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Inhibition of Sialyltransferases of Murine Lymphocytes by Disaccharide Nucleosides

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Two disaccharide nucleosides, 5-fluoro-2′,3′-isopropylidene-5′-*O*-(4-*N*-acetamido-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-*glycero*-α-D-*galacto*-octapyranosyl) uridine (KI-8110) and 2′,3′-di-*O*-acetyl-5′-*O*-(4-*N*-acetamido-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-*glycero*-α-D-*galacto*-octapyranosyl)inosine (KI-8115) decreased incorporation of sialic acid into glycoconjugates on the murine lymphocyte surface. Since sialidase (EC 3.2.1.18) activity, CMP-NeuAc hydrolase (EC 3.1.4.40) activity and the incorporation of galactose or *N*-acetylgalactosamine into cell-surface glycoconjugates were all unaffected by these disaccharide nucleosides, the observed decrease in incorporation of sialic acid into glycoconjugates is considered to reflect the inhibition of sialyltransferase (EC 2.4.99.1) on the lymphocyte surface. KI-8110 and KI-8115 specifically inhibited the transfer of sialic acid to exogenous desialylated glycoproteins or exogenous desialylated glycolipids. Namely, KI-8100 and KI-8115 inhibited the transfer of sialic acid to desialylated fetuin, porcine submaxillary mucin (PSM) and bovine submaxillary mucin (BSM), all of which have Ser(Thr)-linked oligosaccharide chains, but did not inhibit transfer to desialylated α_1-acid glycoprotein, which has only Asn-linked oligosaccharide chains. KI-8110 and KI-8115 inhibited the transfer of sialic acid to desialylated gangliosides GA_1 and GA_2, but not GA_3. By using *p*-nitrophenyl-β-D-galactoside as an acceptor and cytidine 5′-monophosphate-[^{14}C]*N*-acetylneuraminic acid (CMP-[^{14}C]NeuAc) as a donor, two types of sialyl derivatives, α-sialosyl-(2→3)-*p*-nitrophenyl-β-D-galactoside and α-sialosyl-(2→6)-*p*-nitrophenyl-β-D-galactoside, were produced in murine lymphocytes. The production of α-sialosyl-(2→3)-*p*-nitrophenyl-β-D-galactoside was specifically inhibited by KI-8110 and KI-8115. KI-8110 and KI-8115 competitively inhibited sialic acid transfer by lymphocyte sialyltransferase to desialylated fetuin as an exogenous acceptor. The apparent K_i values were 2.3 mM for KI-8110 and 2.5 mM for KI-8115. When cytidine 5′-diphosphate (CDP), a known inhibitor of sialyltransferase, was incubated with lymphocytes for 24 h at 37 °C, the inhibitory activity of CDP was dramatically decreased. However, such incubation did not affect the inhibitory activity of KI-8110 or KI-8115.

Keywords——disaccharide nucleoside; sialic acid; murine lymphocyte; sialyltransferase inhibitor; sugar linkage

Carbohydrate chains are known to play essential roles in structurally diverse glycoproteins and glycolipids on the plasma membrane and are considered to be carriers in biological information exchange between cells. The non-reducing termini of carbohydrate chains are generally occupied by sialic acid. Sialic acid is considered to play important roles in various biological phenomena of cells: modification of the negative charge[1] and permeability[2] of the cell membrane, cell–cell recognition and interaction,[3] contact inhibition,[4] crypticity of immunogenic loci[5] and regulation of the activity of some ectoenzymes.[6] Furthermore, recent studies have suggested that there is a close relationhsip between cell–

