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Synthesis and biological evaluation of D-gluconhydroximo-1,5-lactam and its oxime-substituted derivatives as pharmacological chaperones for the treatment of Gaucher disease†‡

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D-Gluconhydroximo-1,5-lactam and its oxime-substituted derivatives were prepared and assessed for inhibition and pharmacological chaperone (PC) activities in Gaucher disease cell lines derived from N370S. The most active compound, *O*-(D-glucopyranosylidene) amino-*Z*-*N*-dodecylcarbamate (**38**), gave a nearly 2.0-fold increase in N370S β-GCase activity at 12.5 μM with no inhibition to other commercially available glucosidases. Docking studies of ligand–enzyme interactions have also been conducted to account for the results of enzyme activity increase. All these results demonstrate that compound **38** is a promising PC for the treatment of GD.

1. Introduction

Lysosomal storage disorders (LSDs)[1] are usually caused by deficiency of certain lysosomal enzymes, resulting in excessive storage of corresponding substrates in lysosomes. Representing the most common LSD, Gaucher disease (GD)[2] is caused by recessive mutation in β-glucocerebrosidase (GCase; EC 3.2.1.45), which is responsible for the hydrolysis of glucosylceramide to glucose and ceramide in the lysosome.[3] Although mutant GCase proteins can be synthesized and functionalized in ER at normal levels, they are not as stable as the wild-type. Misfolded GCase is easy to accumulate and degrade due to the efficient quality-control system in the ER; only correctly folded proteins can be transported to the Golgi compartment for further maturation. There are mainly three clinical types of GD disease which differ in the severity of neurological involvement.[4] Type 1 GD is the most common and known as the non-neuronopathic or adult variant, accounting for more than 90% of all cases. Type 2 and type 3 variants involve central nervous system affections. These problems are attributed to the deficiency of glucocerebrosidase (GCase) and ultimately lead to the abnormal accumulation of GlcCer substrates in lysosomes of GD patients.

Currently, two therapeutic approaches are used for LSDs: substrate reduction therapy (SRT)[5] and enzyme replacement therapy (ERT).[6] SRT drugs, such as NB-DNJ (*N*-butyl-1-deoxynojirimycin, Zavesca), aim to inhibit glucosylceramide synthase (GS) and decrease GlcCer production. However, GS is indispensable in the process of biosynthetic glucosylceramide, thus Zavesca was only approved for the treatment of type 1 GD.[7] In contrast, ERT aims to maintain the levels of GCase activity in patients by supplementing recombinant human GCase.[8] This therapy approach is also not suitable for types 2 and 3 GD owing to the disability to cross the blood–brain barrier.[9]

Recently, pharmacological chaperone therapy (PCT) has attracted much attention for the treatment of GD and other LSDs.[10] It is assumed that under inhibition concentrations, small molecules such as competitive inhibitors could specifically bind to mutant enzymes, assist in folding, then promote trafficking to lysosomes. Once the inhibitor and mutant-enzyme complexes get into the lysosome, the acidic and substrate-rich environment of the lysosome will favor dissociation and release active enzymes. Liberated mutant enzymes

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then perform their catalytic function in the lysosome.[11] The dissociated chaperones are either secreted out to the extracellular environment or recycled to interact with other mutant enzymes to fulfill their functions (Fig. 1).

A large number of iminosugar-type glycomimetics with GCase PC activity have recently been reported as chaperones, such as *N*-nonyl-deoxynojirimycin (NN-DNJ) (**1**),[12] α-1-*C*-nonyl-1-deoxynojirimycin (α-1-C-nonyl-DIX) (**2**),[13] isofagomine (IFG) and its derivatives (**3**, **4**, and **5**),[14] polyhydroxylated lactam (**6**),[15] bicyclic isourea (**7**), guanidines (**8**)[16] and bicyclic nojirimycin analogues with sp^2 iminosugar structures (NOI-NJ) (**9**, **10**, and **11**)[17] (Fig. 2). All of these compounds exhibit high performance as pharmacological chaperones for the therapy of GD by increasing the cellular GCase catalytic activity. Previously, we have found that C2-substituted 3,3-dimethyl-*N*-phenyl-4-amide-1-butyl glucoimidazole showed an excellent PC activity with a 2.1-fold increase in N370S lymphoblasts at 2.5 μM and a 1.2-fold increase in L444P at 0.5 μM after three-day incubation.[18]

