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Non-iminosugar glucocerebrosidase small molecule chaperones

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Abstract

Small molecule chaperones are a promising therapeutic approach for the Lysosomal Storage Disorders (LSDs). Here, we report the discovery of a new series of non-iminosugar glucocerebrosidase inhibitors with chaperone capacity, and describe their structure activity relationship (SAR), selectivity, cell activity pharmacokinetics.

Introduction

Gaucher disease is an autosomal recessive disorder that results from DNA mutations affecting the expression, function or translocation properties of the lysosomal glucocerebrosidase (EC 3.2.1.45, GCCase).¹ As a result of these changes, glucosylceramides, the enzyme's natural substrates, accumulate in lysosomes of macrophages, resulting in diverse disease manifestations.²⁻⁴

Many mutant forms of GCCase, including the prevalent Gaucher mutation N370S, are still functional. However, the altered amino acid sequence has a strong impact on the protein structure, preventing its proper folding to be recognized by the corresponding lysosomal conformation recognition transporters in the endoplasmic reticulum (ER).⁴ As consequence of misfolding, mutant GCCase may accumulate in the ER, and is eventually tagged for proteasome-mediated breakdown. Small molecule chaperones can bind with these mutants, inducing or accelerating proper folding, therefore are able to facilitate the translocation of the enzyme to the lysosome.⁵⁻⁷

Previous studies have shown that several iminosugar inhibitors of lysosomal hydrolases also display chaperone capacity.^{5,8-12} However, iminosugars tend to have selectivity, and their therapeutic window is very small.¹³⁻¹⁵ Therefore, alternative chaperone scaffolds are highly desirable. Herein, we present a new series of non-iminosugar small molecule chaperones.

Results and discussion

Previously, we described an approach for improving high-throughput screening techniques by executing assays for lysosomal hydrolases in a tissue-homogenate environment, which

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better emulated the cell's native environment.^{16,17} Using this tissue homogenate protocol,¹⁸ we identified compound ML156 (**8i**) with potent GCase inhibitory activity. In addition, this compound was highly selective when tested with other hydrolases, including alpha-glucosidase and alpha-galactosidase. It did not exhibit auto-fluorescence, and it displayed inhibitory activity in isolated enzyme assays. Moreover, this compound effectively inhibited the hydrolysis of 4-methylumbelliferone β -D-glucopyranoside (blue substrate), resorufin- β -glucopyranoside (red substrate) and a fluorescent ceramide substrate.

As an initial evaluation of the potential chaperone capacity of ML156 (**8i**), we evaluated its effect on GCase function when the enzyme was incubated at 51 °C for 60 minutes (Figure 2).¹⁹ Other known GCase chaperones, such as Isfagomine, have demonstrated their capacity to thermostabilize this enzyme. In the absence of compound, the enzyme lost most of its hydrolytic capacity within 1 hour. In contrast, in the presence of ML156 (**8i**) or Isfagomine as a control molecule, the enzyme was able to retain considerable function, indicating its ability to stabilize the functional conformation of GCase (Figure 2). These data suggested that members of this chemical series had potential as chemical chaperones.

The synthesis of the selected compound ML156 (**8i**) and its analogues for SAR exploration is detailed in Schemes 1 to 3. As shown, reaction of 2-bromopyridines **1a-c** with an excess of a diaminoalkane gave the corresponding 2-aminopyridines **3a-e**. The primary amino group of **3a-e** was then protected by a trityl group, yielding **4a-e**. Subsequent selective alkylation of **4a-e** with appropriate alkyl halides in dimethylformamide using lithium hexamethyldisilazide (LiHMDS) as a deprotonating agent afforded **5a-f** and **5h-i**, which were readily converted to amine **6a-i** by trifluoroacetic acid mediated trityl group deprotection. The desired ureas **8j-k** and thioureas **8a-i** and **8l** were prepared by a multicomponent one-pot reaction of **6**, **7**, and 1,1'-carbonyldiimidazole or 1,1'-thiocarbonyldiimidazole, followed by removal of the trityl protecting group (Scheme 1).

Additional analogues were synthesized employing a Suzuki reaction of **5a** with phenyl boronic acid, followed by trityl group deprotection to afford amine **9**. Thiourea **10** was formed by a similar one-pot reaction of **9**, **7a**, and 1,1-thiocarbonyldiimidazole, followed by deprotection of the trityl group (Scheme 2).

Multicomponent reactions of **6a**, **11**, and 1,1-thiocarbonyldiimidazole yielded the corresponding thiourea **12**. Trifluoroacetic acid mediated Boc-deprotection of **12** afforded the free amine **13a**. Capping of the amine with an acetyl group gave the final compound **13b** (Scheme 3).

All analogues were tested in an assay using an N370S spleen homogenate assay (AID2590)¹⁸ to evaluate their inhibitory activity (Table 1). The resynthesized hit compound **8i** displayed the same activity (0.6 μ M) as the purchased sample (entries 1-2).

