels seen in the mice following coadministration suggest the potential for reduced dose and/or administration frequency of ERT while maintaining the same level of efficacy.[5,40] Recent shortages in Fabry ERT supplies underscore the importance of investigating this possibility.

Collectively, our data indicate that AT1001 increases the stability and total cellular and tissue levels of exogenous rhα-Gal A, translating to greater enzyme activity and substrate turnover *in situ*. In our cell-based studies, the effect of AT1001 coincubation on rhα-Gal A uptake and GL-3 reduction was seen in cultured fibroblasts derived from different male Fabry patients. These cell lines harbor different *GLA* mutations, differ by the presence or absence of detectable endogenous α-Gal A activity, and show different increases of endogenous α-Gal A in the presence of AT1001,[17] suggesting that the beneficial effects of rhα-Gal A and AT1001 coadministration may not depend on the specific *GLA* genotype. As such, AT1001 coadministration may provide an improved therapeutic strategy that is broadly applicable to a diverse population of Fabry patients. Based on these encouraging findings, phase 2 clinical investigations have been initiated.

MATERIALS AND METHODS

Materials. Male Fabry patient-derived (C52S, c.155 G>C; R301Q, c.902 G>A; L300P, c.899 T>C) and normal human fibroblast cell lines (CRL2076) were from sources described previously.[17] All cell culture reagents were purchased from Invitrogen (Carlsbad, CA), except for characterized fetal bovine serum, which was purchased from HyClone (Waltham, MA). AT1001 (1-deoxygalactonojirimycin hydrochloride; migalastat hydrochloride; GR181413A) was synthesized by Cambridge Major Laboratories (Germantown, WI). rhα-Gal A (agalsidase β; Fabrazyme) was purchased from Genzyme. Rabbit antihuman α-Gal A polyclonal antibody, CR0020, for western blots in cells and tissues was provided by Shire HGT (Cambridge, MA). Rabbit antihuman cyclophilin A and antihuman actin polyclonal antibodies were purchased from Millipore (Billerica, MA) and Invitrogen, respectively. Mouse antihuman GL-3 monoclonal antibody was purchased from Seikagaku (Tokyo, Japan). Horseradish peroxidase conjugated goat anti-rabbit secondary antibody was purchased from ThermoPierce (Rockford, IL). *GLA* knockout (KO) mice were obtained from Dr Robert Desnick (Mt Sinai University, New York, NY). Wild-type C57BL/6 mice and Sprague-Dawley rats (carotid artery cannulated) were purchased from Taconic Farms (Germantown, NY). Animal husbandry and all experiments were conducted under Institutional Animal Care and

Use Committee approved protocols. All other reagents were purchased from Sigma Aldrich (St Louis, MO) unless noted otherwise.

Thermal stability assay. The stability of rhα-Gal A was assessed using a modified fluorescence thermostability assay[25] on a Realplex Mastercycler qRT-PCR system (Eppendorf, Hamburg, Germany) in either neutral pH buffer (25 mmol/l sodium phosphate, 150 mmol/l sodium chloride, pH 7.4) or acidic pH buffer (25 mmol/l sodium acetate, 150 mmol/l sodium chloride, pH 5.2). Briefly, rhα-Gal A (2.5 µg) was combined with SYPRO Orange and various concentrations of AT1001 in a final reaction volume of 25 µl. A thermal gradient was applied to the plate at a rate of 1 °C/minute, during which time the fluorescence of SYPRO Orange was continuously monitored. The fluorescence intensity at each temperature was normalized to the maximum fluorescence after complete thermal denaturation. For time-dependent denaturation assays, reactions were incubated at 37 °C for up to 24 hours, with SYPRO Orange fluorescence intensity monitored at the indicated time points and normalized to the maximum fluorescence after complete thermal denaturation.

