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α-1-*C*-Octyl-1-deoxynojirimycin as a pharmacological chaperone for Gaucher disease

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Abstract—The most common lysosomal storage disorder, Gaucher disease, is caused by inefficient folding and trafficking of certain variants of lysosomal β-glucosidase (β-Glu, also known as β-glucocerebrosidase). Recently, Sawker et al. reported that the addition of subinhibitory concentrations (10 μM) of the pharmacological chaperone *N*-nonyl-1-deoxynojirimycin (NN-DNJ) (**10**) to Gaucher patient-derived cells leads to a 2-fold increase in activity of mutant (N370S) enzyme [*Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15428]. However, we found that the addition of NN-DNJ at 10 μM lowered the lysosomal α-glucosidase (α-Glu) activity by 50% throughout the assay period in spite of the excellent chaperoning activity in N370S fibroblasts. Hence, we prepared a series of DNJ derivatives with an alkyl chain at the C-1α position and evaluated their in vitro inhibitory activity and potential as pharmacological chaperones for Gaucher cell lines. Among them, α-1-*C*-octyl-DNJ (CO-DNJ) (**15**) showed 460-fold stronger in vitro inhibitory activity than DNJ toward β-Glu, while NN-DNJ enhanced in vitro inhibitory activity by 360-fold. Treatment with CO-DNJ (20 μM) for 4 days maximally increased intracellular β-Glu activity by 1.7-fold in Gaucher N370 cell line (GM0037) and by 2.0-fold in another N370 cell line (GM00852). The addition of 20 μM CO-DNJ to the N370S (GM00372) culture medium for 10 days led to 1.9-fold increase in the β-Glu activity without affecting the intracellular α-Glu activity for 10 days. Only CO-DNJ showed a weak β-Glu chaperoning activity in the L444P type 2 variant, with 1.2-fold increase at 5–20 μM, and furthermore maximally increased the α-Glu activity by 1.3-fold at 20 μM. These experimental results suggest that CO-DNJ is a significant pharmacological chaperone for N370S Gaucher variants, minimizing the potential for undesirable side effects such as α-Glu inhibition.

1. Introduction

Gaucher disease is the most common lysosomal storage disorder caused by a deficiency of lysosomal β-glucosidase (β-Glu, known as β-glucocerebrosidase), resulting in the progressive accumulation of glucosylceramide. Clinically, Gaucher disease is classified into three major types based on the absence (type 1) or the presence and severity (types 2 and 3) of central nervous system (CNS) involvement. Type 1 is nonneuronopathic and sometimes called the 'adult' form. Type 2 is the infantile form of the disorder with severe CNS involvement, and type 3 is subacute neuronopathic with typically childhood or early adult onset.[1] The first successful treatment is the enzyme replacement therapy (ERT) for patients with type 1 Gaucher disease. Ceredase in 1991 and its recombinant successor Cerezyme in 1994 were introduced as ERT of this type. However, ERT is not available for types 2 and 3 of Gaucher disease since proteins do not cross the blood–brain barrier, and another problem in this therapy is the cost, which prevents many patients from obtaining this treatment.

In recent years, remarkable progress has been made in developing a molecular therapy for the glycosphingolipid storage disorders.[2–5] There are two novel approaches in this field. One is substrate reduction therapy (SRT) and another is pharmacological (or chemical) chaperone therapy (PCT). The basic concept of SRT is to reduce the substrate influx into the lysosomes by inhibitors of glycosphingolipid synthesis. *N*-Butyl-1-deoxynojirimycin (NB-DNJ, miglustat, Zavesca™) (**7**) is an inhibitor of ceramide-specific glucosyltransferase[6] and has been

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approved for the oral treatment of type 1 Gaucher disease. The concept of PCT is that an intracellular activity of a misfolded mutant enzyme can be restored by administering a competitive inhibitor that serves as a pharmacological chaperone. Such inhibitor appears to act as a template that stabilizes the native folding state in the endoplasmic reticulum (ER) by occupying the active site of the mutant enzyme, thus allowing its maturation and trafficking to the lysosome.[3] The concept of PCT was originally introduced with Fabry disease.[7] Residual α-galactosidase A (α-Gal A) activity in lymphoblasts derived from Fabry patients and in tissues of R301Q α-Gal A transgenic mice was enhanced by treatment with 1-deoxygalactonojirimycin (DGJ), a competitive inhibitor of α-Gal A with a K_i value of 40 nM. Very recently, Yam et al. showed that DGJ induces trafficking of ER-retained R301Q α-Gal A to lysosomes of transgenic mouse fibroblasts and that DGJ treatment results in efficient clearance of the substrate, globotriaosylceramide (Gb3).[8] Sawker et al. reported that the addition of *N*-nonyl-DNJ (NN-DNJ) (**10**) to the fibroblast culture medium of the fibroblasts derived from N370S Gaucher patients led to a 2-fold increase in the β-Glu activity.[9]

Lysosomal glycosidases are glycoproteins with the *N*-linked oligosaccharide chains. One of the functions of *N*-glycans is to assist in the folding of glycoproteins by mediating interactions of the lectin-like chaperone calnexin with nascent glycoproteins.[10,11] Glucose trimming, catalyzed by ER α-glucosidases I and II, begins on the nascent sugar chain and its inhibition by the inhibitors, such as castanospermine (CST) and NB-DNJ, can have significant biological consequences because of the blockage of the normal interaction with calnexin.[12] For example, treatment of cultured cells with CST interferes with folding, secretion, and proper localization of some glycoproteins, and stimulates their rapid degradation.[12–15] Tyrosinase, a key enzyme in melanin biosynthesis, was catalytically inactive when synthesized in the presence of NB-DNJ but was transported correctly to the melanosome.[16] However, inhibition of glucose trimming by ER α-glucosidase inhibitors does not necessarily lead to the impairment in glycoprotein expression. When the murine macrophage cells, expressing significant levels of functional class A scavenger receptors, are cultured in the presence of NB-DNJ, the glucose trimming inhibition does not result in gross misfolding and degradation of this receptor or prevent its transport to the cell surface, following significantly enhanced levels of expression.[17] Thus, it is not possible to predict the precise effects of inhibiting *N*-glycan processing, and the effects of ER processing α-glucosidase inhibitors on glycoproteins should be considered on a case-by-case.

