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ORIGINAL ARTICLE

Chaperone therapy for Krabbe disease: potential for late-onset GALC mutations

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Krabbe disease is an autosomal recessive leukodystrophy caused by a deficiency of the galactocerebrosidase (GALC) enzyme. Hematopoietic stem cells transplantation is the only available treatment option for pre-symptomatic patients. We have previously reported the chaperone effect of N-octyl-4-epi-β-valienamine (NOEV) on mutant G_{M1} β-galactosidase proteins, and in a murine G_{M1}-gangliosidosis model. In this study, we examined its chaperone effect on mutant GALC proteins. We found that NOEV strongly inhibited GALC activity in cell lysates of *GALC*-transfected COS1 cells. *In vitro* NOEV treatment stabilized GALC activity under heat denaturation conditions. We also examined the effect of NOEV on cultured COS1 cells expressing mutant GALC activity and human skin fibroblasts from Krabbe disease patients: NOEV significantly increased the enzyme activity of mutants of late-onset forms. Moreover, we confirmed that NOEV could enhance the maturation of GALC precursor to its mature active form. Model structural analysis showed NOEV binds to the active site of human GALC protein. These results, for the first time, provide clear evidence that NOEV is a chaperone with promising potential for patients with Krabbe disease resulting from the late-onset mutations.
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INTRODUCTION

Krabbe disease (globoid cell leukodystrophy; OMIM #245200) is a rapidly progressive, fatal neurodegenerative disorder caused by a deficiency of galactocerebrosidase (GALC; EC 3.2.1.46). Patients with infantile onset at 3–6 months of age show non-specific symptoms such as stiffness, feeding difficulties and irritability, but soon progress to motor deterioration and cognitive decline. Patients with later onset (⩾7 months) forms usually present with ataxia, weakness, blindness, spastic paraplegia, behavioral problems and dementia.[1]

The human *GALC* gene (OMIM #606890) encoding GALC protein is composed of 17 exons and 669 amino acids.[2] After synthesis of the GALC precursor (80 kDa), it transported into lysosomes for further maturation that results in two fragments, an N-terminal fragment (50 kDa) and a C-terminal fragment (30 kDa; active GALC).[3] To date, more than 140 disease causing mutation have been found worldwide,[1] among which a 30-kb deletion mutation is responsible for >40% of cases in Caucasian patients with Krabbe disease.[4] For Japanese patients, c.635_646delinsCTC, p.T652P, p.R204X and p.P302A are commonly responsible for infantile onset (30%), and p.G270D, p.[I66M+I289V] and p.L618S for late-onset (28%) forms.[5]

The main disease pathology depends on accumulation of psychosine, the major pathologic substrate for Krabbe disease.[6] It is also reported that an impaired protein processing ratio from precursor to mature form is a key factor for the severity of the disease.[5]

To date, there is no available therapy for human Krabbe disease except for hematopoietic stem cells transplantation for pre-symptomatic or very early stage patients.[7] However, it has been reported that systemic enzyme replacement therapy via intraperitoneal or intracerebroventricular injection increased the median lifespan of twitcher mice (a naturally occurring Krabbe disease model).[8,9] Small-molecule chaperone treatments have been investigated using cultured skin fibroblasts (SFs)[10] and cultured transfected cells[11] from Krabbe disease patients. Our group has identified a low molecular weight compound, N-octyl-4-epi-β-valienamine (NOEV) as a potent chaperone for mutant G_{M1} β-galactosidase proteins.[12–14] We also found its effectiveness in a murine G_{M1}-gangliosidosis model.[15,16] In the present study, we further investigated the chaperone effect of NOEV toward mutant GALC proteins.

MATERIALS AND METHODS

Construction of human GALC expression vectors

Wild-type human *GALC* complementary DNA was constructed in a pSVL vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as previously reported.[5] Seven infantile mutations (p.L70P, c.635_646delinsCTC, p.P302A, p.G352R, p.G496S, p.G646A and p.T652P) and four late-onset mutations (p.[I66M+I289V], p.G270D, p.G569S and p.L618S) were introduced by PCR-based mutagenesis. The sequences of all constructs were confirmed by DNA sequencing and restriction enzyme analysis.

