Bioorganic & Medicinal Chemistry 16 (2008) 7330–7336

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

In vitro inhibition of glycogen-degrading enzymes and glycosidases by six-membered sugar mimics and their evaluation in cell cultures

Chinami Kuriyama [a], Ogusa Kamiyama [a], Kyoko Ikeda [a], Fujiko Sanae [a], Atsushi Kato [b], Isao Adachi [b], Tatsushi Imahori [c], Hiroki Takahata [c], Tadashi Okamoto [d,e], Naoki Asano [a,e,*]

[a] Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920-1181, Japan
[b] Department of Hospital Pharmacy, Toyama Medical and Pharmaceutical University, Toyama 930-0194, Japan
[c] Faculty of Pharmaceutical Sciences, Tohoku Pharmaceutical University, 4-4-1 Kamatsushima, Aoba-ku, Sendai 981-8558, Japan
[d] Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe 651-2180, Japan
[e] Cooperative Research Center of Life Science, Kobe Gakuin University, Chuo-ku, Kobe 650-8586, Japan

ARTICLE INFO

Article history:
Received 23 May 2008
Revised 12 June 2008
Accepted 12 June 2008
Available online 18 June 2008

Keywords:
Six-membered sugar mimics
Glycogen phosphorylase
Amylo-1,6-glucosidase
Hepatocytes
Caco-2
Pharmacological chaperone
Noncompetitive inhibitor

ABSTRACT

We investigated in vitro inhibition of mammalian carbohydrate-degrading enzymes by six-membered sugar mimics and their evaluation in cell cultures. 1-Deoxynojirimycin (DNJ) showed no significant inhibition toward glycogen phosphorylase (GP) but was a potent inhibitor of another glycogen-degrading enzyme, amylo-1,6-glucosidase (1,6-GL), with an IC_{50} value of 0.16 μM. In primary rat hepatocytes, the inhibition of glycogen breakdown by DNJ reached plateau at 100 μM with 25% inhibition and then remained unchanged. The potent GP inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) inhibited hepatic glucose production with an IC_{50} value of about 9 μM and the inhibition by D-AB1 was further enhanced in the presence of DNJ. DNJ and α-homonojirimycin (HNJ) are very potent inhibitors of rat intestinal maltase, with IC_{50} values of 0.13 and 0.08 μM, respectively, and also showed a similar strong inhibition toward maltase in Caco-2 cell model system, with IC_{50} value of 0.05 and 0.10 μM, respectively. D-Isofagomine (D-IFG) and L-IFG are competitive and noncompetitive inhibitors of human lysosomal β-glucosidase (β-GL), respectively, with K_i values of 8.4 nM and 6.9 μM. D-IFG increased intracellular β-GL activity by twofold at 10 μM in Gaucher N370S cell line as an 'active-site-specific' chaperone, and surprisingly a noncompetitive inhibitor L-IFG also increased intracellular β-GL activity by 1.6-fold at 500 μM.

1. Introduction

A large number of compounds mimicking the structures of monosaccharides or oligosaccharides have been isolated from plants and microorganisms.[1,2] Such sugar mimics have attracted considerable interest because of their often effective and specific inhibition of various carbohydrate-degrading enzymes involved in a wide range of important biological processes, such as intestinal digestion, hepatic glycogen breakdown, lysosomal catabolism of glycoconjugates, and maturation of the sugar chains in glycoproteins. Although sugar mimics with inhibitory activities toward carbohydrate-degrading enzymes have potential as anti-diabetics, antiobesities, antivirals, and therapeutic agents for some genetic disorders, only some inhibitors, such as acarbose (Glucobay), voglibose (Basen), and miglitol (Glyset) for the treatment of type 2 diabetes and zanamivir (Relenza) and oseltamivir (Tamiflu) as viral agents toward influenza A and B, are on the market.[3] In recent years, combinatorial methods and the rapid generation of large libraries of potential lead compounds have been favored for drug discovery. However, the rapid and practical bioassay is still a key step for the development of new drugs.

Increased hepatic glucose production is an essential feature of fasting hyperglycemia in type 2 diabetes.[4–6] A possible way to suppress hepatic glucose production and to lower blood glucose in type 2 diabetes may be through inhibition of glycogen phosphorylase (GP).[7] GP catalyzes the first step in glycogen breakdown to yield glucose 1-phosphate (glucose-1-P). In the liver, glucose-1-P is mostly converted to glucose, via glucose-6-P, to maintain blood glucose homeostasis.[8] In enzyme assay, Fosgerau et al. reported that a five-membered iminosugar 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) is a potent inhibitor of hepatic GP and is the most potent inhibitor of basal and glucagons-stimulated glycogenolysis ever reported in primary rat hepatocytes, with an IC_{50} value of 1 μM.[9,10] It has been reported that D-AB1 is a potent inhibitor of GP with anti-hyperglycemic effect in ob/ob mice.[9] We previously reported that the structural modification of D-AB1 markedly lowered or abolished the inhibitory activity toward GP.[11] Thus, we as well as Jakobsen et al.[12] also confirmed that GP has a strict structure requirement for inhibitors at the catalytic site. Glycogenolysis

* Corresponding author. Fax: +81 76 229 2781.
E-mail addresses: n-asano@hokuriku-u.ac.jp, naoki22@po.incl.ne.jp (N. Asano).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.06.026

requires the concerted action of GP and debranching enzyme, which possesses both 4-α-glucanotransferase and amylo-1,6-glucosidase (1,6-GL).[13,14] 1-Deoxynojirimycin (DNJ, **1**) (Fig. 1) is known to be a potent inhibitor of 1,6-GL both in vitro and in hepatocytes.[15,16] However, there are few reports on the effect of a combination of a GP inhibitor and a 1,6-GL inhibitor on glucose production in hepatocytes.

Intestinal α-glucosidases catalyze the final step in the digestive process of carbohydrates. Hence, α-glucosidase inhibitors can retard the absorption of dietary carbohydrates to suppress postprandial hyperglycemia. In fact, α-glucosidase inhibitors such as acarbose (Glucobay), voglibose (Basen), and miglitol (Glyset) are on the market as the therapeutic agents of diabetes. We have long used rat brush border membranes prepared from rat small intestines as an enzyme source for the first screening of α-glucosidase inhibitors. However, 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 epiterium.[17] 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.[18–21] The rapid in vitro assay with the enzyme α-glucosidase and evaluation with Caco-2 cells enable more practical selection of potential drug candidates.

