Inhibitory Effect of Validamine, Valienamine and Valiolamine on Activities of Carbohydrases in Rat Small Intestinal Brush Border Membranes

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Three pseudo-aminosugars, validamine, valienamine and valiolamine, produced by *Streptomyces hygroscopicus* subsp. *limoneus* showed potent inhibitory action on rat small intestinal carbohydrase activities such as sucrase, maltase, glucoamylase, isomaltase and trehalase activities, but negligible action on lactase activity and pancreatic α-amylase activity. Where inhibition was seen, kinetic analysis showed fully competitive inhibition of the carbohydrase activities by all three inhibitors. Valiolamine has more potent carbohydrase inhibitory activity than validamine or valienamine, and the apparent K_i values of valiolamine for sucrase, maltase, glucoamylase, isomaltase and trehalase activities were 3.2×10^{-7}, 2.9×10^{-6}, 1.2×10^{-6}, 9.1×10^{-7} and 4.9×10^{-5} M, respectively, which are 10^{-5} to 10^{-3} times smaller than the apparent K_m values.

Keywords pseudo-aminosugar; valiolamine; carbohydrase activity; rat small intestine; competitive inhibitor

Limiting intestinal digestion of dietary carbohydrates by inhibition of intestinal α-glucosidases has been suggested as a possible means of controlling diabetes mellitus and obesity.[2,3] A number of polypeptides and pseudo-saccharides of microbial origin that exhibit a very pronounced inhibitory effect on intestinal α-glucosidases have been reported.[4] Among those, two types of α-glucosidase inhibitors have been more thoroughly studied with respect to chemical investigations and developing medical application. The first type of potent α-glucosidase inhibitor is the pseudo-oligosaccharides, such as acarbose, and its homologues, amylostatins, adiposins, oligostatins and trestatins.[4] The inhibitors are basically α-amylase inhibitors, very effective on α-amylase and less or hardly effective on the other carbohydrases. The second is represented by nojirimycin and 1-deoxynojirimycin,[4] the glucose analogues in which the ring oxygen is replaced with a nitrogen atom. They are very effective on α- and β-glucosidases, however, significantly less on α-amylases. 1-Deoxynojirimycin is well suited as a starting material for chemical derivation. Extensive efforts to obtain compounds with superior inhibitory activity and, furthermore, to develop them as anti-hyperglycemic medicines have been made.[4]

Subsequently, as a new inhibitor group, we isolated the pseudo-aminosugars, validamine, valienamine and valiolamine, in which the ring oxygen is replaced with a carbon atom (Chart 1), from the fermentation broth of *Streptomyces hygroscopicus* subsp. *limoneus* IFO 12703, which is a producer of validamycin.[5–7] In the previous paper,[7] we reported that these pseudo-aminosugars show strong inhibition, selectively, on α-glucosidases, sucrase and maltase solubilized from porcine intestinal mucosa. However, *in vivo* effect of these pseudo-aminosugars on carbohydrases can not be examined in porcine. Furthermore, porcine show more sensitive carbohydrases inhibitory effects on these inhibitors than rat (unpublished data), and the apparent K_m values for sucrase of porcine and rat were 3.0×10^{-3} M[7] and 3.4×10^{-2} M (unpublished data), respectively. On the other hand, the inhibitory mechanisms of these pseudo-aminosugars on glucoamylase, isomaltase, trehalase and lactase activities are not known.

In this paper we describe the inhibitory effect of these pseudo-aminosugars on rat small intestinal carbohydrase (sucrase, maltase, glucoamylase, isomaltase, trehalase and lactase) activities and rat pancreatic α-amylase activity.

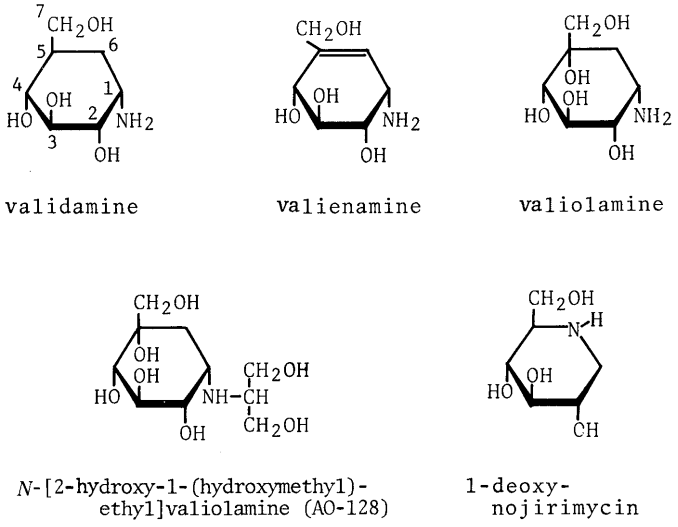

Chart 1. Structures of Validamine, Valienamine, Valiolamine, *N*-[2-Hydroxy-1-(hydroxymethyl)ethyl]valiolamine (AO-128) and 1-Deoxynojirimycin

