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Calystegine B_3 as a specific inhibitor for cytoplasmic α-mannosidase, Man2C1

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Cytoplasmic α-mannosidase (Man2C1) has been implicated in non-lysosomal catabolism of free oligosaccharides derived from *N*-linked glycans accumulated in the cytosol. Suppression of Man2C1 expression reportedly induces apoptosis in various cell lines, but its molecular mechanism remains unclear. Development of a specific inhibitor for Man2C1 is critical to understanding its biological significance. In this study, we identified a plant-derived alkaloid, calystegine B_3, as a potent specific inhibitor for Man2C1 activity. Biochemical enzyme assay revealed that calystegine B_3 was a highly specific inhibitor for Man2C1 among various α-mannosidases prepared from rat liver. Consistent with this *in vitro* result, an *in vivo* experiment also showed that treatment of mammalian-derived cultured cells with this compound resulted in drastic change in both structure and quantity of free oligosaccharides in the cytosol, whereas no apparent change was seen in cell-surface oligosaccharides. Calystegine B_3 could thus serve as a potent tool for the development of a highly specific *in vivo* inhibitor for Man2C1.

Keywords: cytosol/mannosidase/free oligosaccharides/swainsonine/calystegine.

Abbreviations: BSA, bovine serum albumin; DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; DMEM, Dulbecco's modified eagle media; ER, endoplasmic reticulum; FBS, foetal bovine serum; HPLC, high performance liquid chromatography; KIF, kifunensin; Man2Cl, cytoplasmic α-mannosidase; PA, pyridylamino- or 2-aminopyridine; PNGase, peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase; SWN, swainsonine.

N-Glycosylation is a common co- and post-translational modification of eukaryotic proteins, occurring in the lumen of the endoplasmic reticulum (ER). *N*-glycans have critical effects on glycoproteins' physicochemical properties, such as solubility or stability of proteins, as well as physiological properties such as their bioactivities or intra- and inter-cellular trafficking (*1*, *2*). The biosynthetic pathways of *N*-glycosylation are well understood; genetically modified animal models allow us to assess the importance of specific sugar modifications in greater detail (*3*–*5*). On the other hand, the molecular mechanism of catabolic pathways for *N*-glycans is relatively less understood, other than the involvement of lysosomal glycosidases (*6*, *7*). For instance, the molecular mechanism of the catabolic pathway for cytosolic-free oligosaccharides in eukaryotic cells has only been clarified recently (*8*–*12*). While cytoplasmic peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase [PNGase; (*13*)] can release *N*-glycans from misfolded glycoproteins (*14*–*17*), how these free glycans are processed in the cytosol seems to differ among organisms (*18*–*21*). In mammalian cells, two cytoplasmic glycosidases, *i.e.* endo-β-*N*-acetylglucosaminidase (*22*) and cytoplasmic α-mannosidase (Man2C1) (*23*–*25*), are purportedly involved in processing and degradation of free oligosaccharides, but the physiological function of their processing remains unknown. For instance, while the suppression of Man2C1 expression reportedly results in apoptosis (*26*, *27*), any direct connection between free oligosaccharide processing and apoptosis remains to be determined.

To provide more insight into the physiological function of Man2C1, development of its specific inhibitor is imperative. We previously reported the isolation of various polyhydroxylated alkaloids, potent glycosidase inhibitors, from various plant sources (*28*–*32*). Among them, calystegine B_3 was found to be a highly specific inhibitor for Man2C1. Indeed, *in vivo* study showed that treatment with calystegine B_3 could result in accumulation of unprocessed oligosaccharides in HeLa cells, without affecting cell-surface glycan structures. This result is in sharp contrast to the case with swainsonine (SWN) a broad-spectrum inhibitor for various Class II α-mannosidases (*33*). All results taken together, calystegine B_3 has been shown to have possibilities as a basis compound for a specific *in vivo* inhibitor for Man2C1.

Materials and Methods

Animals and materials

Male Wistar rats with body weight of 250–300 g were obtained from Japan SLC Inc. (Hamamatsu, Japan). Calystegines A_5, B_2, B_3 and C_2 were isolated as described previously (*28*, *29*). SWN was isolated from *Ipomoea carnea* (*30*); 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) were isolated from *Angylocalyx pynaertii* (*34*), unless noted otherwise. Kifunensin (KIF) was purchased from Funakoshi Corp (Tokyo, Japan).

Protein determination

Protein concentration was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO, USA) used as a standard.

