Title

Identification of pyrimidinyl piperazines as non-iminosugar glucocerebrosidase (GCase) pharmacological chaperones

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Keywords

Glucocerebrosidase

Pharmacological Chaperone

Gaucher disease

Parkinson's disease

Abstract

Glucocerebrosidase (GCase) is a lysosomal enzyme encoded by the GBA1 gene, loss of function variants of which cause an autosomal recessive lysosomal storage disorder, Gaucher disease (GD). Heterozygous variants of GBA1 are also known as the strongest common genetic risk factor for Parkinson's disease (PD). Restoration of GCase enzymatic function using a pharmacological chaperone strategy is considered a promising therapeutic approach for PD and GD. We identified compound **4** as a GCase pharmacological chaperone with sub-micromolar activity from a high-throughput screening (HTS) campaign. Compound **4** was further optimised to ER-001230194 (compound **25**). ER-001230194 shows improved ADME and physicochemical properties and therefore represents a novel pharmacological chaperone with which to investigate GCase pharmacology further.

Glucocerebrosidase (GCase), encoded by the GBA1 gene, is a lysosomal sugar hydrolase which plays a central role in the metabolism of glucosylceramide by catalysing the cleavage of the β-glycosidic bond.[1] Mutations in the GBA1 gene cause an autosomal recessive lysosomal storage disorder known as Gaucher disease (GD).[2] Heterozygous variants of GBA1 represent the strongest common genetic risk factor for Parkinson's Disease (PD) and reduced GCase activity can be observed in sporadic PD patients.[3] Modulation of GCase is therefore a logical and promising drug target for the treatment of GD and PD. Currently, enzyme replacement therapy (ERT) and substrate reduction therapy (SRT) are used to treat GD, but these approaches still have limitations in terms of poor brain penetrance[4] and side effects potentially related to reduced substrate levels.[5]

One major consequence of many GBA variants is protein misfolding within the endoplasmic reticulum, resulting in a reduction of correctly trafficked and folded mature protein and hence a reduction in lysosomal GCase enzymatic activity.[3] Conceptually, pharmacological chaperones (PC) are an interesting approach to restore GCase function.[6, 7] A PC should bind to the mutant GCase protein, enhance its correct folding, processing and trafficking, to increase functional GCase levels within the lysosome. A number of iminosugars have been studied as pharmacological chaperones of GCase and other lysosomal enzymes,[8] but the iminosugar structure can lead to unfavourable physicochemical properties due to high polarity and multiple hydrogen-bonding donors[9] as well as off-target activities at other enzymes that recognize the sugar structure.[10] To mitigate the consequence of such concerns, several non-iminosugar PCs have been reported by other researchers (Compound **1**-**3**, Figure 1).[11, 12, 13]

We established an assay using a patient-derived fibroblast cell line bearing the N370S GBA mutation to report on the GCase pharmacological chaperoning activity of our compounds. In this assay, intact cells were treated with test compounds for 5 days, lysed and evaluated for GCase activity (as a surrogate for GCase protein levels) by measuring cleavage of the artificial fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG).[14] Fluorescence measurements were normalised to the maximal response of the known GCase PC Isofagomine (IFG) (100%). The concentration of each compound producing 50% of its maximal response (Emax) was calculated as an AC_{50}. Under these conditions, IFG displayed consistent activity with an AC_{50} of 0.3 μM, which is in agreement with previous reports.[11, 15]

Compound **4** was initially identified as a potent GCase PC (AC_{50} 0.09 μM), although its solubility and human microsomal stability were sub-optimal. Initial structure-activity-relationship (SAR) exploration of **4**, through analogue searches, led to the diaminopyrimidine **5** which showed comparable chaperoning activity and with significantly lower molecular weight. Here we report the results of our investigation of this series culminating in the identification of compound **25**, a potent GCase chaperone with promising *in vitro* ADME characteristics.