Several positions of the hit compound ML156 (**8i**) were explored for SAR, yielding the following results: (a) Decreasing the size of the R₁ substituent gradually weakened the activity (entries 1-5). (b) At the R₂ position, placing a phenyl group at C5 resulted in better activity than placing the phenyl group at C4 (**10** vs **8c**). (c) Ureas had activities similar to the thioureas (**8i** vs **8j** and **8a** vs **8k**). (d) Decreasing the distance between the thiourea group and the pyridyl moiety from 3C to 2C resulted in a small decrease in activity (**8i** vs **8d**). On the other hand, increasing this carbon chain from 3C to 4C slightly increased the activity (**8e** vs **8a**). However, shortening 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 was found to be very important for optimal activity. Replacing the imidazole with a free amino group (**13a**), an acetamide (**13b**), or a methyl group (**25**)

resulted in significant reduction or complete loss of activity. (f) Selective methylation of the thiourea NH between the thiourea and pyridine resulted in a 2-fold loss of activity (**26** vs **8a**). Methylation of the other thiourea NH between the thiourea and imidazole resulted in a 10-fold loss of activity (**27** vs **8a**). (g) Replacing the pyridine ring with a benzene ring (**24** vs **8a**), as shown in entry 21, yielded a similar level of activity.

Our lead compound (ML156, **8i**) has been previously described in the literature as an agonist of the Somatostatin receptor SST4.²⁰ For this reason, we decided to evaluate several analogues of the series in a human SST4 receptor binding affinity assay (Table 2). When comparing data from the receptor binding assay and the GCase assay, it was clear that the SARs did not correlate with each other. For example, compound **24** was about 10-fold more potent than compound **27** in the N370S spleen homogenate GCase assay, but **24** was about 5-fold less potent than **27** in the SST4 binding affinity assay. These results suggest that the SST4 binding affinity of this series could be further diluted without diminishing the GCase activity through additional structure modifications.

We then evaluated the GCase chaperone capacity of our best compounds, as well as the known chaperone of Isofagomine with laser scanning confocal microscopy in both wild type fibroblasts and fibroblasts from patients with genotype N370S/N370S.²¹ The cells were treated with 1 μ M of the selected compounds, let rest for five days, and then stained both with an Alexa555-labeled antibody for GCase (red) and an Alexa488-labeled antibody for the lysosomal marker LAMP-1 (green). Figure 3 shows high magnification images resulting from these translocation studies. Increased co-localization between LAMP-1 (green), representing the lysosomes, and the N370S GCase enzyme (red) is clearly observed after treatment with 1 μ M of **24** (Figure 3C) or **8i** (Figure 3D) compared to vehicle-only treatment (Figure 3A). These compounds show translocation capacities that are similar to the iminosugar GCase chaperone Isofagomine (Figure 3B), which has been advanced to clinical trials. In addition, these compounds display a lower GCase inhibitory activity when compared to Isofagomine (IC_{50} = 80 nM, using the N370S tissue homogenate assay with the blue substrate), and therefore, they may offer a wider therapeutic window.

We further evaluated the stability of our chemical series under *in vitro* conditions. Compounds were incubated in mouse liver microsomes for 60 minutes in the presence of NADPH. After incubation with our lead compound (ML156, **8i**), 58% of the parent molecule remained, suggesting that this compound is metabolically stable. This promising result prompted us to evaluate the *in vivo* pharmacokinetic profile of ML156 (**8i**). Table 3 shows the levels and pharmacokinetic parameters of ML156 (**8i**) in plasma following intraperitoneal administration of the compound to mice at a dose of 30 mg/kg.

The high stability of ML156 (**8i**) is shown in Table 3. This compound maintained plasma levels above the GCase IC_{50} for more than 8 hours. Additional pharmacokinetic studies disclosed that reasonable levels of the compound also reached the brain (please see the supporting information). This finding is very encouraging, suggesting that this compound could have utility in the treatment of the neuronopathic forms (Type 2 and 3) of Gaucher disease.

Conclusions

We have reported the discovery and initial SAR study of a novel non-iminosugar small molecule chemical series with encouraging GCase chaperone activity. Initial pharmacokinetic studies of IP administered ML156 (**8i**) show good penetration of this molecule in plasma, brain and liver. The level of the compound in brain is especially important, as the current standard-of-care, enzyme replacement therapy with recombinant

GCase, has no effect on the neurological manifestations of the disease due to its poor CNS distribution. Therefore, this series provides an excellent lead candidate for further development and testing in animal models of Gaucher disease. Further *in vivo* evaluation is currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Concentration-response of lead compound ML156 (**8i**) in the primary screening assay for inhibition using tissue homogenate from a patient with genotype N370S/N370S. The IC_{50} of this compound, measured in triplicate on three different days, was 580 ± 30 nM.

Figure 2. Thermofunctional stabilization studies with ML156 (**8i**) compared to the iminosugar Isofagomine.

Figure 3. Translocation studies of N370S GCase in Gaucher fibroblasts (genotype N370S/ N370S). Gaucher fibroblasts were stained for LAMP-1 (green) and GCase (red) in the presence of (A) DMSO (vehicle), (B) 1 μ M Isofagomine, (C) presence 1 μ M of compound **24**, (D) 1 μ M of compound ML156 (**8i**). Scale bar is 5 μ m.