Purification of various α-mannosidases from rat liver

Soluble, cytoplasmic α-mannosidase was prepared from cytosolic extract of rat livers and purified essentially as described previously (*35*). In brief, enzyme fractions were concentrated using ammonium sulfate precipitation (40% saturated); enzymes were purified using a combination of concanavalin A-Sepharose (GE Healthcare Life Sciences Japan, Tokyo), cobalt-chelating Sepharose (GE Healthcare Life Sciences Japan) and DEAE-trisacryl columns (Sepracor, France). Active fractions for neutral α-mannosidase were pooled, concentrated using ultrafiltration and used as an enzyme fraction. Specific activity of the partially purified enzyme fraction was 14 nmol/min/mg protein.

Golgi-enriched fraction from rat liver was obtained by repeated centrifugation, as described previously (*36*). Enzymes were further purified using cellulose-phosphate columns, as reported (*37*). Briefly, 1 M KH_2PO_4 solution was added to the golgi-enriched fraction in order to adjust the pH to 5.8, and the solution was dialysed against 10 mM potassium phosphate buffer (pH 5.8) containing 5 mM $MgCl_2$ and 0.1% Triton X-100. The fraction was applied to a cellulose phosphate column (1 × 15 cm; Sigma Chemical Co.). α-Mannosidases I and II were adsorbed to the column; it was further washed by 130 ml of 10 mM potassium phosphate buffer (pH 5.8) containing 5 mM $MgCl_2$ and 0.1% Triton X-100. A fraction containing α-mannosidase Ia but devoid of α-mannosidase II activity was then eluted by 130 ml of 10 mM potassium phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$ and 0.1% Triton X-100. Finally, the α-mannosidase II-enriched fraction was eluted using 500 ml of 10 mM potassium phosphate buffer (pH 7.2) containing 5 mM $MgCl_2$, 500 mM NaCl and 0.1% Triton X-100. Specific activities of the partially purified enzyme fractions were 2.0 nmol/min/mg protein for golgi α-mannosidase Ia and 1.9 nmol/min/mg protein for α-mannosidase II.

Lysosomal α-mannosidase was partially purified from rat liver homogenates according to the method of Opheim and Touster (*38*) using cellulose-phosphate and hydroxyapatite chromatography. Specific activity of the partially purified enzyme fraction was 25 nmol/min/mg protein.

Enzyme assay

Cytoplasmic α-mannosidase. Enzyme fraction (50 µl) consisting of 200 mM sodium phosphate buffer (pH 6.5), 2 mM $CoCl_2$ and 1 mg/ml BSA with or without inhibitors was pre-incubated at 0°C for 30 min. Then 200 µl of *p*-nitrophenyl-α-mannopyranoside (final conc. 2 mM) was added, and the solution was incubated at 37°C for 30 min. The reaction was stopped by adding 2 ml of 400 mM Na_2CO_3; absorbance at 400 nm was measured to determine released *p*-nitrophenol amounts.

Golgi α-mannosidase Ia. The enzyme fraction (10 µl) consisting of 200 mM sodium acetate buffer (pH 5.5) and 1 mg/ml BSA with or without inhibitors was pre-incubated at 0°C for 30 min. To this solution, 50 pmol of the substrate (PA-M6B; Manα1-2Manα1-3[Manα1-3(Manα1-6)Manα1-6]Manβ1-4GlcNAcβ1-4GlcNAc-PA; 10 pmol/µl; Takara Bio Inc., Shiga, Japan) was added, and the mixture was incubated at 37°C for 60 min. The reaction was centrifuged at 13,000*g* for 5 min, and the supernatant was subjected to the high performance liquid chromatography (HPLC) analysis.

Golgi α-mannosidase II. The enzyme fraction (50 µl), consisting of 200 mM sodium acetate buffer (pH 5.5) and 1 mg/ml BSA with or without inhibitors was pre-incubated at 0°C for 30 min. Then 200 µl of *p*-nitrophenyl-α-mannopyranoside (final conc. 2 mM) was added, and the solution was incubated at 37°C for 30 min. The reaction was stopped by adding 2 ml of 400 mM Na_2CO_3, and absorbance at 400 nm was measured to determine the amount of *p*-nitrophenol released.

