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Tetrahedron: *Asymmetry* 16 (2005) 223–229
Tetrahedron: *Asymmetry*

The L-enantiomers of D-sugar-mimicking iminosugars are noncompetitive inhibitors of D-glycohydrolase?

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Received 1 November 2004; accepted 25 November 2004
Available online 22 December 2004

Abstract—1,4-Dideoxy-1,4-imino-L-arabinitol (L-AB1) and 2,5-dideoxy-2,5-imino-L-mannitol (L-DMDP) are much more potent inhibitors of isomaltase than their D-enantiomers. D-Enantiomers inhibited isomaltase in a competitive manner, whereas L-enantiomers were noncompetitive inhibitors of the enzyme. Similarly D-isofagomine and L-isofagomine were competitive and noncompetitive inhibitors of human lysosomal β-D-glucosidase (β-glucocerebrosidase), with K_i values of 0.016 and 5.7 μM, respectively. The multiple inhibition analysis of β-glucocerebrosidase by D-isofagomine and L-isofagomine indicated that the D-enantiomer best fits the catalysis site of β-glucocerebrosidase, while the L-enantiomer has a favorable interaction with a regulatory site other than the active site. Our recent and present results suggest that D-iminosugars are competitive inhibitors of D-glycohydrolases but their L-enantiomers are noncompetitive inhibitors.
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1. Introduction

The ability of enzymes to discriminate between enantiomers is vital for organisms. The enzyme can distinguish between the desired substrate and its mirror image (the enantiomer). This is because the enzyme has an active site that will only accept one of the enantiomers but reject the other. For instance, α-D-glucosidase cleaves the terminal D-glucosyl residue of the substrate but does not cleave the L-glucosyl residue. Enzyme inhibitors can provide information about the mechanism of action and binding topography of the active sites of enzymes. It has been known that the substitution of the ring oxygen of the pyranose or the furanose form of a sugar by nitrogen to give an iminosugar (or azasugar) leads to a class of compounds, which are powerful and specific inhibitors of glycohydrolases.[1–3] These iminosugars bind specifically to the active sites of glycohydrolases by mimicking the corresponding natural substrates and generally inhibit them in a competitive manner. Currently, these glycohydrolase-inhibiting iminosugars are arousing great interest as chemotherapeutic agents for treating diabetes, obesity, viral diseases and as therapeutic agents for some genetic disorders.[4–6] Hence, a variety of synthetic approaches have been carried out to assemble this class of compounds. Many syntheses have focused on D-iminosugars with D-*gluco*, D-*manno*, and D-*galacto* configurations because their target enzymes are essential for survival and existence of all living organisms.[1,7] L-Iminosugars, except 1,5-dideoxy-1,5-imino-L-fucitol (1-deoxyfuconojirimycin)[8] and 1,5-dideoxy-1,5-imino-L-rhamnitol (1-deoxyrhamnojirimycin)[9] mimicking natural L-sugars, have attracted little attention due to their presumed lack of biological activity profile. However, there is an interesting early report on the biological activities of an L-iminosugar. 1,4-Dideoxy-1,4-imino-D-arabinitol (D-AB1), isolated from the seeds of *Angylocalyx pynaertii* (Leguminosae), is a much more powerful inhibitor of yeast α-D-glucosidase,[10] while its synthetic L-enantiomer (L-AB1) is much more potent inhibitor of mammalian α-D-glucosidases.[11,12] Very recently, it has been reported that L-DMDP (2,5-dideoxy-2,5-imino-L-mannitol), synthesized from

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doi:10.1016/j.tetasy.2004.11.067

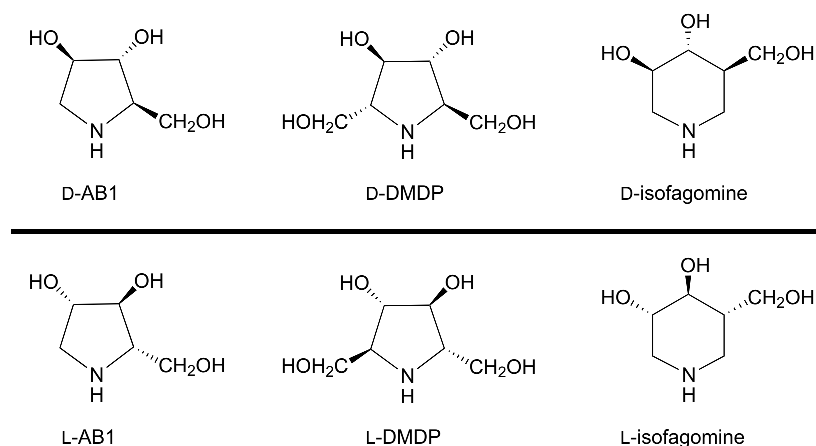

Figure 1. Structures of D- and L-iminosugars.

