APPLIED MICROBIOLOGY, June 1969, p. 825-829

Vol. 17, No. 6
Printed in U.S.A.

Antiviral and Antitumor Antibiotics

XIV. Effects of Ascochlorin and Other Respiration Inhibitors on Multiplication of Newcastle Disease Virus in Cultured Cells

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Received for publication 30 December 1968

Antiviral activity on Newcastle disease virus was examined with some respiration inhibitors including ascochlorin, rotenone, antimycin A_3, piericidin A, dicoumarol, 2,4-dinitrophenol, pentachlorophenol, and fatty acids. Of the chemicals tested, ascochlorin and rotenone showed significant inhibitory effect on the viral growth in cultured cells as determined by the plate and tube assay methods. Dose response of ascochlorin and rotenone was observed in rate and final yield of hemagglutinin synthesis. Ratio of infectivity for hemagglutinin was nearly equal at any test concentrations of ascochlorin. Thus, the possibility of inhibition of virus maturation by the antibiotic was excluded. Ascochlorin had no activity on virus particles and on viral adsorption onto host cells.

Searching for antiviral antibiotics, we isolated a new antibiotic, ascochlorin, from a culture of *Ascochyta viciae* (14). Some other strains also produced the antibiotic (*unpublished data*). Ascochlorin was active on multiplications of both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses in cultured cells. Studies on the biological properties of the antibiotic found that it has inhibitory effect on mitochondrial respiration (*unpublished data*). Kalicheva et al. (4) reported the inhibitory action on the growth of tobacco mosaic virus of some respiration inhibitors such as antimycin A_3, azide, and cyanide. These results have led us to the examination of antiviral activity of respiration inhibitors on the multiplication of Newcastle disease virus (NDV). Employing an NDV-chick embryo fibroblast (CEF) system, we examined the effects on the viral reproduction of respiration inhibitors belonging to two groups which consist of inhibitors of electron transfer and uncouplers. Antimycin A_3, piericidin A, rotenone, and ascochlorin belong to the first group, and dicoumarol, 2,4-dinitrophenol, pentachlorophenol, and fatty acids to the second (5). Among inhibitors tested, rotenone exhibited antiviral activity which was similar to that of ascochlorin. Some biological aspects of the antiviral activity of ascochlorin are dealt with in this paper also.

MATERIALS AND METHODS

Virus-cell system. The Miyadera strain of NDV and primary cultures of CEF were employed, and the proliferation methods were described previously (11).

Assay of antiviral activity. Antiviral activity was examined by the plate or tube assay method or both. The agar-diffusion plaque-inhibition method of Herrmann et al. (3) was used for the plate assay, and diameters of plaque-free, virus-inhibited and cytotoxic zones caused by test compounds on infected monolayer cultures of CEF in petri dishes were determined after 2 days of incubation at 38.5 C. (1). The tube assay method was the same as reported previously (11), and antiviral activity was determined by observation of cytopathic effect after virus infection and by titration of virus production.

Test for virucidal effect. NDV was suspended in the medium containing a test compound, and the suspension was incubated at 38.5 C. At designated time intervals, titrations were performed and infectivity was measured in plaque-forming units (PFU; 11).

Action on viral adsorption onto host cells. NDV was added to suspensions of monodispersed CEF in the medium containing a test compound and was held at 5 C with occasional stirring. After 2 hr of incubation, viruses adsorbed to CEF were removed by centrifugation (900 × *g* for 15 min), and residual infectivity in the supernatant fluid was titrated (11).

Titration of virus. After virus was produced, it was titrated to determine plaque formation and hemagglutinin production. PFU and hemagglutinin units (HAU) were determined for cell-associated virus released by three cycles of freezing and thawing in a dry ice-acetone bath (11).

Chemicals. Rotenone and piericidin A were obtained through the courtesy of M. Matsui and N. Takahashi, respectively, of this department. Antimycin A_3 was purchased from Kyowa Hakko Co.,

Tokyo, Japan, and the other respiration inhibitors, i.e., dicoumarol, 2,4-dinitrophenol, pentachlorophenol, and fatty acids were obtained from Tokyo Kasei Co., Tokyo, Japan. Aliphatic dicarboxylic acids were also products of Tokyo Kasei Co.