Lysosomal α-mannosidase. The enzyme fraction (50 µl), consisting of 200 mM sodium acetate buffer (pH 4.5), 0.1% Triton X-100 and 1 mg/ml BSA with or without inhibitors was preincubated at 0°C for 30 min. Then 200 µl of *p*-nitrophenyl-α-mannopyranoside (final conc. 2 mM) was added; the solution was incubated at 37°C for 30 min. The reaction was stopped by adding 2 ml of 400 mM Na_2CO_3; absorbance at 400 nm was measured to determine released amounts of *p*-nitrophenol.

Measurement of IC_{50} value

The IC_{50} values for inhibitors (concentration causing 50% reduction of control activity) were determined by linear regression analysis from the plot of the logarithm of inhibitor concentration versus percentage of the remaining activity.

Cell culture

Cells were maintained in Dulbecco's Modified Eagle Media (Nacalai Tesque Co., Kyoto, Japan), supplemented with 10% foetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 100 U/ml penicillin and 100 ng/ml streptomycin (Nacalai Tesque Co.). Cells were incubated in a humidified incubator at 37°C under 5% CO_2 atmosphere.

To examine the effects of mannosidase inhibitors on structures and quantities of free oligosaccharides, cells were incubated with indicated concentrations of SWN (Wako Pure Chemical Industries, Ltd, Osaka, Japan) or calystegine B_3 in the presence of serum for 24 h.

Isolation of 2-aminopyridine (PA)-labelled free oligosaccharides recovered from cytosol

To extract free oligosaccharides from the cytosolic fraction of the cells, four dishes (100 mm diameter) of HeLa cells, treated with either 100 µM SWN (Wako Pure Chemical Industries Ltd) or 1 mM calystegine B_3, or no-inhibitor control, for 24 h, were washed with cold PBS twice, harvested and lysed in 800 µl of lysis buffer [10 mM Hepes/NaOH buffer (pH 7.4), 5 mM DTT, 250 mM mannitol, 1 mM EDTA (pH 8.0), 1× complete protease inhibitor cocktail (Roche Diagnostics K. K., Tokyo, Japan) and 1 mM Pefabloc (Roche Diagnostics K. K.)]. The cells were homogenized and centrifuged at 1,000*g* for 10 min at 4°C to remove nuclei and unbroken cells. The supernatant was then centrifuged at 100,000*g* for 1 h at 4°C, and the cytosol fraction was recovered from supernatant. To precipitate proteins, 1.5 v of ethanol was added to the supernatant, mixed well and centrifuged at 17,000*g* for 20 min at 4°C. The supernatant, which contains the free oligosaccharides, was desalted using a PD-10 column (GE Healthcare Life Sciences Japan) according to manufacturer's instructions. Desalted fractions were evaporated and pyridylaminated with 20 µl 2-amino pyridine reagent at 90°C for 1 h, followed by reduction reaction of 20 µl borane-dimethylamine at 80°C for 1 h. PA-labelled sugars were purified using a MonoFas spin column (GL Sciences Inc, Tokyo, Japan) as described previously (*21*). PA sugars thus obtained were dissolved into 100–200 µl water for HPLC analysis.

High performance liquid chromatography analysis

Analysis of the golgi α-mannosidase Ia reaction product by HPLC was carried out with a Bondaspher 15C18 (Nihon Waters K. K.). Column temperature was maintained at 40°C; samples were eluted by 100 mM acetic acid-triethylamine (pH 4.0). Flow rate was 1.0 ml/min and the elution was monitored by fluorescence detector (excitation 320 nm; emission 400 nm); reaction product (Manα1-3[Manα1-3(Manα1-6)Manα1-6]Manα1-4GlcNAcβ1-4GlcNAc-PA) was quantitated.

Size fractionation HPLC of cytosol-free oligosaccharides was performed by a method reported previously (*21*). For reversed-phase HPLC, a dual gradient system was used for separation and quantitation of free oligosaccharides (*39*). Detailed method for structural determination of newly identified glycans, M5C′, M6E′ and M7E′ (structures of these glycans were shown in Fig. 2C) will be reported elsewhere.

