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REVIEW

Glycosidase inhibitors: update and perspectives on practical use

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About 40 years have passed since the classical glycosidase inhibitor nojirimycin was discovered from the cultured broth of the *Streptomyces* species. Since then, over 100 glycosidase inhibitors have been isolated from plants and microorganisms. Modifying or blocking biological processes by specific glycosidase inhibitors has revealed the vital functions of glycosidases in living systems. Because enzyme-catalyzed carbohydrate hydrolysis is a biologically widespread process, glycosidase inhibitors have many potential applications as agrochemicals and therapeutic agents. Glycosidases are involved in the biosynthesis of the oligosaccharide chains and quality control mechanisms in the endoplasmic reticulum of the *N*-linked glycoproteins. Inhibition of these glycosidases can have profound effects on quality control, 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 past and current applications of glycosidase inhibitors to agricultural and medical fields and the prospect for new therapeutic applications are reconsidered.

Key words: agrochemicals/antidiabetic agents/antiviral agents/genetic disorders/glycosidase inhibitors

Agrochemicals

Trehalase inhibitors

In 1968 the validamycin family was detected by the researchers of Takeda Chemical Industries in greenhouse assay for the treatment of sheath blight disease of rice plants caused by the fungus *Rhizoctonia solani*. Validamycin A (**1**, Figure 1), the major and most active component of the complex, was isolated from the cultured broth of *Streptomyces hygroscopics* var. *limoneus* (Iwasa *et al*., 1970, 1971; Horii *et al*., 1972). Takeda commercialized validamycin A for the control of the pathogen in rice and other plants as well as damping off diseases in vegetable seedlings. The structure of validamycin A contains the pseudodisaccharide, validoxylamine A (**2**, Figure 1), and an additional glucose unit. Validamycin A is not fungicidal or fungistatic but reduces the maximum rate of hyphal extension and increases hyphal branching without affecting the organism's specific growth rate (Nioh and Mizushima, 1974; Trinci, 1985; Robson *et al*., 1988). In 1987, validoxylamine A was found to be a powerful inhibitor of trehalases of *R. solani* and other organisms (Asano *et al*., 1987; Kameda *et al*., 1987). Furthermore, validamycin A was found to be much more readily taken up into the fungal cell than validoxylamine A and to be intracellularly converted into a powerful inhibitor, validoxylamine A, by β-glucosidase (Asano *et al*., 1987). Interestingly, validamycin A is readily transported into the fungal cell by the common uptake system with β-oligosaccharides, such as laminarioligosaccharides and cellooligosaccharides (Asano *et al*., 1991). Validamycin A is a prodrug that is converted within the fungal cell and suppresses breakdown of intracellular trehalose at a very low concentration of 0.1 μg/ml. Trehalose is well known as a storage carbohydrate, and trehalase plays the essential role in the transport of glucose in insects and fungi (Elbein, 1974; Defaye *et al*., 1980; Thevelein, 1984; Elbein *et al*., 2003). The pathogen *R. solani* contains the remarkable trehalose level of 10% (dry weight) (Asano *et al*., 1987) and is one of the most typical examples of a fungus that grows rapidly by transporting nutrients from the basal part to the tip part through long stretches of hypae (Misato *et al*., 1977). Validamycin A is practically nontoxic, and the mode of action described gives it a favorable biological selectivity because vertebrates do not depend on the hydrolysis of trehalose.

All powerful trehalase inhibitors reported to date are the pseudodisaccharide type of inhibitors, such as validoxylamine A (**2**, Figure 1), MDL 25637 (α-homonojirimycin-7-*O*-β-D-glucopyranoside) (**3**, Figure 1) (Anzeveno *et al*., 1989), trehazolin (**4**, Figure 1) (Ando *et al*., 1991), salbostatin (**5**, Figure 1) (Vértesy *et al*., 1994), and casuarine-6-*O*-α-D-glucopyranoside (**6**, Figure 1) (Kato *et al*., 2003). These compounds are powerful competitive inhibitors of porcine kidney trehalases, with K_i values in the nanomolar range (Salleh and Honek, 1990; Vértesy *et al*., 1994; Kyosseva *et al*., 1995; Kato *et al*., 2003). There are two subsites, one for catalysis and one for recognition, on the active center of porcine kidney trehalase (Asano *et al*., 1996). The extremely high affinity of pseudodisaccharide inhibitors derives from the synergistic interactions of an alkaloid unit and a sugar (or cyclitol) unit with two subsites (Asano *et al*., 1996). Trehalose is a blood sugar in insects and a major storage sugar in fungi and yeast. Therefore trehalase inhibitors are expected to have potential as insecticides or fungicides.

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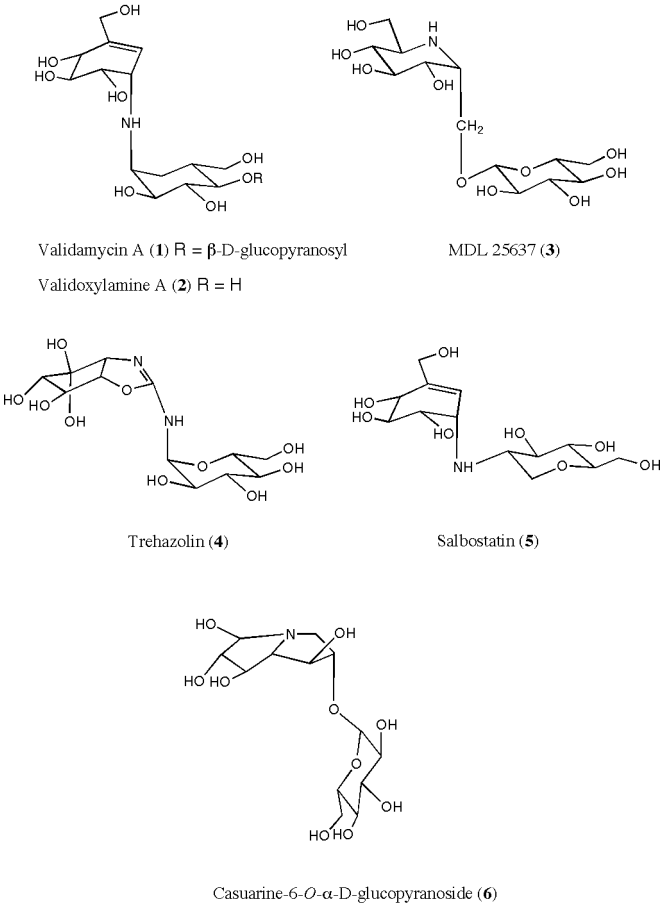


Fig. 1. Structures of trehalase inhibitors validamycin A (**1**), validoxylamine A (**2**), α-homonojirimycin-7-*O*-β-D-glucopyranoside (MDL 25637) (**3**), trehazolin (**4**), salbostatin (**5**), and casuarine-6-*O*-α-D-glucopyranoside (**6**).

