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Potent chemical chaperone compounds for G_{M1}-gangliosidosis: *N*-substituted (+)-conduramine F-4 derivatives †

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The development of second-generation valienamine-type chemical chaperones for G_{M1}-gangliosidosis is described. Several *N*-substituted (+)-conduramine F-4 derivatives were designed as novel chemical chaperones based on a lead chaperone compound, *N*-octyl-4-*epi*-β-valienamine, to decrease the inhibitory activity while increasing the enzyme enhancement activity. Among the derivatives synthesized during this study, a conduramine derivative with an *N*-cyclohexylmethyl group has demonstrated significant enhancement of R201C mutated β-galactosidase activity.

Introduction

Small amounts of certain glycosidase inhibitors can benefit the treatment of some genetic lysosomal storage disorders (LSDs); this strategy is called pharmacological chaperone therapy[1]. Stacking an inhibitor in the pocket of a mutated enzyme stabilizes the three-dimensional structure; this stabilization prevents degradation during transport via endoplasmic reticulum, enhancing the activity of the enzyme in the lysosome[2]. Therefore, the development of 'suitable' glycosidase inhibitors would generate novel pharmacological chaperones (PCs) for LSDs. The design of PCs for various LSDs has been based on mimicking the substrate structures or high-throughput screenings[3]. Interestingly, many of the former type of PCs consist of pseudo-sugar moiety and long alkyl chain as aglycone moiety. For example, the authors have developed PCs with a transition-state analogue for the hydrolysis of sugars (half-chair conformation), valienamine structure, and *n*-octyl alkyl side chain: *N*-octyl-β-valienamine (NOV)[4] for Gaucher disease, which results from glucocerebrosidase deficiency, and *N*-octyl-4-*epi*-β-valienamine (NOEV[5], **1**) for G_{M1}-gangliosidosis (Fig. 1).

NOV **NOEV (1)**

Fig. 1. Pharmacological chaperone reagents, *N*-octyl-β-valienamine (NOV) and *N*-octyl-4-*epi*-β-valienamine (NOEV).

Among LSDs, G_{M1}-gangliosidosis is a very rare disease caused by the mutation of lysosomal β-galactosidase. G_{M1} ganglioside and its asialo derivative G_{A1} ganglioside are accumulated in the central nervous system due to weak activity of the enzyme. Unfortunately, enzyme replacement therapy, which is available for some LSDs, may not provide benefit for treatment of G_{M1}-gangliosidosis because intravenously injected enzymes hardly penetrate the blood brain barrier. No effective medical treatment is available for G_{M1}-gangliosidosis at this time. Several research groups, including that of the authors, have been engaged in developing pharmacological chaperone reagents for G_{M1}-gangliosidosis, including NOEV and derivatives of 1-deoxygalactonojirimycin[6]. NOEV possesses remarkable enhancement activities for mutated lysosomal β-galactosidase *in vitro*[7] and *in vivo* with blood brain barrier penetration for mice[8].

Although NOEV is a potent chaperone drug candidate for G_{M1}-gangliosidosis, we found that the mutated enzyme enhancement activity of this compound decreased at higher concentrations, as described later. The strong inhibitory action of NOEV might disrupt its enhancement activity. In addition, the non-inhibitory chaperone reagents developed by Patnaik et al[9] and the allosteric chemical chaperone developed by Porto et al[10] were recently reported to have avoided inhibitory activity relative to the previous PCs. In this paper, the chemical structure of the lead chaperone compound was modified to reveal the requirements for moderate inhibitory activity while improving the enzyme enhancement activity. The chaperone assays were carried out using mouse fibroblast cells with human normal β-galactosidase or human mutant R201C β-galactosidase, which causes juvenile G_{M1}-gangliosidosis.