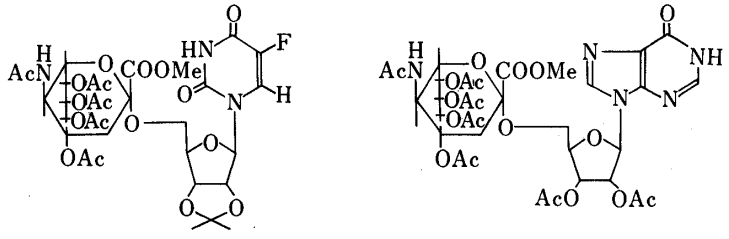
surface sialic acid content or sialyltransferase activity and the growth[7] or metastatic potential[8] of tumor cells. In addition, the amount of surface sialic acid has been correlated with host defensive activity against viral infection or tumor cells.[9] Thus, since sialylation of the cell surface plays crucial roles in various biological phenomena, the possibility of altering cell surface sialylation by modification of sialic acid metabolism with drugs becomes very interesting. As we reported previously,[10] two newly synthesized disaccharide nucleosides, KI-8110 and KI-8115, can enhance the induction of suppressor T cells by concanavalin A (Con A) and can also induce suppressor T cells by themselves. One way to examine the mechanism of induction of suppressor T cells by these disaccharide nucleosides may be to investigate the effect of KI-8110 and KI-8115 on the sialic acid metabolism of lymphocytes, since Con A-induced suppressor T cells are selectively labeled by peanut agglutinin,[11] which binds to sialic acid free β-galactosyl residues. In this paper, we describe the effects of these disaccharide nucleosides on the sialic acid metabolism of murine lymphocytes, and show that these substances inhibit sialyltransferase.

Materials and Methods

Reagents——The two disaccharide nucleosides, 5-fluoro-2′,3′-isopropylidene-5′-*O*-(4-*N*-acetamido-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-*glycero*-α-D-*galacto*-octapyranosyl)uridine (KI-8110) and 2′,3′-di-*O*-acetyl-5′-*O*-(4-*N*-acetamido-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-*glycero*-α-D-*galacto*-octapyranosyl)inosine (KI-8115) (Fig. 1) were synthesized by Kijima *et al.*[10] The starting materials for the above disaccharide nucleosides, 4-*N*-acetamido-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl-D-*glycero*-α-D-*galacto*-octapyranoside (acetylated derivative of sialic acid, ADSA), 5-fluoro-2′,3′-isopropylidene uridine (FIU) and 2′,3′-di-*O*-acetylinosine (DAI), were also synthesized by Kijima *et al.*[10] Cytidine 5′-diphosphate (CDP), fetuin type IV, ganglioside type III and *p*-nitrophenyl-β-D-galactoside (NphGal) were purchased from Sigma (St. Louis, Mo., U.S.A.). α_1-Acid glycoprotein was kindly provided by Dr. K. Schmid (Boston University School of Medicine, Boston, Mass., U.S.A.). Porcine submaxillary mucin (PSM) was prepared as described by Katzman and Eyler,[12] and bovine submaxillary mucin (BSM) as described by Tettamanti and Pigman.[13] Standard samples of gangliosides were kindly supplied by Dr. A. Suzuki (Tokyo Metropolitan Institute of Medical Science, Tokyo) and Prof. Seyama (School of Medicine, University of Tokyo, Tokyo). These glycoproteins were desialylated by treatment with 0.05 M H_2SO_4 at 80 °C for 1 h and glycolipids by treatment with 1 M formic acid at 100 °C for 1 h. Cytidine 5′-monophosphate-*N*-acetyl[4,5,6,7,8,9-^{13}C]neuraminic acid (CMP-[^{14}C]NeuAc, 247 mCi/mmol) and uridine diphosphate-*N*-acetyl-D-[1-^{14}C]galactosamine (UDP-[^{14}C]GalNAc, 47 mCi/mmol) were purchased from New England Nuclear (Boston, Mass., U.S.A.), uridine diphosphate-D-[6-^{3}H]galactose (UDP-[^{3}H]Gal, 16.3 Ci/mmol) from the Radiochemical Centre (Amersham, U.K.), 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (4-MU-NeuAc) and sialidase from *Arthrobacter ureafaciens* were purchased from Nakarai Chemical Co. (Kyoto).