Hydroximo-lactones (lactone oximes) and the corresponding *N*-phenylcarbamates are strong and competitive glycosidase inhibitors with the K_i between 10^{-5} and 10^{-8} μM.[19] In addition, modification of an oxime with phenyl isocyanate can dramatically improve its inhibitory ability towards glycosidase. Unprotected aldonhydroximo-lactams are analogues of aldonolactones; thus related D-gluconhydroximo-1,5-lactam (GHL) and derivatives may also be potential inhibitors of glycosidases and may be useful for treating other diseases.[20] In this paper, a series of D-gluconhydroximo-1,5-lactam analogues bearing hydrophobic substituents were synthesized and biologically evaluated as GCase chaperones for GD therapy. We expect that these modified analogues could present the greatest affinity with mutant GCase at the neutral pH of ER, resulting in normal folding and formation of a stable chaperone–enzyme complex followed by trafficking to the lysosome without degradation. Once the mutant enzyme–inhibitor complex was in the lysosome, excessive storage of the substrates in the lysosome promotes the dissociation of the complex, which can efficiently increase the GCase activity for the treatment of GD.

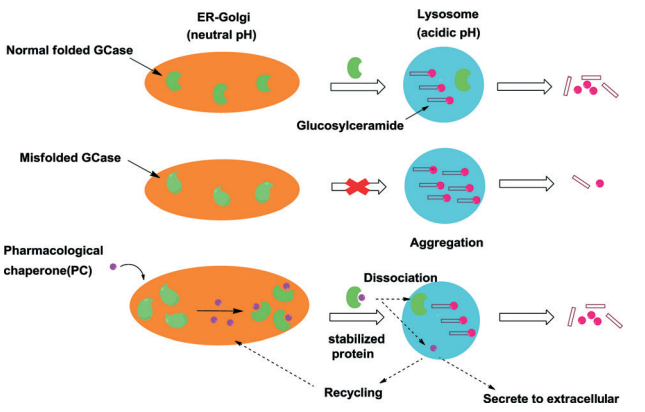


Fig. 1 The proposed mechanism of pharmacological chaperones in treatment of GD and other LSDs.

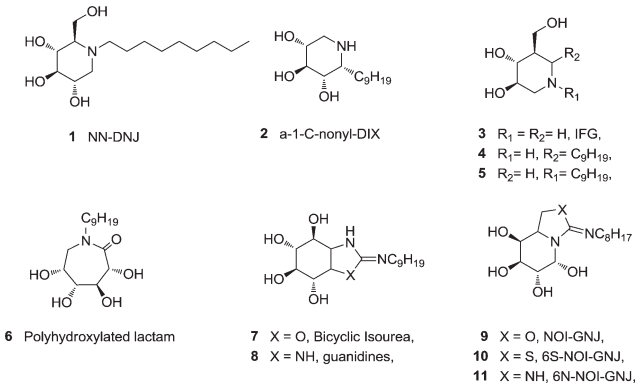
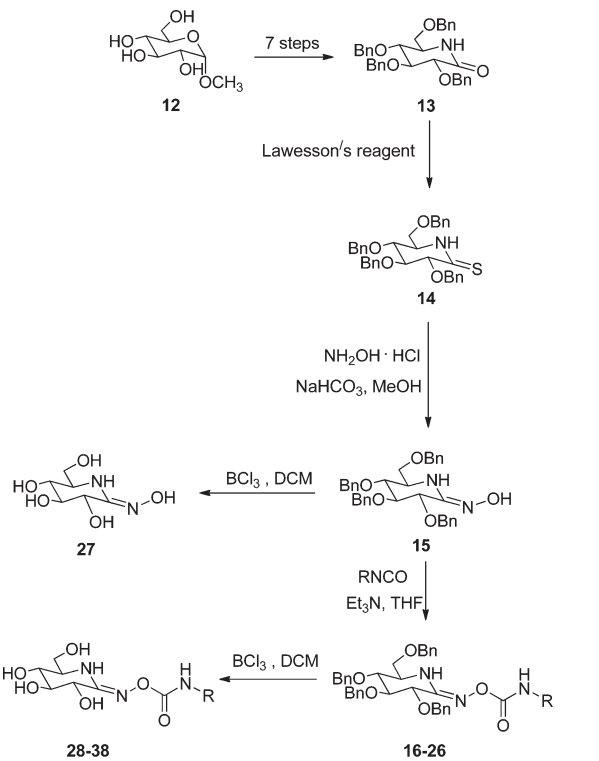


Fig. 2 Chemical structures of pharmacological chaperones for GCase.

