

**Title: Identification of Modulators of the N370S Mutant Form of Glucocerebrosidase as a Potential Therapy for Gaucher Disease**

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**Chemistry Center Name & PI:** NIH Chemical Genomics Center, Dr. Christopher P. Austin

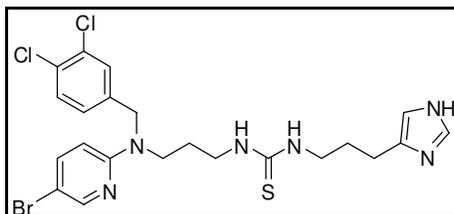
**Assay Sunbmitter & Institution:** NIH Human Genome Research Institute, Dr. Ellen Sidransky

**PubChem Summary Bioassay Identifier (AID):** 2593

**Abstract:**

Known glycosidase chaperone molecules belong to the aminosugar class. Because iminosugar inhibitors work by mimicking the transition state of the glycosidic cleavage, they tend to be poorly selective. This has hampered their advance in clinical development. Alternative scaffolds with chaperone activity are quite desirable. Here, we present a new non-aminosugar series of glucocerebrosidase inhibitors having chaperone capacity. The probe, ML156 (CID 9893924), is able to inhibit the hydrolytic activity of the N370S mutant form of glucocerebrosidase. Most importantly, ML156 (CID 9893924) increased glucocerebrosidase translocation to the lysosome in Gaucher patient-derived fibroblasts homozygous for the N370S mutation, and can be used to study ER-lysosomal trafficking of clinically relevant GC mutants *in vitro*. This probe may be a useful lead for the pre-clinical development of a chemical chaperone of glucocerebrosidase.

### Probe Structure & Characteristics:

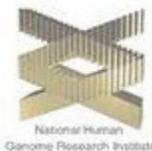


**ML156**

CID/ML#	Target Name	IC <sub>50</sub> /EC <sub>50</sub> (nM) [SID, AID]	Anti-target Name(s)	IC <sub>50</sub> /EC <sub>50</sub> (μM) [SID, AID]	Fold Selective	Secondary Assay(s) Name: IC <sub>50</sub> /EC <sub>50</sub> (nM) [SID, AID]
9893924/ ML156	N370S GC	580nM [89449177, 2590]	Alpha- glucosidase  Alpha- galactosidase	> 57μM [2921544, 2577]  > 57μM [2921544, 2578]	>100-fold	Chaperone activity in N370S GC fibroblasts: 500nM [2921544,2587]

### Recommendations for scientific use of the probe:

Known glycosidase chaperone molecules belong to the aminosugar class. Because iminosugar inhibitors work by mimicking the transition state of the glycosidic cleavage, they tend to be poorly selective. This has hampered their advance in clinical development. Alternative scaffolds with chaperone activity are quite desirable. Here, we present a new non-aminosugar series of glucocerebrosidase inhibitors having chaperone capacity. The probe is able to inhibit the hydrolytic activity of the N370S mutant form of glucocerebrosidase. Most importantly, the probe increased glucocerebrosidase translocation to the lysosome in Gaucher patient-derived fibroblasts homozygous for the N370S mutation, and can be used to study ER-lysosomal trafficking of clinically relevant GC mutants *in vitro*. This probe may be a useful lead for the pre-clinical development of a chemical chaperone of glucocerebrosidase.



## 1 Introduction.

Gaucher disease, the most common of the lipidoses, is an autosomal recessive disorder resulting from mutations in the enzyme glucocerebrosidase (EC 3.2.1.45)<sup>1</sup>. The function of glucocerebrosidase (GC) is to hydrolyze beta glycosidic linkages of glucocerebrosides, also called glucosylceramides, in the lysosome<sup>2</sup>. These glycosphingolipids are cell membrane components that maintain the stability of the lipid bilayer, function as cellular recognition elements and play an important role in cellular adherence<sup>3</sup>.

There are more than 200 recognized mutations in the glucocerebrosidase gene<sup>4</sup>. Although many GC mutants are still functional<sup>5</sup>, many affect translocation to the lysosome, which results in protein premature degradation in the ER. The inability of GC protein to reach the lysosome produces accumulation of glucosylceramides in the lysosome, causing tissue-specific lysosomal enlargement, which is characteristic of the disease. Currently, the major FDA approved medication for the treatment of Gaucher disease is the infusion of recombinant human enzyme as enzymatic replacement therapy (ERT). Although ERT successfully reverses some of the disease manifestations, the limited tissue distribution of the infused enzyme to the CNS and lungs and its high cost require the need for improvement<sup>6</sup>.

A proposed alternate therapeutic strategy is the use of small molecular chaperones to restore the cellular function of the mutant enzyme. Small molecules that bind the mutant protein can facilitate its proper folding and increase the translocation of the mutant enzyme to the lysosome<sup>7-8</sup>. Several iminosugar inhibitors of glycosidases have been reported to have chaperone activity<sup>9-20</sup>. For GC, two iminosugars have been clinical evaluated: Genz-112638<sup>21</sup>, currently in clinical trials, and isofagomine (Afeostat<sup>22</sup>), whose development was recently halted during phase II testing. Because iminosugar inhibitors work by mimicking the transition state of the glycosidic cleavage, they tend to be poorly selective<sup>13</sup>. Alternative scaffolds with chaperone activity are quite desirable. In addition, it is important that the compound inhibitory potency is not the

primary determinant of therapeutic potential, because native substrates need to be able to displace the inhibitor after translocation to the lysosome<sup>2, 8, 23</sup>. Thus, the molecules that have moved to clinical testing are not the most potent inhibitors known<sup>24</sup>.

A goal of the current study was to develop a non-iminosugar series with a favorable balance between inhibitory potency and chaperone activity. In the cell, GC activity is modulated through the binding of an allosteric activator, Saposin C<sup>25</sup>. In isolation, the addition of a bile salt is required to induce GC activity<sup>26</sup>. A series of GC inhibitors identified by screening with purified enzyme were found to have reduced or absent activity when tested in tissue homogenate assays. We speculate that this difference in activity is due to nonspecific protein binding and/ or to GC conformational differences between the conformation induced by detergent and that induced by Saposin C. In addition, activity differences are observed between wildtype and mutant enzyme.

As 70 % of Gaucher patients carry the N370S mutation, we focused our efforts on the use of spleen<sup>27</sup> homogenate homozygous for the N370S mutation for screening for GC inhibitors and activators. GC specific activity was evaluated using 4-methylumbelliferone  $\beta$ -D-glucopyranoside. Upon hydrolysis, the blue dye 4-methylumbelliferone (4-MU) was liberated, producing a fluorescent emission at 440 nm when excited at 370 nm. Active compounds were then further characterized in several additional assays to confirm specificity, rule out artifacts, and most importantly, characterize chaperone activity.

## 2 Materials and Methods

### Chemistry

All commercially available reagents and solvents were purchased and used without further purification. All microwave reactions were carried out in a sealed microwave vial equipped with a magnetic stir bar and heated in a Biotage Initiator Microwave Synthesizer. HPLC purification was performed using a Waters semi-preparative HPLC equipped with a Phenomenex Luna<sup>®</sup> C18 reverse phase (5 micron, 30x 75mm) column having a flow rate of 45 ml/min. The mobile phase was a mixture of acetonitrile and H<sub>2</sub>O, each containing 0.1% trifluoroacetic acid. During purification, a gradient of 30% to 80% acetonitrile over 8 minutes was used with fraction collection triggered by UV detection (220nm). <sup>1</sup>H spectra were recorded using either an Inova 400 MHz spectrometer (Varian) or an Inova 300 MHz spectrometer (Varian). Two LCMS methods were used to analyze samples' purity.

Method 1: Agilent 1200 series LC/MS equipped with a Zorbax<sup>™</sup> Eclipse XDB-C18 reverse phase (5 micron, 4.6x 150mm) column having a flow rate of 1.1 ml/min. The mobile phase was a mixture of acetonitrile and H<sub>2</sub>O, each containing 0.05% trifluoroacetic acid. A gradient of 5% to 100% acetonitrile over 8 minutes was used during analytical analysis.

Method 2: Acquity HPLC equipped with a Waters BEH C18, 1.7 micron, 2.1 x 50 mm column; Column Temperature: 45 degrees C; Flow: 0.5ml/min; Solvent A: 0.05% TFA in Water; Solvent B: 0.025% TFA in Acetonitrile; Gradient: 2% to 100% Solvent B over 1.3 minutes; Run Time - 3 min.

### Biology

The recombinant wildtype enzyme, Cerezyme<sup>®</sup>, was obtained from Genzyme Corporation (Cambridge, MA). N370S recombinant glucocerebrosidase was a gift from Dr. Tim Edmunds at Genzyme. Patients' spleens were obtained from splenectomies with informed consent under an NIH-IRB approved clinical protocol. Control spleens were obtained under an NIH protocol. 4-methylumbelliferyl- $\beta$ -D-glucopyranoside (4MU- $\beta$ -glc), a blue fluorogenic substrate, resorufin  $\beta$ -D-glucopyranoside (res- $\beta$ -glc), a red fluorogenic substrate, sodium taurocholate, and the buffer components were purchased from Sigma-Aldrich (St. Louis, MO). Isofagomine and N-nonyl-

deoxynojirimycin (NN-DNJ) were purchased from Toronto Research Biochemicals (Ontario, Canada).

