Development and Medical Application of Unsaturated Carbaglycosylamine Glycosidase Inhibitors

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Abstract: This article reviews synthesis and structures of carbaglycosylamines, a group of carbocyclic sugar analogues. Some unsaturated derivatives are known to be potent glycosidase inhibitors. Among them, *N*-octyl-4-epi-β-valienamine as a lysosomal β-galactosidase inhibitor is currently undergoing a new molecular therapeutic trial (chemical chaperone therapy) for control of the human β-galactosidase deficiency disorder, G_{M1}-gangliosidosis.

Key Words: Carbasugars, aminocyclitols, 5a-carbaglycosylamines, glycosidase inhibitors, chemical chaperone therapy, lysosomal disease, G_{M1}-gangliosidosis.

1. INTRODUCTION

Inhibition of glycosidases may be useful for treatment of diseases [1] such as diabetes, viral and bacterial infections, and inflammation. Among currently important glycosidase inhibitors, validamycin A (**1**) [2] and acarbose (**2**) [3], widely used to control sheath bright of rice plant and to treat diabetes, respectively, feature the same unsaturated branched-chain aminocyclitol, valienamine [4] (**4α**), with a glycoside-like N-linked bond (Fig. **1**). Other related compounds are components of validamycins: validamine (**3α**) and valiolamine [5] (**5α**). They belong to carbasugars [6], carbocyclic analogues of glycofuranoses and pyranoses, where the ring-oxygen atoms are replaced with carbon atoms. The valienamine-portions of **1** and **2** have been shown to play roles by structural mimicking of transition states of glucopyranose residues during hydrolysis of glucosides [6] (Fig. **2**), binding to the active sites of enzymes. Compounds **3α**–**5α** themselves possess more or less notable inhibitory activity toward glycohydrolases. Actually, further development of strong and specific α-glucosidase inhibitors has been carried out extensively through their chemical modification, leading to discovery of voglibose [7], a clinically important medicine for treatment of diabetes, fully compatible with acarbose (**2**) (Fig. **3**). Since then, unfortunately, only very few studies have so far been directed toward these compounds, compared with those on aza sugar glycosidase inhibitors, viz. 1-deoxynojirimycin (DNJ) and related compounds. This situation has thus stimulated our interest in identifying new type of carbaglycosylamine glycosidase inhibitors as therapeutic agents, taking advantage of their structural and biochemical features.

Surprisingly some of these *in vitro* inhibitors were found to induce remarkable expression of mutant lysosomal

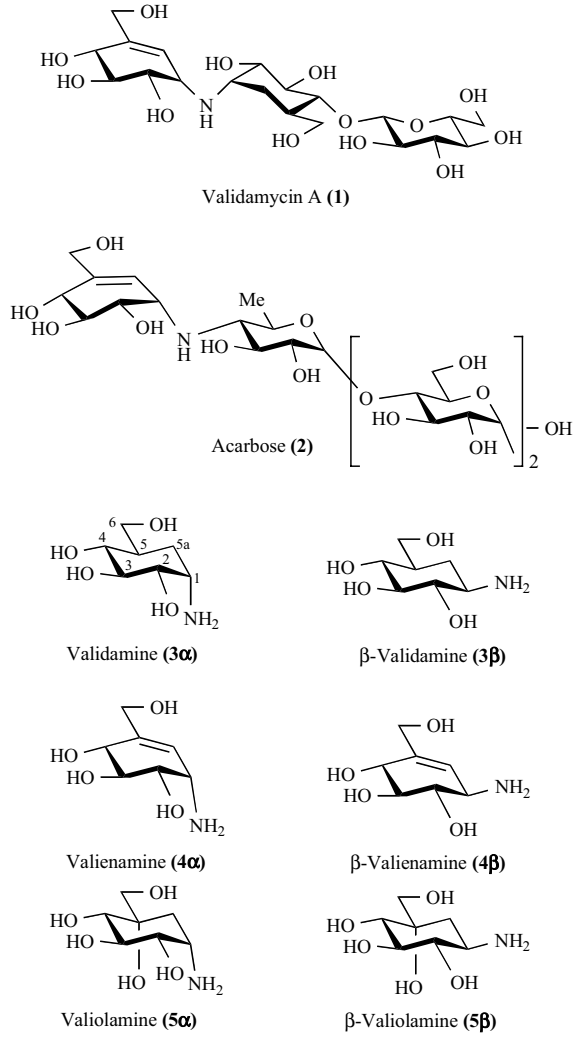

Fig. (1). Validamycin A and acarbose, and some naturally occurring 5a-carbaglycosylamines **3α**–**5α** and the 1-epimers (β-anomers) **3β**–**5β** of biological interest.

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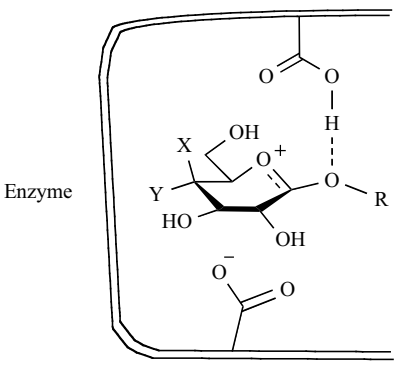
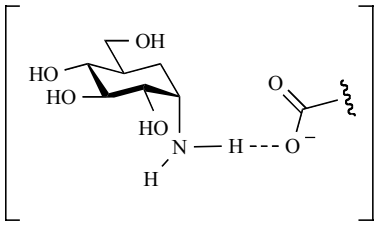
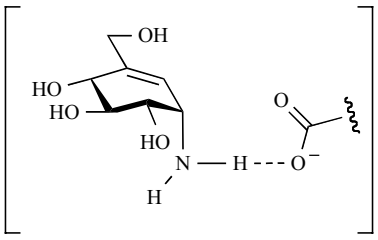


Putative transition-state of hydrolysis of glucopyranosides (X = H, Y = OH) and galactopyranosides (X = OH, Y = H)



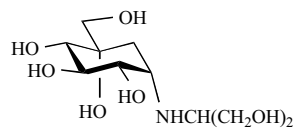
Ground-state α-glucosidase inihibitor: validamine **(3α)**



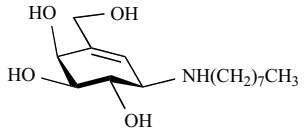
Transition-state α-glucosidase inhibitor: valienamine **(4α)**

Fig. (2). Hypothetical transition states for the cleavage of glycosidic bonds and binding of glycosidase inhibitors of 5a-carbaglycosylamine type to active sites of enzymes.

enzymes and to correct pathological intracytoplasmic storage of substrates in some human disorders. We therefore started a systematic survey of compounds exhibiting biological activity of this type, and found *N*-octyl-4-epi-β-valienamine (NOEV) to be a good candidate for a new molecular therapeutic approach (chemical chaperone therapy), particularly to G_{M1}-gangliosidosis caused by β-galactosidase deficiency [8,9].



Voglibose



NOEV

N-Octyl-4-epi-β-valienamine

Fig. (3). Voglibose and NOEV.

2. DESIGN AND SYNTHESIS OF CARBAGLYCOSYLAMINE GLYCOSIDASE INHIBITORS

2.1. Structure-Inhibitory Activity Relationships

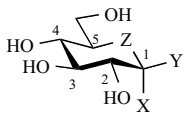
The active core of validamycin A (**1**), dicabadisaccharide validoxylamine A [10], resembles the substrate trehalose, the structure of which is thought to adopt a transition state for hydrolysis by trehalase. The unsaturated derivative of N-linked 5a,5a'-dicarba-α,α-trehalose, composed of **3α** and **4α**, possesses strong inhibitory activity against trehalase [11]. On the other hand, the active core of α-amylase inhibitor **2** is thought to be a maltose-type N-linked pseudodisaccharide, composed of **4α** and 4-amino-4,6-dideoxy-D-glucopyranose.