Enzyme inactivation assay in whole blood. rhα-Gal A (1 nmol/l) was incubated with 1 µmol/l AT1001 for 10 minutes on ice in human whole blood (Lampire, Pipersville, PA). Reactions were then transferred to 37 °C, with aliquots removed and diluted 500× (to ensure minimum inhibition of rhα-Gal A by AT1001) at the indicated times and incubated for an additional 1 hour at 37 °C in rhα-Gal A reaction buffer (3 mmol/l 4-methylumbelliferyl-α-D-galactopyranoside, 0.1% Triton X-100, 0.1 mol/l citrate phosphate, pH 4.6). Reactions were stopped by the addition of an equal volume of 0.5 mol/l sodium carbonate (pH 10.5). Liberated 4-MU was measured on Victor^3 plate reader (Perkin Elmer, Waltham, MA) at 355 nmol/l excitation and 460 nmol/l emission, with fluorescence plotted as a function of time. Data were normalized to the activity at time zero.

Western blot analysis in Fabry patient fibroblasts. Fabry fibroblasts were seeded in a T-75 flask at a density of one million cells in growth medium (Dulbecco's modified Eagle medium + 15% fetal bovine serum) and incubated at 37 °C, 8% CO_2 overnight. The cells were then incubated with rhα-Gal A (0.5 nmol/l) alone, AT1001 alone (1,000 µmol/l), or rhα-Gal A (0.5 nmol/l) and AT1001 (10, 100, or 1,000 µmol/l) for 5 hours. The cells were then washed three times with growth medium, and maintained in growth medium at 37 °C, 8% CO_2 for 2 days. After, the cells were washed twice with Dulbecco's phosphate-buffered saline and lysed (5 minutes in 200 µl of 0.5% Triton X-100, 27 mmol/l citric acid monohydrate, 46 mmol/l phosphate buffer, pH 4.6). The protein concentration in the lysates was determined using a MicroBCA Protein Assay Kit (ThermoPierce) according to the manufacturer's instructions. Ten to fifteen microgram of total protein were loaded per lane and blotted as described previously,[17] except that the anti-α-Gal A primary antibody, CR0020, was diluted 1:2,000, and the anti-cyclophilin A loading control antibody was diluted 1:4,000. The net intensity of the α-Gal A band was normalized to the net intensity of the cyclophilin A band in each lane. The ratio of α-Gal A to cyclophilin A was compared across lanes to calculate the relative increase in α-Gal A protein after coincubation.

α-Gal A activity assay in Fabry patient fibroblasts. Fabry patient fibroblasts were seeded in sterile, clear-bottom, 96-well black plates (Corning Costar, Corning, NY) at 10,000 cells/well, and incubated at 37 °C, 8% CO_2 for 1–2 hours. The cells were then incubated with rhα-Gal A (0.5 nmol/l) alone, or rhα-Gal A (0.5 nmol/l) and AT1001 (0.1, 1, 10, 100, or 1,000 µmol/l) for 5 hours. The cells were washed three times with growth medium, and then maintained in growth medium at 37 °C, 8% CO_2 for 2 days. After, the cells were washed twice with Dulbecco's phosphate-buffered saline, and lysed in 35 µl 0.2% Triton X-100, 27 mmol/l citric acid monohydrate, 46 mmol/l phosphate buffer, pH 4.6, for 5 minutes. Enzyme activity was measured as described previously, and is expressed as the nanomoles of 4-MU liberated per mg protein/hour (nmol/mg protein/hour).[17]