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Transfection and cell culture

COS1 cells were prepared to 70–80% confluence in 3.5-cm dishes on the day prior to transfection. Cell transfection was performed with 2 µl of TransFectin Lipid Reagent (Bio-Rad Laboratories K.K., Tokyo, Japan) and 4 µg of plasmids including empty vector (mock), wild-type *GALC* complementary DNA and *GALC* mutant constructs. The transfection reaction was performed for 24 h and the medium was then changed to standard culture medium of Dulbecco's Modified Eagle Medium (Life Technologies, Carlsbad, CA, USA) with 10% fetal calf serum for an additional 96 h with or without NOEV (synthesized by Central Research Lab, Seikagaku, Tokyo, Japan). Human SFs from a healthy subject and four patients with Krabbe disease (Table 1) were cultured with or without NOEV for 96 h, as described for COS1 cell culture.

Table 1 List of patients whose skin fibroblasts were treated with NOEV

Patient no.	*Phenotype*	*Genotype*	*Reference*
1	Infantile	c.[635_646delinsCTC];p.[T652P]	Hossain *et al.*[5]
2	Juvenile	p.[P302A];[L618S]	Hossain *et al.*[5]
3	Adult	p.[G270D];[G352R]	Hossain *et al.*[5]
4	Adult	p.[G43R];[I66M+I289V]	Hossain *et al.*[5]

Measurement of GALC activity

GALC enzyme activity was measured as previously reported.[5] In short, cells in 3.5-cm dishes were washed with phosphate-buffered saline and trypsinized, then cell pellets were sonicated in deionized distilled water. Protein concentrations were measured by Lowry's method.[17] Cell lysates were incubated with substrate, 6-hexadecanoylamino-4-methylumbelliferyl-β-D-galactopyranoside (Slater & Frith, Norwich, UK), in citrate-phosphate buffer (pH 4.2) at 37 °C for 1 h. Fluorescence (excitation at 385 nm/emission at 450 nm) was measured with a microplate reader.