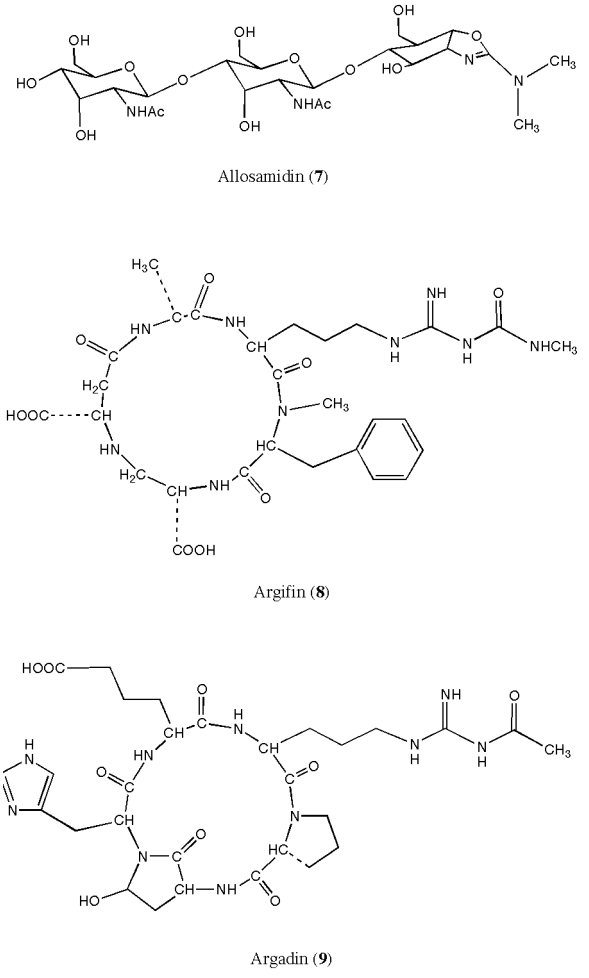


Fig. 2. Structures of chitinase inhibitors allosamidin (**7**), argifin (**8**), and argadin (**9**).

In fact, validoxylamine A has an insecticidal activity when injected (10 μg) into tobacco cut worm larvae (Asano *et al.*, 1990), and trehazolin also shows an insecticidal activity toward silkworm larvae (50–100 μg, Ando *et al.*, 1995). However, these trehalase inhibitors are not practical as insecticides because they have poor percutaneous absorption through insect skin. The design of trehalase inhibitors or prodrugs with good percutaneous absorption will lead to practical use as insecticides.

Chitinase inhibitors

Chitin, linear polymers of β1,4-linked *N*-acetylglucosamine, occurs in fungi and many invertebrates, including insects. Chitin turnover is regulated by two enzymes, chitin synthase and chitinase. Polyoxins interfere with the fungal cell wall synthesis and the insect ecdysis *in vivo* by inhibiting chitin synthase (Cohen and Cashida, 1982; Gooday, 1989). Polyoxins are commercially produced via fermentation. Polyoxin B finds application against many plant fungal pathogens, and polyoxin D is marketed as the Zn salt to control rice sheath blight *R. solani*. Chitinase hydrolyzes chitin into oligomer of *N*-acetylglucosamine and is the key enzyme for the ecdysis of insects. This enzyme has also been proposed as an attractive target for the development of fungicides, insecticides, and antimalarials.

The first chitinase inhibitor, allosamidin (**7**, Figure 2), was isolated from the mycelial extract of *Streptomyces* sp. no. 1713 (Sakuda *et al.*, 1987). Allosamidin is a potent inhibitor of insect chitinase, with an IC_{50} value in the submicrogram/ml range. When allosamidin at a dose of more than 4 μg was injected to the larvae of silkworm and armyworm, all larvae failed in larval ecdysis to the next stage and death followed (Sakuda *et al.*, 1987). Allosamidin derivatives are able to kill *Lucilia cuprina* blowfly larvae *in vitro* systems after contact or feeding (Blattner *et al.*, 1997). Quite recently, allosamidin has been found to be a competitive inhibitor of the fungal chitinase CiX1 from *Coccidioides immitis*, with a K_i value of 60 nM (Bortone *et al.*, 2002). *C. immitis*, a pathogenic fungus that causes coccidioidomycosis or San Joaquin Valley fever (Drutz and Catanzaro, 1978), is most commonly found in the deserts of the southwestern United States and Central and South America. During the early 1990s, the incidence of coccidioidomycosis in California increased dramatically. About 60% of infections resolve without ever causing symptoms and are only recognized by a positive coccidioidin skin test. The major antigen for antibodies

used in this test is *C. immitis* chitinase CiX1 (Yang *et al.*, 1996). In the remaining 40% of patients, symptoms range from mild coldlike or flulike symptoms to severe pneumonia. Antifungal agents, such as amphotericin B and triazole compounds, are available for treating coccidioidomycosis. However, amphotericin B must be given intravenously and is considerably toxic. If allosamidin is effectively taken up into the fungal cell *in vivo*, it may become a reasonable therapeutic agent without side effects for coccidioidomycosis.

In the course of the screening for new insecticides, two new cyclopentapeptide chitinase inhibitors, argifin (**8**, Figure 2) (Omura *et al.*, 2000) and argadin (**9**, Figure 2) (Arai *et al.*, 2000), were found from the fungal cultures of *Gliocladium* sp. FTD-0668 and *Clonostachys* sp. FO-7314, respectively. Argadin potently inhibited the blowfly *Lucilia cuprina* chitinase, with IC_{50} values of 150 nM at 37°C and 3.4 nM at 20°C. This inhibitory potential was about 30-fold more potent than that of argifin and 9-fold weaker than that of allosamidin at 20°C (Arai *et al.*, 2000). Interestingly, the structures of these cyclic peptides complexed with chitinase B from *Serratia marcescens* showed in detail how the peptide backbone and side chains of the inhibitors mimic the interactions of the enzyme with the substrate chitooligosaccharides (Houston *et al.*, 2002). Both argifin and argadin interact with side chains (Asp-142, Glu-144, and Tyr-214) in the chitinase active site that are conserved completely and required for catalytic activity in family 18 chitinases (van Aalten *et al.*, 2000, 2001; Papanikolau *et al.*, 2001). Allosamidin inhibits chitinase B with a K_i value of 450 nM, whereas argifin (**8**) shows weaker inhibition ($K_i = 33$ μM) and argadin (**9**) shows about 20-fold stronger inhibition ($K_i = 20$ nM) than allosamidin (Houston *et al.*, 2002). Argifin has a more open and oval shape than argadin, and there is only one intramolecular hydrogen bond in argifin, but four in argadin. These results may explain the 1000-fold greater inhibition by argadin over argifin. This interesting approach based on the structures of enzyme–inhibitor complexes leads to the development of more potent inhibitors with possible chemotherapeutical potential.

