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CHARACTERISTICS OF THE ANTIVIRAL EFFECT OF 9-(S)-(2, 3-DIHYDROXYPROPYL)ADENINE

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Summary. — 9(S)-(2,3-Dihydroxypropyl)adenine (DHPA) at 1–3 mg/ml completely inhibited the replication of vaccinia virus in ZP cells; the analogue did not inactivate the virus. Formation of a virus component sensitive to DHPA preceded by 2 hr the formation of infectious vaccinia virus. It seems likely that the component inhibited by DHPA is viral mRNA. The virus inhibitory effect was not reverted by numerous nucleotides, nucleosides or amino acids. DHPA was not metabolized during the latent period of vaccinia virus replication cycle either in uninfected ZP or chick embryo cell cultures.

Key words: 9-(S)-(2,3-dihydroxypropyl)adenine; mechanism of action; inhibition of virus multiplication; vaccinia virus

Introduction

The variation of sugar moiety in nucleosides revealed many substances with antiviral activity (for review, see De Clercq, 1979). Recently, a novel type of nucleoside analogues was developed in which the sugar moiety is replaced by an aliphatic chain resembling portion of the sugar moiety. Of these analogues, acycloguanosine [9-(2-hydroxyethoxymethyl)guanine] (Elion *et al.*, 1977; Schaeffer *et al.*, 1978) and 9-(S)-(2,3-dihydroxypropyl)adenine (DHPA) were shown to be the most promising virus inhibitors. Inhibition of multiplication of several DNA and RNA viruses by DHPA was described (De Clercq *et al.*, 1978; Kára *et al.*, 1979) and relationships of the structure of DHPA and its virus inhibitory activity were studied (De Clercq and Holý, 1979). In our laboratories a strong antiviral effect of the combination of DHPA and 6-azauridine was found, though limited thus far to chick embryo cell cultures (Rada and Holý, 1980).

The aim of the present work was to study the site of action of this analogue in the virus replication cycle and other characteristics of this action, since so far no data about the mechanism of action of DHPA were available.

Materials and Methods

Cells and virus. ZP cells, a cell line derived by Szántó (1960) from rabbit lungs, were grown either as monolayers or in suspension in Earle's medium with increased glucose content (0.6 %) and supplemented with 0.1 % yeastolate and 10 % calf serum. Chick embryo cells (CEC) used as monolayers or cell suspensions were grown in medium M-199 supplemented with 10 % calf serum. The WR strain vaccinia virus was obtained from Dr. N. P. Salzman, National Institute of Allergy and Infectious Diseases, Bethesda, U.S.A. The stock was prepared by infection of HeLa cell monolayers, distributed in tubes and stored at −60 °C. Virus titres were determined by plaque assay in CEC and expressed in plaque-forming units (PFU).

Virus inhibitory experiments in ZP cell cultures were arranged as described by Salzman *et al.* (1963). The suspension of ZP cells (5×10^6 cells per ml) was infected with 10 PFU of vaccinia virus per cell. Adsorption proceeded at 37 °C for 1 hr. The suspension was then washed three times with medium to remove unadsorbed virus. The infected cells were resuspended at a density of 2×10^5 cells per ml. Unless otherwise stated, the cultures were incubated for 24 hr. All virus samples were subjected to 4 cycles of freezing and thawing prior to virus titration.

DHPA action on vaccinia virus replication cycle in ZP cells. The cells were pelleted 1 hr after infection to remove unadsorbed virus, resuspended in medium and replicate cultures were established. The rate of formation of infectious virus was determined by sampling one culture at various times. New infectious virus was detected 6 to 8 hr after infection and the rise in titre was logarithmic for the next several hours. To other cultures, DHPA (3 mg/ml) was added at various times after infection; all cultures were sampled at 24 hr and titrated. Thus, any virus formed after addition of the inhibitor must have contained the DHPA — sensitive component of the virus synthesized prior to the addition of DHPA.