D-gulonolactone, is a more powerful and more specific α-D-glucosidase inhibitor than the enantiomeric natural product DMDP (D-DMDP).[13] Whether these L- and D-iminosugars bind to the active sites of D-glycohydrolases and the type of inhibition shown are very important problems.

Recently, we synthesized enantiospecifically the D- and L-enantiomers of 1-deoxynojirimycin (DNJ) and 1-deoxygalactonojirimycin (DGJ),[14,15] and carefully examined the nature of their inhibition of D-glycohydrolases by Lineweaver–Burk plots.[16] The kinetic analysis showed that D-DNJ mimicking D-glucose is a competitive inhibitor of rice α-D-glucosidase with a K_i value of 5.7 nM, whereas the enantiomer L-DNJ is a noncompetitive inhibitor (K_i = 4.5 μM) of the enzyme. Similarly, D-galactose-mimicking D-DGJ and the enantiomer L-DGJ are competitive (K_i = 3.5 nM) and noncompetitive (K_i = 7.3 μM) inhibitors of coffee bean α-D-galactosidase, respectively. These results prompted us to re-examine the nature of inhibition of D-glycohydrolases by D- and L-iminosugars. Herein the inhibitory activities of D- and L-iminosugars (Fig. 1) toward a variety of glycosidases and kinetic analyses are described.

2. Results and discussion

2.1. Preparation of D- and L-iminosugars

Synthetic L-AB1 and L-DMDP were prepared from D-xylose and D-gulonolactone, according to the literature.[11,13] Both enantiomers of isofagomine were synthesized via the epoxidation of homochiral *N*-Boc-5-hydroxy-3-piperidene.[17] We isolated D-AB1, D-DMDP, and D-DNJ from *Adenophora triphylla* var. *japonica* (Campanulaceae) according to the literature.[18]

2.2. D-Glycohydrolase inhibition

The IC_{50} values of the D- and L-enantiomers of the iminosugars are shown in Table 1. D-AB1 and D-DMDP have been known to be potent inhibitors of yeast α-D-glucosidase, with IC_{50} values of 0.18 and 1.1 μM, respectively, while L-AB1 is a good inhibitor (IC_{50} = 10 μM) of the enzyme but L-DMDP is not an inhibitor.[11,12] Interestingly, L-AB1 and L-DMDP are more potent inhibitors than their D-enantiomers of mammalian intestinal α-D-glucosidases, especially of isomaltase.[12,13] Although D-isofagomine has been reported to be a potent inhibitor of almond β-D-glucosidase,[19] there is no report on D-glycohydrolase inhibition by the L-enantiomer. The present work shows that L-isofagomine is a weak inhibitor of almond β-D-glucosidase (IC_{50} = 70 μM) and bovine liver β-D-galactosidase (IC_{50} = 250 μM) but does not inhibit the other D-glycohydrolases tested.

Inhibitory activity of D- and L-iminosugars toward human lysosomal D-glycohydrolases is shown in Table 2. L-AB1 and L-DMDP potently inhibit mammalian digestive α-D-glucosidases with IC_{50} values ranging from 1 to 0.05 μM (Table 1), but they were found to be weak inhibitors of human lysosomal α-D-glucosidase with IC_{50} values of 31 and 70 μM, respectively. On the other hand, D-isofagomine was a more potent inhibitor of human lysosomal β-D-glucosidase (β-glucocerebrosidase) than almond β-D-glucosidase, with an IC_{50} value of 0.058 μM, and L-isofagomine also more effectively inhibited β-glucocerebrosidase than almond β-glucosidase, with an IC_{50} value of 9.0 μM. Although D- and L-isofagomines were also inhibitors of bovine liver β-D-galactosidase with IC_{50} values of 3.0 and 250 μM, respectively, they showed no significant inhibition of human lysosomal β-D-galactosidase.