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We have been interested in modifying the structure of NOEV to generate a novel pharmacological chaperone based on the strategy shown in Fig. 2. First, the pseudo-β-galactose moiety of NOEV was simplified, which was expected to lower its enzyme inhibitory activity and streamline its preparation. Therefore, the C-5 function of NOEV was modified to form the dehydroxy (**2**) derivative, which is a previously reported 6-deoxy derivative of NOEV[11], and the dehydroxymethyl (**3**) derivative, which is *N*-octyl-(+)-conduramine F-4. Some conduramines and their derivatives have shown moderate inhibitory activity toward glycosidases[12]. Moreover, to increase the enzyme enhancement activity, the *N*-substituent of **3** was modified. Because the space adjacent to the active site of β-galactosidase is hydrophobic[6f], a functional group such as an alkyl or aryl group is desirable for interactions in this region.

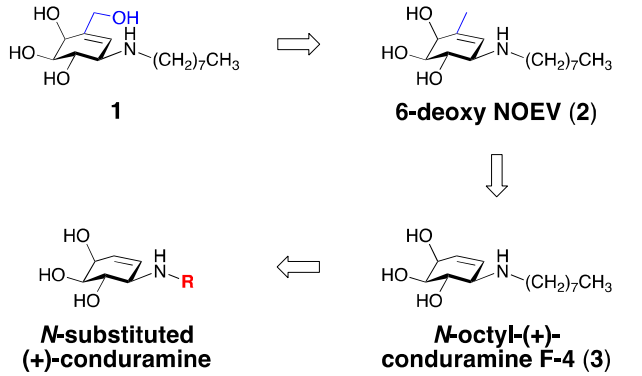
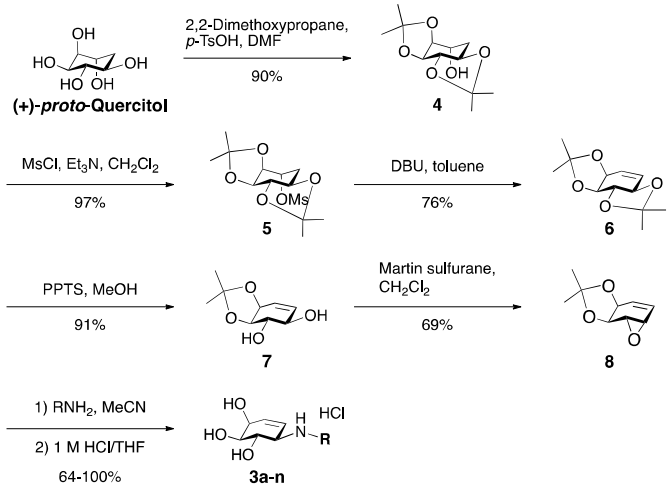


Fig. 2. Strategy of modification of NOEV (**1**) to decrease the inhibitory activity while increasing the enzyme enhancement activity.

Results and discussion

Synthesis of *N*-substituted (+)-conduramine F-4 derivatives

The synthesis of various *N*-substituted (+)-conduramine F-4 derivatives is shown in detail in Scheme 1. Although the synthesis of (+)-conduramine F-4 was described previously[13], we report a more concise route toward (+)-conduramine F-4 derivatives starting from (+)-*proto*-quercitol.



Scheme 1. Synthesis of the *N*-substituted (+)-conduramine F-4 derivatives from (+)-*proto*-quercitol.

First, an *O*-isopropylidenation[14] of (+)-*proto*-quercitol with 2,2-dimethoxypropane in the presence of an acid catalyst in DMF afforded diacetonide **4** at 90% yield. Afterwards, the remaining hydroxyl group on **4** was sulfonylated, providing mesylate **5** (97%). Treating **5** with excess DBU in refluxing toluene produced cyclohexene **6** at 76% yield. The appearance of two ring protons at δ 5.78 and 6.16 in the ^{1}H-NMR spectrum of **6** supported the assigned alkene structure. Next, the trans-isopropylidene group of **6** could be removed selectively by using catalytic pyridinium-*p*-toluenesulfonate (PPTS) in methanol, giving diol **7** in high yield (91%). The subsequent epoxidation of **7** was accomplished under conditions described by Martin et al[15]; treatment with slight excess of Martin sulfurane generated epoxide **8** at 69% yield. Alternatively, **8** was also obtained in a moderate yield (59%) under Mitsunobu conditions with PPh_3 and di-isopropyl azodicarboxylate (DIAD). Incorporating various amine functionalities into the C-1 position of **8** was performed via simple addition reactions with alkylamines. Because the allylic C-1 carbon atom of the epoxide was more reactive, the amination reaction occurred in a regio- and stereo-selective fashion. After considering the solubility of these compounds for successive biological assays, we intended to isolate the pure amine hydrochlorides directly. Silica-gel column chromatography was followed by treatment with hydrochloric acid/aqueous THF to afford the *N*-substituted (+)-conduramine F-4 derivatives as the HCl salts (**3a**-**n**) at 64 to 100% yields.

