Liquid Chromatography - Tandem Mass Spectrometry Determination of AT2220 in Rodent Plasma and Tissues

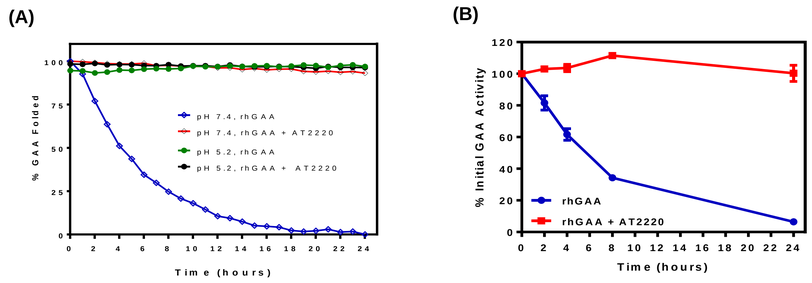
Leo B. Dungan, Hui H. Chang, Rick Hamler, Nastry Brignol, Su Xu, Richie Khanna, John J. Flanagan, David J. Lockhart, Kenneth J. Valenzano, and Elfrida R. Benjamin

Amicus Therapeutics, 1 Cedar Brook Drive, Cranbury, NJ 08512

Introduction

Pompe disease is an inherited lysosomal storage disease that results from deficiency in acid α-glucosidase (GAA) activity, and is characterized by accumulation of lysosomal glycogen primarily in heart and skeletal muscles. While recombinant human GAA (rhGAA; alglucosidase alfa; Lumizyme; Genzyme-Sanofi) provides some clinical benefits, the infused enzyme tends to be unstable at neutral pH/body temperature, shows insufficient uptake in key tissues, and can elicit immune responses that affect tolerability and efficacy. The pharmacological chaperone AT2220 (1-deoxynojirimycin HCl, duvoglustat HCl) has been shown to improve the pharmacological properties of rhGAA via direct binding and stabilization, leading to increased enzyme uptake and glycogen reduction in *Gaa* knock-out (KO) mice when co-administered (prior to rhGAA) or co-formulated with rhGAA. Liquid chromatography tandem mass spectrometric (LC-MS/MS) quantitation of AT2220 in animal plasma and tissues is challenging due the compound's polarity, low molecular weight, and high baselines observed in mass spectra. A LC-MS/MS method has been developed for the quantitation of AT2220 in rodent plasma, and the disease-relevant tissues heart and skeletal muscle (quadriceps). This method has enabled *in vivo* preclinical studies of AT2220 co-formulated or co-administered with rhGAA.

1. AT2220 Prevents rhGAA Denaturation and Loss of Activity *in vitro* and *ex vivo*



A. The stability of rhGAA (1 μM) ± AT2220 (50 μM) was measured as a function of time in buffers of neutral and acidic pH at 37 °C using SYPRO® Orange, which binds to exposed hydrophobic residues when proteins denature. **B**. The activity of rhGAA (0.5 μM) ± AT2220 (50 μM) was also measured in human blood (~ pH 7.4) at 37 °C in a 4-MUG (4-methyl-umbelliferyl-alpha-D-glucopyranoside) assay. While rhGAA remained stable at pH 5.2 (a condition mimicking the acidic environment of the lysosome), the protein quickly unfolded at pH 7.4 ($t_{1/2}$ ≈ 3 hr) and lost its activity by the end of a 24-hr period. Co-incubation with AT2220 stabilized rhGAA, preventing its pH-, temperature-, and time-dependent denaturation and inactivation, thus allowing the protein to retain its structural integrity and activity over a 24-hr period.

2. LC-MS/MS Method for Quantifying AT2220 in Rodent Plasma and Muscle

Sample Preparation:

Step I: For tissue homogenate, accurately weigh 25 to 35 mg of rodent muscle tissue into a FastPrep® tube. Add Lysing Matrix (MP Biomedicals, LLC) to each tube. Add 7 μL deionized water (dH_2O) per mg of tissue and homogenize using FastPrep 24® instrument.