Effect of DHPA on the virion. Suspensions of vaccinia virus (1×10^6 PFU/ml) in normal medium and in medium containing DHPA in concentrations of 1 and 5 mg/ml were incubated at 37 °C, and the virus titre was determined.

Reversal of the inhibitory effect. The agar-diffusion plaque-inhibition method (Rada and Blaškovič, 1966) was used. CEC monolayers in 10 cm dishes were infected with a virus dose producing semiconfluent plaques. After agar overlay had solidified, glass cylinders were placed in the agar overlay and solutions of DHPA (0.24 M; central cylinder) or of the effectors (6×10^{-2} M; lateral cylinders placed 10 mm from the central one) were applied. At the concentration used, DHPA formed a plaque-free concentric zone of 40 mm diameter. Cultures with substances tested for reversal were examined for a change of the shape of this zone.

DHPA metabolism in vivo. ZP cell suspensions were collected by low speed centrifugation and resuspended at 1×10^7 cells per ml (or 5×10^7 cells per ml with secondary CEC). Vaccinia virus (5—10 PFU per cell) was added and adsorption proceeded at 37 °C for 1 hr. The cultures were then washed twice to remove unadsorbed virus, the cells resuspended at a concentration of 1×10^7 cells per ml and after 1, 2 or 3 hr after infection exposed for 45 min to 2 MBq of [8-^{3}H]-DHPA per ml (molar activity 20 GBq/mmol).

The cells were then harvested by centrifugation, the medium deproteinized by trichloroacetic acid precipitation at 0 °C and the respective supernatants ($1.5—2 \times 10^6$ count/min per ml) analyzed by paper chromatography in the solvent system 2-propanol — conc. aqueous ammonia — water (7:1:2) with DHPA, its 3'-phosphate and 9-(S)-(2,3-dihydroxypropylhypoxanthien as standards. The radioactivity distribution on chromatograms was detected by the Packard Model 7200 scanner and by liquid scintillation in the toluene-based scintillation mixture. Cell pellets were washed three times with saline at 0 °C and precipitated twice with trichloroacetic acid. Both supernatants after trichloroacetic acid precipitation were combined ($5—7 \times 10^5$ count/min per ml) and analyzed similarly to the samples of cultivation media.

Materials. Uridine, guanosine monohydrate, adenosine 5'-monophosphate, guanosine 5'-monophosphate disodium salt. 4 1/2 H_2O, adenosine 5'-triphosphate disodium pentahydrate, quanosine 5'-triphosphate trilithium dihydrate were purchased from Calbiochem, U.S.A. Adenosine, xanthosine, inosine 5'-monophosphate disodium salt and inosine 5'-triphosphate trisodium salt were products of Koch-Light, England. Cytidine was obtained from Reanal, Hungary, 2'-deoxythymidine and folic acid were purchased from Lachema, Czechoslovakia. 2'-Deoxyuridine was obtained from Fluka A.G., Switzerland, adenosine 3'-phosphate (free acid) from Serva (F.R.G.). L-Alanine, L-valine, L-leucine, L-isoleucine, L-threonine, L-cyteine and L-methionine were products of Nutritional Biochemicals Corp., U.S.A. [8-^{3}H]-9(S)-(2,3-Dihydroxypropyl) adenine (20 GBq/mmol) was prepared by Dr. J. Černý, Radioisotope Laboratories, Czechoslovak Academy of Sciences, Prague.

Results and Discussion

DHPA caused no significant alteration in growth or morphology of ZP cells at concentrations from 1—3 mg/ml ($0.5-1.5\times10^{-5}$ M). At a concentration of 10 mg/ml (5×10^{-5} M), the cells ceased to multiply and the pH of the medium was slightly more alkaline than that in an untreated control culture. This concentration also caused cell rounding or shrinking and formation of elongated cytoplasmic processes. However, the vaccinia virus yield was reduced by more than 99 % at a concentration of 1 mg/ml (Fig. 1). The titre of virus suspension exposed to DHPA at this concentration or at 5 mg/ml