In the case of furanose-mimicking (or five-membered) iminosugars with the ring oxygen replaced by a nitrogen, the fact that the L-enantiomers such as L-AB1 and L-DMDP more potently inhibit plant and mammalian α-D-glucohydrolases than do the D-enantiomers is very interesting from the biological and biochemical points of view. On the other hand, the α-D-glycohydrolase inhibition by six-membered L-iminosugars such as L-DNJ and L-DGJ is 100–4000-fold less potent than that by their D-enantiomers.[16] Isofagomine is an isoiminosugar (or 1-*N*-iminosugar) with the anomeric carbon replaced by a nitrogen and the ring oxygen by a methylene group. L-Isofagomine as well as L-DNJ and L-DGJ was

Table 1. Concentration of D- and L-iminosugars giving 50% inhibition of D-glycohydrolase activities

Enzyme	IC$_{50}$ (μM)					
	D-AB1	L-AB1	D-DMDP	L-DMDP	D-Isofagomine	L-Isofagomine
α-D-Glucosidase						
Yeast	0.18[a]	10[a]	1.1[b]	—[b,c]	150[e]	—[e]
Rice	250	1.7	370[b]	1.5[b]	—	—
Rat intestinal maltase	55[d]	1.3	—[d]	1.4	—	—
Rat intestinal sucrase	230	1.7	81[b]	0.1[b]	—	—
Rat intestinal isomaltase	5.8[d]	0.08	75[b]	0.05[b]	115	—
β-D-Glucosidase						
Almond	200[a]	—	17[b]	—[b]	0.1[e]	70
α-D-Mannosidase						
Jack bean	100[a]	—	—	—	250[e]	—
β-D-Mannosidase						
Rat epididymis	290	—	14	—	—	—
α-D-Galactosidase						
Coffee bean	—[a]	—[a]	—[a]	—	—[e]	—
β-D-Galactosidase						
Bovine liver	—	—	4.6[b]	—[b]	3.0	250
Amyloglucosidase						
Aspergillus niger	400	—	85[b]	—[b]	—	—

[a] Taken from Ref. 11.
[b] Taken from Ref. 13.
[c] —: less than 50% inhibition at 1000 μM.
[d] Taken from Ref. 27.
[e] Taken from Ref. 19d.

Table 2. Concentration of D- and L-iminosugars giving 50% inhibition of human lysosomal D-glycohydrolase activities

Enzyme	IC$_{50}$ (μM)					
	D-AB1	L-AB1	D-DMDP	L-DMDP	D-Isofagomine	L-Isofagomine
α-D-Glucosidase	900	31	—[a]	70	—	—
β-D-Glucosidase	160	—	340[b]	—[b]	0.058	9.0
α-D-Mannosidase	320	—	—	—	—	—
β-D-Mannosidase	—	—	100	—	—	—
α-D-Galactosidase	—	—	—	—	—	—
β-D-Galactosidase	—	—	—	—	—	—

[a] —: less than 50% inhibition at 1000 μM.
[b] Taken from Ref. 13.

an over 100-fold weaker inhibitor of β-glucocerebrosidase than D-isofagomine.

2.3. Kinetic analyses

It is not expected that an inhibitor and its mirror image (enantiomer) dominate the same active site of the enzyme. We recently reported[16] that D-DNJ and D-DGJ inhibit competitively D-glycohydrolases, whereas their L-enantiomers inhibit the enzymes in a noncompetitive manner. Hence, the mode of inhibition of D- and L-iminosugars used in this study was investigated by Lineweaver–Burk plots. Figure 2 shows the Lineweaver–Burk plots of D-AB1 and L-AB1 inhibition of yeast α-D-glucosidase and rat intestinal isomaltase. D-AB1 inhibited yeast α-D-glucosidase and rat intestinal isomaltase in a competitive manner with K_i values of 0.16 (Fig. 2A) and 2.4 μM (Fig. 2C), respectively, whereas L-AB1 was a noncompetitive inhibitor of the enzymes with K_i values of 11.5 (Fig. 2B) and 0.07 μM (Fig. 2D), respectively. Similarly, L-DMDP was also a noncompetitive inhibitor of rice α-glucosidase (Fig. 3B) and rat intestinal isomaltase (Fig. 3D) with K_i values of 0.1 and 0.023 μM, respectively. Thus, the removal of either of two CH_2OH groups in L-DMDP to give L-AB1 weakened the inhibitory potency toward rat intestinal isomaltase by 3-fold. D-Isofagomine is known to be a potent competitive inhibitor of almond β-glucosidase with a K_i value of 0.11 μM.[19b] In the present work, D-isofagomine was found to show a much stronger affinity (K_i = 0.016 μM) for human lysosomal β-D-glucosidase (Fig. 4A) than the almond enzyme, and L-isofagomine was a noncompetitive inhibitor of the human enzyme with a K_i value of 5.7 μM, as seen in Fig. 4B. These kinetic analyses indicate that L-iminosugars are bound to the site other than the active site of D-glycohydrolase and the shape of the active site consequently changes so that substrate can no longer fit there. Competitive inhibitors of lysosomal glycohydrolases have been noted as potential drugs (called chemical chaperones) for the treatment of lysosomal storage disorders, which are caused by deficiencies in lysosomal enzymes.[20–22] Hence, D-isofagomine may be