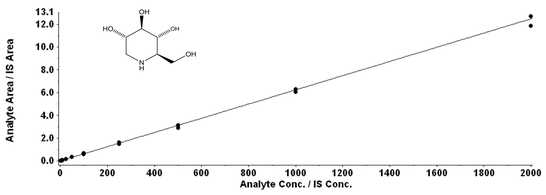
Step II: Transfer 50 μL plasma or 100 μL tissue homogenate (from Step I) into a labeled microcentrifuge tube. Add 50 μL dH_2O containing 500 ng/mL AT2220-$^{13}C_6$ internal standard. Add 600 μL 5 mM HCl in 95:5 (MeOH:H_2O). Shake on a multi-tube vortexer for approximately 2 minutes. Centrifuge for 10 minutes at room temperature at 20,000 x g. Transfer supernatant to a 96-well, 2 mL, square top, plate. Add 750 μL 5 mM HCl in dH_2O to each well.

Step III: Transfer the mixture to a pre-conditioned Waters Oasis® MCX (30 mg) SPE 96-well plate. Wash with 1000 μL 5 mM HCl in dH_2O. Wash with 750 μL 2.5 mM HCl in 1:1 (MeOH:dH_2O). Wash with 750 μL 0.1% formic acid in MeOH. Wash with 750 μL MeOH. Elute the analyte with 2 x 375 μL of 1% NH_4OH in 90/10 (MeOH:dH_2O). Place sample plate in a TurboVap 96® (Biotage) and evaporate eluant to dryness under N_2. Reconstitute the sample with 120 μL mobile phase A. Inject 40 μL of the extract onto the HPLC column for LC-MS/MS analysis.

HPLC Conditions:
Equipment: Shimadzu LC-30AD Pumps and SIL-30AC Autosampler.
Mobile Phase A: 95/5 acetonitrile/H2O + 0.5% formic acid + 5 mM ammonium formate.
Mobile Phase B: 5/47.5/47.5 acetonitrile/MeOH/H2O + 0.5% formic acid + 5 mM ammonium formate.
Elution: Isocratic, 30% mobile phase B. Flow Rate: 0.7 mL/min. Total run time: 7.2 minutes.
HPLC column: Mac-Mod Halo HILIC Silica 2.7 μm, 4.6x150 mm.

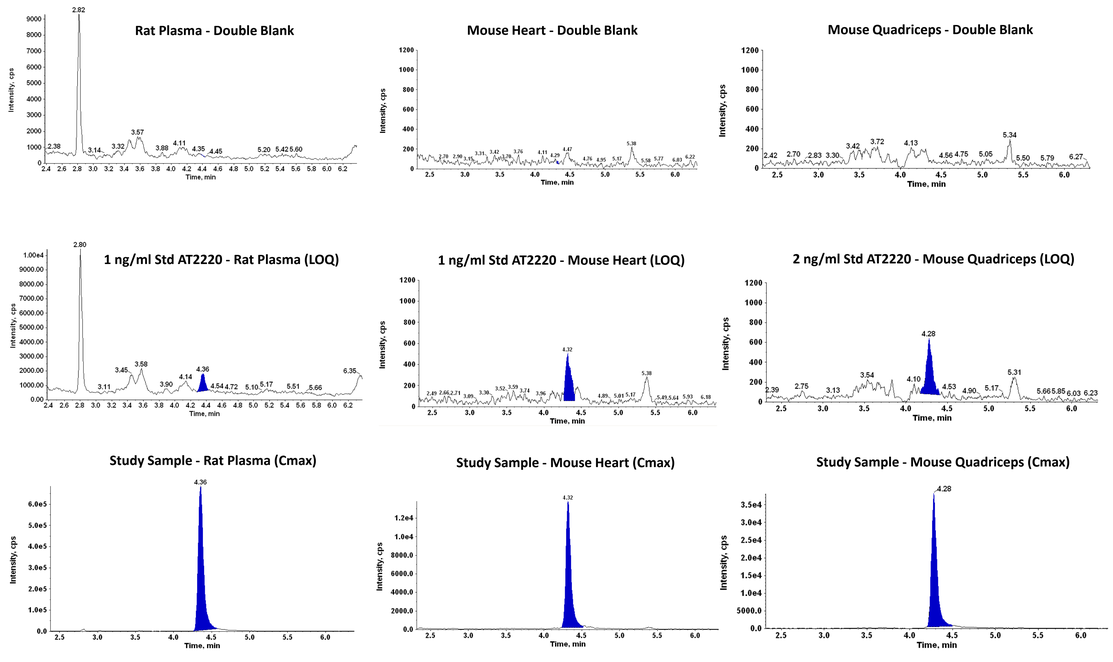
Mass Spectrometry Parameters and Conditions:
Mass Spectrometer: ABSciex 5500QTRAP LC-MS/MS system – APCI ionization operated in positive ion mode.
MRM Transitions: AT2220, 163.98 / 80.0, Internal Standard (AT2220-$^{13}C_6$) 170.10 / 85.1
DP (volts): 50, CE (volts): 26, CXP (volts): 10, EP (volts): 10