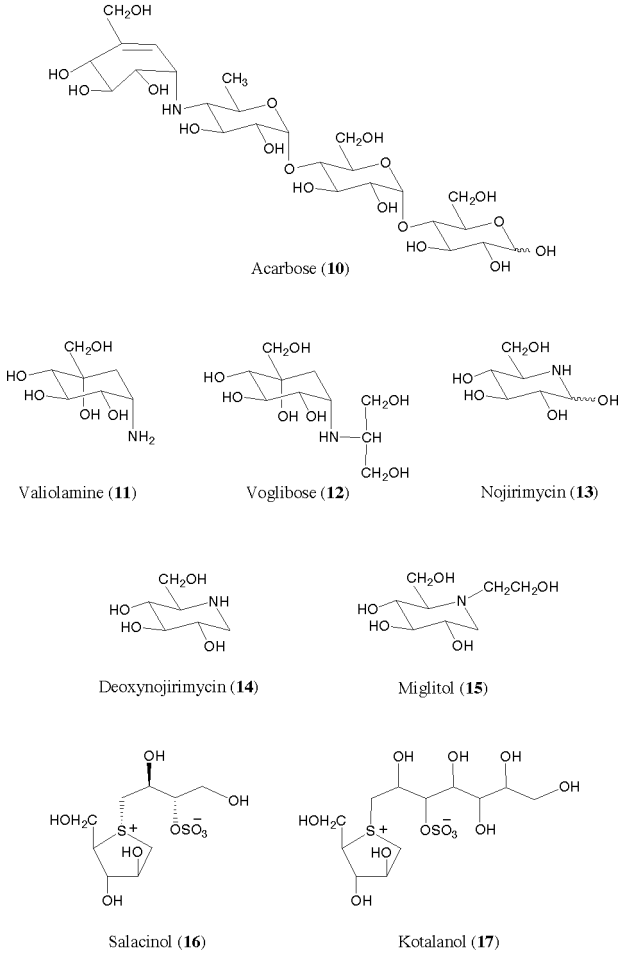


Fig. 3. Structures of digestive α-glucosidase inhibitors acarbose (**10**), valiolamine (**11**), voglibose (**12**), nojirimycin (**13**), 1-deoxynojirimycin (**14**), miglitol (**15**), salacinol (**16**), and kotalanol (**17**).

Antidiabetic agents

Digestive α-glucosidase inhibitors

In the 1970s, it was realized that inhibition of all or some of the intestinal disaccharidases and pancreatic α-amylase by inhibitors could regulate the absorption of carbohydrate and these inhibitors could be used therapeutically in the oral treatment of the non–insulin-dependent diabetes mellitus (type II diabetes). Bayer's researchers found that the *Actinoplanes* strain SE 50 yields a potent sucrase inhibitor, acarbose (**10**, Figure 3), which inhibits pig intestinal sucrase with an IC_{50} value of 0.5 μM (Schmidt *et al.*, 1977). Acarbose was also effective in carbohydrate loading tests in rats and healthy volunteers, reducing postprandial blood glucose and increasing insulin secretion (Puls *et al.*, 1977). After intensive clinical development, acarbose (Glucobay) was introduced onto the market in Germany in 1990 for the treatment of diabetes and has since been successfully marketed in Europe and Latin America. In 1996, acarbose was introduced onto the market in the United States under the brand name Precose. Thus acabose provided the physician with the first new therapeutic principle for the treatment of dibetes in nearly 40 years.

In 1984, the validamycin A–producing organism *Streptomyces hygroscopicus* var. *limoneus* was reported to coproduce valiolamine (**11**, Figure 3), which is a potent inhibitor of pig intestinal maltase and sucrase with IC_{50} values of 2.2 and 0.049 μM, respectively (Kameda *et al.*, 1984). Numerous *N*-substituted valiolamine derivatives were synthesized to enhance its α-glucosidase inhibitory activity *in vitro* and the very simple derivative voglibose (AO 128) (**12**, Figure 3), which was obtained by reductive amination of valiolamine with dihydroxyacetone, was selected as the potential oral antidiabetic agent (Horii *et al.*, 1986). Its IC_{50} values toward maltase and sucrase were 0.015 and 0.0046 μM, respectively. Voglibose (Basen) has been commercially available for the treatment of diabetes in Japan since 1994.

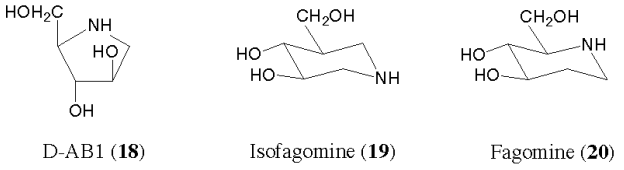
In 1966 nojirimycin (**13**, Figure 3) was discovered as the first glucose analog with the nitrogen atom in place of the ring oxygen (Inoue *et al.*, 1966). Nojirimycin was first described as an antibiotic produced by *Streptomyces*

roseochromogenes R-468 and *S. lavendulae* SF-425 and was shown to be a potent inhibitor of α- and β-glucosidases from various sources (Niwa *et al*., 1970). However, because this iminosugar with the hydroxyl group at C-1 is fairly unstable, it is usually stored as bisulfite adducts or it may be reduced by catalytic hydrogenation with a platinum catalyst or by $NaBH_4$ to 1-deoxynojirimycin (DNJ) (**14**, Figure 3) (Inoue *et al*., 1967). DNJ was later isolated from the roots of mulberry trees and called molanoline (Yagi *et al*., 1976). DNJ is also produced by many strains in the genera *Bacillus* and *Streptomyces* (Schmidt *et al*., 1979; Murao and Miyata, 1980; Ezure *et al*., 1985). Despite the excellent α-glucosidase inhibitory activity *in vitro*, its efficacy *in vivo* was only moderate (Junge *et al*., 1996). Therefore, a large number of DNJ derivatives were prepared in the hope of increasing the *in vivo* activity. Thus miglitol (BAY m 1099) (**15**, Figure 3) was selected as the most favorable inhibitor out of a large number of *in vitro* active agents. Miglitol differs from acarbose (**10**, Figure 3) in that it is almost completely absorbed from the intestinal tract and may possess systemic effects in addition to the effects in the intestinal border (Joubert *et al*., 1987, 1990). In 1996, miglitol (Glyset) was granted market clearance by the U.S. Food and Drug Administration (FDA) and was introduced onto the market in 1999 as a more potent second-generation α-glucosidase inhibitor with fewer gastrointestinal side effects.