Inhibitory and chaperone activity

The inhibitory and enhancement activities of the compounds for normal human β-galactosidase and R201C mutated human β-galactosidase, respectively, are shown in Table 1. Regarding the enzyme enhancement activity, R201C is often used to select pharmacological chaperone candidates for mutant human β-galactosidase. First, NOEV•HCl (**1a**) has an IC_{50} = 1.7 μM, while 6-deoxy-NOEV•HCl (**2a**) and *N*-octyl-(+)-conduramine F-4.•HCl (**3a**) have IC_{50} = 46 μM and 120 μM, respectively. Therefore, the inhibitory activity was decreased based on the functional groups at C-5, and the inhibitory activity of **3a** could be remarkably diminished to 1.4% compared to **1a**. The enhancement activity of the mutant R201C β-galactosidase induced by treatment with both **1a** (2 μM) and **2a** or **3a** (20 μM) was almost identical, producing an approximately 5-fold increase compared to the additive-free system. However, the enzyme enhancement activity mediated by **2a** and **3a** increased relative to their concentration at 0.2, 2 and 20 μM, while for **1a**, the concentration was nearly optimal at 2 μM and decreased at 20 μM (Graph 1). The strong inhibitory action of **1a** may influence its enzyme enhancement activity at the higher concentration, or the high concentration of **1a** was participating in an unknown process that stabilized the conformation of the R201C mutant through protein

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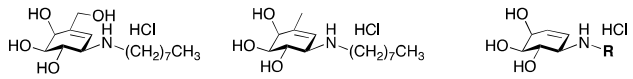
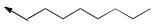
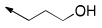
translocation. During the chemical modification of **1a**, simplifying its pseudo-β-galactose moiety decreased its strong inhibition activity to approximately one-hundredth, preserving the activity of the chaperone at concentrations up to 20 μM.



1a **2a** **3a-n**

compd	R	IC_{50} (μM)[a]	Enhancement (fold)[b]
1a	—	1.7	3.6 (4.6)[c]
2a	—	46	5.2
3a		120	5.4
3b		50	2.0
3c		19	4.0
3d		56	4.6
3e		43	1.6
3f		>1000	0.9
3g		41	4.6
3h		15	7.4
3i		60	8.5
3j		180	1.5
3k		>1000	1.2
3l		490	1.4
3m		28	4.6
3n		86	4.2

[a]IC_{50} for normal human β-galactosidase determined at pH 4.5. [b]Comparison between the addition of 20 μM of compounds and the additive-free system. [c]For the addition of 2 μM of **1a**.

Table 1. Structure-activity relationships for NOEV•HCl (**1a**), 6-deoxy-NOEV•HCl (**2a**), and *N*-substituted (+)-conduramine derivatives (**3a-n**).

Graph 1 also shows the enhanced R201C β-galactosidase activity after adding several *N*-substituted (+)-conduramine F-4 hydrochlorides at various concentrations (0.2, 2 and 20 μM). The enhancement activity of all of the conduramine derivatives did not decrease up to 20 μM, indicating that the desired decrease in inhibitory activity after trimming the pseudo-sugar moiety was successful; this decrease most likely occurred by reducing the interaction between compounds and enzyme through decreased mutual hydrogen bonding.

