TUNICAMYCIN, A NEW ANTIBIOTIC. III

REVERSAL OF THE ANTIVIRAL ACTIVITY OF TUNICAMYCIN BY AMINOSUGARS AND THEIR DERIVATIVES

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The antiviral activity of tunicamycin, an antibiotic containing glucosamine, was partially reversed with some aminosugars and their derivatives. N-Acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, and N-hexanoyl- and N-heptanoyl-D-glucosamines were the most potential competitors among all tested. D-Glucosamine, D-mannosamine and D-galactosamine were not as effective as their derivatives because of their toxicity. Pentoses, hexoses, methylpentoses and the other sugars examined were not as active as the above-mentioned aminosugar derivatives. Reversal of the antiviral activity of tunicamycin with aminosugars and their derivative was partial, and full reversal was not observed with any sugar or any combination of sugars examined. Cholesterol did not affect the antiviral activity of tunicamycin, and Mg^{++} had only a slight effect.

Tunicamycin is an antiviral antibiotic produced by *Streptomyces lysosuperificus*. Two moles of glucosamine were produced from one mole of tunicamycin on acid hydrolysis. Treatment with periodate markedly decreased the antiviral activity of tunicamycin within a few minutes[1]. Studies on the mechanism of action of tunicamycin reveal that it interferes with sugar uptake into acid-insoluble fractions. Syntheses of protein and nucleic acids are affected as a result of the inhibition (manuscript in preparation). These results suggest that glucosamine in the tunicamycin molecule plays an important role in the action of the antibiotic. The effect of sugars on the antiviral activity of tunicamycin was examined. Some derivatives of glucosamine, galactosamine and mannosamine were found to partially reverse the activity of tunicamycin against the multiplication of Newcastle disease virus (NDV) in cultured cells. The effect of sugars and sugar derivatives on NDV multiplication will be also discussed in this paper.

Materials and Methods

Virus, cell and medium: The MIYADERA strain of NDV was prepared as described previously[2]. Monolayer cultures of chick embryo fibroblasts (CEF) in rubber capped test tubes were employed as a host cell for virus multiplication. Cell culture was carried out according to the previously reported method[2]. Glucose was depleted from the medium in some cases in examination of effect of sugars on the antiviral activity of tunicamycin.

Virus titration: Hemagglutinin units (HAU) titration was employed as a sole method of quantitation of virus multiplication. Procedure of HAU titration was the same as reported previously[2].

Chemicals: Tunicamycin was prepared from cultures of *Streptomyces lysosuperificus*[1]. The same lot of three times recrystallized tunicamycin was used throughout the experiment. N-Benzoyl derivatives of glucosamine and galactosamine, mannosamine and galactosamine were obtained from Nakarai Chemicals Ltd., Kyoto. N-Acetyl derivatives of glucosamine and galactosamine, glucose-6-phosphate, chondroitin sulfate A and C, galactose-6-sulfate and 5-keto-fructose were purchased from Seikagaku Kogyo Co., Tokyo. Others were the products of Tokyo Chemical Co., Tokyo.

Results and Discussion

1. Effect of Aminosugars and their Derivatives

On hydrolysis of tunicamycin with HCl, glucosamine was found in the hydrolysate as revealed by column chromatography on Dowex 50 (H^+ type)[1]. Incorporation of radioactive glucosamine into acid-insoluble fractions was inhibited by the antibiotic at relatively low concentrations (manuscript in preparation). On the basis of these observations, the effect of aminosugars and their derivatives on the antiviral activity of tunicamycin against NDV was studied.

Cell cultivation and virus multiplication have hitherto been carried out in the presence of 1 mg/ml glucose. In a study of the competing effect of aminosugars against the antiviral activity of tunicamycin, glucose was studied to see if it affects the reversal effect of aminosugars. As shown in Table 1, the presence of glucose had a little influence on the competition of glucosamine, galactosamine and their derivatives, and the reversal effect of N-acetylaminosugars was better in the presence of glucose than in the absence of the sugar. Therefore reversal tests were carried out in the presence of glucose in the following.

All the aminosugars tested reversed to some extent the antiviral activity of tunicamycin, except N-benzoyl derivatives of glucosamine and galactosamine. Effects of N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine and N-alkylglucosamines were remarkable among all.