Accordingly, by analogy with structure and inhibitory activity relationships deduced by consideration of the active compounds (**1**, **2**, and **3α–5α**), the corresponding 5a-carbaglycosylamines (**3** and **6α,β–14α,β**) and analogues with naturally common β-*gluco*, α,β-*galacto*, α,β-*manno*, and α,β-*fuco*-configurations [12] (Fig. **4**), have been nominated as leads for development of new biological active compounds such as enzyme-inhibitors of structurally related glycosidases and/or glycosyltransferases.

2.2. Chemical Modification of Methyl Acarviosin

Acarviosin (**15a**), the active core of acarbose (**2**) [13], is a very potent α-glucosidase inhibitor, with activity attributable to structural features resembling the transition state associated with hydrolysis of maltose. We have attempted to ascertain the relationship between the stereochemistry of **3α** and inhibitory activity against α-glucosidase. Acarviosin was chosen as a suitable lead for this purpose, chemical modification of its aglycone portion being first carried out, giving the 6-hydroxyl derivative **15b** and two methyl ethers **15c–d** [14] (Fig. **5**). Decrease of inhibitory potency was observed for all derivatives prepared. However the 1,6-anhydride **16a** derived unintentionally by base-treatment of the 6-tosylate derivative of **15b** was found to possess activity as high as **15a** or greater [15]. Three dehydroxy derivatives **16b–d**, obtained by consecutive removal of the hydroxyl groups of the anhydroglucopyranose residue, were found to have increased activity relative to decrease of hydrophilicity of the aglycone. These results suggested that improvement of the activity might be readily achieved by incorporation of simple hydrophobic functions of alkyl and phenylalkyl groups into the aglycone of **3α**. Secondly, the 2'-epimer **17** of methyl α-acarviosine was prepared [16], its unsaturated aminocyclitol part structurally in accord with an α-mannopyranose residue. By analogy, pseudodisaccharide **17** was expected to show inhibitory activity toward α-mannosidase, and was finally shown to be a mild α-mannosidase inhibitor. On the same basis, two acarviosin analogues, the 1-epimer (**18a**) and its 2-acetamido-2-deoxy derivative (**18b**), were thus designed and synthesized [17]. Structures of their unsaturated aminocyclitol moieties corresponded to the postulated transition-state mimics of β-D-glucose and *N*-acetyl-β-D-glucosamine residues, respectively, in hydrolysis of the respective glycosides. However, disappointingly, the pseudodisaccharides **18a,b** did not possess any inhibitory activity toward the respective commercially available β-glucosidase and chitinase forms.

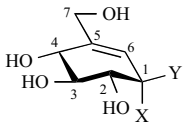
Glc



Validamine

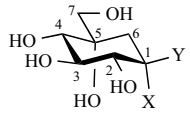
Glucopyranosylamine: Z = O

Validamine **(3α,β)**: Z = CH_2



(4α,β)

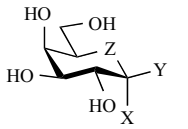
Valienamine



(5α,β)

Valiolamine

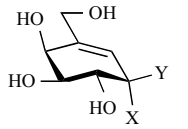
Gal



4-Epivalidamine

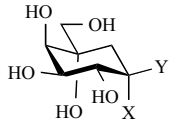
Galactopyranosylamine: Z = O

4-Epivalidamine **(6α,β)**: Z = CH_2



(9α,β)

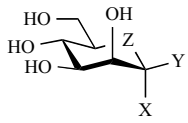
4-Epivalienamine



(10α,β)

4-Epivaliolamine

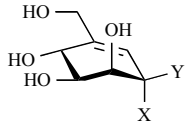
Man



2-Epivalidamine

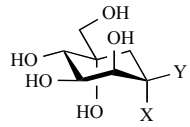
Mannopyranosylamine: Z = O

2-Epivalidamine **(7α,β)**: Z = CH_2



(11α,β)

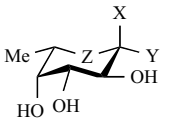
2-Epivalienamine



(12α,β)

2-Epivaliolamine

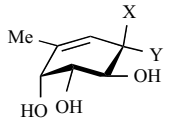
Fuc



7-Deoxy-4-epivalidamine

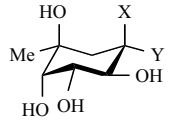
Fucopyranosylamine: Z = O

7-Deoxy-4-epivalidamine **(8α,β)**: Z = CH_2



(13α,β)

7-Deoxy-4-epivalienamine



(14α,β)

7-Deoxy-4-epivaliolamine

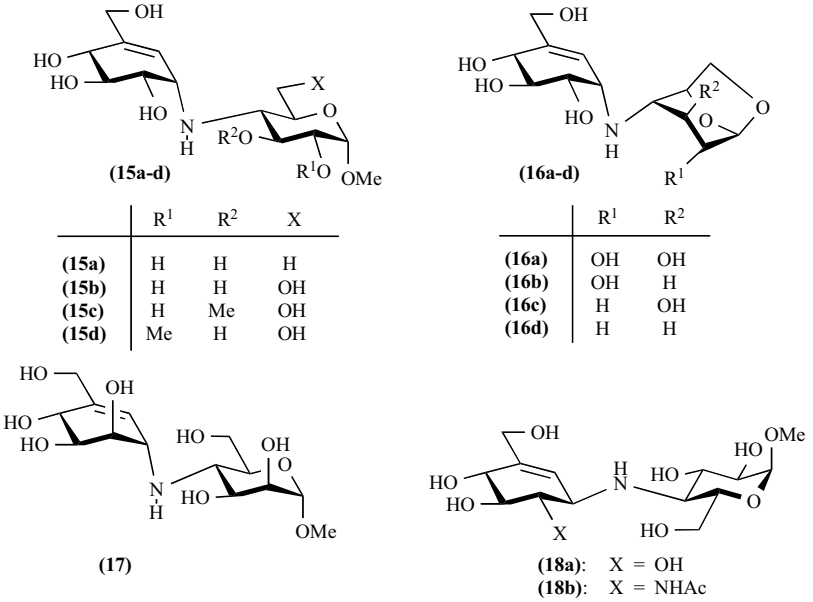
Fig. (4). Biologically interesting carbaglycosylamines and derivatives (α: X = NH_2, Y = H; β: X = H, Y = NH_2).

2.3. Design and Synthesis of Carbaglycosylceramides

In 1991, some glycosylamides **19a** were demonstrated [18] to possess significant potential as immunomodulators of responses to *Escherichia coli* (Fig. **6**). We therefore became interested in ready preparation of glycosylamide analogues, the sugar moieties being replaced with carbasugars. The resulting *N*-(5a-carba-β-glucopyranosyl)-*N*-octadecyldodecanamide (**19b**) and related carbasugar analogues [19] possessed similar biological activity to true sugar congeners and we therefore anticipated development of biologically interesting carbasugar derivatives for basic research into glycolipids. Referring to natural occurring glucosyl and galactosylceramides, we first elaborated a total synthesis of 5a-carbaglucosylceramides **20a–c**, where 5a-carba-β-D-glucopyranose residues were bonded to ceramide-chains through ether, thioether, and imino linkages, respectively [20]. Among the carbaglycosylceramides obtained, the N-linked analogue **20c**, together with the galactosyl analogue **21** later provided, were observed to possess weak but distinct inhibitory activity against the corresponding gluco and galactocerebrosidases (mouse liver). Encouraged by these results, incorporation of unsaturated-bonds into the carbasugar residues was attempted in the hope of increasing their potential and selectivity. Carbaglycosylceramide analogues **22a** and **22b**, featuring valienamine and its 4-epimer, were thus prepared and demonstrated to have very potent and specific inhibitory activity (IC_{50} 0.3 and 2.7 μM) toward the respective gluco and galactocerebrosidases [21] (Fig. **6**). The configuration at C-4 of the valienamine moiety was found to be a critical point for differential recognition by the respective enzymes, as with gluco and galactopyranose residues.