Immunofluorescence detection of GL-3 in Fabry patient fibroblasts. Fabry patient fibroblasts were seeded at 10,000 cells/well in glass-bottom 96-well black plates (Thermo Scientific Nunc, Pittsburgh, PA) that were coated with 10 µg/ml of human plasma fibronectin (Invitrogen) for 1 hour at 37 °C. The cells were incubated at 37 °C, 8% CO_2 for 1–2 hours to promote attachment, then incubated with rhα-Gal A in the absence or presence of AT1001, followed by washing as described for the α-Gal A activity assay. The cells were maintained in growth medium at 37 °C, 8% CO_2 for 10 days. After, the cells were washed twice with Dulbecco's phosphate-buffered saline, and GL-3 analysis by immunostaining was performed as previously described.[17] For data analysis, the GL-3 fluorescence intensity was divided by the actin fluorescence intensity to obtain the GL-3 to actin fluorescence ratio (referred to as the "GL-3 level" in the cell-based experiments). Fabry fibroblast GL-3 levels were normalized to the percentage of GL-3 levels in normal untreated fibroblasts (defined as 0%) and untreated Fabry fibroblasts (of the same cell line) measured in parallel (defined as 100%).

AT1001/rhα-Gal A coadministration studies in rats. Eight-week-old male Sprague-Dawley rats were administered either vehicle (water) or AT1001 (3 mg/kg) via oral gavage. Thirty minutes later, vehicle (saline) or rhα-Gal A (10 mg/kg) was administered via bolus tail vein injection. Whole blood was collected into lithium heparin tubes from the carotid artery cannula at the indicated time points. Plasma was collected by centrifuging blood at 2,700*g* for 10 minutes at 4 °C, and was used for measurement of α-Gal A activity as described below.

Coadministration studies in GLA KO mice. In the studies shown in **Figure 4** and **Table 2**, 12-week old male *GLA* KO mice were administered rhα-Gal A (0.1, 0.3, 1, or 3 mg/kg) via bolus tail vein injection. Mice were coadministered vehicle (water) or AT1001 (30, 100, or 300 mg/kg) via oral gavage, 30 minutes before, and 2 hours after, rhα-Gal A administration. Mice were euthanized 3 or 7 days after rhα-Gal A administration as indicated. In the studies shown in **Figure 5**, 12-week old male *GLA* KO mice were administered rhα-Gal A (1 mg/kg) via bolus tail vein injection. Mice were coadministered vehicle (water) or AT1001 (3 or 10 mg/kg, which result in plasma C_{max} values of ~10 µmol/l and 30 µmol/l, respectively, that are roughly equivalent to the C_{max} values seen in humans following oral administration of 150 and 450 mg AT1001, respectively) via oral gavage, 30 minutes before rhα-Gal A administration. Mice were killed 1 or 7 days following rhα-Gal A administration. Heart, kidney, and skin (shaved and removed from the lower ventral side of the neck) were quickly removed, rinsed in cold phosphate-buffered saline, blotted dry, and stored on dry ice. Samples of heart and kidney were stored in 4% paraformaldehyde for immunohistochemical analysis. Tissue α-Gal A activity, protein, and GL-3 levels were assessed using methodologies described previously.[19] Baseline α-Gal A activities in C57BL/6 mouse skin, heart, and kidney were 9 ± 0.5, 2.5 ± 0.2, and 8 ± 0.4 nmol/mg protein/hour, respectively; baseline activities in *GLA* KO mouse tissues were 0.5 ± 0.04, 0.1 ± 0.04, and 0.3 ± 0.02, nmol/mg protein/hour, respectively. Baseline GL-3 levels in C57BL/6 mouse skin, heart, and kidney were 5 ± 0.6, 8.4 ± 1, and 74 ± 7 µg/g tissue weight, respectively; baseline levels in *GLA* KO mice were 1,500 ± 123, 387 ± 22, and 503 ± 24 µg/g tissue, respectively. For plasma α-Gal A activity measurements, samples were diluted 50-fold before assay; ~10 µg of total protein was used for western blotting.

Data analysis. Determinations of statistical significance were conducted using Excel 2003 (Microsoft, Redmond, WA) or GraphPad Prism, version 5 (San Diego, CA) as defined in the figure and table legends. Linear trends for dose-dependence were calculated using a one-way ANOVA in GraphPad Prism. The half-life ($t_{1/2}$) of rhα-Gal A in plasma was calculated using a nonlinear one-phase exponential decay curve fitting function in GraphPad Prism.