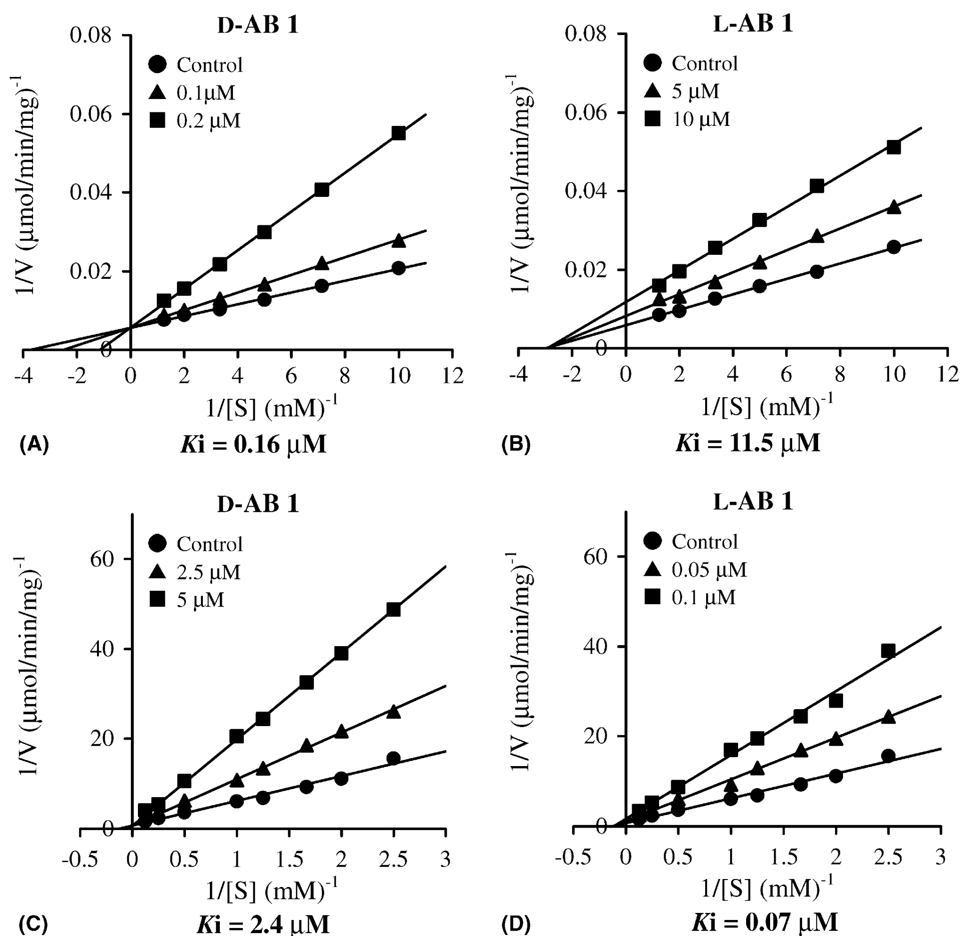

Figure 2. The Lineweaver–Burk plots of D-AB1 and L-AB1 inhibition of yeast α-D-glucosidase (A and B) and rat intestinal isomaltase (C and D).

a chemical chaperone that treat Gaucher's disease caused by the inefficient folding and trafficking of certain variants of β-glucocerebrosidase.

