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A Fluorescent sp^2-Iminosugar With Pharmacological Chaperone Activity for Gaucher Disease: Synthesis and Intracellular Distribution Studies

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Gaucher disease (GD) is the most prevalent lysosomal-storage disorder, it is caused by mutations of acid β-glucosidase (β-glucocerebrosidase; β-Glu). Recently, we found that bicyclic nojirimycin (NJ) derivatives of the sp^2-iminosugar type, including the 6-thio-*N*′-octyl-(5*N*,6*S*)-octyliminomethylidene derivative (6S-NOI-NJ), behaved as very selective competitive inhibitors of the lysosomal β-Glu and exhibited remarkable chaperone activities for several GD mutations. To obtain information about the cellular uptake pathway and intracellular distribution of this family of chaperones, we have synthesized a fluorescent analogue that maintains the fused piperidine–thiazolidine bicyclic skeleton and incorporates a dansyl group in the *N*′-substituent, namely 6-thio-(5*N*,6*S*)-[4-(*N*′-dansylamino)butyliminomethylidene]nojirimycin (6S-NDI-NJ). This structural modification does not significantly modify the biological activity of the glycomimetic as a chemical chaperone. Our study showed that 6S-NDI-NJ is mainly located in lysosome-related organelles in both normal and GD fibroblasts, and the fluorescent intensity of 6S-NDI-NJ in the lysosome is related to the β-Glu concentration level. 6S-NDI-NJ also can enter cultured neuronal cells and act as a chaperone. Competitive inhibition studies of 6S-NDI-NJ uptake in fibroblasts showed that high concentrations of D-glucose have no effect on chaperone internalization, suggesting that it enters the cells through glucose-transporter-independent mechanisms.

Introduction

Gaucher disease (GD), the most prevalent lysosomal storage disorder, is caused by mutations in the gene encoding for acid β-glucosidase (β-Glu; glucocerebrosidase, EC 3.2.1.45).[1] These mutations lead to significant protein misfolding during translation in the endoplasmic reticulum and then to a reduction in enzyme trafficking to the lysosome.[2] The deficiency of lysosomal β-Glu results in progressive accumulation of glucosylceramide in macrophages, which often leads to hepatosplenomegaly, anemia, bone lesions, respiratory failure, and, in the most severe manifestations of the disease, central nervous system (CNS) involvement.

At present there are two therapeutic strategies for GD namely, enzyme replacement therapy (ERT) and substrate reduction therapy (SRT). ERT has been achieved by intravenous administration of macrophage-targeted recombinant β-Glu.[3] SRT however, can be realized by oral administration of *N*-(*n*-butyl)-1-deoxynojirimycin (NB-DNJ, miglustat, Zavesca®), which inhibits glucosylceramide synthase and thereby decreases the biosynthesis of glucosylceramide, the natural substrate of β-Glu.[4] Both therapies have been proven to be effective for visceral, hematologic, and skeletal abnormalities.[5–7] However, the efficacy of these therapies for neurological manifestations is limited.[8–11] Bone marrow transplantation can also reverse the disease, but thus far gene therapy strategies have been unsuccessful.

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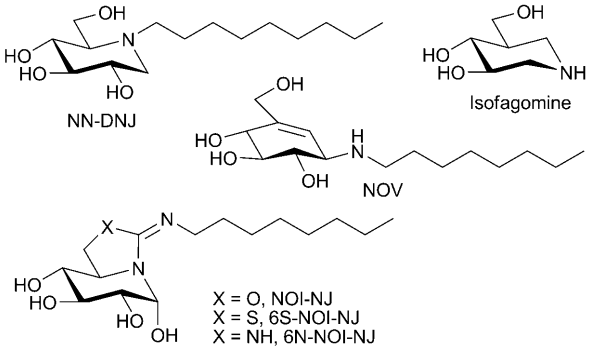
It has been recently found that compounds that act as inhibitors of a lysosomal glycosidase can also stabilize the properly folded structure of the enzyme, thus rendering them suitable for an alternative therapeutic concept, namely *chemical chaperone therapy*. Active-site-directed chemical chaperones stabilize mutant forms of lysosomal enzymes, such as β-Glu, as they pass through the secretory pathway, evading endoplasmic-reticulum-associated degradation (ERAD). Once at the lysosome, the excess of substrate displaces the chaperone from the active site and the enzyme recovers its hydrolytic activity.[12] Several years ago we proposed the carbasugar-type glucomimetic *N*-octyl-β-valienamine (NOV), a potent inhibitor of β-Glu, as a potential chemical chaperone for the treatment of neuronopathic GD.[13–17] NOV could increase the protein level and enzyme activity of mutant β-Glu in cultured cells with several mutation profiles, including homozygotic F231I, N188S, N370S, and G202R mutants. Nitrogen-in-the-ring glucomimetics of the iminosugar or 1-azasugar families, such as *N*-(*n*-nonyl)nojirimycin (NN-DNJ) or isofagomine, respectively (Scheme 1), have also shown great promise as chemical chaperones although they have been proposed in general for non-neuronopathic forms of GD (type 1 GD).[18–24] Compounds having chemical structures that are unrelated to carbohydrates have been additionally added to the list of potential chemical chaperones.[25,26]



Scheme 1. Chemical structures of chaperones for β-Glu.

Recently, we found that bicyclic sugar-shaped compounds that incorporate a pseudoamide-type (isourea, isothiourea, or guanidine) endocyclic nitrogen atom with substantial sp^2 character (sp^2-iminosugars), such as 5*N*,6*O*-[*N*′-(*n*-octyl)iminomethylydene]nojirimycin (NOI-NJ) or its 6-thio or 6-amino-6-deoxy analogues (6S-NOI-NJ and 6N-NOI-NJ),[27–29] behaved as very selective competitive inhibitors of human lysosomal β-Glu and exhibited remarkable chaperone activities for several Gaucher mutations.[30] Interestingly, a comparative study with the classical iminosugar NN-DNJ indicated that sp^2-iminosugars are significantly more efficient for mutations associated with neuronopathic forms of GD (types 2 and 3 GD), which was ascribed to their ability to bind to the mutant β-Glu with a considerably higher affinity at neutral rather than at acidic pH.[30] It was assumed that these compounds would act by rescuing the mutant enzyme at the endoplasmic reticulum thus facilitating trafficking and finally dissociating at the lysosome. To substantiate this hypothesis we have now synthesized a fluorescent sp^2-iminosugar analogue bearing a dansyl group namely, 6-thio-(5*N*,6*S*)-[4-(*N*′-dansylamino)butyliminomethylidene]nojirimycin (6S-NDI-NJ; see Scheme 2), as a probe to unravel the cellular uptake mechanisms and intracellular distribution of this type of glycomimetic. It has been previously shown that the introduction of fluorescent probes such as dansyl residues onto lateral chains in classical iminosugars results in compounds that might exhibit similar or even enhanced glycosidase-inhibitory activity.[31–33] The utility of the fluorescently labeled iminosugar inhibitors for the construction of sensors to detect glycosidase binding[34] and as chemical chaperones[35] has been demonstrated, illustrating the potential of the approach in glycobiology.

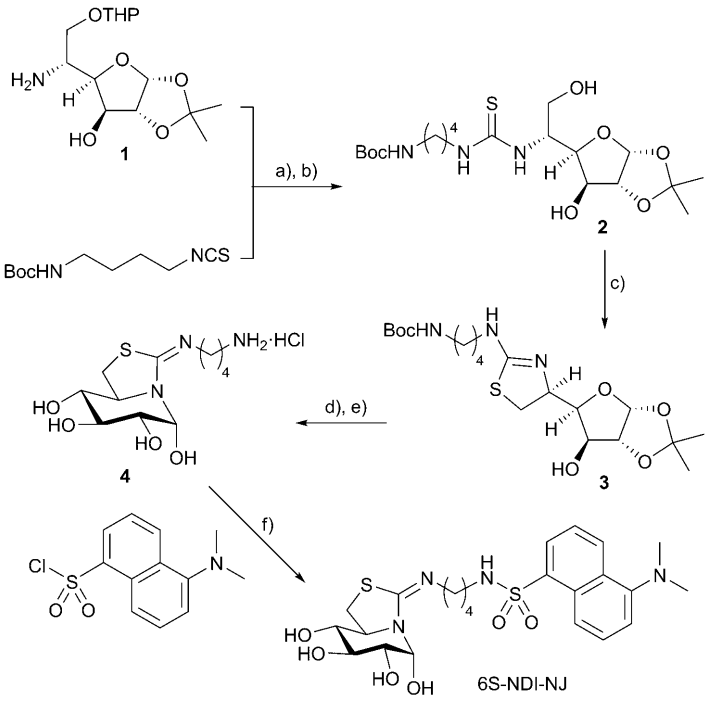
Results

6S-NDI-NJ can be synthesized from a D-glucofuranose precursor following the general method disclosed for bicyclic sp^2-iminosugars

The coupling reaction of 5-amino-5-deoxy-α-D-glucocofuranose derivative **1**[28] with 4-(*tert*-butyloxycarbonylamino)butyl isothiocyanate afforded the corresponding thiourea adduct, which was transformed into the desired *vic*-thioureidoalcohol **2** by removing the tetrahydropyranyl protecting group at O-6. Activation of the primary hydroxyl in **2** by formation of the corresponding methanesulfonate ester resulted in spontaneous cyclization to the key aminothiazoline pseudo-*C*-nucleoside precursor **3**. Simultaneous acid-catalyzed removal of the *tert*-butoxycarbonyl and isopropylidene groups and final neutralization afforded the bicyclic NJ derivative **4**, which bears a terminal free amino group at the exocyclic substituent and was characterized as the corresponding hydrochloride salt. Finally a sulfonamide-forming reaction with dansyl chloride afforded the target compound 6S-NDI-NJ in 98% yield (Scheme 2).