SUPPLEMENTARY MATERIAL

Figure S1. Fabry fibroblast cell lines show high baseline GL-3 levels that are decreased after incubation with rhα-Gal A.

Figure S2. Concentration- and time-dependence of rhα-Gal A-mediated effects on Fabry fibroblasts.
Figure S3. The effects of rhα-Gal A and AT1001 in Fabry fibroblasts (C52S) are dependent on the timing of coincubation.
Figure S4. rhα-Gal A dose-dependently increases in α-Gal A activity and decreases in GL-3 levels in *GLA* KO mice.
Table S1. Coadministration of AT1001 at doses that yield clinically achievable exposures in humans increases the tissue levels of rhα-Gal A in *GLA* KO mice.

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REFERENCES

1. Brady, RO, Gal, AE, Bradley, RM, Martensson, E, Warshaw, AL and Laster, L (1967). Enzymatic defect in Fabry's disease. Ceramidetrihexosidase deficiency. *N Engl J Med* **276**: 1163–1167.
2. Desnick, RJ, Ioannou, YA and Eng CM (2001). *α-Galactosidase A deficiency: Fabry disease*. In: *The Metabolic and Molecular Bases of Inherited Disease*. New York, NY: McGraw Hill, pp. 3733–3774.
3. Askari, H, Kaneski, CR, Semino-Mora, C, Desai, P, Ang, A, Kleiner, DE *et al.* (2007). Cellular and tissue localization of globotriaosylceramide in Fabry disease. *Virchows Arch* **451**: 823–834.
4. Branton, MH, Schiffmann, R, Sabnis, SG, Murray, GJ, Quirk, JM, Altarescu, G *et al.* (2002). Natural history of Fabry renal disease: influence of alpha-galactosidase A activity and genetic mutations on clinical course. *Medicine (Baltimore)* **81**: 122–138.
5. Eng, CM, Banikazemi, M, Gordon, RE, Goldman, M, Phelps, R, Kim, L *et al.* (2001). A phase ½ clinical trial of enzyme replacement in fabry disease: pharmacokinetic, substrate clearance, and safety studies. *Am J Hum Genet* **68**: 711–722.
6. Schiffmann, R, Kopp, JB, Austin, HA 3rd, Sabnis, S, Moore, DF, Weibel, T *et al.* (2001). Enzyme replacement therapy in Fabry disease: a randomized controlled trial. *JAMA* **285**: 2743–2749.
7. West, M, Nicholls, K, Mehta, A, Clarke, JT, Steiner, R, Beck, M *et al.* (2009). Agalsidase alfa and kidney dysfunction in Fabry disease. *J Am Soc Nephrol* **20**: 1132–1139.
8. Hughes, DA (2008). Early therapeutic intervention in females with Fabry disease? *Acta Paediatr Suppl* **97**: 41–47.
9. Schiffmann, R, Floeter, MK, Dambrosia, JM, Gupta, S, Moore, DF, Sharabi, Y *et al.* (2003). Enzyme replacement therapy improves peripheral nerve and sweat function in Fabry disease. *Muscle Nerve* **28**: 703–710.
10. Thurberg, BL, Rennke, H, Colvin, RB, Dikman, S, Gordon, RE, Collins, AB *et al.* (2002). Globotriaosylceramide accumulation in the Fabry kidney is cleared from multiple cell types after enzyme replacement therapy. *Kidney Int* **62**: 1933–1946.
11. Bénichou, B, Goyal, S, Sung, C, Norfleet, AM and O'Brien, F (2009). A retrospective analysis of the potential impact of IgG antibodies to agalsidase beta on efficacy during enzyme replacement therapy for Fabry disease. *Mol Genet Metab* **96**: 4–12.
12. Bodensteiner, D, Scott, CR, Sims, KB, Shepherd, GM, Cintron, RD and Germain, DP (2008). Successful reinstitution of agalsidase beta therapy in Fabry disease patients with previous IgE-antibody or skin-test reactivity to the recombinant enzyme. *Genet Med* **10**: 353–358.