Flow cytometry

HeLa cells were treated with either 100 µM SWN or 1 mM calystegine B_3 for 24 h. Cells were washed with PBS, collected and were suspended with PBS containing 10 mg/ml BSA and 0.1% sodium azide (FACS solution) to make up a concentration of 1×10^7 cells/ml. Cells were stained with biotinylated Con A (Vector Lab; 100 µg/ml)/streptavidin Alexa fluor 488 conjugate (Invitrogen Corp. Japan, Tokyo) or FITC-labelled MAM lectin (Seikagaku Biobusiness Corp., Tokyo, Japan; 40 µg/ml). Labelled cells were analysed using

LSR flow cytometer (BD Japan, Tokyo) and the results were analysed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Results and Discussions

Effect of iminosugars on rat liver α-mannosidases: identification of calystegine B_3 as a specific inhibitor for cytoplasmic α-mannosidase

α-Mannosidases are classified into Classes I and II families with distinct sequences and enzymatic properties (*33*). Class I α-mannosidases specifically cleave α1, 2-mannose residues and do not utilize aryl-α-D-mannosides as substrates. These α-mannosidases are inhibited by iminopyranose analogues such as KIF. Class II α-mannosidases cleave α1,2-, α1,3- and α1,6-mannose residues, and can hydrolyse a synthetic substrate, *p*-nitrphenyl-α-D-mannopyranoside; they are inhibited by iminofuranose analogues such as SWN and DIM. SWN has a broad-spectrum inhibitory effect on Class II α-mannosidases such as golgi II and lysosomal α-mannosidases and Man2C1 (*23*, *40*–*43*). Thus, particular precautions should be taken to assess SWN's effect on animal cells.

Calystegines were first discovered as secondary metabolites of some restricted plants and implicated in the establishment and maintenance of specific plant–bacterium relationships (*44*, *45*). Calystegines have now been shown to broadly occur in the plant families Convolvulaceae, Solanaceae and Moraceae (*28*, *29*, *46*, *47*). Calystegines possess three structural features in common: a nortropane ring system; 2–4 hydroxyl groups varying in position and stereochemistry and a novel aminoketal functionality, which generates a tertiary hydroxyl group at the bicyclic ring bridgehead. The C2, C3 and C4 -OH groups and ring heteroatom in the six-membered ring of (+)-calystegine B_2 are configurationally analogous to the C4, C3 and C2 -OH groups and ring heteroatom of the α-glucosidase inhibitor 1-deoxynojirimycin. These structural similarities suggested that calystegines might also have glycosidase inhibitory activities. We previously reported calystegine B_2 to be a potent competitive inhibitor of human lysosomal β-glucosidase and α-galactosidase, with K_i values of 0.49 and 30 μM, respectively (*42*), while 2-*epi*-calystegine B_2 (calystegine B_3) is a 76-fold weaker inhibitor of β-glucosidase and a very weak inhibitor of human lysosomal α-mannosidase, with an IC_{50} value of 620 μM. Other than calystegine B_3, no calystegines significantly inhibited human lysosomal α-mannosidase.

To examine in more detail the specificity of calystegines towards α-mannosidases, their inhibitory effects on Class II α-mannosidases (golgi α-mannosidase II, lysosomal α-mannosidase and cytoplasmic α-mannosidase) were examined (Table I). To this end, three Class-II α-mannosidases were partially purified from rat liver. Golgi mannosidase Ia (*37*) was used to compare calystegines' inhibitory effect on a Class I α-mannosidase.

KIF is known to inhibit the conversion of high-mannose oligosaccharides to complex oligossacharides by blocking processing of ER α-mannosidase I or golgi α-mannosidase I. Structures of inhibitors used are shown in Fig. 1. As shown in Table I, this compound had inhibitory effects only on Class I golgi α-mannosidase Ia, but had no effects on any Class II enzymes examined. In sharp contrast, SWN was a powerful inhibitor for all Class II enzymes tested, but had no effect on golgi α-mannosidase Ia (Table I). DAB, an inhibitor for rat epididymis (lysosomal) α-mannosidase (*34*), exhibited a broad-spectrum inhibition profile, affecting activities of golgi α-mannosidases Ia, II and lysosome α-mannosidase. However, DAB did not have an inhibitory effect on cytoplasmic α-mannosidase (Table I). Among the various calystegines examined, we found that calystegine B_3 inhibited cytoplasmic α-mannosidase in a highly specific manner (IC_{50}: 8.7 μM) (Table I). Interestingly, this compound did not have any significant inhibitory effects towards other α-mannosidases tested. We therefore compared the inhibitory profile with other calystegines that had structural configurations similar to calystegine B_3 (Table I). Calystegine C_2 (6-α-hydroxylcalystegine B_3) inhibited cytoplasmic α-mannosidase with an IC_{50} value of 60 μM, but also had a weak inhibitory activity on lysosomal α-mannosidase (Table I). This result suggests that the α-hydroxylation of C6 lowers the specificity towards Man2C1. On the other hand, 2-*epi*-calystegine B_3 (calystegine B_2) and 2-deoxy-calystegine B_3 (calystegine A_5) did not have any inhibitory effects against all α-mannosidases tested. These results suggested that the axially oriented -OH group at C2 is the essential feature for calystegines to be recognized by Man2C1.