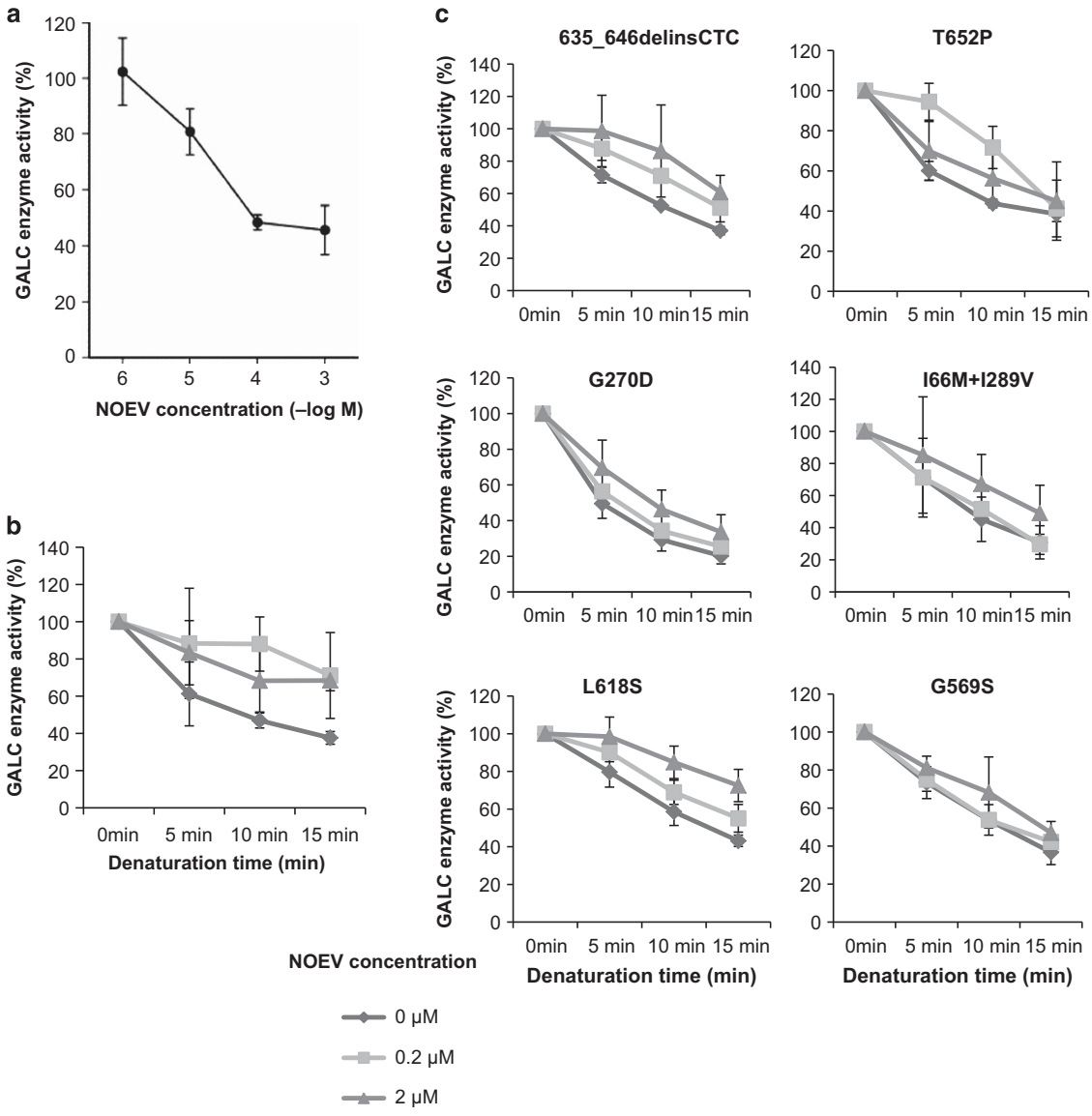

Figure 1 *In vitro* inhibition and stabilization of human GALC proteins. (**a**) GALC activity in cell lysate from wild-type *GALC*-transfected COS1 cells was determined after incubation with various concentrations of NOEV. (**b**) Wild-type and (**c**) mutant GALC-transfected cell lysates were incubated at pH 7.0 and 48 °C for the indicated time and GALC activity was measured. Each point represents the mean ± s.e.m. of duplicates obtained in at least three independent experiments. Values are expressed relative to the activity in the absence of NOEV as 100%. GALC, galactocerebrosidase; NOEV, N-octyl-4-epi-β-valienamine.

Inhibition and stabilization of GALC *in vitro*

Cell lysates from wild-type and mutant-transfected COS1 cells were used for *in vitro* analysis. For inhibition assay, lysates were mixed with 6-hexadecanoylamino-4-methylumbelliferyl-β-D-galactopyranoside substrate in the absence or presence of increasing concentrations of NOEV. For heat stability test, lysates were incubated in citrate-phosphate buffer (pH 7.0) at 48 °C for the time indicated, and then kept on ice to terminate the reaction. GALC activity was measured as described above.