6S-NDI-NJ shows a lysosomal glycosidase inhibition profile similar to that of 6S-NOI-NJ

The inhibitory activity of 6S-NDI-NJ was first checked on lysosomal glycosidases by using lysates from normal human fibroblasts. Strong inhibition of β-Glu and no or weak inhibition of other lysosomal enzymes, such as α-glucosidase, α-galactosidase, β-galactosidase, and β-hexosaminidase, was observed (Figure 1). We next analyzed the inhibitory potencies on F213I/F213I, N370S/N370S, and L444P/L444P mutant β-Glu. The IC_{50} values of 6S-NDI-NJ on F213I/F213I, L444P/L444P and normal β-Glu were very similar, whereas the corresponding IC_{50} value on N370S/N370S mutant β-Glu turned out to be significantly higher. A comparison of β-Glu inhibition at pH 7.0 and 5.2 indicated that, although 6S-NDI-NJ strongly inhibits F213I, N370S, L444P, and normal acid β-Glu at both neutral and acidic pH, all of the β-Glu were more sensitive to inhibition at pH 7.0 than at pH 5.2 (Table 1). Overall, these results indicate that the behav-



Scheme 2. Synthesis of 6-thio-(5*N*,6*S*)-[4-(*N*′-dansylamino)butyl]iminonojirimycin (6S-NDI-NJ). a) Et_3N, py; b) TsOH, 70%; c) MsCl, py, 78%; d) TFA/H_2O (9:1); e) OH^- resin, 82%; f) DMF, Et_3N, 98%.

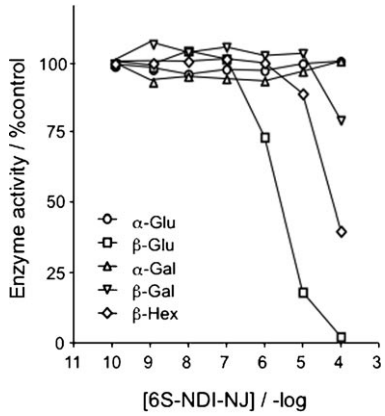


Figure 1. Effects of 6S-NDI-NJ on lysosomal enzyme activities in lysate from human normal fibroblasts. Enzyme activity in normal cell lysates was determined in the absence or presence of increasing concentrations of chaperones. Each point represents means of triplicate determinations obtained in a single experiment. Values were expressed relative to the activity in the absence of compounds (100%).

Table 1. Inhibition of normal and mutant β-Glu by 6S-NDI-NJ at neutral and acidic pH.

Cell lysates	IC_{50} (pH 5.2) [μM]	IC_{50} (pH 7) [μM]
H37	1.287	0.37
F213I/F213I	2.221	0.93
N370S/N370S	29.38	1.15
L444P/L444P	2.318	2.1

ior of 6S-NDI-NJ towards lysosomal glycosidases and mutant β-Glu is very similar to that previously observed for 6S-NOI-NJ.

6S-NDI-NJ showed no cytotoxicity on GD cells

To evaluate the cytotoxicity of 6S-NDI-NJ, we cultured normal and GD human fibroblasts and neuronal cells in the absence and presence of 6S-NDI-NJ at 0.3, 1.0, 3.0, 10, and 30 μM concentrations for four days, and then the cell viabilities were assayed. The results indicated the absence of cytotoxicity in all cases, even at the maximum concentration of 30 μM.

6S-NDI-NJ enhances β-Glu activity in human GD fibroblasts

To evaluate the enzyme activity enhancement of 6S-NDI-NJ, normal as well as F213I/F213I, N370S/N370S, and L444P/L444P mutant human fibroblasts were cultured in the absence and presence of 6S-NDI-NJ at 0.3, 1.0, 3.0, 10, and 30 μM for four days before performing the intact cell lysosomal β-Glu assay (Figure 2a). Contrary to the β-Glu inhibition assay, which is carried out in cell lysates, in intact cells, the chemical-chaperone-promoted ERAD-evading mechanism might operate. The measured enzyme activities then represent the balance between the chemical chaperone and the enzyme inhibition activities of 6S-NDI-NJ at each concentration. In F213I/F213I mutant cells, treatment with 10 and 30 μM MTD111 resulted in 100–200% increase of β-Glu activity. In N370S/N370S mutant fibroblasts, treatment with 30 μM 6S-NDI-NJ resulted in about 70% increase of β-Glu activity. Lower concentrations of the fluorescent sp^2-iminosugar failed to improve enzyme activity in both mutant cells. No effect was observed either in the L444P/L444P mutant or the normal cell line H37.

The optimal concentration of 6S-NDI-NJ from the above results (30 μM) was selected to carry out a ten-day time-course analysis of chaperone activities by using H37 (normal) and F213I/F213I, N370S/N370S and L444P/L444P (mutant GD) human fibroblasts. For F213I/F213I and N370S/N370S cells, β-Glu activity increased in a time-dependent manner in the presence of the fluorescent chaperone; it reached a peak on days 3–5, then decreased slightly and came to a plateau at about 50–100% increase in the last few days. When cells were deprived of 6S-NDI-NJ on day 4, the activity gradually decreased to the basal level within 1–4 days. No effects were observed in the L444P/L444P cell line. In normal H37 cells, the β-Glu activity slightly increased in the first few days in the presence of 30 μM 6S-NDI-NJ, then dropped back to the basal level (Figure 2B).

To investigate the specificity of the chaperone activity of 6S-NDI-NJ among lysosomal glycosidases, mutant Gaucher and normal fibroblasts were treated with 30 μM 6S-NDI-NJ for four days, and then the cell lysates were screened for α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, and β-

