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Review

Sugar-mimicking glycosidase inhibitors: bioactivity and application

N. Asano

Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920 – 1181 (Japan), Fax: +81 76 229 2781, e-mail: n-asano@hokuriku-u.ac.jp

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Abstract. A large number of compounds mimicking the structures of monosaccharides or oligosaccharides have been discovered from natural sources. Such sugar mimics inhibit carbohydrate-degrading enzymes because of a structural resemblance to the sugar moiety of the natural substrate. Carbohydrate-degrading enzymes are involved in a wide range of important biological processes, such as intestinal digestion, posttranslational processing of the sugar chain of glycoproteins, their quality control mechanisms, lysosomal catabolism of glycoconjugates, and some viral infections. It has now been realized that inhibitors of the enzymes have enormous therapeutic potential in diabetes and lysosomal storage disorders. In this review, the general bioactivity, current applications, and the prospects for new therapeutic applications are described.

Keywords. Six-membered sugar mimics, five-membered sugar mimics, α-glucosidase inhibitor, glycogen-degrading enzyme inhibitor, diabetes, glucosylceramide synthase inhibitor, pharmacological chaperone, lysosomal storage disorder.

Introduction

Great interest in sugar-mimicking glycosidase inhibitors has been aroused in recent years because it has been elucidated that such compounds and their derivatives have enormous therapeutic potential in many diseases such as diabetes, viral infection, and lysosomal storage disorders [1 – 3]. Sugar-mimicking glycosidase inhibitors are classified into three major structural classes: iminosugars, thiosugars, and carba-glycosylamines. Furthermore, their derivatives and oligosaccharides containing such sugar mimics are also included in this category. Iminosugars and thiosugars are sugar mimics with respectively a nitrogen atom or a sulfur atom in place of the ring oxygen of monosaccharides, and carba-glycosylamines are glycosylamines in which the ring oxygen is replaced with a methylene group. The biological properties can be explained by their structural resemblance to the terminal sugar moiety in the natural substrates. Glycosidases are involved in a wide range of important biological processes, such as intestinal digestion, posttranslational processing of the sugar chain of glycoproteins, quality-control systems in the endoplasmic reticulum (ER) and the ER-associated degradation (ERAD) mechanism, and the lysosomal catabolism of glycoconjugates. Hence, inhibition of these glycosidases can have profound effects on carbohydrate catabolism in the intestines, maturation, transport, and secretion of glycoproteins, and can alter cell-cell or cell-virus recognition processes. This principle is the basis for the potential use of glycosidase inhibitors in viral infection, cancer, and genetic disorders. In this review, the general bioactivity of

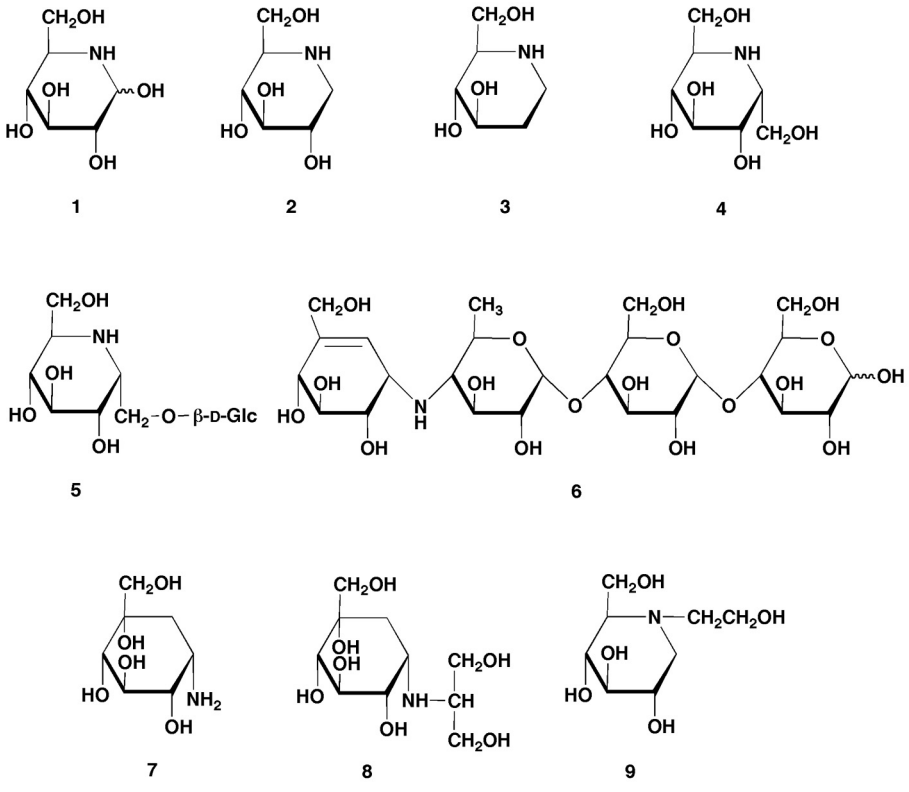

Figure 1. Structures of six-membered sugar mimics. ***1***, nojirimycin (NJ); ***2***, 1-deoxynojirimycin (DNJ); **3**, fagomine; ***4***, α-homonojirimycin (HNJ); ***5***, 7-O-β-D-glucopyranosyl-HNJ (Glc-HNJ); ***6***, acarbose; ***7***, valiolamine; ***8***, voglibose; ***9***, miglitol.

sugar-mimicking glycosidase inhibitors, their current applications, and the prospects for new therapeutic applications are described.

Diabetes and α-glucosidase inhibitors

Over 40 years have passed since nojirimycin (NJ) (Fig. 1.1**)** was discovered as the first natural glucose mimic, with a nitrogen atom in place of the ring oxygen [4]. NJ was originally described as an antibiotic produced by *Streptomyces* species and was shown to be a potent inhibitor of α- and β-glucosidases from various sources [4, 5]. However, because this iminosugar is fairly unstable, it is usually stored as a bisulfite adduct or may be reduced by catalytic hydrogenation with a platinum catalyst or by $NaBH_4$ to 1-deoxynojirimycin (DNJ) (Fig. 1.2) [5]. DNJ was later isolated from the roots of mulberry trees and called molanoline [6], and was further found to be produced by many strains in the genera *Bacillus* and *Streptomyces* [7–9]. Although DNJ is a potent inhibitor of all kinds of α-glucosidases tested, 1,2-dideoxynojirimycin, fagomine (Fig. 1.3), is a much weaker inhibitor of α-glucosidases than DNJ [10] but has an interesting activity that DNJ lacks. The effect of fagomine on immunoreactive insulin (IRI) release was investigated with the perfused pancreas of normal rats [11]. The 8.3 mM glucose-induced IRI release was increased in the presence of fagomine in a concentration-dependent manner. In 1988, α-homonojirimycin (HNJ) (Fig. 1.4) was isolated from the neotropical liana, *Omphalea diandra* (Euphorbiaceae), as the first example of a naturally occurring DNJ derivative with a carbon substituent at C-1 [12]. However, before the isolation of the natural product, 7-*O*-β-D-glucopyranosyl-α-HNJ (Glc-HNJ) (Fig. 1.5) had been designed and prepared as a potential drug for the treatment of diabetes [13, 14]. The α-glucosidase inhibitory activities of HNJ are known to be identical to those of DNJ [15].