RESULTS AND DISCUSSION

Antiviral activity of inhibitors of electron transfer in mitochondrial respiration. Ascochlorin has been shown to inhibit respiratory electron transfer in vitro (M. Morimoto et al., *in preparation*). Antimycin A_3, piericidin A, and rotenone belong to this class of inhibitors (5). Drug dose responses of the antiviral activity of these compounds were determined by the agar-diffusion method (Table 1). Antimycin A_3 and piericidin A were very toxic for cultured cells, and their effect on the viral growth was not as significant as that of ascochlorin and rotenone in this assay method. The latter two had rather high chemotherapeutic indexes in vitro; the ratios of the maximal concentration tolerated by cultured cells to the minimal virus-inhibitory concentration were higher than 50. The site of inhibition of respiration by piericidin A [I. Vallin and H. Löw, 7th International Congress of Biochemistry (Tokyo), p. 897, 1967] and rotenone (6) is the same, but piericidin A is

TABLE 1. *Antiviral activity of inhibitors of respiratory electron transfer as determined by the agar-diffusion method*[a]

Compounds	Concn (μg/ml)	CTZ[b]	AVZ[c]
		mm	*mm*
Rotenone	3000.00	28.0	35.6
	600.00	21.9	33.4
	120.00	16.4	25.9
	24.00	—	20.3
	4.80	—	16.8
	0.96	—	10.9
	0.19	—	—
Antimycin A_3	22.000	32.6	—
	4.400	25.7	—
	0.960	19.4	—
	0.176	12.8	13.5
	0.035	—	—
Piericidin A	7000.000	34.4	39.5
	1400.000	28.6	36.5
	280.000	29.5	34.0
	56.000	25.7	30.1
	11.200	21.6	26.3
	2.240	16.8	19.9
	0.450	12.0	17.8
Ascochlorin	3000.00	27.0	38.5
	600.00	23.8	34.1
	120.00	—	29.8
	24.00	—	27.0
	4.80	—	24.5
	0.96	—	18.3
	0.19	—	—

[a] Monolayer cultures of CEF in petri dishes (90 mm in diameter) were infected with NDV to form about 3,000 plaques per dish, the soft agar medium was overlaid, and paper discs [8 mm in diameter with about 25 μliters of methanol solution adsorbed per disc (Toyo Roshi Co., Tokyo, Japan)] impregnated with solutions of the drug in methanol were put on the hardened overlayers. After 2 days of incubation at 39 C, antiviral activity and cytotoxicity were determined.

[b] Diameter of the cytotoxic zone (CTZ) caused by the chemicals.

[c] Antiviral zone (AVZ) in which plaque formation after viral growth was suppressed.

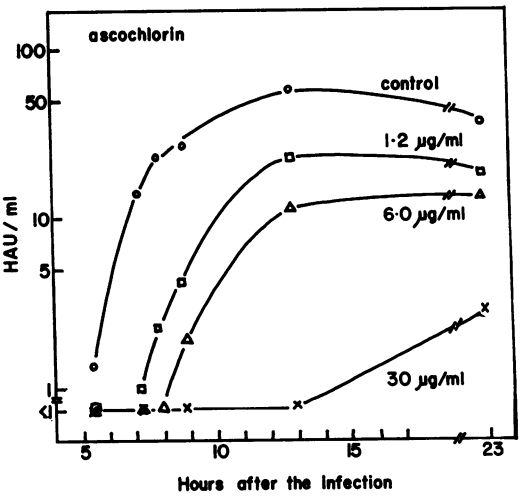

FIG. 1. *Antiviral activity of ascochlorin on NDV as determined by HAU titration.*

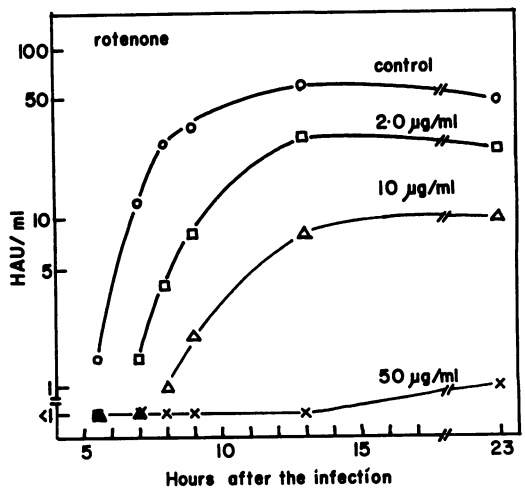

FIG. 2. *Antiviral activity of rotenone on NDV as determined by HAU titration.*

TABLE 2. *Effect of ascochlorin on production of infective NDV and of hemagglutinin*[a]