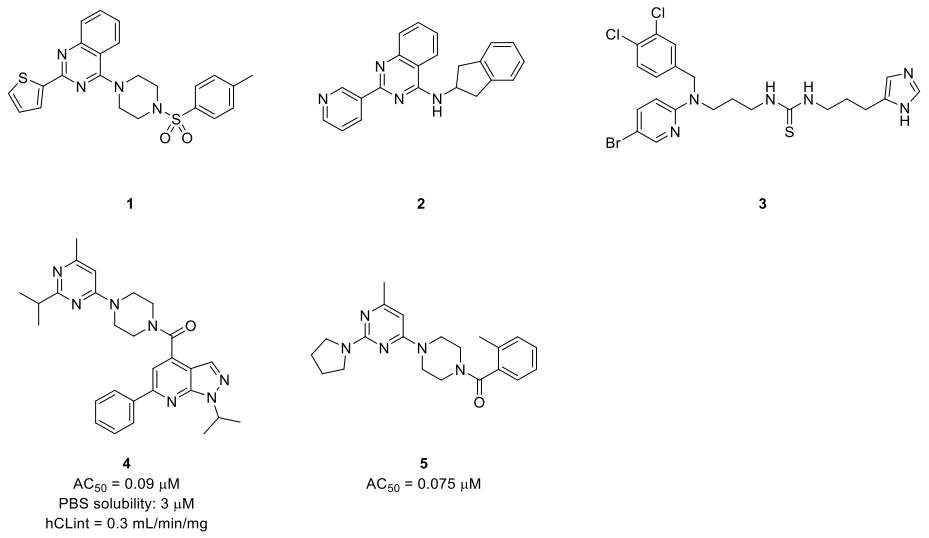
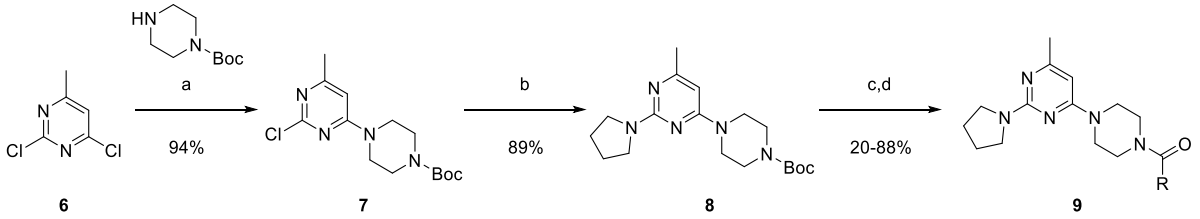


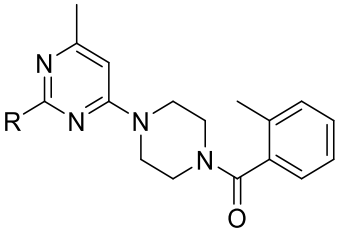
Figure 1. Structure of representative non-iminosugar GCase pharmacological chaperons and early HTS hits

The general synthetic route for the preparation of diaminopyrimidine analogues is described in Scheme **1**. Dichloropyrimidine **6** was treated with 1-Boc-piperidine, affording compound **7** as the major product which is easily separable from the minor isomers by column chromatography. The second SNAr reaction took place by heating compound **7** with amines in DMA, affording compound **8** in high yield. After Boc deprotection with HCl-dioxane, the piperidine was acylated with an acid chloride or carboxylic acid/EDCI to generate the final product **9**. This versatile route allowed for the rapid exploration of SAR simply by changing the combination of amines and acylating agents.



Scheme 1. General method for synthesis of pyrimidine compounds. Reagents and conditions: a) 1-Boc-piperazine, N,N-diisopropylethylamine, THF, rt; b) pyrrolidine, DMA, 120 °C; c) 4N HCl in 1,4-dioxane, rt; d) RCOCl, Et_3N, DCM, rt or RCOOH, EDCI, HOBt, N,N-diisopropylethylamine, DMF, rt

Exploration of the SAR began by synthesising the hybrid compound **10**, where the pyrrolidine substituent on the pyrimidine core was replaced by the iPr group present in compound **4**, but no significant change of activity was observed (Table 1). Considering synthetic feasibility, we decided to concentrate on diaminopyrimidines for further SAR exploration. Replacement of the pyrrolidine with smaller amines reduced the chaperoning activity (compound **11**, **12**), while the larger piperidine- and azepane-substituted analogues (**13** and **14**) had comparable or more potent activity than compound **5**. In contrast, the morpholine (**15**) and difluoropyrrolidine (**16**) analogues showed significantly reduced activity.