As described above, D- and L-iminosugars were found to be competitive and noncompetitive inhibitors of D-glycohydrolases, respectively. However, when two inhibitors with a different mode of action coexist in the reaction mixture, it is important to prove that they bind to different sites of the enzyme. A graphical method has been devised to analyze the multiple inhibition of an enzyme by two inhibitors.[23,24] This method distinguishes whether two inhibitors interact with the same site or different sites of the enzyme. Dixon plots ($1/v$ vs I) run parallel to the line obtained with one inhibitor alone if the two inhibitors compete with each other at the same site, whereas they cross to the left side of the $1/v$ axis if they do not compete with each other. Figure 5A shows β-glucocerebrosidase as a function of various concentrations of D-isofagomine in the presence and absence of a fixed concentration of L-isofagomine. D-Isofagomine and L-isofagomine do not compete with each other since the lines describing the inhibition by D-isofagomine in the presence and absence of L-isofagomine cross at the left side of the $1/v$ axis. D-DNJ is a powerful inhibitor of human lysosomal α-D-glucosidase (IC_{50} = 0.04 μM) and is also a weak inhibitor of β-glucocerebrosidase (IC_{50} = 240 μM).[16] From the Lineweaver–Burk plots of D-DNJ inhibition of β-glucocerebrosidase, D-DNJ was found to inhibit the enzyme in a competitive manner with a K_i value of 79 μM (data not shown). As shown in Figure 5B, D-DNJ and D-isofagomine compete with each other at the same site (active site) since the line obtained with D-isofagomine in the presence of D-DNJ is parallel to that obtained with D-isofagomine alone. Thus, we found that D-isofagomine best fits the active site of β-D-glucosidase, while L-isofagomine has a favorable interaction with the regulatory site other than the active site.

3. Conclusion

In summary, L-AB1 and L-DMDP were more potent inhibitors of plant and mammalian α-D-glucosidases than their D-enantiomers and their mode of action was of a noncompetitive type, whereas their D-enantiomers were competitive inhibitors of α-D-glucosidases. D-isofagomine was a stronger inhibitor of human lysosomal β-D-glucosidase (β-glucocerebrosidase) than almond β-D-glucosidase and inhibited β-D-glucosidase in a competitive manner. The multiple inhibition studies of β-glucocerebrosidase by D-isofagomine (a competitive inhibitor) and L-isofagomine (a noncompetitive inhibi-