In the 1970s, it was realized that inhibition of all or some intestinal disaccharidases and pancreatic α-amylase by inhibitors could regulate the absorption of monosaccharides and that these inhibitors could be used therapeutically in the oral treatment of the non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes). Bayer's researchers found that *Actinoplanas utahences* produces a potent sucrase inhibitor, acarbose (Fig. 1.6), with an IC_{50} value of 0.5 μM [16]. In 1990, after intensive clinical development, acarbose became the first α-glucosidase inhibitor as a therapeutic agent for type 2 diabetes to be launched in Germany under the name Glucobay and has since been successfully marketed in Europe and Latin America. It is now sold in the United States as Precose and in Canada as Prandase. In 1984, the validamycin-

producing organism *Streptomyces hygroscopicus* var. *limoneus* was reported to coproduce valiolamine (Fig. 1.7), which is a potent inhibitor of pig intestinal maltase and sucrase with IC_{50} values of 2.2 and 0.049 μM, respectively [17]. Numerous N-substituted valiolamine derivatives were synthesized to enhance its inhibitory activity *in vitro* and the very simple derivative voglibose (Fig. 1.8) was selected as the potential oral anti-diabetic agent [18]. Voglibose was launched in 1994 in Japan under the brand name Basen. The final commercially available anti-diabetic agent miglitol (Fig. 1.9) is the N-hydroxyethyl derivative of DNJ designed to delay the digestion of ingested carbohydrates, thereby resulting in a smaller rise in blood glucose concentration following meals. Despite the excellent *in vitro* α-glucosidase inhibitory activity of DNJ, its efficacy *in vivo* was only moderate [19]. Therefore, a large number of DNJ derivatives were prepared in the hope of increasing the *in vivo* activity. Among them, miglitol was selected as the most favorable inhibitor. The most striking feature of miglitol is that it is almost completely absorbed from the intestinal tract. Hence, it should be pointed out that it may possess systemic effects in addition to the effects in the intestinal border [20, 21], but there is no evidence that systemic absorption of miglitol contributes to its therapeutic effect.

An enzyme source for the first screening of α-glucosidase inhibitors greatly affects the susceptibility of inhibitors. Hence, as the second screening for the selected inhibitors, it is required to evaluate their possible inhibitory effect on human enzymes. The Caco-2 cell line is derived from a human colonic carcinoma and has the ability to express most of the morphological and functional characteristics normally associated with the human intestinal epithelium [22]. Caco-2 cell monolayers are well utilized as a culture model of human intestinal cells for the drug transport systems and the effect of α-glucosidase inhibitors [23–26]. The rapid *in vitro* assay with the enzyme α-glucosidase and evaluation with Caco-2 cells enable more practical selection of potential drug candidates. The experiment with Caco-2 cells is based on the assumption that maltose added to the apical side of the Caco-2 monolayer is hydrolyzed to yield glucose by α-glucosidases expressed in Caco-2 cells. In our recent study (Figure 2), DNJ and HNJ showed the IC_{50} values of 0.06 and 0.10 μM, respectively, toward Caco-2 maltase, while Glc-HNJ was a 10-fold weaker inhibitor of Caco-2 maltase than HNJ (Figure 2A). Voglibose (Basen), miglitol (Glyset), and acarbose (Glucobay) commercially available as anti-diabetic agents inhibited Caco-2 maltase with IC_{50} values of 0.07, 1, and 5 μM, respectively (Figure 2B). These results indicate that the inhibitory potentials of DNJ and HNJ toward human maltase are identical to that of voglibose.

Three α-glucosidase inhibitors acarbose (Fig. 1.6), voglibose (Fig. 1.8), and miglitol (Fig. 1.9), are widely used in the treatment of patients with type 2 diabetes. The inhibitory potential of voglibose toward intestinal α-glucosidases is the best of these three drugs, as shown in Figure 2B, while the inhibitory activity of DNJ toward human maltase with Caco-2 cells is also identical to that of voglibose (Figure 2A). However, the efficacy of DNJ *in vivo* is only moderate despite its excellent α-glucosidase inhibitory activity *in vitro* [19]. The initial occurrence of DNJ was due to reduction by catalytic hydrogenation of NJ (Fig. 1.1) with a platinum catalyst or by $NaBH_4$ to DNJ [5]. Ten years after its initial chemical preparation, DNJ was discovered from roots of mulberry trees [6], and the cultured broth of various species of the genera *Bacillus* and *Streptomyces* [7–9]. Although the discovery of the inhibitory effect on mammalian α-glucosidases opened the possibility of a therapeutic application for DNJ, it had already lost its 'novelty' as an anti-diabetic agent. The possibility of preventing the onset of diabetes using dietary supplements and/or herbal medicines has attracted increasing attention. It has been known that DNJ and HNJ occur in mulberry leaves and the Thai medicinal plant "Non tai yak" (*Stemona tuberosa*), respectively [27, 28]. These traditional herbal medicines would be candidates for diabetes care and prevention.

Diabetes and glycogen-degrading enzyme inhibitors

In mammals glycogen exists both as a cytosolic and a lysosomal form, and is broken down by different pathways. The cytosolic form is cleaved by glycogen phosphorylase (GP) and the debranching enzyme amylo-1,6-glucosidase (1,6-GL), while the lysosomal form is degraded by a single enzyme, acid (lysosomal) α-glucosidase. The liver is the predominant source of blood glucose. It is generally recognized that the hepatic glucose output in type 2 diabetes is elevated and thus significantly contributes to hyperglycemia [29–31]. A possible way to suppress hepatic glucose production and lower blood glucose in type 2 diabetes may be through inhibition of GP [32]. Fosgerau et al. reported that in an enzyme assay a five-membered iminosugar 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) (Fig. 3.10) is a potent inhibitor of hepatic GP and is the most potent inhibitor of basal and glucagon-stimulated glycogenolysis ever reported in primary rat hepatocytes, with an IC_{50} value of 1 μM [33, 34]. It has been reported that D-AB1 is a potent inhibitor of GP with an anti-hyperglycemic effect in ob/ob mice [33].