Cells——Splenic lymphocytes were obtained from pathogen-free Balb/c strain female mice (7 to 8 weeks of age, Charles River Japan, Kanagawa) using the Ficoll–Urografin technique.[14]

Glycosyltransferase Activity of the Intact Cells——Sialyltransferase activity was measured according to the method of Painter *et al.*[15] The incubation mixture contained 10^7 cells, 2 μM CMP-[^{14}C]NeuAc and assay buffer composed of 0.12 M NaCl–1 mM $MgCl_2$–0.01 M phosphate-buffered saline (PBS) (pH 6.5) in a total volume of 100 μl. The reaction was carried out at 37 °C in a shaking water bath for 2 h, and terminated by addition of 2 ml of ice-cold 1% phosphotungstic acid (PTA)–0.5 N HCl. The acid-insoluble fraction was collected by centrifugation, then washed



KI-8110 KI-8115

Fig. 1. The Structures of KI-8110 and KI-8115

twice with 2 ml of 1% PTA and once with ice-cold 5% trichloroacetic acid (TCA). The washed pellet was extracted twice with chloroform–methanol (2 : 1, v/v). The extracts were pooled and dried in a scintillation vial. The pellets were solubilized with 0.5 N KOH and neutralized with 0.5 N HCl, then transferred to scintillation vials. Galactosyltransferase activity and *N*-acetylgalactosaminyltransferase activity were measured according to the procedures described above except that 0.12 M NaCl–10 mM $MnCl_2$–0.1 M sodium cacodylate solution was used as a buffer, with 2 μM UDP-[^{3}H]Gal for galactosyltransferase assay and 2 μM UDP-[^{14}C]GalNAc for *N*-acetylgalactosaminyltransferase assay as substrates. For measurement of [^{14}C]NeuAc incorporation into the exogenous acceptor, the assay was carried out as described above except that desialylated glycoproteins (2 mg/ml), desialylated gangliosides (2 mg/ml) or NphGal (10 mg/ml) were added to the incubation mixture. When desialylated gangliosides were used as an acceptor, the lipid was extracted with chloroform–methanol (2 : 1, v/v). The gangliosides were separated by thin layer chromatography on a Silicagel 60 plate (Merck, Darmstadt, Germany) in chloroform–methanol–2.5 N ammonia water (60 : 40 : 9, v/v) including 0.2% $CaCl_2$.[16] The distribution of radioactive gangliosides was determined by cutting 0.5 cm bands along the chromatographic path. When NphGal was used as the acceptor, the separation of sialylated products was carried out according to the method of Bauvois *et al.*[17] Briefly, the incubation mixture was centrifuged at 175 *g* for 10 min, and then the supernatant was applied to Whatman 3 MM paper. The sialylated products were separated by descending paper chromatography in ethyl acetate–pyridine–water (10 : 4 : 3, v/v). The radioactive pattern was determined by cutting 1 cm bands along the chromatographic path. The radioactivity was determined by counting in a liquid scintillation system. Intact cells were used as the enzyme source unless otherwise stated.

Sialyltransferase Activity of Cell Homogenate——Cells were suspended in 10 mM PBS (pH 6.5) containing 1 mM $MgCl_2$, 0.05% Triton X-100 (Nakarai Chemical Co., Kyoto) and 1 mM dithiothreitol (Boehringer Mannheim, Germany). After brief sonication using a Sonifier Cell Disrupter (Branson Sonic Power Co., Conn., U.S.A.), the homogenate was centrifuged at 1500 g for 10 min. Sialyltransferase activity in the supernatant was measured according to the method described above using desialylated PSM as the acceptor.

Sialidase Activity of the Intact Cells——Sialidase activity of the intact cells was measured according to the method of Nojiri *et al.*[18] with minor modifications. The incubation mixture contained 10^7 cells, 0.5 mM 4-MU-NeuAc and assay buffer composed of 0.12 M NaCl–0.01 M PBS (pH 6.4) in a total volume of 100 μl. The reaction was performed at 37 °C in a shaking water bath for 1 h, and terminated by adding 3.5 ml of 0.1 M 2-amino-2-methyl-1-propanol–HCl buffer (pH 10.3). The sialidase activity was determined by measuring the fluorescence intensity of released 4-MU on a fluorescence spectrophotometer with excitation at 360 nm and emission at 440 nm, using 4-MU as a standard.

Sialidase Activity of Cell Homogenate——Total sialidase activity of the cells was also measured according to the method described above. Cells were suspended in ice-cold distilled water, allowed to stand in ice for 10 min and then disrupted by brief sonication. The incubation mixture contained the sonicated cell homogenate (200 μg of protein), 0.5 mM 4-MU-NeuAc and assay buffer composed of 75 mM sodium acetate buffer (pH 4.0) in a total volume of 100 μl. The reaction and determination of sialidase activity were carried out under the same condition described above.