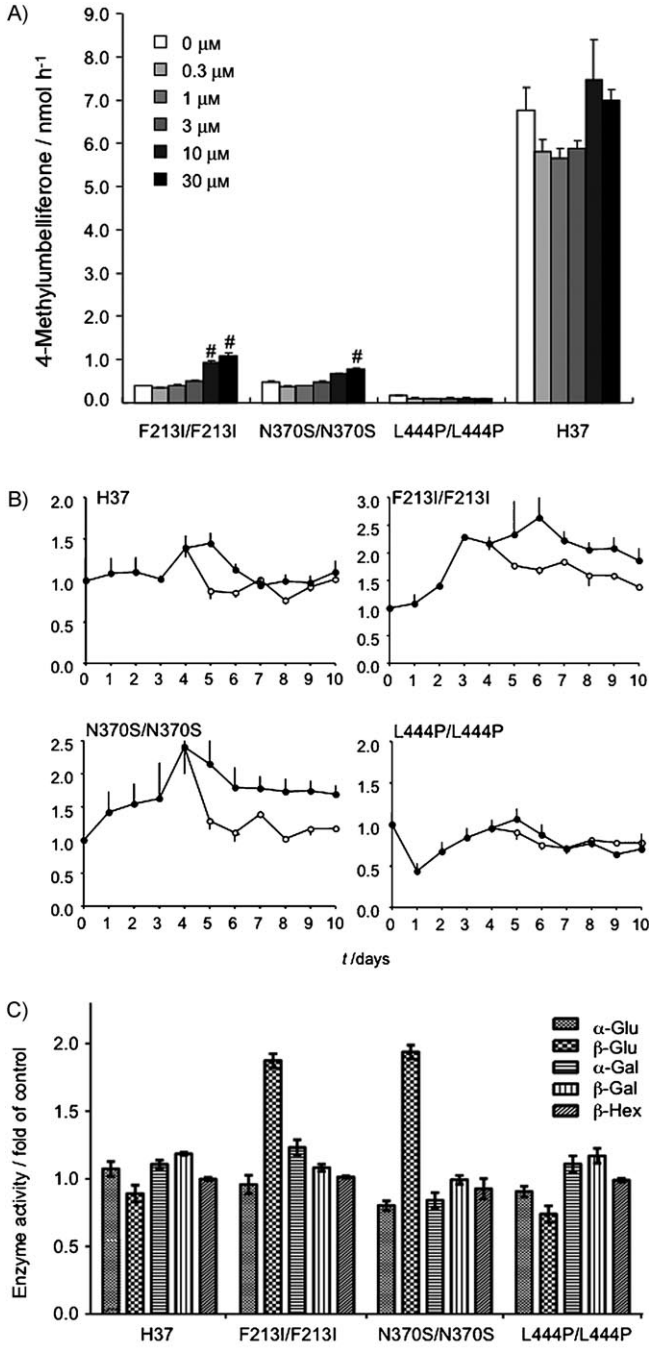


Figure 2. Chaperone activities of 6S-NDI-NJ on mutant β-Glu in fibroblasts. A) Intact cell enzyme assay. Cells were cultured for 4 d in the absence or presence of increasing concentrations of 6S-NDI-NJ. Lysosomal β-Glu activity was estimated in intact cells as described in Experimental Section. Each bar represents the mean ± S.E.M. of 3 determinations each done in triplicate. $^{\#}p < 0.01$ highly significantly statistically different from the values in the absence of the compound (t test). B) Time course. Cells were cultured in the presence of 30 μM 6S-NDI-NJ up to 10 d respectively (●). A subset of cells was cultured with chaperones for 4 d, washed and further cultured without the drug for 6 d (○). β-Glu activity in cells was determined at the indicated time in triplicate. C) The influence of chemical chaperones on activity of lysosomal enzymes. Mutant Gaucher and normal fibroblasts were treated with 30 μM 6S-NDI-NJ for 4 d, and cell lysates were screened for α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, and β-hexosaminidase in triplicate. The activity of treated cells was normalized against the activity of untreated cells. Data shown are the average of triplicate wells for a representative experiment, and the error bars correspond to the standard deviation.

hexosaminidase activities. For F213I/F213I and N370S/N370S GD cells, an increase in the activity of β-Glu was observed, whereas the activities of the other lysosomal enzymes remained essentially unaffected. 6S-NDI-NJ did not influence the activity of either of the lysosomal enzymes in L444P/L444P mutant and normal cell lines (Figure 2C). Western blot experiments further supported that treatment of F213I/F213I and N370S/N370S GD cells with 30 μM 6S-NDI-NJ for four days caused a significant increase in the protein level of mutant β-Glu (data not shown).

6S-NDI-NJ attenuated pH and heat-dependent loss of mutant β-Glu activity in vitro

Sawkar et al. reported that efficient chemical chaperones are capable of preventing pH-dependent in vitro degradation of several mutant β-Glu.[46] We also observed a similar effect for NOV on F213I mutant β-Glu.[14,15] Here, we compared stability at pH 7 and 37 °C and heat-dependent stability at 48 °C of F213I/F213I, N370S/N370S, L444P/L444P and normal β-Glu in the absence and in the presence of 6S-NDI-NJ (Figure 3). When F213I/F213I cell lysates were incubated at 37 °C at pH 7, mutant β-Glu activity rapidly diminished to less than 60% of the initial level after 1 h. In stark contrast, only marginal decreases of mutant β-Glu activity occurred in N370S/N370S cell lysates, with more than 90% activity retained after 1 h incubation at pH 7, whereas the β-Glu activity in normal and L444P/L444P mutant cell lysates remained unaltered under identical conditions. The decreases of F213I/F213I and N370S/N370S mutant β-Glu activities at neutral pH were attenuated by 6S-NDI-NJ in a dose-dependent manner.

In separate experiments, the lysates were heat-denatured (48 °C) at neutral pH and assayed for residual enzyme activity. All β-Glu variants lost activity to some extent under thermal denaturation, with only 20–30% of the initial activity remaining for F213I/F213I and N370S/N370S mutants and 40–50% for L444P/L444P and H37 β-Glu after 1 h. At 10 μM concentration 6S-NDI-NJ fully prevented the heat-induced loss of β-Glu activity in the lysates of L444P/L444P and H37 cells, whereas 30 μM 6S-NDI-NJ was needed in the case of N370S/N370S mutant β-Glu and just 70% activity was retained for F213I/F213I after 1 h heating at 48 °C under the same conditions (Figure 3).

6S-NDI-NJ can be internalized in and released from living fibroblasts

The rate of internalization and release from the cells is expected to be an important parameter for the identification of good chemical chaperones for pharmacological applications. After four days incubation with 30 μM 6S-NDI-NJ, the concentration of the fluorescent sp^2-iminosugar in the cells increased gradually for all F213I/F213I, N370S/N370S, L444P/L444P (mutant), and H37 (normal) fibroblasts. We found that after replacing the culture medium, the intensity of fluorescence in the living cells rapidly decreased with time. Most of the fluorescent chaperone was already released into the external medium after one day (Figure 4), and fluorescence was almost undetectable in

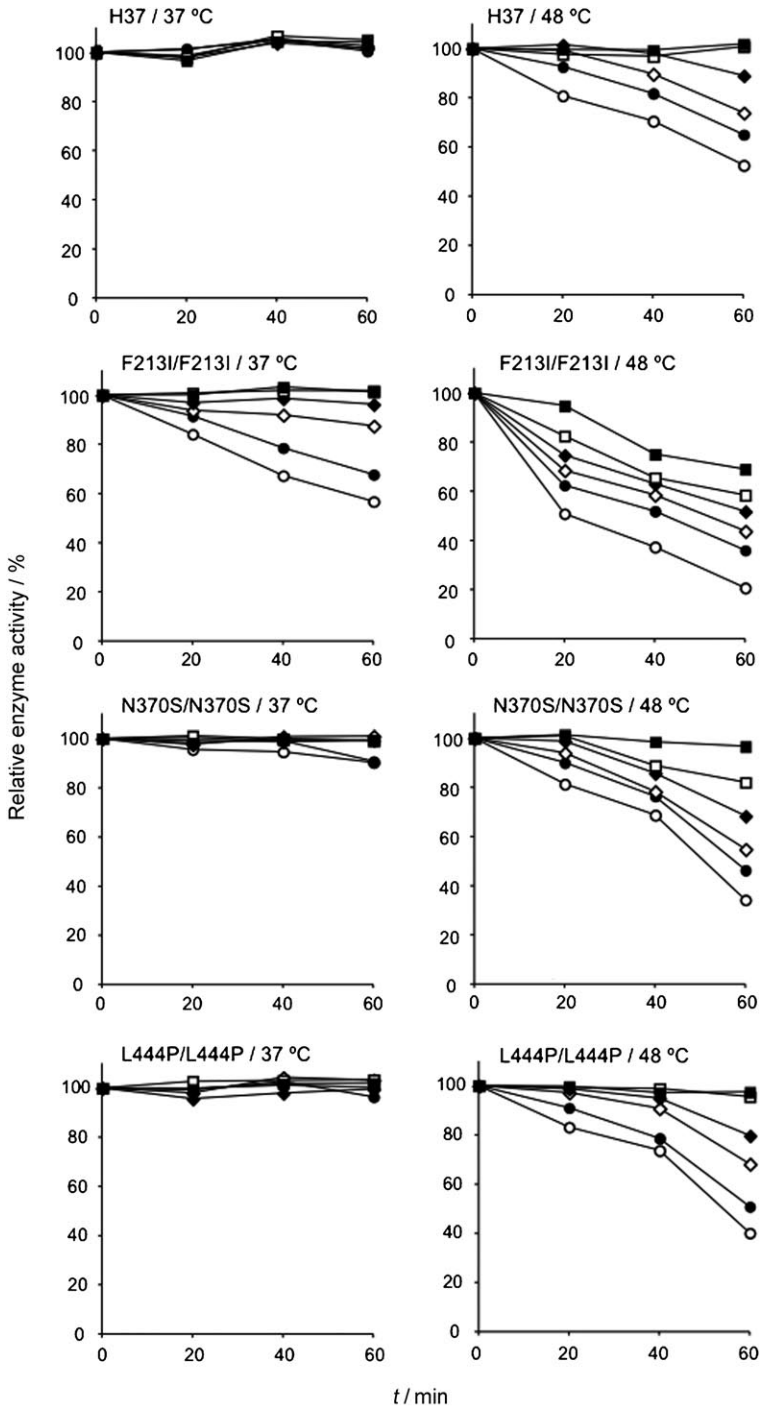


Figure 3. Effects of 6S-NDI-NJ on time-dependent loss of mutant β-Glu activity in vitro at pH 7 at 37 and 48 °C. Cell lysates were incubated in 0.1 M citrate-phosphate buffer at pH 7 and at 37 and 48 °C for the indicated time. Each point represents mean values of triplicate determinations obtained in a single experiment. Values are expressed as relative to the activity before the incubation (100%). ○: 0, ●: 0.3, ◇: 1.0, ◆: 3.0, □: 10.0, ■: 30 μM 6S-NDI-NJ.

the cells after six days. These data suggest that 6S-NDI-NJ can enter and be released from living GD and normal fibroblasts depending on its concentration in the external medium.

6S-NDI-NJ co-localizes in lysosomes in human GD fibroblasts

To explore the intracellular distribution of the chaperone, we have used organelle markers for the endoplasmic reticulum (ER) and the lysosome in combination with confocal microscopy. We found that β-Glu immunoreactivity in F213I/F213I, N370S/N370S, and L444P/L444P mutant fibroblasts are significantly lower than in normal cells. Normal β-Glu co-localized with the lysosomal-related organelle marker LAMP2, which is consistent with its expected localization mainly in the lysosomes. A similar analysis suggested that very few mutant β-Glu localized in the lysosomes of F213I/F213I, N370S/N370S, and L444P/L444P fibroblasts (Figure 5 A).