NN-DNJ has been reported to be an effective pharmacological chaperone to increase the activity of mutant β-Glu in Gaucher cells,[9] while it is also known that this compound, similarly to NB-DNJ, is a potent inhibitor of ER processing α-glucosidases, and hence has potential as anti-viral agents to inhibit folding and trafficking of viral envelope glycoproteins.[18,19] Inhibitors targeting a host function such as ER processing α-glucosidases must be carefully considered in terms of side-effects since they may inhibit folding, secretion, and trafficking of other glycoproteins in patient's cells or may inhibit directly lysosomal α-glucosidase (α-Glu) after being taken up into cells. Although NN-DNJ has been shown to increase the trafficking and intracellular activity of N370S mutant β-Glu,[9] there is no statement on the influence on other glycoproteins, especially for α-Glu. Recently, examination of a series of DNJ analogues on the residual activities of various β-Glu variants has revealed that the nature of the alkyl moiety greatly influences their chaperoning activity: NB-DNJ is inactive, DNJ derivatives with *N*-nonyl and *N*-decyl chains are active, and *N*-dodecyl-DNJ is predominantly inhibitory.[20] Therefore, we prepared a series of *N*-alkyl and α-1-*C*-alkyl-DNJ derivatives in order to assess their β-Glu chaperoning activity and cellular α-Glu inhibitory activity in Gaucher cell lines. Herein, we report that α-1-*C*-octyl-DNJ (CO-DNJ) (**15**) is an excellent pharmacological chaperone increasing N370S β-Glu activity without inhibiting intracellular α-Glu activity.

2. Results

2.1. In vitro inhibition of β-Glu and α-Glu

Naturally occurring iminosugars DNJ (**1**) and *N*-methyl-DNJ (**5**) were isolated from *Morus alba* (Moraceae),[21] α-homonojirimycin (α-HNJ, **2**) and β-homonojirimycin (β-HNJ, **3**) from *Stemona tuberosa* (Stemonaceae),[22] and α-1-*C*-methyl-DNJ (**11**, α-7-deoxyhomonojirimycin) from *Scilla sibirica* (Hyacinthaceae)[23] according to the literature (Fig. 1). The *N*-alkyl derivatives of DNJ were prepared by treatment with the appropriate alkyl bromide and K_2CO_3 in DMF, and the α-1-*C*-alkyl-DNJ derivatives were prepared according to the synthetic route using a cross-metathesis reaction as the key step reported previously.[24–26] The β-Glu activity was measured with commercially available β-glucocerebrosidase (Ceredase) as the enzyme source and 4-methylumbelliferyl (4-MU) β-D-glucoside as the substrate. The α-Glu activity was assayed with the cell lysate of normal human fibroblasts (GM05659) as an enzyme source and 4-MU α-glucoside as substrate at pH 4.5. The IC_{50} values of iminosugars and their alkyl derivatives toward β-Glu and α-Glu are shown in Table 1. Natural iminosugars DNJ, *N*-methyl-DNJ, α-HNJ, β-HNJ, and α-1-*C*-methyl-DNJ showed weak or null in vitro inhibition of β-Glu. Introduction of the butyl group to the C-1β position of DNJ abolished its inhibition toward β-Glu, whereas introducing this group to the C-1α position slightly improved its inhibitory activity. The DNJ derivatives with a alkyl chain longer than the butyl group at the C-1α or the imino group remarkably enhanced the β-Glu inhibitory activity with increasing chain length, whereas α-Glu was less sensitive to inhibition by the DNJ derivatives with the elongated chain than β-Glu. *N*-Octyl-DNJ (NO-DNJ) (**9**) and NN-DNJ were 290-fold and 360-fold stronger inhibitors of β-Glu than DNJ, respectively, and CO-DNJ and α-1-*C*-nonyl-DNJ (CN-DNJ) (**16**) showed 460-fold and 890-fold stronger inhibition toward β-Glu than DNJ.

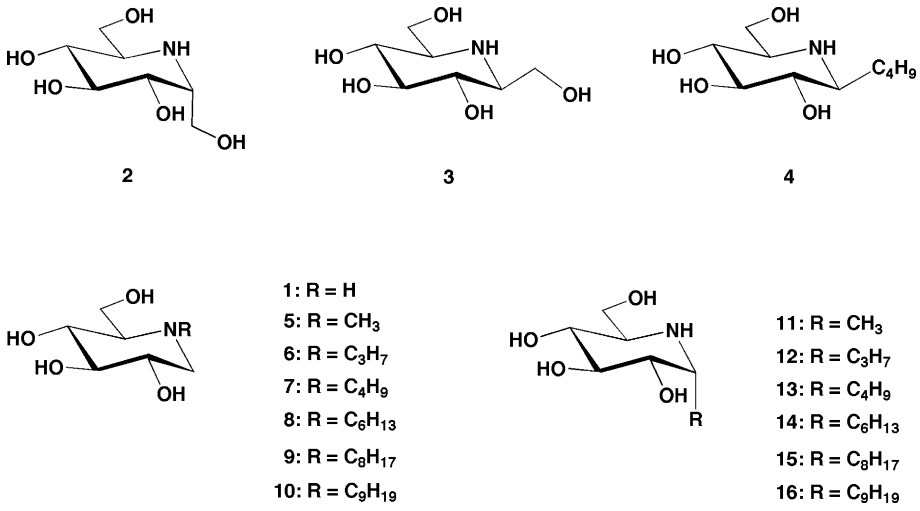

Figure 1. Structures of iminosugars and their alkyl derivatives. **1**, 1-deoxynojirimycin (DNJ); **2**, α-homonojirimycin (α-HNJ); **3**, β-HNJ; **4**, β-1-*C*-butyl-DNJ; **5**, *N*-methyl-DNJ; **6**, *N*-propyl-DNJ; **7**, *N*-butyl-DNJ (NB-DNJ); **8**, *N*-hexyl-DNJ; **9**, *N*-octyl-DNJ (NO-DNJ); **10**, *N*-nonyl-DNJ (NN-DNJ); **11**, α-1-*C*-methyl-DNJ; **12**, α-1-*C*-propyl-DNJ; **13**, α-1-*C*-butyl-DNJ; **14**, α-1-*C*-hexyl-DNJ; **15**, α-1-*C*-octyl-DNJ (CO-DNJ); **16**, α-1-*C*-nonyl-DNJ (CN-DNJ).