Effect of Disaccharide Nucleosides on Hydrolysis and Breakdown of CMP-[^{14}C]NeuAc——Breakdown of radioactive CMP-NeuAc was determined by incubation of CMP-[^{14}C]Neu with the sialyltranseferase activity assay system described above. After incubation for 2 h at 37 °C, hydrolysis was terminated by the addition of 1 ml of ice-cold 95% ethanol. The particulate material was removed by centrifugation at 2500 *g* and then the supernatant was spotted onto Whatman No. 1 chromatography paper. The samples were allowed to separate overnight in 1 M ammonium acetate–95% ethanol (3 : 7, v/v). Chromatograms were cut as before, and the radioactivity of free [^{14}C]NeuAc was determined in a liquid scintillation system.

Results

Effect of the Disaccharide Nucleosides on Sugar Transfer to Endogenous Acceptors

Disaccharide nucleosides KI-8110 and KI-8115 inhibited 20—30% of the sialic acid incorporation into murine lymphocyte glycoproteins which were used as endogenous acceptors, while the starting materials used in synthesis of these disaccharide nucleosides, ADSA, FIU and DAI, did not significantly inhibit the incorporation (Table I). KI-8110 and KI-8115 also inhibited sialic acid incorporation into the glycolipid of murine lymphocytes (Table I). In this assay, there was no significant loss of cell viability during the incubation period. CDP, a well-known sialyltransferase inhibitor, also inhibited sialic acid transfer to cellular glycoproteins and glycolipids of murine lymphocytes. On the cell surface, galactosyl and *N*-acetylgalactosaminyl residues of glycoconjugates are generally sialylated. Inhibition of galactose or *N*-acetylgalactosamine transfer to cell-surface glycoconjugates may decrease

TABLE I. Effects of KI-8110 and KI-8115 on [^{14}C]NeuAc Incorporation in Cellular Glycoconjugates as Endogenous Acceptors

Treatment		[^{14}C]NeuAc incorporated into	
		Glycoproteins	Glycolipids
		dpm/10^7 lymphocytes[a]	
None		95.1± 6.3	55.6± 2.5
ADSA	10^{-3} M	94.6± 3.5	49.2± 6.4
FIU	10^{-3} M	87.4±11.9	50.8± 7.9
DAI	10^{-3} M	95.8± 5.4	51.3± 6.1
KI-8110	10^{-3} M	76.2± 6.9[c]	43.9± 4.3[c]
KI-8115	10^{-3} M	69.6± 2.4[d]	48.4± 5.0[b]
CDP	10^{-3} M	68.1±11.9[c]	37.3±12.2[b]

a) Mean±S.D. of 4 determinations. b) $p<0.05$. c) $p<0.01$. d) $p<0.001$.

TABLE II. Effects of KI-8110 and KI-8115 on [^{3}H]Galactose and [^{14}C]*N*-Acetylgalactosamine Incorporations into Cellular Glycoproteins

	[^{3}H]Gal incorporated	[^{14}C]GalNAc incorporated
	dpm/10^7 lymphocytes[a]	
Control	1733± 92	827±64
KI-8110 10^{-3} M	1608±106	766±69
KI-8115 10^{-3} M	1613± 93	704±87

a) Mean±S.D. of 3 determinations.

TABLE III. Effects of KI-8110, KI-8115 and CDP on [^{14}C]NeuAc Incorporation into Desialylated Glycoproteins as Exogenous Acceptors

Treatment	[^{14}C]NeuAc incorporated into				
	Asialo-α_1-acid glycoprotein[b]	Asialo-fetuin[a]	Asialo-BSM[a]	Asialo-PSM[a]	Asialo-PSM[b]
	dpm/mg protein added[c]				
Control	936±14	852±55	1065± 48	860±144	1462± 3
KI-8110 10^{-3} M	836± 6[d]	646±79[d]	718±109[e]	624± 44	918± 26[f]
KI-8115 10^{-3} M	1003±28	631±50[e]	512±16[f]	514±178	905±113[e]
CDP 10^{-3} M	256± 2[f]	78±36[f]	97±26[f]	320±28[f]	882± 98[f]

a) Sialyltransferase activity of intact lymphocytes was measured.
b) Sialyltransferase activity of the supernatant from lymphocyte homogenate was measured.
c) Mean±S.D. of 3 determinations.
d) $p<0.05$. e) $p<0.01$. f) $p<0.001$.

sialic acid incorporation. However, the incorporation of galactose or *N*-acetylgalactosamine into the glycoproteins of murine lymphocytes was not inhibited by KI-8110 or KI-8115 (Table II). Therefore, the decrease of sialic acid transfer is not due to inhibition of galactose or *N*-acetylgalactosamine transfer by KI-8110 and KI-8115.