Table I. Effects of various compounds on α-mannosidases isolated from rat liver.

	IC_{50} (μM)						
				Calystegines			
	DAB	KIF	SWN	B_3	A_5	B_2	C_2
Cytosol	NI	NI	4.5	8.7	NI	NI	60
Golgi II	140	NI	0.013	NI	NI	NI	NI
Lysosome	290	NI	0.040	NI	NI	NI	240
Golgi Ia	390	2.0	NI	NI	NI	NI	NI

DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; KIF, kifunensine; SWN, swainsonine; NI: <50% inhibition at 500 μM.

In vivo *treatment of calystegine B_3 caused accumulation of cytosolic-free oligosaccharides, but did not alter cell-surface oligosaccharide structures*

Having confirmed that calystegine B_3 can be a very potent and specific inhibitor for α-mannosidase, the *in vivo* effect of calystegine B_3 against mammalian-cultured cells was examined. To this purpose, HeLa cells were treated either with 100 μM SWN or 1 mM calystegine B_3 for 24 h. After free oligosaccharides were isolated, structural isomers were separated and quantitated by two-dimensional HPLC method as reported previously (*39*). As shown in Fig. 2A, treatments with SWN as well as calystegine B_3 resulted in accumulation of larger oligosaccharides corresponding to $Man_{6-9}GlcNAc_{1-2}$, as judged by size-fractionation HPLC. The peaks were fractionated according to the number of hexose residues as indicated (Fig. 2A), and

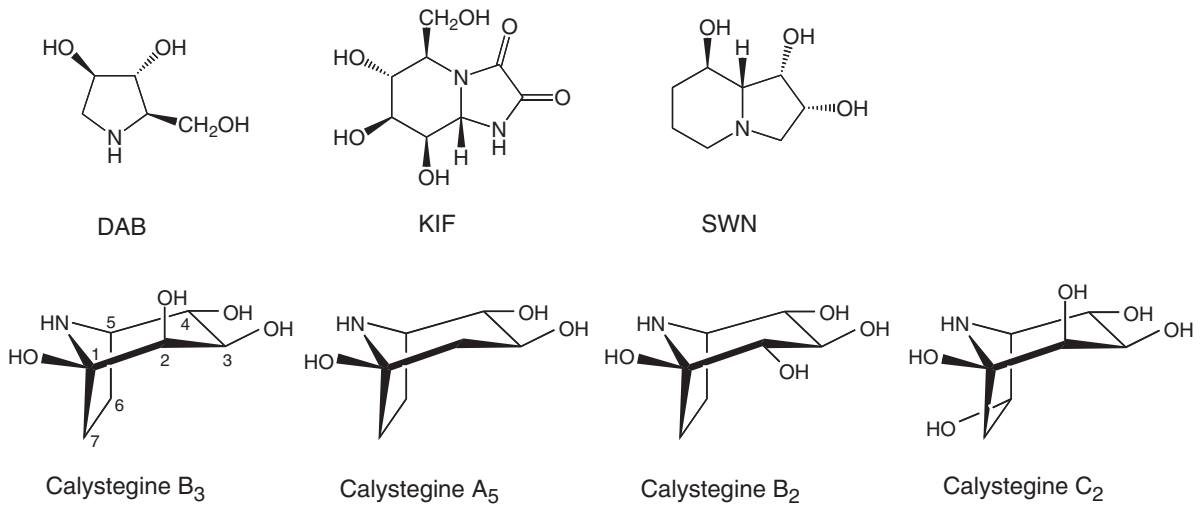

Fig. 1 Structures of known mannosidase inhibitors and calystegines used in this study. DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; KIF, kifunensin; SWN, swainsonine.

