

**Probe Report**

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

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**Assay Submitter & Institution:** National Human Genome Research Institute, Dr. Ellen Sidransky

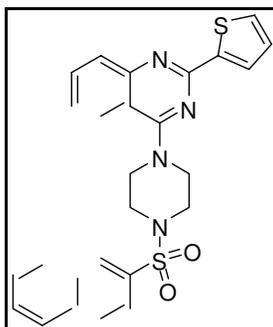
**PubChem Summary Bioassay Identifier (AID):** 2593

**Abstract:**

In this report, ML155 (CID 40225210) is described as a probe that is able to inhibit the hydrolytic activity of the N370S mutant form of glucocerebrosidase, as well as wild type glucocerebrosidase, in tissue homogenate assays. The probe does not inhibit purified glucocerebrosidase, but the cellular activity of the enzyme is known to be dependent on interactions with other factors, such as Saposin C. 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 clinical development of a chemical chaperone of glucocerebrosidase.

### Probe Structure & Characteristics:



**ML155**

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]
40225210/ ML155	N370S GC*	330 nM [85267237, 2590]	Alpha- glucosidase	>57μM [85267237, 2577]	>100-fold	Chaperone activity in N370S GC fibroblasts: 500 nM [85267237, 2587]
40225210/ ML155			Alpha- galactosidase	> 57μM [85267237, 2578]	>100-fold	

\* GC = glucocerebrosidase, also known as beta-glucosidase.

### Recommendations for scientific use of the probe:

The probe is able to inhibit the hydrolytic activity of the N370S mutant form of glucocerebrosidase, as well as wild type glucocerebrosidase, in tissue homogenate assays. The probe does not inhibit purified glucocerebrosidase, but the cellular activity of the enzyme is known to be dependent on interactions with other factors, such as Saposin C. 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 clinical development of a chemical chaperone of glucocerebrosidase.



## 1 Introduction

Gaucher disease is an autosomal recessive disorder resulting from mutations in the enzyme Glucocerebrosidase (EC 3.2.1.45; also known as acid beta-glucosidase) and affects 1 in 50,000 live births<sup>1</sup>. The function of Glucocerebrosidase (GC) is to hydrolyze the beta glucosidic linkage of glucocerebrosidases, also called glucosylceramides<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>. The deficiency of GC due to the genetic mutations results in the accumulation of glucosylceramides in lysosomes.

There are more than 200 recognized mutations of the GC gene<sup>4</sup>. Although many GC mutants are still functional<sup>5</sup>, conformational differences in mutant proteins reduce their recognition by transporters, resulting in ER protein accumulation and premature degradation in endosome. The inability of mutant proteins to reach their site of action causes lysosomal accumulation of glucosylceramides and lysosomal enlargement. Ultimately, this leads to liver and spleen enlargements, as well as the neurological symptoms in type 2 and type 3 Gaucher disease.

Currently, the only FDA approved treatment for Gaucher disease is enzyme replacement therapy using the human recombinant glucocerebrosidase, Cerezyme. Although this approach does address some aspects of the disease, the limited tissue distribution of the infused enzyme, for example poor CNS penetration, reduces its therapeutic benefits in type 2 and type 3 Gaucher patients<sup>6</sup>. Small molecule chaperone therapy has been proposed as an alternative therapeutic strategy for Gaucher disease. The binding of small molecules to mutant protein may facilitate proper folding and translocation of the mutant protein to the lysosome<sup>7-8</sup>. Several imino sugar inhibitors of glycosidases have been reported to have chaperone activity<sup>9-20</sup>. Isofagomine is an imino sugar that can act as a chaperone for glucocerebrosidase, but was recently withdrawn from clinical trial testing.

Imino sugars inhibit glycosidases by mimicking the transition state of the glycosidic cleavage, and as such tend to be poorly selective<sup>13</sup>. Therefore, it would be desirable to develop alternative, more selective series with chaperone activity. It is also important to remark that molecules with very potent inhibitors may have a lower probability of ultimately producing a therapeutic effect or an increase in GC activity in the lysosome. Though the GC protein translocation is increased, the tightly bound inhibitors may not be easily displaced by the natural substrate, and may even inhibit any residual activity of the enzyme<sup>2, 8, 21</sup>. Previous chaperone molecules that have entered clinical trials are all potent GC inhibitors. Our goal is to identify a non-imino sugar series with modest inhibitory capacity and good chaperone activity.

GC activity is modulated in cells through the binding of an allosteric activator, Saposin C<sup>27</sup>. In the GC enzyme assay with a purified enzyme, the addition of sodium taurocholate, a bile salt, is required to activate the enzyme<sup>23</sup>. Assays by our group and others that screen for inhibitors of GC using purified enzyme have identified several interesting inhibitor chemotypes, but most have reduced or no activity when tested using tissue homogenate that contains both GC and Saposin C, as well as other components that may be required for GC function in cells. We speculate that this variation in activity is due to the GC conformational differences between the active conformation induced by detergent and the one induced by Saposin C and/or other factors in cells. Additionally, 70% of Gaucher patients carry the N370S mutation, and therefore we developed an assay utilizing the spleen<sup>28</sup> homogenate derived from a GC patient homozygous for the N370S mutation in the primary screen. This assay uses a fluorogenic substrate, 4-methylumbelliferone  $\beta$ -D-glucopyranoside, to monitor glucocerebrosidase specific activity, and the spleen tissue homogenate as the GC enzyme preparation. Upon hydrolysis, 4-methylumbelliferone (4-MU) is liberated, which produces a fluorescent emission at 440 nm when excited at 370 nm.

## 2 Materials and Methods

Unless otherwise stated, all reactions were carried out under an atmosphere of dry argon or nitrogen in dried glassware. Indicated reaction temperatures refer to those of the reaction bath, while room temperature (rt) is noted as 25°C. All solvents were of anhydrous quality purchased from Aldrich Chemical Co. and used as received. Commercially available starting materials and reagents were purchased from Aldrich, TCI and Acros and were used as received. Analytical thin layer chromatography (TLC) was performed with Sigma Aldrich TLC plates (5x 20cm, 60 Å, 250µm). Visualization was accomplished by irradiation under a 254nm UV lamp.