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 endoplasmic reticulum (ER) and successive degradation.[22] Lysosomes are membrane-bound cytoplasmic organelles that serve as a major degradative compartment in eukaryotic cells. The degradative function of lysosomes is carried out by more than 50 acid hydrolases contained within the lumen.[23] The glycosphingolipid (GSL) storage diseases are genetic disorders in which a mutation of one of GSL glycohydrolases blocks GSL degradation, leading to lysosomal accumulation of undegraded GSL.[24] One of novel approaches in developing a molecular therapy for GSL storage diseases is a pharmacological chaperone therapy.[3,25] The concept of pharmacological chaperone therapy is that an intracellular activity of misfolded mutant enzymes can be restored by administering competitive inhibitors that serve as pharmacological chaperones. However, targets for the pharmacological chaperoning are limited to a mutation which renders the protein unstable but not inactive. Such 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.[25] A number of competitive inhibitors toward lysosomal glycosidases have potential as active-site-specific chaperones for GSL storage diseases. However, there is no report on the chaperoning activity of noncompetitive inhibitors.

We here report the effects of six-membered sugar mimics on glucose production in hepatocytes, on α-glucosidase activity with Caco-2 cells, and on chaperoning activity with fibroblasts derived from Gaucher patients.

2. Results

2.1. Preparation of six-membered sugar mimics

Naturally occurring iminosugars DNJ (**1**) and fagomine (**2**) were isolated from *Morus alba* (Moraceae),[26] and α-homonojirimycin (HNJ, **3**) and 7-*O*-β-D-glucopyranosyl-HNJ (Glc-HNJ, **4**) were prepared from *Stemona tuberosa* (Stemonaceae).[27] Miglitol (**5**) was chemically prepared by heating DNJ with 2-bromoethanol in the presence of anhydrous K_2CO_3 in DMF according to the literature.[28] 1,5-Dideoxy-1,5-imino-D-xylitol (**6**) was purchased from Industrial Research Limited (Lower Hutt, New Zealand). Both D-isofagomine (D-IFG, **7**) and L-IFG (**8**) were enantiospecifically synthesized according to the literatures.[29,30] Valiolamine (**9**) was isolated from a fermentation broth of *Streptomyces hygroscopicus* var. *limoneus* according to the literature[31] and voglibose (**10**) was obtained by reductive amination of valiolamine with dihydroxyacetone.[32] Acarbose (**11**) was purified from Glucobay Tab 100 mg using Dowex 50W-X2 (H^+ form).

2.2. Effects of six-membered sugar mimics on glycogen-degrading enzymes

We investigated the inhibitory activity of six-membered sugar mimics toward glycogen-degrading enzymes, GP and 1,6-Glu. The results are shown in Table 1. The five-membered iminosugar D-AB1 has been identified as a potent inhibitor of rabbit muscle and liver GP and displays anti-hyperglycemic effect in obese mice.[9,10] Recently, Oikonomacos et al. have reported that D-AB1-binds at the catalytic site of GP b from X-ray crystallographic investigation.[33] The six-membered iminosugar D-IFG inhibits liver GP a with an IC_{50} value of 0.7 μM, and basal and glucagon-stimulated glycogen breakdown in hepatocytes with IC_{50} values of 3.0 and 2.0 μM, respectively.[12] In the present study, D-IFG (**7**) showed an IC_{50} value of 5.8 μM toward GP b, whereas its enantiomer L-IFG (**8**) showed no significant inhibition even at 400 μM. DNJ (**1**), a strong inhibitor of mammalian α-glucosidases, was not an inhibitor of GP b, while its 2-deoxy derivative fagomine (2) is a weak inhibitor with an IC_{50} value of 200 μM.[12] Thus, D-AB1 is the most potent inhibitor ever reported and GP b has a strict structure requirement for inhibitors at the catalytic site. On the other hand, the six-membered iminosugars showed much more potent inhibition toward another glycogen-degrading enzyme 1,6-GL than the five-membered sugar mimics.[11] DNJ and HNJ (**3**) potently inhibited the enzyme with IC_{50} values of 0.19 and 0.11 μM, respectively, and the *N*-hydroxyethyl derivative **5** of DNJ retained its potency toward 1,6-GL, as seen in the *N*-hydroxyethyl derivative of D-AB1.[11].

2.3. Inhibition of glycogen breakdown by iminosugars in primary rat hepatocytes

GP inhibitors have been developed and studied as a potential therapy for improving hyperglycemia associated with type 2 diabetes.[34,35] D-AB1 has been shown to be the most potent inhibitor of GP both in enzyme assay and primary rat hepatocytes.[9–11] We investigated the effect of a 1,6-GL inhibitor on glucose production in primary rat hepatocytes (Fig. 2). D-AB1, a potent inhibitor of GP b with an IC_{50} value of 0.43 μM, inhibited glucagons-stimulated glucose production dose-dependently with an IC_{50} value of about 9 μM, whereas DNJ, a potent inhibitor of 1,6-GL with an IC_{50} value of 0.19 μM, reached plateau at 100 μM with 25% inhibition and then remained unchanged. However, the inhibition of hepatic glucose production by D-AB1 was significantly enhanced in the presence of 100 μM DNJ. This result suggests that 1,6-GL inhibitors in combination with GP inhibitors may lower the high glucose level in type 2 diabetes.