## 2.1 Assays

PubChem AID	Type	Target	Conc. Range	Samples Tested	Notes
2101	Primary qHTS	N370S GC	57.5 $\mu$ M – 0.7nM	326,770	Tissue, blue
2590	Confirmatory	N370S GC	54 $\mu$ M – 0.01nM	320	Tissue, blue
2613	Secondary	N370S GC	57.5 $\mu$ M – 0.3nM	83	Tissue, red
2592	Secondary	Wildtype GC	57.5 $\mu$ M – 0.3nM	21	Tissue, blue
2588	Secondary	Wildtype GC	50 $\mu$ M – 0.1nM	152	Tissue, red
2595	Secondary	Wildtype GC	77 $\mu$ M – 0.3nM	52	Purified, blue
2597	Secondary	N370S GC	77 $\mu$ M – 0.3nM	52	Purified, blue
2596	Secondary	N370S GC	230 $\mu$ M – 0.1nM	94	Purified, natural substrate
2577	Anti-target	Alpha-glucosidase	57.5 $\mu$ M – 0.3nM	70	Purified, blue

<b>2578</b>	Anti-target	Alpha-galactosidase	57.5µM – 0.3nM	70	Purified, blue
<b>2587</b>	Tertiary	N370S GC	100µM – 10nM	4	Immunostaining of fibroblast lysosomes
<b>2589</b>	Tertiary	Wildtype GC	100µM – 10nM	4	Immunostaining of fibroblast lysosomes
<b>2593</b>	Summary	N370S GC			

**Table 1.** Assay protocols and data deposited into PubChem.

**AID 2101:** qHTS assay for activators and inhibitors of N370S Mutant Form of Glucocerebrosidase

This is a fluorogenic enzyme assay with 4-methylumbelliferyl-beta-D-glucopyranoside as the substrate and the glucocerebrosidase from spleen homogenate of a N370S Gaucher patient as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 4-methylumbelliferone, can be excited at 365 nm and emits at 440 nm; this can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation.

The human spleen tissue was homogenized using a food blender at the maximal speed for 5 minutes, followed by 10 passes in a motor-driven 50ml glass-Teflon homogenizer. The homogenate was centrifuged at 1000 ×g for 10 min. The supernatant was then filtered using a 40µm filter and aliquots of resultant spleen homogenate were frozen at -80°C until use. (Assay buffer: 50mM citric acid (titrated with potassium phosphate to pH 5.0), 100mM potassium chloride, 10mM sodium chloride, 1mM magnesium chloride, 0.01% Tween-20.)

1536-well assay protocol:

- (1) Add 2  $\mu$ l/well of spleen homogenate (27 $\mu$ g final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.5nM to 58 $\mu$ M.
- (3) Add 2 $\mu$ l of substrate (1mM final).
- (4) Incubate at 37° C for 40 min.
- (5) Add 2 $\mu$ l stop solution (1M NaOH and 1M Glycine mixture, pH 10)
- (6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2590:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Primary Screen Confirmation

This is a fluorogenic enzyme assay with 4-methylumbelliferyl-beta-D-glucopyranoside as the substrate and N370S glucocerebrosidase from human spleen homogenate as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 4-methylumbelliferone, can be excited at 365 nm and emits at 440 nm; this can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation. (Assay buffer: 50mM citric acid (titrated with potassium phosphate to pH 5.0), 100mM potassium chloride, 10mM sodium chloride, 1mM magnesium chloride, 0.01% Tween-20.)

1536-well assay protocol:

- (1) Add 2  $\mu$ l/well of spleen homogenate (27 $\mu$ g final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.5nM to 58 $\mu$ M.
- (3) Add 2 $\mu$ l of substrate (1mM final).
- (4) Incubate at 37C for 40 min.
- (5) Add 2 $\mu$ l stop solution (1M NaOH and 1M Glycine mixture, pH 10).
- (6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2613:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Activity in N370S Spleen Homogenate Using a Red Fluorescent Substrate

This is a fluorogenic enzyme assay with resorufin-beta-D-glucopyranoside as the substrate and mutant N370S glucocerebrosidase from human spleen homogenate as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 4-methylumbelliferone, can be excited at 365 nm and emits at 440 nm; this can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation. (Assay buffer: 50mM citric acid (titrated with potassium phosphate to pH 5.0), 100mM potassium chloride, 10mM sodium chloride, 1mM magnesium chloride, 0.01% Tween-20.)

1536-well assay protocol:

- (1) Add 2 µl/well of spleen homogenate (27µg final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.5nM to 58µM.
- (3) Add 2µl of substrate (1mM final).
- (4) Incubate at 37C for 40 min.
- (5) Add 2µl stop solution (1M NaOH and 1M Glycine mixture, pH 10).
- (6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2592:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Activity in Non-Mutant Spleen Homogenate

This is a fluorogenic enzyme assay with 4-methylumbelliferyl-beta-D-glucopyranoside as the substrate and wildtype glucocerebrosidase from human spleen homogenate as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 4-methylumbelliferone, can be excited at 365 nm and emits at 440 nm; this can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation. (Assay buffer: 50mM citric

acid (titrated with potassium phosphate to pH 5.0), 100mM potassium chloride, 10mM sodium chloride, 1mM magnesium chloride, 0.01% Tween-20.)

1536-well assay protocol:

- (1) Add 2  $\mu$ l/well of spleen homogenate (27 $\mu$ g final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.5nM to 58 $\mu$ M.
- (3) Add 2 $\mu$ l of substrate (1mM final).
- (4) Incubate at 37C for 40 min.
- (5) Add 2 $\mu$ l stop solution (1M NaOH and 1M Glycine mixture, pH 10).
- (6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2588:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Activity in Non-Mutant Spleen Homogenate Using a Red Fluorescent Substrate

This is a fluorogenic enzyme assay with resorufin-beta-D-glucopyranoside as the substrate and wildtype glucocerebrosidase from human spleen homogenate as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 4-methylumbelliferone, can be excited at 365 nm and emits at 440 nm; this can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation. (Assay buffer: 50mM citric acid (titrated with potassium phosphate to pH 5.0), 100mM potassium chloride, 10mM sodium chloride, 1mM magnesium chloride, 0.01% Tween-20.)

1536-well assay protocol:

- (1) Add 2  $\mu$ l/well of spleen homogenate (27 $\mu$ g final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.5nM to 58 $\mu$ M.
- (3) Add 2 $\mu$ l of substrate (1mM final).
- (4) Incubate at 37C for 40 min.
- (5) Add 2 $\mu$ l stop solution (1M NaOH and 1M Glycine mixture, pH 10).

(6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2595:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Purified Non-mutant Glucocerebrosidase

This assay characterizes compound activity using purified wildtype enzyme instead of mutant enzyme from tissue homogenate. Compound activity differences may be due to the absence of endogenous cofactors found in tissue homogenate, as well as inherent differences in enzyme stability and catalytic activity between the N370S and wildtype forms of the protein. This is a fluorogenic enzyme assay, with 4-methylumbelliferyl-beta-D-glucopyranoside as the substrate and purified wildtype glucocerebrosidase as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 4-methylumbelliferone, can be excited at 365 nm and emits at 440 nm; this can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation. (Assay buffer: 50mM citric acid (titrated with potassium phosphate to pH 5.0), 100mM potassium chloride, 10mM sodium chloride, 1mM magnesium chloride, 0.01% Tween-20.)

1536-well assay protocol:

- (1) Add 2 µl/well of glucocerebrosidase enzyme solution (5nM final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.5nM to 58µM.
- (3) Add 2µl of substrate (1mM final).
- (4) Incubate at 37C for 40 min.
- (5) Add 2µl stop solution (1M NaOH and 1M Glycine mixture, pH 10).
- (6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2597:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Purified N370S Glucocerebrosidase

This assay characterizes compound activity using purified mutant enzyme instead of tissue homogenate. Compound activity differences may be due to the absence of endogenous cofactors found in tissue homogenate. This is a fluorogenic enzyme assay, with 4-methylumbelliferyl-beta-D-glucopyranoside as the substrate and purified N370S glucocerebrosidase as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 4-methylumbelliferone, can be excited at 365 nm and emits at 440 nm; this can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation. (Assay buffer: 50mM citric acid (titrated with potassium phosphate to pH 5.0), 100mM potassium chloride, 10mM sodium chloride, 1mM magnesium chloride, 0.01% Tween-20.)

1536-well assay protocol:

- (1) Add 2 µl/well of glucocerebrosidase enzyme solution (5nM final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.5nM to 58µM.
- (3) Add 2µl of substrate (1mM final).
- (4) Incubate at 37C for 40 min.
- (5) Add 2µl stop solution (1M NaOH and 1M Glycine mixture, pH 10).
- (6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2596:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Purified N370S Glucocerebrosidase Cleavage of Glucosylceramide

This assay characterizes compound activity using purified, mutant enzyme cleavage of the native substrate for the enzyme instead of mutant enzyme from tissue homogenate. Compound activity differences may be due to the absence of endogenous cofactors found in tissue homogenate, as well as inherent differences in enzyme stability and catalytic activity using the difference substrates.

This is an enzyme assay using glucosylceramide from soy (Avanti Polar Lipids Catalog #: 131304P) as the substrate and recombinant human glucocerebrosidase as the enzyme

preparation. Upon hydrolysis of the substrate, the glucose product can be detected using the Amplex Red Glucose Oxidase Assay Kit (Invitrogen catalog #: A22189). The product of this reaction can be read with a fluorescence plate reader with an excitation at 573 nm and an emission at 610 nm. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation. (Assay buffer for enzyme reaction: 50mM citric acid (titrated with potassium phosphate to pH 5.9), 0.01% Tween-20 (pH 5.9 is an optimal condition for this enzyme assay) and assay buffer for Amplex Red reaction: Tris-HCl, pH 7.5.)

1536-well assay protocol for the glucocerebrosidase assay:

- (1) Add 2 µl/well glucocerebrosidase enzyme solution (5nM final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.7nM to 77µM.
- (3) Add 1µl of glucosylceramide solution (50µM final).
- (4) Incubate at 37C for 40 min.
- (5) Add 2 µl Tris-HCl buffer with Amplex Red reagents.
- (6) Incubate 45 min at room temperature.
- (7) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=573 nm and Em=610nm.