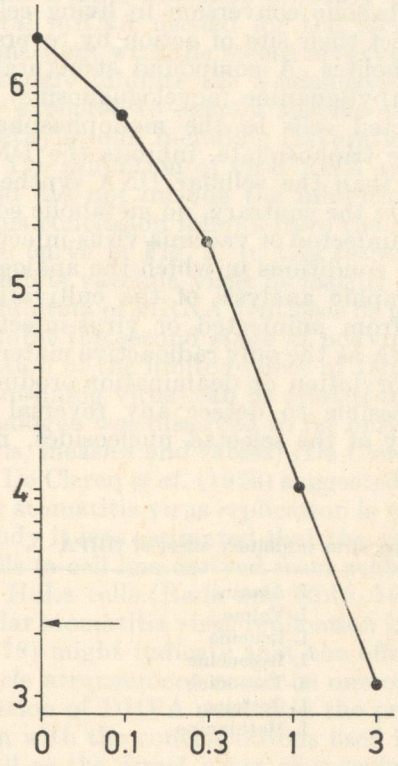

Fig. 1.

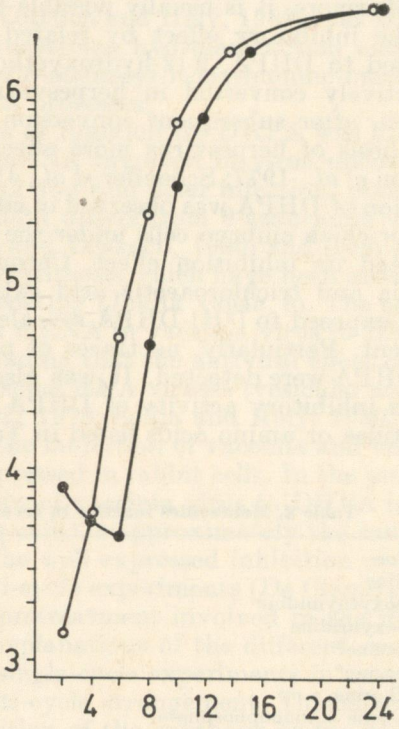

Fig. 2.

Fig. 1. Relationship between DHPA concentration and single-cycle yield of vaccinia virus from ZP cell cultures.
Abscissa: DHPA mg/ml; ordinate: log PFU/ml (arrow indicates titre at 1 hr after infection)

Fig. 2. The curve of the formation of a vaccinia virus component sensitive to inhibition by DHPA and the curve of virus formation.
Abscissa: hr after infection; ordinate: virus titre in log PFU/ml
● Virus titres in untreated control cultures
○ Virus titres obtained at 24 hr, after the addition of DHPA at the times indicated by the position of the point

Table 1. Lack of inactivation of vaccinia virus in the presence of DHPA

Time (hr)	PFU/ml		
	Medium alone	DHPA 1 mg/ml	DHPA 5 mg/ml
0	9.5×10^5	1.1×10^6	1.4×10^6
3	1.1×10^6	1.4×10^6	1.0×10^6

did not differ from the untreated control virus (Table 1); thus the antiviral effect of DHPA cannot be due to its action upon the virion outside the cell.

Most nucleoside analogues undergo metabolic conversion in living cells. Furthermore, it is usually possible to detect their site of action by reversal of the inhibitory effect by related metabolites. A compound structurally related to DHPA, 9-(2-hydroxyethoxymethyl)guanine (acycloguanosine), is selectively converted in herpesvirus-infected cells to the monophosphate which, after subsequent conversion to the triphosphate, inhibits the DNA synthesis of herpesvirus more effectively than the cellular DNA synthesis (Elion *et al.*, 1977; Schaeffer *et al.*, 1978). On the contrary, no metabolic conversion of DHPA was observed in either uninfected or vaccinia virus-infected ZP or chick embryo cells under the above conditions in which the analogue exerted its inhibition effect. Chromatographic analysis of the cultivation media and trichloroacetic acid extracts from uninfected or virus-infected cells exposed to [^{3}H]-DHPA revealedDHPA as the only radioactive material present. Partiularly, no traces of phosphorylation or deamination products of DHPA were detected. It was also impossible to detect any reversal of ivrus inhibitory activity of DHPA by any of the selected nucleosides, nucleotides or amino acids listed in Table 2.