3. Representative Calibration Curve of AT2220 in Rat Plasma

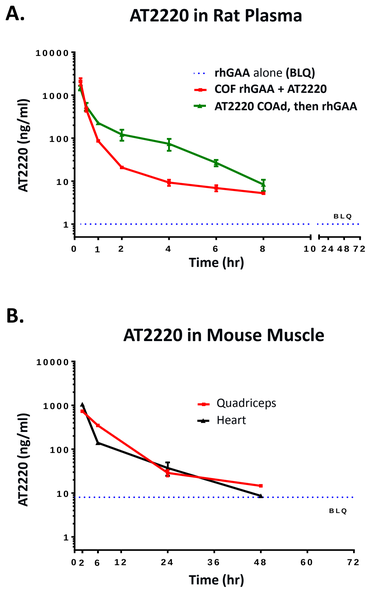


- Heparinized plasma from male Sprague Dawley rats was spiked with known concentrations of AT2220 in order to generate an 11-point calibration set. After SPE extraction and LC-MS/MS analysis, the resulting AT2220 concentrations were plotted using linear regression with $1/x^2$ weighting.
- For rat plasma, the correlation coefficient (R) is R = 0.9982.
- The rat plasma AT2220 assay range is 1.00 - 2000 ng/mL (6.13nM - 12.3 μM).
- Inter-day mean accuracy (%Bias) ranged from -1.06 – 15.7 and inter-day precision (%CV) ranged from 3.91 – 11.1.
- The assay range for mouse heart and quadriceps tissue homogenate is 1.00 – 2000 ng/mL (6.13 nM - 12.3 μM) and 2.00 – 2000 ng/mL (12.3 nM - 12.3 μM), respectively. This equates to 8.00 – 16000 ng/g tissue (49.0 nM – 98.1 μM) and 16.0 – 16000 ng/g tissue (98.0 nM – 98.1 μM), respectively.
- R values, mean accuracy, and precision were similar for the mouse heart and quadriceps tissue analysis (data not shown).

4. LC-MS/MS Chromatograms of AT2220 Extracted from Rat Plasma and Disease-Relevant Mouse Tissues



5. AT2220 Levels were Detected in Rat Plasma and Disease-Relevant Mouse Tissues

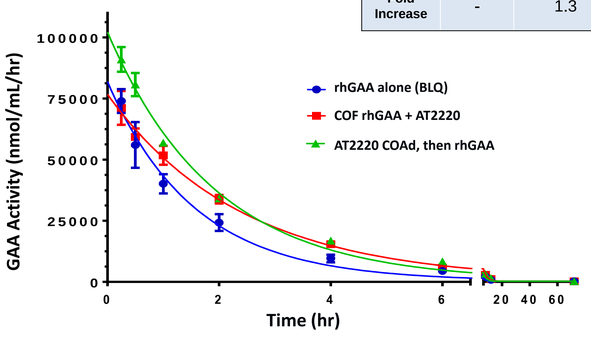


A. Eight-week-old male Sprague Dawley rats (n=3/group) were given a single tail vein bolus administration of rhGAA (20 mg/kg) ± co-formulated (COF) AT2220 (10 mg/kg), or 30 minutes following an AT2220 (10 mg/kg) oral co-administration (COAd). Plasma samples were taken at various time points and AT2220 levels were measured by LC-MS/MS. AT2220 levels were detected up to 8 hrs when administered as co-formulation or co-administration with rhGAA.

B. Wild-type mice were given a single oral gavage administration of AT2220 (30 mg/kg). Tissue samples were collected at various time points following the oral dose, flash frozen, and AT2220 levels measured by LC-MS/MS. AT2220 levels were BLQ for all 72 hour samples.