Effect of the Disaccharide Nucleosides on Sialic Acid Transfer to Exogenous Acceptors

As shown in Table III, when desialylated α_1-acid glycoprotein, which contains only Asn-

TABLE IV. Effects of KI-8110 and KI-8115 on [^{14}C]NeuAc Incorporation into Desialylated Gangliosides as Exogenous Acceptors

Treatment	[^{14}C]NeuAc incorporated into		
	GA_1	GA_2	GA_3
	dpm/mg lipid added[a]		
Exp. 1 None	2788	525	350
KI-8110 10^{-3} M	1988	438	456
KI-8115 10^{-3} M	2150	396	488
Exp. 2 None	1338	210	201
KI-8110 10^{-3} M	963	103	209
KI-8115 10^{-3} M	1038	171	241

a) In each experiment, assays were performed in duplicate and the values are averages.

TABLE V. Linkage Specificity of Inhibition by KI-8110 and KI-8115 in [^{14}C]NeuAc Transfer to *p*-Nitrophenyl-β-D-galactoside

Treatment	Sialylated products	
	[^{14}C]NeuAcα2→6NphGal	[^{14}C]NeuAc2→3NphGal
	dpm/mg NphGal added[a]	
Exp. 1 None	191	136
KI-8110 10^{-3} M	192	97
KI-8115 10^{-3} M	191	75
Exp. 2 None	251	174
KI-8110 10^{-3} M	253	124
KI-8115 10^{-3} M	216	102

a) In each experiment, assays were performed in duplicate and the values are averages.

linked oligosaccharide chains, was used as an exogenous acceptor, KI-8110 inhibited sialic acid incorporation only a little and KI-8115 did not inhibit this incorporation. KI-8110 and KI-8115 apparently inhibited the incorporation of sialic acid into desialylated PSM and BSM, both of which have only Ser(Thr)-linked oligosaccharide chains. The incorporation of sialic acid into desialylated fetuin, which has both Asn-linked and Ser(Thr)-linked oligosaccharide chains, was also inhibited by KI-8110 and KI-8115. The results suggest that the inhibitory effect of KI-8110 and KI-8115 on sialic acid incorporation into desialylated glycoproteins is specific for *O*-glycosylproteins. CDP strongly inhibited sialic acid transfer to all exogenous acceptors tested.

When desialylated gangliosides were used as exogenous acceptors, specificity of inhibition by KI-8110 and KI-8115 was also observed. KI-8110 and KI-8115 inhibited the incorporation of sialic acid into GA_1 and GA_2. but did not inhibit the incorporation into GA_3 (Table IV).

Next, to investigate the linkage specificity of the inhibition by KI-8110 and KI-8115, NphGal was used as an acceptor of sialic acid and the sialylated products were analyzed. Two types of sialylated products, α-sialosyl-(2→3)-*p*-nitrophenyl-β-D-galactoside and α-sialosyl-(2→6)-*p*-nitrophenyl-β-D-galactoside, were identified and it was found that KI-8110 and KI-8115 specifically inhibited the production of α-sialosyl-(2→3)-*p*-nitrophenyl-β-D-galactoside (Table V).

When cell homogenate was used, instead of intact cells, as an enzyme source, KI-8110

TABLE VI. Effects of KI-8110 and KI-8115 on the Sialidase Activity of Lymphocytes

Treatment	Released 4 MU/mg protein of	
	Cell homogenate	Intact cells
	nmol[a]	
None	11.4±0.3	0.18±0.08
KI-8110 10^{-3} M	11.2±0.2	0.21±0.04
10^{-4} M	11.1±0.3	0.18±0.03
KI-8115 10^{-3} M	11.5±0.1	0.19±0.04
10^{-4} M	11.5±0.1	0.21±0.01

a) Mean±S.D. of 4 determinations.