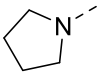
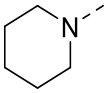
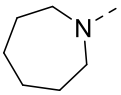
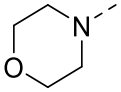
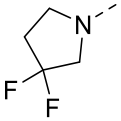
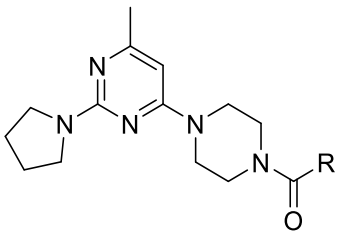
Compound	R	Fib AC_{50} (μM)	Fib E_{max} (%)
5		0.075	119
10		0.15	148
11		0.44	104
12		0.63	109
13		0.17	137
14		0.029	126
15		5.2	114
16		1.4	90

Table 1. SAR of amine substituents on pyrimidine core

Next, the N-acyl piperazine substituent was investigated (Table 2). The simple N-benzoyl compound **17** showed a comparable AC_{50} to compound **5**. Other *ortho* and *meta* substituted derivatives made no significant impact on the chaperoning activity (compounds **18**-**21**). Exploration of the *para*-position revealed that 4-substituted benzenes possess more potent chaperoning activities (compound **21**-**25**). In particular, the *tBu* analogue **25** which exhibited a single-digit nanomolar AC_{50} (9.6 nM). Replacement of the benzene ring with heteroaromatic groups or an acetyl group significantly diminished activity, suggesting the importance of a lipophilic interaction at this site (compound **26**-**28**). Interestingly, the cyclohexyl amide **29** and phenylacetyl amide **30** exhibited weaker activities than benzoyl amide **17** despite having similar lipophilicities, which may imply that the planarity of this substituent is important.



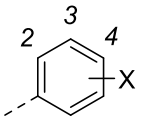
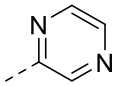
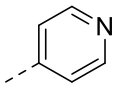
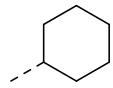
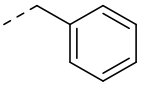
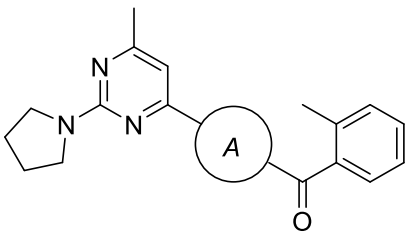
Compound	R	X	Fib AC_{50} (μM)	Fib E_{max} (%)
5		2-Me	0.075	119
17		H	0.072	105
18		2-F	0.17	93
19		2-CF_3	0.10	156
20		2-Cl	0.063	149
21		3-Me	0.13	141
22		4-Me	0.070	89
23		4-Cl	0.040	137
24		4-Ph	0.058	118
25		4-*t*Bu	0.0096	121
26		-	5.3	78
27		-	6.8	>120
28	Me	-	7.1	60
29		-	0.36	69
30		-	0.46	86

Table 2. SAR of N-acyl groups on piperazine

We then turned our attention to investigate SAR of the piperazine unit (Table 3). Ring-expanded, fused, and spirocyclic isosteric piperazine replacements were synthesized (compounds **31**-**33**)[16] but all led to a significant reduction in chaperoning activity.



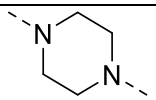
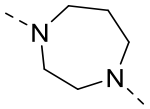
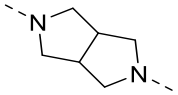
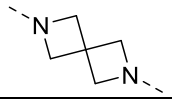
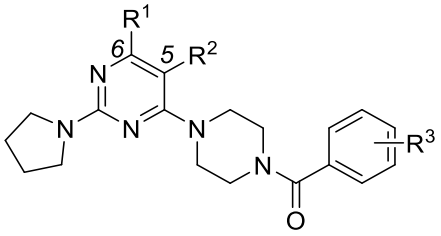
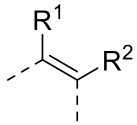
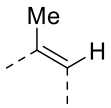
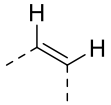
Compound	Ring *A*	Fib AC_{50} (μM)	Fib E_{max} (%)
5		0.075	119
31		1.8	95
32		0.66	89
33		2.1	>70