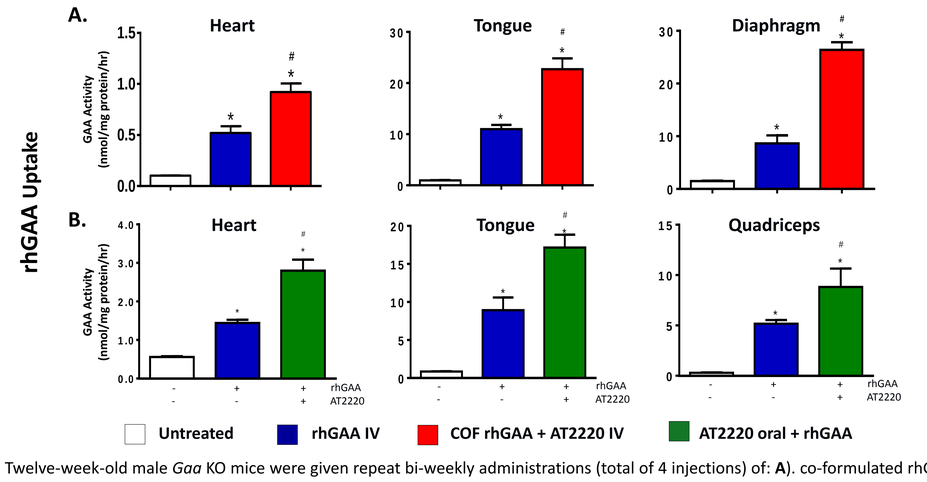
6. Co-formulation or Co-administration of AT2220 Increases the Circulating Levels of Active rhGAA in Rats

	rhGAA	rhGAA + AT2220	AT2220 oral, then rhGAA
AUC	167112	215452	253925
Fold Increase	-	1.3	1.5



Eight-week-old male Sprague-Dawley rats (n=3/group) were given a single administration of rhGAA via tail vein bolus injection. The injections were given with or without AT2220 through various routes of administration. 1) rhGAA alone (20 mg/kg) blue, 2) rhGAA (20 mg/kg) + co-formulated AT2220 (10 mg/kg) red or 3) rhGAA (20 mg/kg) 30 minutes following an AT2220 (10 mg/kg) oral co-administration green. Plasma GAA activity was measured at various time points. Co-formulated and co-administered AT2220 with rhGAA increased the total GAA plasma activity (AUCs) by 1.3 fold and 1.5 fold, respectively.

7. Co-formulation or Co-administration of AT2220 Leads to Greater Enzyme Uptake Compared to rhGAA Alone in Disease-Relevant Tissues of *Gaa* KO mice



Twelve-week-old male *Gaa* KO mice were given repeat bi-weekly administrations (total of 4 injections) of: **A**). co-formulated rhGAA (20 mg/kg) ± AT2220 (30 mg/kg) via bolus tail vein injection (upper panels); or **B**). rhGAA (20 mg/kg) via bolus tail vein injection ± oral AT2220 (30 mg/kg), 30 minutes before IV (lower panels). Tissues were analyzed for GAA activity (using 4-MUG) 7 days post-last IV injection. Significantly greater rhGAA uptake (up to 2.5-fold) was observed in multiple disease-relevant tissues when AT2220 was administered in combination with rhGAA (as co-formulation or co-administration), compared to rhGAA IV alone. The bar graphs represent the mean ± SEM of six mice per group. *p<0.05 vs. untreated and # p<0.05 vs. rhGAA IV alone; t-test

Conclusions

- AT2220 increases the physical stability of rhGAA at neutral pH and body temperature *in vitro*.
- AT2220 maintains rhGAA in a stable active form *ex vivo* in human whole blood at neutral pH and body temperature.
- A LC-MS/MS method was developed for the quantitation of AT2220 in rodent plasma and disease-relevant heart and quadriceps tissue. The method showed good linearity, dynamic range, accuracy and precision, allowing reliable AT2220 quantitation.
- AT2220 levels were measured in plasma up to 8 hours from rats given a single tail vein bolus administration of rhGAA (20 mg/kg) ± co-formulated AT2220 (10 mg/kg), or 30 minutes following an AT2220 oral (10 mg/kg) co-administration.
- AT2220 levels were measured in heart and quadriceps tissue from wild-type mice given a single oral dose (30 mg/kg) and were not detectable (BQL) after 72 hours.
- In rats, oral co-administration and IV administration of co-formulated AT2220 + rhGAA increases the exposure of rhGAA 1.5 fold and 1.3 fold respectively.
- Up to 2.5 fold greater enzyme uptake was achieved in disease-relevant tissues of *Gaa* KO mice given four bi-weekly IV bolus injections of rhGAA (20 mg/kg) ± co-formulated AT2220 (30 mg/kg) or orally co-administered AT2220 (30 mg/kg).
- The LC-MS/MS method allowed for the analysis of technically challenging, disease-relevant rodent muscle tissues, and enabled measurement of AT2220 after co-formulation or co-administration with rhGAA in preclinical studies.