ANTIVIRAL ACTIVITY OF BREFELDIN A AND VERRUCARIN A

Sir :

In a primary screening using paper-disc agar-diffusion plaque-inhibition method by HERRMAN'S[1], we selected two fungi, FL-24 and No. 38, from soil samples, which produced antiviral antibiotics effective against Newcastle disease virus strain Miyadera (NDV). The fungi were aerobically cultured in a medium containing glucose, peptone and yeast extract as the main nutrients. The active principles were extracted from both mycelia and culture filtrates and isolated in crystalline form by silica gel column chromatography. Infrared and ultraviolet absorption spectra, melting point and molecular weight determined by mass spectroscopy of the antibiotic produced by FL-24 were virtually identical with those of brefeldin A[2]. The antibiotic produced by No. 38 was found to be verrucarin A[3] from infrared, ultraviolet and mass spectra comparisons.

Brefeldin A and verrucarin A are antibiotics with antifungal and cytotoxic activity *in vitro* as well as antitumor activity *in vivo* against some experimental tumors but the antiviral activity of these antibiotics was previously unknown.

Table 1. Antiviral activity of brefeldin A in plaque-inhibition agar-diffusion method.

Concentration (mcg/ml)	Antiviral activity	
	CTZ	PIZ
2,000	17	70
400	12	61
100	—	58
25	—	46
6	—	40
1.5	—	24
0.4	—	15

CTZ : Cytotoxic zone in diameter (mm)
PIZ : Plaque inhibited zone (mm)

The cell monolayer used was primary chick embryo fibroblast which were infected with Newcastle disease virus strain Miyadera after confluence. The paper-disc used was the product of Toyo Kagaku Co., Ltd., and was 8 mm in diameter. Antiviral activity is expressed as diameter of plaque-free protected zones with viable cells together with inner cytotoxic zone.

Table 2. Antiviral activity of verrucarin A in plaque-inhibition agar-diffusion method.

Concentration (mcg/ml)	Antiviral activity	
	CTZ	PIZ
2,000	49	70
500	42	65
125	38	57
31	32	48
8	28	40

CTZ : Cytotoxic zone in mm diameter
PIZ : Plaque inhibited zone in mm diameter
The method was the same as in Table 1.

In this communication, the antiviral activity of brefeldin A and verrucarin A in both the agar-diffusion plaque-inhibition and tube culture methods is described.

The antiviral activity of these antibiotics is remarkable *in vitro*, especially with the agar-diffusion plaque-inhibition method. It is characteristic of brefeldin A that it forms large plaque-free protected zones without cytotoxicity (Table 1). In this assay system brefeldin A is effective against herpes simplex virus strain HF (HSV) as well as NDV so that both DNA and RNA viruses are included in its antiviral spectrum. The paper-disc used absorbed only 0.05 ml of solvent per disc so 0.1 mg of brefeldin A gave a 70 mm diameter zone free from plaque formation by NDV. The effective dose, therefore, is low compared with tenuazonic acid[4] and trichothecin[5].

In contrast to brefeldin A, verrucarin A is a powerful cytotoxic antibiotic to primary chick embryo fibroblast monolayer (Table 2). At a concentration of 2 mg/ml, it formed a plaque-inhibited zone of 75 mm diameter and a cytotoxic zone of 50 mm diameter within the plaque-inhibited zone. We confirmed in another experiment that the minimum inhibitory dose is 0.01 mcg/ml. As far as antiviral activity is concerned, verrucarin A is the most active agent yet known. It is also effective against HSV in this assay system.

Initial experiments to determine the susceptibility of HeLa cells to the toxic effect of brefeldin A and verrucarin A were carried out in triplicate tubes. After 24 and 48 hours' exposure to the antibiotics, cell monolayer were vitally stained with neutral red, graded for degradation and the

Table 3. Inhibition of cytopathic effect by brefeldin A and verrucarin A in tube culture.

		Cytopathic effect of HeLa cells					
		Vaccinia virus			NDV		
		48 hrs.	72 hrs.	96 hrs.	48 hrs.	72 hrs.	96 hrs.
Brefeldin A	0.0150 mcg/ml	− − −	− − −	+ + +	− − −	− − −	− − −
	0.0075 〃	− − −	+ + +	+ + +	+ + +	‡ ‡ ‡	‡‡ ‡‡ ‡‡
Verrucarin A	0.0025 〃	− − −	− − −	− − +	− − −	− − −	− − −
	0.0013 〃	− − −	− − −	+ + +	− − −	− − −	− − −
Control		+ + +	‡ ‡ ‡	‡‡ ‡‡ ‡‡	+ + +	‡ ‡ ‡	‡‡ ‡‡ ‡‡

CPE was graded as follows : − no CPE, + 1~25 % CPE, ‡ 26~50 % CPE, ‡‡ 51~75 % CPE, ‡‡‡ 76~100 % CPE. Inhibition of CPE were read by direct microscopic observation. Triplicate tubes were used in each concentration.

LD_{50} was calculated.

The LD_{50} of brefeldin A for HeLa cells was approximately 0.03 mcg/ml and that of verrucarin A was 0.005 mcg/ml. Experiments were performed to determine effective concentrations in serial dilution tests starting with one half the LD_{50}. Cytopathic effect (CPE) of the cell layer caused by virus infection was determined once a day through direct microscopic observation.

As shown in Table 3, brefeldin A completely inhibited CPE of NDV at a concentration of 0.015 mcg/ml. However, the effect was not so impressive as expected from the results in the agar-diffusion plaque-inhibition method, because CPE of HeLa cells was observed at a concentration of 0.0075 mcg/ml or below so that the chemotherapeutic index of brefeldin A is approximately one. Although the antibiotic inhibits plaque formation by HSV in the agar-diffusion assay system, it was almost ineffective against vaccinia virus strain DII in tube culture.

Verrucarin A is able to inhibit CPE caused by infection of both NDV and vaccinia virus strain DII at concentrations between 0.0025 and 0.0013 mcg/ml ; the chemotherapeutic index is two in this assay system. As the effective concentration is surprisingly low, verrucarin A may exhibit a different mode of action on mammalian cells.

It is the common characteristics of these two antibiotics that they form large plaque-free protected zones on primary chick embryo fibroblast monolayer and inhibit CPE of HeLa cells infected with either NDV orvaccinia virus at low concentrations.

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