Current scientific evidence demonstrates that much of the morbidity and mortality of diabetes can be eliminated by aggressive treatment with diet, exercise, and new pharmacological approaches to achieve better control of blood glucose level. Furthermore, the possibility of preventing the onset of diabetes using dietary supplements and/or herbal medicines has attracted increasing attention. *Salacia reticulata* Wight, known as kothalahimbutu in Singhalese and distributed in Sri Lanka and Indian forests, has been used as a supplementary food in Japan to prevent obesity and diabetes. Traditionally, ayurvedic medicine advised that a person suffering from diabetes should drink water left overnight in a mug carved from kothalahimbutu wood. Salacinol (**16**, Figure 3) and kotalanol (**17**, Figure 3) have been identified as α-glucosidase-inhibiting components from the water-soluble fraction of the roots and stems of *S. reticulata* (Yoshikawa *et al*., 1997, 1998). The IC_{50} values of salacinol toward rat intestinal maltase, sucrase, and isomaltase are 3.2, 0.84, and 0.59 μg/ml, respectively. The inhibitory activities toward maltase and sucrase are nearly equal to those of acarbose (**10**, Figure 3) and that toward isomaltase is much more potent than that of acarbose. Kotalanol shows a more potent inhibitory activity than salacinol and acarbose toward surase. Furthermore salacinol has been found to more strongly inhibit the increase of serum glucose levels in sucrose-loaded rats than acarbose (Yoshikawa *et al*., 1998). The use of dietary supplement to prevent or treat diabetes will increase dramatically as knowledge about bioactive components of food in health increases.

Glycogen phosphorylase inhibitors

In type II diabetes, hepatic glucose production is increased (Defronzo *et al*., 1992). A possible way to suppress hepatic glucose production and lower blood glucose in type II diabetes patients may be through inhibition of hepatic glycogen phosphorylase (Martin *et al*., 1991). In enzyme assays, 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) (**18**, Figure 4), which was first isolated from the fruits of the legume *Angylocalyx boutiquenus* (Nash *et al*., 1985), was found to be a potent inhibitor of hepatic glycogen phosphorylase (Fosgerau *et al*., 2000). D-AB1 further inhibited hepatic glycogen breakdown *in vivo* and displayed an accompanying antihyperglycemic effect, which was most pronounced in obese mice (Fosgerau *et al*., 2000). In primary rat hepatocytes, D-AB1 was also shown to be the most potent inhibitor ever reported ($IC_{50} = 1$ μM) of basal and glucagon-stimulated glycogenolysis (Andersen *et al*., 1999). Recently the synthetic piperidine alkaloid isofagomine (**19**, Figure 4), (3R,4R,5R)-5-hydroxymethylpiperidine-3,4-diol, has been reported to potently inhibit hepatic glycogen phosphorylase with an IC_{50} value of 0.7 μM, and to prevent basal and glucagon-stimulated glycogen degradation in cultured hepatocytes with IC_{50} values of 2–3 μM (Jakobsen *et al*., 2001). However, its *N*-substitution always resulted in a loss of activity compared to the parent compound, and fagomine ((2*R*,3*R*,4*R*)-5-hydroxymethylpiperidine-3,4-diol) (**20**, Figure 4) was a weak inhibitor of this enzyme, with an IC_{50} value of 200 μM (Jakobsen *et al*., 2001). Inhibitors of glycogen phosphorylase would be a beneficial target to attack in the development of new antihyperglycemic agents because such inhibitors have not yet been on the market for the treatment of type II diabetes.



D-AB1 (**18**) Isofagomine (**19**) Fagomine (**20**)

Fig. 4. Structures of glycogen phosphorylase inhibitors D-AB1 (**18**), isofagomine (**19**), and fagomine (**20**).

Antiviral agents

Processing α-glucosidase I inhibitors

The biosynthesis of the oligosaccharide chains of the *N*-linked glycoproteins is initiated by the cotranslational transfer of a common oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$) to the glycosylation site (Asn-X-Ser/Thr) of newly synthesized polypeptides in the endoplasmic reticulum (ER) (Elbein, 1987, 1991). The *N*-glycan chain is modified by a series of reactions within the ER and Golgi apparatus, including trimming by the action of specific processing α-glucosidases and α-mannosidases and elongation by the addition of fucose, galactose, *N*-acetylglucosamine, sialic acids, and sulfate catalyzed by glycosyltransferases and sulfotransferases. The viral envelope glycoproteins are often essential for virion assembly and secretion and/or infectivity. Compounds that interfere with the glycosylation processes of viral glycoproteins can be expected as antiviral agents. In fact, α-glucosidase inhibitors such as DNJ

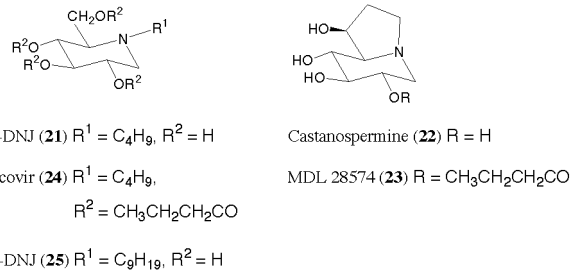


Fig. 5. Structures of processing α-glucosidase inhibitors NB-DNJ (**21**), castanospermine (**22**), 6-*O*-butanoylcastanospermine (MDL 28574) (**23**), glycovir (SC 49483) (**24**), and NN-DNJ (**25**).

(**14**, Figure 3), *N*-butyl-DNJ (NB-DNJ) (**21**, Figure 5), castanospermine (**22**, Figure 5), and 6-*O*-butanoylcastanospermine (MDL 28574) (**23**, Figure 5) inhibit human immunodeficiency virus (HIV) replication and HIV-mediated syncytium formation *in vitro* (Karpas *et al*., 1988; Fu *et al*., 1993; Ficher *et al*., 1995; Taylor *et al*., 1991). The anti-HIV activity (EC_{50}) determined by syncytial counts of compounds **14**, **21**, **22**, and **23** are 560, 56, 29, and 1.1 μM, respectively, but these compounds are not as potent as zidovudine ($EC_{50} = 0.1$ μM) (Taylor *et al*., 1991). These sugar analogs showing anti-HIV activity have the common property that they are potent processing α-glucosidase inhibitors but not processing α-mannosidase inhibitors. The activities of these inhibitors toward processing α-glucosidase I correlate well with the antiviral effects (Taylor *et al*., 1991; Sunkara *et al*., 1989). The *in vivo* data obtained to date do not promise practical use of processing α-glucosidase I inhibitors as anti-HIV agents. Problems exist in achieving therapeutic serum concentrations of inhibitors needed to inhibit α-glucosidase I sufficiently and side effects such as diarrhea occur. With respect to diarrhea, the prodrug of NB-DNJ, glycovir (SC 49483) (**24**, Figure 5), was developed as a candidate anti-HIV agent. This prodrug is NB-DNJ tetrabutanoate, which will be converted into active NB-DNJ after it passes through the intestines, avoiding diarrhea (Cook *et al*., 1995). However, it is believed that morphologic changes in various tissue cells were the result of nonspecific inhibition of host α-glucosidases by the prodrug, causing clinically silent perturbation in host cell glycoprotein processing and/or glycoprotein transport (Khan *et al*., 1996).