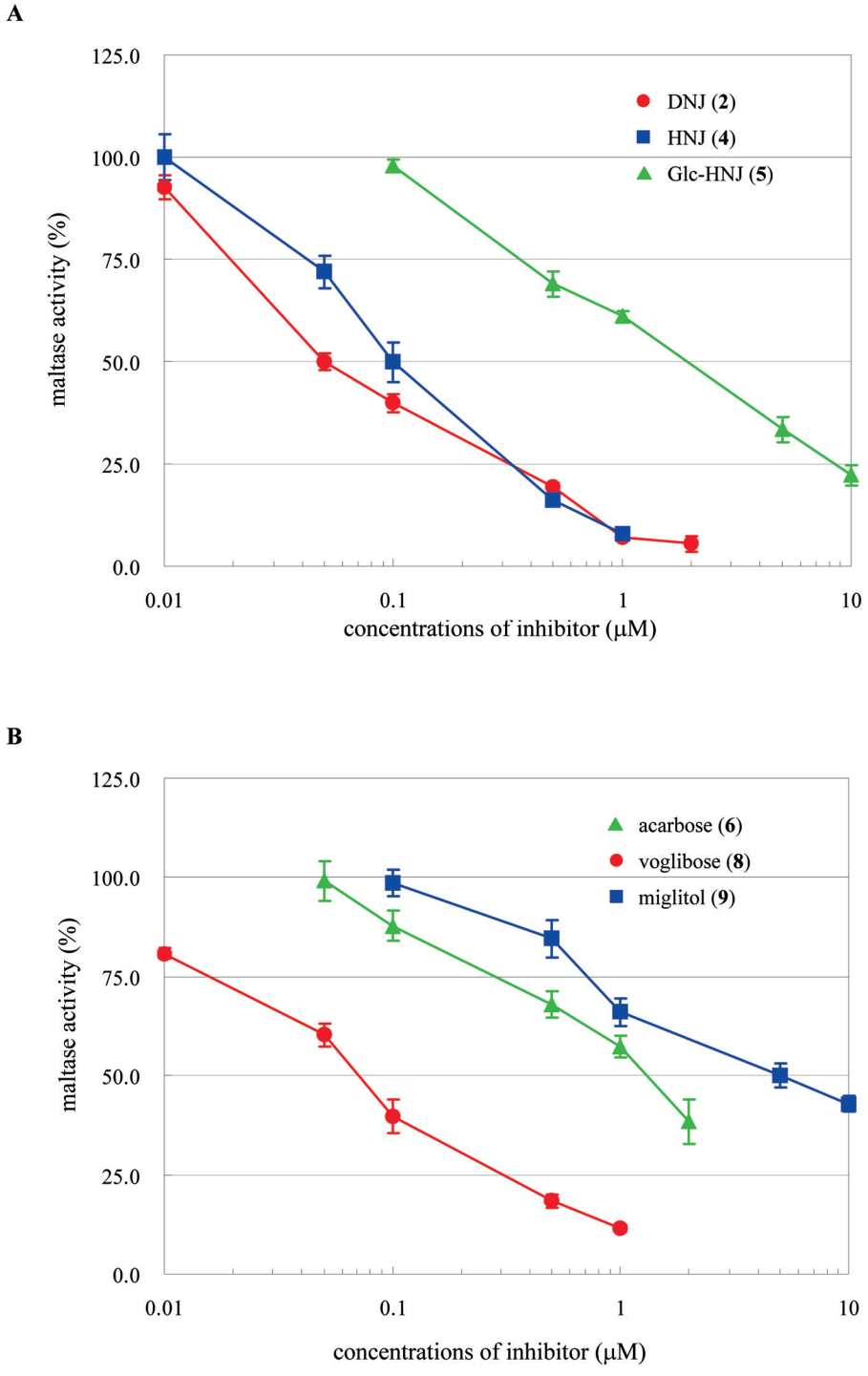

Figure 2. Maltase inhibitory activity of sugar mimics at the apical side of Caco-2 monolayer. Caco-2 cells were incubated in the presence of *(A)* DNJ, HNJ, Glc-HNJ or *(B)* acarbose, voglibose, miglitol for 2 h. Mean values ± SD are shown for triplicate experiment. (This figure was published in [42])

The inhibitory activity of five-membered sugar mimics toward GP has been investigated [35]. D-AB1 potently inhibited rabbit muscle GP b with an IC_{50} value of 0.43 μM, whereas its enantiomer L-AB1 (Fig. 3.11) showed no inhibition toward the enzyme. Introduction of the hydroxymethyl group to the C-1 position of D-AB1 to give 2,5-dideoxy-2,5-imino-D-mannitol (Fig. 3.12) and 2,5-dideoxy-2,5-imino-D-glucitol (Fig. 3.13) abolished its inhibition. The epimerization at C-2 to give 1,4-dideoxy-1,4-imino-D-ribitol (D-DRB) (Fig. 3.14) lowered its inhibition 60-fold and the removal of the 2-OH group of D-AB1 to give 1,4-imino-1,2,4-trideoxy-D-arabinitol (CYB-1) (Fig. 3.15) showed null inhibition of GP. Replacement of the ring nitrogen by a sulfur atom to give 1,4-anhydro-4-thio-D-arabinitol (D-ATA) (Fig. 3.16) completely abolished its inhibition of GP,

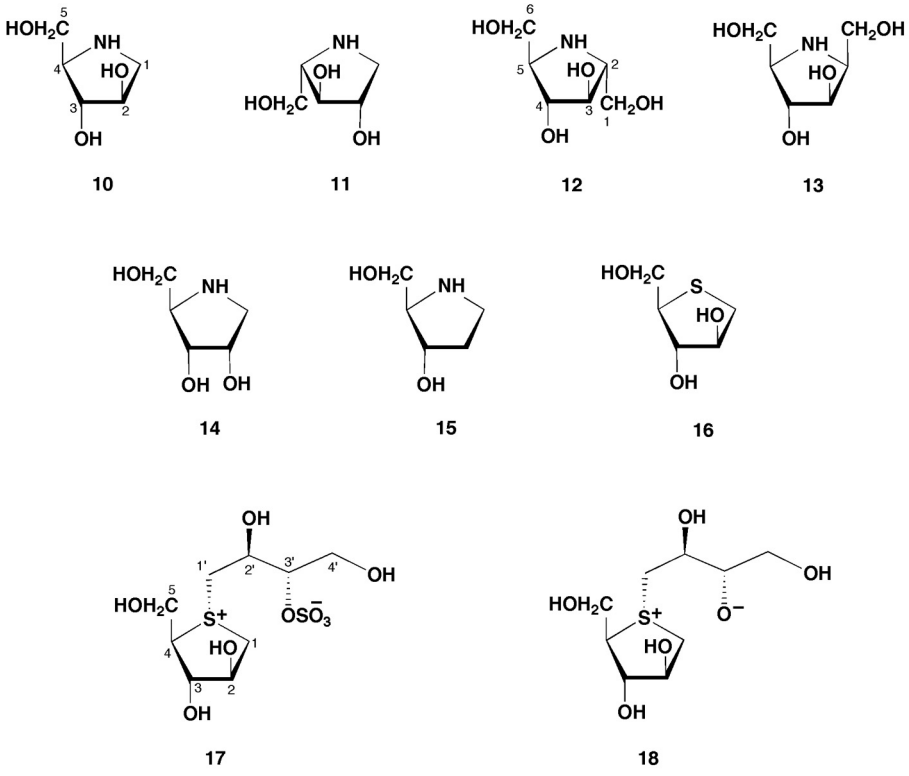

Figure 3. Structures of five-membered sugar mimics. ***10***, 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1); ***11***, 1,4-dideoxy-1,4-imino-L-arabinitol (L-AB1); ***12***, 2,5-dideoxy-2,5-imino-D-mannitol (DMDP); ***13***, 2,5-dideoxy-2,5-imino-D-glucitol (DIG); ***14***, 1,4-dideoxy-1,4-imino-D-ribitol (D-DRB); ***15***, 1,4-imino-1,2,4-trideoxy-D-arabinitol (CYB-1); ***16***, 1,4-anhydro-4-thio-D-arabinitol (D-ATA); ***17***, salacinol; ***18***, 1,4-Dideoxy-1,4-{(*S*)-[(2S, 3S)-2,4-dihydroxy-3-butyl]epi-sulfoniumylidene}-D-arabinitol inner salt.