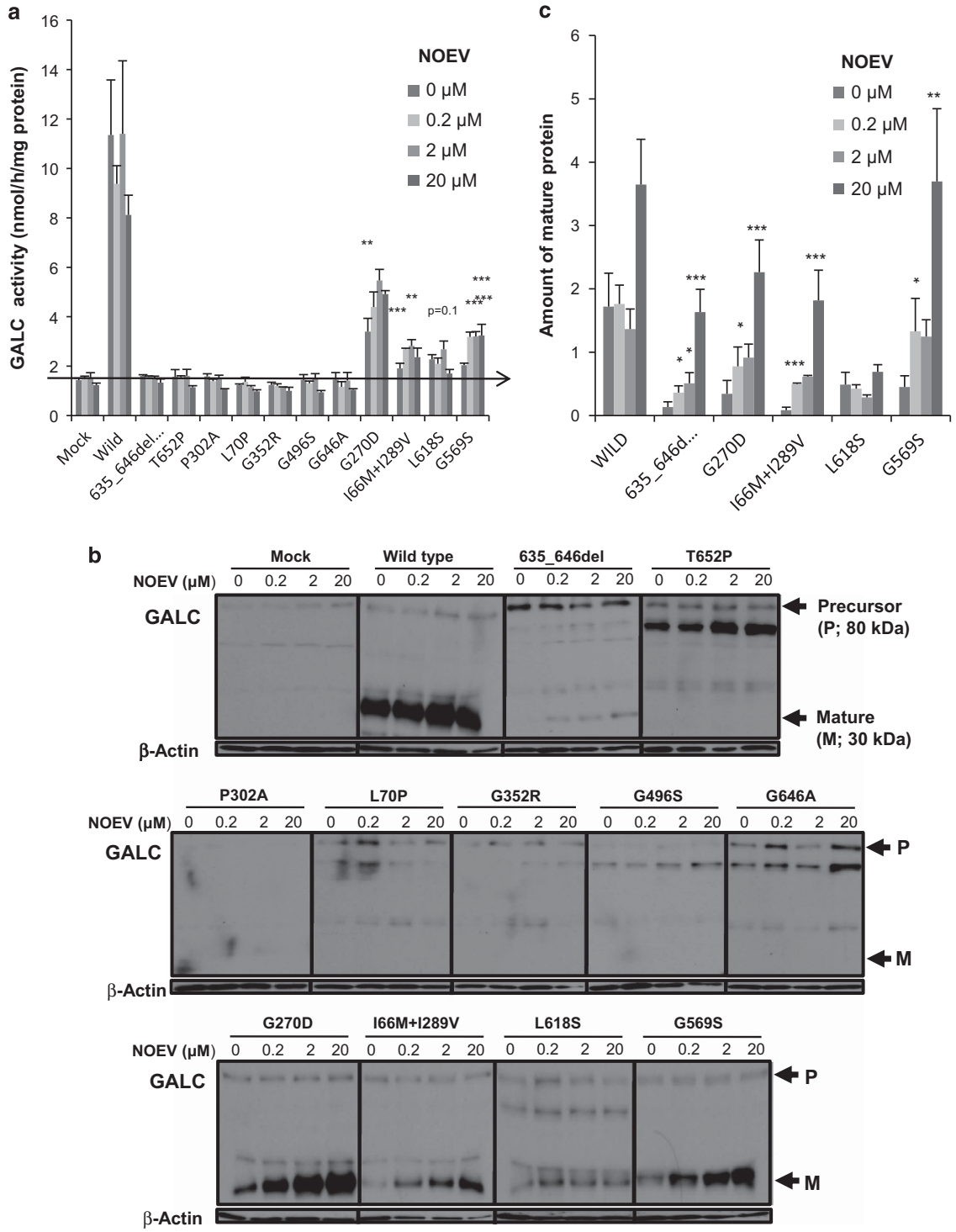

Figure 2 Effects of NOEV on human GALC mutant proteins in cultured COS1 cells. (**a**) GALC enzyme activity in lysate from COS1 cells expressing human wild-type and mutant GALC with or without NOEV treatment. (**b**) Intracellular effects of NOEV on maturation of GALC mutants. Western blot shows a high degree of influence by NOEV on GALC maturation. (**c**) Amount of mature protein was determined following division by the amount of control β-actin protein. Each bar represents the mean ± s.e.m. of duplicates obtained in three determinations. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$ (Student's *t*-test) compared with values in the absence of NOEV. GALC, galactocerebrosidase; NOEV, N-octyl-4-epi-β-valienamine.