2. Results and discussion

2.1 Synthesis

A successful synthetic strategy toward D-gluconhydroximo-1,5-lactam derivatives is shown in Scheme 1. Initially, 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose-lactam (**13**) was synthesized from commercially available methyl-α-D-glucopyranose (**12**) as previously reported.[21] Treatment of lactam **13** with Lawesson's reagent afforded the corresponding glucothionolactam (**14**) in 92% yield.[22] Compound **14** was then treated with hydroxylamine hydrochloride to generate a 10 : 1 mixture of the hydroximo-lactam (**15**) and the manno-isomer. The mixture was separated by flash column chromatography to afford the desired product 2,3,4,6-tetra-*O*-benzyl-D-gluconhydro-



Scheme 1 Synthesis of the D-gluconhydroximo-1,5-lactam **27** and its oxime-substituted derivatives **28**–**38**.

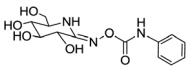
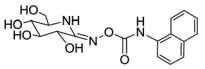
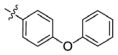
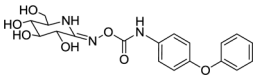
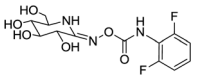
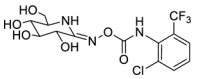
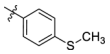
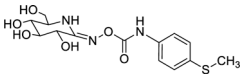
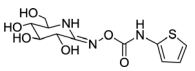
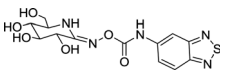
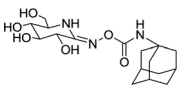
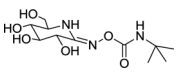
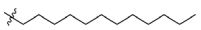
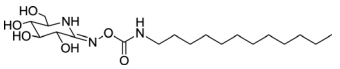
ximo-lactam (**15**) in a yield of 83%. Compound **15**, a key intermediate, was reacted with various isocyanates in the presence of Et_3N to afford the corresponding products. Catalytic hydrogenolysis ($Pd(OH)_2/C$, H_2) for removal of the benzyl protective groups would break the oxime bond (C═N–O bond). Alternatively, the experiments were carried out with BCl_3 in anhydrous CH_2Cl_2 at −78 °C for 3 h and then 0 °C for 12 h. A low temperature was crucial in this step to obtain the ultimate products in high yields (73–85%). The synthesis of the D-gluconhydroximo-1,5-lactam **27** and its oxime-substituent derivatives **28–38** is summarized in Table 1. All the synthesized compounds were characterized using ^1H, ^{13}C NMR and HRMS.

2.2 Glycosidase inhibition

To determine the selectivity profile, synthesized compounds **27–38** were tested as inhibitors against a panel of commercially available glucosidases, including α-glucosidase from baker's yeast, β-glucosidase from almonds, β-galactosidase from *E. coli*, and α-galactosidase from green coffee beans. Inhibition data for NN-DNJ and IFG were also included for comparative purposes. The corresponding IC_{50} values are listed in Table 2. All synthesized compounds exhibited a poor inhibition for α/β-galactosidase with the IC_{50} values above 100 μM, which were selectively against glucosidases.

As shown in Table 2, all synthesized compounds exhibited comparatively effective inhibition of α and β-glucosidases. The lack of selectivity may be ascribed to the C═N bond of oxime at C-1. Among analogues bearing an aromatic or a heterocyclic substitute, **31–35** with IC_{50} values below 10 μM showed a slightly stronger inhibitory potency than others for β-glucosidase. **34** (IC_{50} = 2.68 μM) showed a 17-fold inhibitory potency increase compared to **37** for β-glucosidase from baker's yeast and **29** (IC_{50} = 3.06 μM) showed a 28-fold inhibitory potency increase compared to **37** for α-glucosidase from baker's yeast (both except for **38**), respectively. Subtle chemical modifications in the positions of aromatic substituents for **31–33** and in the size of the aromatic or heterocycle for **34** and **35**, which were 4–12-fold more potent than their parent **28**, indicate that the presence of heteroatoms in the aromatic substituent could slightly improve the binding affinity for β-glucosidase. In addition, parent **27** and **38** showed weak or no inhibition for all assayed glycosidases.