Table 1. Inhibition of lysosomal β-glucosidase (β-glucocerebrosidase) and α-glucosidase by 1-deoxynojirimycin (DNJ) derivatives

Compound	IC_{50} (μM)	
	β-Glucosidase	α-Glucosidase
DNJ (**1**)	240	1.0
α-Homonojirimycin (**2**)	—	1.0
β-Homonojirimycin (**3**)	—	—
β-1-*C*-butyl-DNJ (**4**)	—	—
N-Methyl-DNJ (**5**)	150	1.0
N-Propyl-DNJ (**6**)	700	56
N-Butyl-DNJ (**7**)	270	5.0
N-Hexyl-DNJ (**8**)	13	4.0
N-Octyl-DNJ (NO-DNJ, **9**)	0.82	2.1
N-Nonyl-DNJ (NN-DNJ, **10**)	0.66	1.5
α-1-*C*-Methyl-DNJ (**11**)	900	28
α-1-*C*-Propyl-DNJ (**12**)	400	270
α-1-*C*-Butyl-DNJ (**13**)	100	31
α-1-*C*-Hexyl-DNJ (**14**)	4.2	12
α-1-*C*-Octyl-DNJ (CO-DNJ, **15**)	0.50	5.0
α-1-*C*-Nonyl-DNJ (CN-DNJ, **16**)	0.27	4.8

'—,' less than 50% inhibition at 1000 μM.

DGJ and NN-DNJ are competitive inhibitors and active-site-specific chaperones.[7,9] We previously reported that a potent inhibitor shows an effective chaperoning activity and less potent inhibitors require higher concentrations to achieve the same effect.[27] Hence, the mode of inhibition and inhibition constant of the DNJ derivatives were determined by the Lineweaver–Burk plots (Table 2). All of DNJ and its alkyl derivatives inhibited β-Glu in a competitive manner. The *N*-butylation and α-1-*C*-butylation of DNJ decreased the affinity for β-Glu, but the introduction of an alkyl chain longer than the butyl group remarkably enhanced the affinity. Addition of the alkyl chain to the C-1α position (pseudo-anomeric position) showed a slightly better affinity than that to the imino group. The K_i values for CO-DNJ and CN-DNJ are 0.28 and 0.20 μM, respectively. For a comparison, those for NO-DNJ and NN-DNJ are 0.42 and 0.30 μM, respectively.

2.2. Effects of DNJ derivatives on α-Glu and β-Glu activities in Gaucher fibroblasts

With respect to the compounds showing potent in vitro inhibitory activity toward β-Glu, we investigated the influence of selected DNJ derivatives on β-Glu and α-Glu activities in Gaucher fibroblasts cultured for 4 days. Treatment with NO-DNJ and NN-DNJ caused 2.3-fold maximal increase at 50 μM (Fig. 2C) and 2.4-fold maximal increase at 10 μM (Fig. 2D), respectively, in the β-Glu activity of an N370S cell line (GM00372), while CO-DNJ and CN-DNJ maximally increased the activity by 1.7-fold at 20 μM (Fig. 2A) and 1.7-fold at 2.5 μM (Fig. 2B), respectively. Although CN-DNJ showed a chaperoning activity at low concentrations, it was inhibitory at concentrations higher than 10 μM. It should be noted that addition of NO-DNJ at only 5 μM led to 50% inhibition of the α-Glu activity, and that NN-DNJ inhibited α-Glu in a dose-dependent manner and decreased the activity by 70% at 50 μM. On the other hand, the α-1-*C*-alkyl derivatives slightly inhibited α-Glu activity at concentrations higher than 10 μM. Another N370S cell line (GM00852) was less sensitive to the *N*-alkyl-DNJ derivatives and CN-DNJ than GM00372 cell line (Figs. 3B–D). CO-DNJ maximally increased β-Glu activity in this cell line by 2.0-fold at 20 μM without inhibiting α-Glu activity, rather a slight enhancement of α-Glu activity was observed over a concentration range of 10–20 μM (Fig. 3A). NO-DNJ and NN-DNJ dose-dependently lowered α-Glu activity, with 30% and 50% inhibition at 50 μM, respectively.

The β-Glu and α-Glu activities of an L444P variant, which characterize a more severe disease phenotype than N370S, were measured using Gaucher disease type 2 patient-derived cell line (GM00877) after incubation with the *N*-alkyl- and α-1-*C*-alkyl-DNJ derivatives for

Table 2. Measured K_i values and inhibition mode of human lysosomal β-glucosidase

Compound	K_i[a] (μM)	Inhibition[b]
DNJ (**1**)	79	Competitive
N-Butyl-DNJ (**7**)	116	Competitive
N-Hexyl-DNJ (**8**)	5.5	Competitive
N-Octyl-DNJ (NO-DNJ, **9**)	0.42	Competitive
N-Nonyl-DNJ (NN-DNJ, **10**)	0.30	Competitive
α-1-*C*-Butyl-DNJ (**13**)	110	Competitive
α-1-*C*-Hexyl-DNJ (**14**)	2.3	Competitive
α-1-*C*-Octyl-DNJ (CO-DNJ, **15**)	0.28	Competitive
α-1-*C*-Nonyl-DNJ (CN-DNJ, **16**)	0.20	Competitive

[a,b]Inhibition constant (K_i) and inhibition mode were determined by the Lineweaver–Burk plots.

4 days. None of candidate compounds tested to date shows effective β-Glu chaperoning activity for L444P mutation.[9,20,28] It has been reported that L444P β-Glu is not chaperoned by any of the DNJ analogues and is at least 50% inhibited at concentrations higher than 25 μM.[20] In the present study, none of the *N*-alkyl- and α-1-*C*-alkyl-DNJ derivatives likewise showed distinct chaperoning activity in L444P cell line. In particular, the nonyl-DNJ derivatives weakly inhibited the cellular β-Glu activity at concentrations exceeding 5 μM (Figs. 4B and D). Interestingly, CO-DNJ showed a weak chaperoning activity toward both α-Glu and β-Glu over the concentration range assayed (Fig. 4A), whereas NO-DNJ and NN-DNJ dose-dependently inhibited the cellular α-Glu activity, with 40% and 70% inhibition at 50 μM, respectively (Figs. 4C and D).