each fraction was subjected to the dual gradient, reversed phase HPLC (*39*) for separation/quantitation of each structural isomer. Structures were determined by comparing their elution positions with those of standard samples, and were further confirmed by determining their elution positions on amino column after (i) α-mannosidase and (ii) α1,2-mannosidase digestions. Quantitation of data also revealed a significant increase in total free oligosaccharides by treatment with SWN (2.2-fold) or calystegine B_3 (1.5-fold), compared with untreated control (Fig. 2B). Moreover, detailed structural analysis revealed that larger and unprocessed glycans accumulated more in SWN or calystegine B_3-treated cells (Table II). Detailed structural analysis indeed showed that the amount of larger Gn1-type glycans (bearing single GlcNAc at their reducing termini) such as M8A′ or M9A′ (detailed structures were shown in Fig. 2C) was significantly increased upon treatment with these compounds. On the other hand, the amount of M5B′, which is predicted to be the major and final product by Man2C1 (*23*, *43*), was decreased (Table II). These results clearly indicated that calystegine B_3 as well as SWN could inhibit the processing of free oligosaccharides by Man2C1 *in vivo*. It was interesting to note that amount of Gn2-glycans (bearing *N*,*N*′-diacetylchitobiose at their reducing termini) was relatively unaffected by these treatments. This result is consistent with the previous finding that the cytoplasmic α-mannosidase prefers Gn1-glycans to Gn2-glycans as substrates (*35*, *41*). Similar results were observed by other mammalian cells, such as pancreatic carcinoma-derived PK-59 cells, suggesting that the effect of drug was not cell line-dependent (W. Li and S. Tadashi, unpublished results).

SWN, unlike calystegine B_3, could inhibit not only Man2C1 but also other Class II mannosidases, *i.e.* lysosomal α-mannosidases (Man2B1/2B2) and golgi α-mannosidase IIs (Man2A1/2A2) (Table I; 23, 33). Therefore, treatment with SWN could drastically change cell-surface glycan structures, because it could prevent transformation of high mannose- or hybrid-type glycans into complex-type glycans. To examine the effect of inhibitors on cell-surface glycan structures, FACS analysis was carried out. As shown in Fig. 3, significantly increased staining with Con A was observed in SWN-treated cells. This observation was expected, as Con A has a high affinity for high mannose-type glycans, which are predicted to increase by SWN treatment. Consistent with this finding, reduced cell-surface sialic acid was also apparent in SWN-treated cells, as shown by MAM-lectin staining, which recognize terminal α-2,6-linked sialic acids (Fig. 3). In sharp contrast, none of these drastic changes was seen in calystegine B_3-treated cells (Fig. 3). These results, together with *in vitro* specificity studies, strongly suggested that calystegine B_3 has a highly specific inhibitory effect on Man2C1 but not other Class II α-mannosidases localized in lysosomes and golgi.

Discussion

It is widely accepted that α-mannosidases in mammalian cells play important roles in posttranslational processing of the sugar chains on glycoproteins, quality-control systems in the ER and the lysosomal catabolism (*2*, *33*, *48*). Numerous studies demonstrated the functional importance of golgi, ER and lysosome-localized enzymes in such biological contexts. On the other hand, the biological significance of Man2C1, if any, is relatively unexplored, other than its involvement in catabolism of free oligosaccharides in the cytosol (*23*). In this connection, it should be noted that Man2C1 expression levels have been reported to be tightly associated with apoptosis, although the relevant mechanism remains to be characterized in detail (*26*, *27*). This observation clearly offers an important clue that functional importance of Man2C1 should also be evaluated by careful analysis. To this purpose, a specific inhibitor for this enzyme will be an invaluable tool. A potent inhibitor for Man2C1, which causes accumulation of free oligosaccharides in mammalian cells, was recently reported, although its effects