Antibiotic concn (μg/ml)	Percentage		
	PFU	HAU	% PFU/HAU
40.00	1.25	1.1	1.1
20.00	3.75	12.5	0.3
10.00	18.0	17.5	1.0
5.00	20.0	17.5	1.2
2.50	30.5	21.3	1.4
1.25	40.5	42.5	1.0
0.625	46.8	42.5	1.1
0.313	64.8	72.5	0.9

[a] Monolayer cultures of CEF in test tubes were infected at an input multiplicity of 50 PFU/cell, and ascochlorin was added after 2-hr adsorption period at 5 C. Production of infective virus and of hemagglutinin after 17 hr of infection was determined after releasing cell associated viruses by freezing and thawing and was expressed as a per cent of that of the controls.

very toxic for animals and fishes whereas rotenone has rather low toxicity for animals (7). The difference in in vitro toxicity determined by the plate assay method was similar to the observation in vivo.

Results of the antiviral activity of ascochlorin and rotenone were determined by the tube assay method (Fig. 1–2). A drug dose response was observed in both rate and final yield of hemagglutinin production. This observation differed from the results with xanthocillin X monomethylether (11) and trichothecin (12) which were found to be inhibitors of protein synthesis (12, 13). In the latter case, the viral growth was suppressed completely at concentrations of these drugs not toxic for cultured cells and the drug dose response was observed only at lower concentrations in the lengthening of the lag period before the detectable onset of hemagglutinin synthesis; no difference between antibiotic-treated and control samples was apparent in rate or final yield after the beginning of the synthesis.

Rather good correlation was found between production of infective virus and production of hemagglutinin, that is the ratio of PFU to HAU was nearly equal at various concentrations of ascochlorin (Table 2). Thus the possibility of inhibition of virus maturation by the antibiotic was excluded. As expected from the mode of action of ascochlorin on mitochondrial respiration, the antibiotic neither had virucidal effect on free NDV particles at 38.5 C (Fig. 3) nor influenced viral adsorption onto host cells (Table 3). It may be concluded from these results that inhibition of the viral growth by ascochlorin

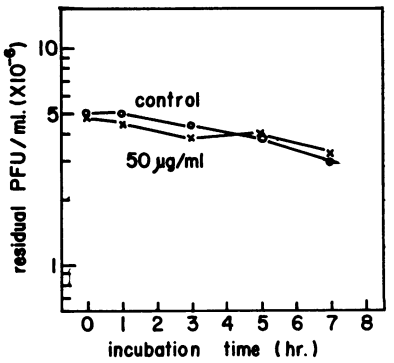

FIG. 3. *Virucidal effect of ascochlorin on NDV as measured by plaque formation.*

TABLE 3. *Effect of ascochlorin on NDV adsorption onto CEF*[a]

Antibiotic concn (μg/ml)	Residual PFU[b]
40	15.8
20	17.2
10	16.5
Control	17.0

[a] NDV was added to the suspension of monodispersed CEF in the medium, and the suspension was held at 5 C with occasional stirring. After 2 hr of incubation, adsorbed viruses were removed by centrifugation (900 × *g* for 15 min). Residual infectivity in the centrifugation supernatant fluid was titrated and was expressed in per cent of input infectivity.

[b] Residual PFU is expressed as the per cent of input PFU.

occurs after viral adsorption and before maturation. Interruption of the energy-generating system by inhibition of respiration may be partly, if not totally, the cause of the inhibition of the virus reproduction.

Antiviral activity of uncouplers of mitochondrial respiration. Dicoumarol, 2,4-dinitrophenol, pentachlorophenol, and fatty acids are representatives of uncouplers of mitochondrial respiration (5). These chemicals had no effect on the viral growth in the agar-diffusion method at concentrations at which most antibiotics exert their activity (1, 8–10, 12). At higher test concentrations, pentachlorophenol and some fatty acids exhibited antiviral activity (Tables 4–6).

The units of concentration in Table 5 are mixed, but the densities of the fatty acids expressed in per cent (v/v) are in a range of 0.90–0.94. Thus, since the values expressed in per cent v/v are nearly equal to those in w/v per cent, direct comparison of the values may be possible.

TABLE 4. *Antiviral activity of dicoumarol, pentachlorophenol, and 2,4-dinitrophenol as determined by the agar-diffusion method*

Compounds	Concn (μg/ml)	CTZ[a] (mm)	AVZ[a] (mm)
Dicoumarol	5,600	—	—
	1,120	—	—
Pentachlorophenol	3,680	25.6	27.8
	736	11.7	15.2
	147	—	—
2,4-Dinitrophenol	1,864	—	—
	373	—	—

[a] Same as in Table 1.