Western blotting

Western blotting was performed as previously reported.[5] In short, whole cell lysates were mixed with sample buffer containing protease inhibitor and SDS. Samples (20 μg per lane) were applied to a 10% polyacrylamide gel. After SDS-PAGE, protein was transferred to a 0.45-μm polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA) followed by blocking with 5% skimmed milk solution. Rabbit polyclonal anti-GALC antibody (51051-2-AP, Proteintech, Chicago, IL, USA) was used at 1:2000 dilution as the primary antibody followed by the secondary antibody conjugated with horseradish peroxidase at 1:5000 dilutions. Detection was performed by chemical luminescence (SuperSignal West Dura, Thermo Fisher Scientific, Tokyo, Japan).

Structural modeling of the complex of human GALC with NOEV to its active site

A structural model of the complex of mouse GALC with NOEV bound to its active site was built based on the structural information of the complex for mouse GALC and galactose (PDB: 3ZR6) using modeling software, Modeller,[18] with energy minimization. The structure of mouse GALC was then replaced with that of the human GALC model previously constructed by homology modeling.[5] Auto Dock Vina (Molecular Graphics Laboratory, La Jolla, CA, USA), a grid-based docking program,[19] was used to dock NOEV to the human GALC structure according to the method described previously.[20,21]

Statistical analysis

Experiments were performed three times each in duplicate. Data were presented as means ± s.e.m. Comparison was conducted with a Student's *t*-test. Results were considered statistically significant when $P<0.05$.

RESULTS

In vitro effects of NOEV on GALC enzyme activity

NOEV inhibited wild-type GALC activity in cell lysates of transiently expressed COS1 cells with an IC_{50} value 0.089 μM at pH 4.2 (Figure 1a). To evaluate the effects of NOEV on enzyme stabilization *in vitro*, wild-type and mutant whole cell lysates were incubated with different concentrations of NOEV and were then subjected to heat inactivation (48 °C) at pH 7.0 (Figure 1b and c). In the absence of NOEV, the activities of both wild-type and mutant GALC enzyme activity were decreased to 20–30%. NOEV induced stabilization of wild-type, infantile and late-onset mutant proteins at 0.2 or 2 μM.

Effects of NOEV on overexpressed GALC mutant proteins

We checked the effects of NOEV on seven infantile and four late-onset GALC mutants using transient expression in COS1 cells. After transfection, cells were incubated with increasing NOEV concentrations for 96 h and GALC activity was then measured in whole cell lysates (Figure 2a). All the late-onset mutants (p.G270D, p.[I66M +I289V], p.L618S and p.G569S) responded to NOEV and exhibited higher activity. The maximum response was obtained with NOEV 2 μM for all the late-onset mutants except p.G569S, which had maximum activity at NOEV 20 μM. After subtracting mock, activity was increased 1.4- to 3.3-fold and residual activity was 12–40% of wild-type activity.

Chaperone effects of NOEV in cultured human SFs

To determine the chaperone effect of NOEV in cultured human SFs, cells were cultured in normal Dulbecco's Modified Eagle Medium with various concentrations of NOEV for 96 h, and GALC enzyme activity of cell lysates was then measured. We found a chaperone effect with NOEV for all four patients (Figure 3). GALC activity versus normal was increased 1.16- to 1.44-fold. The maximum response was obtained with NOEV 0.01 μM for all patients except patient 4, who had maximum activity at NOEV 0.2 μM. However, statistical significance was only found for patient 3 (p.[G270D];[G352R]).