13. Tesmoingt, C, Lidove, O, Reberga, A, Thetis, M, Ackaert, C, Nicaise, P *et al.* (2009). Enzyme therapy in Fabry disease: severe adverse events associated with anti-agalsidase cross-reactive IgG antibodies. *Br J Clin Pharmacol* **68**: 765–769.
14. Fan, JQ and Ishii, S (2003). Cell-based screening of active-site specific chaperone for the treatment of Fabry disease. *Meth Enzymol* **363**: 412–420.
15. Asano, N, Ishii, S, Kizu, H, Ikeda, K, Yasuda, K, Kato, A *et al.* (2000). *In vitro* inhibition and intracellular enhancement of lysosomal alpha-galactosidase A activity in Fabry lymphoblasts by 1-deoxygalactonojirimycin and its derivatives. *Eur J Biochem* **267**: 4179–4186.
16. Fan, JQ, Ishii, S, Asano, N and Suzuki, Y (1999). Accelerated transport and maturation of lysosomal alpha-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nat Med* **5**: 112–115.
17. Benjamin, ER, Flanagan, JJ, Schilling, A, Chang, HH, Agarwal, L, Katz, E *et al.* (2009). The pharmacological chaperone 1-deoxygalactonojirimycin increases alpha-galactosidase A levels in Fabry patient cell lines. *J Inherit Metab Dis* **32**: 424–440.
18. Ishii, S, Chang, HH, Yoshioka, H, Shimada, T, Mannen, K, Higuchi, Y *et al.* (2009). Preclinical efficacy and safety of 1-deoxygalactonojirimycin in mice for Fabry disease. *J Pharmacol Exp Ther* **328**: 723–731.
19. Khanna, R, Soska, R, Lun, Y, Feng, J, Frascella, M, Young, B *et al.* (2010). The pharmacological chaperone 1-deoxygalactonojirimycin reduces tissue globotriaosylceramide levels in a mouse model of Fabry disease. *Mol Ther* **18**: 23–33.
20. Yam, GH, Bosshard, N, Zuber, C, Steinmann, B and Roth, J (2006). Pharmacological chaperone corrects lysosomal storage in Fabry disease caused by trafficking-incompetent variants. *Am J Physiol, Cell Physiol* **290**: C1076–C1082.
21. Schiffmann, R, Fernhoff, P, Germain, D, Hughes, D, Mehta, A, Nicholls, K *et al.* Long-term safety, tolerability, and assessments of renal function in adult Fabry patients receiving treatment with AT1001 (migalastat hydrochloride), a pharmacological chaperone. In: Presented at the 7th Annual WORLD Symposium, 2011, Las Vegas, NV.
22. Valenzano, KJ, Khanna, R, Powe, AC, Boyd, R, Lee, G, Flanagan, JJ *et al.* (2011). Identification and characterization of pharmacological chaperones to correct enzyme deficiencies in lysosomal storage disorders. *Assay Drug Dev Technol* **9**: 213–235.
23. Porto, C, Cardone, M, Fontana, F, Rossi, B, Tuzzi, MR, Tarallo, A *et al.* (2009). The pharmacological chaperone N-butyldeoxynojirimycin enhances enzyme replacement therapy in Pompe disease fibroblasts. *Mol Ther* **17**: 964–971.
24. Shen, JS, Edwards, NJ, Hong, YB and Murray, GJ (2008). Isofagomine increases lysosomal delivery of exogenous glucocerebrosidase. *Biochem Biophys Res Commun* **369**: 1071–1075.
25. Niesen, FH, Berglund, H and Vedadi, M (2007). The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat Protoc* **2**: 2212–2221.
26. Schiffmann, R, Germain, DP, Castelli, J, Shenker, A and Lockhart, DJ (2008). 768/T. Phase 2 clinical trials of the pharmacological chaperone AT1001 for the treatment of Fabry disease. In: *768/T. Phase 2 Clinical Trials of the Pharmacological Chaperone AT1001 for the Treatment of Fabry Disease*.