and the derivatives, salacinol (fig. 3.17) and the desulfonated compound (Fig. 3.18) of salacinol, also showed no inhibition of the enzyme. Thus, it was shown that the structural modification of D-AB1 markedly lowers or abolishes the inhibitory activity toward GP, i.e. GP has a strict structure requirement for inhibitors at the catalytic site [35, 36]. GP exists in two interconvertible forms: a dephosphorylated form (GP b) and a Ser14-phosphorylated form (GP a). GP a is a homodimer of 97 kDa subunits and has two conformational states, the T state (inactive) and the R state (active) [37, 38]. Adenosine 5′-monophosphate (AMP) activates GP b by binding to the nucleoside-activator site [37]. Recently, Oikonomakos et al. reported on the binding of D-AB1 with GP b by X-ray crystallographic investigation [39]. The X-ray structure of D-AB1 in complex with rabbit muscle GP b shows that the inhibitor binds tightly at the catalytic site in the presence of the substrate phosphate, and induces a conformational change that characterizes the R state conformation of the enzyme. Their findings suggest that D-AB1 functions as an oxocarbenium ion transition-state analogue.

Glycogen degradation is catalyzed by two enzymes: glycogen phosphorylase (GP) and the debranching enzyme [40]. The debranching enzyme possesses both activities of 4-α-glucanotransferase and amylo-1,6-glucosidase (1,6-GL). GP catalyzes the sequential phosphorolysis of α-1,4-linked glucose units until four glucosyl units remain before an α-1,6 branch point, and yields glucose-1-P [41]. The 4-α-glucanotransferase activity of the branching enzyme removes a maltotriosyl unit from the α-1,6 branch and attaches it through an α-1,4-glucosidic bond to the free C-4 of the main chain. Thus, it allows the continued release of glucose-1-P by GP. The single remaining α-1,6-linked glucosyl unit is then removed as free glucose by the debranching enzyme 1,6-GL activity. In the liver, glucose can be produced from glucose-1-P by the successive actions of phosphoglucomutase and glucose-6-phosphatase. D-AB1 is the most potent inhibitor of GP to date, and has been shown to inhibit glycogen breakdown both *in vitro* and *in vivo* [33, 34]. However, D-AB1 has not yet come into practical use as an anti-diabetic agent. This is probably due to its lesser efficacy in human bodies. A combination of a potent GP inhibitor and a potent debranching enzyme inhibitor might be more effective for inhibition of glycogen breakdown. D-AB1 and DNJ are potent inhibitors of GP and 1,6-GL, with IC_{50} values of 0.43 and 0.19 μM, respectively [35, 42]. D-AB1 inhibited glucagon-stimulated glucose production dose-dependently with an IC_{50} value of 9 μM, whereas administration of DNJ reached a plateau at 100 μM with 25 % inhibition and then remained unchanged, as shown in Figure 4 [42]. However, the inhibition of hepatic glucose production by D-AB1 was significantly enhanced in the presence of 100 μM DNJ. This

result suggests that GP inhibitors in combination with 1,6-GL inhibitors might lower the high glucose level in type 2 diabetes.

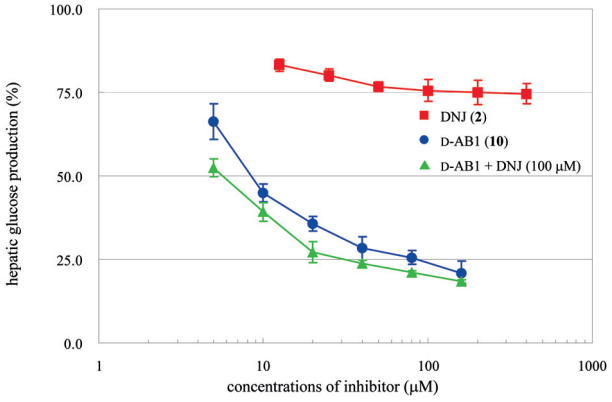

Figure 4. Effect of glycogen phosphorylase inhibitor (D-AB1) and amylo-1,6-glucosidase inhibitor (DNJ) on glucagon-induced glucose production in primary rat hepatocytes. (This figure was published in [42])

Protein folding and quality control mechanisms

The lumen of the ER provides a highly specialized compartment for the folding and oligomeric assembly of secretory proteins, plasma membrane proteins, and proteins destined for the various organelles of the vacuolar system. Protein conformational maturation is a complex process determined not only by the amino acid sequence but also by post and cotranslational modifications, by the intralumenal milieu, and by a variety of chaperones and folding enzymes [43, 44]. The ER possesses efficient quality control mechanisms to ensure that transport is limited to correctly folded and assembled proteins [45]. In many cases, misfolded proteins are recognized to be undesirable by a group of proteins called heat shock proteins, and consequently directed to protein degradation machinery in the cells [46–48]. This involves conjugation to the protein ubiquitin, which acts as a tag that directs the proteins to proteasomes, where they are degraded into their constituent amino acids.

Genetically inherited diseases are often characterized by missense mutations and short in-frame deletions or insertions that give rise to proteins that fail to achieve their properly folded state. Detailed studies of the fate of mutant proteins in human genetic diseases have shown that impaired or aberrant folding of mutant polypeptides typically results in prolonged retaining in the ER and degradation by intracellular proteases before the functional conformation is acquired [47, 49]. Cystic fibrosis (CF) and α_1-antitrypsin (α_1-AT) deficiency are well-known heritable diseases that fall under this category [50, 51]. CF is a human genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR), which functions as a chloride channel in the plasma membrane [52, 53]. The most common mutation observed in patients with CF (over 90%) is a deletion of Phe-508 (ΔF508) of CFTR. This deletion leads to retention of the mutant in the ER, implicating the defect of its trafficking [54]. Many CFTR folding mutants, including the ΔF508 mutant, are potentially functional at the cell surface if they can be induced to fold correctly [55]. The defective folding in the ΔF508 mutant is temperature-sensitive and expression at low temperature (27°C) yields functional molecules at the cell surface. Mutant α_1-antitrypsin Z (α_1-ATZ) protein is associated with the development of chronic liver injury and hepatocellular carcinoma in hereditary α_1-AT deficiency. Only about 15% of newly synthesized α_1-ATZ proteins are secreted into the blood and extracellular fluid, and the remainder is retained in the ER [56, 57]. However, this secreted mutant protein retains about 80% of the functional activity of its wild-type counterpart [58, 59]. These observations raise the possibility that a functional molecule that can elicit the proper folding and trafficking of the mutant protein may be a promising drug for the treatment of these genetic disorders.