Chromatography on silica gel was performed using forced flow (liquid) of the indicated solvent system on Biotage KPSil pre-packed cartridges and using the Biotage SP-1 automated chromatography system. <sup>1</sup>H NMR spectra were recorded on a Varian Inova 400 MHz spectrometer. Chemical shifts are reported in ppm with the solvent resonance as the internal standard (CDCl<sub>3</sub> 7.27 ppm, DMSO-*d*<sub>6</sub> 2.49 ppm, for <sup>1</sup>H NMR). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, sep = septet, quin = quintet, br = broad, m = multiplet), coupling constants, and number of protons. Low resolution mass spectra (electrospray ionization) were acquired on an Agilent Technologies 6130 quadrupole spectrometer coupled to an Agilent Technologies 1200 series HPLC. The HPLC retention times were recorded through short standard gradient 4% to 100% acetonitrile (0.05% TFA) over 3 minutes (t<sub>1</sub>) or long standard gradient 4% to 100% acetonitrile (0.05% TFA) over 7 minutes (t<sub>2</sub>) using Luna C<sub>18</sub> 3 micron 3x 75mm column with a flow rate of 0.800 ml/min. High resolution mass spectral data was collected in-house using an Agilent 6210 time-of-flight mass spectrometer, also coupled to an Agilent Technologies 1200 series HPLC system.

## 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
2671	Confirmatory	N370S GC	54 $\mu$ M – 0.01nM	142	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

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

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

**Assay details and protocol:** 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, which 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 50 ml glass-Teflon homogenizer. The homogenate was centrifuged at 1000 ×g for 10 min. The supernatant was then filtered using a 40 µm 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.

## 2.2 Probe Chemical Characterization

Structural verification information of probe SID: 40225210/ ML155

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  ppm 3.28 (m, 4H), 3.84 (m, 4H), 7.16 (dd, 1H, J=1.6, 5.2 Hz), 7.42 (m, 1H), 7.729 (m, 6H), 7.80 (dd, 1H, J= 2.0, 8.8 Hz), 7.90 (m, 2H), 8.07 (d, 1H, J= 8.0 Hz), 8.20 (m, 2H), 8.49 (s, 1H).

LC/MS (Agilent system) Retention time  $t_1$  (short) = 3.22 min

Purity: UV<sub>220</sub> > 99%, UV<sub>254</sub> > 99%; MS m/z 487.1 (M+H);

Column: 3x 75mm Luna C18, 3 micron

Run time: 4.5 min (short)

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: 220 nm, 254 nm

The corresponding CID is 40225210 and is commercially available for purchase from the following vendors:

Order number: ken-705035 and registry number: 1090708-11-4

Aurora Fine Chemicals LLC

7929 Silverton Ave.

Suite 609

San Diego, CA, 92126

USA

Phone: +1 858 549 4700

Fax: +1 858 549 4701

Email: [aurora@aurorafinechemicals.com](mailto:aurora@aurorafinechemicals.com)

Web: <http://www.aurorafinechemicals.com>

Order number: T6239298 and registry number: 1090708-11-4

Enamine

23 Alexandra Matrosova Street

Kiev, 01103

Ukraine

Phone: +380 44 537 32 18

Fax: +380 44 537 32 53

Email: [enamine@enamine.net](mailto:enamine@enamine.net)

Web: <http://www.ename.net>

Order number: T6239298 and registry number: 1090708-11-4

Ambinter

50, avenue de Versailles

Paris, F-75016

France

Phone: (33-1) 45 24 48 60

Fax: (33-1) 45 24 62 41

Email: [contact@ambinter.com](mailto:contact@ambinter.com)

Web: <http://www.ambinter.com>

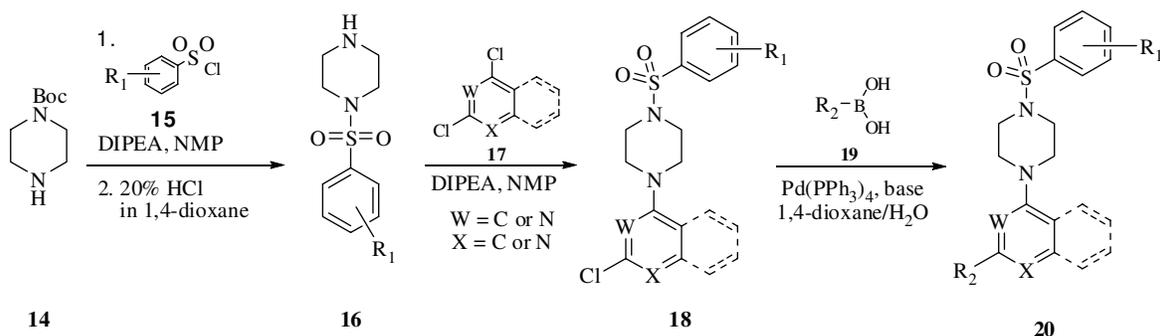
MLS ID	SID	CID	NCGC ID	ML	Type
MLS003429232	85267237	40225210	NCGC00182292	155	Probe
MLS002699813	85267406	44246455	NCGC00186073		Analog
MLS002699814	85267276	4787756	NCGC00182327		Analog
MLS002699815	85267403	25708371	NCGC00186069		Analog
MLS002699816	85267407	44246456	NCGC00186444		Analog
MLS002699817	85267408	44246457	NCGC00186445		Analog
MLS002699818	92386624	45136810	NCGC00187483		Analog
MLS000393962	104219315	2477651	MLS000393962		Analog
MLS000393919	22410873	4035156	MLS000393919		Analog

## 2.3 Probe Preparation

With the confirmatory data in hand, we embarked on systematic SAR modifications based on commercially available quinazolines as shown in Table 1. Scheme 1 discloses the synthesis strategy for some of the modifications at the quinazoline core<sup>26</sup>.