We selected CO-DNJ as the most promising pharmacological chaperone that did not affect the cellular α-Glu activity in Gaucher cell lines. Longer time-course study using N370S cell line (GM00372) was conducted for CO-DNJ and NN-DNJ. Each compound was added to the culture medium at concentrations (20 μM for CO-DNJ and 10 μM for NN-DNJ) causing the maximal increase in the cellular β-Glu activity and incubated for 10 days. The β-Glu activity was increased 1.9-fold by CO-DNJ and 2.3-fold by NN-DNJ in a time-dependent manner up to day 10 (Fig. 5). The cellular α-Glu activity remained quite unaffected throughout the assay period in the presence of CO-DNJ (20 μM), whereas addition of NN-DNJ (10 μM) lowered the activity by 50% on day 2 and the inhibition levels remained constant for the duration of this experiment (Fig. 5B).

NN-DNJ shows a better β-Glu chaperoning activity than CO-DNJ in Gaucher cell lines but significantly impairs the cellular α-Glu activity. Further experimental approaches are needed to ascertain whether the loss of the cellular α-Glu activity is due to gross misfolding, degradation, and trafficking impairment of α-Glu derived from processing α-glucosidase inhibition by the *N*-alkyl-DNJ derivatives, or due to direct inhibition of

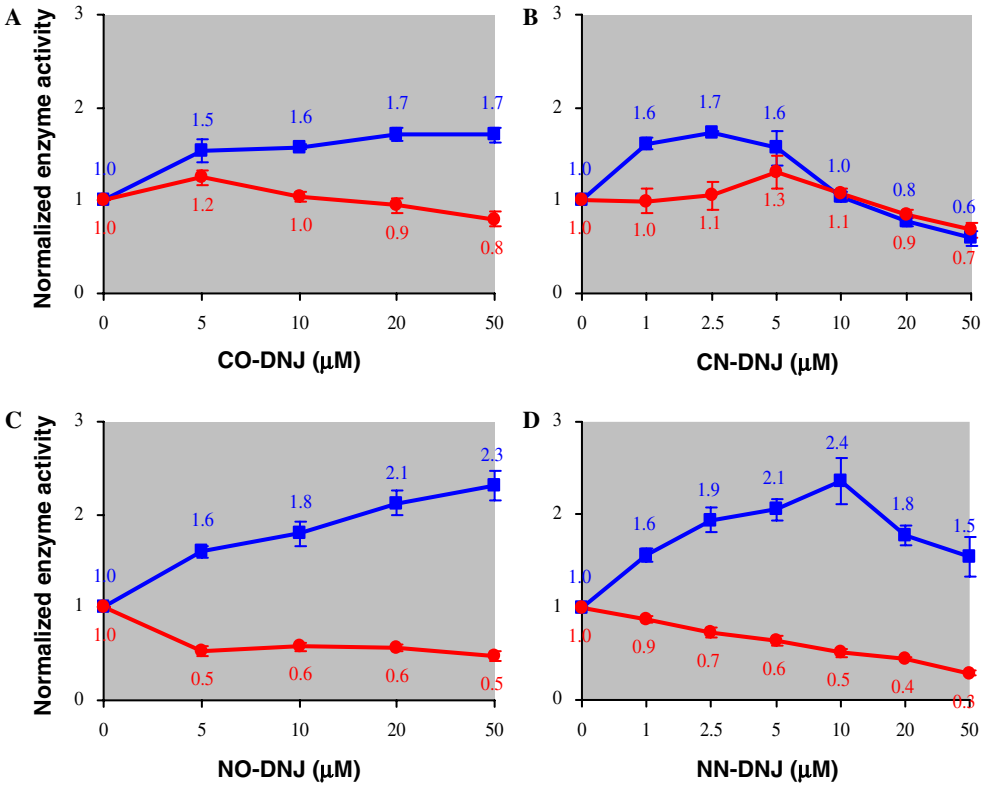

Figure 2. The influence of the alkylated DNJ derivatives on lysosomal α-glucosidase (red line) and β-glucosidase (blue line) activities in N370S Gaucher fibroblasts (GM00372). The fibroblasts were incubated in the presence of (A) CO-DNJ, (B) CN-DNJ, (C) NO-DNJ, or (D) NN-DNJ over a concentration range of 0–50 μM for 4 days. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1. Mean values ± SD are shown for triplicate experiments.

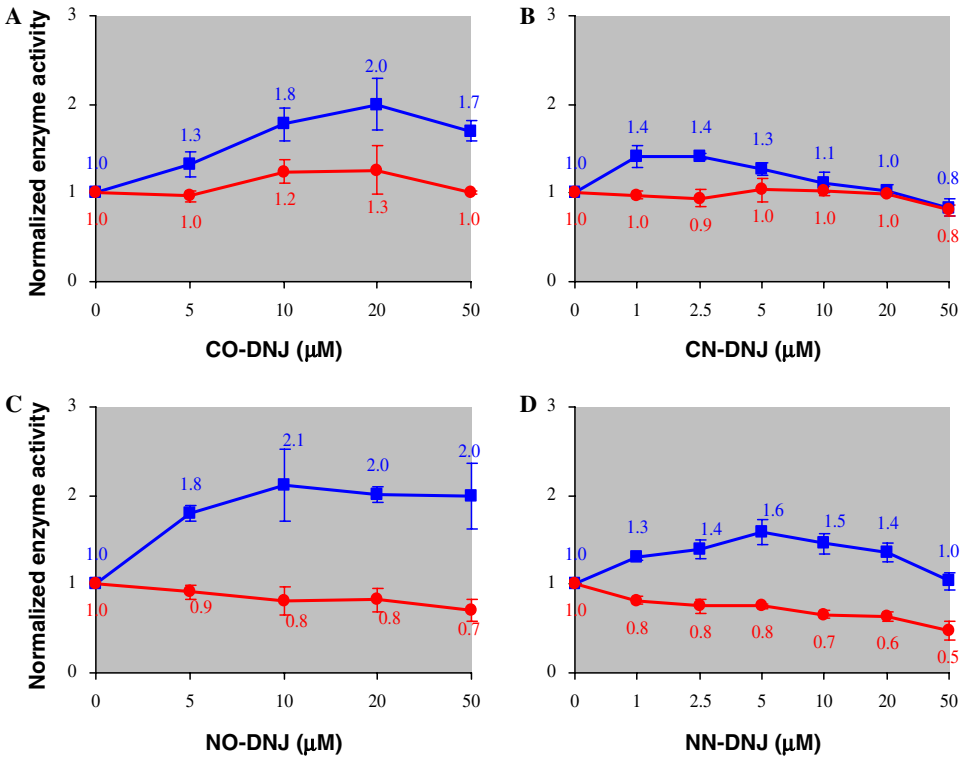

Figure 3. The influence of the alkylated DNJ derivatives on lysosomal α-glucosidase (red line) and β-glucosidase (blue line) activities in N370S Gaucher fibroblasts (GM00852). The fibroblasts were incubated in the presence of (A) CO-DNJ, (B) CN-DNJ, (C) NO-DNJ, or (D) NN-DNJ over a concentration range of 0–50 μM for 4 days. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1. Mean values ± SD are shown for triplicate experiments.