Lysosomal storage disorders and treatment

Enzyme replacement therapy

Lysosomes are membrane-bound cytoplasmic organelles that break down macromolecules for recycling in eukaryotic cells. The degradative function of lysosomes is carried out by more than 50 acid-dependent hydrolases contained within the lumen. Among these macromolecules, glycosphingolipid (GSL) is an essential cell membrane component containing polysaccharide chains. The lysosomal storage disorders are due to inherited deficiency of individual enzymes, and organ damage arises from progressive accumulation of the substrates for the deficient enzyme. The successful treatment for such diseases to date is enzyme replacement therapy (ERT). The concept of ERT as treatment for lysosomal storage disorders was proposed by de Duve and Wattiaux [60] in 1966 and the first successful treatment was that for patients with Type 1 Gaucher disease in 1990 [61]. An important breakthrough for the spread of ERT was the passage of the Orphan Drug Act in the US, which provided numerous incentives to industry to undertake the commercial development of the treatment of rare diseases [62, 63]. ERT is now available for Gaucher disease, Fabry disease, Pompe disease, mucopolysaccharidosis Type I (MPS I) (Hurler and Hurler-Scheie syndrome), and mucopolysaccharidosis Type II (MPS

II) (Hunter syndrome). Injectable enzymes for ERT are as follows: imiglucerase (Cerezyme) for the treatment of Type 1 Gauche disease (adult type) is a recombinant form of the naturally occurring glucocerebrosidase; agalsidase beta (Fabrazyme) for the treatment of Fabry disease is a recombinant form of the naturally occurring α-galactosidase A; laronidase (Aldurazyme) for the treatment of MPS I is recombinant human α-I-iduronidase; alglucosidase alpha (Myozyme) for the treatment of Pompe disease is recombinant human acid α-glucosidase; idursulfase (Elaprase) for the treatment of MPS II is recombinant human iduronate 2-sulfatase. However, several questions remain to be answered. The long-term effectiveness of most of the treatments has not yet been established. Furthermore, this ERT is only useful in diseases in the absence of neuropathology since enzymes do not cross the blood-brain barrier. Another problem of this therapy is the cost, which prevents many patients from obtaining this treatment.

Substrate reduction therapy

As long as the synthesis of substrate continues under the decreased corresponding enzyme activity, the pathological accumulation of undegraded substrate in the lysosomes proceeds. The aim of substrate reduction therapy (SRT) is to reduce GSL substrate influx into the lysosomes by inhibitors of GSL synthesis. N-Butyl-DNJ (NB-DNJ, miglustat, Zavesca) (Fig. 5.19) is an inhibitor of ceramide-specific glucosyltransferase (glucosylceramide synthase) and inhibits the first committed step in GSL biosynthesis [64, 65]. N-Butyl-1-deoxygalactonojirimycin (NB-DGJ) (Fig. 5.20) has been found to be a more specific inhibitor of the enzyme without inhibition of N-linked oligosaccharide processing α-glucosidases [66]. Clinical trials with miglustat have been performed in adult patients with Type 1 Gaucher disease (the non-neuronopathic variant), using orally administered doses ranging from 50–200 mg three times a day. These clinical studies showed that treatment with miglustat promoted reduction in liver and spleen volumes and stabilization or improvement in blood counts [67]. Miglustat received marketing authorization from the European Agency for the Evaluation of Medicinal Products in November 2002 and subsequently FDA granted approval for its use in Type 1 Gaucher disease patients in the US under the orphan drug regulatory procedures [68]. Although there are many lysosomal storage diseases in which the central nervous system is affected, ERT is not applicable to such diseases. On the other hand, SRT using a small molecule such as miglustat may be effective in the treatment of neuronopathic forms of lysosomal storage diseases. Miglustat was used for treatment of Niemann-Pick C disease in animal models, and reduction of ganglioside accumulation, delayed onset of neurological symptoms, and increased survival were observed in the drug-treated animals [69]. Recent reports demonstrate its efficacy in the treatment of neurological symptoms in Niemann-Pick C patients [70, 71]. However, this drug appears to have an additional potential use combined with ERT, since this drug's weakness as a monotherapy is known. Furthermore, this combination therapy could be much less expensive because it leads to significantly lower doses of each drug. In clinical trials with miglustat at a target plasma concentration of 10 μM, it has been reported that patients suffered peripheral neuropathies, dose-dependent tremors, paresthesias, and cognitive dysfunction [67, 72, 73]. On the other hand, it should be noted that the isomer NB-DGJ does not show adverse gastrointestinal effects observed by miglustat [72].

Another prototype of GSL synthesis inhibitors is a ceramide-based inhibitor. D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (Fig.5.21) inhibits glucosylceramide synthase and produces the reversible depletion of cellular GSLs in cultured cells and animal models [74, 75]. PDMP is a more potent inhibitor of glucosylceramide synthase than miglustat but has an undesirable side effect of increasing intracellular ceramide level, resulting in cytotoxicity [76]. PDMP has been found to have two sites of action, one for the inhibition of glucosylceramide synthase and one for the inhibition of 1-O-acylceramide synthase [77]. Although the inhibition of glucosylceramide synthase resulted in the cellular depletion of GSLs, that of 1-O-acylceramide synthase resulted in the elevation of intracellular ceramide levels and inhibition of cell growth *in vitro* [77, 78]. Subsequently, D-threo-1-phenyl-2-palmitoilamino-3-pyrrolidino-1-propanol (P4) (Fig. 5.22), 4′-hydroxy-P4 (pOH-P4) (Fig. 5.23), and 3′,4′-ethylenedioxy-P4 (EtDO-P4) (Fig. 5.24) have been developed using PDMP as the lead compound [79]. pOH-P4 inhibited glucosylceramide synthase at an IC_{50} value of 90 nM and the inhibitory potential of EtDO-P4 was identical to that of pOH-P4 [79]. These inhibitors resulted in the inhibition of GSL synthesis in cultured cells at concentrations that did not significantly raise intracellular ceramide levels or inhibit cell growth. In 2001, Genzyme released preclinical data supporting Genz-78132 (EtDO-P4) as the second-generation substrate reduction agent and announced that Genz-78132 is 100–5000 times more potent *in vitro* for substrate inhibition than the first-generation inhibitors NB-DNJ, NB-DGJ, and N-(5-adamantane-1-yl-methoxypentyl)-DNJ (AMP-DNJ) (Fig. 5.25). Very recently, Genz-112638, which is a structural homologue of P4 formulated a tartrate salt, has been found to more potently and more specifically inhibit the target enzyme with an IC_{50}