**AID 2577:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Alpha-Glucosidase Counterscreen

To characterize compound selectivity, selected hits from the primary screen were screened against purified alpha-glucosidase, a related sugar hydrolase. Alpha-glucosidase is responsible for hydrolysis of terminal, non-reducing 1,4-linked alpha-D-glucose residues with release of alpha-D-glucose. This is a fluorogenic enzyme assay with 4-methylumbelliferyl-alpha-D-pyranoside as the substrate and human alpha-glucosidase as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 1,4-methylumbelliferone, can be excited at 365 nm and emits at 440 nm; this can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). The AC<sub>50</sub> values were determined from concentration-response data

modeled with the standard Hill equation. (Assay buffer: 50mM citric acid (titrated with potassium phosphate to pH 5.0), 0.005% Tween-20, pH 5.0 (pH 5.0 is an optimal condition for this enzyme assay).)

1536-well assay protocol for the human alpha-glucosidase:

- (1) Add 2 $\mu$ l/well of enzyme (4nM final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.7nM to 77 $\mu$ M.
- (3) Add 1 $\mu$ l of substrate (400 $\mu$ M final).
- (4) Incubate at room temperature for 20 min.
- (5) Add 2 $\mu$ l stop solution (1M NaOH and 1M Glycine mixture, pH 10).
- (6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2578:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Alpha-Galactosidase Counterscreen

To characterize compound selectivity, selected hits from the primary screen were screened against purified alpha-galactosidase, a related sugar hydrolase. Alpha-galactosidase is a homodimeric glycoprotein that hydrolyzes the terminal alpha-galactosyl moieties from glycolipids and glycoproteins. This is a fluorogenic enzyme assay with 4-Methylumbelliferyl alpha-D-galactopyranoside as the substrate and human alpha-galactosidase as the enzyme preparation. Upon the hydrolysis of this fluorogenic substrate, the resulting product, 1,4-Methylumbelliferone, can be excited at 365 nm and emits at 440 nm. This fluorescence can be detected by a standard fluorescence plate reader. Data were normalized to the controls for basal activity (without enzyme) and 100% activity (with enzyme). AC<sub>50</sub> values were determined from concentration-response data modeled with the standard Hill equation. (Assay buffer: 50mM citric acid (titrated with potassium phosphate to pH 4.5), 0.005% Tween-20, pH 4.5 (pH 4.5 is an optimal condition for this enzyme assay).)

1536-well assay protocol for the human alpha-galactosidase:

- (1) Add 2 $\mu$ l/well of enzyme (12nM final).
- (2) Add 23nl compounds in DMSO solution. The final titration was 0.7nM to 77 $\mu$ M.

- (3) Add 1µl of substrate (80µM final).
- (4) Incubate at room temperature for 20 min.
- (5) Add 2µl stop solution (1M NaOH and 1M Glycine mixture, pH 10).
- (6) Detect the assay plate in a ViewLux plate reader (PerkinElmer) with Ex=365 nm and Em=440nm.

**AID 2587:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Chaperone Activity in Gaucher Fibroblasts after Multi-day Incubation with Compound

This assay attempts to quantitate translocated glucocerebrosidase protein in patient-derived fibroblasts following extended compound incubation. The fibroblasts tested in this experiment were homozygous for N370S glucocerebrosidase.

Primary dermal fibroblasts derived from skin biopsies from two previously described N370S/N370S Gaucher patients (Goker-Alpan *et al*, 2008)<sup>28</sup> and a control were seeded in Lab-Tek 4 chamber slides (Fisher Scientific, Pittsburgh, PA). After compound treatment, fibroblasts were fixed in 3% paraformaldehyde. The cells were permeabilized with 0.1 % Triton-X for 10 min. and blocked in PBS containing 0.1% saponin, 100µM glycine, 0.1% BSA and 2% donkey serum, followed by incubation with mouse monoclonal anti-LAMP1 or LAMP-2 (1:100, Developmental Studies Hybridoma bank, University of Iowa, Iowa City, IA) and the rabbit polyclonal anti-GCase R386 antibody (1:500); the cells were washed and incubated with secondary donkey anti-mouse or anti-rabbit antibodies conjugated to ALEXA-488 or ALEXA-555, respectively (Invitrogen, Carlsbad, CA), washed again, and mounted in VectaShield with DAPI (Vector Laboratories, Burlingame, CA).

Cells were imaged with a Zeiss 510 META confocal laser-scanning microscope (Carl Zeiss, Microimaging Inc., Germany) using an Argon (458, 477, 488, 514 nm) 30 mW laser, a HeNe (543 nm) 1 mW laser, and a laser diode (405 nm). Low and high magnification images were acquired using a Plan-Apochromat 20X/0.75 objective and a Plan-Apochromat 100x/1.4 oil DIC objective, respectively. Images were taken with the same laser settings, and all images shown are collapsed z-stacks.

**AID 2589:** qHTS Assay for Inhibitors and Activators of N370S glucocerebrosidase as a Potential Chaperone Treatment of Gaucher Disease: Chaperone Activity in Non-Gauche Fibroblasts after Multi-day Incubation with Compound

This assay attempts to quantitate translocated glucocerebrosidase protein in patient-derived fibroblasts following extended compound incubation. The fibroblasts tested in this experiment contain wildtype glucocerebrosidase.

Protocol Summary:

- (1) Seeded 3000 cells/well in 96-well plates using DMEM (10% FBS), with only medium in outer wells to eliminate the edge effect.
- (2) Next day, replaced medium with OptiMem (2% FBS) and spiked in various concentrations of compound.
- (3) 2 days later, replaced with fresh OptiMem, and again spiked each well the same as before.
- (4) 3 days later, washed 1x with PBS.
- (5) Incubated with 3% Paraformaldehyde for 15 min, replaced with PBS.
- (6) Aspirated PBS and added block solution (5% goat serum + 0.1% saponin + 15 mg/ml glycine in PBS) and incubated at RT for 40 min.
- (7) Aspirated block solution and added r386 antibody solution to stain glucocerebrosidase (20µl Ab in 19ml of 5% goat serum, 0.1% saponin in PBS) (~1:1000 dilution).
- (8) Left at 4C overnight.
- (9) Next day, washed 3x with block solution (no glycine). Waited 10 min between washes.
- (10) Added mix of secondary antibody solution (1:100 dilution of Cy-3 for GC, 1:100 dilution of FITC for LAMP2, 1:5000 dilution of Hoechst) in 5% goat serum solution and incubated 1 hr.
- (11) Rinsed 3X with PBS, waiting 5 minutes between washes.
- (12) Visualized using a fluorescence microscope and the intensity of fluorescence in the selected areas were calculated.

LC-MS hydrolysis experiment

This assay uses liquid chromatography linked to a mass spectrometer to assess the ability of glucocerebrosidase in the spleen homogenate to cleave its natural substrate (glucosylceramide). The substrate has a fluorescent tag, which allows the cleavage to be measured; however, it is not

believed to have a role in the enzymatic reaction. This assay most closely reflects the physiological condition in the body.

Chromatography was performed using an Agilent HPLC. The Agilent 1200 LC was equipped with a quaternary pump, a G1315 diode array detector, and a G1321 Fluorescent Detector. A 4.6x 250 mm Agilent Eclipse Plus C18 (5 micron) at ambient temperature was used at a flow rate of 1.8 ml/min with a gradient of 85/15 (methanol/0.1% formic acid in water) to 100 methanol over 10 minutes. Compounds were monitored using fluorescence detection with an excitation wavelength of 505 nanometers and emission wavelength at 540 nanometers.

#### Caco-2 Permeability and Microsomal Stability

Analytical signal was optimized for each compound by ESI positive or negative ionization mode. A MS2 SIM scan was used to optimize the precursor ion, and a product ion analysis was used to identify the best fragment for analysis and to optimize the collision energy. Samples were analyzed by LC/MS/MS using either an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent), or an ABI2000 mass spectrometer coupled with an Agilent 1100 HPLC and a CTC PAL chilled autosampler, all controlled by Analyst software (ABI). After separation on a C18 reverse phase HPLC column (Agilent, Waters, or equivalent) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode.

CaCo-2 cells grown in tissue culture flasks are trypsinized, suspended in medium, and the suspensions were applied to wells of a collagen-coated BioCoat Cell Environment in 24-well format (BD Biosciences) at 24,500 cells per well. The cells are allowed to grow and differentiate for three weeks, feeding at 2-day intervals.

For Apical to Basolateral (A->B) permeability, the test agent is added to the apical (A) side and amount of permeation is determined on the basolateral (B) side; for Basolateral to Apical (B>A) permeability, the test agent is added to the B side and the amount of permeation is determined on the A side. The A-side buffer contains 100µM Lucifer yellow dye, in Transport Buffer (1.98 g/l glucose in 10mM HEPES, 1x Hank's Balanced Salt Solution) pH 6.5, and the B-side buffer is Transport Buffer, pH 7.4. CaCo-2 cells are incubated with these buffers for 2 h., and the receiver

side buffer is removed for analysis by LC/MS/MS. To verify that CaCo-2 cell monolayers are properly formed, aliquots of the cell buffers are analyzed by fluorescence to determine the transport of the impermeable dye Lucifer Yellow.

Data are expressed as permeability ( $P_{app}$ ):

$$P_{app} = \frac{dQ/dt}{C_0 A}$$

where  $dQ/dt$  is the rate of permeation,  $C_0$  is the initial concentration of test agent, and  $A$  is the area of the monolayer. In bidirectional permeability studies, the asymmetry index (AI) or efflux ratio is also calculated:

$$AI = \frac{P_{app}(B \rightarrow A)}{P_{app}(A \rightarrow B)}$$

An  $AI > 1$  indicates a potential substrate for PGP or other active transport.