2.4. Modification of Carbaglycosylceramides: Synthesis of Potent β-Gluco and Galactocerebrosidase Inhibitors

The above lead compounds thus made possible our aim of structurally more simple carbaglycosylceramide analogues with high potency.



	R^1	R^2	X
(15a)	H	H	H
(15b)	H	H	OH
(15c)	H	Me	OH
(15d)	Me	H	OH

	R^1	R^2
(16a)	OH	OH
(16b)	OH	H
(16c)	H	OH
(16d)	H	H

Fig. (5). Chemical modification of methyl acarviosin (**15a**).

Several *N*-alkyl-β-valienamines **23a–d**,**f**,**h** were designed and prepared systematically to determine, if possible, any relationship between chain length of aliphatic functions and inhibitory activity [22] (Fig. **6**). Actually, the *N*-octyl derivative **23c** was found to possess about 10-fold greater potency than the parent carbaglucosylceramide **22a**, indicating the possibility of replacing the ceramide moiety by simple hydrophobic aliphatic chains without affecting the activity (Table **1**). Similarly, some double-strand type *N*,*N*-dialkyl derivatives **24a–g**, prepared by reduction of the corresponding

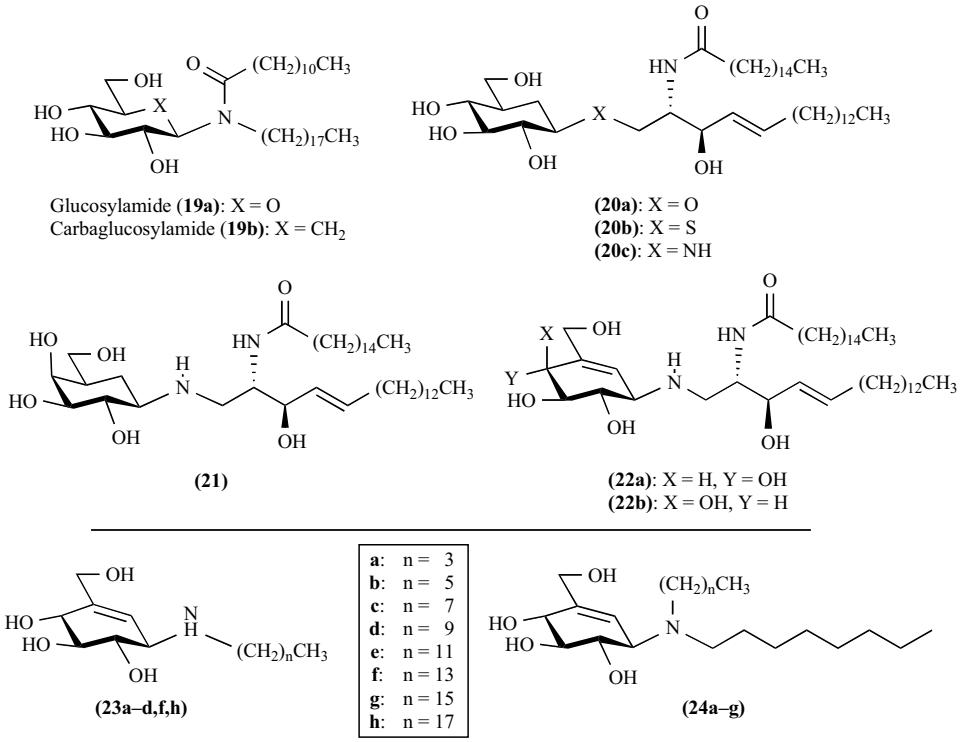


Fig. (6). 5a-Carbaglucosyl and galactosylceramides, and some *N*-alkyl- and *N*,*N*-dialkyl-β-valienamines.

Table 1. Inhibitory Activity (IC_{50}, μM) of Some *N*-Alkyl-β-valienamine Homologues Against α-Glucosidase (Baker's Yeast) and β-Glucocerebrosidase (Mouse Liver)

Compound	α-Glucosidase	Glucocerebrosidase
3α	100	NI
3β	100	NI
22a	NT	0.3
23a	NI	11
23b	50	0.3
23c	17	0.03
23d	NT	0.07
23f	NT	0.12
23h	NI	0.3

NI: No inhibition ($<10^{-3}$ M); NT: Not tested.

alkylamide derivatives, were also revealed to be as potent as their parents [23]. However, we were rather disappointed by the unexpected fact that *N*-octyl-4-epi-β-valienamine (**26b**) [8] prepared at the same time did not show any significant improvement in potency toward galactocerebrosidase. On the other hand, interest in studying actions of inhibitors toward glycosidases and glycosyltransferases prompted us to provide hybrid-inhibitors with functions targeting inhibition of both glucosylceramide synthase and glucocerebrosidase under controlled conditions. Thus, PDMP (IC_{50} 23 μM, mouse liver) [24] was chosen as a potent synthase-inhibitor, and coupling of all stereoisomers of PDMP with carbaglycosylamines **3β**, **4α** and **4β** *via* N-linkages afforded four respective *N*-(2-decyamino-3-hydroxy-3-phenylprop-1-yl)-β-valienamines **25a–c** [25] (Fig. **7**). Interestingly, all coupled compounds were shown to be strong β-glucocerebrosidase inhibitors, while the PDMP moiety abrogated activity against

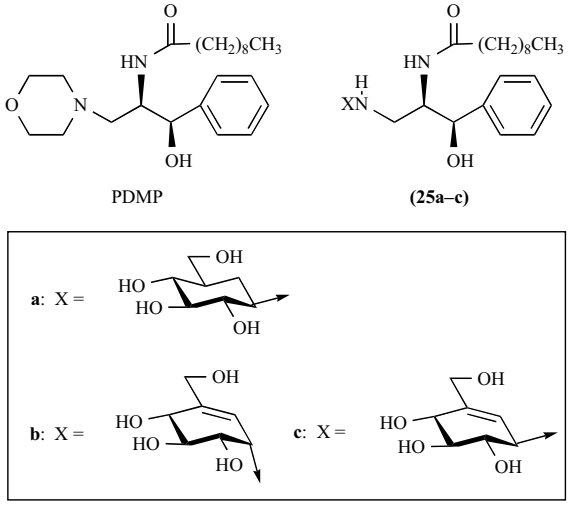


Fig. (7). The glucosylceramide synthase inhibitor PDMP and its hybrids composed of 5a-carbaglucosylamines.

glucosylceramide synthase: e.g. **25c** corresponding to the (2*R*,3*R*)-isomer of PDMP possessed inhibitory activity IC_{50} 0.7 μM against glucocerebrosidase (mouse liver).

After almost two years, strong and specific inhibitory activity (IC_{50} 0.3 μM, human G_{M1} β-galactosidase) was observed for *N*-octyl-4-epi-β-valienamine (**26b**), which was then selected as a new candidate for chemical chaperon therapy of human genetic diseases [9]. Taking advantage of the available data, we have concentrated our efforts on developing effective synthetic routes to 4-epi-β-valienamine derivatives in order to facilitate screening of as many homologous compounds as possible [26] (Fig. **8**). Inhibitory assay results for four such homologues **26a–d** thus prepared are listed in Table **2**.