We further examined the intracellular localization of 6S-NDI-NJ in GD and normal fibroblasts after incubation for four days by using a 30 μM concentration. In all cases it was found that the fluorescent sp^2-iminosugar co-localized with β-Glu (Figure 5 A) and LAMP2 (Figure 5 B). In contrast, no co-localization was observed with calnexin, a protein marker for the ER (Figure 5 B). This suggests that the internalized fluorescent chaperone is essentially bound to the enzyme and that the 6S-NDI-NJ:β-Glu complex is mainly distributed in lysosome-related organelles. The high affinity of 6S-NDI-NJ towards β-Glu probably triggers internalization of the chemical chaperone, which is probably the reason for the much lower concentration of 6S-NDI-NJ detected in the chaperone-insensitive L444P/L444P mutant fibroblasts. Thus, 6S-NDI-NJ could increase the fluorescent intensity of β-Glu in F213I/F213I and N370S/N370S fibroblasts, but did not affect L444P/L444P mutant fibroblasts (Figure 5 A), which is consistent with the results from the β-Glu activity assay and the Western blot. High 6S-NDI-NJ-specific fluorescent intensities were observed in F213I/F213I, N370S/N370S, and normal fibroblasts but not in L444P/L444P fibroblasts after the four-day treatment (Figure 5 B). Most probably, the intensity of the 6S-NDI-NJ specific fluorescence in the lysosome is related to the β-Glu protein level in this organelle.

6S-NDI-NJ exhibits chaperone activity on neuronal cells

Chaperone therapy with sp^2-iminosugars was mainly proposed for neuronopathic GD. Thus, previous results with the parent compound NOI-NJ evidenced good properties regarding oral availability and ability to enhance the β-Glu activity in tissues, including brain, as well as the lack of acute toxicity at high doses in normal mice.[30] We have now examined the effects of 6S-NDI-NJ on normal neuronal cells, which were differentiated from P19 mouse embryonic carcinoma cells by retinoic acid. βIII-Tubulin and MAP2 antibodies were used to identify immature

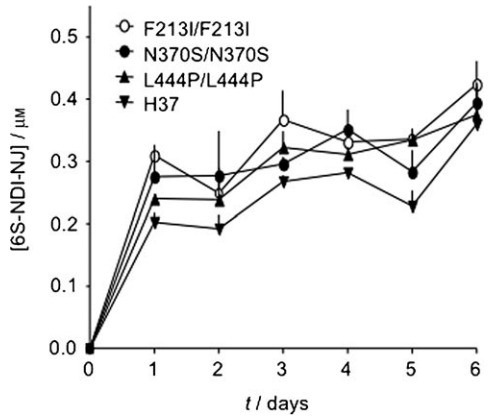


Figure 4. Expulsion of 6S-NDI-NJ from living fibroblasts. Living GD fibroblasts were incubated with 30 μM 6S-NDI-NJ for four days, then washout. The cells were further cultured for 1–6 d. The medium was aspirated for measurement of 6S-NDI-NJ concentration at indicated time. 6S-NDI-NJ medium was measured as described in experimental section.

and mature neurons respectively. We found that 6S-NDI-NJ can enter both immature and mature neurons after four days' incubation (Figure 6B). Additionally, 6S-NDI-NJ showed no toxicity on neuronal cells at a maximum concentration of 10 μM, whereas both intact and lysate assays showed that 6S-NDI-NJ enhanced β-Glu activities of neuronal cells, the maximum chaperone activity being reached at 1 μM concentration (Figure 6A). At higher concentrations the chaperone effect is counterbalance by the inhibitory effect, which becomes predominant at 10 μM.

High concentrations of glucose do not competitively inhibit cellular uptake of 6S-NDI-NJ

At present, there is no information about the cellular uptake pathways of chemical chaperones. Considering the structural similarity of the polyhydroxylated cyclic nojirimycin framework and D-glucose, it seemed reasonable that this family of compounds might share the cell-uptake mechanisms through glucose-specific transporters. Notwithstanding, we found that the presence of D-glucose at 25, 50, and 100 mM in the incubation medium had no influence on the intracellular fluorescent intensities of 6S-NDI-NJ in the human normal fibroblasts (data not shown). The absence of competitive inhibition by D-glucose in the internalization of 6S-NDI-NJ suggests that the chemical chaperone enters the cells through glucose-transporter-independent mechanisms.

Discussion

To circumvent the limitations of ERT and SRT to neurological manifestations, new therapeutic avenues for GD have been explored in recent years.[5,6] Chemical chaperone therapy is a promising approach because of its potential for simple oral administration, penetration of the blood–brain barrier, and low cost. Chaperones are small molecules that can specifically bind to a misfolded protein and help it fold into the correct three-dimensional shape. This allows the protein to be properly trafficked from the ER and distributed to the lysosome in the cell, thereby increasing enzyme activity and cellular function and reducing substrate and stress on cells.[47,48] Up to now, a broad battery of chemical chaperones has been developed for GD, including both sugar-like and non-sugar-related molecules. Recently, we found that bicyclic derivatives featuring a 2-imino-oxazolidine, -thiazolidine, or imidazolidine five-membered ring fused to a polyhydroxylated pyperidine cycle, termed sp^2-iminosugars, behaved as selective competitive inhibitors of the lysosomal β-Glu and exhibited significant chaperone activity for several neuronopathic GD mutations.[30]

Although the concentration of mutant GD proteins in the lysosome has been shown to be enhanced by the presence of chemical chaperones, thereby supporting the proposed rescuing mechanism, to the best of our knowledge there is no direct information available about the intracellular distribution and cellular uptake pathways of such compounds. To clarify these questions, we have synthesized a fluorescently tagged version of the previously reported bicyclic nojirimycin sp^2-iminosugars NOI-NJ, 6S-NOI-NJ, and 6N-NOI-NJ, namely 6-thio-(5*N*,6*S*)-[4-(*N*′-dansylamino)butyliminomethylidene]nojirimycin (6S-NDI-NJ). Actually, the general approach previously developed for the synthesis of bicyclic nojirimycin derivatives of the sp^2-iminosugar type was purposely conceived to allow introduction of molecular diversity in the structure at a relatively low synthetic cost.

In the molecular design of 6S-NDI-NJ, we took advantage of the information previously obtained from X-ray crystallography and thermodynamic studies on bicylic sp^2-iminosugars in complex with recombinant human β-glucocerebrosidase[29] or with the β-glucosidase from the extremophile microorganism *Thermotoga maritima*,[28] an enzyme that belongs to the same clan GHA as β-Glu in the CaZy classification.[48] The octyl chain at the exocyclic nitrogen of NOI-NJ, 6S-NOI-NJ, or 6N-NOI-NJ (Scheme 1) in the enzyme–inhibitor complexes was found to occupy a hydrophobic pocket at the entrance of the active site in all cases, while maintaining substantial flexibility. It was then inferred that structural modifications at this portion of the molecule would not affect the extensive hydrogen-bond network involving the hydroxyl groups and would be well tolerated as far as its hydrophobic nature is preserved. The 6-thio-5*N*,6*S*-(alkyliminomethylidene)nojirimycin bicyclic core was chosen in view of the good chaperone activities previously obtained with 6S-NOI-NJ for several mutant β-Glu. Moreover, the isothiourea segment can be generated with high efficiency from a *vic*-hydroxythiourea intermediate **2**, which is readily accessible from the known 5-amino-5-deoxy-D-glucofuranose derivative **1** by activation of the OH function as a sulfonate ester followed by nucleophilic displacement by the thiocarbonyl sulfur atom (→**3**). The subsequent furanose→pyranose rearrangement is a very efficient process that affords the desired bicyclic sp^2-iminosugar core **4**. The terminal amino group in the N-alkyl substituent in **4** was then exploited for the incorporation of the fluorescent probe in the structure by coupling reaction with dansyl chloride (Scheme 2).