Table 2. Metabolites inactive in reversal of the virus inhibitory effect of DHPA

Uridine	L-Alanine
Cytidine	L-Valine
2'-Deoxythymidine	L-Leucine
2'-Deoxyuridine	L-Isoleucine
Adenosine*	L-Threonine
Guanosine*	L-Cysteine
Xanthosine	L-Methionine
Adenosine 5'-monophosphate	
Adenosine 3'-monophosphate	Folic acid
Guanosine 5'-monophosphate*	
Inosine 5'-monophosphate	
Adenosine 5'-triphosphate	
Guanosine 5'-triphosphate*	
Inosine 5'-triphosphate	

* These metabolites caused opposite changes of the shape the plaque-free zones, i.e. enlargement of the inhibition of vaccinia virus plaque formation produced by DHPA. In separate control cultures guanosine 5'-triphosphate itself (without DHPA) caused a plaque-free zone of 43 mm. Smaller virus inhibitory zones in control cultures (without DHPA) were also found with guanosine 5'-monophosphate (34 mm), guanosine (29 mm) and adenosine (25 mm).

The experiments performed to determine the site of DHPA action in the virus replication cycle revealed that the synthesis of a viral component sensitive to the drug starts within 2 hr after infection and precedes by 2 hr the formation of infectious virus (Fig. 2). A comparison of this curve obtained with DHPA with the curve obtained with vaccinia virus and 6-azauridine (Rada and Blaškovič, 1966) suggests that the virus component inhibited by DHPA has the same time course as the formation of vaccinia mRNA as detected by 6-azauridine inhibition. On the other hand, fluorodeoxyuridine or iododeoxyuridine inhibit viral DNA and curves obtained with these inhibitors precede by 3 hr the formation of infectious virus. Thus the curve obtained with DHPA indicates probably the rate of formation of the last species of viral mRNA needed for the formation of infectious virus. This finding is consistent with the recent observation (Votruba and Holý, 1980) that DHPA strongly inhibits the S-adenosyl-L-homocysteine hydrolase action and can be expected to exert a specific effect on S-adenosyl-L-methionine-mediated methylations, including maturation RNA processes.

The inhibition of viral RNA synthesis or function by DHPA was limited and did not include the inhibition of mRNA involved in virus uncoating. This conclusion follows from the initial (2 and 4 hr after infection) points of the curve in Fig. 2. In this respect, DHPA differs from 6-azauridine which inhibits vaccinia virus uncoating (Rada and Doskočil, 1980) and from other inhibitors of mRNA synthesis or protein synthesis which have been shown to inhibit the second stage of poxvirus uncoating (Joklik 1964*a*, *b*). The explanation of the limited effect of DHPA on mRNA in vaccinia virus (a DNA—containing virus) can be related to the finding that the antiviral effect of the analogue was observed so far only with some RNA viruses (vesicular stomatitis, measles and rabies) (De Clercq *et al.*, 1978; Sodja and Holý, 1980).

De Clercq *et al.* (1978) suggested that the inhibition of vaccinia and vesicular stomatitis virus replication is well expressed in rabbit cells. In the present study it was estimated that the sensitivity of vaccinia virus to DHPA in ZP cells (a cell line derived from rabbit lung cells) is approximately the same as in HeLa cells (Rada and Holý, 1980). The well expressed inhibition of vesicular stomatitis virus replication in multi-cycle experiments (De Clercq *et al.*, 1978) might indicate that the effect of pretreatment involved in the multi-cycle arrangement could be one of the explanations of the different concentration of DHPA needed in the present single-cycle experiments in comparison with the concentrations used in multi-cycle arrangement. This effect, as well as the direct proof of specific inhibition of the synthesis or function of viral RNA still remains to be established.

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