Almost all of the N-substituted aminosugars examined reversed the antiviral activity of tunicamycin, and they all showed less inhibitory activity against the multiplication of NDV than the corresponding free aminosugars. Free aminosugars were themselves antivirals at concentrations higher than 2.5 mg/ml, and virus yield was about one-hundredth to one-fifteenth of the controls at a drug concentration of 10 mg/ml, at which N-substituted aminosugars scarcely showed antiviral activity. Their competing effect against the antiviral activity of tunicamycin was not outstanding in comparison with their derivatives. BEKESI *et al.*[3] reported inhibitory effect of aminosugars and sugar derivatives on the viability and transplantability of Sarcoma 180 ascites tumor cells. They observed that free aminosugars such as glucosamine, galactosamine and mannosamine have stronger cytocidal and anti-transplantation activities than their N-acetyl derivatives. Similar effects were observed in virus multiplication as discussed above. The antiviral activity of the free aminosugars may be caused by their cytocidal effect on CEF. Thus the effect of aminosugars on ascites tumor cells may not be selective.

N-Benzoyl substitution decreased both the antiviral and reversing activities of

Table 1. Effect of aminosugars on the antiviral activity of tunicamycin in the presence and absence of glucose

Sugars	Concentration mg/ml	% HAU			
		−Glucose		+Glucose (1 mg/ml)	
		−TM[a]	+TM	−TM	+TM
D-Glucose	20			100	<0.6
	10			100	<0.6
	5.0			100	<0.6
	2.5			100	<0.6
	1.25			100	<0.6
	0 (control)	100	<0.6		
D-Glucosamine	20	1.3	1.3	0.8	0.8
	10	1.8	1.3	25	0.9
	5.0	13	6.0	NT[b]	NT
	2.5	42	8.8	NT	NT
	1.25	70	8.8	NT	NT
D-Galactosamine	20	1.3	0.9	1.1	1.3
	10	1.8	0.8	0.8	<0.6
	5.0	13	1.3	NT	NT
	2.5	70	<0.6	NT	NT
	1.25	100	0.9	NT	NT
D-Mannosamine	20	NT	NT	1.6	0.8
	10	NT	NT	2.6	1.3
	5.0	NT	NT	7.2	2.6
	2.5	NT	NT	86	0.8
	1.25	NT	NT	100	<0.6
N-Acetyl-D-glucosamine	20	100	10	100	20
	10	100	7.0	100	12
	5.0	100	5.0	NT	NT
	2.5	100	2.5	NT	NT
	1.25	100	1.3	NT	NT
N-Acetyl-D-galactosamine	20	100	13	100	28
	10	100	10	100	28
	5.0	100	8.5	NT	NT
	2.5	100	5.0	NT	NT
	1.25	100	2.5	NT	NT

Confluent monolayer cultures of CEF in test tubes were infected with NDV at an input multiplicity of 10 plaque-forming units per cell. After a 2-hour period of adsorption at room temperature, the infected cells were re-fed with fresh medium containing sugars at various concentrations. Glucose (1 mg/ml) was deleted from the medium in one group of experiments. Tunicamycin (0.5 mcg/ml) was added simultaneously with the sugars. The re-fed cultures were incubated at 39℃ for 24 hours. HAU titration was carried out after three cycles of freezing-and-thawing in a dryice-acetone bath.[2] HAU were expresssed as % of the controls. Results with N-acetyl derivatives of glucosamine and galactosamine are included in this Table for a comparison of effect of glucose on reversal effects of aminosugars. The procedures were the same in Tables 1 to 10.

a. Tunicamycin b. Not tested

Table 2. Effect of N-benzoyl derivatives of glucosamine and galatosamine on NDV multiplication and the antiviral activity of tunicamycin.

Compounds	Concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
N-Benzoyl-D-glucosamine	5.0	100	<0.9
	2.5	100	<0.9
	1.25	100	<0.9
N-Benzoyl-D-galactosamine	5.0	100	<0.9
	2.5	100	<0.9
	1.25	100	<0.9

Table 3. Effect of N-acetyl-aminosugars on NDV multiplication and the antiviral activity of tunicamycin

Sugars	Concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
N-Acetyl-D-glucosamine	80	13	2.5
	40	50	6.3
	20	100	13
	10	100	35
	5.0	100	13
N-Acetyl-D-galactosamine	80	13	6.3
	40	70	13
	20	100	25
	10	100	13
	5.0	100	16
N-Acetyl-D-mannosamine	50	50	16
	25	85	7.0
	12.5	100	7.0
	6.25	100	5.0
	3.13	100	3.5

aminosugars (Table 2).

Among N-acetyl derivatives of glucosamine, galactosamine and mannosamine, the former two reversed to a little more extent than the last (Table 3). N-Acetyl-glucosamine and N-acetyl-galactosamine have been found in many glycoproteins and glycolipids of animal origins, but no description of the presence of N-acetyl-mannosamine can be found. Difference in the reversing activity of the three N-acetyl-aminosugars may be partly explained by their presence or absence in animal cells.