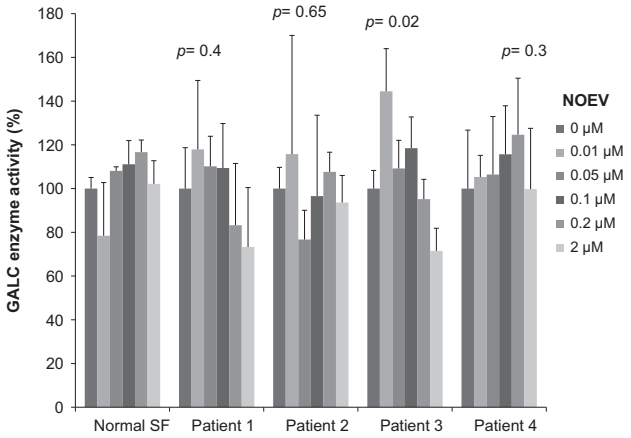

Figure 3 Chaperone effects of NOEV in cultured human skin fibroblasts. Each bar represents the mean ± s.e.m. of duplicates obtained in three determinations. *$P<0.05$ compared with values in the absence of NOEV. GALC, galactocerebrosidase; NOEV, N-octyl-4-epi-β-valienamine.

Intracellular effects of NOEV for maturation of GALC mutants

To evaluate the chemical chaperone effect of NOEV in transiently expressed COS1 cells and cultured human SFs, NOEV-treated whole cell lysates were used for western blotting. For overexpressed COS1 cells, we found that the amount of GALC precursor protein (80 kDa) did not changed by NOEV treatment. However, the expression level of mature GALC protein (30 kDa) for all late-onset mutants and one infantile mutant (c.635_646delinsCTC) markedly increased with NOEV in a concentration-dependent manner (Figure 2b and c), which suggested that mutant precursors were effectively transported to lysosomes for further maturation. We could not detect the 50 kDa fragment that was expected to show similar expression to the 30 kDa fragment. For SFs, we found that 30-kDa fragment expression was increased at relatively high NOEV concentrations for the healthy subject but no band was detected for the patients with Krabbe disease (data not shown).

Structural model of the complex of human GALC and NOEV

To elucidate the interaction between GALC and NOEV, we built a structural model of NOEV bound to the active site of human GALC (Figure 4a). The overview of the complex of human GALC and NOEV is shown in Figure 4b. The locations of the amino acid residues involved in the substitutions examined in this study are indicated in the figure. Among the residues involved in substitutions, I289 is thought to be located near E258, one of the catalytic residues, and the others being far from the active site.

DISCUSSION

Several lysosomal diseases such as Gaucher disease,[22–26] G_{M1} gangliosidosis,[12–16] G_{M2} gangliosidosis[22,27] and Fabry disease[28] are treated with small molecules that assist the folding of mutant enzymes and thereby preventing their early degradation by endoplasmic reticulum-associated degradation. These small molecules are known as proteostasis regulators.[29]

In this study, we examined the chaperone effects of NOEV and confirmed its proteostatic effect in stabilizing all types of mutant