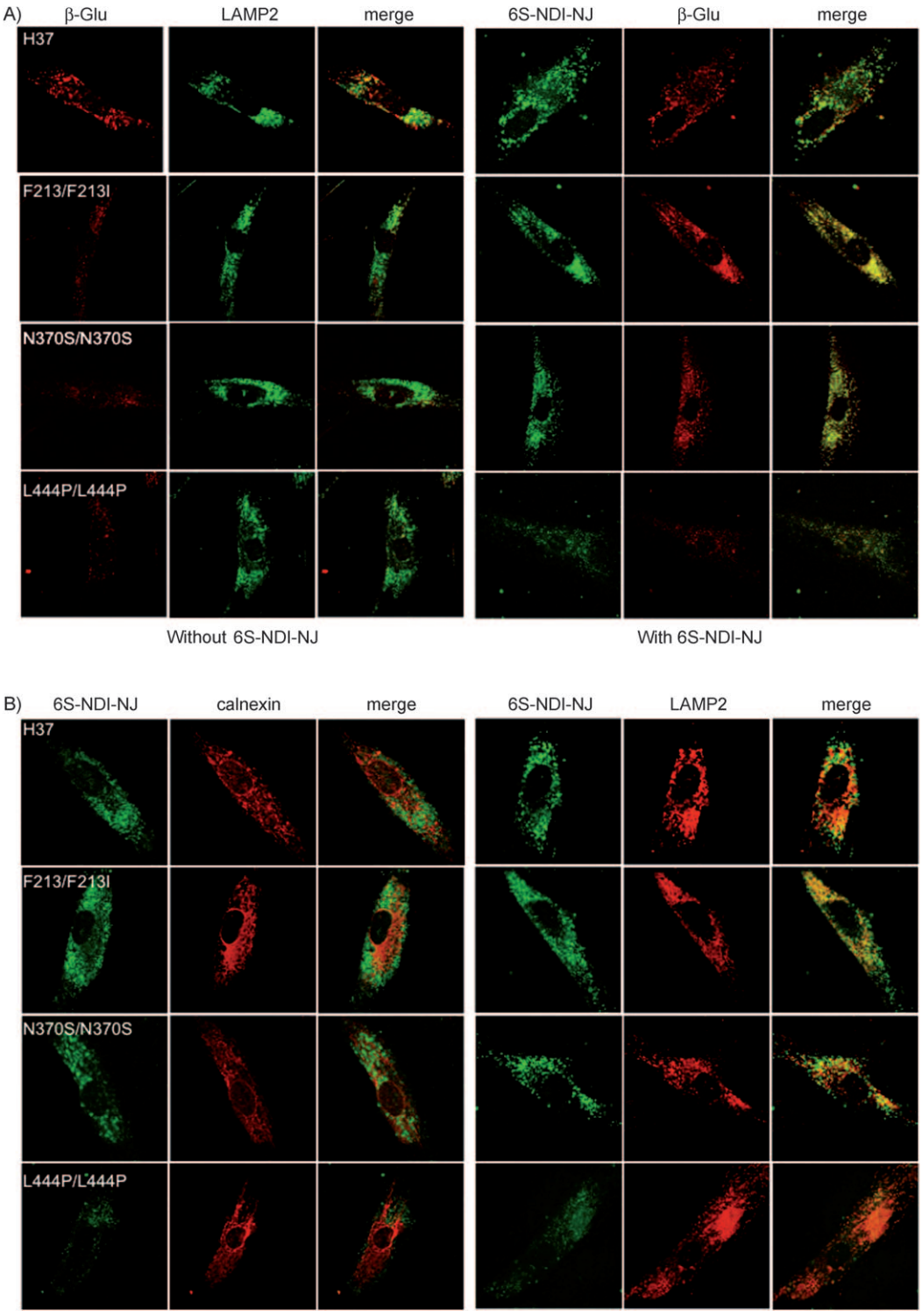


Figure 5. Intracellular distribution of 6S-NDI-NJ in GD fibroblasts. A) Cells in the absence or the presence of 6S-NDI-NJ (30 μM) were double stained with anti β-Glu and LAMP2 antibody or anti β-Glu antibody. B) The cells were cultured for 4 d in the presence of 6S-NDI-NJ (30 μM) and stained with anti-calnexin antibody or anti-LAMP2 antibody. Bound antibodies were visualized with different secondary antibody. The images obtained with a confocal microscope are shown.

First, we confirmed that the insertion of the dansyl group was not detrimental for the biological activity. According to the collected data, 6S-NDI-NJ is a specific inhibitor of β-Glu (Figure 1), exhibits no cytotoxicity at the maximum assayed concentration of 30 μM in human fibroblasts, and can enhance β-Glu activities in human F213I/F213I and N370S/N370S GD fibroblasts (Figure 2). All these characteristics are similar to those previously reported for 6S-NOI-NJ.[30] Moreover, fluores-

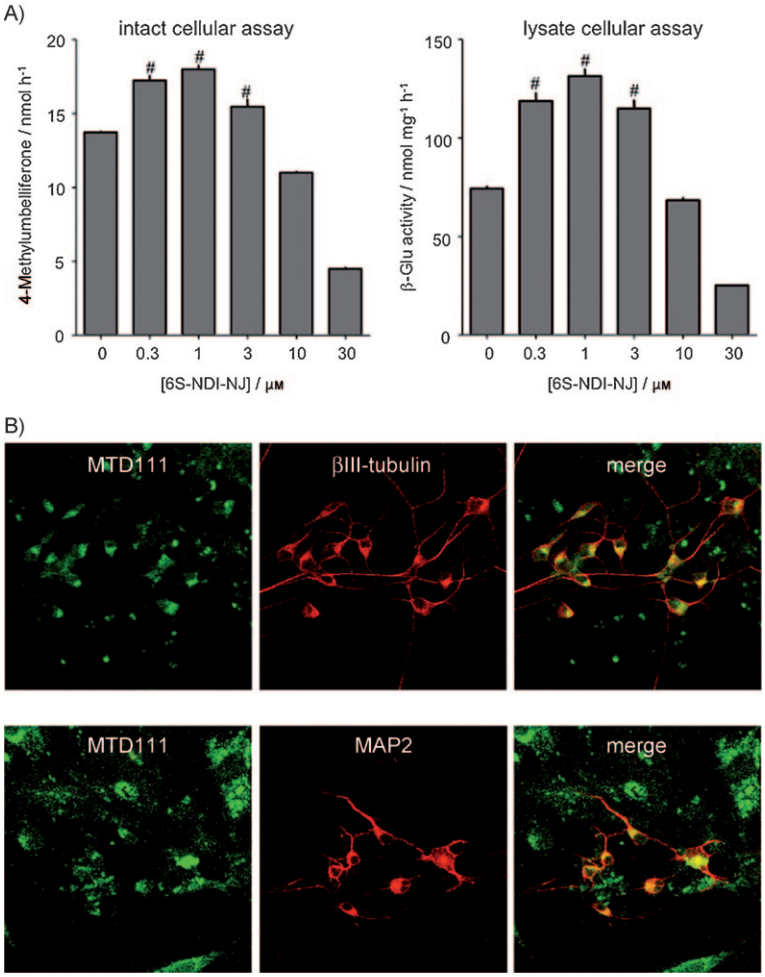


Figure 6. The effect of 6S-NDI-NJ on neuronal cells differentiated from P19 EC cells. A) Enzyme assay. Neuronal cells were cultured for 4 d in the absence or presence of increasing concentrations of 6S-NDI-NJ. β-Glu activity was estimated in intact cells and lysate as described in the Experimental Section. $^{\#}p < 0.01$ highly significant, statistically different from the values in the absence of the compound (t-test). B) The immature and mature neuronal cells were cultured for 4 d in the presence of 6S-NDI-NJ (30 μM) and stained with anti-βIII-tubulin antibody and anti-MAP2 antibody. Bound antibodies were visualized with a different secondary antibody. Shown are the images obtained with a confocal microscope.

cence spectroscopy measurements showed that the spectral properties of 6S-NDI-NJ in aqueous and organic media were well suited for studies of the interactions of this molecule with cultured cells.

Prior to examining the ability of 6S-NDI-NJ to increase the concentration of mutant β-Glu in the lysosome, we examined its capacity to attenuate time-dependent loss of mutant β-Glu activity in vitro at pH 7 at 37 and 48 °C. This protecting effect has been shown to be an indication of good chaperone capabilities, and was ascribed to its efficiency in forcing a correct folding.[46,49] The GD mutants F213I/F213I and N370S/N370S are not stable in the neutral pH environment of the ER and undergo endoplasmic-reticulum-associated degradation (ERAD) to a great extent. In vitro experiments showed that the degradation rate at pH 7.0 at either 37 °C or 48 °C in vitro dramatically decreased in the presence of the dansyl derivative 6S-NDI-NJ. On the contrary, the L444P/L444P mutant protein was found to be as stable as the wild-type (WT) β-Glu at pH 7.0 at 37 °C and also showed similar activity, in agreement with previous results by Sawkar et al.[46] Heat denaturation at 48 °C was also efficiently attenuated by 6S-NDI-NJ in the WT and the L444P/L444P mutant. Nevertheless, the Ig-like domain in which the L444P mutation is located is not tolerated, and, although it does not cause instability associated with the neutral pH, the mutant β-Glu is still subjected to ERAD leading to degradation of the entire enzyme. Actually, the results in Figure 2 further supports that location of the mutation responsible for GD in the catalytic domain of β-Glu (domain III), which is the case of the F213I/F213I and N370S/N370S but not of the L444P/L444P mutant, is a prerequisite for the pharmacological rescue of the protein by an active-site-directed chemical chaperone.

Fluorescence microscopy evidenced that the dansyl-tagged chaperone 6S-NDI-NJ enters the cells upon incubation. Conversely, washout of the culture medium resulted in a gradual decrease of fluorescence intensity in the living cells (data not shown) and a concomitant increase in the concentration of 6S-NDI-NJ in the fresh culture medium (Figure 4). The sp^2-iminosugar can therefore be internalized and further released from fibroblasts into the surrounding medium.