Pubchem CID	Pubchem SID	Compound number	R1	R2	N370S AC <sub>50</sub> (μM)	Compound number	R1	R2	N370S AC <sub>50</sub> (μM)	Pubchem CID	Pubchem SID
2520263	22408868	1			Inactive	8			1.27	2573787	22409679
2514355	22407428	2			Inactive	9			0.45	2477651	22404737
2467471	22407911	3			Inactive	10			25.29	6224211	24833921
2469159	24825474	4			79.97	11			25.29	2460930	24833315
2469099	24825528	5			63.52	12			6.35	2563217	57581505
2469096	24833571	6			63.52	13			0.33	40225210	85267237
2566280	24832668	7			79.97						

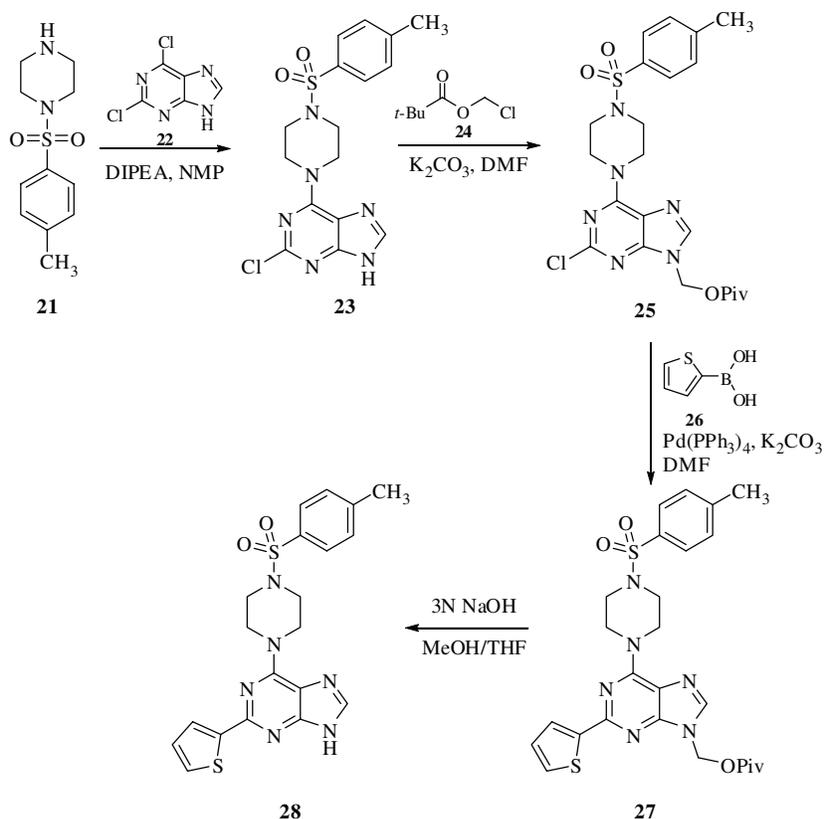
**Table 1:** SAR of commercial quinazolines



**Scheme 1:** General strategy for modification in the functional core

Commercially available Boc protected piperazine **14** was sulphonylated, using the sulphonyl chloride **15** in the presence of a suitable base such as Diisopropylethylamine, followed by a quantitative deprotection of the Boc functional group to yield intermediate **16**. The next step involves the selective chloro displacement at the core ring **17** to produce compound **18**, followed by a Suzuki cross-coupling reaction with an aromatic or heteroaromatic boronic acid **19** to produce final compound **20**. Application of this synthetic strategy allowed us the synthesis of analogs having quinazoline, pyrimidine and isoquinoline cores.

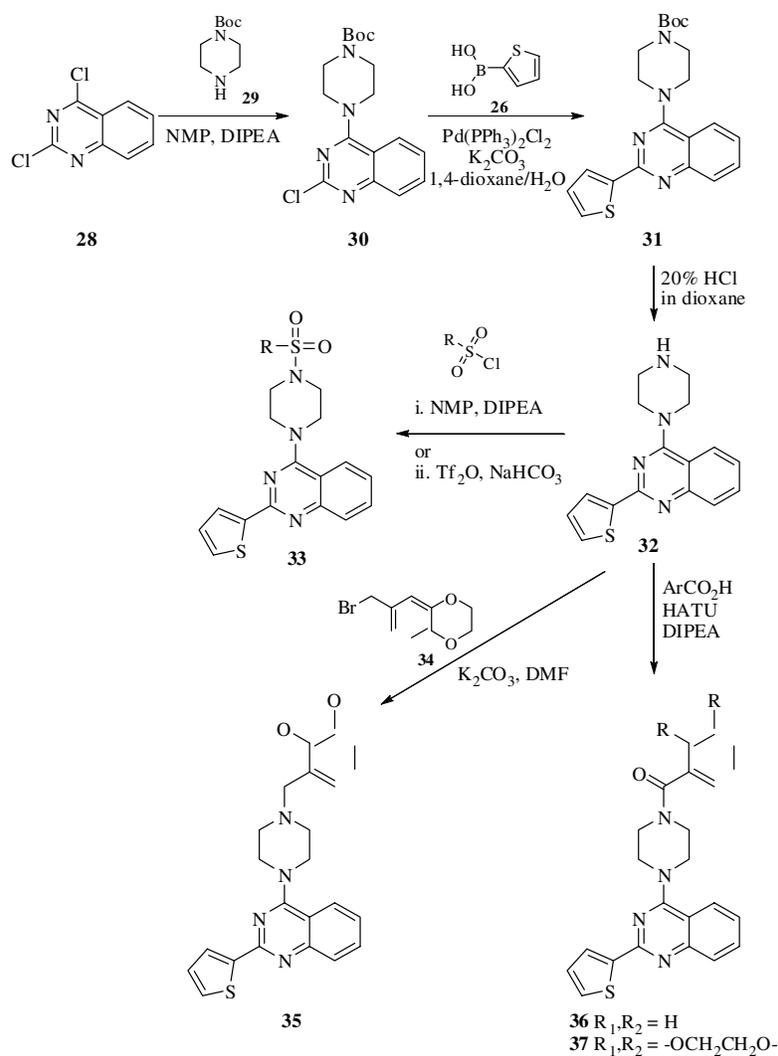
The synthesis of analogs with a purine core, Scheme 2, required the selective protection of the ring NH with a methyl pivalate functional group, followed by cross coupling and deprotection. Numerous attempts of carrying out Suzuki reactions with the free purine core or using a Boc protecting group failed to yield the intended coupling product.