Structure-activity relationships for conduramine derivatives summarized in Table 1 indicate that the enhancement activity increases relative to the length of the linear alkyl chain from *n*-butyl (**3b**), *n*-pentyl (**3c**), *n*-hexyl (**3d**) to *n*-octyl (**3a**), although the *n*-decyl derivative (**3e**) exhibited a poor enhancement. Incorporating a hydroxyl group in the alkyl chain (**3f**) remarkably eliminated the inhibitory and enhancement activities. This may suggest that the hydrophobic interactions between the side chain part and the peripheral region around the active site of the enzyme dominate the onset of both inhibitory and enhancement activities and/or the hydrophobicity of the side chain is essential for the cell membrane permeability. The C4 isobutyl group (**3g**) induced better enhancement activity than the C4 *n*-butyl group (**3b**). In addition, elongating the terminus of the side chain from C4 isobutyl (**3g**) to C6 2-ethylbutyl (**3h**) increased the enhancement activity from 4.6-fold to 7.4-fold, surpassing that of *n*-octyl conduramine **3a**. Thus, in this case, *N*-CH_2-branched alkyl chains contributed strongly to the enzyme enhancement activities. Furthermore, bridging the branched chains of **3h** to form a cyclohexylmethyl group (**3i**) enhanced the R201C mutated β-galactosidase activity in cell lysates 8.5-fold, attaining the highest increase. When assuming that compound **3i** possesses the optimal structure within the present series, **3a** and **3d** should exert a strong activity enhancement toward the mutant enzyme when the *N*-C6 and C8 aliphatic chains undulate and adopt a conformation that resembles the cyclohexylmethyl function of **3i**. Moreover, **3i** showed moderate IC_{50} values (60 μM) against normal human β-galactosidase compared to that of **1a** (1.7 μM). Replacing the cyclohexyl group with a phenyl group (**3i** → **3j**) decreased both the inhibitory and enhancement activities. The predominance of the *N*-CH_2-structure in the side chains was confirmed because the activity of the 1-ethylpropylamino (**3k**) and cyclohexylamino (**3l**) compounds decreased drastically. Elongating the alkyl chains (**3g** → **3m**) further induced no remarkable

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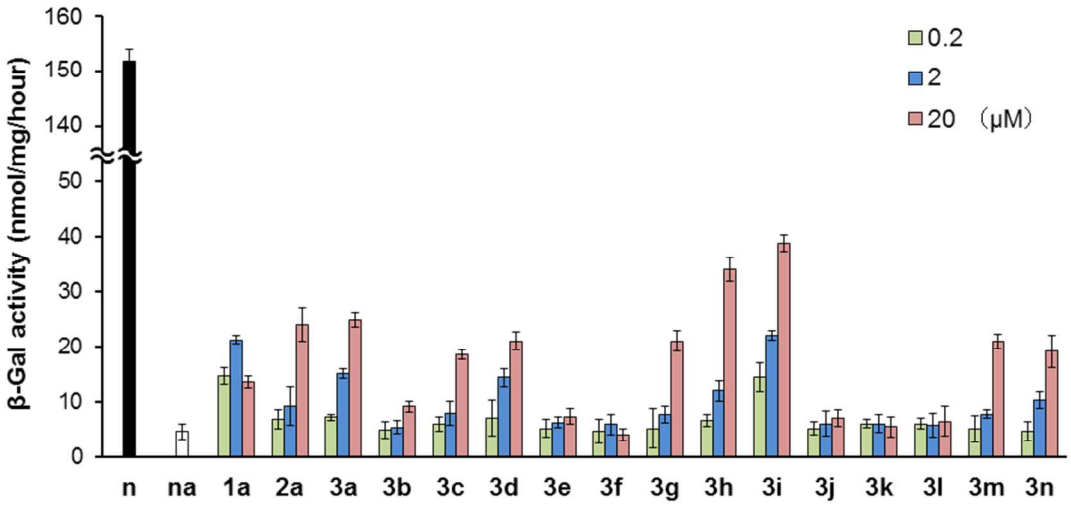

n: normal human β-galactosidase. na: no added compounds.

Graph 1. Enzyme enhancement activity of NOEV•HCl (**1a**), 6-deoxy-NOEV•HCl (**2a**), and *N*-substituted (+)-conduramine F-4 derivatives (**3a-n**) with mutated R201C human β-galactosidase. Each bar represents the means±S.E.M. of three independent assays performed in triplicate.

effects on the enhancement activity, but phenethylamine (**3n**) was superior to benzylamine (**3j**).