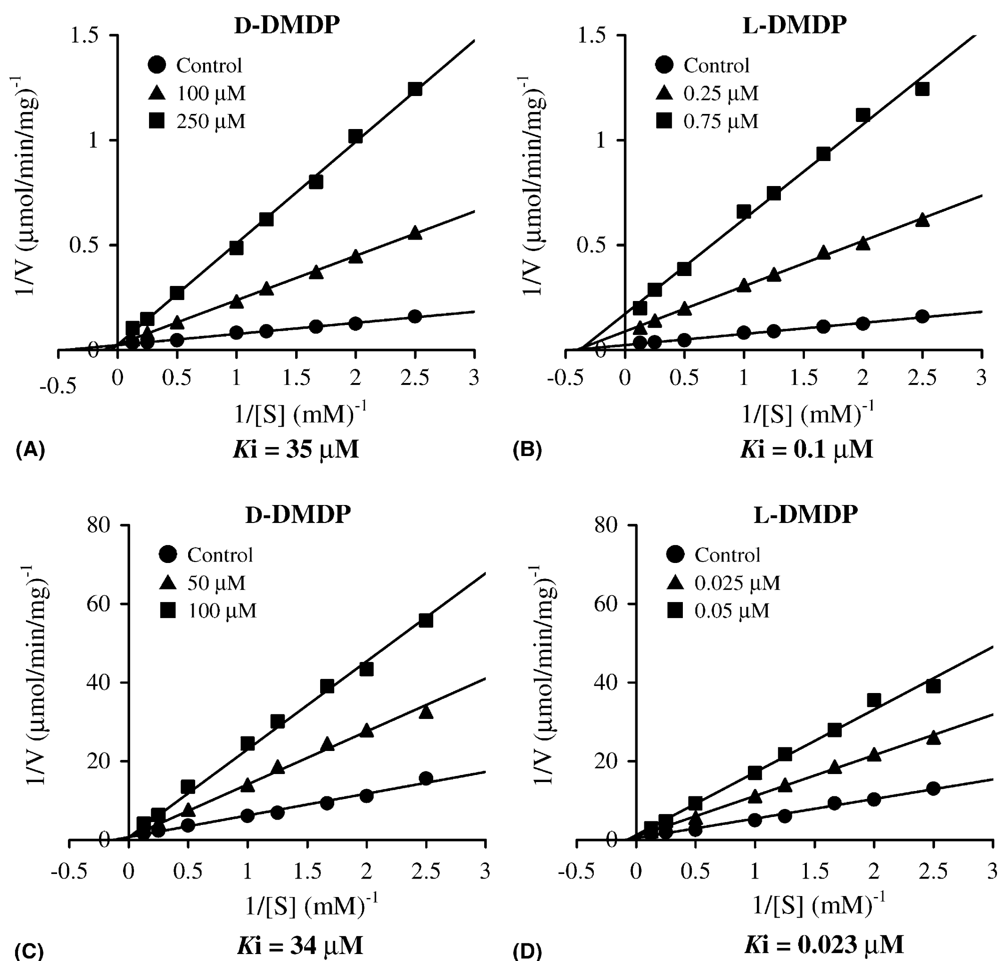

Figure 3. The Lineweaver–Burk plots of D-DMDP and L-DMDP inhibition of rice α-D-glucosidase (A and B) and rat intestinal isomaltase (C and D).

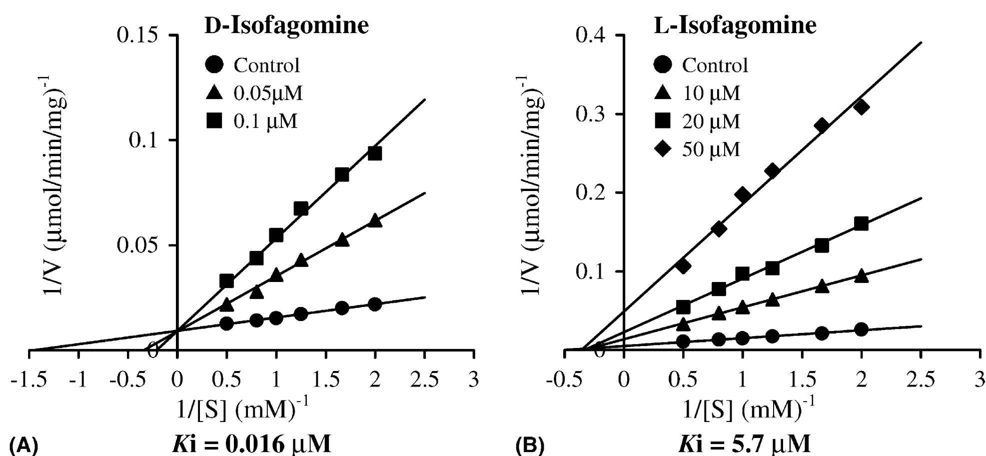

Figure 4. The Lineweaver–Burk plots of D-isofagomine and L-isofagomine inhibition of human lysosomal β-D-glucosidase (β-glucocerebrosidase).

tor) indicated that they do not compete with each other at the same site but two competitive inhibitors, D-isofagomine and D-DNJ, compete with each other at the same site. Our recent and present studies suggest that D-iminosugars are competitive inhibitors of D-glycohydrolases but their L-enantiomers are noncompetitive inhibitors of the enzymes.

4. Experimental

4.1. General

The purity of free base iminosugars was checked by HPTLC on Silica Gel $60F_{254}$ (E. Merck) using the solvent system PrOH–AcOH–H_2O (4:1:1), and a