**Scheme 2:** Synthesis of purine analogs

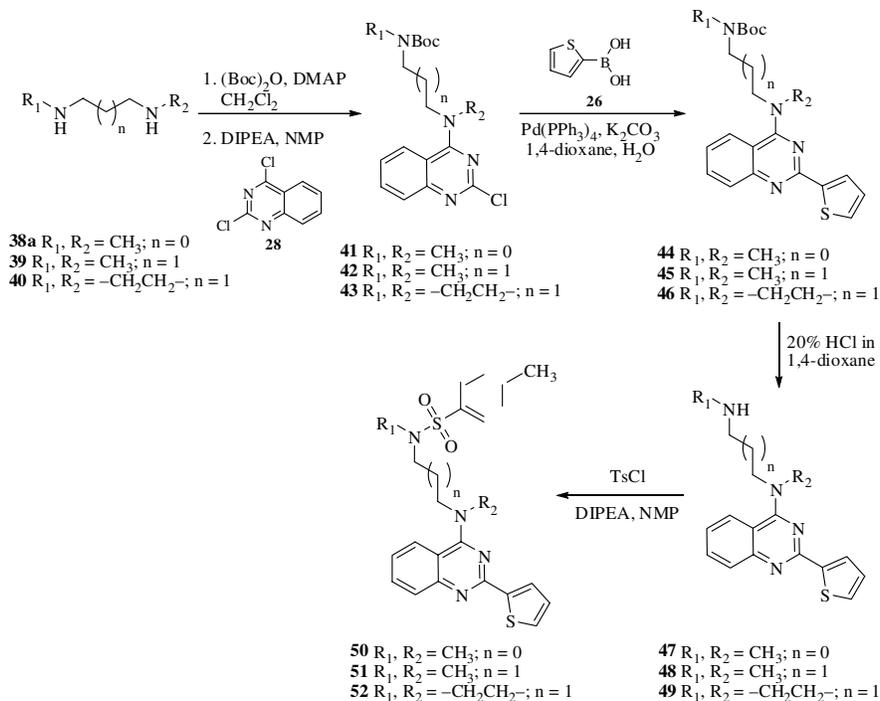
For the SAR study of the sulphonamide portion of the molecule, we modified the synthetic procedure toward a more convenient methodology that allowed us to introduce variation at the

last step of the synthesis. Scheme 3 shows how sulphonylation, carboxylic coupling and alkylation of the key intermediate **32** provided a variety of final compounds.

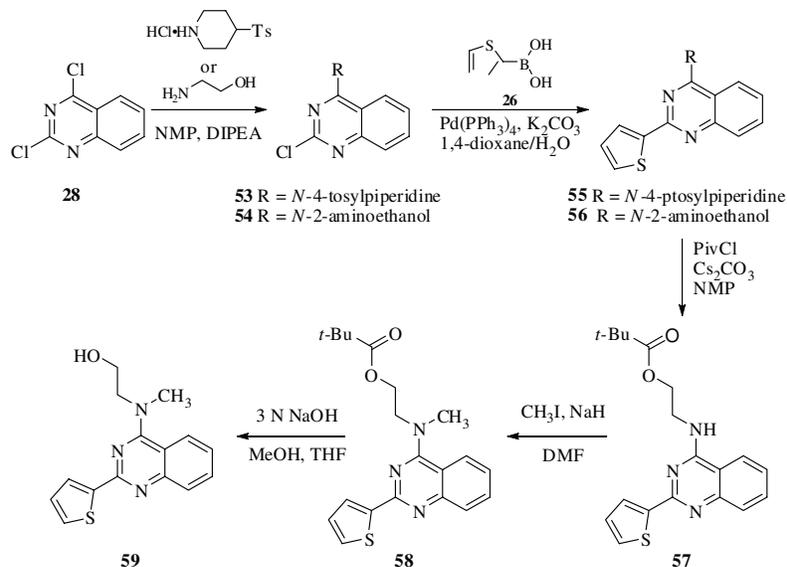


**Scheme 3:** Synthesis of analogs with modification of the aromatic sulphonamide

We also synthesized a number of analogs testing the flexibility of the diamine linker, as well as the activity of other amine-linked functionalities attached to the piperidine ring. Schemes 4 and 5 present example syntheses of some of these compounds, which were performed in a similar fashion to the previously described compound syntheses.



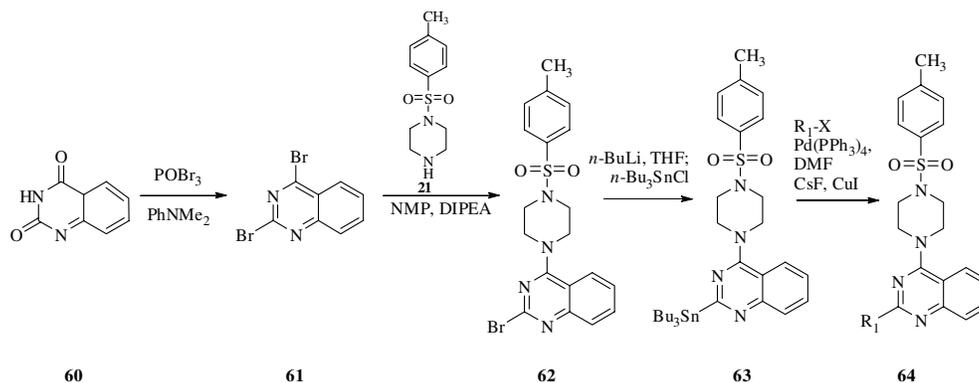
**Scheme 4:** Synthesis of analogs with a flexible linker