TABLE VII. Effects of KI-8110 and KI-8115 on Degradation of CMP-[^{14}C]NeuAc

Treatment	CMP-[^{14}C]NeuAc degraded (dpm)[a]	
	Exp. 1	Exp. 2
None	12366	8230
KI-8110 10^{-3} M	13036	8573
KI-8115 10^{-3} M	12384	7781

a) In each experiment, assays were performed in duplicate and the values are averages.

and KI-8115 also inhibited the incorporation of sialic acid into desialylated PSM, and the inhibitory effect of KI-8110 on the sialyltransferase activity of the cell homogenate was more potent than that on sialyltransferase activity of intact cells (Table III). It seemed that the susceptibilities of sialyltransferase of intact cells and that of cell homogenate to these inhibitors were different.

Effect of Disaccharide Nucleosides on Sialidase Activity

Since the incorporation of sialic acid into desialylated glycoconjugate was affected by the sialidase activity, the influence of KI-8110 and KI-8115 on sialidase activity was examined. In experiments with cell homogenate, 11.4 nmol of 4-MU per mg protein was released from 4-MU-NeuAc after incubation for 1 h at 37 °C. In this assay system, KI-8110 and KI-8115 had no significant effect on the hydrolysis of 4-MU-NeuAc (Table VI).

When intact cells were used as the enzyme source, sialidase activity was much lower than that of the cell homogenate and 0.18 nmol of 4-MU-NeuAc per mg protein was hydrolyzed. Similarly, the sialidase activity of intact cells was not affected by either KI-8110 or KI-8115 (Table VI).

Effect of Disaccharide Nucleosides on Breakdown of CMP-[^{14}C]NeuAc

Since the breakdown of CMP-NeuAc during sialyltransferase assay may also induce a decrease of sialic acid incorporation, the breakdown of CMP-[^{14}C]NeuAc (CMP-hydrolase activity) was determined. The addition of KI-8110 or KI-8115 to the sialyltransferase assay medium did not have any significant effect on the breakdown of CMP-[^{14}C]NeuAc (Table VII).

Competitive Inhibition of Sialyltransferase

The type of inhibition and the K_i were determined graphically by using teh method of

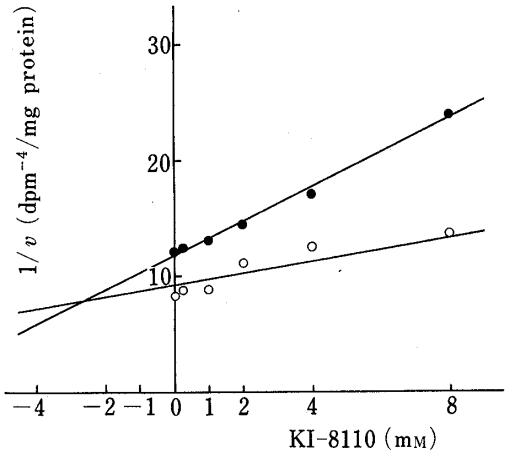


Fig. 2. Dixon Plot of KI-8110 Inhibition of Sialyltransferase on the Lymphocyte Surface Using 2 Concentrations of CMP-[^{14}C]NeuAc Substrate [2 μM (●—●) and 4 μM (○—○)]

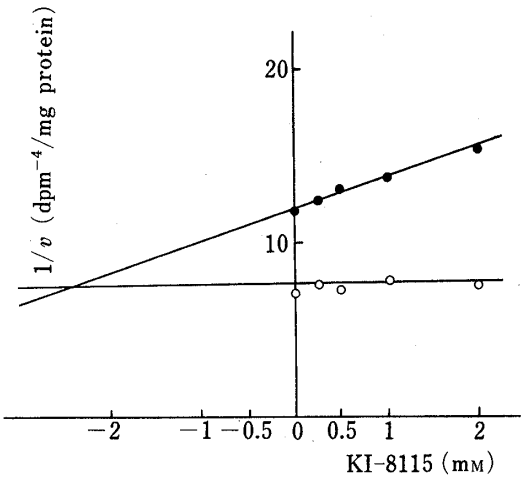


Fig. 3. Dixon Plot of KI-8115 Inhibition of Sialyltransferase on the Lymphocyte Surface Using 2 Concentrations of CMP-[^{14}C]NeuAc Substrate [2 μM (●—●) and 4 μM (○—○)]