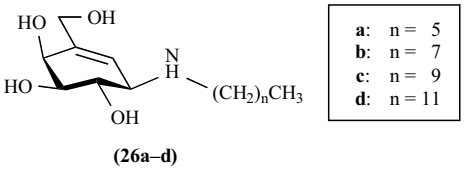


Fig. (8). Some *N*-alkyl-4-epi-β-valienamines.

2.5. Structure-Inhibitory Activity Relationships of Unsaturated Carbaglycosylamine Glycosidase Inhibitors

Free glycosylamines as well as *N*-alkyl derivatives, namely, simple N-glycosides, are often chemically unstable in aqueous solution, undergoing mutarotation accompanied by hydrolytic cleavage to give rise to equilibrium mixtures of sugars and ammonia or amines [27]. Since carbaglycosylamines are comparatively stable polyhydroxylated (hydroxymethyl)cyclohexylamines, they might be expected to play roles as non-hydrolyzable mimics of glycopyranosylamines of biological interest. Taking advantage of their chemical and biochemical features, preferential utilization as active lead compounds in biological systems and/or building blocks of complex glycoconjugate molecules has been targeted. Moreover, in addition to the chemical stability, efficient modification of the stereochemical nature of carbaglycosylamines may be achieved by unsaturation at C-5 and C-5a, hydroxylation at C-5 and/or C-5a, and so on (as seen in Fig. **4**), without appreciably altering their characteristic features as close mimics of particular hexopyranoses, possibly leading to improvement of biological potential.

As shown by the inhibitory activity of 4-epi-β-valienamines (**9β**) and the *N*-alkyl derivatives **23a–d** (Table **2**), inclusion of hydrophobic *N*-alkyl chains into **9β**, is very important for improving its potential significantly, which would suggest that, in attempts to develop new such analogues and mimics, additional modification of their physicochemical nature might be advisable, for instance for the purpose of generating strong binding to active sites of enzymes or peptides. With **4β** and **9β**, our present knowledge suggests that a simple eight-carbon chain may be sufficient.

2.6. Novel Synthetic Routes to Carbaglycosylamines of Biological Interest

Valienamines (**4α,β**) were first totally synthesized [28] from the conjugate alkadiene (**33**), derived from the *endo*-adduct **27**(***S***) of furan and acrylic acid (Fig. **9**). Di-*O*-iso-

Table 2. Inhibitory Activity (IC_{50}, μM) of 4-Epi-α- and β-valienamines 9α,β, and Some *N*-Substituted Derivatives 26a-d Against Four Glycosidases

Compound	α-Glucosidase[a]	β-Galactosidase[b]	β-Glucosidase[c]	α-Mannosidase[d]
9α	56	NI	NI	370
9β	12	NI	NI	190
26a	207	2.3	1.2	NI
26b	3.1	0.87	3.1	NI
26c	1.9	0.13	2.5	NI
26d	4.4	0.01	0.87	NI
DMJ	NT	NT	NT	150

NI: No Inhibition (>10^{-3} M); NT: Not tested.

[a]Green Coffee Beans; [b]Bovine liver; [c]Almonds; [d]Jack Beans.

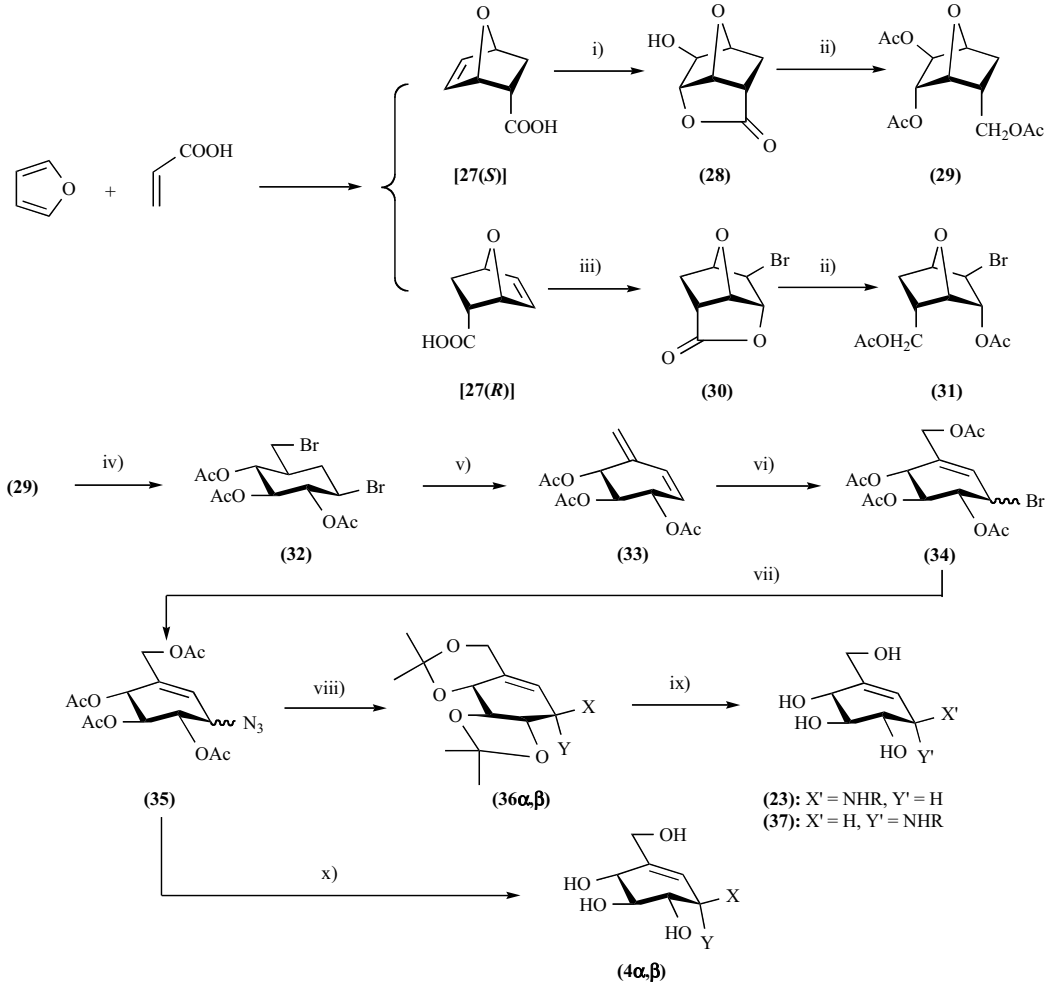


Fig. (9). Synthesis of 5a-carbaglycopyranosylamines with β-*gluco* configurations, starting from the Diels-Alder *endo*-adduct of furan and acrylic acid (α: X = H, Y = NH_2; β: X = NH_2, Y = H). *Conditions and reagents*: i) H_2O_2, HCOOH; ii) $LiAlH_4$/THF; Ac_2O/Pyr; iii) Br_2, Na-HCO_3/H_2O; iv) 15% HBr/AcOH, 80 °C; v) DBU/toluene; vi) Br_2, AIBN, AcOH; AcONa, aq. MCS; vii) NaN_3/aq. DMF; viii) NaOMe/MeOH; DMP, *p*-TsOH/DMF; H_2S, or Ph_3P/aq. *p*-dioxane; ix) RCOCl/Pyr; $LiAlH_4$/THF; aq.AcOH; acidic resin treatment; x) NaOMe/MeOH; H_2S, aq. DMF.