**Scheme 5:** Synthesis of additional analogues with a flexible linker

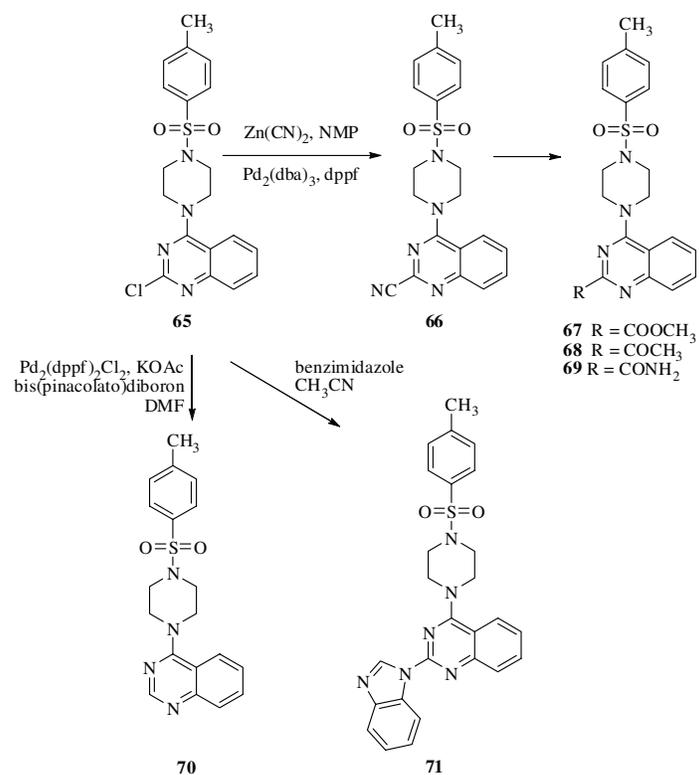
Regarding the synthesis of analogs with aromatic and heteroaromatic modifications at the 2 position, Scheme 6 shows the synthetic methodology involving the production of the organotin **63** as a convenient intermediate for last step diversification using Stille coupling. Thus, 2,4

quinazoline dione, **60**, was converted in 2,4-dibromoquinazoline **61** with phosphorus oxybromide. Selective halogen displacement with substituted piperidine **21** and Lithium halogen exchange followed by reaction with tributyl tin chloride yielded compound **63**. Cross coupling reaction between **63** and heteroaromatic halogens yielded final compounds **64**.

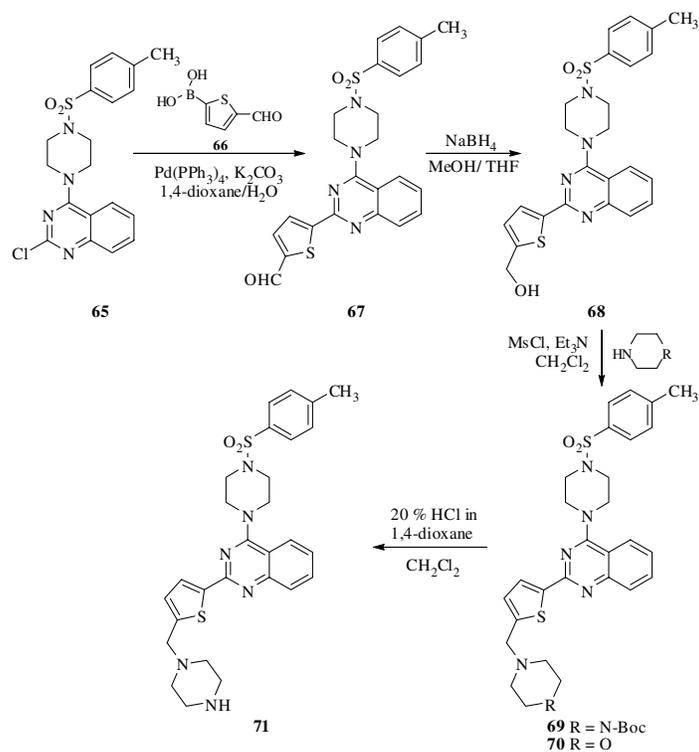


**Scheme 6:** Synthesis of analogs with modifications at the 2 position.

Additional modifications at the same position 2 were obtained as show in Schemes 7 and 8. Thus, cross-coupling reaction with Zinc cyanide catalyzed by Palladium yielded intermediate **66**. Hydrolysis and esterification of nitrile **66** yielded final compounds **67-69**. Reduction of starting material **65** produced compound **70**, and displacement of the chloro at position 2 yielded final compound **71**.



**Scheme 7:** Additional modifications at the 2 position



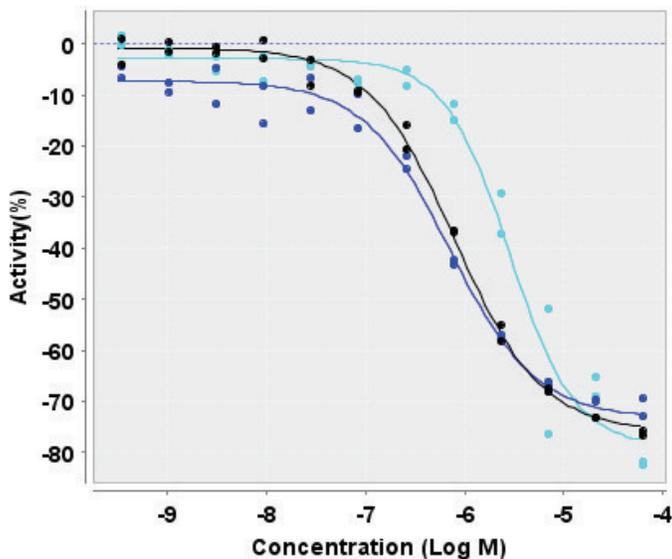
**Scheme 8:** Synthesis of thiophene analogs with improved solubility

### 3.1 Summary of Screening Results

1300 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).

83 of the more potent, high confidence inhibitors and activators were re-ordered for confirmation in N370S GC spleen homogenate using the resorufin-based 'red' fluorescent substrate (AID 2613). Several quinazoline analogs were confirmed to be active, including MLS000393962 (CID 2477651), which is compound **9** below. Based on similar activity in this secondary assay, we chose to develop the SAR of the series by purchasing available analogs from commercial vendors. Table 1 discloses the inhibitory activity of compounds from the primary screen and the initial SAR-by-catalog approach. The activity is reported as N370S spleen homogenate AC<sub>50</sub>, concentration necessary to inhibit 50 % of the 4-methylumbelliferone  $\beta$ -D-glucopyranoside hydrolysis.