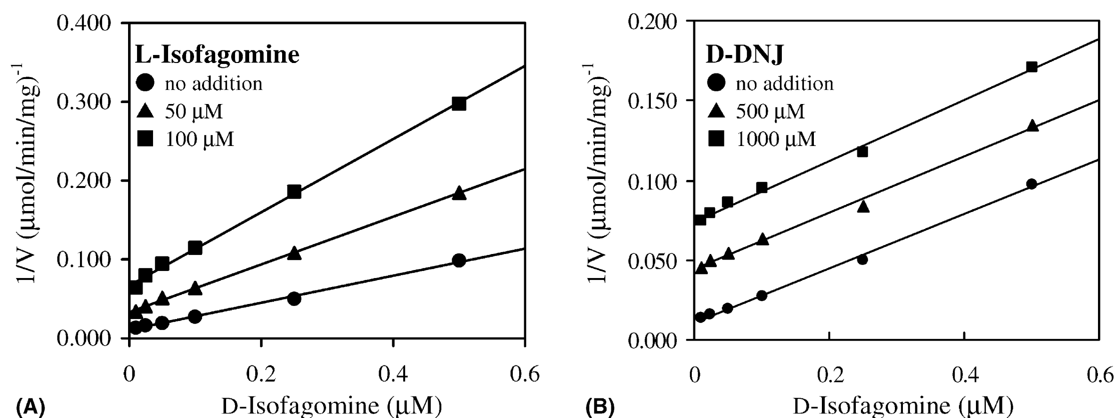

Figure 5. Multiple inhibition of β-glucocerebrosidase by two inhibitors: (A) lack of competition between D-isofagomine and L-isofagomine; (B) mutual competition between D-isofagomine and D-DNJ.

chlorine–*o*-tolidine reagent was used for detection. Optical rotations were measured with a Jasco DIP-370 digital polarimeter (Tokyo, Japan). ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a JEOL ECP-500 spectrometer (Tokyo, Japan). FABMS were measured using glycerol as a matrix on a JEOL JMS-700 spectrometer. The spectroscopic data of the prepared iminosugars were completely identical with those reported.

4.2. Biological assay methods

The enzymes α-D-glucosidases (from rice, assayed at pH 5.0; from yeast, pH 6.8), β-D-glucosidase (from almond, pH 5.0), α-D-mannosidase (from jack bean, pH 4.5), α-D-galactosidase (from coffee bean, pH 6.5), β-D-galactosidase (from bovine liver, pH 6.8), amyloglucosidase (1,4-α-D-glucan glucohydrolase, from *Aspergillus niger*, pH 4.5), *p*-nitrophenyl glycosides, and disaccharides were purchased from Sigma Chemical Co. The rat epididymal fluid was purified from epididymis according to the method of Skudlarek et al.,[25] and assayed at pH 5.2 for β-D-mannosidase using *p*-nitrophenyl-glycosides. Brush border membranes prepared from rat small intestine according to the method of Kessler et al.[26] were assayed at pH 5.8 for rat intestinal maltase, sucrase, and isomaltase using maltose, sucrose, and isomaltose as substrates, respectively. For the activity of rice α-glucosidase and rat intestinal enzymes, the reaction mixture (0.2 mL) contained 25 mM disaccharide and the appropriate amount of enzyme, and the incubations were performed for 10–30 min at 37 °C. The reaction was stopped by heating at 100 °C for 3 min. After centrifugation (600 g; 10 min), 0.05 mL of resulting reaction mixture was added to 3 mL Glucose B-test Wako (Wako Pure Chemical Ind.). The absorbance at 505 nm was measured to determine the amount of the released D-glucose. Other glycosidase activities were determined using an appropriate *p*-nitrophenyl glycoside as substrate at the optimum pH of each enzyme. The reaction mixture (1 mL) contained 2 mM of the substrate and the appropriate amount of enzyme. The reaction was stopped by adding 2 mL of 400 mM Na_2CO_3. The released *p*-nitrophenol was measured spectrometrically at 400 nm. Human lysosomal α-D-galactosidase (Fabrazyme) and β-D-glucosidase (Ceredase) were purchased from Genzyme (Boston, MA) and assayed at pH 5.5. The cell lysate of normal human fibroblasts (GM00498B), which were cultured in MEM medium (Gibco) supplemented with 15% fetal bovine serum and antibiotics at 37 °C under 5% CO_2, was used as the source of lysosomal α-D-glucosidase, α-D-mannosidase, β-D-mannosidase, and β-D-galactosidase. The reaction mixture consists of 50 μL of 0.15 M sodium phosphate–citrate buffer (pH 4.5), 50 μL of 2% Triton X-100 (Sigma Chemical Co.), 30 μL of the enzyme solution, and 20 μL of an inhibitor solution or H_2O. The reaction mixture was pre-incubated at 0 °C for 10 min and started by the addition of 50 μL of 6 mM 4-methylumbelliferyl glycoside (Sigma Chemical Co.) (1 mM in the case of β-D-galactoside), followed by incubation at 37 °C. The reaction was stopped by the addition of 2 mL of 0.1 M glycine buffer (pH 10.6). Liberated 4-methylumbelliferone was measured (excitation 362 nm, emission 450 nm) with a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

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