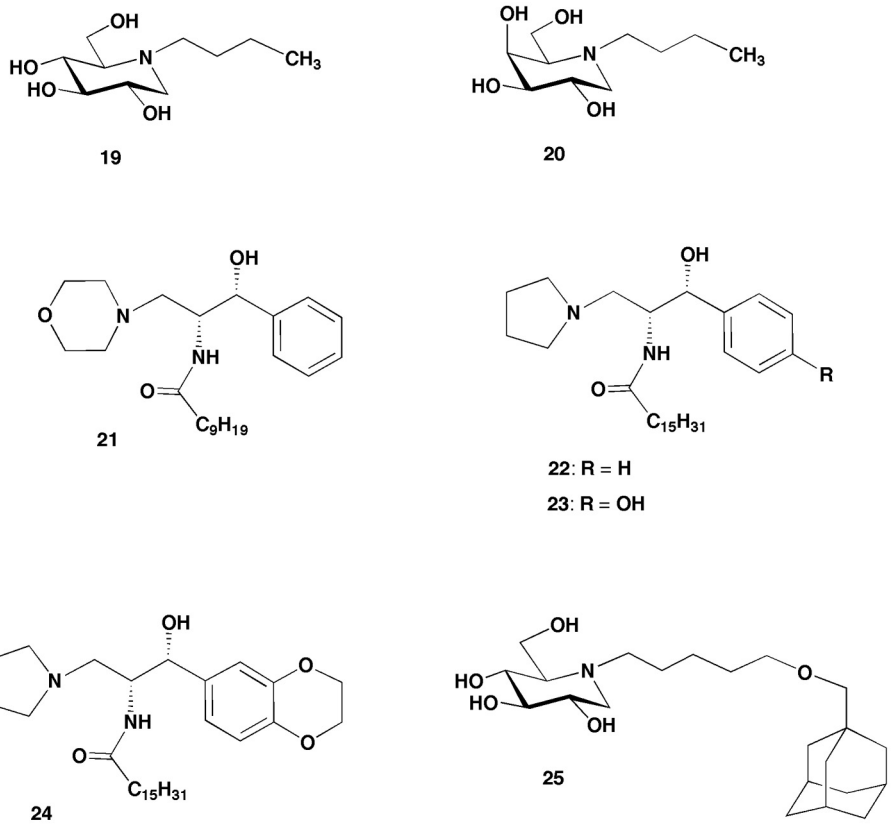

Figure 5. Structures of glucosylceramide synthase inhibitors. ***19***, N-butyl-DNJ (NB-DNJ, miglustat, Zavesca); ***20***, N-butyl-1-deoxygalactonojirimycin (NB-DGJ); ***21***, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP); ***22***, D-threo-1-phenyl-2-palmitoyl-3-pyrrolidino-1-propanol (P4); ***23***, 4′-hydroxy-P4 (pOH-P4); ***24***, 3′,4′-ethylenedioxy-P4 (EtDO-P4); ***25***, N-(5-adamantane-1-yl-methoxypentyl)-DNJ (AMP-DNJ).

value of 24 nM [80]. A Phase II study of Genz-112638 in Type 1 Gaucher patients is presently underway.

Pharmacological chaperone therapy

In recent years, the concept of 'pharmacological chaperone therapy' has been proposed in the treatment for lysosomal storage disorders. The concept that an intracellular activity of mutant enzymes can be restored by administering competitive inhibitors serving as pharmacological chaperones was first introduced with Fabry disease, as described later [81]. These inhibitors appear to act as a template that stabilizes the native folding state in the ER by occupying the active site of the mutant enzyme, thus allowing its maturation and trafficking to the lysosome [82, 83]. Fabry disease is a lysosomal storage disorder caused by deficiency of α-galactosidase A (α-Gal A) resulting in lysosomal accumulation of the substrate globotriaosylceramide (Gb3). Residual α-Gal A activity in lymphoblasts derived from Fabry patients and in tissues of R301Q α-Gal A transgenic mice was enhanced by treatment with 1-deoxygalactonojirimycin (DGJ) (Fig. 6.26) [81]. To establish the concept of using competitive inhibitors as specific pharmacological chaperones, a number of naturally occurring and chemically synthesized DGJ derivatives were tested for intracellular enhancement of a mutant α-Gal A activity in Fabry lymphoblasts [84]. Consequently, it was found that DGJ and α-homogalactonojirimycin (HGJ) (Fig. 6.27) are competitive inhibitors of α-Gal A with K_i values of 0.040 and 0.17 μM, respectively, and addition of DGJ and HGJ at 100 μM to culture medium of Fabry lymphoblasts increases the intracellular α-Gal A activity by 14- and 5.2-fold, respectively. Recently, Yam et al. showed that DGJ induces trafficking of ER-retained R301Q α-Gal A to lysosomes of transgenic mouse fibroblasts and that DGJ treatment results in efficient clearance of Gb3 [85]. Furthermore, they showed the usefulness of a pharmacological chaperone for correction of the lysosomal storage in Fabry fibroblasts harbouring different mutations with residual enzyme activity, such as T194I and V390fsX8 [86].

In 2002, Sawkar et al. reported that N-nonyl-DNJ (NN-DNJ) (Fig. 6.28) is a potent inhibitor of lysosomal β-glucosidase (glucocerebrosidase, GCase), with an IC_{50} value of 1 μM, and that the addition of a subinhibitory concentration (10 μM) of this compound to a fibroblast culture medium leads to a 2-fold increase in the mutant (N370S) enzyme activity [87]. In 2005, examination of the effect of a series of DNJ analogues on the residual activities of various GCase variants revealed that the nature of the alkyl moiety greatly influences their chaperoning activity: NB-

DNJ is inactive, the DNJ derivatives with N-nonyl and N-decyl chains are active, and N-dodecyl-DNJ is predominantly inhibitory [88]. However, it is also known that NN-DNJ is a potent inhibitor of ER processing α-glucosidases such as NB-DNJ and hence has potential as anti-viral agents to inhibit folding and trafficking of viral envelope glycoproteins [89, 90]. Inhibitors targeting a host function such as ER processing α-glucosidases must be carefully considered in terms of side-effects, since they may inhibit folding, secretion, and trafficking of other glycoproteins in patients' cells or may directly inhibit lysosomal α-glucosidase after being taken up into cells. In fact, addition of NN-DNJ at 10 μM lowered the cellular lysosomal α-glucosidase activity by 50 % throughout the assay period (10 days), in spite of the excellent chaperoning activity for the mutant GCase [91]. The inhibition of lysosomal α-glucosidase as the side effect may induce storage of glycogen in the lysosomes, as observed in Pompe disease. On the other hand, α-1-C-octyl-DNJ (CO-DNJ) (Fig. 6.29) is also a potent inhibitor of GCase, with a K_i value of 0.28 μM, and treatment with CO-DNJ (20 μM) for four days maximally increased intracellular GCase activity by 1.7-fold in the Gaucher N370S cell line (GM0037) and by 2.0-fold in another N370S cell line (GM00852), minimizing the potential for undesirable side effects such as lysosomal α-glucosidase inhibition [91]. Fan et al. have assessed the efficacy of pharmacological chaperoning activity on a variety of iminosugars [92]. Among them, D-isofagomine (D-IFG) (Fig. 6.30) was the most potent inhibitor of GCase *in vitro* and the most effective pharmacological chaperone capable of increasing residual GCase activity in N370S fibroblasts. Intracellular GCase activity increased approximately 2-fold when cells were incubated with D-IFG. The effective concentration for D-IFG was determined to be 10–50 μM. D-IFG (Plicera) is currently in Phase II clinical trials for Gaucher disease, and Amicus Therapeutics has very recently presented positive data from Phase II clinical trials of Plicera. Yu et al. have recently reported that the cellular activity of N370S and G202R GCase in fibroblasts is increased by 2.5- and 7.2-fold with D-IFG-based pharmacological chaperones N-adamantanyl-4-((3R,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)-butanamide (Fig. 6.31) and N-adamantanyl-4-((3R,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)-pentanamide (Fig. 6.32), respectively, the best enhancements observed to date [93].