propylidene derivatives (**36α**,**β**) of the isomeric valienamines **4α**,**β**, obtained from the azides **35** in the course of studies on total synthesis of validamycins [29], acarbose [30], and methyl epiacarviosins [15], have effectively been subjected to N-alkylation processes for production of derivatives **23a**–**h** of **4α**. On routine treatment with alkanoyl chloride in pyridine, the amine **36β** was readily converted to the corresponding *N*-alkanoyl derivative. Six derivatives thus obtained were reduced with lithium aluminum hydride in THF, followed by acid hydrolysis, to afford the *N*-alkyl-β-valienamines (**23a**–**d**,**f**,**h**) [22] in 80–85% yields. For example, *N*-octyl-4-epi-β-valienamine (**26b**) was first obtained by epimerization at C-4 of *N*-octyl-β-valienamine (**23c**) *via* multi-step reactions [8]. Thus, selective reduction of the 4-keto derivative, provided by oxidation of the 4-OH unprotected derivative of **23c**, could be carried out under careful conditions to improve acceptable selectivity for the 4-epimer.

Production of a large quantity of NOEVs is now needed for further development of possible oral medicines applicable for chaperone therapy of genetic diseases caused by lysosomal accumulation. Versatile key compounds can be envisaged for combinatrial preparation of a homologous series of *N*-alkyl-4-epi-β-valienamines [31] (Fig. **10**). Thus, the 3-epimeric alkadiene **40** of **33** was designed and synthesized by conventional dehydrobromination of the dibromide **39** derived from the tribromide **38**. The 2,3-*O*-isopropylidene acetate **41** was converted into the dibromides, which were subjected without isolation to selective acetolysis at the primary site to give an isomeric mixture **42** of the reactive allylic bromides. The mixture was found to offer convenient precursors for preparation of a number of *N*-alkyl-4-epi-β-valienamine homologues. Thus, the α-allyl bromide was considered to be attacked by alkylamine in a S_N2 fashion to mainly give β-amino compound, while, on the other hand, the β-allyl bromide might produce a similar mixture of products through neighboring participation with the 3-acetoxyl group at C-4 to form a 3,4-acetoxonium ion, followed by upside attack of nucleophiles. The mixture **42** readily undergoes substitution reactions with nucleophiles, such as azide anions, alkyl and phenylalkyl amines, etc. to afford various *N*-substituted β-epivalienamines selectively. Since general synthetic intermediates **33** and **40** may also be obtainable starting from readily available biomaterial containing glucose, galactose, etc., we should pay particular attention to what kind of OH-protecting groups may be employed in individual reaction sequences.

Recently, bio-oxidation of (–)-*vibo*-quercitol derived by bioconversion [32] of *myo*-inositol gave a quantity of (–)-2-deoxy-*scyllo*-inosose (**45**) [33]. This has already been employed to allow establishment of a new convenient route for carbaglycosylamines through crystalline spiro epoxy **46**, methylene compounds **47**, and the alkadiene **33** [34] (Fig. **11**).

VALIENAMINES AS CHEMICAL CHAPERONES FOR MEDICAL APPLICATIONS

3.1. Historical Background

A large number of inherited diseases have been identified and registered during the past 40 years [35]. Many of them

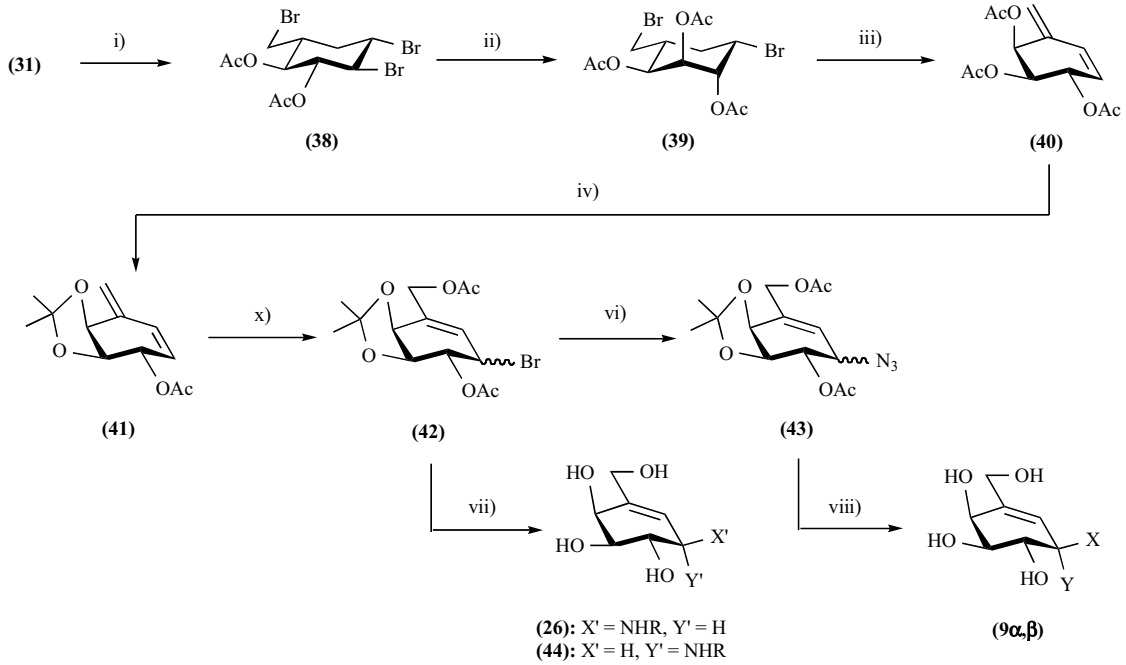


Fig. (10). Convenient synthesis of 5a-carbaglycopyranosylamines with β-*galacto* configuration, starting from the Diels-Alder *endo*-adduct of furan and acrylic acid (α: X = H, Y = NH_2; β: X = NH_2, Y = H). *Conditions and reagents*: i) 15% HBr/AcOH, 80 °C; ii) NaOMe/MeOH; 1% H_2SO_4/aq. acetone; Ac_2O/Pyr; iii) DBU/toluene, 60 °C; iv) NaOMe/MeOH; DMP, *p*-TsOH/DMF; Ac_2O/Pyr; v) Br_2, AIBN, toluene; AcONa/MCS; Ac_2O/Pyr; vi) NaN_3/DMF; vii) RNH_2/*i*-PrOH; aq. AcOH; 4 M HCl; acidic resin treatment; viii) NaOMe/MeOH; H_2S or Ph_3P/aq. *p*-dioxane; acidic resin treatment.