TABLE 5. *Antiviral activity of saturated fatty acids as determined by the agar-diffusion method*

CH_3-$(CH_2)_n$-COOH (n)	Name	Concn[a]	CTZ[b]	AVZ[b]
		%	mm	mm
3	*N*-valeric acid	4.0 v	17.8	—
		0.8 v		—
4	*N*-caproic acid	4.0 v	19.8	±
		0.8 v		—
5	Enanthioic acid	4.0 v	23.8	—
		0.8 v		—
6	*N*-caprylic acid	4.0 v	L[c]	
		0.8 v	L	
7	Perargonic acid	4.0 v	L	
		0.8 v	L	
8	*N*-capric acid	1.5 w	43.2	—
		0.3 w	18.0	—
9	*N*-undecanoic acid	2.0 w	39.2	—
		0.4 w	15.7	—
10	Lauric acid	1.0 w	17.3	—
		0.2 w	—	—
11	*N*-tridecanoic acid	10.0 w	9.6	12.6
		2.0 w	—	—
12	Myristic acid	1.0 w	—	13.6
		0.2 w	—	—
13	Pentadecyclic acid	1.5 w	—	13.9
		0.3 w	—	—
14	Palmitic acid	8.0 w	—	13.6
		1.6 w	—	—
15	Margaric acid	5.0 w	—	12.2
		1.0 w	—	—
16	Stearic acid	5.0 w	—	12.2
		1.0 w	—	—

[a] Concentrations are expressed in v/v% (v) or in w/v% (w).
[b] Same as in Table 1.
[c] Large.

Saturated fatty acids of short chain length, especially C_8–C_{11}, were toxic for cultured cells, and cytotoxicity decreased when the chain length increased (Table 5). A similar response in vivo in

TABLE 6. *Antiviral activity of unsaturated fatty acids as determined by the agar-diffusion method*

Name	Concn[a]	CTZ[b]	AVZ[b]
	%	mm	mm
Crotonic acid (C_4)	7.5 w	—	16.7
	1.5 w	—	—
Elaidic acid (C_{18})	9.0 w	—	11.6
	1.8 w	—	—
Vaccenic acid (C_{18})	9.0 w	—	13.3
	1.8 w	—	—
Oleic acid (C_{18})	30.0 v	—	20.6
	6.0 v	—	—
Linoleic acid (C_{18})	7.0 v	31.2	—
	1.2 v	13.9	±
Linolenic acid (C_{18})	1.5 v	19.2	—
	0.3 v	—	—
Erucic acid (C_{22})	8.0 v	—	12.0
	1.6 v	—	—

[a] Same as in Table 5.
[b] Same as in Table 1.

TABLE 7. *Antiviral activity of saturated aliphatic dicarboxylic acids as determined by the agar-diffusion method*

HOOC-(CH_2)-COOH	Name	Concn (w/v%)	CTZ[a]	AVZ[a]
			mm	mm
0	Oxalic acid	2.50	14.5	21.1
		0.25	—	—
1	Malic acid	2.50	24.3	38.3
		0.25	—	—
2	Succinic acid	2.00	11.4	18.5
		0.20	—	—
3	Glutaric acid	2.50	12.6	22.2
		0.25	—	—
4	Adipic acid	3.50	20.2	27.4
		0.35	—	—
5	Pimeric acid	3.00	14.9	20.8
		0.30	—	—
6	Subelic acid	2.00	10.4	16.2
		0.20	—	—
7	Azelaic acid	4.00	13.1	20.8
		0.40	—	—
8	Sebecic acid	3.00	13.6	21.8
		0.30	—	—

[a] Same as in Table 1.

toxicity and antitumor activity of fatty acids was observed (G. Tamura, K. Ando, K. Kato, S. Suzuki, K. Suzuki, K. Kodama, and K. Arima. J. Antibiot., *in press*). Myristic and pentadecyclic acids were the most active of all tested. Eisler and von Metz (2) examined the effects of fatty acids on *Pasteurella pestis* and observed that lauric and myristic acids were the most active and the effect of lauric acid was more marked

than that of myristic acid. In the case of antiviral activity, myristic acid had the greatest activity of all. At the dosages tested, lauric acid was not active on virus reproduction and was more cytotoxic than myristic acid. The discrepancy between the anti*Pasteurella* and antiviral activities may be partly explained by the difference in cytotoxicity.

The unsaturated fatty acids did not appear to have greater antiviral activity than the saturated fatty acids (Table 6).