The third targeting enzyme of pharmacological chaperone therapy is acid α-glucosidase (GAA), which catalyzes hydrolysis of the α-1,4- and α-1,6-glucosidic bonds of oligosaccharides and glycogen to yield glucose. Pompe disease (glycogen storage disease Type II) is due to mutations of the GAA gene, and its deficiency results in generalized tissue glycogen accumulation and secondary cardiac and skeletal

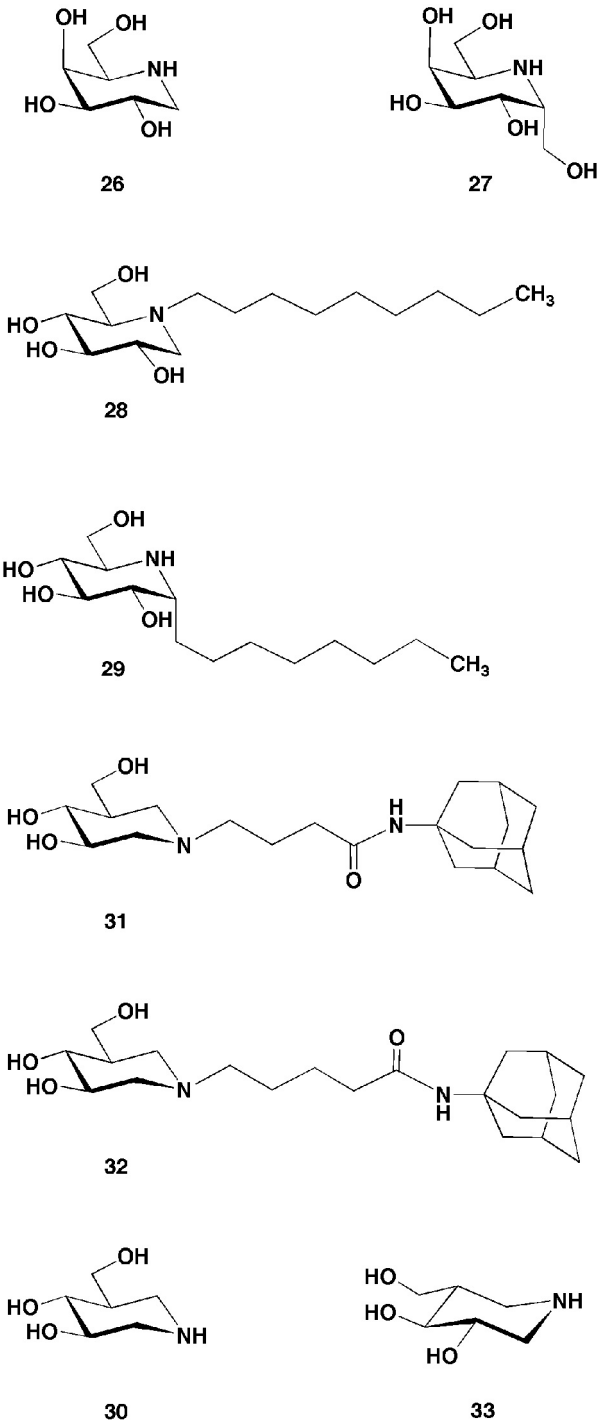

Figure 6. Structures of pharmacological chaperones. ***26***, 1-deoxygalactonojirimycin (DGJ); ***27***, α-homogalactonojirimycin (HGJ); ***28***, N-nonyl-DNJ (NN-DNJ); ***29***, α-1-C-octyl-DNJ (CO-DNJ); ***30***, D-isofagomine (D-IFG); ***31***, N-adamantanyl-4-((3R,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)-butanamide; ***32***, N-adamantanyl-4-((3R,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl)piperidin-1-yl)-pentanamide; ***33***, L-isofagomine (L-IFG).

muscle destruction [94]. Recombinant human GAA (Myozyme) has been developed for ERT for the treatment of Pompe disease [95, 96]. Recently, there have been some potential approaches to use pharmacological chaperones for treating Pompe disease [97–99]. Parenti et al. have reported the effects of DNJ and NB-DNJ on residual GAA activity in fibroblasts from eight patients with different forms of Pompe disease (two classic infantile, two non-classic infantile onset, four late-onset forms) [98]. A 1.8–5.6-fold increase of residual GAA activity after treatment with NB-DNJ was observed in fibroblasts from three patients carrying at least one allele with the L552P mutation and from a patient carrying the missense mutation G549R and a splicing mutation on the second allele, while the GAA residual activity after culturing the same fibroblast cell lines in the presence of DNJ was enhanced by 1.3–7.5-fold. Furthermore, immunofluorescence studies in HEK293T cells overexpressing the L552P mutation showed an improved trafficking of the mutant enzyme to lysosomes after NB-DNJ treatment. Yoshimizu et al. found that, among the iminosugars, DNJ most strongly binds to GAA. By means of the binding parameters and thermodynamics of the interaction of iminosugars with recombinant GAA and using constructed structural models of the catalytic domain of the enzyme with iminosugars, they could show that the active site of the enzyme is almost completely occupied by DNJ [99]. These results provide a rationale for an alternative treatment, other than ERT, for Pompe disease. In June 2008, Amicus Therapeutics announced the initiation of the Phase II study of DNJ (AT2220), designed to enroll 18 adult patients diagnosed with Pompe disease.

To date, all successful pharmacological chaperones for treatment of lysosomal storage disorders have been active-site-directed competitive inhibitors. Such competitive inhibitors stabilize the conformation of the mutant enzymes by occupying their catalytic sites and allow resumption of their processing, leading to maturation and trafficking to the lysosomes. Very interestingly, the first report on a chaperoning activity by a noncompetitive inhibitor has just been published [42]. D-IFG (Fig. 6.30) is a very strong inhibitor of human GCase and inhibits the enzyme in a competitive manner, with a K_i value of 0.016–0.025 μM [100, 101]. It is known to be an effective active site-specific chaperone for some Gaucher variants [92, 93,102]. The L-enantiomer (L-IFG) (Fig. 6.33) of D-IFG has been found to be a noncompetitive inhibitor of GCase, with a K_i value of 5.7 μM [100]. Surprisingly, the noncompetitive inhibitor L-IFG showed a chaperoning activity in a dose-dependent manner in N370S Gaucher fibroblasts, with a 1.6-fold increase at 500 μM (Fig. 7B), while treatment with 10 μM D-IFG caused a 2-fold increase in GCase activity compared with untreated cells (Fig. 7A) [42]. Furthermore, it was investigated whether a combination effect is manifested upon N370S Gaucher fibroblasts when competitive and noncompetitive inhibitors are administered simultaneously. However, combined administration with 10 μM D-IFG and 100 μM L-IFG to the cells showed only the chaperoning activity obtained with the competitive inhibitor D-IFG alone (Fig. 7C). The finding in the present study that a noncompetitive inhibitor also showed a chaperoning activity is very important, because a noncompetitive inhibitor may act as a pharmacological chaperone toward the variant with a mutation that destabilizes a domain distinct from the catalytic domain. It further suggests that ligands with pharmacological selectivity, functioning as pharmacological chaperones, may be able to rescue misfolded proteins including receptors.

