

THE PHARMACOLOGICAL CHAPERONE AT2101 INCREASES β -GLUCOCEREBROSIDASE LEVELS IN MACROPHAGES AND LYMPHOBLASTS DERIVED FROM GAUCHER PATIENTS

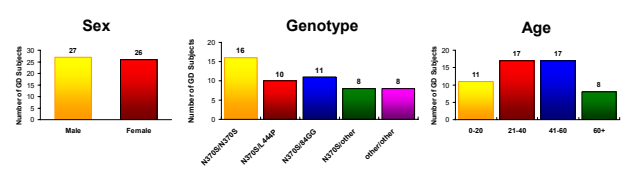


Corey W. Pine¹, Brian E. Ranes¹, Frank Insinga¹, Karin Ludwig¹, John Flanagan¹, Gregory A. Grabowski², Neal J. Weinreb³, Gregory M. Pastores⁴, Daniel Gruskin⁵, Paige Kaplan⁶, Hung Do¹, David J. Lockhart¹ and **Brandon A. Wustman¹** ¹Amicus Therapeutics, 6 Cedar Brook Drive, Cranbury, NJ; ²Division of Human Genetics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; ³University Research Foundation for Lysosomal Storage Diseases Inc, Northwest Oncology Hematology Associates, Coral Springs, FL; ⁴Departments of Neurology and Pediatrics, New York University School of Medicine, New York, NY; ⁵Departments of Human Genetics and Pediatrics, Emory University School of Medicine, Atlanta, GA; ⁶Section of Metabolic Diseases, Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA

Introduction

Gaucher disease (GD) is caused by a deficiency of lysosomal glucocerebrosidase (GCase). Deficient GCase activity leads to an accumulation of glucosylceramide (GlcCer) and the development of symptoms such as anemia, thrombocytopenia, hepatosplenomegaly, bone necrosis, infarcts and osteoporosis, and in some cases, neuroopathic disease. The pharmacological chaperone AT2101 (isofagomine-tartrate) selectively binds and stabilizes mutant (N370S/N370S) GCase in the ER and increases its trafficking to the lysosome. In Phase 1 trials, AT2101 was well tolerated with no serious adverse events and increased GCase levels in white blood cells (WBC) up to ~3.5-fold. To evaluate the effects of AT2101 on different GCase variants, we conducted an ex vivo response study using patient-derived macrophages and Epstein Barr Virus (EBV)-transformed lymphocytes. We also screened plasma for potential biomarkers associated with inflammation, bone metabolism, multiple myeloma and neurodegeneration. The study was conducted on samples from 53 Gaucher patients enrolled at 5 sites in the United States.

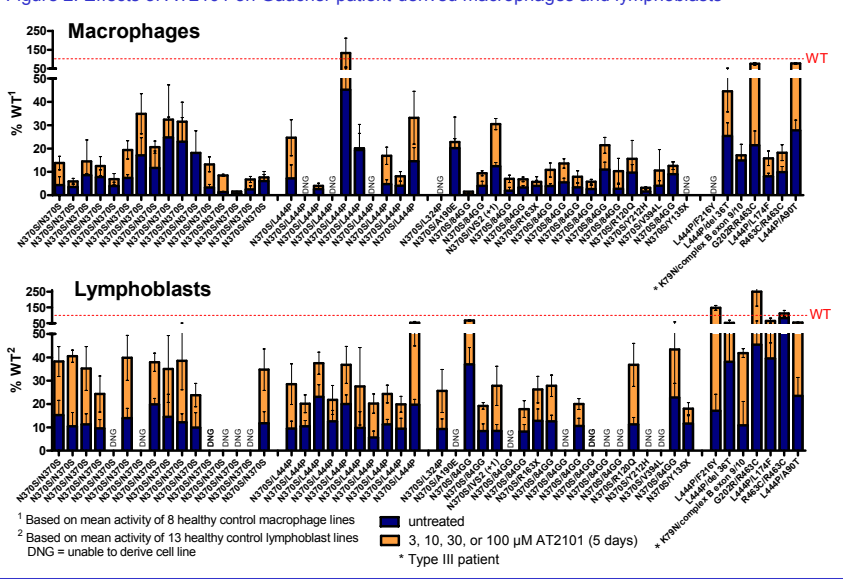
Figure 1. Demographics of Gaucher patients in the ex vivo response study