TABLE VIII. Sialyltransferase Activities of Lymphocytes Incubated with KI-8110, KI-8115 or CDP for 24 h

Treatment	[^{14}C]NeuAc incorporated/mg fetuin (dpm)[a]
None	511 ± 33
KI-8110 10^{-3} M	430 ± 9[b]
KI-8115 10^{-3} M	408 ± 48[b]
CDP 10^{-3} M	807 ± 44[c]

a) Mean ± S.D. of 3 determinations. *b*) $p<0.05$. *c*) $p<0.001$.

Dixon.[19] In this assay, intact lymphocytes were used as a source of sialyltransferase and desialylated fetuin was used as an acceptor for sialic acid. As shown in Figs. 2 and 3, KI-8110 and KI-8115 competitively inhibited sialic acid transfer to desialylated fetuin. The apparent K_i for KI-8110 was 2.3 mM and that for KI-8115 was 2.5 mM.

KI-8110 and KI-8115 inhibited the transfer of sialic acid to endogenous acceptors (Table I) but the non-linear Dixon plot precluded a K_i determination (data not shown). This may be because of the existence of some factors which affect sialyltransferase activity, but further work is necessary on this problem.

Effect of Preincubation with Intact Lymphocytes on Inhibitory Activity for Sialyltransferase

Incubation of KI-8110 and KI-8115 with intact lymphocytes for 24 h caused only partial loss of inhibitory activity, whereas the inhibitory activity of CDP was completely lost after a 24 h incubation (Table VIII). Indeed, the inhibitory activity of CDP for sialyltransferase was lost within 6 h (data not shown). Therefore, the inhibitory activity of CDP was more labile than those of KI-8110 and KI-8115.

Discussion

Disaccharide nucleosides KI-8110 and KI-8115, which consist of nucleoside and sialic acid moieties, inhibited sialic acid incorporation into endogenous cellular acceptors or into

added desialylated glycoproteins and glycolipids (Tables I, III and IV). Since cells are impermeable to nucleoside sugars, it is likely that in our assay system, which uses intact lymphocytes, enzymes related to sialic acid incorporation into endogenous or exogenous acceptors are located at the cell surface. Sialyltransferase, which catalyzes the transfer of sialic acid from CMP-NeuAc to glycoconjugates, is apparently localized at the cell surface.[20] The subcellular localization of sialidase, which participates in the catabolism of sialo compounds, remains unclear. CMP-NeuAc synthetase is a nuclear enzyme[21] and CMP-NeuAc hydrolase, which degrades CMP-NeuAc, was reported to be localized in the plasma membrane.[22] The enhancement of CMP-NeuAc hydrolase and sialidase activities may reduce apparent [^{14}C]sialic acid incorporation in our assay system. However, as shown in Tables VI and VII, KI-8100 and KI-8115 did not enhance the activities of these enzymes. Furthermore, the inhibition of galactose and *N*-acetylgalactosamine transfer to cell glycoconjugates was not responsible for the decrease of sialic acid incorporation into endogenous cellular acceptors, since the incorporation of galactose or *N*-acetylgalactosamine into cell surface glycoconjugate was not inhibited by KI-8110 and KI-8115 (Table II). These results indicate that the decrease of sialic acid incorporation was due to inhibition of lymphocyte surface-associated sialyltransferase by KI-8110 and KI-8115.