Table 1 D-Gluconhydroximo-1,5-lactam and its oxime-substituted derivatives

Isocyanate	Product	Yield[a]
		82%
		76%
		78%
		81%
		84%
		85%
		74%
		81%
		73%
		83%
		85%

[a] Yields reported for the removal of benzyl protective groups after purification on silica gel.

2.3 Effects of compounds on the activity of GCase and activity enhancement assays in human N370S lymphoblasts

Most pharmacological chaperones are competitive inhibitors of their target enzymes. In this study, the chemically synthesized compounds were evaluated as inhibitors of recombinant GCase (imiglucerase, Cerezyme) at pH = 5.2 and pH = 7.0 and the corresponding IC_{50} values for **27–38** are shown in Table 3, which showed that the differences in IC_{50} measured at pH = 7.0 were not remarkable compared with the values at pH = 5.2. As controls, inhibition data for NN-DNJ and IFG were also included which were better pharmacological chaperones for mutant GCase. The K_i values tested at pH = 5.2 indicated that parent **27** and analogues **28** and **29** bearing a phenyl or a naphthyl substituent showed general inhibitory potency. Subtle chemical modification in the position of aromatic substituents with electron-withdrawing groups of fluorine or chloride atom for **31** and **32** or electron-donating groups of methylthio or thiazole for **33** and **35** did not significantly improve the binding affinity. Alkyl substituents such as *tert*-butyl substituted **37** showed weak inhibitory potency with K_i = 30.08 μM, while adamantane substituted **36** showed slightly higher potency than **37** with K_i = 1.52 μM. Compound **30**, bearing a 4-phenoxyphenylcarbamate substituent, was found to be the strongest inhibitor against imiglucerase and showed more than 600-fold increased inhibition compared to **37** with K_i = 0.05 μM. This dramatic enhancement of inhibitory potency was partially attributed to the electrostatic interactions involving the π–π stacking of the phenoxyphenyl substituent with the enzyme. Additionally, both compounds **34** and **38** showed strong inhibition of imiglucerase with subnanomolar potency (K_i = 0.64 and 0.14 μM, respectively).

Table 2 Glycosidase inhibitory activities (IC_{50}) for the synthetic derivative inhibitors against commercial glycosidase

Compd	β-Glucosidase (almond)	α-Glucosidase (baker's yeast)	β-Galactosidase (*E. coli*)	α-Galactosidase (green coffee beans)
27	80.3	>100	>100	>100
28	31.08	70.97	>100	>100
29	10.70	3.06	>100	>100
30	14.36	22.02	>100	>100
31	7.59	4.93	>100	>100
32	5.82	18.85	>100	>100
33	8.38	7.03	>100	>100
34	2.68	14.65	>100	>100
35	6.21	7.40	>100	>100
36	14.39	4.09	>100	>100
37	45.95	87.05	>100	>100
38	>100	>100	>100	>100
NN-DNJ	>100	>100	>100	>100
IFG	>100	>100	>100	>100

Comparative enzyme activity enhancement assays were conducted in human lymphoblasts derived from Gaucher disease patients homozygous for N370S mutation. The cells were incubated with compounds **27–38** or NN-DNJ and IFG at a range of concentrations from 3.125 to 100 μM for three days and then lysed. The increase in enzyme activities and the corresponding concentrations of compounds are summarized in Table 3 and Fig. 3. MTT assays were also carried out for intact wild type of CCC-ESF-1 fibroblast cell line at 100 μM, the highest concentration in activation assays, and no cytotoxicity was observed (Table S1†).

All synthetic compounds containing various carbamate modifications displayed at least 1.2-fold GCase activity increase compared to untreated cells, which was attributed to the inherent hydrophilicity and affinity with GCase. Moreover, even unmodified parent compound **27** can improve the GCase activity by nearly 1.3-fold in N370S mutant cells. In addition, NN-DNJ and IFG gave maximal enhancement of 1.41- and 1.38-fold at 12.5 and 50 μM which are in agreement with the results previously reported.[15,23]

Table 3 Inhibitory activity against imiglucerase and maximum observed increase in GCase activity using compounds **27–38** and the positive controls (NN-DNJ and IFG)