In contrast to the heavily glycosylated HIV envelope glycoproteins, the envelope glycoproteins of hepatitis B virus (HBV) contain only two glycosylation sites (Mehta *et al*., 1998). However, the HBV glycoproteins are sensitive to inhibitors of the *N*-linked glycosylation pathway. In this virus, correct glycosylation appears to be necessary for processes involved in transport of the virus out of the host cell. *In vitro* treatment of HBV with NB-DNJ results in a high proportion of virus particles being retained inside the cells (Block *et al*., 1994). Recent work has shown that *N*-nonyl-DNJ (NN-DNJ) (**25**, Figure 5) reduced the viremia in chronically infected woodchucks in a dose-dependent manner (Block *et al*., 1998). NN-DNJ is 100–200 times more potent than NB-DNJ in inhibiting HBV in cell-based assays (Mehta *et al*., 1998). Furthermore NN-DNJ, compared with NB-DNJ, exhibits a prolonged hepatic retention of bovine viral diarrhea virus, a tissue culture surrogate of human hepatitis C virus (Zitzmann *et al*., 1999). A single drug against HBV and hepatitis C may be of great therapeutic value. However when processing α-glucosidase inhibitors are used as antiviral agents, it remains to be determined what effects occur on host cell glycoprotein processing and/or glycoprotein transport.

Neuraminidase inhibitors

The influenza pandemic of 1918–19, known as Spanish flu that killed more than 20 million people, prompted a substantial research effort to find a preventive agent and/or cure for this oldest and most common disease. There are two major surface antigen proteins, hemagglutinin and neuraminidase (sialidase; NA), which are seen as spikes covering the surface of the viral particle (Winn and Westenfeld, 1995). In addition to these two proteins, influenza A virus has a small hydrophobic protein (M2) that functions as an ion channel selective for monovalent ions (Pinto *et al*., 1992). Until the approval of new agents (NA inhibitors) in 1999, there were two options available: vaccines and the antiviral drugs amantadine and rimantadine. The M2 inhibitors, amantadine and rimantadine, are both clinically effective for the treatment of influenza , but not influenza B. The use of these drugs are of limited usefulness because of the lack of activity against influenza B virus and the emergence of drug-resistant virus, often occurring as rapidly as 2 days after start of therapy (Hayden and Couch, 1992; Aoki, 1998).

NA performs a vital function in the final stage of the viral life cycle. NA-deficient influenza virus is still infective, but the budding virus particles form aggregates or remain bound to the infected cell surface (Liu *et al*., 1995). That is, the lack of NA makes it difficult for virus to travel from one cell to another. Hence, viral NA is a potential and promising molecular target for anti-influenza drugs. The first substrate-based NA inhibitor described was Neu5Acen (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; DANA) (**26**, Figure 6) (Meindl and Tuppy, 1969). DANA is a reasonably potent inhibitor of influenza NA with a K_i value in the micromolar range, but it is not selective for influenza NA (Meindl *et al*., 1974; Holzer *et al*., 1993). Despite *in vivo* less efficacy of DANA (Palese and Schulman, 1977) and the possibility that such compounds may not be suitable candidates as anti-influenza drugs, considerable research effort has been devoted to the development of substrate-based NA inhibitors. Introduction of the trifluoroacetamido group in place of the acetamido of DANA to give 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA) (**27**, Figure 6) resulted in a slight improvement in inhibitory activity ($K_i = 0.8$ μM) toward influenza NA (Palese *et al*., 1974), but it was ineffective in animal models of influenza infection (Palese and Schulman, 1977).

A breakthrough for rational drug design was certainly the deciphering of influenza NA's three-dimensional structure in 1983 (Varghese *et al*., 1983; Colman *et al*., 1983). Computational analysis of the catalytic site revealed that replacement of the C-4 OH group in DANA by an amino group, to give 4-amino-4-deoxy-DANA (**28**, Figure 6), would be

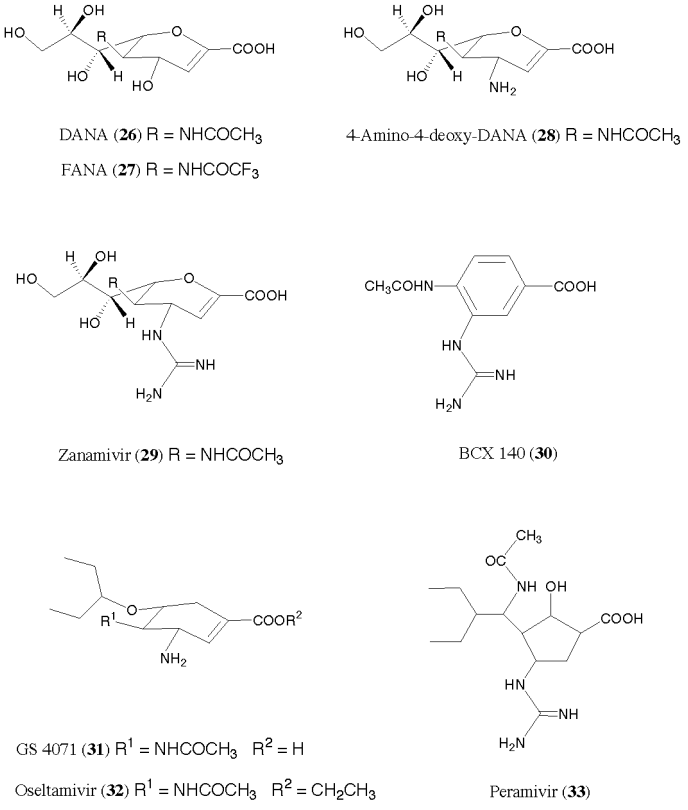


Fig. 6. Structures of NA inhibitors DANA (**26**), FANA (**27**), 4-amino-4-deoxy-DANA (**28**),4-deoxy-4-guanidino-DANA (zanamivir) (**29**), 4-(acetylamino)-3-guanidinobenzoic acid (BCX 140) (**30**), oseltamivir carboxylate (GS 4071) (**31**), oseltamivir (GS 4104) (**32**), and peramivir (BCX 1812, RWJ 270201) (**33**).

beneficial (von Itzstein *et al*., 1993, 1996). This approach further predicted a higher affinity of the 4-deoxy-4-guanidino derivative of DANA, zanamivir (**29**, Figure 6), for the binding site (von Itzstein *et al*., 1993). Zanamivir (Relenza) inhibits NA of both influenza A and B strains in the subnanomolar range (Taylor and von Itzstein, 1994; von Itzstein *et al*., 1994) and received FDA approval in 1999.

