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Note

Inhibition of bacterial and viral sialidases by 3-fluoro-*N*-acetylneuraminic acid

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A sialidase (neuraminidase; EC 3.2.1.18) that speficically hydrolyzes glycosidic bonds formed by nonreducing terminal *N*-acetylneuraminic acid has been suggested to play an important role in influenza virus infection or in the budding out of the virus from infected cells [1]. In our search for drugs for the treatment of influenza we synthesized fluorinated sialic acid derivatives and examined them for inhibitory effects on various bacterial and viral sialidases.

1. Experimental

Materials.—5-Acetamido-5-deoxy-3-fluoro-β-D-*erythro*-L-*gluco*-2-nonulopyranosonic acid (3-fluoro-NeuAc, 3FNA) and 5-acetamido-2,5-dideoxy-2,3-difluoro-D-*erythro*-α-L-*gluco*-2-nonulopyranosonic acid (NeuAc2, $3F_2$, DFNA, 2-fluoro-3FNA) were synthesized according to a previous report [2]. *N*-Acetyl-2,3-didehydro-2-deoxyneuraminic acid (NeuAc2en, DDNA; sodium salt) was synthesized according to Meindl and Tuppy [3].

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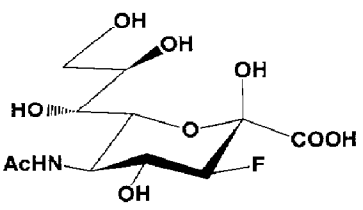
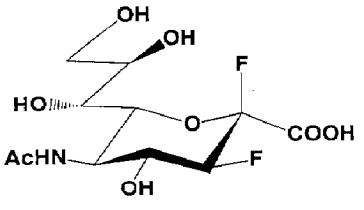


3FNA



DFNA

Sialidases from bacterial sources (*Arthrobacter ureafaciens*, *Vibrio cholerae*, *Clostridium perfringens*, and *Streptococcus* sp.), exoglycosidases, *N*-acetyl-α-neuraminic acid 4-methylumbelliferyl glycoside (4-MU-NeuAc), bovine colostrum *N*-acetylneuraminlactose (sialyllactose; ~85% was NeuAcα2-3Galβ1-4Glc, the remainder NeuAcα2-6Galβ1-4Glc) and other substrates and chemicals of reagent grade were obtained commercially. Concentrated suspensions of influenza A virus (A/PR/8/34 strain), hemagglutinating virus of Japan (HVJ; z strain) and Newcastle disease virus (NDV; Miyadera strain) were kindly supplied by Dr. K. Iwasaki (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and suspensions of 9 other strains of influenza A virus (A/R1/5'/57, A/Aichi/68, A/turkey/Wisconsin/66, A/turkey/England/63, A/turkey/Ontario/68, A/duck/Alberta/76, A/duck/England/56, A/equine/Prague/56, and A/equine/Miami/63) by Dr. K. Nakajima (Faculty of Medical Science, Nagoya City University, Nagoya, Japan).

Assay for bacterial sialidases.—(*a*) With 4-MU-NeuAc according to Meyers et al. [4]. The assay mixture contained 100 μL each of enzyme solution, 0.2 M sodium acetate (pH 4.2 for sialidase of *A. ureafaciens*; 5.5 for *V. cholerae*; 6.5 for *Streptococcus* sp., and 5.0 for *C. perfringens*), 0.5 mM 4-MU-NeuAc, and sample solution in 10-mL glass tubes. After incubation for 15 min at 37°C the reaction was terminated by adding 3.6 mL of 200 mM glycine–NaOH (pH 10.4). The 4-methylumbelliferone (4-MU) released was fluorometrically determined at 440 nm after excitation at 360 nm.

(*b*) With sialyllactose. The assay mixture contained 50 μL each of enzyme solution, 0.2 M sodium acetate as used in (*a*), 0.25 mM sialyllactose and sample solution in 10-mL glass tubes. For *V. cholerae* and *Streptococcus* sp. sialidases, $CaCl_2$ (final concn 5 mM) was added as the enzyme activator. After incubation at 37°C for 30 min (for *A. ureafaciens* and *Streptococcus* sp.) or 60 min (for *V. cholerae* and *C. perfringens*), the *N*-acetylneuraminic acid (NeuAc) released was determined by the thiobarbituric acid method according to Aminoff [5].