Compound	IC_{50} (μM)		K_i[a] (μM)	GCase activity increase[b]
	pH 5.2	pH 7.0		p.N370S
27	5.84	65.81	4.28	1.28
28	12.50	19.32	11.55	1.49
29	7.21	1.43	7.94	1.47
30	0.32	3.32	0.05	1.17
31	16.82	21.87	17.37	1.35
32	18.12	23.51	12.34	1.32
33	9.15	17.19	8.12	1.19
34	0.84	13.20	0.64	1.36
35	5.75	12.46	6.38	1.23
36	1.85	8.37	1.52	1.35
37	29.06	21.50	30.08	1.30
38	0.70	5.74	0.14	1.93
NN-DNJ	1.03	0.79	0.62	1.41
IFG	0.74	1.52	0.22	1.38

[a] The inhibition was competitive in all cases (determined at pH 5.2). [b] N370S lymphoblasts from Gaucher disease patients were incubated in the presence of each of the compounds for 3 days before enzyme assay.

The carbamate attached to the aromatic substituents of **28–35** or the presence of oxygen, nitrogen, sulfur or fluoride atoms in the aromatic substituents of **31–35** reinforces the hydrogen interaction with the enzyme pocket, which led to a moderate increase in GCase activity (1.2- to 1.4-fold). Interestingly, though **35** showed an intermediate K_i value, it elicited a better improvement in GCase activity with a 1.5-fold increase. It is worth noting that compound **38** was an efficient inhibitor against GCase *in vitro* and also showed the most impressive improvement in N370S lymphoblast cells, with a nearly 2.0-fold increase at 12.5 μM. It can be concluded that both the hydrophilic aminocyclitol and the long hydrophobic chain promoted the high affinity with GCase in N370S mutant cells.

As shown in Fig. 3, the concentrations for maximum activity increase for each compound were varied over the entire concentration range. Most compounds followed this rule: a relative GCase activity increase improved with higher concentration, however, thereafter the activity gradually decreased. Only compound **27** did not reach the peak even at 100 μM.

2.4 Molecular docking studies

In order to investigate the chaperone activity, docking studies were performed using the reported NN-DNJ–GCase complex (PDB ID: 2V3E) as a template for docking studies. Molecular operating environment (MOE) was employed to perform the docking simulation to study the structure–activity relationships of **38**, which showed the highest activity, increased by 1.93-fold, among the tested compounds in this work (Fig. 4). The results in Fig. 4A showed that **38** was positioned at the center of the binding pocket with an inward-facing conformation similar to NN-DNJ,[24] which places the oxime at the position of the anomeric carbon in a native glycoside. The "half chair form" $^1C_4$ sugar moiety of compound **38** showed the best docked orientation with the enzyme-active sites, in which all the hydroxyl moieties in **38** showed hydrogen bond interactions mainly with important residues N396, W381, D127, W179, N234 and E235. These interactions seemed to

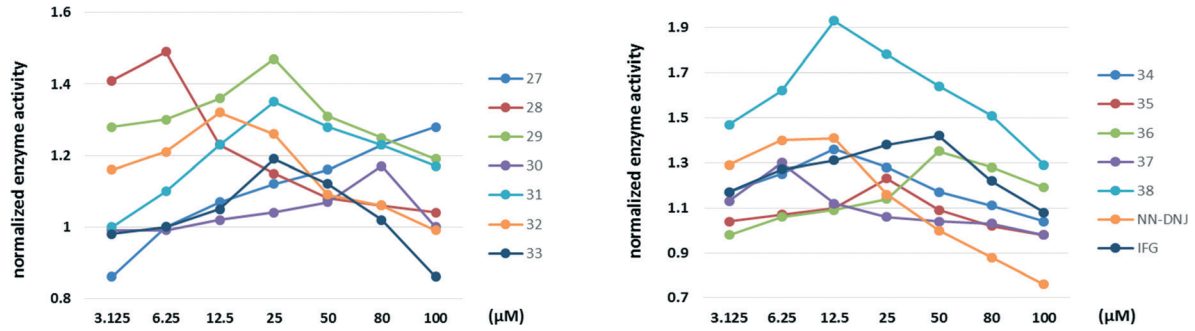


Fig. 3 Increase in GCase activity in N370S lymphoblasts from Gaucher disease patients over a range of concentrations from 100–3.125 μM. The relative activity in the lysates from treated cells was normalized to the activity in untreated cells. NN-DNJ is *N*-nonyl-1-deoxynojirimycin and IFG is isofagomine.