There has also been considerable effort expended in the search for noncarbohydrate-based zanamivir analogs, resulting in the development of benzoic acid mimics of zanamivir (Singh *et al*., 1995; Williams *et al*., 1995; Chand *et al*., 1997; Sudbeck *et al*., 1997). Among benzoic acid derivatives, the most potent inhibitor was 4-(acetylamino)-3-guanidinobenzoic acid (BCX 140) (**30**, Figure 6), with an IC_{50} value of 2.5 μM (Singh *et al*., 1995). BCX 140 was oriented in the active site of the NA in a manner that was not predicted from the reported active site binding of zanamivir with NA (Chand *et al*., 1997). Unfortunately, BCX 140 did not show *in vivo* activity when dosed intranasally in a mouse model of influenza. Although it is not clear why BCX 140 was not active *in vivo*, it is possible that it may be removed rapidly from lung by metabolism and/or absorption (Chand *et al*., 1997).

Zanamivir must be administered intranasally or by inhalation because of low oral bioavailability due to the positive charge of the guanidino group. The carbocyclic zanamivir mimetic, oseltamivir carboxylate (GS 4071) (**31**, Figure 6), inhibits influenza NA with equal potency ($IC_{50} = 1$ nM) to zanamivir (Kim *et al*., 1997) and was also approved by the FDA in 1999. Oseltamivir carboxylate is more lipophilic than zanamivir and lacks a guanidino group but still suffers from low oral bioavailability due to a negatively charged carboxylate moiety (Li *et al*., 1998). Hence oseltamivir (GS 4104) (Tamiflu) (**32**, Figure 6), the ethyl ester of **31**, was developed to improve oral bioavailability (Li *et al*., 1998). Oseltamivir is a prodrug metabolized by liver esterases to its active form **31** and was approved by the FDA in October 1999. Furthermore, the FDA has recently expanded the labeling for this compound to include the prevention of influenza. More recently, the tetrasubstituted cyclopentane derivative, peramivir (BCX 1812, RWJ 270201) (**33**, Figure 6), has been synthesized and found to be a potent inhibitor of influenza A and B NA, with IC_{50} values of <1 and <10 nM, respectively (Chand *et al*., 2001). These IC_{50} values are comparable or superior to those of zanamivir. BCX 1812 is orally active and is in Phase III clinical trials in North America and Europe. Thus protein crystals enable scientists to determine the three-dimensional structure of the enzyme and to build drugs designed to fit its active site. The success of the structure-based drug design through computational analysis provides encouragement for future efforts targeted at other diseases.

Molecular therapy for human genetic disorders

Ceramide glucosyltransferase (glucosylceramide synthase) inhibitors: substrate deprivation therapy for lysosomal storage diseases

Glycosphingolipid (GSL) storage diseases are relatively rare hereditary disorders that are severe in nature and frequently fatal (Neufeld, 1991). Possible strategies for the treatment of these lysosomal storage diseases include enzyme replacement therapy, gene therapy, substrate deprivation, and bone marrow transplantation. The only successful treatment for such diseases to date is the enzyme replacement therapy for patients with type 1 Gaucher disease (Barton *et al*., 1990). This disease results from mutations in the glucocerebrosidase gene, which leads to the storage of glucosylceramide (GlcCer). Genzyme introduced Ceredase in 1991 and its recombinant successor Cerezyme in 1994. In 2001, Fabrazyme was launched in the European Union for the treatment of Fabry diseases and has just received FDA approval in April 2003. Fabry disease is a rare hereditary disorder caused by the deficiency of the essential enzyme α-galactosidase A (α-Gal A), resulting in renal failure along with premature myocardial infarction and strokes (Brady *et al*., 1967; Desnick *et al*., 1995). The greatest problem in enzyme replacement is the cost, which can prevent many patients from obtaining treatment.

There is a new approach that may be generally applicable to the GSL storage disorders. As long as the biosynthesis of substrates continues, the pathological accumulation of undegraded substrates in the lysosomes proceeds. If substrate influx into the lysosomes could be reduced by inhibition of GSL biosynthesis, it should be possible to positively influence the severity as well as the onset of these diseases

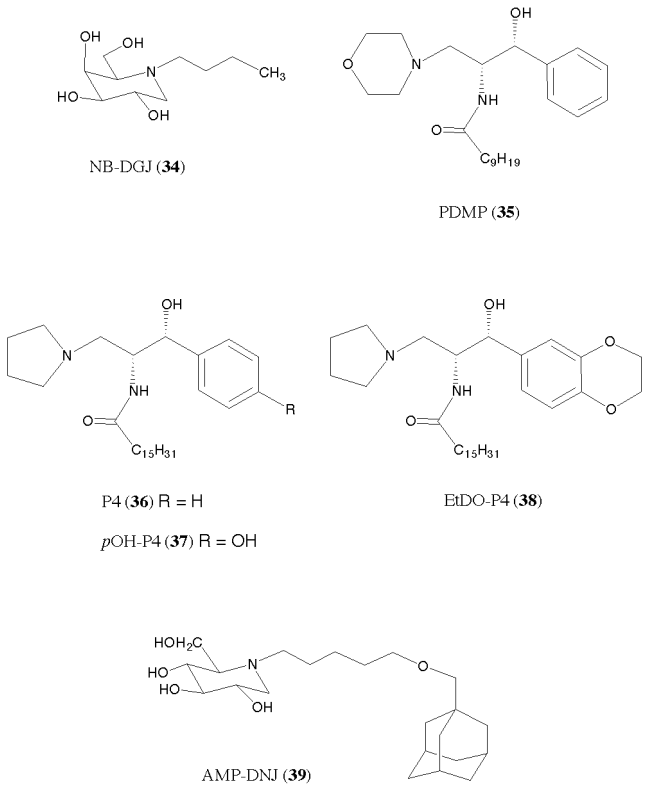


Fig. 7. Structures of ceramide glucosyltransferase (glucosylceramide synthase) inhibitors NB-DGJ (**34**), PDMP (**35**), P4 (**36**), *p*OH-P4 (**37**), EtDO-P4 (**38**), and AMP-DNJ (**39**).

with the aid of inhibitors. Tifft and Proia (2000) have reviewed significant progress in the use of synthesis inhibitors as therapy for the GSL lysosomal storage diseases. There are two classes of GSL synthesis inhibitors in possible therapeutic agents for the treatment.