Assay for viral sialidases.—The assay mixture contained 25 μL each of virus suspension, 0.2 M sodium acetate (pH 5.5) or 0.2 M MES (2-morpholinoethanesulfonic acid, pH 6.5 for the influenza virus A/PR/8/34 strain), 80 mM $CaCl_2$ (final concn 20 mM), 0.5 mM 4-MU-NeuAc or sialyllactose, and sample solution in 10-mL glass tubes. The mixture was incubated and assayed as described for the bacterial enzymes.

Other exoglycosidases were assayed according to Yu et al. [6] using the appro-

Table 1
Effects of 3FNA and DFNA on *A. ureafaciens* sialidase activity

Compound	Concn (μM)	Inhibition (%)	
		4-MU-NeuAc as substrate	Sialyllactose as substrate
3FNA	1	14	15
	10	49	37
	100	82	89
DFNA	1	8	9
	10	3	−9
	100	4	−3

priate 4-MU glycoside instead of PNP glycoside as the substrate. *N*-Acetylneuraminate pyruvate-lyase was assayed by the established method [7].

2. Results and discussion

As shown in Table 1, 3FNA dose-dependently inhibited the activity of *A. ureafaciens* sialidase by more than 80% at 100 μM. On the other hand, DFNA showed almost no inhibitory effect. 3FNA (100 μM) also significantly inhibited sialidases from three other bacteria and from mouse spleen homogenate (Table 2).

In contrast to DDNA, a well-known sialidase inhibitor [8], which inhibited the 4 different bacterial sialidases to almost the same extent, 3FNA (0.1 mM) inhibited the enzyme from different sources to different extents in our sialyllactose assay. Lineweaver–Burk and Dixon plots of the *A. ureafaciens* sialidase reaction indicated that the inhibition of 4-MU-NeuAc hydrolysis by 3FNA was competitive (Fig. 1), with an apparent K_i value of 2.4 μM.

As shown in Table 3, 3FNA also exerted potent inhibitory effect on sialidase activity of influenza A virus (PR/8/34 strain), but at up to 1 mM concentration,

Table 2
Effect of 3FNA and DDNA on sialidases of various origins

Sialidase	Inhibition (%)		
	4-MU-NeuAc as substrate	Sialyllactose as substrate	
	3FNA 100 μM	3FNA 100 μM	DDNA 100 μM
A. ureafaciens	82	88	98
Streptococcus sp.	82	58	95
C. perfringens	75	42	80
V. cholerae	60	27	85
Mouse spleen homogenate	86	n.t. [a]	n.t.

[a] Not tested

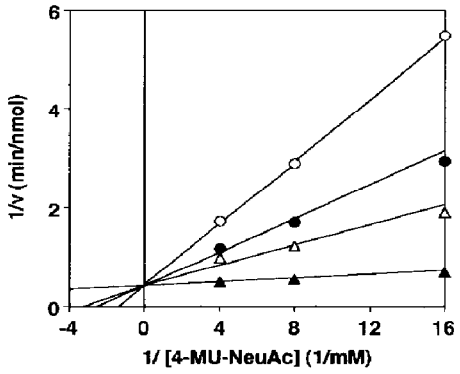


Fig. 1. Lineweaver–Burk plot of the inhibition of sialidase (*A. ureafaciens*) by 3FNA. 4-MU-NeuAc was used as the substrate. 3FNA was added at final concentrations of 0 (▲), 20 (△), 40 (●), and 80 μM (○). Each value represents the mean of triplicate experiments.

Table 3
Effect of 3FNA, DDNA, and NeuAc on viral sialidase activities

Virus	Inhibition (%)						
	4-MU-NeuAc as substrate				Sialyllactose as substrate		
	3FNA		DDNA	NeuAc	3FNA		DDNA
	100 μM	1 mM	100 μM	1 mM	100 μM	1 mM	100 μM
Influenza A virus (PR/8/34 strain)	82	n.t.[a]	92	27	91	n.t.	97
HVJ (z strain)	0	0	88	0	0	5	73
NDV (Miyadera strain)	9	41	90	27	11	58	92

[a] Not tested

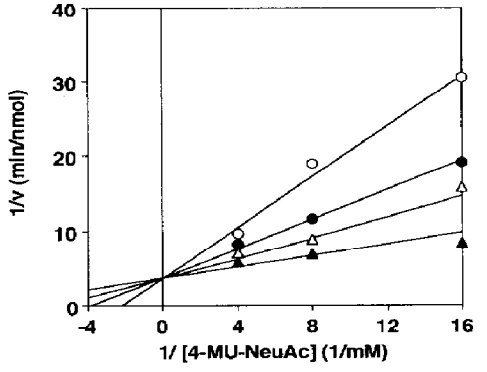


Fig. 2. Lineweaver–Burk plot of the inhibition of influenza A virus (strain PR/8/34) sialidase activity by 3FNA. 4-MU-NeuAc was used as the substrate. 3FNA was added at final concentrations of 0 (▲), 10 (△), 20 (●), and 40 μM (○). Each value represents the mean of triplicate experiments.