Materials and Methods

Preparation of Rat Intestinal Brush Border Membranes and Pancreatic α-Amylase Brush border membranes prepared from the intestine of male Wistar rats (250—300 g), by the method of Kessler *et al.*,[8] were used as the source of intestinal carbohydrase.

Pancreata were homogenized in 4 vol. of 50 mM phosphate buffer (pH 7.0) and used as the source of pancreatic α-amylase.

Protein was determined by the method of Lowry *et al.*[9] using bovine serum albumin as the standard.

Assay of Carbohydrase and α-Amylase Activity The sucrase, maltase, isomaltase, glucoamylase and trehalase activities were assayed by the method of Dahlqvist.[10]

The lactase (neutral β-galactosidase) activity was measured in the presence of *p*-chloromercuriphenylsulfonic acid which completely inhibits the acid β-galactosidase.[11]

The enzyme (rat intestinal brush border membrane) was preincubated at 37 °C for 10 min in the absence or presence of the inhibitors in 0.1 M maleate buffer (pH 5.8). The reaction was started by the addition of respective substrates. Aliquots were withdrawn at selected times and the D-glucose liberated was determined with the "Glucose B-test" (Wako Pure

Chemical). For inhibitory studies, sucrose, maltose, soluble starch, palatinose, trehalose and lactose were used as substrates for sucrase, maltase, glucoamylase, isomaltase, trehalase and lactase activities, respectively. The final substrate concentrations for kinetic studies were 10—100 mM for sucrose and lactose, 1—10 mM for maltose, 2—20 mM for palatinose, 4—20 mM for trehalose, and 1—10 mg/ml for soluble starch. Values for K_m, K_i and V_{max} were determined from the Lineweaver–Burk plot.

α-Amylase activity was assayed by the method of Bernfeld.[12]

Preparation of Validamine, Valienamine and Valiolamine Validamine, valienamine and valiolamine were prepared as described in the previous paper.[7]

Chemicals All the substrates (sucrose, maltose, palatinose, soluble starch, trehalose and lactose) used were from commercial sources and were of the best grade available. Sucrose, soluble starch, palatinose, lactose and Glucose B-test were purchased from Wako Pure Chemical; maltose, trehalose and *p*-chloromercuriphenylsulfonic acid were from Nakarai Chemical; 1-deoxynojirimycin was from Boehringer Mannheim.

Results and Discussion

In this study we compared three pseudo-aminosugars (validamine, valienamine and valiolamine) by their specificity of inhibition and affinity on rat small intestinal carbohydrases *in vitro*. The inhibitory effects of 1-deoxynojirimycin on intestinal sucrase and maltase solubilized from porcine mucosa have been previously reported[4] and therefore were used in the present study as a reference inhibitor.

Preliminary results demonstrated that in the presence of valiolamine, the activity of rat intestinal brush border membrane carbohydrases (sucrase, maltase, glucoamylase, isomaltase and trehalase) was not linear during the first 10—15 min of assay. A similar phenomenon was reported in the inhibition of rabbit intestinal sucrase activity by acarbose and 1-deoxynojirimycin, which were shown as slow and tight inhibitors of sucrase.[13] Therefore, the activity of the carbohydrases was determined when a constant rate of glucose production in the assay mixture was observed (between 20 and 40 min after the start of the assay incubation).

Table I shows the kinetic constants (apparent K_m and V_{max} values) for activities of carbohydrases in rat small intestinal brush border membrane.

To characterize the mechanism of inhibition by validamine, valienamine and valiolamine, kinetic studies were performed. Figure 1 shows Lineweaver–Burk plots of sucrase, maltase, isomaltase, trehalase and glucoamylase activities with and without valiolamine. The inhibition of these carbohydrase activities by valiolamine was fully competitive. These carbohydrase activities were also competitively inhibited by validamine and valienamine (data not shown).