Results & Discussion

The study included 26 males with type I GD, 1 male with type II GD, and 26 females with type I representing 18 different genotypes (Fig. 1). Patients ranged in age from 7 to 83 years; 50 of 53 patients were receiving enzyme replacement therapy and blood was drawn immediately prior to enzyme infusion. Macrophages were successfully derived from 46 of 53 patients and incubation with AT2101 (3, 10, 30 or 100 μ M) for 5 days increased GCase levels in macrophages from 42 of 46 patients (mean = 2.6-fold; range: 1.4- to 6.5-fold; p value < 0.05 for 29 of 42; Fig. 2). Lymphoblasts cell lines were also derived from 38 of 53 patients and incubation with AT2101 significantly increased (p value < 0.05) GCase levels from all 38 patients (mean = 2.7-fold; range: 1.4- to 8.6-fold; Fig. 2), including 3 of the 4 macrophages which showed no response to AT2101. Residual activity levels and response to AT2101 were more consistent in lymphoblasts when measured in lymphoblasts compared to macrophages, potentially due to the variability in macrophage viability between different patients. Analysis of untreated WBCs yielded reduced GCase activity compared to controls and normal GlcCer levels (most patients were receiving ERT) (Fig. 3). Plasma levels of human pulmonary and activation-regulated chemokine (PARC, CCL18) and chitotriosidase activity have been shown to correlate with disease severity and response to treatment. In this study, PARC and chitotriosidase activity remained significantly elevated in most GD patients compared to controls (Fig. 3). Markers of osteoclast (tartrate-resistant acid phosphatase, TRAP 5b) and osteoblast (bone-specific alkaline phosphatase, BAP) activities were abnormal for 44 of 53 patients. Elevated TRAP 5b activity was observed for 14 males and 8 females, while 11 males and 16 females had lower than normal BAP levels (Fig. 4). These results suggest that bone metabolism is altered in most patients, favoring osteoclast activity and bone resorption. Interestingly, proinflammatory cytokines and chemokines IL-8, IL-17, VEGF and MIP-1 α were elevated in some patients compared to controls, and a correlation was observed between IL-17 and VEGF levels (Fig. 5). These cytokines and chemokines have also been implicated in the pathogenesis of multiple myeloma, which may be relevant to GD since it has been reported that Gaucher patients have an increased risk for developing multiple myeloma. Plasma levels of other proinflammatory (IL-1 [α , β], IL-6, IL-7, IL-12p40, IL-12p70, IL-15, IL-17, fractalkin, EGF, TG β , sCD40L, GM-CSF, eotaxin, sCD14, IP-10, IFN- γ , G-CSF, MIP-1 β , TNF α , HSP60, HSP70), anti-inflammatory (IL-1ra, IL-2, IL-4, IL-5, IL-10, IL-13) and cardiovascular (CRP, SAA, SAP) markers were unremarkable for most GD patients when compared to controls. Since multiple studies have identified mutations in Gba, the gene that encodes for GCase, as a potential risk factor for synucleinopathies, we screened for plasma levels of α -synuclein. Surprisingly, GD patients showed elevated levels of total α -synuclein compared to controls (Fig. 6). The plasma level of α -synuclein has been investigated as a potential biomarker for Parkinson's disease; however, contradictory results have been published and the usefulness is still a matter of debate. Finally, we screened patient cell lines and transfected COS7 cells for response to AT2101 and found that many GCase variants can be rescued including those with mutations far from the enzyme's active site (Fig. 7).

Figure 2. Effects of AT2101 on Gaucher patient-derived macrophages and lymphoblasts



1 Based on mean activity of 8 healthy control macrophage lines
2 Based on mean activity of 13 healthy control lymphoblast lines
DNG = unable to derive cell line
* Type III patient