Table 4. Effect of N-alkyl-glucosamines on NDV multiplication and the antiviral activity of tunicamycin

Compounds	Concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
N-Propionyl-D-glucosamine	40	70	2.5
	20	70	3.5
	10	70	1.8
	5.0	100	1.1
	2.5	100	0.8
N-Butyryl-D-glucosamine	40	85	2.5
	20	100	3.5
	10	100	0.8
	5.0	100	<0.6
	2.5	100	<0.6
N-Valeryl-D-glucosamine	40	35	2.5
	20	70	4.4
	10	70	1.8
	5.0	100	1.1
	2.5	100	<0.6
N-Hexanoyl-D-glucosamine	20	100	25
	10	100	18
	5.0	100	4.4
	2.5	100	1.7
N-Heptanoyl-D-glucosamine	10	60	13
	5.0	100	6.3
	2.5	100	2.5

Table 5. Combined effect of N-acetyl-aminosugars on NDV multiplication and the antiviral activity of tunicamycin

Sugar combinations	Total concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
N-Acetyl-D-glucosamine N-Acetyl-D-galactosamine	20	85	25
	10	100	18
	5.0	100	7.0
	2.5	100	6.3
	1.25	100	6.3
N-Acetyl-D-glucosamine N-Acetyl-D-galactosamine N-Acetyl-D-mannosamine	40	70	25
	20	100	30
	10	100	18
	5.0	100	5.0
	2.5	100	7,0

The same weight of each N-acetyl-aminosugar was mixed and inoculated simultaneously. Sugar concentrations were expressed as the total weight of the mixed sugars.

N-Alkyl-glucosamines showed relatively strong reversing effect (Table 4). N-Hexanoyl-glucosamine had the most significant activity in this series of derivatives, and its effect was quite similar to that of N-acetyl-aminosugars. N-Heptanoylglucosamine also had a relatively significant effect against the antiviral activity of tunicamycin, but its reversing activity was limited because of its lower solubility in water than the other N-alkylaminosugars tested. The other N-alkyl-glucosamines showed a competing effect against the antiviral activity of tunicamycin, but their effect was one-third to one-sixth of that of N-hexanoyl- and N-heptanoyl-glucosamines.

In these experiments, sugars were administrated individually. N-Acetyl-aminosugars are found in combination in natural origins. To increase the recovery ratio, some combinations of N-acetyl-aminosugars were inoculated to NDV-infected cell cultures. No synergistic effect was detected, and the effect observed was additive as shown in Table 5.

2. Effect of Pentoses

Arabinose and xylose were added to NDV-infected cell cultures. Neither of these sugars had any effect on NDV multiplication or on the antiviral activity of tunicamycin (Table 6).

3. Effect of Methylpentoses

Fucose belongs to this group of sugars. L-Fucose is found in membrane of animal cells and has a growth retarding effect on cultured mouse cells[4]. But both L- and D-fucoses had no effect on the multiplication of NDV in CEF at concentrations examined (Table 7). The antiviral activity of tunicamycin was only slightly affected by the sugars at relatively high concentrations.

Table 6. Effect of pentoses on NDV multiplication and the antiviral activity of tunicamycin.

Sugars	Concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
L-Arabinose	20	100	<0.6
	10	100	<0.6
	5.0	100	<0.6
D-Xylose	20	100	<0.6
	10	100	<0.6
	5.0	100	<0.6

Table 7. Effect of methylpentoses on NDV multiplication and the antiviral activity of tunicamycin

Sugars	Concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
L-Fucose	20	100	1.5
	10	100	0.8
	5.0	100	<0.6
D-Fucose	20	100	1.5
	10	100	<0.6
	5.0	100	<0.6

Table 8. Effect of aldohexoses and a ketohexose on NDV multiplication and the antiviral activity of tunicamycin

Sugars	Concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
D-Galactose	20	100	<0.6
	10	100	<0.6
	5.0	100	<0.6
D-Mannose	20	100	3.5
	10	100	0.9
	5.0	100	<0.6
D-Fructose	40	70	<0.6
	20	100	<0.6
	10	100	<0.6

Table 9. Effect of miscellaneous sugars on NDV multiplication and the antiviral activity of tunicamycin