NB-DNJ (**21**, Figure 5) and *N*-butyl-1-deoxygalactonojirimycin (NB-DGJ) (**34**, Figure 7) were discovered to be specific inhibitors of glucosyltransferase-catalyzed biosynthesis of glucosylceramide (Platt *et al*., 1994a,b). Inhibitors with no effect on the maturation of *N*-linked oligosaccharides or on lysosomal glucocerebrosidase and with oral availability and low toxicity would be certainly a candidate toward the molecular therapy of glucosylceramide-based GSL storage disorders. Oxford GlycoSciences performed multiple clinical studies using NB-DNJ in type 1 Gaucher disease patients in the United Kingdom and Europe in 1999–2000. The result of this clinical trial indicates that NB-DNJ (OGT 918) is effective in depleting GSLs and leads to an improvement in clinical parameters measuring disease progression (Cox *et al*., 2000). Oxford GlycoSciences has filed a marketing approval application for Zavesca (formerly known as Vevesca or OGT 918) with the European Agency for the Evaluation of Medicinal Products. In 2002, Zavesca received a positive opinion recommending approval in the European Union for use in patients with mild to moderate type 1 Gaucher disease. On the other hand, the FDA rejected approval for Zavesca, indicated that Oxford GlycoSciences has not provided sufficient support for the safety and efficacy of the developmental compound and cited the necessity for the company to address these issues in further clinical trials. Because the drug's weakness as a monotherapy is pointed out, this drug's best hope seems to demonstrate clinical benefit in combination with Cerezyme. In combination with Zavesca, Cerezyme treatment could become much less expensive and less traumatic because is administered as an intravenous infusion and Zavesca is orally available as a pill.

The prototype of another class of GSL synthesis inhibitors is a ceramide-based inhibitor. D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (**35**, Figure 7) inhibits glucosylceramide synthase (GlcT-1) and produce the reversible depletion of cellular GSLs (Abe *et al*., 1992). PDMP is a more potent inhibitor of GlcT-1 than NB-DNJ but has an undesirable side effect of increasing intracellular ceramide levels, resulting in cytotoxicity (Abe *et al*., 1996). PDMP has been found to have two sites of action, one for the inhibition of GlcT-1 and one for the inhibition of 1-*O*-acylceramide synthetase (Abe and Shayman, 1998). Although the inhibition of GlcT-1 resulted in the cellular depletion of GSLs (Abe *et al*., 1995), that of 1-*O*-acylceramide synthetase resulted in the elevation of intracellular ceramide levels and inhibition of cell growth *in vitro* (Abe and Shayman, 1998; Lee *et al*., 1999). Subsequently, D-*threo*-1-phenyl-2-palmitoilamino-3-pyrrolidino-1-propanol (P4) (**36**, Figure 7), 4′-hydroxy-P4 (*p*OH-P4) (**37**, Figure 7), and 3′,4′-ethylenedioxy-P4 (EtDO-P4) (**38**, Figure 7) have been developed using PDMP as the lead compound (Lee *et al*., 1999). *p*OH-P4 and EtDO-P4 blocked GlcT-1 activity at concentrations that had little effect on 1-*O*-acylceramide synthetase activity and resulted in the inhibition of GSL synthesis in cultured cell without the elevation of intracellular ceramide levels and inhibition of cell growth (Lee *et al*., 1999). In 2001 Genzyme released preclinical data supporting Genz-78132 (EtDO-P4) as the second-generation substrate-deprivation agent. The company has reports 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) (**39**, Figure 7), and is at least 20 times more potent *in vivo* than AMP-DNJ, the most potent compound (Overkleeft *et al*., 1998). Although there is no supporting clinical data, Genz-78132 may allow the administration of small quantities of a drug to achieve the desired therapeutic effect, minimizing the potential for toxic side effects.

Lysosomal glycosidase inhibitors: chemical chaperone therapy for lysosomal storage diseases

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. Their 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 (Gething and Sambrook, 1992; Helenius *et al*., 1992). The ER possesses efficient quality control mechanisms to ensure

that transport is limited to properly folded and assembled proteins (Hurtley and Helenius, 1989). Recent experimental data show that some human genetic diseases are due to mutations in proteins that influence their folding and lead to retaining of mutant proteins in the ER and successive degradation (Bychkova and Ptitsyn, 1995; Welch and Brown, 1996).

Genetically inherited diseases are often characterized by specific point mutations or deletions that give rise to proteins that fail to achieve their properly folded state. In some cases, the mutations are relatively minor and result in the protein exhibiting only a partial loss of its normal activity. In the classic type of α_1-antitripsin deficiency (the most common genetic cause of enphysema in adults and liver diseases in children) patients with the Z mutation secrete only about 15% of newly synthesized proteins into the extracellular fluid and the remainder of the mutant protein is retained in the ER (Teckman and Perlmutter, 1996). However, this secreted mutant protein retains about 80% of the functional activity of its wild-type counterpart (Bathurst *et al*., 1984; Ogushi *et al*., 1987). Although some of the retained mutant protein is eventually degraded, the remainder tends to form aggregates in the ER (Lomas *et al*., 1992; Yu *et al*., 1995). Over time the accumulation of the aggregate interferes with the normal functional activities of the hepatocyte.

Another example of a disease involving abnormal protein trafficking is cystic fibrosis (CF). CF is a human genetic disease caused by mutations in CF transmembrane conductance regulator (CFTR), which functions as a chloride channel in the plasma membrane (Sferra and Collins, 1993; Welsh and Smith, 1993). The most common mutation observed in patients with CF (over 70%) 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 (Cheng *et al*., 1990). The defective folding in the ΔF508 CFTR mutant is temperature sensitive. The processing of the ΔF508 CFTR reverts toward that of wild type as the incubation temperature is reduced below 30°C. When the processing defect is corrected, cAMP-regulated Cl^- channels appear in the plasma membrane (Denning *et al*., 1992). Hence, the major problem in α_1-antitrypsin and CF appears to be a failure of the newly synthesized mutant proteins to exit from the ER and to move to their proper locale. These observations raise the possibility that a functional compound that can elicit the proper folding and trafficking of the mutant protein might prove to be effective strategy for the treatment of the genetic disorders.