2.4. Effect of six-membered sugar mimics on rat intestinal α-glucosidases

The intestinal oligo- and disaccharidases are fixed components of the brush border membranes of the intestinal wall. These enzymes digest dietary carbohydrates to monosaccharides which are absorbed through the intestinal wall. They include maltase, isomaltase, sucrase, lactase, trehalase, and hetero-β-glucosidase. As

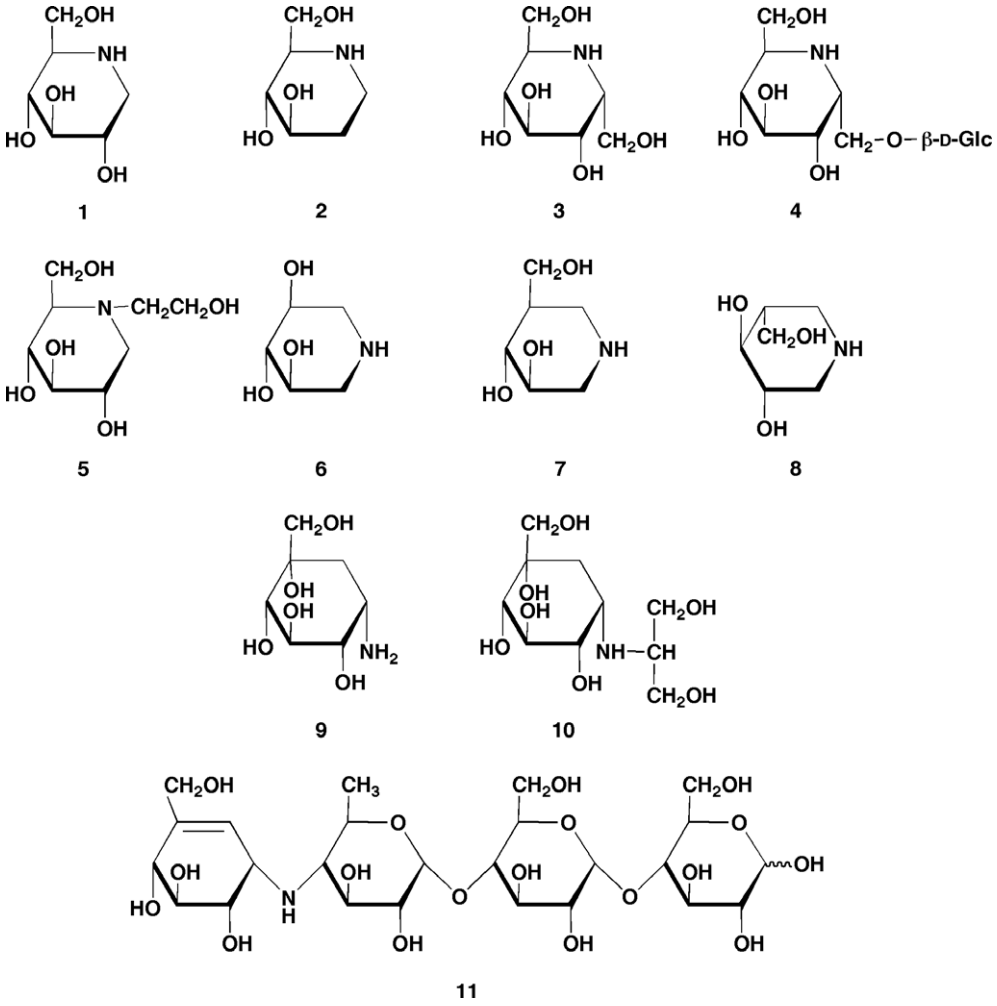

Figure 1. Structures of six-membered sugar mimics. **1**, 1-deoxynojirimycin (DNJ); **2**, fagomine; **3**, α-homonojirimycin (HNJ); **4**, 7-*O*-β-D-glucopyranosyl-HNJ (Glc-HNJ); **5**, miglitol; **6**, 1,5-dideoxy-1,5-imino-D-xylitol;**7**, D-isofagomine (D-IFG); **8**, L-isofagomine (L-IFG); **9**, valiolamine;**10**, voglibose;**11**, acarbose.

Table 1
Concentration of six-membered sugar mimics giving 50% inhibition of glucosidases, glycogen phosphorylase b (GP b), and amylo-1,6-glucosidase (amylo-1,6-Glu)

Enzyme	IC_{50} (μM)										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Gut α-glucosidase											
Rat maltase	0.13	820	0.08	0.38	1.3	—[a]	—[a]	—[a]	18	0.11	0.35
Rat isomaltase	0.16	460	0.70	0.60	1.2	—[a]	—[a]	—[a]	2.1	0.16	380
Rat sucrase	0.21	90	0.17	2.6	0.11	—[a]	—[a]	—[a]	0.69	0.07	0.56
Human lysosome											
α-Glucosidase	1.0	—[a]	1.0	18	0.35	—[a]	—[a]	—[a]	57	5.6	42
β-Glucosidase	240	—[a]	—[a]	—[a]	84	2.3	0.06	9.0	—[a]	—[a]	—[a]
Rabbit GP b	—[b]	200	—[b]	—[b]	—[b]	—[b]	5.8	—[b]	—[b]	—[b]	—[b]
Rabbit 1,6-GL	0.19	2.1	0.11	6.1	0.39	—[a]	480	—[a]	31	70	—[a]

[a] Less than 50% inhibition at 1000 μM.
[b] Less than 50% inhibition at 400 μM.

seen in Table 1, the inhibitory potential of DNJ and HNJ toward rat maltase and sucrase was identical to that of voglibose (**10**) commercially available as an anti-diabetic agent. *N*-Hydroxyethyl-DNJ (miglitol, **5**) showed about twofold stronger inhibition toward sucrase than DNJ, whereas its inhibitory activity toward maltase and isomaltase decreased by about 10-fold. Introduction of the β-D-glucopyransoyl group to the 7-OH group of HNJ to give Glc-HNJ (**4**) fairly retained its inhibitory potential toward maltase and isomaltase but lowered that toward sucrase by 10-fold. Valiolamine (**9**) is a potent inhibitor of pig intestinal maltase and sucrase, with IC_{50} values of 2.2 and 0.049 μM, respectively.[31] In the present study, it showed the IC_{50} values of 18 and 0.69 μM, respectively, toward rat intestinal maltase and sucrase, and voglibose obtained by reductive amination of valiolamine with dihydroxyacetone showed a very potent inhibitory activity toward rat maltase, isomaltase, and sucrase, with IC_{50} values of 0.11, 0.16, and 0.07 μM, respectively. Acarbose (**11**) was a slightly weaker inhibitor of maltase and sucrase than DNJ and HNJ.