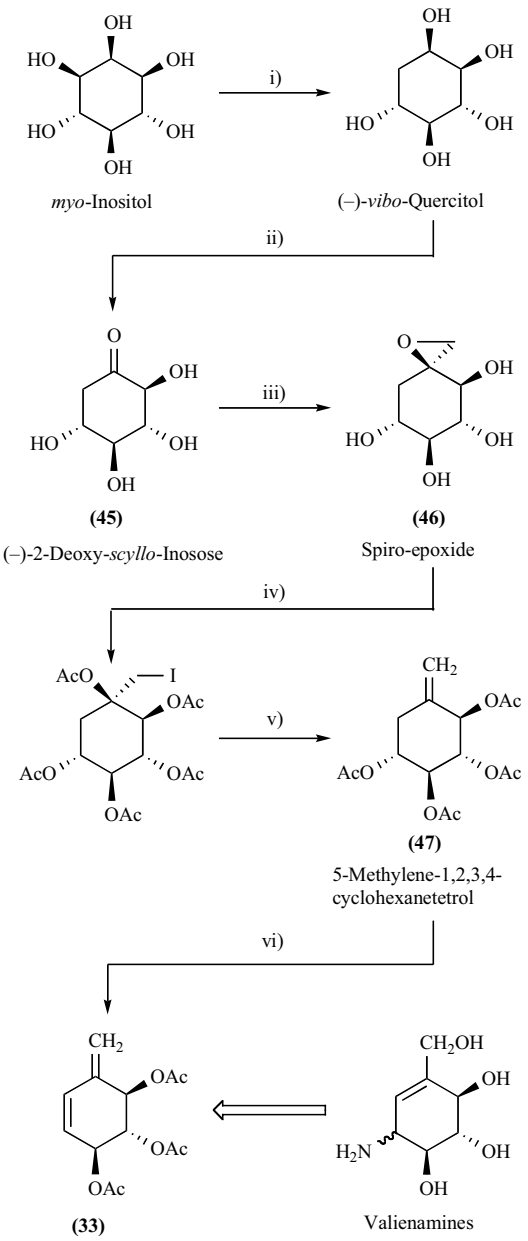


Fig. (11). Convenient synthesis of 5a-carbaglycopyranosylamines starting from optically active deoxyinositol produced by bioconversion of *myo*-inositol. *Conditions and reagents*: i) Bioconversion; ii) Bio-oxidation; iii) CH_3N_2/MeOH, Et_2O; iv) HI, AcOH; v) Zn, AcOH; vi) Br_2/AcOH; Zn/toluene.

are expressed clinically as progressive central nervous system diseases in children (neurodegenerative diseases). Unfortunately, molecular approaches have not yet been successful for prevention or cure of brain pathology in these diseases, although secondary brain dysfunctions caused by metabolic abnormalities in other tissues are currently available for clinical practice, such as with phenylketonuria, a hepatic enzyme disease treated by low phenylalanine diet, and congenital hypothyroidism, a hormone deficiency treated by thyroid hormone supplementation.

For more than 15 years we have performed molecular analyses of β-galactosidase deficiency disorders (β-galactosidosis) caused by various mutations of the gene coding for a lysosomal enzyme β-galactosidase [36]. In this article we define the term β-galactosidase as the enzyme encoded by a gene on chromosome 3 (GLB1) catalyzing hydrolysis of ganglioside G_{M1} (G_{M1} galactosidase). Another enzyme catalyzing hydrolysis of galactocerebroside (galactosylceramide) encoded by a different gene on chromosome 14 (GALC) will be described as galactocerebrosidase in this article. Clinical expression of β-galactosidase deficiency is variable, with a wide range of ages of onset (from infancy to adulthood), involving mainly the central nervous system (G_{M1}-gangliosidosis) or the skeletal system (Morquio B disease). After cloning cDNA for this enzyme [37], we performed extensive mutation analysis [38,39].

At present only symptomatic therapy is available for human β-galactosidosis patients and the reported results of animal experiments have been disappointing. For example, allogeneic bone marrow transplantation did not modify the subsequent clinical course or cerebral enzyme activity in a Portuguese water dog affected with G_{M1}-gangliosidosis [40]. Amniotic tissue transplantation was not effective in a patient with Morquio B disease [41]. Enzyme replacement therapy conducted for Gaucher disease and other lysosomal storage diseases is not available at present for β-galactosidosis. An experiment to inhibit G_{M1} synthesis resulted in reduction of the G_{M1} content in the mouse brain, but not G_{A1} (a derivative of G_{M1}) [42]. Clinical effects were not confirmed in this study. Clearly more evaluation is necessary of therapeutic trials in this direction.

We earlier established that mutant proteins of another lysosomal enzyme (α-galactosidase A) did not exhibit catalytic activity simply because of molecular instability in culture cells from patients with hereditary deficiency of this enzyme (Fabry disease) [43]. Subsequently the unstable protein was found to have a defect in molecular folding, resulting in rapid degradation after biosynthesis [44]. We therefore started trials to stabilize the mutant protein in living cells, and, in fact, galactose (the α-linked terminal sugar of the carbohydrate branch in the substrate molecule) was an excellent inducer to express the mutant α-galactosidase A gene in cultured lymphoblasts at high concentrations in the culture medium, although the sugar was rapidly catabolized after being taken up by the culture cells [45]. We searched for more potent inducers of mutant gene expression among commercially available chemical compounds structurally similar to galactose and showed 1-deoxygalactonojirimycin (DGJ) to be the best candidate for a new molecular approach to Fabry disease therapy [46].

Simultaneously we developed a new disease model knockout (KO) mouse, a counterpart of human G_{M1}-ganglio-sidosis, using a genetic engineering technique of homologous recombination for specific destruction of the β-galactosidase gene [47,48]. This was then employed to survey various synthetic compounds for therapeutic potential. We thereby identified a number of valienamine derivatives exerting the same activities as human enzymes with regard to competitive inhibition *in vitro* and molecular stabilization and catalytic activity expression *in situ* [8,21-23,31] (Fig. **12**).

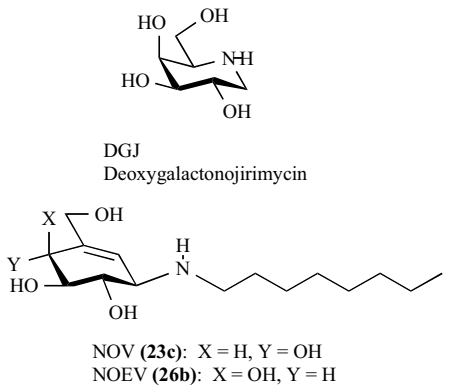


Fig. (12). 1-Deoxygalactonojirimycin (DGJ), and *N*-octyl-β-valienamine (NOV) and 4-epi-β-valienamine (NOEV).

After preliminary screening, two compounds were chosen as possible candidates for chemical chaperone therapy: *N*-octyl-4-epi-β-valienamine (NOEV) for β-galactosidase deficiency disorders (particularly G_{M1}-gangliosidosis) [9], and *N*-octyl-β-valienamine (NOV) for β-glucosidase deficiency disorders (Gaucher disease) [49].

3.2. Concept of Chaperone Therapy

In general, molecular events in hereditary enzyme deficiency disorders may be expected to in value charges in various processes, like biosynthesis, intracellular turnover, and catalytic function. Three possible causes of defects in mutant gene expression in somatic cells can be listed: (1) biosynthetic defects; (2) extremely low or completely deficient catalytic activity of the expressed mutant protein; and (3) expression of unstable mutant protein with normal or near-normal catalytic activity.

We tested these possibilities in Fabry disease in our experiments described above, and found a surprisingly high frequency of the third possibility for mutant α-galactosidase A proteins. They were unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus, and became rapidly degraded without appropriate molecular folding [43,44].

An exogenous substrate analogue compound of low molecular weight that inhibits an enzyme activity *in vitro* binds to the misfolded mutant lysosomal protein as a molecular chaperone in the endoplasmic reticulum/Golgi apparatus of cells, resulting in formation of a molecular complex at neutral pH. The catalytically active mutant gene is thereby stabilized, and the protein-chaperone complex is safely transported to the lysosome, where it dissociates under the acidic conditions, the mutant enzyme remains stabilized, and its catalytic function is expressed (Fig. **13**). We have already confirmed that this principle is valid for α-galactosidase A (Fabry disease), β-galactosidase (G_{M1}-gangliosidosis), and β-glucosidase (Gaucher disease).