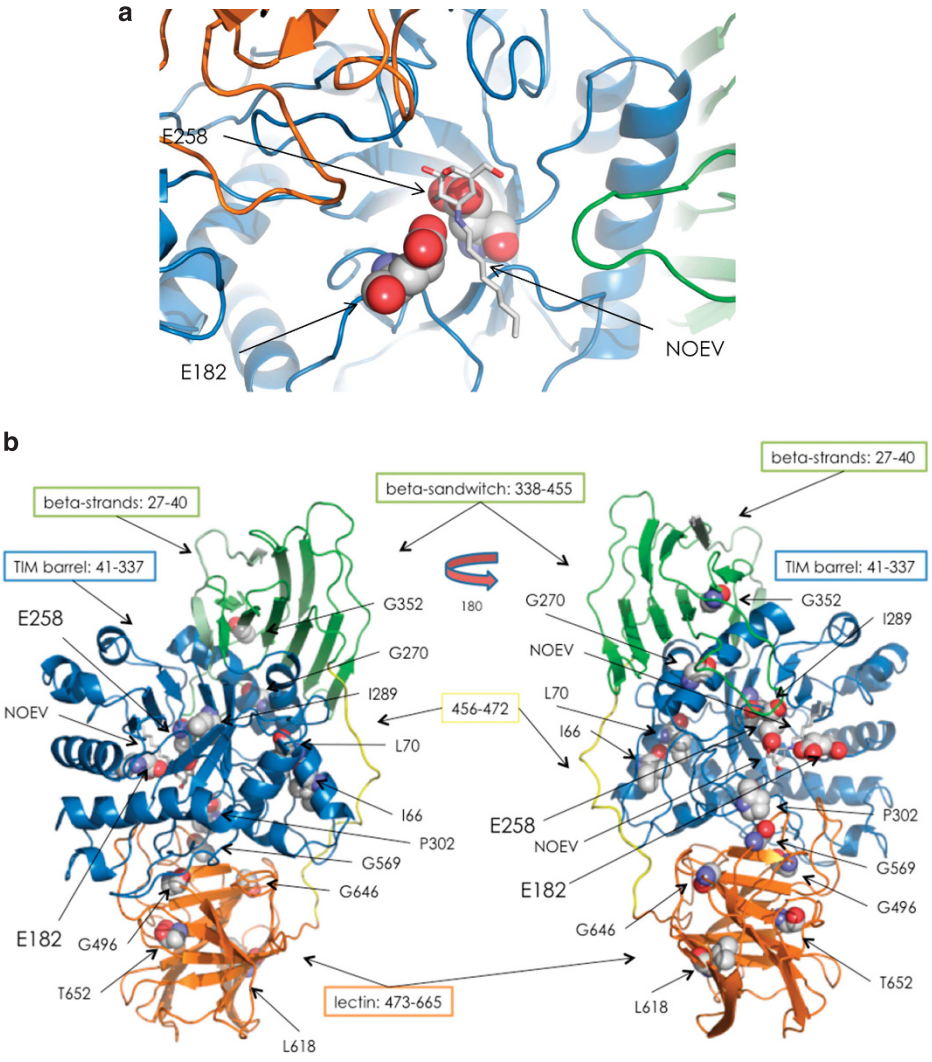

Figure 4 Docking model of human GALC and NOEV binding to the enzyme's active site. (**a**) The backbone of the GALC structure is shown as a ribbon. The catalytic residues (E182 and E258) are shown as space filling models, and NOEV as a stick model. (**b**) Overview of the human GALC structure and the residues involved in substitutions. The backbone of the enzyme is shown as a ribbon (the β-sandwich domain, green; the triosephosphate isomerase (TIM) barrel domain containing the active site, blue; and the lectin domain, orange). The catalytic residues (E182 and E258) and the residues involved in substitutions (I66, L70, I289, G270, P302, G352, G496, G569, L618, G646 and T652) are presented as space filling models, and NOEV as a stick model. GALC, galactocerebrosidase; NOEV, N-octyl-4-epi-β-valienamine.

GALC proteins at pH 7 *in vitro*. It is suggested that NOEV binds with the misfolded protein in neutral medium and prevented endoplasmic reticulum-associated degradation. When we examined the intracellular effects of NOEV, we found higher GALC enzyme activity for late-onset mutants in both a transient expression system and cultured SFs of patients, with an increased amount of mature active protein (Figures 2 and 3). It is suggested that NOEV supported the transportation of the mutant proteins to lysosomes and helps effective maturation. As NOEV is less effective for most infantile mutations, this implies that infantile mutations are generally severe mutations, which induce earlier and more rapid degradation even in the presence of NOEV. One infantile mutation, c.635_646delinsCTC, had a higher maturation ratio with NOEV treatment (Figure 2). However, GALC activity was not enhanced remarkably, possibly because the specific activity was much lower than for late-onset mutations. For late-onset mutations, protein maturation increased under the influence of NOEV in a dose-dependent manner (Figure 2b and c). However, maximum enzyme activity was found at a concentration of 2 μM for most of the late-onset mutants. For GALC enzyme activity measurement, we used whole cell lysates, which contained both of the precursor and mature form, and we could not separate their activities. It has been reported that the precursor form has only one third of the activity of the mature form.[3] Another possibility is that the whole cell lysates contain NOEV and the high concentration can cause inhibition of the activity *in vitro*.

