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SHORT COMMUNICATION

Anti-herpes Activity of Deoxythymidine Analogues: Specific Dependence on Virus-Induced Deoxythymidine Kinase

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SUMMARY

DE CLERCQ, E., KRAJEWSKA, E., DESCAMPS, J. & TORRENCE, P. F. (1977) Anti-herpes activity of deoxythymidine analogues: specific dependence on virus-induced deoxythymidine kinase. *Mol. Pharmacol.*, 13, 980-984.

In both primary rabbit kidney and human skin fibroblasts, the replication of a particular herpes simplex virus, HSV-1 (strain KOS), is markedly inhibited by a wide variety of pyrimidine deoxyribo- and arabinonucleosides, while another herpes simplex virus, HSV-2 (strain 333), is not affected by any of the pyrimidine nucleoside analogues. The differential responsiveness of the two herpes virus strains to the antiviral effects of the nucleoside analogues could be accounted for by the presence or absence of viral deoxythymidine kinase in the infected cell, since HSV-1 (KOS) was found to induce deoxythymidine-deoxycytidine kinase activity in both primary rabbit kidney and human skin fibroblasts, whereas HSV-2 (333) failed to do so.

Pyrimidine nucleoside analogues such as thymine arabinoside (1), 5-bromo-2'-deoxycytidine, 5-iodo-2'-deoxycytidine (2), and 5-ethyl-, 5-propyl-, and 5-allyl-2'-deoxyuridine (3) have been reported to inhibit the replication of herpes simplex virus at concentrations which did not prove toxic for the host cell. Since bromodeoxycytidine is readily phosphorylated to its 5'-monophosphate by extracts of HSV[4]-infected cells but not by extracts of uninfected cells (4), the selective inhibitory effects of the 5-halogenated deoxycytidine analogues on HSV replication were thought to result from the action of a virus-induced pyrimidine deoxynucleoside kinase (2). The necessity for a specific virus-induced kinase has also been invoked to explain the selective anti-herpes activity of ara-T (1) and 5-ethyl-, 5-propyl-, and 5-allyl-2'-deoxyuridine (3).

Recently we have observed that the herpes type 2 (HSV-2) strain 333, unlike the herpes type 1 (HSV-1) strain KOS, appeared completely resistant to a wide variety of pyrimidine nucleoside analogues when assayed in both primary rabbit kidney and human skin fibroblast cultures. This finding offered a unique opportunity to assess directly the requirement of a virus-coded kinase for the anti-herpes activity of nucleoside analogues.

The HSV-1 strain KOS was originally obtained from W. E. Rawls (Baylor College of Medicine, Houston). The HSV-2

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[4] The abbreviation used is: HSV, herpes simplex virus; the abbreviations of the deoxynucleoside analogues and arabinosides are defined in Table 1.

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strain 333 was originally obtained from F. Rapp (Pennsylvania State University, Hershey). Both herpes virus strains were propagated for several generations in primary rabbit kidney cells.

The nucleoside analogues used in this study included 5-iodo-, 5-bromo-, 5-chloro-, 5-fluoro-, 5-cyano-, 5-ethyl-, 5-trifluoromethyl-, 5-hydroxymethyl-, 5-thiocyanato-, 5-hydroxy-, 5-allyloxy-, 5-propynyloxy-, and 5-carboxamidomethyloxy-2′-deoxyuridine as well as the arabinosides of cytosine, uracil, thymine, and adenine. All deoxyuridines and arabinosides listed, albeit to a variable extent, inhibited the cytopathic effects of HSV-1 (KOS) in both primary rabbit kidney and human skin fibroblast cells (Table 1). Most inhibitory was ara-C, which inhibited the cytopathic effects of HSV-1 at 0.02–0.07 μg/ml. Other compounds which exhibited significant activity against HSV-1 were IUdR, BrUdR,

TABLE 1

Differential sensitivities of HSV-1 (KOS) and HSV-2 (333) to inhibitory effects of various deoxythymidine analogues