Intracellular distribution studies of 6S-NDI-NJ showed that in GD and normal fibroblasts the chaperone co-localized with β-Glu and was mainly distributed in lysosome-associated organelles, the dansyl-associated fluorescent intensity correlating with the β-Glu protein level. These data are in agreement with the observed binding selectivity of 6S-NDI-NJ towards β-Glu. It should be noted that once in the lysosome the chaperone might attenuate β-Glu activity by their enzyme-inhibitory activity. Although iminosugar and sp^2-iminosugar-type chemical chaperones both exhibit generally higher affinities for WT and mutant β-Glu at neutral than at acidic pH, this feature has previously been found to be significantly more pronounced in the case of the sp^2-iminosugars.[30,50] This probably results in comparatively stronger binding to the mutant enzymes at the ER, where the chaperone activity must operate for rescuing the enzyme from ERAD, than in the lysosome. Because they act as competitive inhibitors, once in the lysosome the high concentrations of substrate accumulated in GD cells will compete with the inhibitor in binding to the enzyme.[45]

Given that chemical chaperone therapy by using sp^2-iminosugar glycomimetics was proposed mainly for neuronopathic GD, it was of interest to investigate the effect of 6S-NDI-NJ in neurons. For this purpose, we have used cultured normal neuronal cells, which were differentiated from P19 embryonal carcinoma cells. We found that 6S-NDI-NJ could enter immature and mature neurons after four-days incubation and enhanced β-Glu activities, with a maximum chaperone effect at 1 μM

concentration, compared to 30 μM in fibroblasts. It seems that neuronal cells are much more sensitive to chaperone therapy. Because a much lower concentration of chaperone in the CNS than in peripheral tissues is expected upon oral administration,[17] the possibility to use relatively high doses of sp^2-iminosugars might result in optimal chaperone activity simultaneously in both types of tissues, making them potentially more practical for the treatment of neuronopathic GD.

In addition to intracellular localization studies, the fluorescently labeled chemical chaperone 6S-NDI-NJ provides an excellent opportunity to explore the preferred cellular uptake pathways of sp^2-iminosugars. Considering that the piperidine ring in 6S-NDI-NJ has a hydroxylation profile of stereochemical complementarity with that of D-glucose, it seemed reasonable to consider that it might share identical internalization mechanisms. Because D-glucose is a polar molecule that requires transport proteins to cross biological membranes, this would imply that the chaperone and the monosaccharide compete for the same transporters. Notwithstanding, we found that high concentrations of D-glucose did not result in competitive inhibition of 6S-NDI-NJ uptake, suggesting instead that the sp^2-iminosugar enters the cells through glucose-transporter-independent pathways. The amphiphilic character of 6S-NDI-NJ and the previously studied sp^2-iminosugar chemical chaperones, with a hydrophilic polyhydroxylated moiety and a hydrophobic substituent in the lateral chain, might facilitate passive diffusion across the cell membrane. Concentration gradient and β-Glu binding affinity would then trigger uptake and release of 6S-NDI-NJ. The fact that the chaperone co-localizes with acid β-Glu in lysosome-related organelles and that its concentration in the cell is closely related to the concentration of the enzyme is consistent with this mechanism. Alternative endocytic routes would be expected to lead primarily to localization of the internalized fluorescent chaperone in endosomes, independently of the β-Glu concentration. Further research using specific endocytosis inhibitors is currently underway to fully ascertain this aspect.

In summary, the present body of work provides evidence for the rescuing of GD β-Glu with mutations at the catalytic domain by sp^2-iminosugars, facilitating trafficking from the ER to the lysosome. The concentration of the mutant enzyme in the lysosome correlated with the intracellular concentration of 6S-NDI-NJ both in fibroblasts and in neuronal cells, which underlines the importance of the internalization process in the efficiency of a chemical chaperone. Actually, when screening chemical chaperones we often found that some candidates with very low IC_{50} values against the target enzyme exhibited disappointingly low chaperone activity when using intact cells. Their inability to cross the cell membrane attenuates their chaperone activity. The amphiphilic character of the sp^2-iminosugar chemical chaperones seems to favor a passive diffusion pathway that facilitates cell internalization. Structural modifications aimed at optimizing the hydrophilic/hydrophobic balance might further increase their ability to cross the cell membrane and improve their chaperone activity. Although further work is needed to fully unravel the relationships between molecular structure, cellular uptake pathways, and chaperone activity, these results should help to improve the present strategies for the development of efficient chemical chaperones.

Experimental Section

Materials: Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies Inc (Gibco BRL, MD, USA). Reagents and solvents were purchased from commercial sources and used without further purification. 6S-NDI-NJ was synthesized in our laboratories by following the reaction sequence described hereinafter. A stock solution of the compound was prepared in DMSO at 30 mM and stored at −30 °C.

Spectroscopic and chromatographic techniques: Optical rotations were measured with a JASCO P-2000 polarimeter, by using a sodium lamp ($\lambda = 589$ nm) at 22 °C in 1 cm or 1 dm tubes. NMR spectroscopy experiments were performed at 300 (75.5) and 500 (125.7) MHz by using Bruker DMX300 and DRX500 spectrometers. 1D TOCSY as well as 2D COSY and HMQC spectroscopy experiments were carried out to assist in signal assignment. In the FABMS spectra, the primary beam consisted of Xe atoms with a maximum energy of 8 keV. The samples were dissolved in *m*-nitrobenzyl alcohol or thioglycerol as the matrices, and the positive ions were separated and accelerated over a potential of 7 keV. NaI was added as cationizing agent. Thin-layer chromatography was performed on E. Merck precoated TLC plates, silica gel 30F-245, with visualization by UV light and by charring with 10% H_2SO_4 or 0.2% (*w/v*) cerium(IV) sulfate-5% ammonium molybdate in 2 M H_2SO_4 or 0.1% ninhydrin in EtOH. Column chromatography was performed on Chromagel (SdS silica 60 AC.C 70–200 μm). Elemental analyses were performed at the Servicio de Microanálisis del Instituto de Investigaciones Químicas de Sevilla.

Synthesis: The dansyl-tagged sp^2-iminosugar 6S-NDI-NJ was synthesized from 5-amino-5-deoxy-1,2-di-*O*-isopropylydene-6-*O*-tetrahydropyranyl-α-D-glucofuranose (**1**) as indicated in Scheme 2. Compound **1** was generated from the corresponding 5-azido derivative[36] (700 mg, 2.13 mmol) by dissolution in MeOH (12 mL) and hydrogenation at atmospheric pressure for 1 h by using 10% Pd/C (234 mg) as a catalyst, and was used without further purification.[28]

5-[*N'*-(4-*tert*-Butoxycarbonylaminobutyl)thioureido]-5-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (2): Et_3N (1.6 mL, 11.7 mmol) and 4-(*tert*-butoxycarbonylamino)butyl isothiocyanate (433 mg, 2 mmol) were added to a solution of **1** (2.13 mmol) in pyridine, (12 mL), and the mixture was stirred at RT for 18 h. The solvent was removed under reduced pressure, and the residue was co-evaporated several times with toluene. The resulting syrup was dissolved in CH_2Cl_2/MeOH (1:1, 42 mL) and *p*-toluenesulfonic acid (69 mg, 0.16 mmol) was added. The mixture was stirred for 2 h at RT, then diluted with CH_2Cl_2 (15 mL), washed with sat. aq $NaHCO_3$ (2×15 mL), dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified by column chromatography using 30:1→15:1 CH_2Cl_2/MeOH as eluent to give **2** (629 mg, 70%). $R_f = 0.33$ (15:1, CH_2Cl_2/MeOH); $[\alpha]_D = +45.5$ ($c = 1.0$ in CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$, 313 K): $\delta = 6.96$ (br s, 1H; N'H), 6.72 (br d, $J_{NH,5} = 7.8$ Hz, 1H; NH), 5.92 (d, $J_{1,2} = 3.6$ Hz, 1H; H-1), 5.07 (br s, 1H; OH), 4.82 (br s, 1H; NH), 4.58 (d, 1H; H-2), 4.54 (m, 1H; H-5), 4.19 (d, $J_{3,4} = 1.9$ Hz, 1H; H-3), 4.09 (dd, $J_{4,5} = 9.8$ Hz, 1H; H-4), 4.03 (dd, $J_{6a,6b} = 11.3$ Hz, $J_{5,6a} = 3.1$ Hz, 1H; H-6a), 3.80 (dd, $J_{5,6b} = 3.0$ Hz, 1H; H-6b), 3.47 (m, 2H; CH_2NHCS), 3.11 (m, 2H; CH_2NHCO), 1.60 (m, 4H; CH_2), 1.49 (s, 3H; CMe_2), 1.31 (s, 3H; CMe_2), 1.49 ppm (s, 9H; CMe_3); ^{13}C NMR (75.5 MHz, $CDCl_3$, 313 K): $\delta = 181.8$ (CS), 156.7 (CO), 111.6 (CMe_2), 104.9 (C-1), 84.7 (C-2), 79.8 (CMe_3), 79.7 (C-4), 73.8 (C-3), 62.4 (C-6),

53.7 (C-5), 44.1 (CH_2NHCS), 40.2 (CH_2NHCO), 28.4 (CMe_3), 27.4 (CH_2), 26.7, 26.0 (CMe_2), 25.7 (CH_2); MS (FAB): *m/z* (%): 472 (100) $[M+Na]^+$, 450 (15) $[M+H]^+$; IR: $\tilde{\nu}_{max}$ = 3342, 2933, 1682, 1549, 1367, 1254, 1165, 1075 cm^{-1}; UV: $\lambda_{max}(CH_2Cl_2)$ = 247 nm (ε_{mM} = 12.3); elemental analysis calcd (%) for $C_{19}H_{35}N_3O_7S$: C 50.76, H 7.85, N 9.35, S 7.13; found: C 50.78, H 7.93, N 9.28, S 6.96.