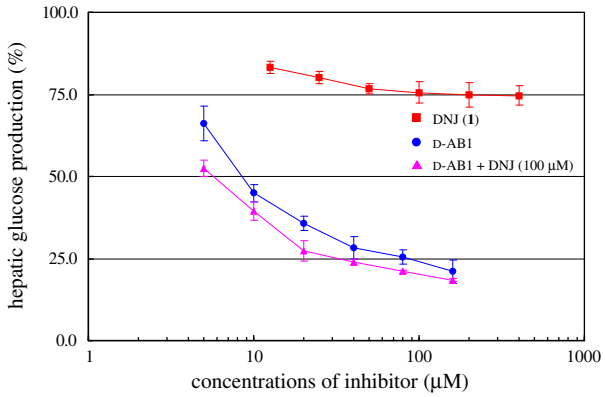

Figure 2. Effect of GP inhibitor (D-AB1) and 1,6-GL inhibitor (DNJ) on glucagon-induced glucose production in primary rat hepatocytes.

2.5. Inhibitory activity of six-membered sugar mimics using Caco-2 cells

As seen in the inhibitory activities of valiolamine, the enzyme origin greatly affects the susceptibility of inhibitors to assay enzymes. Hence, it is necessary to evaluate their inhibitory activities using human enzymes for more practical use. The experiment that we adopted is based on the assumption that maltose added to the apical side of Caco-2 monolayer is hydrolyzed to yield glucose by α-glucosidases expressed in Caco-2 cells. Inhibitory activity of DNJ, HNJ, and Glc-HNJ toward Caco-2 maltase is shown in Figure 3A. DNJ and HNJ showed the IC_{50} values of 0.06 and 0.1 μM, respectively, toward Caco-2 maltase, while Glc-HNJ was a 10-fold weaker inhibitor of Caco-2 maltase than HNJ (Fig. 3A). 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 (Fig. 3B). These results indicate that the inhibitory potentials of DNJ and HNJ toward human maltase are identical to that of voglibose.

2.6. Effect of D-IFG and L-IFG on lysosomal β-glucosidase activity in Gaucher fibroblasts

D-IFG is a very strong inhibitor of human lysosomal β-glucosidase (also known as β-glucocerebrosidase) and inhibits the enzyme in a competitive manner, with a K_i value of 0.016–0.025 μM.[36,37] Furthermore, D-IFG is also known to increase lysosomal β-glucosidase activity by 2- to 3-fold in Gaucher fibrobrasts with the N370S missense mutation as an active-site-specific chaperone.[38,39] Very interestingly, the L-enantiomer (L-IFG, **8**) of D-IFG has been found to be a noncompetitive inhibitor of human lysosomal β-glucosidase, with a K_i value of 5.7 μM.[36] In the present experiment, D-IFG (a competitive inhibitor) and L-IFG (a noncompetitive inhibitor) were incubated with N370S Gaucher fibroblasts for four days, followed by assays of cell lysates for lysosomal β-glucosidase. Treatment with 10 μM D-IFG caused twofold increase in lysosomal β-glucosidase activity compared with untreated cells and no significant change was seen in the enzyme activity at concentrations higher than 10 μM (Fig. 4A). Surprisingly, the noncompetitive inhibitor L-IFG also showed a chaperoning activity in a dose-dependent manner, with 1.6-fold increase at 500 μM (Fig. 4B). This is the first report that a noncompetitive inhibitor showed a chaperoning activity on patient cells. We further investigated whether a combination effect was manifested upon N370S Gaucher fibroblasts when competitive and noncompetitive inhibitors were 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. 4C).

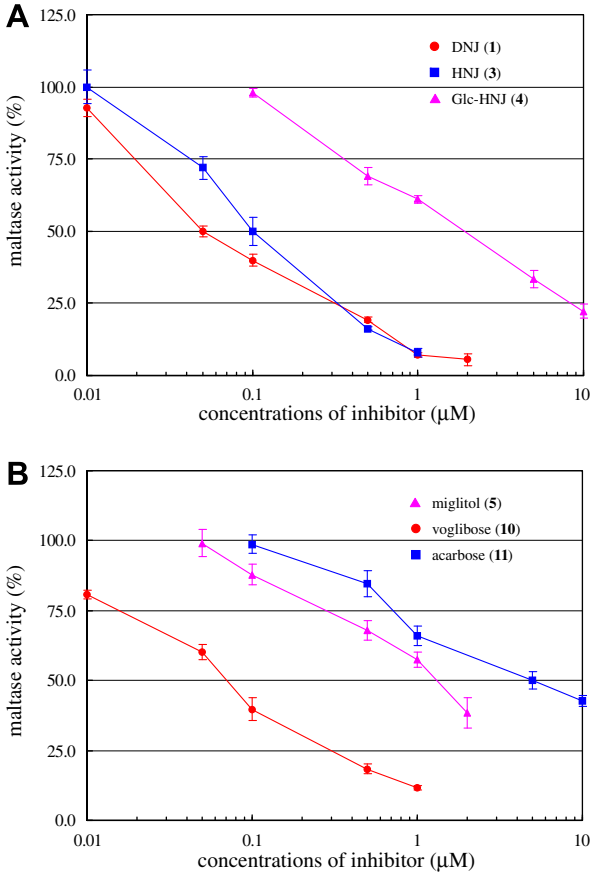

Figure 3. Maltase inhibitory activity of six-membered sugar mimics at the apical side of Caco-2 monolayer. Caco-2 cells were incubated in the presence of (A) DNJ (**1**), HNJ (**3**), Glc-HNJ (**4**) or (B) miglitol (**5**), voglibose (**10**), acarbose (**11**) for 2 h. Mean values ± SD are shown for triplicate experiments.