KI-8110 and KI-8115 inhibited sialyltransferase in a competitive fashion (Figs. 2 and 3). The K_i values are nearly the same for KI-8110 (2.3 mM) and KI-8115 (2.5 mM). Various nucleotides and nucleotide analogs have been reported to be inhibitors of sialyltransferase.[23] Those containing the pyrimidine base cytosine, *e.g.*, CTP, CDP and CMP, inhibit serum sialyltransferase competitively.[23a] The other nucleotides, *e.g.*, UTP, UDP, UMP, ATP, and AMP, show noncompetitive-type inhibition.[23a] CTP and CDP have been shown to be the most effective inhibitors of serum sialyltransferase, having K_i values of 16 and 19 μM, respectively. CDP was also shown to be more effective than KI-8110 and KI-8115 in our assay system (Tables I and III). Many kinds of sialyltransferase have been reported and their acceptors or linkage specificities have been defined.[24] Since the biological roles played by sialylglycoconjugates may depend on sialylation of a specific glycoconjugate or type of linkage, it is important to assess the specificity of sialyltransferase inhibition. Transfer of sialic acid to desialylated fetuin, PSM or BSM as an exogenous acceptor was inhibited by both KI-8110 and KI-8115 (Table III), but sialylation of α_1-acid glycoprotein was inhibited only slightly or not at all by either KI-8110 or KI-8115 (Table III). It is known that α_1-acid glycoprotein has only Asn-linked oligosaccharide chains, that PSM and BSM have only Ser(Thr)-linked oligosaccharide chains, and that fetuin has both Asn-linked and Ser(Thr)-linked oligosaccharide chains. Therefore, the inhibition by KI-8110 and KI-8115 seems to be specific for sialylation in *O*-glycosylproteins. Furthermore, KI-8110 and KI-8115 also showed specificity in the inhibition of sialylation of added desialylated gangliosides. Sialylation of GA_1, and GA_2 was inhibited by KI-8110 and KI-8115, but that of GA_3 was not (Table IV). Namely, KI-8110 and KI-8115 preferentially inhibit the sialylation of gangliosides having longer oligosaccharide chains. Sialylation of monosialo- or disialoganglioside was also inhibited by KI-8110 and KI-8115 (unpublished data). Eppler *et al.*[25] have shown that sialylation of GA_3 is less effectively inhibited by cytidine nucleosides than is that of GM_3 or GD_3. KI-8110 and KI-8115 may also have some specificity in the inhibition of sialylation of monosialo- and disialogangliosides but this has not yet been examined. CDP, a known sialyltransferase inhibitor, did not show any specificity in the inhibition of the sialylation of exogenous acceptors (Table III).

It has been shown that in *N*-glycosylproteins, sialic acid can be added in an $\alpha(2\rightarrow6)$ or $\alpha(2\rightarrow3)$ linkage to the terminal galactose.[24a,b,26] In *O*-glycosylproteins, the $\alpha(2\rightarrow6)$-sialyltransferase incoprorates sialic acid in an $\alpha(2\rightarrow6)$ linkage onto *N*-acetylgalactosamine and the $\alpha(2\rightarrow3)$-sialyltransferase incorporates $\alpha(2\rightarrow3)$-linked sialic acid onto terminal

galactose.[24c-e] When NphGal was used as an acceptor, linkage specificity of inhibition by KI-8110 and KI-8115 was observed (Table V). These substances specifically inhibited the production of α-sialosyl-(2→3)-*p*-nitrophenyl-β-D-galactoside. These results suggest that KI-8110 and KI-8115 specifically inhibit the transfer of sialic acid to the terminal galactose of *O*-glycosylproteins in an α(2→3) linkage. However, Bauvois *et al.*[17] have reported that both types of sialyltransferase activity towards *p*-nitrophenyl-β-D-galactoside are probably involved in the transfer of sialic acid to terminal galactose of *N*-glycosylproteins. The linkage specificity of inhibition by KI-8110 and KI-8115 should be confirmed by using desialylated *O*-glycosylproteins as an acceptor. This problem is now under investigation.

Even after 24 h incubation with lymphocytes, the inhibitory activities of KI-8110 and KI-8115 against sialyltransferase were not significantly reduced (Table VIII). However, inhibitory activity of CDP was lost within 6 h under the same conditions. Inhibitory activity of KI-8110 or KI-8115 was thus more stable than that of CDP. Other cytidine derivatives, which are known to be sialyltransferase inhibitors, may also be labile as a result of metabolic conversion. In conclusion, we suggest that the disaccharide nucleosides KI-8110 and KI-8115 should be useful tools to investigate the effect of altering cell sialylation by modification of sialic acid metabolism on various biological properties of the cells, in view of their specific inhibitory activity for various sialyltransferases and their stability in cell cultures.

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