Table 3. Replacement of piperidine with other bioisosteres

Finally, the importance of substitution at the 5- and 6-position of the pyrimidine ring was explored (Table 4). Removal of the methyl group at position 6 led to a significant decrease of chaperoning activity (compound **34**). Replacement by an electron-withdrawing trifluoromethyl group resulted in complete loss of activity (compound **35**), possibly due to the decreased basicity of pyrimidine core[17], in contrast to the hydroxymethyl derivative (compound **36**) which partially restored the activity of compound **5**. Introduction of a methyl group at the 5-position restored activity (compound **34** vs **37**) but the 5,6-dimethyl analogue **38** exhibited slightly weaker activity than mono-methylated analogues. Interestingly, fusing the substituents at position-5 and 6, as in the quinazoline **39,** restored chaperoning activity. The cyclopentane-fused compound **40** slightly improved activity, suggesting not only lipophilicity but the size or shape of substituents could be important factors. The 4-*t*-butyl benzoyl derivative **41** was synthesized based on SAR described in Table 2 and showed a further improvement in chaperoning activity with an AC_{50} of 5.6 nM. Ring expansion from cyclopentane to cyclohexane did not lead to further improvements in activity (compound **42**). The cyclic ether analogues (compounds **43**-**45**) were prepared in an attempt to mitigate the lipophilic character of carbocycles but resulted in a significant loss of activity.



Compound		R^3	Fib AC_{50} (μM)	Fib E_{max} (%)
5		2-Me	0.075	119
34		2-Me	0.68	81

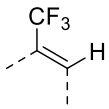
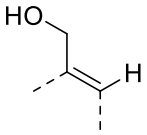
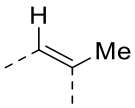
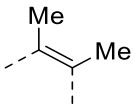
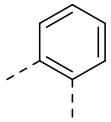
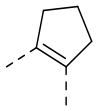
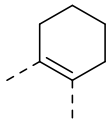
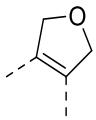
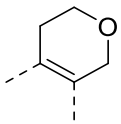
35[a]		2-Me	>25	-
36		2-Me	0.22	112
37		2-Me	0.12	134
38		2-Me	0.25	145
39		2-Me	0.079	134
40		2-Me	0.030	139
41		4-*t*Bu	0.0056	126
42		4-*t*Bu	0.0088	114
43		2-Me	0.54	107
44		4-*t*Bu	0.15	119
45		2-Me	0.41	91

Table 4. SAR of substituents on 5- and 6-positions of pyrimidine. [a] HCl salt was used

The physicochemical and *in vitro* ADME properties of selected compounds are summarized in Table 5. The original HTS hit compound **4** showed low solubility in PBS and high clearance in human liver microsomes. As expected from a smaller molecular size and lower calculated lipophilicity, compound **5** exhibited significant improvements in both solubility and human microsomal stability without diminishing chaperoning activity. Although introduction of the *t*-Bu group significantly increased the lipophilicity, pyrimidine **25**, one of the most potent compounds identified by hit optimisation, still showed good solubility, acceptable human microsomal stability and MDR1 flux ratio.[18] In contrast, the increased lipophilicity of quinazoline **39** resulted in instability in human microsomes. The most potent, fused carbocyclic derivative **41** was more lipophilic and showed significantly lower solubility in PBS. Consequently, compound **25** (ER-001230194) was selected for further pharmacological evaluation as it possessed the most balanced profile of activity and drug-likeness.

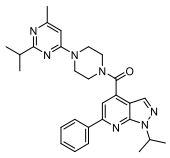
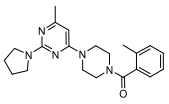
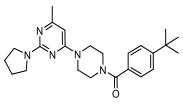
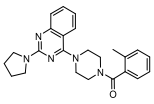
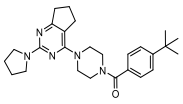
					
Compound	**4**	**5**	**25**	**39**	**41**
MW[a]	483.62	365.48	407.56	401.51	433.60
clogD	4.93	3.10	4.13	4.72	5.12
Fib AC_{50}[b]	0.090	0.075	0.0096	0.079	0.0056
solubility[c]	3	94	68	85	2
hCLint[d]	0.341	0.104	0.172	0.789	N.T[f]
CFR[e]	1.26	1.38	2.00	2.19	N.T[f]

Table 5. Physicochemical and ADME parameters of selected compounds. [a] Molecular weight; [b] AC_{50} values in μM; [c] PBS solubility in μM; [d] Intrinsic clearance in human liver microsomal stability in mL/min/mg; [e] Corrected flux ratio in MDR1-overexpressing PK1 cells compared to control LLC-PK1 cells; [f] Not tested.