3. Discussion

Glycogen degradation is catalyzed by two enzymes glycogen phosphorylase (GP) and 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 1,6-GL activity of the debranching enzyme. In the liver, glucose can be produced from glucose-1-P by the successive actions of phosphoglucomutase and glucose-6-phosphatase. GP inhibitors have been developed and studied as a potential therapy for improving hyperglycemia associated with type 2 diabetes.[34,35] D-AB1 is the most potent inhibitor of GP and has been shown to inhibit glycogen breakdown both in vitro and in vivo.[9,10] Anti-hyperglycemic effect in ob/ob mice reported may be due to a combination of GP b and 1,6-GL inhibition since D-AB1 is also a fairly good inhibitor of 1,6-GL, with an IC_{50} value of 8.4 μM.[11] However, D-AB1 has not yet come into practical use as an anti-diabetic agent. It would prob-

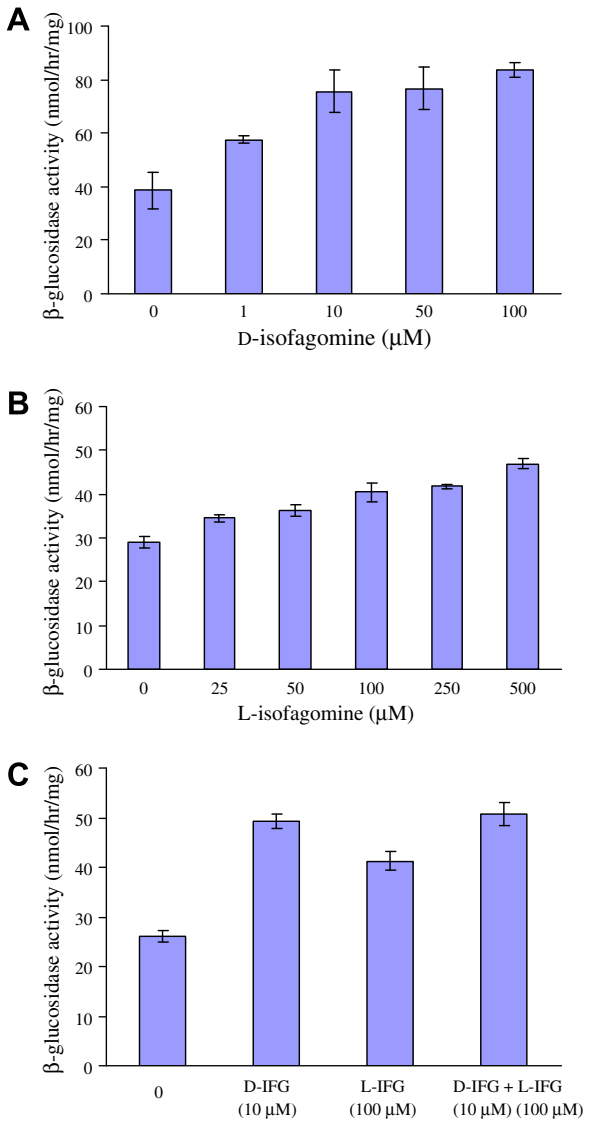

Figure 4. The influence of D-IFG (**7**) and L-IFG (**8**) on lysosomal β-glucosidase activity in N370S Gaucher fibroblasts (GM00372). The fibroblasts were incubated in the presence of (A) D-IFG, (B) L-IFG, or (C) a combination of D-IFG (10 μM) and L-IFG (100 μM) for 4 days. Mean values ± SD are shown for triplicate experiments.

ably be due to its lesser efficacy in human bodies. We thought that a combination of a potent GP inhibitor and a much more potent 1,6-GL inhibitor than D-AB1 might be more effective for inhibition of glycogen breakdown. We found that the inhibition of hepatic glucose production by D-AB1 was significantly enhanced in the presence of DNJ which showed over 40-fold stronger inhibition toward 1,6-GL than D-AB1. The present studies suggest that GP inhibitors in combination with 1,6-GL inhibitors might lower the high glucose level in type 2 diabetes.

Three α-glucosidase inhibitors acarbose (**11**), miglitol (**5**), and voglibose (**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 Table 1 and Figure 3B. However, the inhibitory activity of DNJ toward human maltase is also identical to that of voglibose (Fig. 3A). Why has DNJ not yet become an anti-diabetic agent of practical use? There is only one report, explaining the reason for this. Junge et al. explain that the efficacy of DNJ in vivo was only moderate, despite the excellent α-glucosidase inhibitory activity in vitro.[42] Hence, a large number of DNJ derivatives were prepared in the hope of increasing the in vivo activity. Thus, miglitol was selected as the most favorable inhibitor out of a large number of in vitro active agents. However, the answer to the above question may be that there is a preference for 'novelty' in patent over the efficacy of the drug in vivo. The initial occurrence of DNJ was due to reduction by catalytic hydrogenation of nojirimycin with a platinum catalyst or by $NaBH_4$ to DNJ.[43] Ten years after its initial chemical preparation, DNJ was discovered from roots of mulberry trees, and the cultured broth of various species of the genera *Bacillus* and *Streptomyces*.[44–46] Although the discovery of the inhibitory effect on mammalian α-glucosidases opened the possibility of a therapeutic application for DNJ, it had already lost '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 Thai medicinal plant 'Non tai yak' (*S. tuberosa*), respectively.[47,26,27] These traditional herbal medicines would be candidates for diabetic care and prevention.

Protein misfolding is recognized as the root of many genetic disorders. These misfolded proteins are consequently retained in the ER and degraded by ER-associated degradation (ERAD). Competitive inhibitors are often effective active-site-specific chaperones when they are used at subinhibitory concentration. 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 the genetic disease.[48] Since then, many competitive inhibitors of lysosomal glycosidases have been screened as candidates for the treatment of lysosomal storage disorders. 1-Deoxygalactonojirimycin for Fabry disease, DNJ and D-IFG and their alkyl derivatives for Gaucher disease, and DNJ for Pompe disease have been found as effective pharmacological chaperones for lysosomal storage disorders to date.[48–52] The finding in the present study that a noncompetitive inhibitor also showed a chaperoning activity is very important, because it may be effective toward the variant with a mutation that destabilizes a domain distinct from the catalytic domain. Furthermore, the present finding also suggests that ligands with pharmacological selectivity may be able to rescue misfolded proteins, including receptors, as pharmacological chaperones. Many mutations within the coding sequence of the V2 vasopressin receptor (V2R) gene are known to cause nephrogenic diabetes insipidus (NDI).[53] A large number of these mutant receptors fail to fold properly and therefore are not routed to the cell surface. Morello et al. showed that selective, nonpeptidic V2R antagonists dramatically increase cell-surface expression and rescue the function of mutant NDI-V2Rs by promoting their proper folding and maturation.[54] In addition, it has been reported that a certain mutant of the human pituitary vasopressin V3 receptor (V3R) is retained in the ER and a nonpeptide antagonist (SSR149415) behaves as a pharmacological chaperone for the ER-retained mutant V3R.[55,56] Another example of a disease involving abnormal protein trafficking is cystic fibrosis (CF). CF is a human genetic disease caused by mutations in cystic fibrosis transmembrane conductance regulator (CFTR), which functions as a chloride channel in the plasma membrane.[57,58] The most common mutation observed in patients with CF (over 70%) is a deletion of Phe-508 (ΔF508) of CFTR. A pyrazole compound with phenyl rings linked to two sites (Cfpot-532) has been demonstrated to increase ΔF508 CFTR channel activity.[59] Thus, the development of strategies aimed at promoting proper folding and maturation of mutant proteins may become the main stream of therapies for a wide spectrum of diseases. Clinical trials testing effects of pharmacological chaperones are currently underway (Amicus Therapeutics Inc., Cranbury, NJ).