For microsomal stability testing, the test agent is incubated in duplicate with microsomes at 37°C. The reaction contains microsomal protein in 100 mM potassium phosphate, 2mM NADPH, 3mM  $MgCl_2$ , pH 7.4. A control is run for each test agent omitting NADPH to detect NADPH-free degradation. At indicated times, an aliquot is removed from each experimental and control reaction and mixed with an equal volume of ice-cold Stop Solution (0.3% acetic acid in acetonitrile containing haloperidol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20°C, and an additional volume of water is added. The samples are centrifuged to remove precipitated protein, and the supernatants are analyzed by LC/MS/MS to quantitate the remaining parent. Data are reported as % remaining by dividing by the time zero concentration value.

#### Pharmacokinetic Study in Male Swiss Albino Mice

Compound was dosed IP at 20 mg/kg to mice and plasma, brain, liver and tail. Concentrations were determined pre-dose and at 0, 5, 15, 30, 60, 120, 240, 480, 720, 780, 900, 1020, and 1440 minutes, with 3 animals per time point. Male Swiss Albino mice used in this experiment were procured from National Institute of Nutrition (NIN), Hyderabad, India. Animals were acclimatized for three days in an animal holding room. Test article CID was dissolved in DMA,

TEG and Water for Injection in the ratio of 20:40:40, and vortexed. After dosing of each animal, animals were observed for any abnormal behavioral signs exhibited after drug administration.

Each mouse was anesthetized using Isoflurane. Blood was collected through a capillary, guided in retro-orbital plexus. The blood samples were collected in pre-labeled Heparin coated tubes (BD, cat. No.365965). 0.3ml of blood was collected from each mouse at their respective timepoints. After collection of blood samples at each time point, the blood samples were stored on wet ice prior to centrifugation. Blood samples were centrifuged within 15 minutes to separate plasma at 5000 rpm, 4°C for 10 minutes. The plasma was separated and transferred to pre labeled tubes and promptly frozen at  $-80 \pm 10$  °C until bioanalysis. Immediately after blood withdrawal for PK estimation, *in situ* whole body perfusion was performed using chilled saline. The chest and abdomen of the mouse was exposed, the inferior venacava was cut and Intra-cardiac perfusion was performed through an insertion in the left ventricle. Perfusion for each mouse was followed by decapitation for brain collection. The skin over the cranium was incised and deflected. The head was flexed and a cut was made through the muscles and the spinal cord at the junction of the foramen magnum and atlas vertebra. A circumferential incision was carefully made in the cranium using a pair of small scissors. The roof of the cranium was lifted off to expose the meninges and brain. The meninges were removed carefully. Then holding the head with the nose pointing upward, the anterior part of the brain was lifted to separate the brain. Separated brain was immediately weighed and frozen at  $-80 \pm 10$ °C until homogenization. PK parameters are calculated for mean concentration by the non-compartmental model, trapezoid rule (linear interpolation method) using WinNonlin Software Version 4.1.

## 2.2 Probe Chemical Characterization

Structural verification information of probe SID: 89449177/ML156

<sup>1</sup>H NMR (400 MHz, CHLOROFORM-*d*)  $\delta$  ppm 1.85 - 1.99 (m, 4 H), 2.68 (m, 2 H), 3.61 (m, 6 H), 4.60 (s, 2 H), 6.31 (d,  $J=9.19$  Hz, 1 H), 6.81 (s, 1 H), 7.00 (dd,  $J=8.31, 1.86$  Hz, 1 H), 7.23 (d,  $J=1.76$  Hz, 1 H), 7.36 (d,  $J=8.22$  Hz, 1 H), 7.44 (dd,  $J=9.00, 2.54$  Hz, 1 H), 7.52 (s, 1 H), 8.12 (br. s., 1 H); HRMS (ESI):  $m/z$  calcd for C<sub>22</sub>H<sub>25</sub>BrCl<sub>2</sub>N<sub>6</sub>S 554.0422, found 554.0434; LC/MS (Agilent system) Retention time  $t_1$  (short) = 3.33 min and  $t_2$  (long) = 5.25 min; Purity: UV<sub>220</sub> >

99%, UV<sub>254</sub> > 99%; MS m/z 555.1 (M+H); Column: 3x 75 mm Luna C18, 3 micron Run time: 4.5 min (short); 8.5 min (long) Gradient: 4 % to 100 % Mobile phase: Acetonitrile (0.025 % TFA), water (0.05 % TFA). Flow rate: 0.8 to 1.0ml Temperature: 50 °C UV wavelength: 220nm, 254nm.

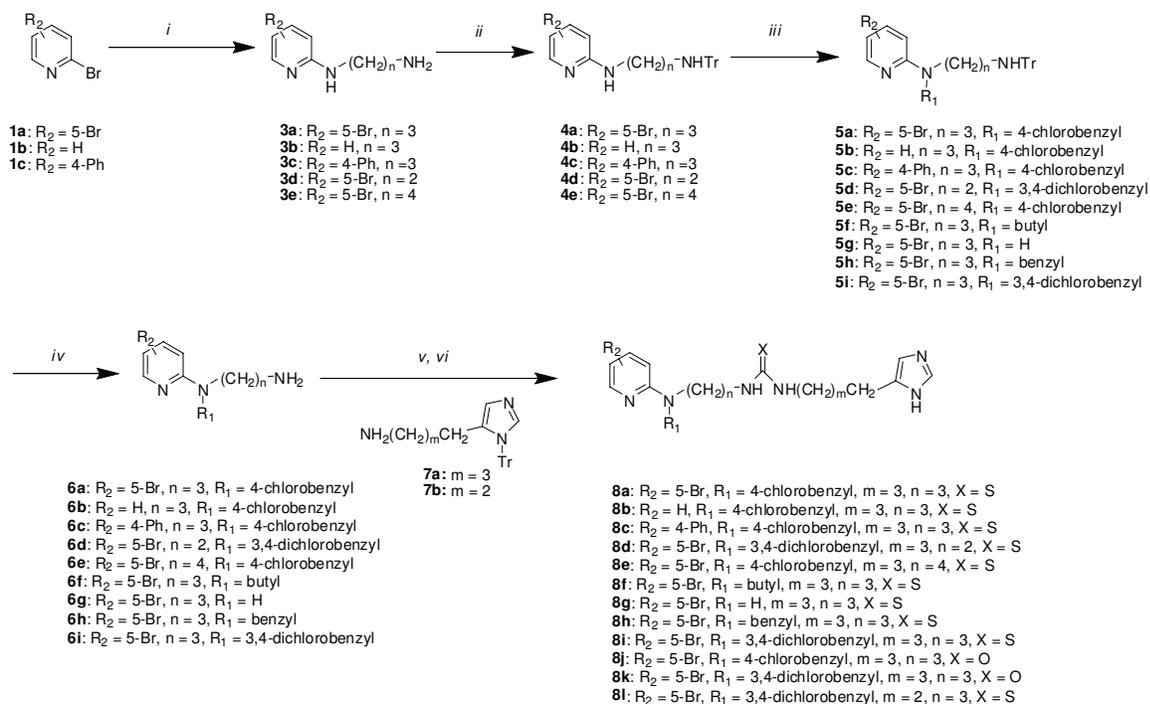
MLS	SID	CID	NCGC ID	ML	Type
MLS002703100	99495629	9893924	NCGC00159568-02	ML156	Probe
MLS002703117	99495645	44820550	NCGC00185835-02		Analog
MLS002703118	99495646	44820554	NCGC00185837-02		Analog
MLS002703119	99495647	44820562	NCGC00185839-02		Analog
MLS002703106	99495635	44820570	NCGC00187945-01		Analog
MLS002703107	99495636	44820542	NCGC00187954-01		Analog
MLS002703108	99495637	44820564	NCGC00187957-01		Analog

**Table 2.** Compounds submitted to the MLSMR.

## 2.3 Probe Preparation

### Synthesis of ML156 and analogs

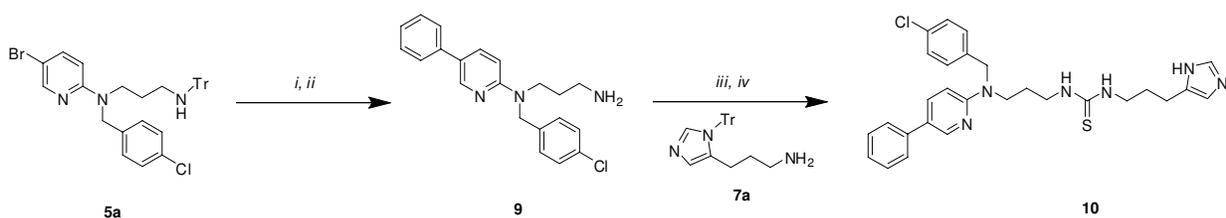
NCGC00159568 (**8i**) and analogs were synthesized as shown in Schemes 1-3. Scheme 1 depicts the synthesis of compounds **8a-l**. Reaction of 2-bromopyridines **1a-c** with an excess of a diaminoalkane gave pyridines **3a-e**. The primary amino group of **3a-e** was protected by a trityl group, giving **4a-e**. Subsequent alkylation of **4a-e** with appropriate alkyl halides in DMF using LiHMDS as a base afforded **5a-f** and **5h-I**, which were converted to amine **6a-i** by TFA-mediated trityl group de-protection. The desired ureas **8k-l** and thioureas **8a-j** were formed by a one-pot reaction of **6**, **7**, and 1,1-thiocarbonyldiimidazole (or 1,1'-carbonyldiimidazole), followed by removal of the trityl protecting group.