The strategy depends on biological activity of chaperone compounds available for each enzyme. In a previous study, we had to add a high dose of galactose (up to 200 mM) to the culture medium of Fabry cells [45]. This is obviously unnatural and deleterious to the physiological function of living cells for long-term treatment, causing an extremely high osmotic pressure of the extracellular fluid, although a short-term human experiment demonstrated a positive therapeutic effect after high-dose intravenous galactose in one Fabry patient [50].

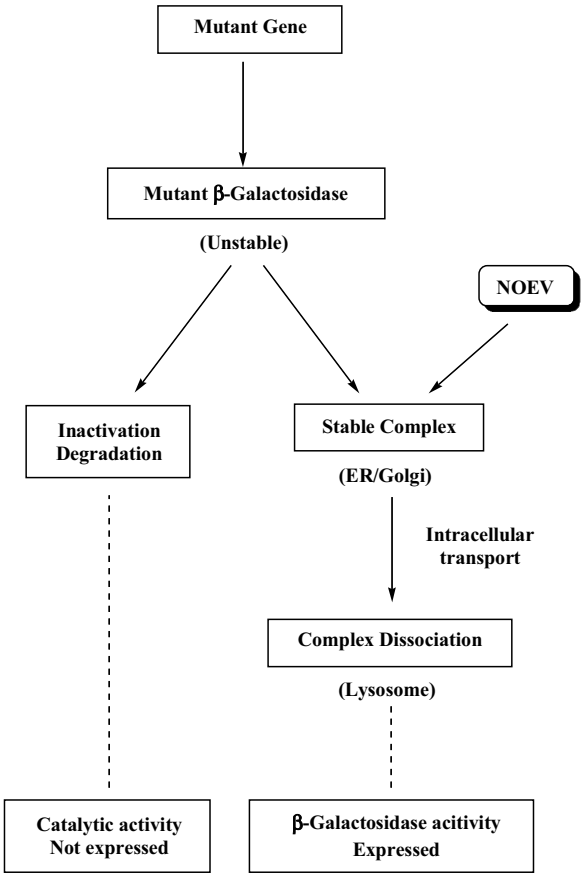


Fig. (13). Principle of chemical chaperone therapy for a β-galactosidase deficiency disorder (G_{M1}-gangliosidosis).

NOEV appears more efficient than DGJ for expression of mutant β-galactosidase activity in G_{M1}-gangliosidosis as compared to that for α-galactosidase A activity in Fabry disease [9,51]. Our calculations indicate that at least 10% of normal enzyme activity is necessary for washout of the storage substrate in lysosomal diseases. The age of onset in patients expressing enzyme activity above this level is theoretically beyond the human life span [Suzuki, unpublished data]. An accurate determination of intracellular chaperone concentrations is technically not feasible at present but we anticipate that the effective NOEV concentrations in human cells and animal tissues are much lower than the IC_{50} for this agent *in vitro*. In fact, the NOEV concentration effective in the culture medium for enhancement of mutant enzyme activity was the same as the IC_{50} in a recent study [52].

3.3. Physicochemical and Biological Characteristics of NOEV

NOEV is a potent inhibitor of lysosomal β-galactosidase *in vitro*. Its structure has been fully assigned by a combination of COSY, TOCSY, and HSQC NMR spectroscopy [9].

It is stable at room temperature, and freely soluble in methanol or DMSO. Solubility in water is limited up to 3–5 μM at room temperature, but the amine hydrochloride is easily soluble in water. The molecular weight is 287.40. The IC_{50} is 0.125 μM toward human β-galactosidase [52]. Addition of NOEV to the culture medium was found to restore mutant enzyme activity in cultured human or murine fibroblasts at low intracellular concentrations, resulting in a marked decrease of intracellular substrate storage [9].

The inhibitory effect of NOEV is much higher toward galactocerebrosidase than β-galactosidase (Fig. **14**). We therefore tried chaperone experiments on cultured fibroblasts from patients with Krabbe disease, caused by galactocerebrosidase deficiency [53]. However, enhancement of the deficient enzyme activity was not achieved under the same culture conditions as for β-galactosidase deficiency (G_{M1}-gangliosidosis). Since galactocerebrosidase is known to be unique for its physicochemical characteristics, intracellular transport, and expression of catalytic activity in somatic cells, a more sophisticated strategy may be necessary for realizing chaperone effects with this disease.

3.4. NOEV Effects on Cultured Human and Mouse Fibroblasts Expressing Mutant Human Genes

We observed heterogeneous responses to NOEV in human cells expressing mutant β-galactosidase [52], in line with results for mouse fibroblasts [9]. However, the degree of enhancement differed for some mutations between human and mouse cells. A common observation was a 5- to 10-fold increase for the R427Q mutation at 0.2 μM of NOEV in the culture medium; and a higher concentration (2 μM) was required for the R201C or R201H mutation for enhancement to the same degree [52].

About one-third of the cells from patients with G_{M1}-gangliosidosis responded to NOEV treatment. Almost all patients with juvenile G_{M1}-gangliosidosis, and some with infantile G_{M1}-gangliosidosis responded to a significantly greater extent. Equivalent or greater effects were achieved with NOEV at a 50-fold lower concentration than with DGJ or *N*-butyl-DGJ [51]. Addition of a ganglioside mixture to the culture medium resulted in a remarkable increase of intracellular G_{M1} in the cells expressing the mutation R201C causing juvenile G_{M1}-gangliosidosis and only a slight increase in the cells expressing the normal human gene. Incubation with NOEV significantly reduced G_{M1} storage in these cells [9].

3.5. Chaperone Therapy in Genetically Engineered G_{M1}-Gangliosidosis Model Mice

A transgenic (Tg) mouse, expressing the human R201C mutation that causes a mild type G_{M1}-gangliosidosis (R201C mouse) based on the KO background [9], was found to have very low β-galactosidase activity in the brain (about 4 % of the wild type activity). They exhibited an apparently normal clinical course for the first 7 months after birth, followed by slowly progressive neurological deterioration, with tremors and gait disturbance and death at 11-18 months of age due to malnutrition and emaciation (life span of normal mice 24-36 months). Neuropathology revealed vacuolated or ballooned neurons, less abundant than in the KO mouse brain [48,54]. Cytoplasmic storage materials were present in pyramidal neurons and brainstem motor neurons, but not in neurons in the other areas of the brain.

Short-term oral administration of NOEV to the R201C model mouse [9] resulted in significant enhancement of enzyme activity in all the tissues examined, including the central nervous system. Immunohistochemical staining revealed an increase in β-galactosidase activity and decrease in G_{M1} and G_{A1} storage. However, mass biochemical analysis did not show substrate reduction in the brain, probably because of the brief duration of treatment and only localized substrate accumulation at the early stage of the disease in this experiment. The compound NOEV was found in a significant amount in the central nervous system by mass spectrometric analysis, at 10% of the level in liver tissue after oral administration of the NOEV solution for 8-16 weeks [Kubo T, unpublished data].