Confluent primary rabbit kidney cultures (in tubes) and human skin fibroblast cultures (in Linbro microtiter plates) were inoculated with 100 $CCID_{50}$/tube or well of either HSV-1 (strain KOS) or HSV-2 (strain 333). After 1 hr of adsorption at 37°, residual virus was removed and the cells were further incubated with Eagle's minimal essential medium (plus 3% calf serum) containing various concentrations of the compounds (200, 100, 40, 20, 10, 4, . . . μg/ml). Cytopathic effect was recorded as soon as it reached 100% in the untreated virus-infected cell cultures. This generally occurred at 2 days for HSV-2 and at 3 days for HSV-1. The minimal inhibitory concentration of the compounds is defined as the concentration required to reduce the virus-induced cytopathic effect by 50%. The HSV-1 and HSV-2 stocks were propagated in primary rabbit kidney cells. Both virus stocks yielded 10^7 $CCID_{50}$/ml when titrated in human skin fibroblast cells.

The sources of the compounds were: IUdR, Ludeco, Brussels; FUdR, Aldrich Chemical Company; F_3TdR, Sigma Chemical Company; EtUdR, see refs. 5 and 6; 5-thiocyanato-2′-deoxyuridine, see refs. 7 and 8; ara-C, Upjohn, Puurs (Belgium); ara-U, either Terra-Marine Bioresearch, La Jolla, Calbiochem, Lucerne, or Sefochem Fine Chemicals, Emek Hayarden (Israel); ara-T, Terra-Marine Bioresearch; ara-A, Parke, Davis (courtesy of Dr. R. Wolf, Parke, Davis Clinical Research Western Europe, München); 5-hydroxy-2′-deoxyuridine, Sefochem Fine Chemicals; 5-bromo-2′-deoxyuridine, Sigma Chemical Company; 5-chloro-2′-deoxyuridine, P-L Biochemicals; 5-cyano-2′-deoxyuridine, Torrence *et al.*, to be published[5] 5-allyloxy-, 5-propynyloxy-, and 5-carboxamidomethyloxy-2′-deoxyuridine, Torrence *et al.*, to be published[6]; 5-hydroxymethyl-2′-deoxyuridine, Calbiochem, La Jolla.

Compound	Minimal inhibitory concentration			
	HSV-1 (KOS)		HSV-2 (333)	
	PRK[a]	HSF[b]	PRK	HSF
	μg/ml		μg/ml	
5-Iodo-2′-deoxyuridine (IUdR)	0.4	0.1	>200	>200
5-Bromo-2′-deoxyuridine (BrUdR)	0.4	0.1	>200	>200
5-Chloro-2′-deoxyuridine (ClUdR)	0.4	0.1	>200	>200
5-Fluoro-2′-deoxyuridine (FUdR)	0.4	0.1	>200	>200
5-Cyano-2′-deoxyuridine	20	150	>200	>200
5-Ethyl-2′-deoxyuridine (EtUdR)	0.7	0.4	>200	>200
5-Trifluoromethyl-2′-deoxyuridine (F_3TdR)	0.4	0.2	>200	>200
5-Hydroxymethyl-2′-deoxyuridine	2	2	>200	>200
5-Thiocyanato-2′-deoxyuridine	20	4	>200	>200
5-Hydroxy-2′-deoxyuridine	4	1	>200	>200
5-Allyloxy-2′-deoxyuridine	4	40	>200	>200
5-Propynyloxy-2′-deoxyuridine	0.7	1	>200	>200
5-Carboxamidomethyloxy-2′-deoxyuridine	40	70	>200	>200
Cytosine arabinoside (ara-C)	0.07	0.02	150	>200
Uracil arabinoside (ara-U)	10	7	>200	>200
Thymine arabinoside (ara-T)	0.4	0.1	>200	>200
Adenine arabinoside (ara-A)	4	1	10	20

[a] Primary rabbit kidney cells.

[b] Human skin fibroblast cells.