We have recently reported that late-onset patients are predominant (59%), and that three late-onset mutations (p.G270D, p.[I66M +I289V] and p.L618S) are responsible for 28% of alleles and 45% patients in Japan.[5] Wenger *et al.*[4] have also reported that the p.G270D mutation is responsible for 50% of adult Caucasian patients with Krabbe disease. Our current study shows that NOEV induces higher activity for all of these three common mutations, as well as for another

late-onset mutation, p.G569S.[5] Lee *et al.*[11] have reported that α-lobeline had chaperone effects for the p.D528N mutation in an overexpression system at a high concentration. However, it was not effective for other mutants and it was not demonstrated using patient cell lines. Ribbens *et al.*[10] also found small-molecule chaperone effects for p.G270D in a homozygous state using SFs. We found significant chaperone effects ($P<0.05$) for NOEV at a low concentration (0.01 μM) using SFs of a patient (patient 3) who has a compound heterozygous state (p.[G270D];[G352R]; Figure 3), even though p.G352R has been reported as a severe infantile mutation.[5] We also found mild chaperone effects using SFs of other patients who also had compound heterozygous mutations such as patient 1, 2 and 4. For patient 1, both of the alleles (c.[635_646delinsCTC];p.[T652P]) are severe infantile mutations.[5] For patient 2 (p.[P302A];[L618S]), p.P302A mutation has been reported as a severe infantile mutation, which is close to the active site and causes severe distortion of the protein.[30] For patient 4 (p.[G43R];[I66M+I289V]), p.G43R mutation has been reported as a severe infantile mutation.[31] A catalytic activity ⩾10% of normal is considered to be effective for the prevention of the neurological phenotype.[32] Therefore, the enhanced GALC activity by NOEV could be high enough to prevent psychosine accumulation, oligodendrocytes damage and subsequent demyelination.[10] A recent study by our group also reported that the degradation rate of a fluorescent substrate (6-hexadecanoylamino-4-methylumbelliferyl-β-D-galactopyranoside) correlated with the psychosine degradation rate.[5] Moreover, NOEV has already been shown to cross the blood brain barrier and effectively improve neurological signs by enhancing enzyme activity in a murine model of G_{M1} gangliosidosis.[16]

Structural analysis revealed that NOEV should occupy the active site pocket of GALC. The amino acid residues involved in substitutions except for I289 are localized far from the active site. Our previous study showed that I289V, a neutral polymorphism, induces a very little structural change and does not affect the structure of the active site.[5] Considering these results, these amino acid substitutions are thought to affect folding of the enzyme protein, which is followed by excessive degradation before maturation. NOEV binding to the active site of the enzyme would therefore correct the defective folding of the mutant GALC proteins resulting from certain specific amino acid substitutions, that is, G270D, I66M+I289V, L618S, and G569S, which would normally lead to the manifestation of late-onset Krabbe disease.

All of our experiments were *in vitro* analysis, and we also could not perform the loading test with psychosine in patients' SF or over-expressed COS1 cells, because of its extreme cytotoxicity and lack of psychosine quantification equipment. Now our current project is aimed to make a transgenic mice with a common late-onset mutation and treat with NOEV. Then we will check the life expectancy of the affected mice, amount of psychosine accumulation in the brain and measure the GALC activity of various organs including brain, liver, thymus, spleen and SF.

In summary, our study is the largest study so far to show that low concentrations of NOEV can be effective for enhancing mutant GALC activity. NOEV would be potentially effective in covering 28% alleles and 45% patients in Japan with Krabbe disease. NOEV should therefore be considered as a therapeutic alternative for late-onset Krabbe disease in Japan and warrants further investigation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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