α-Glu after the uptake of an inhibitor into the cells. If this is due to inhibition of the early glucose-trimming steps in *N*-glycan processing and ensuing prevention of the interaction of glycoproteins with calnexin, addition of *N*-alkyl-DNJ to the culture medium may influence the activity of other lysosomal glycosidases. The DNJ derivatives with the octyl and nonyl chains are inhibitors of both α-Glu and β-Glu (Table 1) but not of lysosomal α-mannosidase (α-Man) (data not shown). Hence, the change in the α-Man activity was investigated using an N370S cell line (GM00372) when inhibitors were added to the culture medium over a concentration range of 0–50 μM and incubated for 4 days. CO-DNJ and CN-DNJ gave no effect on the α-Man activity over a concentration range assayed (Figs. 6A and B), whereas the *N*-alkyl-DNJ derivatives lowered the activity in a dose-dependent manner (Figs. 6C and D). This result may suggest that the *N*-alkyl-DNJ derivatives inhibit the early glucose-trimming steps in *N*-glycan synthesis and lead to misfolding, degradation, and trafficking impairment of normal lysosomal glycosidases, but the α-1-*C*-alkyl DNJ derivatives do not.

3. Discussion

It was reported that NN-DNJ is a potent inhibitor of lysosomal β-glucocerebrosidase (β-Glu) with an IC_{50} value of 1 μM, and addition of subinhibitory concentration (10 μM) of this inhibitor to a culture medium leads to a 2-fold increase in activity of the mutant (N370S) enzyme in the fibroblasts derived from Gaucher disease patients.[9] Furthermore, it has been revealed that the nature of the *N*-alkyl moiety of the DNJ derivatives and analogues influences the pharmacological chaperoning activity in mutant β-Glu, that is, the short *N*-alkylation (4 carbons) of DNJ produces inactive compounds, the long *N*-alkyl chains (12 carbons) proved to be toxic, and the DNJ derivatives with 8–10 carbon side chains are well tolerated and enhance β-Glu activity in some Gaucher cell lines other than L444P.[9] We prepared the DNJ derivatives with a variety of the alkyl chain length at the pseudo-anomeric position and measured their inhibitory activities toward α-Glu and β-Glu. With respect to selected potent inhibitors of β-Glu, we evaluated their β-Glu chaperoning activity in Gaucher cell lines and the influence on the cellular α-Glu activity. Introduction of the alkyl side chains to the C-1β position and the short chains to C-1α resulted in weak or null inhibition of β-Glu, whereas elongation of the alkyl chain from hexyl to nonyl at C-1α markedly enhanced the β-Glu activity with increasing chain length. On the other hand, α-Glu was less sensitive to inhibition by the DNJ derivative with elongated chain length than β-Glu. *N*-Alkyl-DNJs showed a slightly better β-Glu chaperoning activity than α-1-*C*-alkyl-DNJs in Gaucher cell lines with N370S mutation but significantly impaired the α-Glu activity in the cells. There was seen a difference in chaperoning activity of pharmacological chaperones in the cells derived from different Gaucher

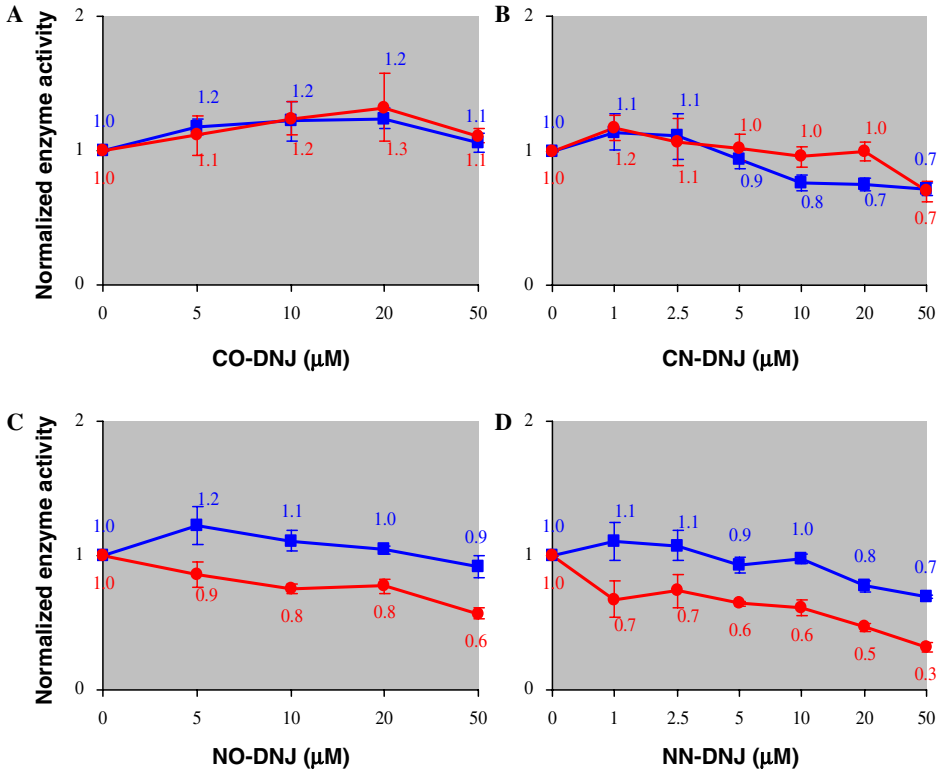

Figure 4. The influence of the alkylated DNJ derivatives on lysosomal α-glucosidase (red line) and β-glucosidase (blue line) activities in L444P Gaucher fibroblasts (GM00877). The fibroblasts were incubated in the presence of (A) CO-DNJ, (B) CN-DNJ, (C) NO-DNJ, or (D) NN-DNJ over a concentration range of 0–50 μM for 4 days. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1. Mean values ± SD are shown for triplicate experiments.