Figure 3. GCase, GlcCer, chitotriosidase and PARC levels in WBCs and plasma

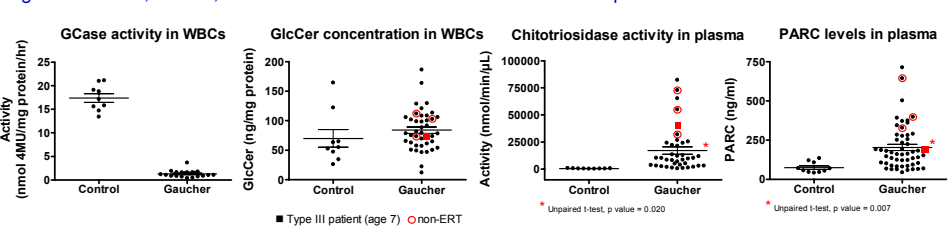
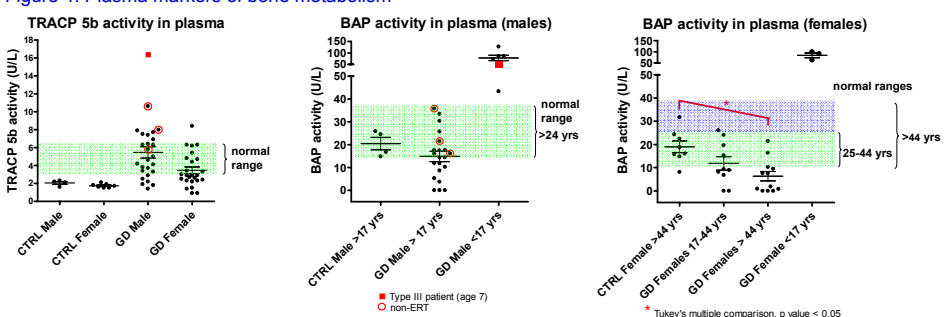


Figure 4. Plasma markers of bone metabolism



Materials & Methods

White blood cells Anticoagulated venous blood was layered onto a Ficoll density gradient, centrifuged and mononuclear cells at the plasma-Ficoll interface were removed, washed and used for the establishment of macrophage and lymphoblast cultures or frozen as pellets for GCase and GlcCer analyses.
Macrophage cultures Cells isolated from the plasma-Ficoll interface were allowed to adhere to the surface of 24-well plates, washed to remove other leukocytes and treated with M-CSF (50 ng/ml, 48-96 hrs) to initiate differentiation in to macrophages.
Lymphoblasts The lymphocytes were transformed with the Epstein Barr Virus and cultured (RPMI 1640 with 10% FBS, 1% PS, 1% L-Glut) for an additional 8-10 weeks to establish pure lymphoblast cultures.
Intracellular activity WBC, macrophages and lymphoblasts derived from patients and healthy controls were cultured with or without AT2101 (3, 10, 30 or 100 μ M) for 5 days, washed and cell lysates were assayed for protein concentration and activity (with and without CBE) using the substrate 4-MU- β -Glc. Activity was expressed as CBE sensitive activity (nmol of 4-MU releasing total protein/hr). Changes in GCase activity were tested for statistical significance using one-way ANOVA (parametric) and Dunnett's multiple comparison tests (95% CI).
Transient expression of GCase in COS7 cells *In vitro* mutagenesis was performed to introduce specific missense mutations and the effects of incubation with AT2101 (2 days) on these GCase variants were assessed by transient expression in COS7 cells.
ELISAs ELISAs for Glucosylceramides were isolated from WBC pellets using solid phase extraction (Bond Elut C18 cartridges) and analyzed by LC-MS/MS with ESI (Shimadzu LC-10 AD and Finnigan TSQ Ultra).
ELISAs ELISAs were purchased from Quidel Corp., SBA Sciences, R&D Systems, Biosource and Assay Designs (Ann Arbor, MI), and manufacturer's instructions for assaying plasma samples were followed for all assays.
Cytokine and cardiovascular panels Plasma from GD patients and healthy controls were screened against a panel of 39 cytokines and 3 cardiovascular markers by Millipore.
GCase crystal structure GCase-AT2101 crystallization studies were carried out as described in Lieberman et al. 2006, Nat. Chem. Biol. 3:101-107.

Figure 5. Plasma levels of proinflammatory cytokines

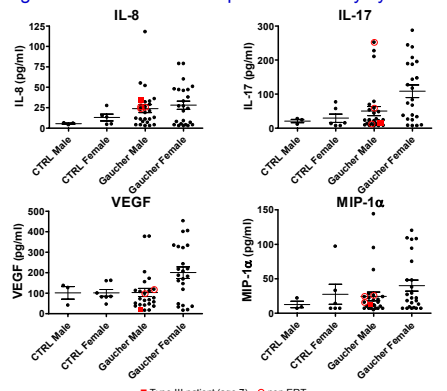


Figure 6. Plasma levels of α -synuclein

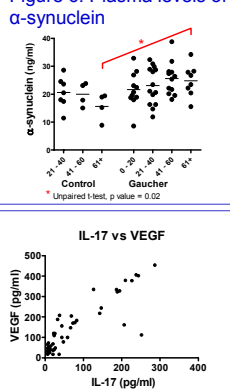


Figure 7. GCase structure with AT2101-responsive mutations