To confirm the effects of compound **25** observed within the 5-day N370S fibroblast chaperoning assay, lysosomal GCase levels were assessed following 5-day compound treatment, as measured using immunocytochemistry (ICC) assessment. As shown in Table 6, both IFG and compound **25** had similar AC_{50} and EC_{50} values in both the chaperoning and ICC assay formats, confirming that the compound driven effects observed within the chaperoning assay was a reliable surrogate of lysosomal GCase protein levels.

In addition to acting as pharmacological chaperones, iminosugars such as IFG that bind the GCase catalytic site are also well known to inhibit GCase enzymatic function.[19, 20] Historically these effects are often measured within isolated GCase biochemical assays, using 4-MUG as the substrate. In our studies using such a cell-free GCase assay, the IC_{50} of IFG was comparable to its chaperoning AC_{50}, whereas compound **25** showed a clear separation (1000-fold) between its IC_{50} and AC_{50} (Table 6). The equipotent chaperoning and inhibitory activity of IFG is at first sight counterintuitive but can potentially be explained by the 4-MUG assay protocol used by many groups to assess GCase chaperoning activity. The 4-MUG assay protocol requires cells to be washed and lysed before the addition of the fluorescent substrate. It is therefore conceivable that IFG and other test compounds are diluted or washed out prior to measuring GCase activity, resulting in an underestimation of inhibitory activity.

To assess the potential inhibitory effects within a cellular environment, compounds were evaluated in a live-cell lysosomal GCase activity assay where the hydrolysis of the cell-penetrant fluorescent substrate PFB-FDGlu is measured.[21] In this format, IFG showed similar inhibitory activity to that observed in the cell-free GCase enzymatic assay but, unexpectedly, compound **25** exhibited in-cell inhibitory activity within the fibroblast PFB-FDGlu assay at much lower concentrations than predicted by the cell-free GCase assay. Similar discrepancies of inhibitory activity between cell-free GCase and cellular PFB-FDGlu data were also observed for compounds **41** and **42**. Although the reason for this discrepancy is unknown, it could be the result of differing inhibitory mechanisms of action of compound 25 and IFG, which are affected by the different biochemical microenvironments within the cell-free GCase and cell-based functional assays. These results also emphasize the importance of assessing compound activities in multiple, orthogonal assays, including in-cell activity assays, rather than relying on single functional read-outs.[22]

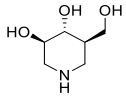
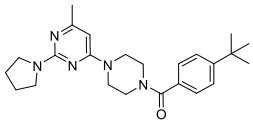
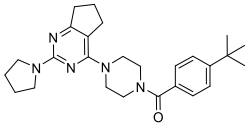
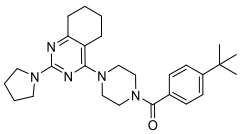
	 **IFG**	 **25 ER-001230194**	 **41**	 **42**
Fib AC_{50}[a]	0.30	0.0096	0.0056	0.0089
Fib ICC EC_{50} [a]	0.39	0.013	N.T[c]	N.T[c]
Cell-free IC_{50}[b]	0.23	9.7	4.2	5.2
Fib PFB-FDGlu IC_{50}[a]	0.41	0.027	0.0037	0.0065

Table 6. Comparison of pharmacological activities of selected compounds. All data are presented in μM. [a] Compound activity was determined in patient-derived fibroblasts bearing the N370S GBA mutation; [b] Inhibitory activity was evaluated in cell-free conditions using recombinant common variant GCase at pH 4.5; [c] Not tested.

In summary, here we report the identification of a potent GCase chaperone ER-001230194 (compound **25**) following optimisation of the hit compound **4** using a cellular assay employing GCase activity in lysates as a surrogate measurement of GCase protein levels. Given the improved ADME and physicochemical properties as well as the improved chaperoning activity, this compound may be a good tool to investigate how pharmacological chaperones can modulate GCase function in a cellular environment and *in vivo*. Although ER-001230194, in the continued presence of compound, also exhibited GCase inhibitory activity in a cellular assay, further studies could be carried out to understand the net benefits of GCase modulation within an *in vivo* setting.

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