The apparent K_i values calculated from the Line-

TABLE I. Kinetic Constants of Carbohydrase Activities in Rat Small Intestinal Brush Border Membranes

Emzyme	Sucrase	Maltase	Glucoamylase	Isomaltase	Trehalase	Lactase
Substrate	Sucrose	Maltose	Sol. starch[a]	Palatinose	Trehalose	Lactose
K_m (M)	3.4×10^{-2}	2.2×10^{-3}	7.7×10^{-3}[b]	5.7×10^{-3}	1.1×10^{-2}	2.4×10^{-2}
V_{max}[c]	85.6	237	119[d]	15.8	10.3	13.7

The apparent K_m and V_{max} values shown are the averages of five determinations with different preparations of brush border membranes. *a*) Soluble starch. *b*) mg/ml. *c*) μmol of hydrolyzed substrate/mg protein/h. *d*) μmol of released glucose/mg protein/h.

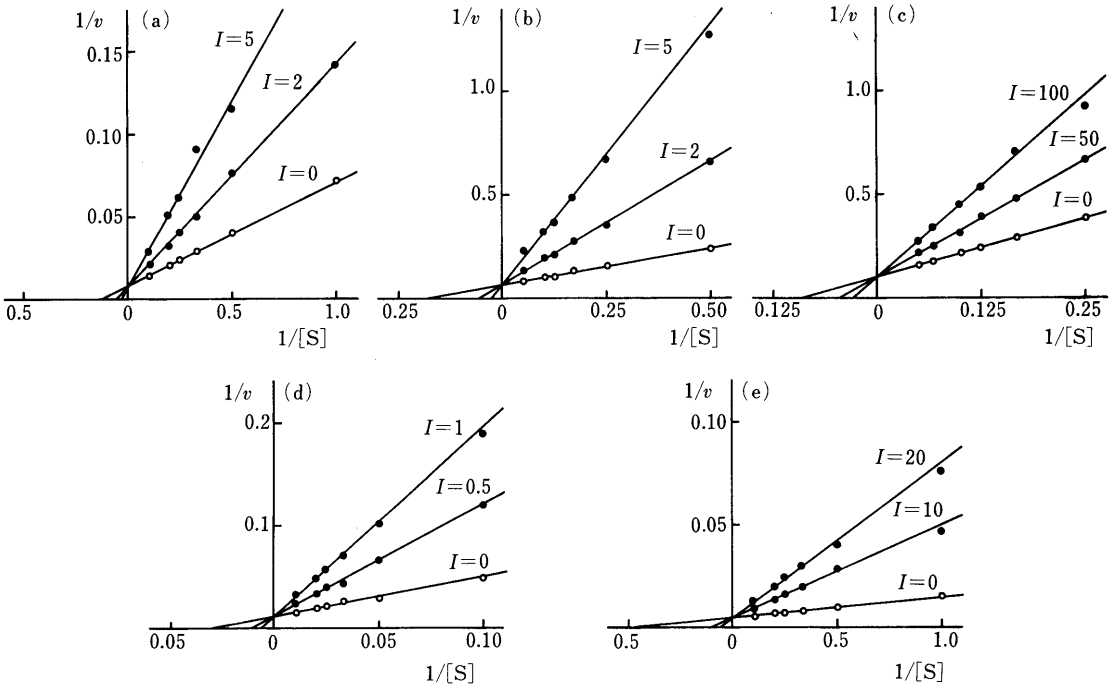

Fig. 1. Lineweaver–Burk Plots of Glucoamylase (a), Isomaltase (b), Trehalase (c), Sucrase (d) and Maltase (e) Activities of Rat Small Intestinal Brush Border Membranes with and without Valiolamine

Ordinate ($1/v$) was expressed as (μmol of released glucose/mg protein/h)$^{-1}$ for (a) or (μmol of hydrolyzed substrate/mg protein/h)$^{-1}$ for (b), (c), (d) and (e). Abscissa (1/[S]) was expressed as (mg/ml)$^{-1}$ for (a) or (mM)$^{-1}$ for (b), (c), (d) and (e). Numerals indicate valiolamine concentration in μM.