27. Lieberman, RL, D'aquino, JA, Ringe, D and Petsko, GA (2009). Effects of pH and iminosugar pharmacological chaperones on lysosomal glycosidase structure and stability. *Biochemistry* **48**: 4816–4827.
28. Eng, CM, Guffon, N, Wilcox, WR, Germain, DP, Lee, P, Waldek, S *et al.*; International Collaborative Fabry Disease Study Group. (2001). Safety and efficacy of recombinant human alpha-galactosidase A–replacement therapy in Fabry's disease. *N Engl J Med* **345**: 9–16.
29. Germain, DP (2010). Fabry disease. *Orphanet J Rare Dis* **5**: 30.
30. Germain, DP, Waldek, S, Banikazemi, M, Bushinsky, DA, Charrow, J, Desnick, RJ *et al.* (2007). Sustained, long-term renal stabilization after 54 months of agalsidase beta therapy in patients with Fabry disease. *J Am Soc Nephrol* **18**: 1547–1557.
31. Keslová-Veselíková, J, Hulková, H, Dobrovolný, R, Asfaw, B, Poupetová, H, Berná, L *et al.* (2008). Replacement of alpha-galactosidase A in Fabry disease: effect on fibroblast cultures compared with biopsied tissues of treated patients. *Virchows Arch* **452**: 651–665.
32. Schiffmann, R, Rapkiewicz, A, Abu-Asab, M, Ries, M, Askari, H, Tsokos, M *et al.* (2006). Pathological findings in a patient with Fabry disease who died after 2.5 years of enzyme replacement. *Virchows Arch* **448**: 337–343.
33. Thurberg, B, Rennke, H, Colvin, R, Dikman, S, Gordon, R, O'Callaghan, M (2008). 96. Pathology of Fabry nephropathy: Renal fibrosis may begin in adolescence. *Mol Genet Metabol* **93**: 39.
34. Yam, GH, Zuber, C and Roth, J (2005). A synthetic chaperone corrects the trafficking defect and disease phenotype in a protein misfolding disorder. *FASEB J* **19**: 12–18.
35. Flanagan, JJ, Rossi, B, Tang, K, Wu, X, Mascioli, K, Donaudy, F *et al.* (2009). The pharmacological chaperone 1-deoxynojirimycin increases the activity and lysosomal trafficking of multiple mutant forms of acid alpha-glucosidase. *Hum Mutat* **30**: 1683–1692.
36. Kornhaber, GJ, Tropak, MB, Maegawa, GH, Tuske, SJ, Coales, SJ, Mahuran, DJ *et al.* (2008). Isofagomine induced stabilization of glucocerebrosidase. *Chembiochem* **9**: 2643–2649.
37. Lee, K, Jin, X, Zhang, K, Copertino, L, Andrews, L, Baker-Malcolm, J *et al.* (2003). A biochemical and pharmacological comparison of enzyme replacement therapies for the glycolipid storage disorder Fabry disease. *Glycobiology* **13**: 305–313.
38. Ioannou, YA, Zeidner, KM, Gordon, RE and Desnick, RJ (2001). Fabry disease: preclinical studies demonstrate the effectiveness of alpha-galactosidase A replacement in enzyme-deficient mice. *Am J Hum Genet* **68**: 14–25.
39. Shu, L and Shayman, JA (2007). Caveolin-associated accumulation of globotriaosylceramide in the vascular endothelium of alpha-galactosidase A null mice. *J Biol Chem* **282**: 20960–20967.
40. Clarke, JT, West, ML, Bultas, J and Schiffmann, R (2007). The pharmacology of multiple regimens of agalsidase alfa enzyme replacement therapy for Fabry disease. *Genet Med* **9**: 504–509.
41. Fabrazyme package insert. In: (eds.) Fabrazyme. Genzyme Corp. (2006).