(4*R*)-2-(4-*tert*-Butoxycarbonylamino)butylamino-4-[(4′*R*)-1′,2′-*O*-isopropylidene-β-L-threofuranos-4′-yl]-2-thiazoline (3): Methanesulfonic chloride (110 μL, 1.42 mmol, 1.2 eq) was added to a solution of the corresponding thioureido derivative **2** (514 mg, 1.14 mmol) in anhyd pyridine (17 mL) at −20 °C under argon. The mixture was stirred for 7 h and allowed to warm to RT. Then, ice–water (30 mL) was added, and the solution was extracted with CH_2Cl_2 (3×20 mL). The combined extracts were washed with iced sat. aq $NaHCO_3$ (25 mL), dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified by column chromatography by using 20:1→10:1 CH_2Cl_2/MeOH as eluent to give **3** (384 mg, 78%). $[\alpha]_D$ = −7.3 (*c* = 1.0 in CH_2Cl_2); R_f 0.46 (CH_2Cl_2/MeOH 7:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.93 (d, $J_{1,2}$ = 3.6 Hz, 1H; H-1), 4.79 (br s, 1H; NH), 4.54 (d, 1H; H-2), 4.42 (m, 1H; H-5), 4.25 (br s, 2H; OH, NH), 4.23 (d, $J_{3,4}$ = 2.5 Hz, 1H; H-3), 4.03 (dd, $J_{4,5}$ = 8.4 Hz, 1H; H-4), 3.51 (dd, $J_{6a,6b}$ = 10.9 Hz, $J_{5,6a}$ = 7.4 Hz, 1H; H-6a), 3.39 (dd, $J_{5,6b}$ = 4.9 Hz, 1H; H-6b), 3.24 (m, 2H; CH_2N), 3.11 (m, 2H; CH_2NHCO), 1.53 (m, 4H; CH_2), 1.48 (s, 3H; CMe_2), 1.30 (s, 3H; CMe_2), 1.33 ppm (s, 9H; CMe_3); ^{13}C NMR(75.5 MHz, $CDCl_3$): δ = 164.6 (CN), 156.1 (CO), 111.5 (CMe_2), 105.1 (C-1), 85.2 (C-2), 81.6 (C-4), 79.3 (CMe_3), 74.9 (C-3), 69.8 (C-5), 44.9 (CH_2N), 40.0 (CH_2NHCO), 37.4 (C-6), 28.4 (CMe_3), 27.3, 26.8 (CH_2), 26.8, 26.1 ppm (CMe_2); IR: $\tilde{\nu}_{max}$ = 3372, 2934, 1696, 1521, 1367, 1251, 1166, 1075 cm^{-1}; MS (FAB): *m/z* (%): 454 (50) $[M+Na]^+$, 432 (100); elemental analysis calcd (%) for $C_{19}H_{33}N_3O_6S$: C 52.88, H 7.71, N 9.74, S 7.43; found: C 52.66, H 7.65, N 9.48, S 7.19.

5-*N*,6-*S*-[*N*′-(4-Amino)butyliminomethylidene]-6-thionojirimycin hydrochloride (4): The 2-amino-2-thiazoline precursor **3** (340 mg, 0.79 mmol) was treated with TFA/H_2O (9:1, 3.5 mL) for 30 min, concentrated under reduced pressure, coevaporated several times with H_2O, neutralized with Amberlite IRA-68 (OH^-) ion-exchange resin, and subjected to column chromatography by using 10:1:1 → 6:3:1 $CH_3CN/H_2O/NH_4OH$ as eluent. The residue was dissolved in dil HCl and freeze-dried to obtain compound **4** as the corresponding hydrochloride (258 mg, 82%). α/β = 1:0.1 (H-1 integration); R_f = 0.22 (6:3:1 $CH_3CN/H_2O/NH_4OH$) $[\alpha]_D$ = −11.3 (*c* = 1.0 in H_2O); α anomer: ^{1}H NMR (500 MHz, D_2O): δ = 5.61 (d, $J_{1,2}$ = 3.7 Hz, 1H; H-1), 4.32 (m, 1H; H-5), 3.76 (m, 2H; H-3, H-6a), 3.62 (dd, $J_{2,3}$ = 9.5 Hz, 1H; H-2), 3.56 (t, $J_{3,4}$ = $J_{4,5}$ = 9.6 Hz, 1H; H-4), 3.46 (m, 3H; H-6b, CH_2N), 3.00 (t, $^3J_{H,H}$ = 7.1 Hz, 2H; CH_2NH$_2$), 1.73 ppm (m, 4H; CH_2); ^{13}C NMR (125.7 MHz, D_2O): δ = 175.7 (CN), 79.0 (C-1), 75.7 (C-4), 74.2 (C-3), 73.1 (C-2), 66.0 (C-5), 50.6 (CH_2N), 41.5 (CH_2NH_2), 33.9 (C-6), 27.7, 26.5 ppm (CH_2); β anomer: ^{1}H NMR (500 MHz, D_2O): δ = 5.02 (d, $J_{1,2}$ = 8.2 Hz, 1H; H-1), 4.08 (m, 1H; H-5), 3.76 (m, 1H; H-3), 3.70 (dd, $J_{6a,6b}$ = 11.6 Hz, $J_{5,6a}$ = 7.6 Hz, 1H; H-6a), 3.60 (m, 1H; H-2), 3.56 (m, 1H; H-4), 3.46 (m, 3H; H-6b, CH_2N), 3.00 (m, 2H; CH_2NH$_2$), 1.73 ppm (m, 4H, CH_2); ^{13}C NMR (125.7 MHz, D_2O): δ = 175.7 (CN), 87.4 (C-1), 77.1 (C-4), 75.8 (C-3), 72.8 (C-2), 69.1 (C-5), 50.5 (CH_2N), 41.5 (CH_2NH_2), 32.5 (C-6), 27.9, 26.5 ppm (CH_2); MS (FAB): *m/z* (%): 314 (40) $[M+Na-HCl]^+$, 292 (90); elemental analysis calcd (%) for $C_{11}H_{22}ClN_3O_4S$: C 40.30, H 6.76, N 12.82, S 9.78; found: C 39.95, H 6.47, N 12.49, S 9.41.

5-*N*,6-*S*-[*N*′-(4-Dansylamino)butyliminomethylidene]-6-thionojirimycin (6S-NDI-NJ): Et_3N (64 μL, 0.21 mmol) and 5-dimethylaminonaphtalene-1-sulfonyl chloride (61.8 mg, 1.1 eq) were added to a solution of **4** (68 mg, 0.21 mmol) in anhyd DMF (15 mL) at 0 °C under argon. The mixture was stirred for 4 h, and the solvent was removed under reduced pressure. The resulting residue was purified by column chromatography by using 90:10:1→60:10:1 CH_2Cl_2/MeOH/H_2O to obtain the target fluorescent sp^2-iminosugar 6S-NDI-NJ (107 mg, 98%). R_f = 0.63 (40:10:1 CH_2Cl_2/MeOH/H_2O); $[\alpha]_D$ = −7.4 (*c* = 0.7 in MeOH); ^{1}H NMR (500 MHz, CD_3CN): δ = 8.52 (d, 1H; dansyl), 8.24 (d, 1H; dansyl), 8.15 (d, 1H; dansyl), 7.60 (m, 2H; dansyl), 7.28 (d, 1H; dansyl), 5.48 (d, $J_{1,2}$ = 3.4 Hz, 1H; H-1), 3.79 (m, 1H; H-5), 3.62 (t, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, 1H; H-3), 3.46 (dd, $J_{6a,6b}$ = 11.2 Hz, $J_{5,6a}$ = 4.4 Hz, 1H; H-6a), 3.40 (dd, 1H; H-2), 3.30 (t, $J_{4,5}$ = 9.3 Hz, 1H; H-4), 3.10 (dd, $J_{5,6b}$ = 7.3 Hz, 1H; H-6b), 3.06 (t, $^3J_{H,H}$ = 10.5 Hz, 2H; CH_2N), 3.00 (m, 2H, CH_2NH), 2.83 (br s, 6H; dansyl), 1.44 (m, 2H; CH_2), 1.34 ppm (m, 2H; CH_2); ^{13}C NMR (125.7 MHz, CD_3CN): δ = 163.1 (CN), 152.0, 135.4, 130.2–129.5, 129.2, 128.4, 123.7, 119.1, 115.5 (dansyl), 76.3 (C-1), 74.3 (C-4), 72.8 (C-3), 71.5 (C-6), 61.1 (C-5), 54.5 (CH_2N), 44.9 (dansyl), 42.5 (CH_2NH), 31.2 (C-6), 26.8, 26.7 ppm (CH_2); HRMS (FAB): *m/z*: 547.1661 $[M+Na]^+$; elemental analysis calcd (%) for $C_{23}H_{32}N_4O_6S_2$ (547.1661): C 52.65, H 6.15, N 10.68, S 12.22; found: C 52.34, H 6.10, N 10.47, S 11.89.