Reagents and conditions: (i) H<sub>2</sub>N(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub> (**2**), pyridine, refluxed; (ii) TrCl, THF, Et<sub>3</sub>N, 0 °C-r.t.; (iii) THF, LiHMDS, R<sub>1</sub>X, r.t.; (iv) TFA, DCM; (v) *i*PrNEt<sub>2</sub>, 7, 1,1'-thiocarbonyldiimidazole (or 1,1'-carbonyldiimidazole), DCM, MW 130 °C; (vi) TFA, DCM

### Scheme 1. Synthesis of compounds 8a-1

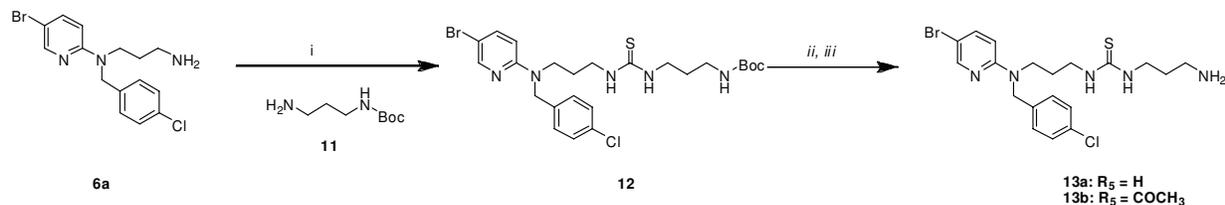
Suzuki reaction of **5a** with phenyl boronic acid followed by trityl group de-protection gave amine **9**. Thiourea **10** was formed by a one-pot reaction of **9**, **7a**, and 1,1'-thiocarbonyldiimidazole, followed by removal of the trityl protecting group (Scheme 2).



Reagents and conditions: (i) PhB(OH)<sub>2</sub>, Na<sub>2</sub>CO<sub>3</sub>, Pd(PPh<sub>3</sub>)<sub>4</sub>, 100 °C, 2 h; (ii) TFA, DMC; (iii) *i*PrNEt<sub>2</sub>, **7a**, 1,1'-thiocarbonyldiimidazole, DCM, MW 150 °C, 10 min; (iv) TFA

### Scheme 2. Synthesis of compound 10

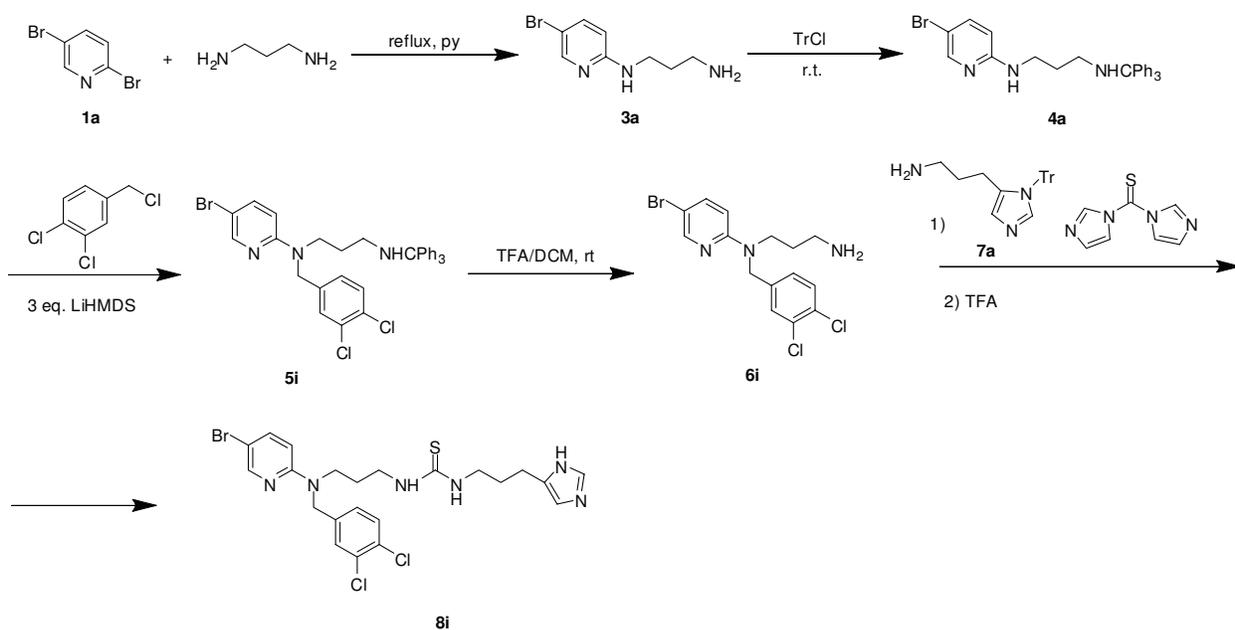
Thiourea **12** was formed by a one-pot reaction of **6a**, **11**, and 1,1-thiocarbonyldiimidazole. TFA mediated Boc-deprotection of **12** afforded **13a**. Compound **13b** was formed by reaction of **13a** with acetic chloride (Scheme 3).



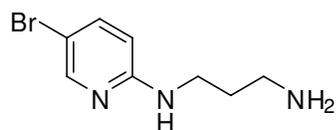
Reagents and conditions: (i)  $i\text{-PrNEt}_2$ , **11**, 1,1'-thiocarbonyldiimidazole, DCM, MW 130 °C, 10 min; (ii) TFA; (iii) for **13b**  $\text{CH}_3\text{COCl}$

**Scheme 3.** Syntheses of compounds 13a-b

**Example: Synthesis of 1-[3-[(5-bromopyridin-2-yl)-(3,4-dichlorophenyl)methyl]amino]propyl-3-[3-(1H-imidazol-5-yl)propyl]thiourea (**8i**)**

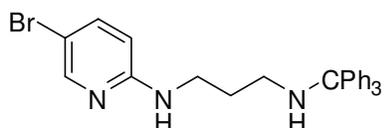


**N<sup>1</sup>-(5-bromopyridin-2-yl)propane-1,3-diamine (3a)**



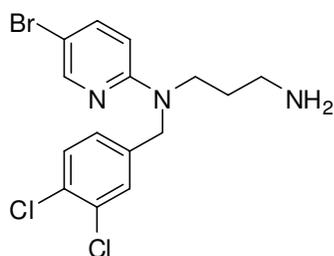
A mixture of 2, 5-dibromopyridine (**1a**, 2.0g, 8.4mmol), pyridine (0.8g, 10.1mmol), and 1, 3-diaminopropane (10 mL) was refluxed for 18 hours. The reaction mixture was evaporated *in vacuo* and 15 mL of anhydrous dichloromethane was added. After filtering off white precipitate, the filtrate was concentrated to yield N<sup>1</sup>-(5-bromopyridin-2-yl)propane-1,3-diamine (**3a**, 1.6g, 83%) as an oil.

#### N<sup>1</sup>-(5-bromopyridin-2-yl)-N<sup>3</sup>-tritylpropane-1,3-diamine (**4a**)



Trityl chloride (7.3g, 26mmol) was added slowly at 0°C to a mixture of N<sup>1</sup>-(5-bromopyridin-2-yl)propane-1,3-diamine (**3a**, 5.0g, 21.7mmol) and triethylamine (2.6g, 26mmol) in anhydrous dichloromethane (50ml). After stirring at room temperature for 1 hour, the reaction mixture was washed with water (3x 10ml). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The residue was washed with methanol to afford N<sup>1</sup>-(5-bromopyridin-2-yl)-N<sup>3</sup>-tritylpropane-1,3-diamine (**4a**, 9.6g, 79%) as a solid.

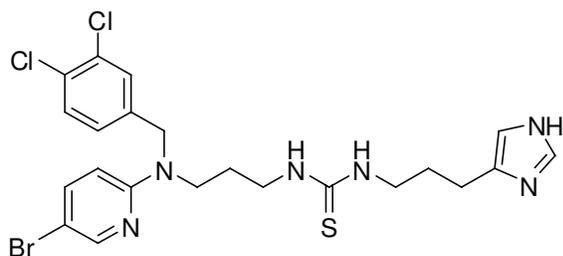
#### N<sup>1</sup>-(5-bromopyridin-2-yl)-N<sup>1</sup>-(3,4-dichlorobenzyl)propane-1,3-diamine (**6i**)



LiHMDS (6ml, 1.0M in THF) was added under nitrogen atmosphere at room temperature to a solution of N<sup>1</sup>-(5-bromopyridin-2-yl)-N<sup>3</sup>-tritylpropane-1,3-diamine (**4a**, 940mg, 2mmol) in anhydrous tetrahydrofuran (10ml). After stirring for a half hour, 1-(bromomethyl)-3,4-dichlorobenzene (2.37g, 10mmol) in 10ml of anhydrous THF was added slowly at room temperature. The solution was stirred at room temperature for another hour after addition. The reaction mixture was then poured into ice water (100ml) and extracted with ethyl acetate (3x 30ml). The combined organic layers were washed with water (20ml), dried over Na<sub>2</sub>SO<sub>4</sub>,

filtered, and concentrated. The crude product was purified by silica column chromatography using hexanes/ethyl acetate (80:1) to yield **5i** (0.72g, 57%). N<sup>1</sup>-(5-bromopyridin-2-yl)-N<sup>1</sup>-(3,4-dichlorobenzyl)-N<sup>3</sup>-tritylpropane-1,3-diamine (**5i**, 629mg, 1.0mmol) was dissolved in DCM/TFA (5ml/ 5ml). The mixture was stirred at room temperature for 1 hour. Volatile was removed under vacuum and the residue was dissolved in methanol (50ml). The methanol solution was washed by hexanes (10x 10ml) and neutralized to pH=7-8 using 1M NaOH aqueous solution. Then the mixture was extracted with DCM (3x 10ml). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated to yield N<sup>1</sup>-(5-bromopyridin-2-yl)-N<sup>1</sup>-(3,4-dichlorobenzyl)propane-1,3-diamine (**6i**, 310 mg, yield: 80 %). <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ ppm 8.26 (d, J = 2.4 Hz, 1H), 7.39 (dd, J = 2.4 Hz, 9 Hz, 1H), 7.34 – 7.25 (m, 1H), 7.20 (d, J = 1.5 Hz, 1H), 6.95 (dd, J = 2.1 Hz, 8.4 Hz, 1H), 6.28 (d, J = 9 Hz, 1H), 4.53 (s, 2H), 3.70 (t, J = 7.3 Hz, 2H), 3.04 (t, J = 6.3 Hz, 2H), 2.11 – 2.07 (m, 2H).