Sugars	Concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
Glucose-6-phosphate	20	2.5	<0.6
	10	1.9	<0.6
	5.0	70	2.5
	2.5	100	1.5
	1.3	100	<0.6
Galactose-6-sulphate	10	1.9	<0.6
	5.0	1.9	<0.6
	2.5	13	<0.6
	1.3	100	<0.6
5-Ketofructose	20	5.0	<0.6
	10	3.0	0.8
	5.0	6.0	<0.6
	2.5	25	1.5
Raffinose	40	100	<0.6
	20	100	<0.6
	10	100	<0.6
	0.5	100	<0.6
Mannitol	40	100	<0.6
	20	100	<0.6
	10	100	<0.6
	0.5	100	<0.6
Chondroitin sulfate A	10	100	2.5
	5.0	100	1.8
	2.5	100	<0.6
Chondroitin sulfate C	20	35	1.5
	10	50	1.8
	5.0	100	1.5
	2.5	100	0.8

4. Effect of Hexoses

Glucose, galactose and mannose are found in glycoproteins of animal origins. These aldohexoses and fructose, a ketohexose, had neither antiviral activity against NDV multiplication nor reversing effect on the antiviral activity of tunicamycin at concentrations below 20 mg/ml except mannose (Tables 1 and 8). BEKESI *et al.*[3] observed cytocidal and anti-transplantation activities on ascites tumor cells with mannose. COX and GESNER[4] have also reported the effect of mannose on a growth of primate cells. No cytotoxic phenomenon was detected with this sugar by direct microscopic observation of confluent monolayer cultures of CEF at the highest drug concentrations tested.

5. Effect of Other Sugars

The effect of miscellaneous sugars examined is summarized in Table 9. Some of the sugars such as glucose-6-phosphate and chondroitin sulfate A and C partially reversed the antiviral activity of tunicamycin, but their effect was not as significant as that of aminosugar derivatives.

Table 10. Effect of $MgCl_2$ and cholesterol on NDV multiplication and the antiviral activity of tunicamycin

Compounds	Concentration mg/ml	% HAU	
		−Tunicamycin	+Tunicamycin (0.5 mcg/ml)
$MgCl_2$	4.0	<0.6	<0.6
	2.0	25	<0.6
	1.0	50	<0.6
	0.5	100	1.5
	0.25	100	0.8
Cholesterol	4.0	100	<0.6
	2.0	100	<0.6
	1.0	100	<0.6

6. Effect of Mg^{++} and Cholesterol

Tunicamycin was found to induce various morphological changes in such bacteria and yeasts as *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megatherium*, *Staphylococcus aureus*, *Sarcina lutea*, *Candida albicans* and *Saccharomyces cerevisiae* without inhibition of protein and nucleic acids syntheses. The antibiotic also inhibits multiplication of SP10 and SP01, bacteriophages of *Bacillus subtilis*, without inhibition of lysis of infected cells. These effects were found to be caused by a disturbance in carbohydrate metabolism involving syntheses of cell wall and cell membrane (manuscript in preparation). Many antibiotics are known to inhibit cell wall synthesis in bacteria or to alter functions of membranes[5]. Bacitracin, D-cycloserine, penicillin, ristocetin and vancomycin inhibit cell wall synthesis. Polyenes, polymixin, some polypeptide antibiotics, and some uncouplers of oxidative phosphorylation interefere with the function of membranes. Disturbance of membrane functions is known to be restored by cholesterol (polyene antibiotics), glucose (gramicidin) or anionic compounds (polymixins). Novobiocin induces filamentation in Gram-negative rod-type bacteria and this effect is reversed by Mg^{++}. Cholesterol did not affect the antiviral activity of tunicamycin, but $MgCl_2$ had a slight effect on it (Table 10). Tunicamycin did not act as an uncoupler of oxidative phosphorylation on rat mitochondria *in vtiro* (unpublished observation).

In conclusion, some sugars, especially aminosugar derivatives such as N-acetyl-glucosamine, N-acetyl-galactosamine, N-acetyl-mannosamine, and N-hexanoyl- and N-heptanoyl-glucosamines, partially reversed the antiviral activity of tunicamycin. No synergistic effect was observed with any combination of N-acetyl-aminosugars in their reversing action. Competition by some sugar analogs against the antiviral activity of tunicamycin is in favor of the observations in isotopic studies, *i.e.*, tunicamycin interferes with sugar metabolism including membrane synthesis and, as a result, inhibits the multiplication of NDV which matures at the plasma membrane and buds from it[6]. A drug which inhibits sugar metabolism including glycoprotein and glycolipid syntheses in both microorganisms and animal cells is not yet known. Tunicamycin may offer a useful tool in studies on membrane synthesis.

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