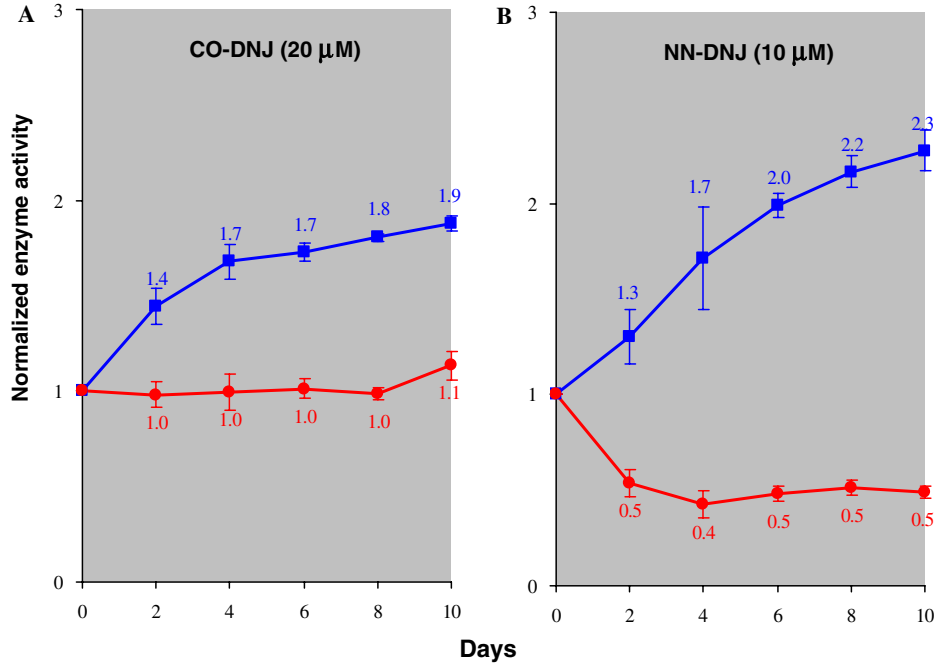

Figure 5. Time course of the lysosomal α-glucosidase (red line) and β-glucosidase (blue line) activities in N370S Gaucher fibroblasts (GM00372) by treatment with α-1-*C*-octyl-DNJ (CO-DNJ) or *N*-nonyl-DNJ (NN-DNJ). The fibroblasts were incubated in the presence of (A) CO-DNJ (20 μM) or (B) NN-DNJ (10 μM) for 10 days. Enzyme activity is normalized to untreated cells, assigned a relative activity of 1. Mean values ± SD are shown for triplicate experiments.

patients with the same N370S mutation. CO-DNJ showed 1.7-fold (GM00372) and 2.0-fold (GM00852) maximal increase in the β-Glu activity at 20 μM, while NN-DNJ maximally enhanced its activity by 2.4-fold at 10 μM in GM00372 and by 1.6-fold at 5 μM in GM00852. Addition of CO-DNJ at 20 μM to the

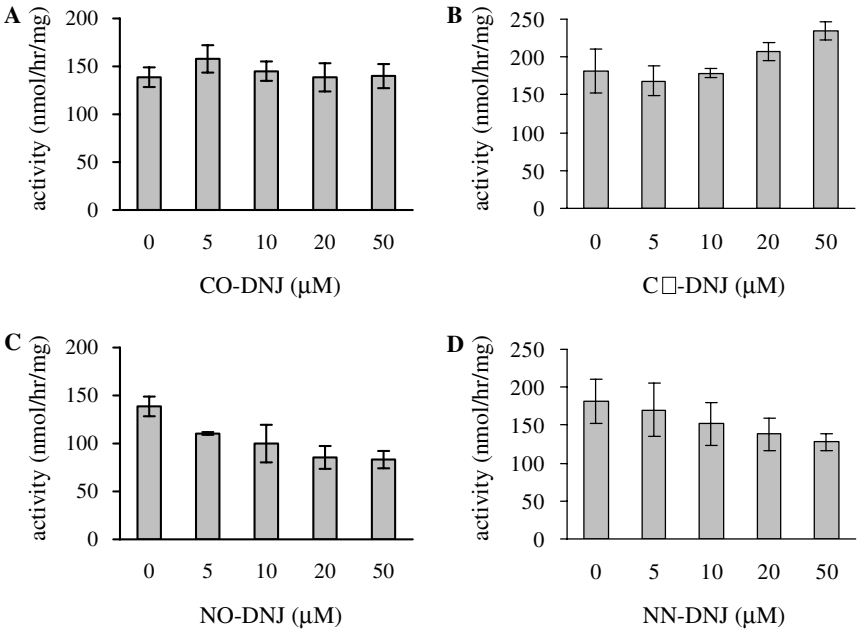

Figure 6. The influence of the alkylated DNJ derivatives on lysosomal α-mannosidase activity in N370S Gaucher fibroblasts (GM00372). The fibroblasts were incubated in the presence of (A) CO-DNJ, (B) CN-DNJ, (C) NO-DNJ, or (D) NN-DNJ over a concentration range of 0–50 μM for 4 days. Mean values ± SD are shown for triplicate experiments.

GM00372 culture medium for 10 days enhanced the β-Glu activity by 1.9-fold without inhibiting α-Glu, whereas that of NN-DNJ at 10 μM lowered the cellular α-Glu activity by 50% throughout the assay period (10 days) in spite of the excellent chaperoning activity. The significant decrease of the α-Glu activity by the *N*-alkyl-DNJ derivatives may be due to the inhibition of the early glucose-trimming steps in *N*-glycan synthesis since they are potent inhibitors of ER processing α-glucosidases I and II, resulting in gross misfolding and degradation, and subsequent mistrafficking to the lysosomes. It has been reported that an L444P variant, which characterizes a more severe disease phenotype than N370S, is not chaperoned by any compounds tested so far.[9,20,28] However, only CO-DNJ showed a weak β-Glu chaperoning activity in L444P and further enhanced the cellular α-Glu activity by 1.3-fold in the same cell line.