4. Materials

4.1. Preparation of six-membered sugar mimics

Naturally occurring iminosugars DNJ (**1**) and fagomine (**2**) were isolated from the bark of *M. alba* (Moraceae),[26] and HNJ (**3**) and Glc-HNJ (**4**) were prepared from the roots of *S. tuberosa* (Stemonaceae),[27] according to the literature. Miglitol (**5**) was chemically prepared by heating DNJ with 2-bromoethanol in the presence of anhydrous K_2CO_3 in DMF according to the literature.[28] 1,5-Dideoxy-1,5-imino-D-xylitol (**6**) was purchased from Industrial Research Limited (Lower Hutt, New Zealand). Both D-IFG (**7**) and L-IFG (**8**) were enantiospecifically synthesized according to the literature.[29,30] Valiolamine (**9**) was isolated from a fermentation broth of *Streptomyces hygroscopicus* var. *limoneus*, which is a producer of the antifungal agent validamycin,[31] and voglibose (**10**) was obtained by reductive amination of valiolamine with dihydroxyacetone,[32] according to the literature. Acarbose (**11**) was prepared from commercially available Glucobay Tab 100 mg. A tablet was dissolved in water and the filtrate was applied to a short column of Dowex 50W-X2 (H^+ form). The column was washed with water and eluted with 0.5 M NH_4OH. The eluate was concentrated and lyophilized to give a powder of acarbose.

4.1.1. 1-Deoxynojirimycin (DNJ, 1)

$[\alpha]_D$ +40.3° (*c* 1.47, H_2O). HRMS (FAB): *m*/*z* 164.0923 $[M+H]^+$ ($C_6H_{14}NO_4$ requires 164.0923).

4.1.2. Fagomine (2)

$[\alpha]_D$ +19.5° (*c* 1.00, H_2O). HRMS (FAB): *m*/*z* 148.0977 $[M+H]^+$ ($C_6H_{14}NO_3$ requires 148.0974).

4.1.3. α-Homonojirimycin (HNJ, 3)

$[\alpha]_D$ +77.3° (*c* 0.57, H_2O). HRMS (FAB): *m*/*z* 194.1025 $[M+H]^+$ ($C_7H_{16}NO_5$ requires 194.1028).

4.1.4. 7-*O*-β-D-Glucopyranosyl-α-homonojirimycin (Glc-HNJ, 4)

$[\alpha]_D$ +24.7° (*c* 0.70, H_2O). HRMS (FAB): *m*/*z* 356.1553 $[M+H]^+$ ($C_{13}H_{26}NO_{10}$ requires 356.1557).

4.1.5. Miglitol (5)

$[\alpha]_D$ −11.0° (*c* 0.55, H_2O). HRMS (FAB): *m*/*z* 208.1183 $[M+H]^+$ ($C_8H_{18}NO_5$ requires 208.1185).

4.1.6. D-Isofagomine (D-IFG, 7)

$[\alpha]_D$ +25.4° (*c* 1.30, EtOH). HRMS (FAB): *m*/*z* 148.0975 $[M+H]^+$ ($C_6H_{14}NO_3$ requires 148.0974).

4.1.7. L-Isofagomine (L-IFG, 8)

$[\alpha]_D$ −23.8° (*c* 1.10, EtOH). HRMS (FAB): *m*/*z* 148.0974 $[M+H]^+$ ($C_6H_{14}NO_3$ requires 148.0974).

4.1.8. Valiolamine (9)

$[\alpha]_D$ +18.8° (*c* 1.00, H_2O). HRMS (FAB): *m*/*z* 194.1029 $[M+H]^+$ ($C_7H_{16}NO_5$ requires 194.1028).

4.1.9. Voglibose (10)

$[\alpha]_D$ +26.2° (*c* 1.00, H_2O). HRMS (FAB): *m*/*z* 268.1395 $[M+H]^+$ ($C_{10}H_{22}NO_7$ requires 268.1396).

4.1.10. Acarbose (11)

$[\alpha]_D$ +167.1° (*c* 1.05, H_2O). HRMS (FAB): *m*/*z* 646.2565 $[M+H]^+$ ($C_{25}H_{44}NO_{18}$ requires 646.2558).

5. Experimental

5.1. General experimental procedures

The purity of samples was checked by HPTLC on Silica Gel $60F_{254}$ (E. Merck) using the solvent systems PrOH/AcOH/H_2O (4:1:1), and a chlorine-*o*-tolidine reagent for **1**–**4** and **7**–**9** and iodine vapor for **5**, **10**, and**11** were used for detection. Optical rotations were measured with a Jasco DIP-370 digital polarimeter (Tokyo, Japan). FABMS were measured using glycerol as a matrix on a JEOL JMS-700 spectrometer.

5.2. Biological assays

Brush border membranes prepared from rat small intestine according to the method of Kessler et al.[60] were used as the source of rat intestinal glucosidases. The activity of rat intestinal α-glucosidases was determined using the appropriate disaccharides as substrates. The released D-glucose was determined colorimetrically using the Glucose CII-test Wako (Wako Pure Chemical Ind.). Human lysosomal α-glucosidase (Myozyme) and β-glucosidase (β-glucocerebrosidase, Cerezyme) were purchased from Genzyme (Boston, MA) and their activities were determined using 4-methylumbelliferyl α-D-glucoside and β-D-glucoside (Sigma Chemical Co.), respectively, as substrates. Liberated 4-methylumbelliferone was measured (excitation 362 nm, emission 450 nm) with a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Glycogen phosphorylase activity was assayed in the direction of glycogen breakdown from the rate of NADPH formation in an assay coupled to phosphoglucomutase and glucose 6-phosphate dehydrogenase.[61] Amylo-1,6-glucosidase was prepared from rabbit skeletal muscles according to the literature[62] and assayed using 6-*O*-α-D-glucosyl-α-cyclodextrin (Wako Pure Chemical Ind.) as substrate.[63,64] The released D-glucose was determined colorimetrically using the Glucose CII-test Wako. Other chemicals used for enzyme assays were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO).