### 3.2 Dose Response Curves for Probe



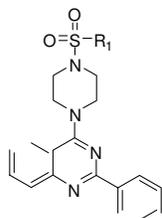
**Figure 1:** Inhibitory curves of compound **13** (CID 40225210), against spleen homogenate homozygous for N370S GC using 4-methylumbelliferyl- $\beta$ -D-glucopyranoside (curve in black) and resorufin  $\beta$ -D-glucopyranoside as substrates (curve in blue), and wildtype spleen homogenate using 4-methylumbelliferyl- $\beta$ -D-glucopyranoside (curve in cyan; AID 2592).

### 3.3 Scaffold/Moiety Chemical Liabilities

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

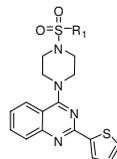
### 3.4 SAR Tables

Tables 3-8 show the capacity of compounds to inhibit the hydrolysis of 4-methylumbelliferone  $\beta$ -D-glucopyranoside. As a positive control, we measured the activity of isofagomine in the same assay ( $AC_{50} = 0.080\mu M$ ).



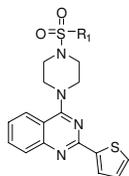
Pubchem CID	Pubchem SID	Compound number	R1	N370S AC <sub>50</sub> (μM)	Compound number	R1	N370S AC <sub>50</sub> (μM)	Pubchem CID	Pubchem SID
2563842	57581506	72		12.67	86		31.83	40225203	85267278
40225166	85267270	73		31.83	87		25.29	40225226	85267262
26451116	85267253	74		12.67	88		20.08	26454973	85267267
40225217	85267255	75		12.67	89		25.29	40039755	85267289
40225294	85267266	76		31.83	90		31.83	4781118	85267274
40087277	85267290	77		15.95	91		40.07	26437257	85267284
26451198	85267256	78		20.08	92		20.08	40225216	85267254
26440758	85267277	79		50.45	93		31.83	26451028	85267250
40225222	85267261	80		15.95	94		100.67	26451000	85267248
40225212	85267246	81		12.67	95		15.85	40225211	85267294
40225287	85267264	82		25.29	96		12.67	40225289	85267279
40225214	85267247	83		12.67	97		25.29	39911974	85267287
26451371	85267258	84		7.99	98		6.35	2563217	57581505
40225219	85267257	85		31.83	99		12.67	6211962	24839374

**Table 3:** Analogs with sulfonamide aromatic modifications having a phenyl at the two position



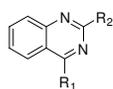
Pubchem CID	Pubchem SID	Compound number	R1	N370S AC <sub>50</sub> (μM)	Compound number	R1	N370S AC <sub>50</sub> (μM)	Pubchem CID	Pubchem SID
4035156	85267271	100		1.26	119		1.27	2477651	85267272
44246410	85267353	101		3.18	120		2.52	45136810	92386624
44246409	85267352	102		2.52	121		2.52	40225286	85267263
26450846	85267234	103		0.32	122		3.18	40225238	85267293
44246456	85267407	104		0.80	123		1.26	40225213	85267238
44246446	85267396	105		1.59	124		0.63	40225220	85267240
44246435	85267384	106		2.00	125		7.99	40225218	85267245
40225165	85267268	107		2.00	126		4.00	40225202	85267285
44246427	85267376	108		0.40	127		2.53	44246454	85267405
44246411	85267354	109		1.00	128		2.53	45136812	92386626
26451103	85267251	110		1.00	129		2.53	26454968	85267295
42909644	85267265	111		3.18	130		5.04	40039753	85267288
17556704	85267291	112		2.00	131		0.32	44246455	85267406
26451399	85267241	113		1.27	132		0.80	40225225	85267233
45136813	92386627	114		1.27	133		1.27	40225215	85267239
26451183	85267243	115		1.00	134		3.18	26451023	85267249
44246443	85267393	116		1.59	135		4.01	26450992	85267242
26440749	85267282	117		1.27	136		1.59	4787756	85267275
40225221	85267259	118		2.01	137		2.35	44246458	85267409

**Table 4:** Analogs with sulfonamide aromatic modifications having a thiophene at the two position



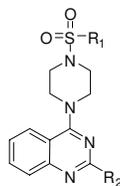
Pubchem CID	Pubchem SID	Compound number	R1	N370S AC <sub>20</sub> (μM)
17564120	85267292	138		2.53
45136815	92386629	139		4.00
44246433	85267382	140		5.04
44246414	85267357	141		1.00
44246418	85267366	142		1.27
44246415	85267358	143		3.18
40225210	85267237	144		0.33
40225288	85267286	145		1.01
4858520	85267280	146		0.50
44246422	85267371	147		0.50
44246428	85267377	148		1.59
44246430	85267379	149		1.26
44246431	85267380	150		2.53
44246412	85267355	151		1.01
44246432	85267381	152		0.50
44246438	85267388	153		1.26
44246436	85267386	154		1.01

**Table 5:** A continuation of Table 4 - analogs with sulfonamide aromatic modifications having a thiophene at the two position



Pubchem CID	Pubchem SID	Compound number	R1	R2	N370S AC50 (μM)	Compound number	R1	R2	N370S AC50 (μM)	Pubchem CID	Pubchem SID
26450846	85267234	103			0.32	173			50.45	45136821	92386635
44246429	85267378	165			2.52	174			10.07	44246444	85267394
44246440	85267390	166			2.52	175			2.00	2459459	85267368
8079890	85267365	167			50.45	176			3.18	44246413	85267356
44246424	85267373	168			1.00	177			79.98	44246420	85267369
44246423	85267372	169			5.04	178			Inactive	45136819	92386633
44246421	85267370	170			50.45	179			6.35	44246457	85267408
2469159	24825474	171			79.98	180			6.35	45136814	92386628
44246441	85267391	172			15.96						