**1-[3-[(5-bromopyridin-2-yl)-[(3,4-dichlorophenyl)methyl]amino]propyl]-3-[3-(1H-imidazol-5-yl)propyl]thiourea (8i)**



1,1'-thiocarbonyldimidazole (28.5mg, 0.16mmol, 1equiv) was added to a solution of **6i** (62.0mg, 0.16mmol, 1equiv) in DCM (1.5ml). The mixture was stirred at room temperature for 3 hours. After that, a solution of **7a** (58.7mg, 0.16mmol, 1equiv) and DIPEA (10.3mg, 0.08mmol) in CH<sub>3</sub>CN (1.5ml) was added. The mixture was heated at 150°C for 10 minutes under microwave irradiation. After removing solvent, the residue was dissolved in 3ml of DCM. 3ml of TFA at 0°C was added to this solution, and the mixture was stirred at room temperature for 2 hours. Volatile was removed and the residue was purified by HPLC to afford **8i** (20.4mg, 23%). <sup>1</sup>H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.85 - 1.99 (m, 4 H), 2.68 (m, 2 H), 3.61 (m, 6 H), 4.60 (s, 2 H), 6.31 (d, J=9.19 Hz, 1 H), 6.81 (s, 1 H), 7.00 (dd, J=8.31, 1.86 Hz, 1 H), 7.23 (d, J=1.76 Hz, 1 H), 7.36 (d, J=8.22 Hz, 1 H), 7.44 (dd, J=9.00, 2.54 Hz, 1 H), 7.52 (s, 1 H), 8.12

(br. s., 1 H); HPLC (method 1):  $t_R = 5.25$  min,  $UV_{220} = 99\%$ ; MS  $m/z$  555.1 (M+H); HRMS (ESI):  $m/z$  calcd for  $C_{23}H_{27}BrClN_5S$  HRMS (ESI):  $m/z$  calcd for  $C_{22}H_{25}BrCl_2N_6S$  554.0422, found 554.0434.

### 3 Results

#### 3.1 Summary of Screening Results

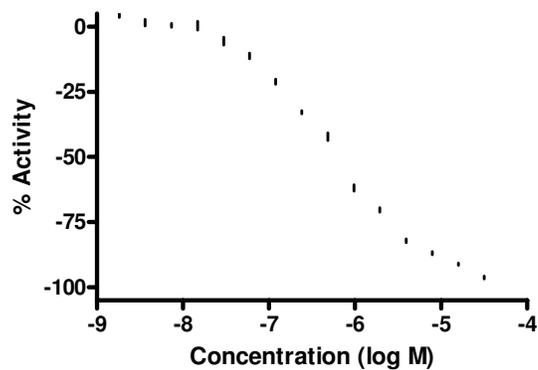
1,300 1536-well plates were screened in qHTS format (most compounds were assayed in titration from 90nM to 57 $\mu$ M final concentration in the primary screen) with a  $Z'$  of 0.80 +/- 0.16 across the entire set of plates, indicating robust performance of the assay. A total of 326,770 compounds were screened<sup>22-23</sup> (AID 2101).

From the primary screen, we identified several series of modulators, including several imino sugar molecules previously reported to inhibit the enzyme, such as isofagomine.

Compound	Series (PubChem CID)	Type	Purified N370S	Purified WT	Spleen N370S	Spleen WT
Isofagomine	447607	Inhibitor	50-60nM	10-15nM	70-80nM	100nM
NCGC00092410	Quinoline 5067281	Inhibitor	560-1000nM	70-80nM	> 10μM	> 10μM
MLS000393962	Quinazoline 2477651	Inhibitor	Inactive	1.6-2.5μM	450nM	1000nM
NCGC00159568	Thiourea 9893924	Inhibitor	2500uM	710nM	580nM	350nM

**Table 3.** Compound representatives of initial hit series. Isofagomine is an imino sugar previously reported to chaperone glucocerebrosidase. NCGC00092410 is a non-imino sugar previously reported to inhibit purified glucocerebrosidase.

### 3.2 Dose Response Curves for Probe



**Figure 1.** Concentration-response of probe, CID 9893924/ML156, in primary screening assay for N370S GC inhibition from tissue homogenate. Compound IC<sub>50</sub>, measured in triplicate on three different days, was 580 +/- 30nM.

### 3.3 Scaffold/Moiety Chemical Liabilities

The probe molecule does not contain any functional groups with potential chemical liabilities.

### 3.4 SAR Tables

Entry	Cmpd. No.	CID	SID	NCGC ID	*	R1	R2	R3	n	m	X	AC50
1	<b>8i</b>	9893924	29215544	NCGC00159568-01	P	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	3	S	0.6±0.1
2	<b>8i</b>	9893924	89449177	NCGC00159568-02	S	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	3	S	0.6±0.1
3	<b>8a</b>	44820550	89449189	NCGC00185835	S	4-Chlorobenzyl	5-Br	4-Imidazole	3	3	S	1.2
4	<b>8f</b>	44820562	89449191	NCGC00185837	S	Butyl	5-Br	4-Imidazole	3	3	S	8.2
5	<b>8h</b>	44820554	89449193	NCGC00185839	S	Benzyl	5-Br	4-Imidazole	3	3	S	5.8
6	<b>8g</b>	44820544	89449192	NCGC00185838	S	H	5-Br	4-Imidazole	3	3	S	46.0
7	<b>8b</b>	44820546	89449194	NCGC00185840	S	3,4-Dichlorobenzyl	H	4-Imidazole	3	3	S	7.3
8	<b>10</b>	44820557	89449208	NCGC00187953	S	4-Chlorobenzyl	5-Ph	4-Imidazole	3	3	S	1.3
9	<b>8c</b>	44820553	89449213	NCGC00187959	S	4-Chlorobenzyl	4-Ph	4-Imidazole	3	3	S	4.0
10	<b>8d</b>	10554498	89449195	NCGC00185841	S	3,4-Dichlorobenzyl	5-Br	4-Imidazole	2	3	S	2.1
11	<b>8e</b>	44820570	89449200	NCGC00187945	S	4-Chlorobenzyl	5-Br	4-Imidazole	4	3	S	0.8
12	<b>8l</b>	44820548	89449197	NCGC00185843	S	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	2	S	18.3
13	<b>8j</b>	5311371	89449196	NCGC00185842	S	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	3	O	0.6
14	<b>8k</b>	44820568	89449205	NCGC00187950	S	4-Chlorobenzyl	5-Br	4-Imidazole	3	3	O	1.0
15	<b>13a</b>	44820542	89449209	NCGC00187954	S	4-Chlorobenzyl	5-Br	NH2	3	3	S	45.0
16	<b>13b</b>	44820564	89449212	NCGC00187957	S	4-Chlorobenzyl	5-Br	NHCOCH3	3	3	S	inactive
17	<b>25</b>	44820567	89449198	NCGC00185844	S	3,4-Dichlorobenzyl	5-Br	CH3	3	3	S	inactive
18	<b>8q</b>	44820543	89449210	NCGC00187955	S	phenylethyl	5-Br	4-Imidazole	3	3	S	2.8
19	<b>8m</b>	44820565	89449201	NCGC00187946	S	4-chlorophenylethyl	5-Br	4-Imidazole	3	3	S	2
20	<b>26</b>	44820569	89449202	NCGC00187947	S							2.8
21	<b>27</b>	44820563	89449204	NCGC00187949	S							18
22	<b>24</b>	44820545	89449203	NCGC00187948	S							1.3

\* P = purchased S = synthesized

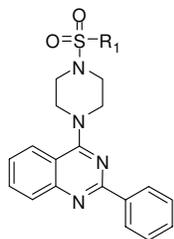
**Table 4.** Structure activity relationship (SAR)

All analogs were tested in a N370S spleen homogenate assay (AID2590) to evaluate their inhibitory activity (Table 4). The hit compound (**8i**) was synthesized in house, and the synthesized sample and the purchased sample had the same activity (entries 1-2). Several positions of the hit compound (NCGC00159568, **8i**) were explored for SAR, and the results are summarized as follows: (a) Decreasing the size of the R1 substituent gradually weakened activity (entries 2-6); (b) At the R2 position, phenyl group at C5 showed better activity than the phenyl group at C4 (entries 8-9); (c) Ureas had similar activities as thiourea (**8i** vs **8j** and **8a** vs **8k**); (d)

While decreasing the distance between the thiourea group and the pyridyl moiety from 4C to 2C resulted in a small decrease in activity (entries 9-11), decreasing the distance between the thiourea group and the imidazole group from 3C to 2C resulted in a 30-fold loss of activity (**8i** vs. **8l**); (e) The imidazole group at the R3 position is important for the activity. Replacing the imidazole with an amino group (entry 15), or an acetamide (entry 16), or a methyl group (entry 17) resulted in significant reduction or complete loss of activity; (f) While replacing one of the thiourea NH with a methyl group as in **26** resulted in loss of 2-fold of activity (entry 20), replacing the other thiourea NH with a methyl group as in **27** resulted in a 10-fold loss of activity (entry 21); (g) Replacing the pyridine ring with a benzene ring as in **24** gave similar activity (entry 22).