Table 4
Effect of 3FNA on the sialidase activities of various influenza A virus strains

Strain	IC_{50} (μM)	
	4-MU-NeuAc as substrate	Sialyllactose as substrate
A/PR/8/34	27	17
A/R1/5'/57	172	155
A/Aichi/68	240	85
A/turkey/Wisconsin/66	215	100
A/turkey/England/63	210	68
A/turkey/Ontario/68	200	48
A/duck/Alberta/76	200	180
A/duck/England/56	210	68
A/equine/Prague/56	800	190
A/equine/Miami/63	380	370

no inhibitory effect on HVJ sialidase activity and weak inhibitory effect on the sialidase of NDV. However, DDNA significantly inhibited all three viral enzymes. The inhibitory effect of 3FNA on the influenza A virus sialidase was competitive (Fig. 2) with an apparent K_i value of 8.0 μM. As shown in Table 4, 3FNA also inhibited the sialidase activities of various influenza A virus strains and its fifty percent inhibition concentration (IC_{50}, estimated by the probit method with log[3FNA(μM)] and log[inhibition (%)] plotted on the x-axis and y-axis, respectively) for the 10 cases examined varied from 27 to 800 μM with 4-MU-NeuAc as the substrate, and from 17 to 370 μM with sialyllactose.

3FNA seems to be a specific inhibitor of sialidase with no effect on any other exoglycosidases, such as *N*-acetyl-β-glucosaminidase, *N*-acetyl-β-hexosaminidase, α-fucosidase, β-galactosidase, β-glucosidase, β-glucuronidase, and α-mannosidase (4-MU glycosides as substrates, data not shown).

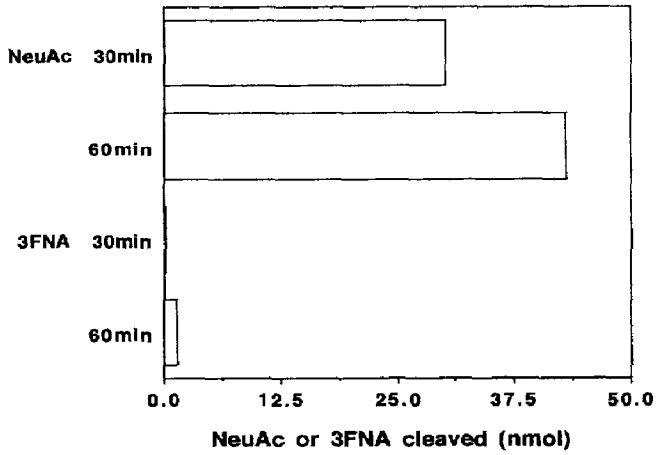


Fig. 3. Susceptibility of 3FNA to *N*-acetylneuraminate pyruvate-lyase. Each value represents the mean of triplicate experiments.

In contrast to NeuAc, 3FNA was only slowly cleaved by *N*-acetylneuraminate pyruvate-lyase (Fig. 3). Furthermore 3FNA, which was reported by Gantt et al. [7] to inhibit this enzyme, showed no retarding effect on it in our assay at concentrations up to 0.1 mM. The reason for the discrepancy between the earlier report [7] and ours is not clear. The difference in the enzyme sources used in these two investigations might have affected the results.

In summary, the selectivity of 3FNA as an inhibitor may be useful for classifying sialidases from various sources, and may favor the in vivo application of this compound. Recently, von Itzstein et al. [9] reported that the newly synthesized NeuAc derivative 4-guanidino-DDNA, having an inhibitory effect on influenza A virus sialidase, dramatically retarded influenza A virus replication in an animal model. Therefore, it is suggested that appropriate modification of NeuAc may produce promising candidates for anti-influenza virus drugs lacking an inhibitory effect on host sialidase.

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