Isolation and culturing of rat hepatocytes and measurements of hepatic glucose production and maltase activity using Caco-2 cells were performed according to the methods described in our recent papers.[65,66] The chaperoning effect of D- and L-isofagomines on lysosomal β-glucosidase activity in the Gaucher cell line GM00372 (Coriell Cell Repositories, Camden, NJ) with an N370S mutation was evaluated according to our previous paper.[51]

References and notes

1. Asano, N.; Nash, R. J.; Molyneux, R. J.; Fleet, D. W. J. *Tetrahedron: Asymmetry* **2000**, *11*, 1.
2. Watson, A. A.; Fleet, G. W. J.; Asano, N.; Molyneux, R. J.; Nash, R. J. *Phytochemistry* **2001**, *56*, 265.
3. Asano, N. *Glycobiology* **2003**, *13*, 93R.
4. DeFronzo, R. A. *Diabetes* **1988**, *37*, 667.
5. Tappy, L. *Diabetes Metab.* **1995**, *21*, 233.
6. Gerich, J. E. *Horm. Metab. Res.* **1992**, *26*, 18.
7. Martin, J. L.; Veluraja, K.; Ross, K.; Johnson, L. N.; Fleet, G. W. J.; Ramsden, N. G.; Bruce, I.; Orchard, M. G.; Oikonomakos, N. G.; Papageorgiou, A. C.; Leonidas, D. D.; Tsitoura, H. S. *Biochemistry* **1991**, *30*, 10101.
8. Newgard, C. B.; Hwang, P. K.; Fletterick, R. J. *Crit. Rev. Biochem. Mol. Biol.* **1989**, *24*, 69.
9. Fosgerau, K.; Westergaard, N.; Quistorff, B.; Grunner, N.; Kristiansen, M.; Lundgren, K. *Arch. Biochem. Biophys.* **2000**, *380*, 274.
10. Andersen, B.; Rassov, A.; Westergaard, N.; Lundgren, K. *Biochem. J.* **1999**, *342*, 545.
11. Minami, Y.; Kuriyama, C.; Ikeda, K.; Kato, A.; Yakebayashi, K.; Adachi, I.; Fleet, G. W. J.; Kettawan, A.; Okamoto, T.; Asano, N. *Bioorg. Med. Chem* **2008**, *16*, 2734.
12. Jakobsen, P.; Lundbeck, J. M.; Kristiansen, M.; Breinholt, J.; Demuth, H.; Pawlas, J.; Torres Candela, M. P.; Andersen, B.; Westergaard, N.; Lundgren, K.; Asano, N. *Bioorg. Med. Chem.* **2001**, *9*, 733.
13. Illingworth, B.; Larner, J.; Cori, G. T. J. *Biol. Chem.* **1952**, *199*, 631.
14. Brown, D. H.; Illingworth, B. *Proc. Natl. Acad. Sci. U.S.A.* **1962**, *48*, 1783.