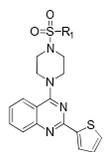
#### **Activity of quinazoline analogs.**

Tables 5-9 show the capacity of compounds to inhibit the hydrolysis of 4-methylumbelliferone  $\beta$ -D-glucopyranoside. As positive control, we measure the activity of Isofagomine in the same assay ( $AC_{50} = 0.080\mu M$ ).



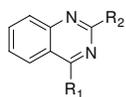
Compound number	R1	N370S AC <sub>50</sub> (μM)	Compound number	R1	N370S AC <sub>50</sub> (μM)	Compound number	R1	N370S AC <sub>50</sub> (μM)
72		12.67	82		25.29	92		20.08
73		31.83	83		12.67	93		31.83
74		12.67	84		7.99	94		100.67
75		12.67	85		31.83	95		15.85
76		31.83	86		31.83	96		12.67
77		15.95	87		25.29	97		25.29
78		20.08	88		20.08	98		6.35
79		50.45	89		25.29	99		12.67
80		15.95	90		31.83			
81		12.67	91		40.07			

**Table 5.** Analogs with sulfonamide aromatic modifications having a phenyl at two positions.



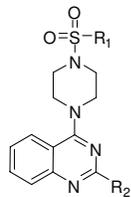
Compound number	R1	N370S AC <sub>50</sub> (μM)	Compound number	R1	N370S AC <sub>50</sub> (μM)	Compound number	R1	N370S AC <sub>50</sub> (μM)
100		1.26	119		1.27	138		2.53
101		3.18	120		2.52	139		4.00
102		2.52	121		2.52	140		5.04
103		0.32	122		3.18	141		1.00
104		0.80	123		1.26	142		1.27
105		1.59	124		0.63	143		3.18
106		2.00	125		7.99	144		0.33
107		2.00	126		4.00	145		1.01
108		0.40	127		2.53	146		0.50
109		1.00	128		2.53	147		0.50
110		1.00	129		2.53	148		1.59
111		3.18	130		5.04	149		1.26
112		2.00	131		0.32	150		2.53
113		1.27	132		0.80	151		1.01
114		1.27	133		1.27	152		0.50
115		1.00	134		3.18	153		1.26
116		1.59	135		4.01	154		1.01
117		1.27	136		1.59			
118		2.01	136		2.35			

**Table 6.** Analogs with sulfonamide aromatic modifications having a thiophene at two positions.



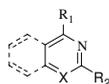
Compound number	R1	R2	N370S AC50 (μM)	Compound number	R1	R2	N370S AC50 (μM)
103			0.32	173			50.45
165			2.52	174			10.07
166			2.52	175			2.00
167			50.45	176			3.18
168			1.00	177			79.98
169			5.04	178			Inactive
170			50.45	179			6.35
171			79.98	180			6.35
172			15.96				

**Table 7.** Analogues with the modifications at the linker.



Compound number	R1	R2	N370S AC <sub>50</sub> (μM)	Compound number	R1	R2	N370S AC <sub>50</sub> (μM)
181		H	20.58	193			Inactive
103			0.32	194			Inactive
182			3.18	195			2.53
183			12.67	196			40.09
184			12.67	197			12.67
185			79.97	198			Inactive
186			4.00	199			Inactive
187			25.29	200			Inactive
188			1.27	201			15.95
189			20.09	202			100.67
190			25.29	203			Inactive
191			200.86	204			Inactive
192			Inactive	205			40.09

**Table 8.** Analogs with modification at the 2 position of the quinazolinone core.



Compound number	Molecular core	R1	R2	N370S AC50 (μM)	Compound number	Molecular core	R1	R2	N370S AC50 (μM)
103				0.32	214				Inactive
206				Inactive	215				Inactive
207				20.08	216				Inactive
208				4.00	217				Inactive
209				3.18	218				Inactive
210				10.31	219				Inactive
211				Inactive	220				Inactive
212				3.18	221				Inactive
213				Inactive	222				Inactive

**Table 9.** Analogs with modifications at the molecular core.

SAR of the series shows very strict requirements for maintaining activity values. Tables 5 and 6 show the activity of analogs with sulfonamide aromatic modifications. In general, potency changes up to 25-fold between aromatic substituents. Derivatives with a single substitution in the para, and especially in the ortho position, tend to provide better activity. The para methyl analog was one of our most potent compounds with an IC<sub>50</sub> of 320nM. As a group, there are not big differences between the activity provide by halogen, electron-withdrawing and electron-donating

functional groups. Bicyclic rings, with combinations of electron-donating groups in meta and para positions (**146**, **147** and **150**) provide the most potent compounds.

We also studied modifications in the piperidine linker. It can be seen that elimination of the aromatic ring of the sulphonamide is detrimental for the activity of the molecule (**172**, **173**, **174**). In addition, replacement of the sulphone by a carbonyl group also reduces the activity (**175** and **176**). This reduction is even greater when the sulphone is eliminated and a benzyl substituent is directly attached to the piperidine ring (**177**). Analogs **178** and **179** show that both nitrogens of the piperidine ring play an important role in maintaining the activity, the one next to the quinazoline ring being the most important. The angle (**180**) and rigidity (**165** and **166**) of the linker is also important for activity.

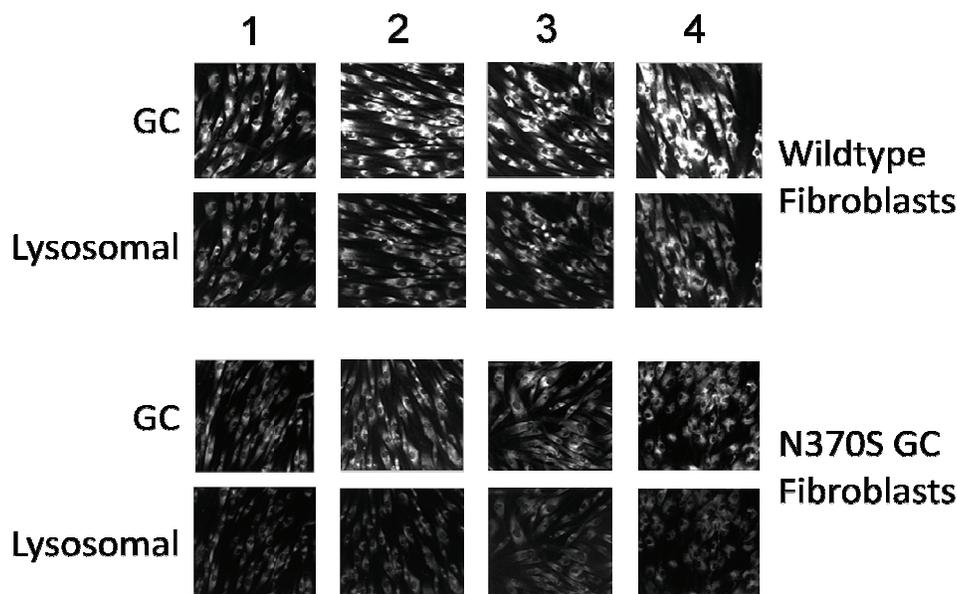
The next modifications that we studied were replacement of the aromatic substituent at the two position of the quinazoline ring. Table 8 shows that complete elimination of the substitution (**181**) reduces the activity more than 60-fold, and its replacement with non-aromatic functional groups, compounds **201** to **205**, have greatly reduced activity. Introduction of an unsubstituted phenyl ring at the 2-position (**197**) increase the activity of the molecule, although all analogues with substituents in this phenyl ring abolish the activity (**198**, **199**, **200**). Replacement of the phenyl aromatic ring by a six member heteroaromatic ring having one or more nitrogens reduces or abolishes the activity (compare **197** with **189** to **194**). The introduction of a five member heteroaromatic ring, unsubstituted 2-thiophene, displays the best activity (**103**) followed by 3-furan (**188**) and 4-thiazone (**186**). In addition, 1-benzoimidazole (**195**) also provides better activity than a simple phenyl ring.

Finally, we studied analogues of the quinazoline core. Replacement of the quinazoline core by purine results in loss of activity, **221** and **222**. Elimination of the quinazoline nitrogens also impacts the activity, reducing (**207**) or completely abolishing it (**206**). Introduction of one additional nitrogen within position 5 or 8 of the quinazoline ring (**208** and **209**) reduced the activity at least 10-fold. Replacement of the quinazoline ring by an unsubstituted pyrimidine ring (**210**) reduces activity 32-fold, or 10-fold if the pyrimidine is bearing methyl groups in positions 5 and 6 (**212**). Introduction of a chloro substituent in position 7 of the quinazoline ring abolished activity (compounds **213** to **220**).

In summary, SAR of the series allowed very narrow functional modification, and most of the functionalities of the hit molecule MLS000393962, such as the quinazoline core, the piperidine ring, the sulphonamide substitution and the thiophene heteroaromatic ring at the two positions were necessary for its activity. Even so, we were able to increase the potency of the molecule to the desirable range. Our most potent compound displays an  $IC_{50}$  of 320nM, which is comparable with isofagomine which was 80nM in our assay.