Considering the side-effect profile, we selected CO-DNJ as the most promising pharmacological chaperone to bind the active site of the mutant β-Glu, stabilize it in the ER, and enable proper trafficking to the lysosomes. Enzyme replacement therapy (ERP) has been the most successful therapeutic approach for lysosomal storage disorders but does not effectively treat the neurological complications because enzymes do not cross the blood-brain barrier. Nonneuronopathic type 1 Gaucher patients have 'Cerezyme' as ERP, whereas type 2 or 3 Gaucher patients have no effective therapy. The increase in mutant β-Glu activity by CO-DNJ may be only double. However, this increase would be very hopeful for type 2 or 3 Gaucher patients. We expect that a number of pharmacological chaperones would be excellent candidates for a new molecular therapy of human genetic disorders.

4. Materials

4.1. Preparation of iminosugars and their alkyl derivatives

Natural DNJ (**1**) and *N*-methyl-DNJ (**5**) were isolated from the root bark of *Morus alba*,[21] α-HNJ (**2**) and β-HNJ (**3**) from *Stemona tuberosa*,[22] and α-1-*C*-methyl-DNJ (**11**) from *Scilla sibirica*[23] according to the literature. The *N*-alkyl-DNJ derivatives *N*-propyl-DNJ (**6**), *N*-butyl-DNJ (NB-DNJ, **7**), *N*-hexyl-DNJ (**8**), *N*-octyl-DNJ (NO-DNJ, **9**), and *N*-nonyl-DNJ (NN-DNJ, **10**) were prepared as follows. Appropriate alkyl bromide (20 μL) and K_2CO_3 (20 mg) were added to a solution of DNJ (10 mg) in DMF. The reaction mixture was heated at 60 °C for 24 h and filtered. The filtrate was diluted with MeOH and applied to a short column of Amberlyst 15 (5 mL), washed with MeOH, and eluted with MeOH/28% aqueous ammonia (4:1). The concentrated eluate was further chromatographed with Dowex 1-X2 (OH^- form) with water for **6**, **7**, and **8** or 70% aqueous MeOH for **9** and **10** as eluent. The 1-*C*-alkyl-DNJ derivatives, β-1-*C*-butyl-DNJ (**4**), α-1-*C*-propyl-DNJ (**12**), α-1-*C*-butyl-DNJ (**13**), α-1-*C*-hexyl-DNJ (**14**), α-1-*C*-octyl-DNJ (CO-DNJ, **15**), and α-1-*C*-nonyl-DNJ (CN-DNJ, **16**), were prepared according to the method reported previously.[24–26] The spectroscopic data of **4**, **13**, and **15** were superimposable with those reported in the literature.[24–26] The structures of **12**, **14**, and **16** were confirmed by ^1H NMR, ^{13}C NMR, and COSY spectroscopic data.

4.1.1. 1-Deoxynojirimycin (DNJ) (1). $[\alpha]_D$ +40.3° (*c* 1.47, H_2O). HRMS (FAB): *m/z* 164.0923 $[M+H]^+$ ($C_6H_{14}NO_4$ requires 164.0923).

4.1.2. α-Homonojirimycin (α-HNJ) (2). $[\alpha]_D$ +77.2° (*c* 0.57, H_2O). HRMS (FAB): *m/z* 194.1026 $[M+H]^+$ ($C_7H_{16}NO_5$ requires 194.1028).

4.1.3. β-Homonojirimycin (β-HNJ) (3). HRMS (FAB): *m/z* 194.1022 $[M+H]^+$ ($C_7H_{16}NO_5$ requires 194.1028).

4.1.4. β-1-*C*-Butyl-DNJ (4). $[\alpha]_D$ +2° (*c* 1.4, H_2O). HRMS (FAB): *m/z* 220.1549 $[M+H]^+$ ($C_{10}H_{22}NO_4$ requires 220.1549).

4.1.5. *N*-Methyl-DNJ (5). $[\alpha]_D$ +12.6° (*c* 1, H_2O). HRMS (FAB): *m/z* 178.1077 $[M+H]^+$ ($C_7H_{16}NO_4$ requires 178.1079).

4.1.6. *N*-Propyl-DNJ (6). $[\alpha]_D$ −15.1° (*c* 1.57, H_2O). HRMS (FAB): *m/z* 206.1393 $[M+H]^+$ ($C_9H_{20}NO_4$ requires 206.1392).

4.1.7. *N*-Butyl-DNJ (NB-DNJ,7). $[\alpha]_D$ −15.9° (*c* 0.77, H_2O). HRMS (FAB): *m/z* 220.1548 $[M+H]^+$ ($C_{10}H_{22}NO_4$ requires 220.1549).

4.1.8. *N*-Hexyl-DNJ (8). $[\alpha]_D$ −15.5° (*c* 0.89, H_2O). HRMS (FAB): *m/z* 248.1860 $[M+H]^+$ ($C_{12}H_{26}NO_4$ requires 248.1862).

4.1.9. *N*-Octyl-DNJ (NO-DNJ, 9). $[\alpha]_D$ −17.4° (*c* 0.18, MeOH). HRMS (FAB): *m/z* 276.2174 $[M+H]^+$ ($C_{14}H_{30}NO_4$ requires 276.2175).

4.1.10. *N*-Nonyl-DNJ (NN-DNJ, 10). $[\alpha]_D$ −14.2° (*c* 1.24, MeOH). HRMS (FAB): *m/z* 290.2334 $[M+H]^+$ ($C_{15}H_{32}NO_4$ requires 290.2335).

4.1.11. α-1-*C*-Methyl-DNJ (11). $[\alpha]_D$ +78.9° (*c* 0.46, H_2O). HRMS (FAB): *m/z* 178.1081 $[M+H]^+$ ($C_7H_{16}NO_4$ requires 178.1079).