**Table 6:** Analogs with the modifications at the linker.



Pubchem CID	Pubchem SID	Compound number	R1	R2	N370S AC <sub>50</sub> (μM)	Compound number	R1	R2	N370S AC <sub>50</sub> (μM)	Pubchem CID	Pubchem SID
9460093	92386619	181		H	20.58	193			Inactive	44246453	85267404
26450846	85267234	103			0.32	194			Inactive	44246437	85267387
44246434	85267383	182			3.18	195			2.53	45136822	92386636
45136817	92386631	183			12.67	196			40.09	44246351	85267226
45136823	92386637	184			12.67	197			12.67	2563842	57581506
44246451	85267401	185			79.97	198			Inactive	42809384	85267230
44246452	85267402	186			4.00	199			Inactive	44246352	85267227
44246447	85267397	187			25.29	200			Inactive	44246356	85267232
44246416	85267359	188			1.27	201			15.95	44246426	85267375
25708371	85267403	189			20.09	202			100.67	44246442	85267392
44246445	85267395	190			25.29	203			Inactive	44246425	85267374
44246449	85267399	191			200.86	204			Inactive	44246419	85267367
44246448	85267398	192			Inactive	205			40.09	8078745	85267360

**Table 7:** Analogs with modification at the 2 position of the quinazolinone core



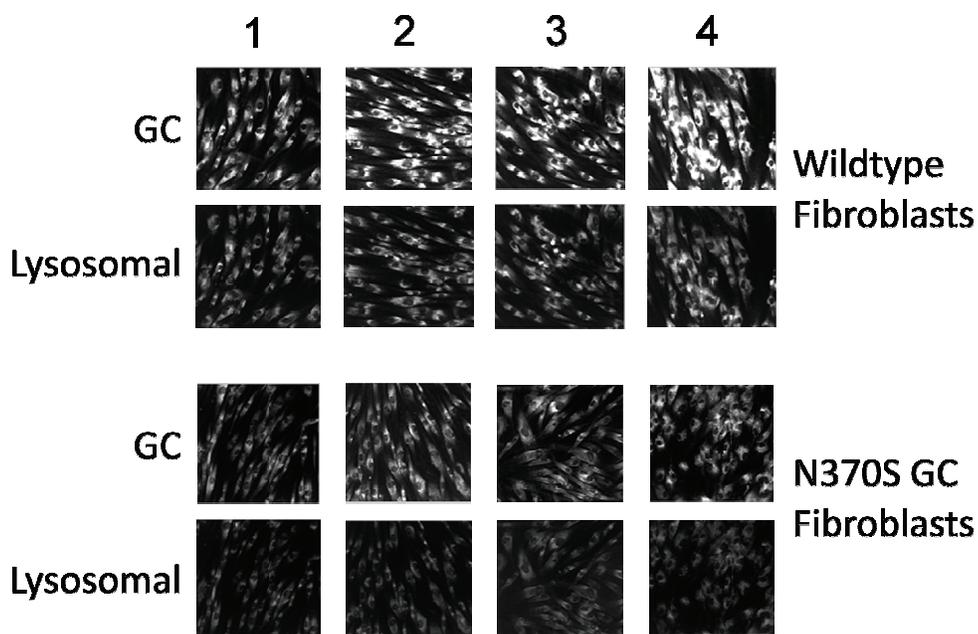
Pubchem CID	Pubchem SID	Compound number	Molecular core	R1	R2	N370S AC50 (μM)	Compound number	Molecular core	R1	R2	N370S AC50 (μM)	Pubchem CID	Pubchem SID
26450846	85267234	103				0.32	214				Inactive	44246345	85267220
45136809	92386623	206				Inactive	215				Inactive	44246347	85267222
45136816	92386630	207				20.08	216				Inactive	44246349	85267224
45136818	92386632	208				4.00	217				Inactive	44246348	85267223
45136820	92386634	209				3.18	218				Inactive	44246350	85267225
45136805	92386618	210				10.31	219				Inactive	44246354	85267229
45136806	92386620	211				Inactive	220				Inactive	44246355	85267231
45136811	92386625	212				3.18	221				Inactive	45136807	92386621
42809381	85267219	213				Inactive	222				Inactive	45136808	92386622

**Table 8:** Analogs with modifications at the molecular core

### 3.5 Cellular Activity

Compound **13** was characterized in a direct assay of chaperone activity using patient fibroblasts; that is, we measured **13**'s ability to increase the translocation of GC to the lysosome<sup>8,16,24,25</sup>. Briefly, fibroblasts obtained from a Gaucher patient homozygous for the N370S mutation and from another donor with wildtype GC were incubated for five days with NCGC00182292 (CID 40225210), in a range of concentrations from 1nM to 50μM, followed by cell fixation and staining with a selective fluorescent GC-antibody (AID2587 and AID2589). Compounds able to increase the ER-Lysosomal traffic show increased fluorescent GC-antibody staining in lysosomes. DMSO and Isofagomine were use as negative and positive controls. Figure 2 shows

the increment of signal in produced by **13**, thereby confirming the chaperone activity of this compound.



**Figure 2:** Chaperone activity of compound **13**, NCGC00182292 (CID 40225210), 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 $\mu$ M Isofagomine (CID 447607), (3) 10 $\mu$ M compound **13**, NCGC00182292 (CID 40225210), and (4) 1 $\mu$ M NCGC00159568 (CID 9893924). Several compounds, including isofagomine (CID 447607) and compound **13**, NCGC00182292 (CID 40225210), show increased lysosomal GC protein after treatment.

### 3.6 Profiling Assays

Selectivity was measured against other two lysosomal hydrolases: alpha glucosidase (AID 2578) and alpha galactosidase (AID 2577). All tested compounds from this quinazoline series show selectivity for inhibiting glucocerebrosidase. None of them show inhibitory activity towards alpha glucosidase or alpha galactosidase at concentrations up to 57 $\mu$ M.