ClUdR, FUdR, EtUdR, F_3TdR, ara-T, and 5-propynyloxy-2′-deoxyuridine. The anti-herpes activity of FUdR in both primary rabbit kidney and human skin fibroblast cells is remarkable, since FUdR has been reported to be inactive against herpes and/or vaccinia in a number of cell culture systems (9–12). It is equally remarkable that ara-U was found to inhibit the herpes cytopathic effects at 7–10 μg/ml; on the basis of scant studies (13), ara-U is generally considered to be pharmacologically inert. A full description of the synthesis and antiviral properties of the novel nucleoside analogues mentioned in Table 1 (e.g., 5-cyano-, 5-allyloxy-, 5-propynyloxy-, and 5-carboxamidomethyloxy-2′-deoxyuridine) will be the subject of forthcoming reports.[5,6] In marked contrast with their inhibitory effects on HSV-1 (KOS) replication, none of the compounds tested, except ara-A, had an appreciable effect on HSV-2 (333) replication (Table 1). The differential sensitivity of HSV-1 (KOS) and HSV-2 (333) toward inhibition by pyrimidine nucleoside analogues was most pronounced for ara-C, which inhibited HSV-1 (KOS) in human fibroblasts at 0.02 μg/ml, yet failed to inhibit HSV-2 (333) at a 10^4-fold higher concentration. Other virus strains, e.g., HSV-1 (strain Lyons) and HSV-2 (strain 196), behaved quite similarly to HSV-1 (KOS) in their sensitivity to the inhibitory effects of IUdR, ara-C, and the other pyrimidine nucleoside analogues listed in Table 1 (data not shown).

How could the differential sensitivities of HSV-1 (KOS) and HSV-2 (333) to inhibition by deoxythymidine analogues be explained? Deoxythymidine kinase-deficient (TK^-) cells, when infected with either herpes simplex virus type 1 or type 2, acquire the ability to synthesize deoxythymidine kinase (14–19). This kinase is coded for by the virus (20, 21). Herpes simplex virus also induces deoxycytidine kinase activity, and both deoxythymidine and deoxycytidine kinase activities are thought to reside in the same molecule (18). To explore whether normal cells would also express these virus-induced kinase activities, primary rabbit kidney and human skin fibroblast cell cultures were infected with either HSV-1 (KOS) or HSV-2 (333) at high multiplicity of infection (greater than 1), and the induction of deoxythymidine and deoxycytidine kinase activities was determined at different times after virus infection. The results of a representative experiment, performed in duplicate, are presented in Fig. 1. When assayed under identical conditions, at the same multiplicity of infection, HSV-1 (strain KOS) effectively increased the levels of deoxythymidine kinase activity (Fig. 1A) and deoxycytidine kinase activity (Fig. 1B) in both primary rabbit kidney and human skin fibroblast cell cultures, whereas neither cell culture displayed any change in these activities upon infection with HSV-2 (333).

The failure of HSV-2 (333) to induce deoxythymidine and deoxycytidine kinase activity in primary rabbit kidney cells does not appear to affect the growth potency of the virus, since HSV-2 (333) and HSV-1 (KOS) grew to the same yield in these cells (10^7 $CCID_{50}$/ml). In fact, HSV-2 (333) appeared to grow considerably faster and to attain its maximum yield earlier than HSV-1 (KOS).

These data point to the necessity of virus-induced deoxythymidine kinase activity for deoxythymidine analogues to be effective as anti-herpes agents. Our data do not necessarily imply that differences in sensitivity of various herpes strains toward inhibition by deoxythymidine analogues can be attributed solely to the presence or absence of virus-induced deoxythymidine (and deoxycytidine) kinase activity. One may indeed hypothesize that HSV-1 (strain KOS) and HSV-2 (strain 333) differ in some other properties besides the induction of deoxythymidine and deoxycytidine kinase activities.

Our findings further suggest that, to exert their selective inhibitory effect on herpes virus multiplication, the deoxythymidine analogues should first be phosphorylated by the viral deoxythymidine

[5] P. F. Torrence, B. Bhooshan, J. Descamps and E. De Clercq, *J. Med. Chem.*, in press (1977).

[6] P. F. Torrence, J. W. Spencer, A. M. Bobst, J. Descamps and E. De Clercq, manuscript submitted for publication (1977).