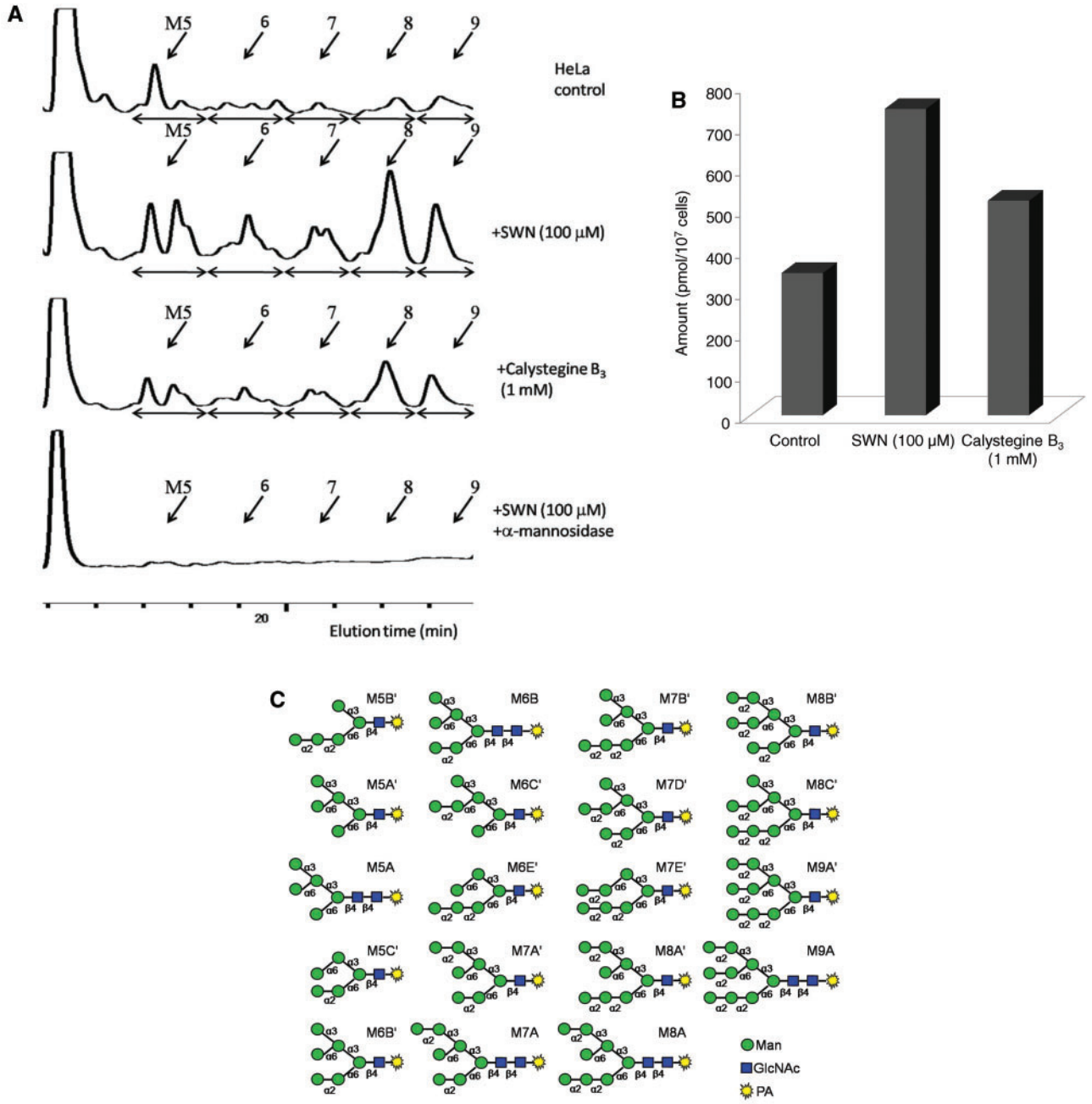

Fig. 2 Effect of calystegine B_3 on structures and amount of free oligosaccharides in HeLa cells. (A) Size-fractionation HPLC profile of free oligosaccharides isolated from control (first row), SWN-treated (second row), calystegine B_3-treated cells (third row) and α-mannosidase digestion of SWN-treated sample (bottom row). Fractions indicated by arrow were pooled, and were subjected to the reversed phase HPLC analysis (*39*) for quantitation of each structural isomer. (B) Effect of inhibitors on amount of free oligosaccharides in HeLa cells. (C) Structures of major free oligosaccharides observed in HeLa cells.

on other α-mannosidases have not been rigorously examined (*49*).

In this study, we identified calystegine B_3 as a potent and specific inhibitor of Man2C1 by assessing its inhibition profile towards various rat liver α-mannosidases. The IC_{50} value for cytoplasmic α-mannosidase was very low (8.7 μM), comparable to SWN (4.5 μM). However, other α-mannosidases so far tested were not significantly inhibited by this compound, further demonstrating its specificity.

In addition to *in vitro* assay, studies using cell lines also demonstrated that calystegine B_3 indeed could serve as a specific inhibitor of Man2C1. A couple of issues, however, remained to be solved for the *in vivo* use of this compound. First, the efficacy of *in vivo* inhibition was far less efficient compared with the *in vitro* study; as much as 1 mM concentration was required for clear inhibition of free oligosaccharide processing, while 100 μM concentration did not exhibit any significant change in amount and structures of oligosaccharides (data not shown). Given the low *in vitro* IC_{50} value (Table I), the poor *in vivo* effect may be attributable to the inefficient incorporation of this compound. Increasing hydrophobicity of this

Table II. Structures and quantitation of free oligosaccharides in control and SWN- or calystegine B_3-treated HeLa cells.