15. Bollen, M.; Vandebroeck, A.; Stalmans, W. *Biochem. Pharmacol.* **1988**, *37*, 905.
16. Bollen, M.; Stalmans, W. *Eur. J. Biochem.* **1989**, *181*, 775.
17. Hidalgo, I. J.; Raub, T. J.; Borchardt, R. T. *Gastroenterology* **1989**, *96*, 736.
18. Boulenc, X.; Marti, E.; Joyeux, H.; Roques, C.; Berger, Y.; Fabre, G. *Biochem. Pharmacol.* **1993**, *46*, 1591.
19. Cogburn, J. N.; Donovan, M. G.; Schasteen, C. S. *Pharm. Res.* **1991**, *8*, 210.
20. Nishioka, T.; Kawabata, J.; Aoyama, Y. *J. Nat. Prod.* **1998**, *61*, 1413.
21. Hansawasdi, C.; Kawabata, J. *Fitoterapia* **2006**, *77*, 568.
22. Cohen, F. E.; Kelly, J. W. *Nature* **2003**, *426*, 905.
23. de Duve, C. In *Lysosome*; de Reuck, A. V. S., Cameron, M. P., Eds.; Chuchill: London, 1963; p 1.
24. Kornfeld, S.; Mellman, I. *Annu. Rev. Cell Biol.* **1989**, *5*, 483.
25. Fan, J.-Q. *Trends Pharmacol. Sci.* **2003**, *24*, 355.
26. Asano, N.; Yamashita, T.; Yasuda, K.; Ikeda, K.; Kizu, H.; Kameda, Y.; Kato, A.; Nash, R. J.; Lee, H.-S.; Ryu, K.-S. *J. Agric. Food Chem.* **2001**, *49*, 4208.
27. Asano, N.; Yamauchi, T.; Kagamifuchi, K.; Shimizu, N.; Takahashi, S.; Takatsuka, H.; Ikeda, K.; Kizu, H.; Chuakul, W.; Kettawan, A.; Okamoto, T. *J. Nat. Prod.* **2005**, *68*, 1238.
28. Yasuda, K.; Kizu, H.; Yamashita, T.; Kameda, Y.; Kato, A.; Nash, R. J.; Fleet, G. W. J.; Molyneux, R. J.; Asano, N. *J. Nat. Prod.* **2002**, *65*, 198.
29. Ouchi, H.; Mihara, Y.; Takahata, H. *J. Org. Chem.* **2005**, *70*, 5207.
30. Mihara, Y.; Ojima, H.; Imahori, T.; Yoshimura, Y.; Ouchi, H.; Takahata, H. *Heterocycles* **2007**, *72*, 633.
31. Kameda, Y.; Asano, N.; Yamaguchi, T.; Matsui, K. *J. Antibiot.* **1987**, *40*, 563.
32. Horii, S.; Fukase, H.; Matsuo, T.; Kameda, Y.; Asano, N.; Matsui, K. *J. Med. Chem.* **1986**, *29*, 1038.
33. Oikonomakos, N. G.; Tiraidis, C.; Leonidas, D. D.; Zoographos, S. E.; Kristiansen, M.; Jessen, C. U.; Nørskov-Lauritsen, L.; Agius, L. *J. Med. Chem.* **2006**, *49*, 5687.
34. Treadway, J. L.; Mendys, P.; Hoover, D. J. *Expert Opin. Investig. Drugs* **2001**, *10*, 545.
35. Somsak, L.; Nagy, V.; Hadady, Z.; Docsa, T.; Gergely, P. *Curr. Pharm. Des.* **2003**, *9*, 1177.
36. Asano, N.; Ikeda, K.; Yu, L.; Kato, A.; Takebayashi, K.; Adachi, I.; Kato, I.; Ouchi, H.; Takahata, H.; Fleet, G. W. J. *Tetrahedron: Asymmetry* **2005**, *16*, 223.
37. Zhu, X.; Sheth, K. A.; Li, S.; Chang, H.-H.; Fan, J.-Q. *Angew. Chem., Int. Ed.* **2005**, *44*, 7450.
38. Chang, H.-H.; Asano, N.; Ishii, S.; Ichikawa, Y.; Fan, J.-Q. *FEBS J.* **2006**, 4082.
39. Steet, R. A.; Chung, S.; Wustman, B.; Powe, A.; Do, H.; Kornfeld, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13813.
40. Cori, C. F.; Larner, J. *J. Biol. Chem.* **1951**, *188*, 17.
41. Brown, D. H.; Whelan, W. J. *Biochem. J.* **1960**, *76*, 264.
42. Junge, B.; Matzke, M.; Stltefuss, J. In *Handbook of Experimental Pharmacology*; Kuhlmann, J., Puls, W., Eds.; Springer-Verlag: New York, 1996; pp 411–482.
43. Inoue, S.; Tsuruoka, T.; Ito, T.; Niida, T. *Tetrahedron* **1967**, *23*, 2125.
44. Yagi, M.; Kouno, T.; Aoyagi, Y.; Murai, H. *Nippon Nogei Kagaku Kaishi* **1976**, *50*, 571.
45. Schmidt, D. D.; Frommer, W.; Müller, E.; Trusscheit, E. *Naturwissenshaften* **1979**, *66*, 584.
46. Murao, S.; Miyata, S. *Agric. Biol. Chem.* **1980**, *44*, 219.
47. Asano, N.; Tomioka, E.; Kizu, H.; Matsui, K. *Carbohydr. Res.* **1994**, *259*, 243.
48. Fan, J.-Q.; Ishii, S.; Asano, N.; Suzuki, Y. *Nat. Med.* **1999**, *5*, 112.
49. Sawker, A. R.; Chen, W.-C.; Beutler, E.; Wong, C.-H.; Balch, W. E.; Kelly, J. W. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15428.
50. Sawker, A. R.; Adamski-Werner, S. L.; Chen, W.-C.; Wong, C.-H.; Beutler, E.; Zimmer, K.-P.; Kelly, J. F. *Chem. Biol.* **2005**, *12*, 1235.
51. Yu, L.; Ikeda, K.; Kato, A.; Adachi, I.; Godin, G.; Compain, P.; Martin, O.; Asano, N. *Bioorg. Med. Chem.* **2006**, *14*, 7736.
52. Okumiya, T.; Kroos, M. A.; Vliet, L. V.; Takeuchi, H.; Van der Ploeg, A. T.; Reuser, A. J. *Mol. Genet. Metab.* **2007**, *90*, 49.
53. Birnbaumer, M.; Seibold, A.; Gilbert, S.; Ishido, M.; Barberis, C.; Antaramian, A.; Brabet, P.; Rosenthal, W. *Nature* **1992**, *357*, 333.
54. Morello, J.-P.; Salahpour, A.; Laperrière, A.; Bernier, V.; Arthus, M.-F.; Lonergan, M.; Petäjä-Repo, U.; Angers, S.; Morin, D.; Bichet, D. G.; Bouvier, M. J. *Clin. Invest.* **2000**, *105*, 887.
55. Robert, J.; Clauser, E.; Petit, P. X.; Ventura, M. A. *J. Biol. Chem.* **2005**, *280*, 2300.
56. Robert, J.; Auzan, C.; Ventura, M. A.; Clauser, E. *J. Biol. Chem.* **2005**, *280*, 42198.
57. Sferra, T. J.; Collins, F. S. *Annu. Rev. Med.* **1993**, *44*, 133.
58. Welsh, M. J.; Smith, A. E. *Cell* **1993**, *73*, 1251.
59. Wang, Y.; Bartlett, M. C.; Loo, T. W.; Clarke, D. M. *Mol. Pharmacol.* **2006**, *70*, 297.
60. Kessler, M.; Acuto, O.; Strelli, C.; Murer, H.; Semenza, G. A. *Biochem. Biophys. Acta* **1978**, *506*, 136.
61. Maddaiah, V. T.; Madsen, N. B. *J. Biol. Chem.* **1966**, *241*, 3873.
62. Taylor, C.; Cox, A. J.; Kernohan, J. C.; Cohen, P. *Eur. J. Biochem.* **1975**, *51*, 105.
63. Tayler, P. M.; Whelan, W. J. *Arch. Biochem. Biophys.* **1966**, *113*, 500.
64. Tabata, S.; Hizukuri, S. *Eur. J. Biochem.* **1992**, *206*, 345.
65. Kato, A.; Nasu, N.; Takebayashi, K.; Adachi, I.; Minami, Y.; Sanae, F.; Asano, N.; Watson, A. A.; Nash, R. J. *J. Agric. Food Chem.* **2008**, *56*, 4469.
66. Minami, Y.; Kuriyama, C.; Ikeda, K.; Kato, A.; Takebayashi, K.; Adachi, I.; Fleet, G. W. J.; Kettawan, A.; Okamoto, T.; Asano, N. *Bioorg. Med. Chem.* **2008**, *16*, 2734.