Future prospects

Over 40 years have passed since the first sugar-mimic nojirimycin (NJ) was discovered from the cultured broth of the *Streptomyces* species and found to be a potent glycosidase inhibitor. The 1-deoxy derivative DNJ, which is a much more stable and potent inhibitor of α-glucosidase than NJ, was found in mulberry trees ten years after the discovery of NJ and then isolated from the cultured broth of bacteria. The N-hydroxyethyl derivative of DNJ, miglitol (Glyset), has now been approved as the second-generation α-glucosidase inhibitor for the treatment of type 2 diabetes. The efficacy, safety, and tolerability of the α-glucosidase inhibitors acarbose and voglibose appear to have been well-established in the treatment of type 2 diabetes. In addition, α-glucosidase inhibitors have been reported to have protective effects for various diabetic complications, including a reduction in cardiovascular events [103, 104]. In the near future, α-glucosidase inhibitors will be approved in many countries for prediabetes treatment since it is considered to be one of the safest and best-tolerated classes of anti-diabetic agents available.

Although inhibitors targeting glycogen phosphorylase (GP) have been developed and studied as a potential therapy for attenuating hyperglycemia associated with type 2 diabetes [33, 34, 105, 106], such anti-diabetic agents targeting hepatic GP are not yet commercially available. In a diabetic mouse model, administration of GP inhibitors has been shown to reduce liver GP activity and to dramatically attenuate hyperglycemia without producing hypoglycemia [107, 108]. Current GP inhibitors have also been shown to be more potent at reducing hepatic glucose output in

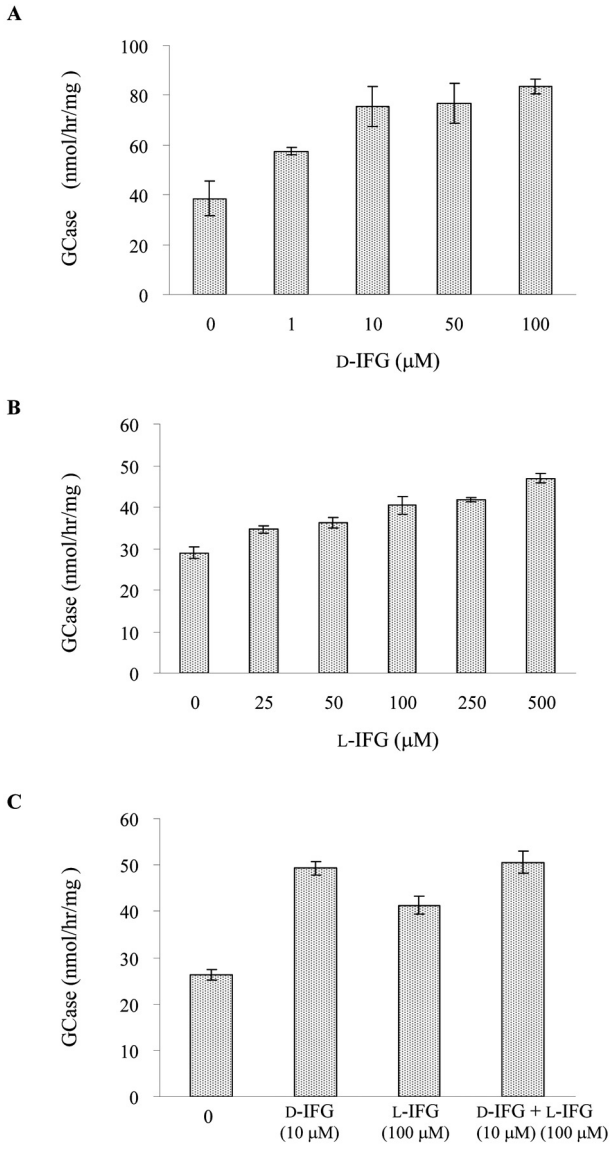

Figure 7. The influence of D-IFG and L-IFG on glucosylcerebrosidase (GCase) activity in N370S Gaucher fibroblasts (GM00372). The fibroblasts were incubated in the presence of *(A)* D-IFG, *(B)* L-IFG, or *(C)* a combination of D-IFG (10 μM) and L-IFG (100 μM) for 4 days. Mean values ± SD are shown for triplicate experiment. (This figure was published in [42])

the presence of high glucose concentrations, thus protecting against rebound hypoglycemia [109]. Furthermore, there is also evidence that GP inhibitors may be cardioprotective, and thus their profile renders them attractive for the treatment of type 2 diabetic patients who typically die from adverse cardiovascular events [110]. In spite of great efforts for drug development, the reasons why such inhibitors have not yet been developed as therapeutic agents are obscure. If the problems in drug development and/or the side effects are clarified, the way would be opened.

Protein misfolding is recognized as the root of many genetic disorders. These misfolded proteins are consequently retained in the ER and degraded by ERAD. Competitive inhibitors are often effective active-site-specific chaperones when they are used at subinhibitory concentrations. They act as a folding template in the ER to facilitate proper folding of mutant proteins, thereby accelerating their smooth escape from the ERAD to maintain a high level of residual enzyme activity. In 1999, Fan et al. reported the first study on a pharmacological chaperone as a new therapeutic strategy for a genetic disease [81]. Since then, many competitive inhibitors of lysosomal glycosidases have been screened as candidates for the treatment of lysosomal storage disorders. To date, DGJ (Fig. 6.26) for Fabry disease, DNJ (Fig. 1.2), D-IFG (Fig. 6.30) and their alkyl derivatives for Gaucher disease, and DNJ for Pompe disease have been found to be effective pharmacological chaperones for lysosomal storage disorders [81, 84, 87, 91, 92, 97]. The finding that a noncompetitive inhibitor also showed a chaperoning activity is very important, because it may be effective toward the variant with a mutation which destabilizes a domain distinct from the catalytic domain [42]. Furthermore, this finding also suggests that ligands with pharmacological selectivity may be able to rescue misfolded proteins, including receptors, as pharmacological chaperones. Now, a number of ligands with pharmacological specificity would be candidates for a new molecular therapy of human genetic disorders. Interestingly, Norez et al. recently found that NB-DNJ (miglustat) (Fig. 5.19) but not NB-DGJ (Fig. 5.20) rescues a mature and functional ΔF508-CFTR in human and mice epithelial cells [111]. Although the mechanism of this effect is not yet known, the elucidation of this mechanism may lead to a new application of sugar-mimicking glycosidase inhibitors for the treatment of human genetic disorders.

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