Esterification of fatty acids decreased their cytotoxicity or antiviral activity as indicated in the cases of lauric, stearic, and oleic acids and their glycerides. Monolaurin and monostearin were both tested at concentrations of 4.0 and 0.8% (w/v) and triolein at 40.0 and 8.0% (w/v). No antiviral activity was present except in the case of monolaurin which had an antiviral zone of 14.2 mm.

Effects of aliphatic dicarboxylic acids on the viral growth were tested for a comparison (Table 7). The dependency of cytotoxicity and antiviral activity on the chain length, which was observed in the cases of the monocarboxylic acids (Table 5), was not detected in the cases of dicarboxylic acids.

In conclusion, the uncouplers of mitochondrial respiration tested were not effective on virus growth at concentrations at which most antivirals are expected to exert their activity. Antiviral activity of some inhibitors of electron transfer in mitochondrial respiration, such as antimycin A_3 and piericidin A, was also not significant, because of their toxicity for cultured cells. The exceptions were rotenone and ascochlorin. These two compounds showed remarkable activity at low concentrations, their toxicity for cultured cells was low, and their chemotherapeutic indexes in vitro were higher than 50. These results are indicative of the presence of effective antivirals among inhibitors of respiratory electron transfer. Some derivatives of rotenone were found to effectively inhibit NDV growth in cultured cells, and their effect on other viruses and the correlation between antiviral and respiration-inhibitory activities are under study now.

LITERATURE CITED

1. Arima, K., A. Takatsuki, S. Suzuki, K. Ando, and G. Tamura. 1968. Antiviral activity of trichothecin. J. Antibiot. (Tokyo) 21:158–159.
2. Eisler, D. M., and E. K. von Metz. 1968. Anti-*Pasteurella pestis* factor. III. Effects of fatty acids on *Pasteurella pestis*. J. Bacteriol. 95:1767–1773.
3. Herrmann, E. C., Jr., J. Gabliks, C. Engle, and P. L. Perlman. 1960. Agar diffusion method for detection and bioassay of antiviral antibiotics. Proc. Soc. Exp. Biol. Med. 103: 625–628.
4. Kalicheva, G. S., L. N. Loginova, and L. N. Saldan. 1966. The effect of some respiration inhibitors on the reproduction of tobacco mosaic virus. Soobsch. Akad. Nauk Gruz. SSR 41:431–434.
5. Kobayashi, S., and K. Tagawa. 1965. Inhibitors of mitochondrial respiration. Protein Nucleic Acid Enzyme 10:1596–1609.
6. Lindahl, P. E., and K. E. Öberg. 1961. The effect of rotenone on respiration and its point of attack. Exp. Cell Res. 23:228–237.
7. Matsunaka, S. 1968. Biochemical aspects of pesticides. Protein Nucleic Acid Enzyme 13:35–46.
8. Miller, F. A., W. A. Rightsel, B. J. Sloan, J. Ehrlich, J. C. French, and Q. R. Bartz. 1963. Antiviral activity of tenuazoic acid. Nature (London) 200:1338–1339.
9. Rightsel, W. A., H. G. Schneider, B. J. Sloan, P. R. Graf, F. A. Miller, Q. R. Bartz, and J. Ehrlich. 1964. Antiviral activity of gliotoxin and gliotoxin acetate. Nature (London) 204:1333–1334.
10. Takatsuki, A., S. Suzuki, K. Ando, G. Tamura, and K. Arima. 1968. New antiviral antibiotics; xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether. I. Isolation and characterization. J. Antibiot. (Tokyo) 21:671–675.
11. Takatsuki, A., G. Tamura, and K. Arima. 1968. New antiviral antibiotics; xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether. II. Biological aspects of the activity. J. Antibiot. (Tokyo) 21:676–680.
12. Takatsuki, A., G. Tamura, and K. Arima. 1969. Mode of action of trichothecin on multiplication of Newcastle disease virus in cultured cells. J. Antibiot. (Tokyo) 22:241–247.
13. Takatsuki, A., G. Tamura, and K. Arima. 1969. Mode of action of xanthocillin X monomethylether on multiplication of Newcastle disease virus in cultured cells. J. Antibiot. (Tokyo) 22:151–160.
14. Tamura, G., S. Suzuki, A. Takatsuki, K. Ando, and K. Arima. 1968. Ascochlorin, a new antibiotic, found by paper-disc agar-diffusion method. I. Isolation, biological and chemical properties of ascochlorin. J. Antibiot. (Tokyo) 21:539–544.