Cell culture: Human skin fibroblasts were cultured in DMEM/10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. One control cell line (H37) and three lines of GD cells that carried the β-Glu mutations F213I/F213I, L444P/L444P, and N370S/N370S, respectively were used.[15] The culture medium was replaced every 2 d with fresh media supplemented with or without chaperone at the indicated concentrations.

In vitro neural cell differentiation: The P19 mouse embryonic carcinoma (EC) cells, obtained from ATCC (American Type Culture Collection), were grown in α-minimum essential medium (α-MEM; Gibco BRL, MD, USA) containing 10% of fetal bovine serum and 1% antibiotic-antimycotic ×100 (Gibco BRL) at 37 °C in the presence of 5% CO_2. For the differentiation process, the P19 cells were removed from the culture flask with 0.25% trypsin solution, and 1×10^6 cells were seeded into 10 mL of α-MEM containing 5% fetal calf serum, and 0.6 μM all-*trans* retinoic acid (Sigma).[37,38] Cells were cultured for one or two weeks to get immature and mature neuronal cells.

Immunoblotting: Except otherwise stated, all procedures were carried out at 4 °C. Fibroblasts were lysed by sonication in PBS supplemented with 1% Triton X-100 and a protease inhibitor cocktail (Roche Diagnostics). After a brief centrifugation to remove insoluble material, the supernatant was collected. For the enzyme assay, 4 μL of the precipitates was used as described below. For immunoblotting, supernatant with the same volume of 2× SDS-PAGE sample buffer was heated at 100 °C for 3 min. SDS-PAGE and Western transfer were carried out as previously described.[14,15] The blots were probed with rabbit polyclonal anti-β-glucosidase and mouse monoclonal anti-α-tubulin antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) and developed by using an ECL kit (GE Healthecare Japan).

In vitro enzyme assay: Lysosomal enzyme activities in cell lysates were determined as described.[39–42] Briefly, cells were scraped into ice-cold H_2O (10^6 mL) and lysed by sonication. Insoluble materials were removed by centrifugation and protein concentrations were determined with a BCA microprotein assay kit (Pierce Biotech, Waltham, MA, USA). The lysate (10 μL) was incubated at 37 °C with the substrate solution (20 μL) in 0.1 M citrate buffer, pH 4.5. The substrates were 4-methylumbelliferone-conjugated α-D-glucopyranoside (for α-glucosidase), α-D-galactopyranoside (for α-galactosidase), β-D-galactopyranoside (for β-galactosidase), and *N*-acetyl-β-D-glucosaminide (for β-hexosaminidase) obtained from Sigma. For

β-Glu, activities in cell lysates were determined by using 4-methylumbelliferone-conjugated β-D-glucopyranoside as a substrate. The lysates 10 μL were incubated at 37 °C with the substrate solution (20 μL) in 0.1 M citrate buffer, pH 5.2, supplemented with sodium taurocholate (0.8% *w/v*). The reactions were terminated by adding 0.2 mL of 0.2 M glycine sodium hydroxide buffer (pH 10.7). The liberated 4-methylumbelliferone was measured with a Perkin–Elmer Luminescence Spectrometer (excitation wave length: 340 nm; emission: 460 nm). One unit of enzyme activity was defined as nmol of 4-methylumbelliferone released per hour and normalized for the amount of protein contained in the lysates (Figure 1).[14,15]

Intact-cell enzyme assay: β-Glu activities in live cells were estimated by the methods described by Sawkar et al.[19] with some modification. Briefly, cells in 96-well assay plates were treated with compounds for 4 d. After washing with PBS, the cells were incubated in PBS (8 μL) and 0.2 M acetate buffer (8 μL; pH 4.0). The reaction was started by addition of 5 mM 4-methylumbelliferyl-β-D-glucoside (10 μL), followed by incubation at 37 °C for 1 h. The reaction was stopped by lysing the cells by the addition of 0.2 M glycine buffer (200 μL; pH 10.7), and the liberated 4-methylumbelliferone was quantified. For neuronal cells, experiments were performed in 35 mm dishes. Every experiment was performed in parallel with cells that had been preincubated with or without conduritol B epoxide (CBE, Toronto Research Chemicals; North York, Ontario, Canada) at 0.5 mM for 1 h. The CBE-sensitive component was ascribed to lysosomal β-Glu, whereas the CBE insensitive component was ascribed to non-lysosomal β-Glu (Figure 2).

Cytotoxicity assay: Cytotoxicity assay was performed by using the colorimetric assay reagent TetraColor One (Seikagaku, Tokyo, Japan),[43] according to the manufacturer's instructions. Cells were seeded on a 96-well assay plate at a density of 3.0×10^4 cells/mL medium and incubated for 4 d with the chaperone. Then, TetraColor One reagent (10 μL) was added to each well, and cells were incubated for another 2 h. The absorbance at 450 nm was measured with a reference wavelength at 630 nm in the microplate reader. Measurement was repeated in triplicate and then averaged for each sample. Results are expressed as mean ± standard deviation (SD).

pH-dependent and heat stability of β-Glu in vitro: Cell lysates were incubated in 0.1 M citrate–phosphate buffer at pH 7 at 37 °C and 48 °C for the time indicated. Incubation was terminated by the addition of 3 volumes of 0.2 M citrate phosphate buffer (pH 5.2), immediately followed by chilling on ice. The enzyme assay was carried out at pH 5.2 as described above (Figure 3).[14]

Measurement of 6S-NDI-NJ release from living fibroblasts: Living GD fibroblasts were incubated with 6S-NDI-NJ in DMEM/10% FBS (30 μL) at 37 °C in 5% CO_2 for 4 d, then the loading solution was replaced with fresh medium after washing twice with DMEM. The cells were cultured for 1–6 d. The medium was aspirated for measurement of the 6S-NDI-NJ concentration. Release of 6S-NDI-NJ from fibroblasts into the surrounding medium was monitored with a Perkin–Elmer Luminescence Spectrometer (excitation wave length: 337 nm; emission: 517 nm) (Figure 4).[44]

Immunofluorescence staining, confocal microscopy and fluorometrical analysis: Fibroblast and neuronal cell lines were grown on glass coverslips in 35 mm dishes. The cells were treated without or with 6S-NDI-NJ (30 μL) at 37 °C in 5% CO_2 for 4 d. Cells were then fixed with 4% formaldehyde for 20 min. After serial washings and permeabilization with 0.1% Triton X-100, rabbit polyclonal anti-glucocerebrosidase antibody (H-300, Santa Cruz, 1:100), rabbit polyclonal anti-calnexin antibody (H70, Santa Cruz, 1:100), mouse monoclonal LAMP-2 antibody (H4B4, Santa Cruz, 1:100), mouse monoclonal anti-β III tubulin antibody (H70, Santa Cruz, 1:100) and rabbit polyclonal anti-MAP2 antibody (H-300, Santa Cruz, 1:100) were applied for 1 h, followed by secondary antibody Alexa Fluor 647 goat anti-rabbit IgG (1:500), Alexa Fluor 647 goat anti-mouse IgG (1:500), Alexa Fluor 546 donkey anti-goat IgG (1:500), Alexa Fluor 546 goat anti-mouse anti-mouse IgG (1:1000) and Alexa Fluor 546 goat anti-rabbit IgG (1:1000) (Invitrogen, Carlsbad, CA, USA). For nuclear staining, cells were incubated with Syto 59 (Invitrogen) for 30 min. Fluorescent images were collected by using a Leica TSC SP2 confocal laser microscope (Leica, Wetzler, Germany).[14,15] For fluorometrical anlysis, fluorescence intensity in randomly selected 25 fields per cells was measured by using an Infinite F500 plate reader (Tecan Japan, Tokyo, Japan; Figures 5 and 6).

Competitive inhibition of 6S-NDI-NJ uptake by glucose in fibroblast: Normal fibroblasts were seeded in 35 mm dishes with coverslips and cultured in DMEM/10% FBS for 2 d. The medium was removed and replaced with 30 μM 6S-NDI-NJ solutions containing 25, 50, and 100 mM glucose (Wako, Tokyo, Japan).[45] After 1 h incubation, cells were washed in PBS (2×) and fixed. Fluorescent images were collected by using confocal laser microscope.

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