### **3.5 Cellular Activity**

To demonstrate chaperone activity, we measured the capacity of ML156 and ML155 to increase the translocation of GC to the lysosome<sup>8, 16, 30, 31</sup>. In this experiment, wildtype and mutant fibroblasts were incubated for five days with compound **8i**, followed by cell fixation and staining with a selective fluorescent GC antibody. Compounds able to promote trafficking from the ER to the lysosome increased the fluorescent lysosomal signal. DMSO and isofagomine were used as negative and positive controls. Figure 2 shows the increment of signal in both cell lines that resulted from treatment, confirming the chaperone capacity of these compounds.



**Figure 2.** Chaperone activity of ML156/CID 9893924/NCGC00159568 and others using wildtype and homozygous, mutant N370S GC fibroblasts. Two genotypes of fibroblasts, fibroblasts homozygous for wildtype GC (top) and fibroblasts homozygous for N370S GC (bottom) were stained both with a Cy3-labeled antibody for GC protein content (first row) and a FITC-labeled antibody specific for lysosomal compartments (LAMP1; second row) after treatment with (1) DMSO vehicle, (2) 10 μM Isogagomine, (3) 10 μM NCGC00182292, and (4) 1 μM ML156/CID 9893924/NCGC00159568. Isogagomine, NCGC00182292 and ML156/CID 9893924/NCGC00159568 show increased lysosomal GC protein after treatment.

### 3.6 Profiling Assays

#### *In vitro* ADME

The result of a microsomal stability study of ML156/CID9893924 is shown in Table 10. After incubation of the molecule in mouse liver microsomes for 60 minutes in the present of NADPH, 58% of the molecule remained, this suggested that ML156/CID9893924 has a reasonable microsomal stability.

CID	test species	test conc (uM)	Plus NADPH Parent remaining			Minus NADPH Parent remaining		
			1 <sup>st</sup>	2 <sup>nd</sup>	mean	1 <sup>st</sup>	2 <sup>nd</sup>	mean
9893924	mouse	5	(%)	(%)	(%)	(%)	(%)	(%)
			59%	57%	58%	99%	105%	102%

**Table 10.** Mouse microsomal stability assay at 60 minutes

*In vivo* Pharmacokinetic Analysis

The pharmacokinetic profiles of ML156/CID9893924 in male Swiss Albino mice after intraperitoneal administration twice at a 12 hour interval of CID9893924 at 20mg/kg dose are shown in Table 11.

Time (h)	CID9893924 Concentration (Mean ± SD)							
	Plasma		Brain		Liver		Tail	
	ng/mL	µM	ng/g	µM	ng/g	µMol/kg	ng/g	µM
0	0	0	0	0	0	0	0	0
0.083	7550.45±4133.97	13.57	156.27±35.04	0.28	114450.60±35495.51	205.72	2382.09±1219.43	4.28
0.25	3718.63±1820.04	6.68	117.00±26.91	0.21	120015.98±31928.46	215.72	1711.93±646.62	3.08
0.5	0.39±0.68	0	144.25±59.45	0.26	172131.23±63886.67	309.39	2757.54±1415.49	4.96
1	2027.95±1490.37	3.65	249.35±85.99	0.45	188379.50±53862.08	338.6	4515.56±419.31	8.12
2	2782.05±2546.66	5	264.68±62.48	0.48	197983.68±86771.35	355.86	3948.02±857.57	7.1
4	3433.14±323.70	6.17	334.73±9.89	0.6	274815.45±33548.02	493.96	4607.28±480.57	8.28
8	3058.29±852.34	5.5	263.30±75.11	0.47	161336.35±97210.11	289.99	4237.98±474.45	7.62
12	68.72±21.67	0.12	25.60±27.39	0.05	3178.62±487.48	5.71	1635.27±1112.20	2.94
13	4356.09±262.37	7.83	65.50±29.60	0.12	96094.83±50368.63	172.72	3048.19±974.44	5.48
15	1332.21±889.37	2.39	114.42±18.10	0.21	121269.48±27551.90	217.97	4746.46±442.57	8.53
17	0.00±0.00	0	0.00±0.00	0	0.00±0.00	0	72.02±47.26	0.13
20	1242.59±477.67	2.23	78.98±24.56	0.14	101618.15±16886.07	182.65	3217.71±2165.76	5.78
24	171.61±126.71	0.31	39.90±23.35	0.07	8024.95±6437.87	14.42	3230.88±1784.48	5.81

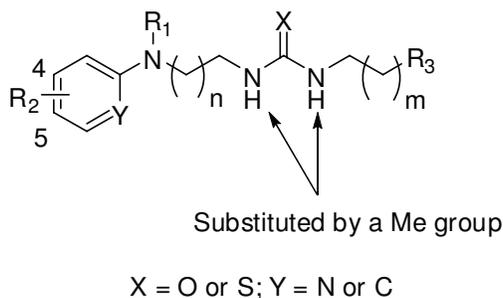
**Table 11.** Concentrations of CID9893924 in plasma, brain, liver and tail; intraperitoneal administration twice at a 12 hour interval of CID9893924 at 20mg/kg dose in male Swiss Albino Mice (mean and SD).

## 4 Discussion

In a screen of 326,770 compounds<sup>26, 29</sup>, we found several series of GC inhibitors.

NCGC00182292, which was one of the most potent compounds in the series, was further characterized in a variety of GC assay formats. Most notable was weak or a complete lack of activity in assays with purified, reconstituted GC protein (AID2597, 2595, 2596). These assays lacked saposin C, an endogenous regulator of GC activity in cells, and so it is unclear how relevant these results are. Unfortunately, GC cleavage of glycosylceramide, the native substrate of GC, can only be examined in purified assay systems, and so it is unknown if this series inhibits cleavage of the native substrate. However, this might be an advantage for a chemical chaperone, as it is preferred that a chaperone does not interfere with substrate processing once protein is translocated to the lysosome.

Among the hits, also a thiourea singleton, NCGC159568 (**8i**), was confirmed. This compound was inactive against the related sugar hydrolases alpha-glucosidase and alpha-galactosidase at a 50 $\mu$ M concentration, and did not exhibit auto-fluorescence. In addition, the compound had very similar activity against GC as determined using an alternate resorufin-based substrate, thereby ruling out nonspecific effects on the fluorescent reporter. More importantly, the chaperone capacity of this compound was confirmed in a translocation assay, in which treatment of compound **8i** resulted in increase of GC translocation to the lysosome. Thus, we initiated a structure-activity relationship (SAR) study around the hit compound, as shown in Figure 3.



**Figure 3.** Structure-activity relationship (SAR) study for CID 9893924 (**8i**)

As described in section 3.4, several positions of the molecule were explored and preliminary structure-activity relationships were obtained for the inhibitory activity. Since ML156/CID 9893924 is a known agonist of the Somatostatin receptor sst4, several analogs were tested in a human SST4 receptor binding assay. As shown in the Table 12, the inhibitory activity observed

in the N370S spleen homogenate assay was not correlated with the activity in the SST4 binding assay. For example, compound **24** is about 10-fold more potent than compound **27** in the N370S spleen homogenate assay, but **24** is about 5 fold less potent than **27** in the SST4 binding assay. These results suggested that the SST4 activity of this series could be removed without diminishing the GC activity through further structure modification.

Entry	NCGC ID	Cmpd. No	CID	SID	GC AC50 (nM)	sst4 Ki (nM)
1	NCGC00159568-03	<b>8i</b>	9893924	89449177	600	17
2	NCGC00185838-01	<b>8g</b>	44820544	89449192	46000	690
3	NCGC00187947-01	<b>26</b>	44820569	89449202	2800	19
4	NCGC00187948-01	<b>24</b>	44820545	89449203	1300	160
5	NCGC00187949-01	<b>27</b>	44820563	89449204	18000	26

**Table 12.** Comparison of GC inhibitory activities with Somatostatin receptor sst4 activity

ML156/CID 9893924 has demonstrated chaperone activity, and preliminary pharmacokinetic studies have demonstrated that the molecule has good exposure in plasma, brain and liver upon IP administration. Exposure in brain is especially significant, as the current standard-of-care, enzyme replacement therapy, has no effect on the neurological component of the disease. In addition, at this high dose, the compound did not exhibit any acute toxicity in initial testing. Thus, this molecule will be tested *in vivo* for the proof of principle study.

#### 4.1 Comparison to existing art and how the new probe is an improvement

As it has been previously mentioned in the introduction, currently described GC chaperone molecules are based on an aminosugar scaffold. The current series presents a new template with very good selectivity versus other glycosidases. In addition, it seems that our series has a lower capacity than isofagomine to inhibit hydrolysis of ceramides maintaining similar chaperone capacity, and therefore it could be expected that there is a potentially better therapeutic window for the series.

## 4.2 Mechanism of Action Studies

In enzyme kinetics assays, the probe, along with other glucocerebrosidase inhibitors such as isofagomine, demonstrated mixed mode inhibition – neither competitive nor non-competitive enzyme kinetics. Obviously, from x-ray crystal structure studies of isofagomine binding, the compound binds in the active site. Thus, the current homogenate-based assays might be ill-suited for such kinetic characterization.

## 4.3 Planned Future Studies

Preliminary pharmacokinetic studies demonstrated that ML156/CID 9893924 has very good exposure in plasma, brain and liver upon IP administration at a 12 hour interval of ML156/CID 9893924 at 20mg/kg dose. We are planning to test this molecule in an animal model for a proof of principle study. In this study, we will dose wt and mutant GC animals for seven days with our inhibitor. After that period, we will stop the treatment with our compound, and sacrifice animals at several time points (1, 2 and 3 days) after treatment to allow the drug to be eliminated. Relevant tissues (blood, liver, brain) will be collected and GC specific activity will be measured. Compounds able to increase the translocation must increase the overall GC production, and therefore should increase the GC specific activity. Upon confirmation in the *in vivo* study, further SAR studies will be performed to remove the Somatostatin receptor sst4 activity.

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