4.1.12. α-1-*C*-Propyl-DNJ (12). $[\alpha]_D$ +60.7° (*c* 0.24, H_2O). ^1H NMR (D_2O): δ 0.93 (t, 3 H), 1.24 (m, 1H), 1.40–1.58 (3H), 2.78 (m, 1H), 3.12 (m, 1H, H-1), 3.18 (dd, 1H, J = 9.2, 9.6 Hz, H-4), 3.53–3.56 (2H, H-3, H-6a), 3.64 (dd, 1H, J = 5.5, 10.1 Hz, H-2), 3.87 (dd, 1H, J = 2.8, 11.5 Hz, H-6b). ^{13}C NMR (D_2O): δ 16.0, 21.6, 29.0, 56.7, 57.9, 64.7, 75.1, 75.5, 77.0. HRMS (FAB): *m/z* 206.1392 $[M+H]^+$ ($C_9H_{20}NO_4$ requires 206.1392).

4.1.13. α-1-*C*-Butyl-DNJ (13). $[\alpha]_D$ +50° (*c* 0.13, H_2O). HRMS (FAB): *m/z* 220.1550 $[M+H]^+$ ($C_{10}H_{22}NO_4$ requires 220.1550).

4.1.14. α-1-*C*-Hexyl-DNJ (14). $[\alpha]_D$ +61.5° (*c* 0.62, H_2O). ^1H NMR (D_2O): δ 0.89 (t, 3H), 1.23–1.47 (8H), 1.50–1.60 (2H), 2.79 (ddd, 1H, J = 2.8, 7.3, 9.2 Hz, H-5), 3.12 (m, 1H, H-1), 3.19 (dd, 1H, J = 9.2, 10.0 Hz, H-4), 3.56 (dd, 1H, J = 7.3, 11.5 Hz, H-6a), 3.56 (t, 1H, J = 10.0 Hz, H-3), 3.67 (dd, 1H, J = 5.5, 10.0 Hz, H-2), 3.92 (dd, 1H, J = 2.8, 11.5 Hz, H-6b). ^{13}C NMR (D_2O): δ 16.2, 24.7, 26.5, 28.1, 31.1, 33.8, 56.7, 58.2, 64.8, 75.2, 75.5, 77.0. HRMS (FAB): *m/z* 248.1863 $[M+H]^+$ ($C_{12}H_{26}NO_4$ requires 248.1862).

4.1.15. α-1-*C*-Octyl-DNJ (CO-DNJ, 15). $[\alpha]_D$ +48.6° (*c* 0.52, MeOH). HRMS (FAB): *m/z* 276.2173 $[M+H]^+$ ($C_{14}H_{30}NO_4$ requires 276.2175).

4.1.16. α-1-*C*-Nonyl-DNJ (CN-DNJ, 16). $[\alpha]_D$ +68.2° (*c* 0.41, MeOH). ^1H NMR (D_2O): δ 0.90 (t, 3 H), 1.28–1.36 (13H), 1.43–1.54 (2H), 1.64 (m, 1H), 2.70 (ddd, 1H, J = 3.2, 7.8, 9.6 Hz, H-5), 3.00 (ddd, 1H, J = 3.2, 5.5, 11.0 Hz, H-1), 3.08 (dd, 1H, J = 8.7, 9.6 Hz, H-4), 3.42 (dd, 1H, J = 8.7, 9.6 Hz, H-3), 3.46 (dd, 1H, J = 7.8, 11.0 Hz, H-6a), 3.58 (dd, 1H, J = 5.5, 9.6 Hz, H-2), 3.88 (dd, 1H, J = 3.2, 11.0 Hz, H-6b). ^{13}C NMR (D_2O): δ 14.5, 23.8, 25.8, 27.4, 30.5, 30.7, 30.8, 30.9, 33.1, 56.2, 57.6, 63.9, 74.3, 74.4, 76.1. HRMS (FAB): *m/z* 290.2335 $[M+H]^+$ ($C_{15}H_{32}NO_4$ requires 290.2331).

5. Experimental

5.1. General experimental procedures

The purity of samples was checked by HPTLC on Silica Gel $60F_{254}$ (E. Merck) using the solvent systems PrOH/AcOH/H_2O (4:1:1) for free base compounds and BuOH/AcOH/H_2O (4:1:1) for their alkyl derivatives, and a chlorine–*o*-tolidine reagent was used for detection. Optical rotations were measured with a Jasco DIP-370 digital polarimeter (Tokyo, Japan). ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a JEOL ECP-500 spectrometer (Tokyo, Japan). Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)propionate (TSP) in D_2O or tetramethylsilane in CD_3OD as internal standard. FABMS were measured using glycerol as a matrix on a JEOL JMS-700 spectrometer.

5.2. Fibroblast culture

Three Gaucher cell lines with β-Glu mutations of GM00372 (N370S), GM00852 (N370S), and GM00877 (L444P), and a normal fibroblast culture (GM05659) were obtained from the Coriell Cell Repositories, Camden, NJ.

5.3. Biological assays

Human β-glucocerebrosidase (Ceredase) was purchased from Genzyme (Boston, MA) and assayed at pH 5.5. The cell lysate of normal human fibroblasts (GM00498B), which were cultured in MEM (Gibco) supplemented with 15% fetal bovine serum and antibiotics at 37 °C under 5% CO_2, was used as the source of lysosomal α-glucosidase (α-Glu) and α-mannosidase (α-Man). The reaction mixture consists of 50 μL of 0.15 M sodium phosphate–citrate buffer (pH 4.5), 50 μL of 2% Triton X-100 (Sigma Chemical Co.), 30 μL of the enzyme solution, and 20 μL of an inhibitor solution or H_2O. The reaction mixture was pre-incubated at 0 °C for 10 min and the reaction was started by the addition of 50 μL of 6 mM 4-methylumbelliferyl glycoside (Sigma Chemical Co.), followed by incubation at 37 °C. The reaction was stopped by the addition of 2 mL of 0.1 M glycine buffer (pH 10.6). Liberated 4-methylumbelliferone was

measured (excitation 362 nm, emission 450 nm) with a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Kinetic parameters were determined by the double-reciprocal-plot method of Lineweaver–Burk at increasing concentrations of 4-MU-β-D-glucoside.

Fibroblasts established from Gaucher patients were cultured in the presence of pharmacological chaperones in a similar manner as described above in normal fibroblasts. After washing with a phosphate buffer twice, the cell pellets were homogenized in a citrate buffer (pH 5.2) containing 0.25% sodium taurocholate and 0.1% Triton X-100 using a microhomogenizer (Physcotron, Niti-on Inc., Chiba, Japan). The supernatant obtained from the homogenate after centrifugation at 10,000*g* for 5 min was subjected to enzyme assays and protein determination.

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