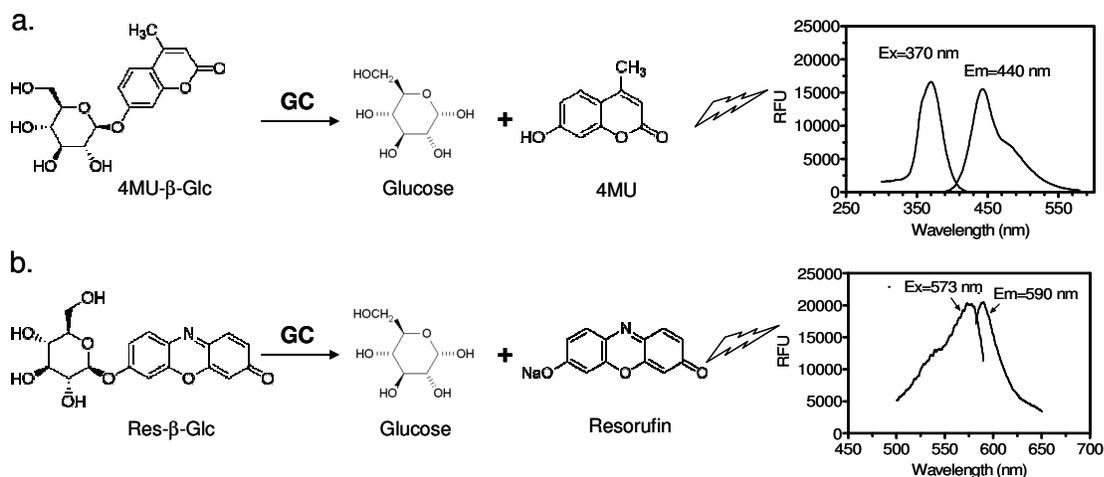
## 4 Discussion

The primary screen for this project assayed for GC enzyme in the context of spleen homogenate derived from a N370S Gaucher patient by monitoring the production of a fluorogenic substrate, 4-methylumbelliferone  $\beta$ -D-glucopyranoside. In addition, several other variations of GC enzyme assays were developed to help eliminate the false positive compounds in the primary screen, including a similar enzyme assay with a red-shifted fluorogenic substrate (resorufin- $\beta$ -D-glucopyranoside) and an enzyme assay with a native substrate (glucosylceramide).

Unfortunately, cleavage of glucosylceramide can only be done in the context of purified enzyme, as this enzyme assay uses a coupled enzyme reaction for detection in which the glucose oxidase/ampex red interacts with glucose, the product of glucosylceramide cleavage, to produce a fluorogenic signal. The strengths and weaknesses of these assays are described in detail below.

In all the assays, activators and auto-fluorescent compounds may both give an apparent increase in signal and quenchers may give a decrease in signal. To eliminate nonspecific compounds, counterscreen enzyme assays with alpha-galactosidase and alpha-glucosidase were used.

Activators and inhibitors were then confirmed using the primary screening assays as well as glucocerebrosidase assays using a red-shifted fluorogenic substrate, and product formation was also directly tracked using LC/MS. Once activity was confirmed, SAR studies were undertaken to characterize chemotypes in more detail.

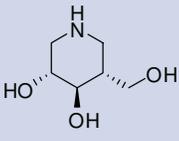
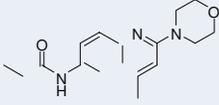
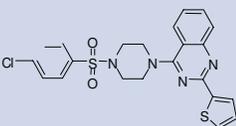
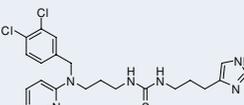


**Figure 3.** Principles of enzyme reactions and product spectrums of two GC enzyme assays. (a) The “Red” GC enzyme assay. The pro-fluorescent substrate Res- $\beta$ -glucopyranoside is hydrolyzed to form two products, glucose

and resorufin, with an excitation peak of 573 nm and an emission peak of 590 nm. This assay is used for the primary screen. (b) The “Blue” GC enzyme assay. The pro-fluorescent substrate 4MU- $\beta$ -Glc is hydrolyzed to form two products, glucose and 4MU, with an excitation peak of 365 nm and an emission peak of 440 nm.

An inhibitory chemical probe for this project is defined as a molecule having  $EC_{50}$  values in lower  $\mu$ M in the cell-based homogenate assay, and being selective against other glycosidic hydrolases (selective –  $EC_{50}$  values > 10 fold against  $\alpha$ -galactosidase and  $\alpha$ -glucosidase).

From the primary screen, we identified several series of modulators, including several imino sugar molecules previously reported to inhibit the enzyme such as isofagomine (Table 9).

Pub Chem CID	Pub Chem SID	Compound	Series	Type	Purified N370S	Purified WT	Spleen N370S	Spleen WT
447607	85267413	 Isofagomine	Imino sugar	Inhibitor	50-60nM	10-15 nM	70-80nM	100nM
5067281	26753330	 NCGC00092410	Quinoline	Inhibitor	560-1000nM	70-80 nM	>10uM	>10uM
2477651	22404737	 MLS000393962	Quinazoline	Inhibitor	Inactive	1.6-2.5uM	450nM	1000 nM
9893924	89449177	 NCGC00159568	Thiourea	Inhibitor	2500uM	710nM	580nM	350nM

**Table 9:** Compound representatives of initial hit series. Isofagomine is an imino sugar previously reported to chaperone glucocerebrosidase. NCGC00092410 (CID 5067281) is a non-imino sugar previously reported to inhibit purified glucocerebrosidase<sup>23</sup>.

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

The probe represents one of two new, non-imino sugar chemotypes that can act as a chaperone of glucocerebrosidase *in vitro*. There are not significant differences in activity between this probe and the other, ML156 (NCGC00159568, CID 9893924). Isofagomine (CID 447607) has been shown to chaperone glucocerebrosidase *in vitro*, but has been abandoned as a candidate for further clinical development (<http://www.gaucher.org.uk/enews.php?id=303>). NCGC00092410 (CID 5067281) is a non-imino sugar previously reported to inhibit purified glucocerebrosidase (Zheng *et al*, Proc. Natl. Acad. Sci. U.S.A. 2007, 104, 13192), but is much less active in tissue homogenate assays, likely indicating non-specific protein binding.

#### **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. It is obvious, from x-ray crystal structure studies of isofagomine binding, that 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**

No further characterization is planned at this time.

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