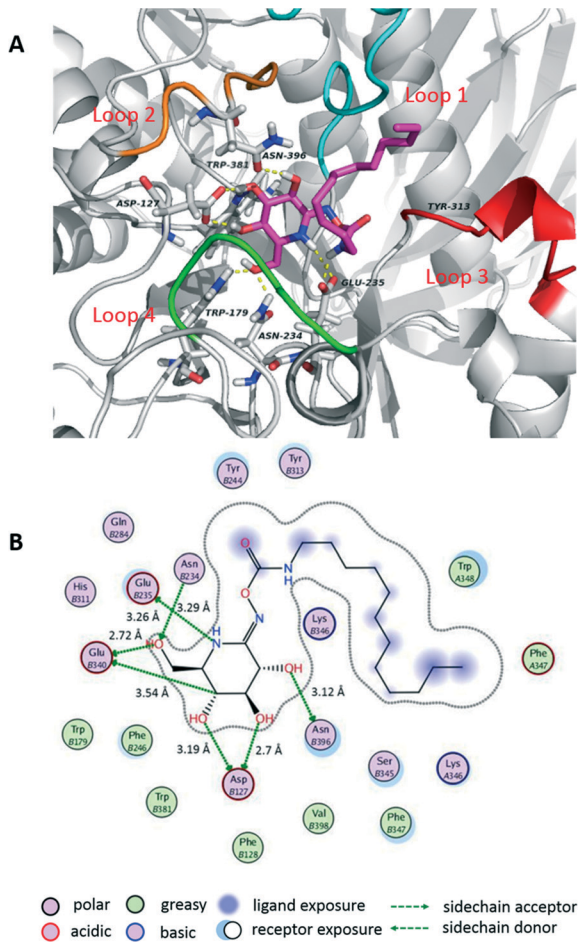


Fig. 4 Best pose obtained for docking **38** to the GCase structure 2V3E. (A) Compound **38** bound to the active site of GCase. The side chains of the residues involved in binding are shown as sticks; loops 1–4 are shown in cyan, orange, red and green, respectively; loop 1 (residues 341–350), loop 2 (residues 393–396), loop 3 (residues 312–319), loop 4 (residues 240–246). (B) Corresponding interaction diagrams of **38** against 2V3E that show strong hydrogen bond interactions between the cyclitol hydroxyl groups and the side chains of residues E235, N234, E340, D127 and D396. Green lines represent hydrogen bonds.

play an essential role in the binding affinity of ligands. The *Z* configuration of **38** facilitated the exocyclic N atom adopt a bent conformation and shared the same acid/base catalytic residue E235 with the endocyclic imino; this extra interaction between **38** and GCase is the possible reason why **38** is a more potent inhibitor than NN-DNJ.

In addition, the corresponding interaction in a 2D image is shown in Fig. 4B. The geometric orientation of **38** was similar to the previously observed structure of NN-DNJ with pGlcCerase. The rigid sp^2 iminosugar imposed a certain restriction to the oxime substituent; this long aliphatic tail was oriented toward and well suited to the entrance of the active site. The docked pose suggested that besides the interactions of the iminosugar moiety with the active site of β-glucosidase, the long and linear chains of the substituent of **38** also established hydrophobic interactions with the residues Y313, Y244, F347 and W348 (indicated by purple filled circles).

According to the crystal structure in Fig. 4B, the distances between loops 1–4 and **38** were greater than IFG[24] and bicyclic glucoimidazoles[18] with each corresponding GCase, which might explain the result that the K_i values in this work were not as good as others at the nanomolar level. It was inferred that just this moderate affinity of compounds with the mutant enzymes assisted the transport of mutant enzymes from the ER to the lysosome and then they efficiently dissociated to recover their catalytic activity.

3. Conclusions

In summary, D-gluconhydroximo-1,5-lactam and its oxime-substituted derivatives have been synthesized and evaluated. With treating GD related cell line N370S, we found that compound **38**, as an efficient pharmacological chaperone for GCase, could effectively promote the activity of the mutant protein with a 1.93-fold increase at 12.5 μM; simultaneously, it has negligible inhibition towards other glycosidases. Detailed computational docking studies exhibited that **38** can stabilize the mutant N370S with high affinity, leading to safe trafficking to the lysosome. In short, compound **38** with advantageous enzymatic and cellular properties, deserves further study as a potent pharmacological chaperone for the treatment of Gaucher disease and other LSDs.

Acknowledgements

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