The inhibitory activity of a compound against an enzyme does not correlate directly with its chaperone activity. However, the IC_{50} values of the derivatives showing good enhancement activity ranged from approximately 10 μM to 100 μM. Future *in vivo* studies will determine whether IC_{50} values ranging from 10~100 μM are acceptable. In addition, most of the derivatives (**3a-n**) showed inhibitory activities against bovine liver β-galactosidase in a similar fashion to those for human β-galactosidase while also demonstrating cross-inhibition for almond β-glucosidase (Supplementary Information, Table S1). However, this cross-inhibition was probably resulted from the loose substrate specificity of the almond glucosidase. We tested the inhibition of human β-glucosidase and human α-galactosidase by the derivatives. As a result, all of the conduramine derivatives (**3a-n**) and also **1a** and **2a** did not inhibit those glycosidases (IC_{50} >1 mM). Thus, both of the conduramine and valienamine derivatives showed high specificity to human β-galactosidase.

Cytotoxicity

Considering therapeutic application of the conduramine derivatives, their cytotoxic effects on human fibroblasts were investigated by lactate dehydrogenase (LDH) assay (Supplementary Information, Graph S1). The highest LDH activity (54%), namely cytotoxicity, was shown by *n*-decyl conduramine derivative (**3e**) at 200 μM, and **3e** at 20 μM also showed modest activity (24%). The cytotoxicity decreased as the length of aliphatic chain shortened; *n*-octyl derivative (**3a**) demonstrated modest activity (25%) at 200 μM whereas *n*-hexyl (**3d**), *n*-pentyl (**3c**), and *n*-butyl (**3b**) showed low activity (<10%) at 200 μM. This suggested that strong amphiphilic properties of the compounds with long alkyl chains caused cytotoxic effect on human fibroblasts. The LDH activities of other derivatives with *N*-branched chains at 200 μM were low (<10%). In addition, except **3e**, almost all of the derivatives showed very small cytotoxicity at 20 μM. The LDH activity of the most effective chaperone compound **3i** was 3.8% at 200 μM and 0.7% at 20 μM, smaller than that of parent **1a** (7.5% at 200 μM and 1.1% at 20 μM).

Conclusions

In summary, we have studied *N*-substituted (+)-conduramine F-4 derivatives as novel PCs for G_{M1}-gangliosidosis. Conduramine was derived from the lead chaperone NOEV based on intentional structural modification. Removing the 5-hydroxymethyl group from NOEV could reduce its strong inhibitory activity, which should mask the enzyme enhancement. The search for a suitable side chain demonstrated that the 2-branched alkylamino group was a preferable chaperone compound for the R201C mutant β-galactosidase. This initial study presented here provided the evidence, at least in part, that these series of compounds as novel potential chaperones for human β-galactosidase deficiency. Further therapeutic study with animal models would be necessary to explore the ability of the compounds to restore

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trafficking the mutant protein into the lysosome, to reduce the substrate burden and to prevent neurological deterioration in the next study. Moreover, the present structural modification strategy could also help the development of novel and potent chaperones for other lysosomal storage diseases.

Currently, the parent NOEV might be replaced by potent *N*-substituted conduramine F-4 derivatives, which are conveniently synthesized from (+)-*proto*-quercitol. Further studies utilizing chiral conduramine derivatives would deepen our insight into biochemically interesting aminocyclitols. In addition, our initial studies suggested that the chaperone effects were mutation specific[6e,7] and many mutants including common mutations such as R201C are currently listed in β-galactosidase for G_{M1}-gangliosidosis. Therefore, examining the enhanced activity spectrum of **3i** is particularly important for future studies. Additional computational calculations and X-ray structure analyses of co-crystallized species will reveal the fine interactions between the enzyme and the compounds with atomic precision. We are also interested in preparing and evaluating the *N*-cyclohexylmethyl derivatives of valienamines.

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† Electronic supplementary information (ESI) available: Experimental protocols, IC_{50} values toward commercially available glycosidases, LDH assay, and copies of NMR spectra. See DOI: 10.1039/c000000x/

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The development of chemical chaperones to decrease the inhibitory activity while increasing the enzyme enhancement activity.