Structure	Amount (pmol/10^7 cells)		
	Control	SWN (100 μM)	Calystegine B_3 (1 mM)
Gn1 glycans			
M5B′[a]	122	68.1	48.3
M5A′	9.39	80.1	46.4
M5C′[b]	6.40	7.18	6.49
M6B′[a]	7.38	90.2	52.0
M6C′	14.9	22.6	15.0
M6E′[b]	4.17	ND	ND
M7A′[a]	17.3	79.3	44.4
M7B′	8.32	33.1	18.4
M7D′	5.41	5.64	2.98
M7E′[b]	10.1	ND	2.24
M8A′[a]	49.0	165	170
M8B′	6.82	19.4	15.6
M8C′	8.94	11.7	7.37
M9A′[a]	21.6	107	52.6
Gn2 glycans			
M5A	19.9	21.5	15.7
M6B	4.19	5.83	2.05
M7A	4.95	7.35	6.28
M8A	20.9	15.5	12.4
M9A	3.31	3.58	2.33

[a]Amount was expressed as a combined sum of GlcNAc and ManNAc form (ca. 10% of the GlcNAc form) in their reducing termini. The ManNAc forms were most likely generated by epimerization of the *N*-acetyl group of the innermost GlcNAc during PA-labelling (*39*). ND, not detectable.
[b]Structures of these isomers were determined based on the elution position of NH2-column as well as MS analysis with or without α-mannosidase or α1,2-mannosidase digestions. Glucose units for these isomers on our reversed-phase HPLC were determined as follows: M5C′, 2.96; M6E′, 3.52; M7E′, 3.32. Detailed method for identification of these isomers will be reported elsewhere.

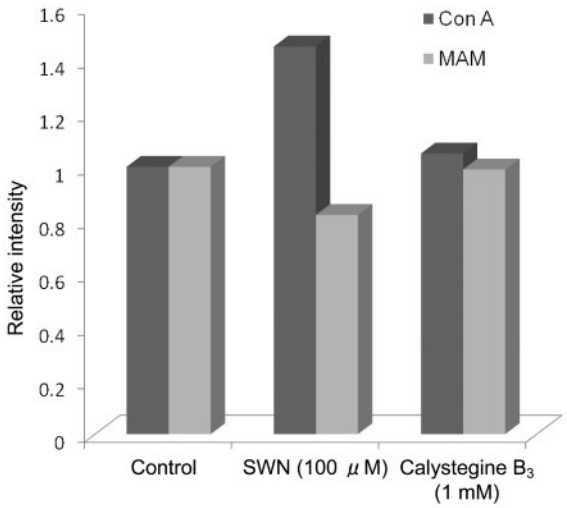

Fig. 3 Analysis of cell-surface glycan structures on SWN- and calystegine B_3-treated HeLa cells using FACS analysis. Relative staining intensity with Con A or MAM lectins for control cells was set to 1.0.

compound, such as *N*-alkylation, may thus result in increasing the *in vivo* efficacy. Despite its inefficiency, however, calystegine B_3's high specificity was evident as cell-surface glycan structures were hardly affected, in sharp contrast to SWN.

The specificity of the newly developed inhibitor should be more extensively examined, especially because calystegine B_3 reportedly inhibits lysosomal β-glucosidase (*42*). While the *in vitro* IC_{50} value against β-glucosidase [76 μM for human lysosomal enzyme (*42*)] was much larger than that of α-mannosidase (8.7 μM, this study), this specificity issue should be further evaluated in detail when developing the *in vivo* inhibitor against Man2C1 using calystegine B_3 as a seed compound, to avoid any side effects.

It should also be noted that, under our experimental conditions, calystegine B_3 did not have any effect on cell growth or morphology (data not shown), although free oligosaccharides accumulated significantly. It is in sharp contrast to the case with the suppression of Man2C1 expression by small interfering RNA or antisense-DNA, where significant delay in cell growth as well as apoptotic phenotype were observed (*26*, *27*). This result suggests that enzymatic activity of Man2C1 is not related to its apoptosis-suppression activity. This result reminds us the case of the cytoplasmic PNGase, which is recently shown to have essential deglycosylation-independent activity in *Neurospora* (*50*) and *Drosophila* (*51*). Whether Man2C1 has an enzyme-independent function in the cell should therefore be carefully examined. A specific inhibitor for this enzyme will be a great tool to distinguish its enzyme-dependent function with any as-yet-unknown enzyme-independent ones.

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Conflict of interest

None declared.

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