Scheme 1.

Reagents and conditions: (i) $\text{H}_2\text{N}(\text{CH}_2)_n\text{NH}_2$ (**2**), pyridine, reflux; (ii) TrCl , Et_3N , THF, 0°C -r.t.; (iii) LiHMDS , THF, R_1X , r.t.; (iv) TFA, DCM; (v) 7, 1,1'-carbonyldiimidazole or 1,1'-thiocarbonyldiimidazole, $i\text{PrNEt}_2$, DCM, μW , 130°C , 10 min; (vi) TFA, DCM.

Scheme 2.

Reagents and conditions: (i) PhB(OH)_2 , Na_2CO_3 , $\text{Pd(PPh}_3)_4$, 100°C , 2 h; (ii) TFA, DCM; (iii) **7a**, 1,1'-thiocarbonyldiimidazole, $i\text{PrNEt}_2$, DCM, $\mu\text{W } 150^\circ\text{C}$, 10 min; (iv) TFA, DCM.

Scheme 3.

Reagents and conditions: Reagents and conditions: (i) **11**, 1,1'-thiocarbonyldiimidazole, $i\text{PrNEt}_2$, DCM, μW 130 °C, 10 min; (ii) TFA, DCM; (iii) for **13b** only, CH_3COCl , TEA, DCM.

Table 1

SAR of the synthesized compounds using the N370S tissue homogenate assay with 4-methylumbelliferone β -D glucopyranoside substrate (blue substrate).

Entry	Cmpd. No			R ₃	n	m	X	IC ₅₀ (μM)
1	ML156, 8i	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	3	S	0.6±0.1
2	8a	4-Chlorobenzyl	5-Br	4-Imidazole	3	3	S	1.2
3	8f	Butyl	5-Br	4-Imidazole	3	3	S	8.2
4	8h	Benzyl	5-Br	4-Imidazole	3	3	S	5.8
5	8g	H	5-Br	4-Imidazole	3	3	S	46.0
6	8b	3,4-Dichlorobenzyl	H	4-Imidazole	3	3	S	7.3
7	10	4-Chlorobenzyl	5-Ph	4-Imidazole	3	3	S	1.3
8	8c	4-Chlorobenzyl	4-Ph	4-Imidazole	3	3	S	4.0
9	8d	3,4-Dichlorobenzyl	5-Br	4-Imidazole	2	3	S	2.1
10	8e	4-Chlorobenzyl	5-Br	4-Imidazole	4	3	S	0.8
11	8l	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	2	S	18.3
12	8j	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	3	O	0.6
13	8k	4-Chlorobenzyl	5-Br	4-Imidazole	3	3	O	1.0
14	13a	4-Chlorobenzyl	5-Br	NH ₂	3	3	S	45.0
15	13b	4-Chlorobenzyl	5-Br	NHCOCH ₃	3	3	S	inactive
16	25	3,4-Dichlorobenzyl	5-Br	CH ₃	3	3	S	inactive
17	8q	phenylethyl	5-Br	4-Imidazole	3	3	S	2.8
18	8m	4-chlorophenylethyl	5-Br	4-Imidazole	3	3	S	2
19	26							2.8

Entry	Cmpd. No	R ₁	R ₂	R ₃	n	m	X	IC ₅₀ (μM)
20	27							18
21	24							1.3

Table 2

Comparison of GCase inhibitory activity with Somatostatin receptor SST4 binding affinity.

Entry	Compound #	GCase IC ₅₀ (nM)	SST4 K (nM)
1	8i	600	17
2	8g	46000	690
3	26	2800	19
4	24	1300	160
5	27	18000	26

Individual and mean plasma concentration-time data of ML156 (**8i**) after intraperitoneal administration of the compound to male C57BL/6 mice at a dose of 30 mg/kg.

Table 3

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL)					Mean (ng/mL)	Mean (μM)	SD	CV(%)
			Individual								
30	IP	0	BQL	BQL	BQL	BQL	BQL	BQL	BQL	N/A	N/A
		0.083	1820	1820	1820	1410	1683	3.03	237	14.1	
		0.25	2420	3010	3010	1640	2357	4.24	687	29.2	
		0.5	2530	2270	2270	3270	2690	4.84	519	19.3	
		1	3740	3770	3770	3150	3553	6.39	350	9.84	
		2	2870	3590	3590	3840	3433	6.17	504	14.7	
		4	2560	2030	2030	2570	2387	4.29	309	12.9	
		8	1190	357	357	714	754	1.35	418	55.5	
		12	118	127	127	80.7	109	0.20	24.5	22.6	
		24	8.04	12.6	12.6	9.98	10.2	0.02	2.29	22.4	
PK parameters		Unit	Estimate								
T _{max}		hr	1.00								
C _{max}		ng/mL	3553								
T _{1/2}		hr	2.57								
AUC _{last}		hr*ng/mL	20600								
AUC _{INF}		hr*ng/mL	20700								