3.6. NOEV Effect on Model Mice: Clinical Assessment

We have established an assessment system for brain function in G_{M1}-gangliosidosis mice [55]. This is a simple modification of neurological tests for human infants and young children, consisting of 11 test items mainly concern-

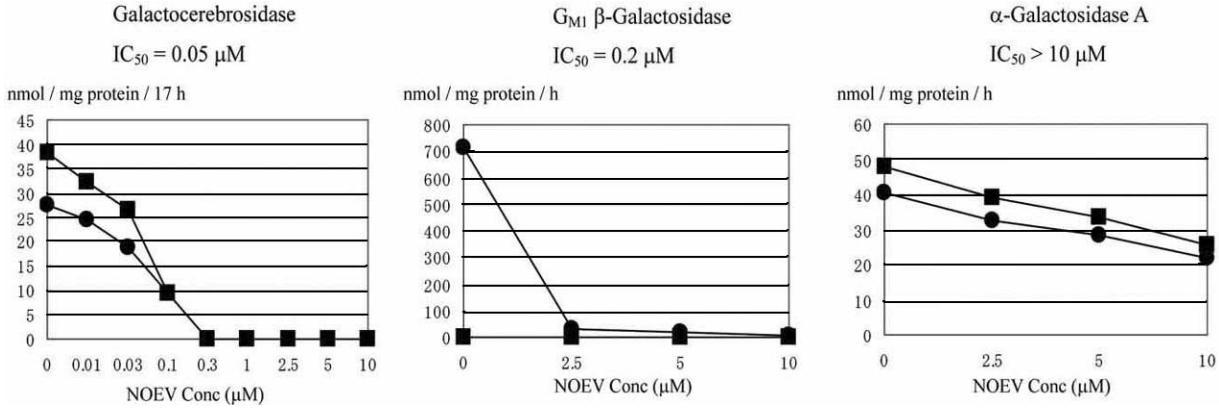


Fig. (14). NOEV effects on three human galactosidases.

Potent inhibitory activity was observed for galactocerebrosidase and (G_{M1}) β-galactosidase, but not for α-galactosidase A. ■-■: control fibroblasts, ●−●: G_{M1}-gangliosidosis fibroblases. Courtesy of Dr. Miho Tabe, SRL Inc, Hachioji, Japan.

ing spontaneous motor and reflex functions. A four-grade scoring system was introduced for each test, and individual and total scores were recorded for each mouse. This clinical test method is useful and sufficiently sensitive to detect early brain dysfunction in disease model mice. NOEV treatment definitely prevented, albeit partially, disease progression [Suzuki, unpublished data]. This provided the first evidence that oral medication can prevent an inherited brain disease in model mice, and we propose that NOEV chaperone therapy should be introduced as a new approach to human G_{M1}-gangliosidosis in the near future.

We have not observed any clear adverse effects on experimental animals during the course of NOEV therapy for up to 6 months, although analytical studies have yet to be completed for pathological, biochemical and pharmacological parameters with this compound.

3.7. Summary: Biological Activities in Human and Mouse NOEV Experiments

NOEV is an *in vitro* competitive inhibitor of both β-galactosidase and galactocerebrosidase, and a mutation-specific enhancer of β-galactosidase in human and mouse fibroblasts. Thus exogenous substrates are digested by the R201C mutant β-galactosidase in mouse fibroblasts in the presence of NOEV.

After oral administration, NOEV is not digested in the mouse gastrointestinal system, goes directly into the bloodstream, and is delivered to the mouse brain through the blood-brain barrier. It enhances the mutant β-galactosidase activity in the brain and liver, and substrates abnormally stored in the brain are digested. Clinically NOEV prevents brain damage, to some extent in mouse G_{M1}-gangliosidosis and is rapidly disposed of after uptake in neural and hepatic cells. Definite adverse effects have not been observed in the R201C mutant mouse after up to 6 months of continuous oral administration.

CONCLUSION

During the past 40 years, a large number of carbasugars have been synthesized and thier structure-function relationships analyzed, some of them being found to be potent inhibitors of glycohydrolases as a result of binding to active sites of the enzyme molecules. Careful investigations have revealed misfolding of mutant enzymes in somatic cells, followed by a rapid protein breakdown and defective expression of catalytic activity. These findings led us to development of a new concept of chemical chaperone therapy to enhance the mutant lysosomal enzyme activity in the presence of a carbasugar as an exogenous molecular chaperone.

The compound NOEV is a good candidate for this new therapeutic approach, particularly for central nervous system pathology, as it is a small molecule delivered directly to the brain from the bloodstream, passing through the blood-brain barrier and inducing expression of enzymes in nerve cells. We are aware at this stage that the approach needs long-term careful evaluation in order to establish optimal dosage and intervals for oral administration, first to mice and then to humans, for prevention of the clinical disease by effective substrate digestion. Possible adverse or toxic effects should also be carefully tested before starting human trials.

This new molecular approach is not justified for all patients with a single lysosomal enzyme deficiency disorder. Biosynthesis of a catalytically active enzyme is prerequisite for chemical chaperone therapy. Our survey indicated that 20-40% of β-galactosidosis (mainly G_{M1}-gangliosidosis) patients will express unstable but catalytically active proteins and respond to NOEV treatment in cultured fibroblasts [52]. Patients of this type are reasonable candidates for chemical chaperone therapy in the near future.

A few related diseases have already been tested, and the validity of this approach has been proven using *in vitro*, *in situ*, or *in vivo* with model animals. At present our studies are focused on diseases with storage of compounds with α- or β-linked glucose or galactose residues at the terminal ends of oligosaccharide chains in substrate molecules: α-glucosidase deficiency (glycogenosis II), β-glucosidase deficiency (Gaucher disease), α-galactosidase A deficiency (Fabry disease), and β-galactosidase deficiency (β-galactosidosis: G_{M1}-gangliosidosis and Morquio B disease). Theoretically, however, this principle can be applied to all other lysosomal diseases, if a specific chaperone compound becomes available for each enzyme in question. We thus hope to extend this approach to other lysosomal diseases in the future. Special drug design technology is mandatory for screening of appropriate inhibitors and bioinformatics analysis is currently progressing in our project.

Further, there may be diseases of other categories which could benefit from this approach. For this purpose, the underlying molecular pathology in somatic cells needs to be well understood in detail, with elucidation of mutant gene expression, mutant protein structure and intracellular transport, and mechanisms of functional expression. We hope that studies in this direction will disclose new aspects of molecular therapy for inherited metabolic diseases with central nervous system involvement in the near future.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AcOH = Acetic acid

DBU = 1,8-Diazabycyclo[5.4.0]undec-7-ene

DGJ = 1-Deoxygalactonojirimycin

DNJ = 1-Deoxynojirimycin

DMAP = 4-Dimethylaminopyridine

DMF = *N*,*N*-Dimethylformamide

DMJ = 1-Deoxymannonojirimycin

DMP = 2,2-Dimethoxypropane

DMSO = Dimethylsulfoxide

i-PrOH = Isopropanol

KO = Knockout

MCS = 2-Methoxyethanol

NOEV = *N*-Octyl-4-epi-β-valienamine

NOV = *N*-Octyl-β-valienamine

p-TsOH = *p*-Toluenesulfonic acid

Pyr = Pyridine

Tg = Transgenic

THF = Tetrahydrofuran

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[12] According to IUPAC-IUBMB Nomenclature of Carbohydrates (Recommendation 1966) (*Carbohydr. Res.* **1997***, 297*, 1), validamine (**3α**) belongs to a family of carbocyclic analogues of glycosylamines and names 5a-carba-α-D-glucopyranosylamine. Concerning its unsaturated derivatives including valienamine (**4α**), we proposed common names 5a-carba-hexenopyranosylamines with carbon numbers, for instance (5,5a), (5a,1) etc., denoting locations of the unsaturation, in order to differentiate them from the 5-deoxy-5-eno derivatives (*Eur. J. Org. Chem*. **1995**, 279). Since the 1-epimer of **4α** would correspond to the β-anomer of parent glucopyranosylamine, compound **4β** is, for convenience, named β-valienamine. On the other hand, all stereoisomers of **4α**, except for the 1-epimer, could be conventionally designated common names epivalienamines with carbon numbers denoting the positions epimerized.

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