TABLE II. Inhibitory Constants (K_i) of Pseudo-aminosugars and 1-Deoxynojirimycin on Activities of Carbohydrases in Rat Small Intestinal Brush Border Membranes

Emzyme Substrate	Sucrase Sucrose	Maltase Maltose	Glucoamylase Sol. starch[a)]	Isomaltase Palatinose	Trehalase Trehalose	Lactase Lactose
K_i (M) for						
Validamine	3.2×10^{-5}	1.8×10^{-4}	1.6×10^{-4}	8.8×10^{-5}	2.7×10^{-4}	NID[b)]
Valienamine	3.0×10^{-4}	9.6×10^{-4}	8.9×10^{-4}	7.6×10^{-4}	8.8×10^{-4}	NID
Valiolamine	3.2×10^{-7}	2.9×10^{-6}	1.2×10^{-6}	9.1×10^{-7}	4.9×10^{-5}	NID
AO-128	2.6×10^{-8}	3.6×10^{-8}	6.1×10^{-8}	2.8×10^{-7}	5.0×10^{-4}	NID
1-Deoxynojirimycin	1.2×10^{-7}	1.2×10^{-7}	2.4×10^{-7}	3.6×10^{-7}	2.8×10^{-5}	2.6×10^{-5}
mU Enzyme[c)]	11.6	10.1	11.8	10.5	10.4	11.4

The apparent K_i value was calculated from the Lineweaver–Burk plots. This value shown is the average of two determinations with different concentrations of inhibitors. *a*) Soluble starch. *b*) No inhibition detected (with 2×10^{-3} M pseudo-aminosugars). *c*) mU Enzyme in incubation volume at 37 °C. One milliunit was defined as 1 nmol of released glucose from starch or hydrolyzed from the other substrates per min at 37 °C.

weaver–Burk plots are summarized in Table II. Valiolamine has stronger inhibitory activity on rat intestinal carbohydrase activities than does validamine or valienamine. The apparent K_i values of valiolamine for sucrase, isomaltase, glucoamylase, maltase and trehalase activities are 10^{-5} to 10^{-3} times smaller than the apparent K_m values, similar to those for 1-deoxynojirimycin. Furthermore, epivaliolamine (C-5 epimer of valiolamine), obtained by isolating the pseudo-aminosugar from the fermentation broth of *S. hygroscopicus*,[14] showed only about 1/1000 the activity of valiolamine (data not shown). These results show that the presence of C-5 hydroxy group and the configuration of C-5 hydroxymethyl and hydroxy groups of valiolamine play a very important role in potency.

Lactase (β-galactosidase) activity was not inhibited, even at 2×10^{-3} M, by these pseudo-aminosugars. These compounds were also ineffective, even at 4×10^{-3} M, on rat pancreatic α-amylase activity. For 1-deoxynojirimycin, the lactase inhibitory activity was shown to be potent and as equally effective as the inhibition of trehalase activity. These findings indicate that valiolamine is a more specific inhibitor of intestinal α-glucosidases than is 1-deoxynojirimycin.

Three pseudo-aminosugars are well suited for chemical derivation because of its structural simplicity and stability. A large number of *N*-substituted derivatives were synthesized[15] and estimated by the measure of inhibitory activity against the rat intestinal carbohydrases (data not shown). The valiolamine derivative was more potent than the corresponding validamine and valienamine derivatives as well as the parent valiolamine (data not shown). We selected *N*-[2-hydroxy-1-(hydroxymethyl)ethyl]valiolamine, one of the *N*-substituted valiolamines, designated AO-128, as a candidate for further development. In the previous paper,[15] we reported that the derivative has much more inhibitory activity on porcine sucrase than does the parent valiolamine. The apparent K_i values of this compound (a fully competitive inhibitor) for rat intestinal brush border membrane carbohydrase activities, such as sucrase, maltase, glucoamylase and isomaltase activities, were found to be 2.6×10^{-8}, 3.6×10^{-8}, 6.1×10^{-8} and 2.8×10^{-7} M, respectively. Thus, rat intestinal carbohydrase inhibitory activity of this compound is greater than that of valiolamine or 1-deoxynojirimycin. On the other hand, the K_i value of this compound for trehalase activity was found to be 5.0×10^{-4} M, which is less active than that of valiolamine or 1-deoxynojirimycin. The ED_{50} values (the doses that suppressed the postprandial blood sugar increase by 50%) were about 0.1 mg/kg in sucrose loading (2.5 g/kg) and 0.5 mg/kg in starch loading (1.0 g/kg) in rats given AO-128 together with the corresponding carbohydrate.[15] This compound is presently undergoing clinical trial as an adjunct to the dietary management of carbohydrate-dependent metabolic disorders such as diabetes, obesity, hyperglycemia and hyperlipemia.

On the other hand, these pseudo-aminosugars also inhibited oligosaccharide trimming glucosidases I and II and lysosomal α-glucosidase from rat liver.[16] Hence pseudo-aminosugars might be useful as a research tool in the investigation of carbohydrate metabolism.

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