Studies on residual α-Gal A activity of mutant enzymes in many Fabry patients revealed that some had kinetic properties similar to those of normal α-Gal A but were significantly less stable (Remeo *et al*., 1975). For example, purified mutant α-Gal A with Q279E mutation detected in Fabry patients with the cardiac variant form had the same K_m and V_{max} as the normal enzyme. However, the mutant enzyme lost most of its catalytic activity after incubation at pH 7.0 at 37°C for 30 min, whereas the normal enzyme was relatively stable under the same conditions (Ishii *et al*., 1993). Both mutant and normal enzymes were stable at pH 5.0 at 37°C for 30 min. Furthermore the intracellular mutant protein formed aggregates in the ER and was quickly degraded (Ishii *et al*., 1996). These results suggest that the enzyme deficiency in this mutant is caused primarily by abortive exit from the ER.

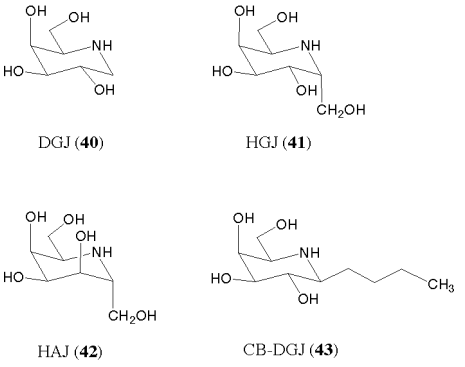


Fig. 8. Structures of lysosomal glycosidase inhibitors DGJ (**40**), HGJ (**41**), HAJ (**42**), and CB-DGJ (**43**).

Recently, 1-deoxygalactonojirimycin (DGJ) (**40**, Figure 8), a potent competitive inhibitor of α-Gal A, effectively enhanced the mutant enzyme activity in lymphoblasts established from Fabry patients with R301Q or Q279E mutation (Fan *et al*., 1999). Furthermore, oral administration of DGJ to transgenic mice expressing a human mutant α-Gal A (R301Q) substantially elevated the enzyme activity in the major organs. DGJ seems to assist the successful transport of the mutant enzyme from the ER to the Golgi apparatus and its correct targeting to the lysosome. It appears that by occupying the catalytic site of the mutant enzyme, DGJ stabilizes its conformation, thus allowing resumption of the glycoprotein processing leading to maturation of the enzyme. Thus a functional compound that can elicit the correct folding of a mutant protein may serve as a specific chemical chaperone for the mutant protein to promote the successful escape from the ER quality control mechanisms (Fan *et al*., 1999). To establish the concept of using competitive inhibitors as specific chemical 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. DGJ, α-homogalactonojirimycin (HGJ) (**41**, Figure 8) (Martin *et al*., 1995), α-homoallonojirimycin (HAJ) (**42**, Figure 8) (Asano *et al*., 1997; Martin *et al*., 1999), and β-1-*C*-butyl-DGJ (CB-DGJ) (**43**, Figure 8) (Ikeda *et al*., 2000) were inhibitors of α-Gal A with IC_{50} values of 0.04, 0.21, 4.3, and 16 μM, respectively, and addition of DGJ, HGJ, HAJ, and CB-DGJ at 100 μM to culture medium of Fabry lymphoblasts increased the intracellular α-Gal A activity by 14-, 5.2-, 2.4-, and 2.3-fold, respectively (Asano *et al*., 2000). These results suggest that more potent inhibitors are more effective chemical chaperone for the mutant enzyme and Fabry disease.

The chemical chaperone therapy established in Fabry disease is also applicable to the other type of lysosomal storage diseases. DGJ (**40**, Figure 8) and NB-DGJ (**34**, Figure 7) are also moderate inhibitors of lysosomal β-galactosidase, with IC_{50} values of 25 μM (Tominaga *et al*., 2001). Both compounds restored mutant enzyme activities in cell

lines of β-galactosidosis obtained by introducing mutant human β-galactosidase cDNAs into knockout mouse fibloblasts (Tominaga *et al*., 2001). However, a survey of more effective compounds is needed for clinical application of this method to β-galactosidosis because relatively high concentrations (0.5–1 mM) were required for mutant enzyme activation.

Gaucher disease is a lysosomal storage disorder caused by deficient lysosomal β-glucocerebrosidase activity. Quite recently, it has been found that NN-DNJ (**25**, Figure 5) is a potent inhibitor of this enzyme, with an IC_{50} value of 1 μM, and that the addition of subinhibitory concentration (10 μM) of NN-DNJ to a fibroblast culture medium leads to a twofold increase in activity of mutant (N370S) enzyme (Sawkar *et al*., 2002). Because clinical data indicate that a small increase in enzyme activity may be effective in treating disease, this twofold increase in activity caused by NN-DNJ chemical chaperoning may be clinically useful. A number of different chemical chaperones that enhance stability of mutant proteins and enable their trafficking to the proper locale would be excellent candidates for a new molecular therapy of human genetic disorders.

Conclusions

The alteration of glycosidase activity by inhibitors *in vivo* is of great interest because of the involvement of glycosidases in a wide range of anabolic and catabolic processes, such as digestion, lysosomal catabolism of glycoconjugates, biosynthesis, ER quality control, and ER-associated degradation of glycoproteins. As reviewed, glycosidase inhibitors could have many kinds of beneficial effects as agrochemicals and therapeutic agents, such as as antifungal agents, insecticides, antidiabetics and antiobesities, antivirals, and therapeutic agents for some genetic disorders. The success of NA inhibitors as anti-influenza drugs was derived from rational designs based on detailed knowledge of the highly conserved active site of influenza virus NA and the interaction with the natural substrate, sialic acid, through computational analysis. In addition, *in vitro* and *in vivo* studies have established the NA inhibitors as effective and safe antiviral drugs. This method will surely become a primary approach for developing other therapeutic agents. There is no doubt that many more glycosidase inhibitors of practical use remain to be discovered.

Abbreviations

AMP-DNJ, *N*-(5-adamantane-1-yl-methoxypentyl)-DNJ; CB-DGJ, β-1-*C*-butyl-DGJ; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; EtDO-P4, 3′,4′-ethylenedioxy-P4; D-AB1, 1,4-dideoxy-1,4-imino-D-arabinitol; DANA, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; DNJ, 1-deoxynojiorimycin; FANA, 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid; FDA, Food and Drug Administration; GSL, glycosphingolipid; HAJ, α-homoallonojirimycin; HBV, hepatitis B virus; HGJ, α-homogalactonojirimycin; HIV, human immunodeficiency virus; NA, neuraminidase; NB-DGJ, *N*-butyl-1-deoxygalactonojirimycin; NB-DNJ, *N*-butyl-DNJ; NN-DNJ, *N*-nonyl-DNJ; P4, D-*threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; ; *p*OH-P4, 4′-hydroxy-P4.

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