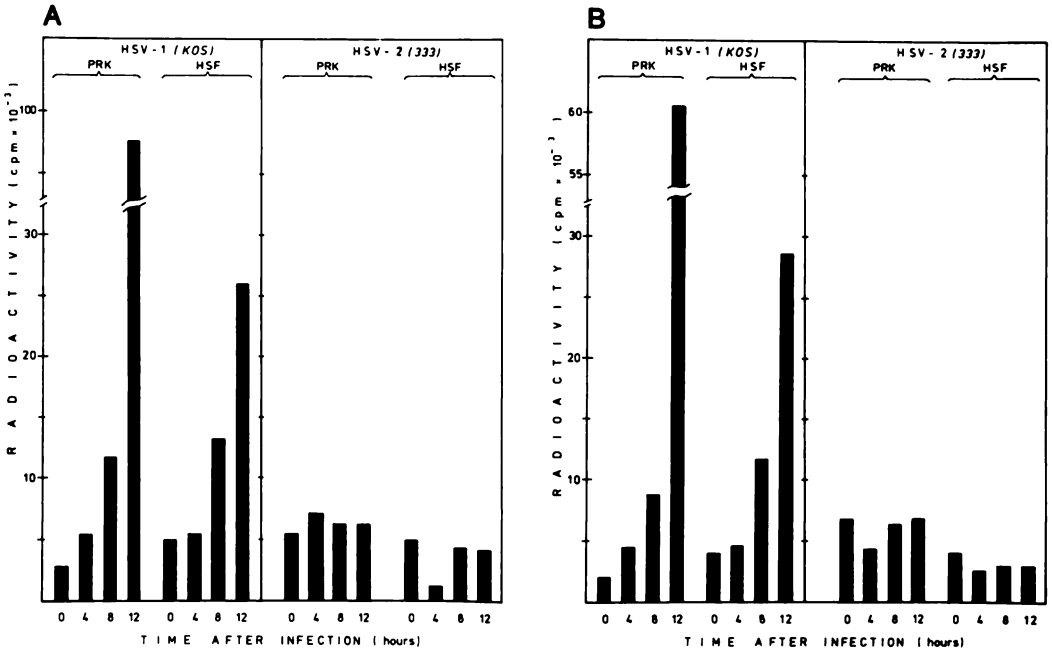

FIG. 1. *Levels of deoxythymidine kinase activity (A) and deoxycytidine kinase activity (B) in primary rabbit kidney (PRK) and human skin fibroblast (HSF) cultures infected with either HSV-1 (strain KOS) or HSV-2 (strain 333)*

Confluent primary rabbit kidney and human skin fibroblast cultures (in Roux bottles) were inoculated with HSV-1 (strain KOS) or HSV-2 (strain 333) at 1.5 $CCID_{50}$/cell. After 1 hr of adsorption at 37°, residual virus was removed and the cells were further incubated at 37° with Eagle's minimal essential medium (plus 10% fetal calf serum). At the times indicated on the abscissa, the cell monolayers were washed four times with phosphate-buffered NaCl and the cells were scraped off with a rubber policeman into 2 ml of 20 mM Tris-HCl (pH 7.3) containing 1 mM EDTA and 3 mM 2-mercaptoethanol. The cell suspensions were sonicated for 20–30 sec at 0° and cleared by centrifugation (30 min, 16,500 rpm), and the supernatant was assayed for deoxythymidine and deoxycytidine kinase activities. The standard assay mixtures contained, in a final volume of 50–60 μl, Tris-HCl (pH 8.0, 5.0 μmoles), ATP (0.25 μmole), $MgCl_2$ (0.5 μmole), NaF (0.5 μmole), 2-mercaptoethanol (0.2 μmole), phosphocreatine (0.3 μmole), creatine kinase (3.0 μg), cell extract (80–100 μg of protein), and 2 nmoles (1 μCi) of either deoxy[*methyl*-^{3}H]thymidine or deoxy[5-^{3}H]cytidine. The mixtures were incubated for 10 min at 37°. The reaction was terminated by immersion of the reaction tubes in a boiling water bath for 2 min, followed by cooling to 0°. The mixtures were spotted onto Whatman DE-81 discs. The filters were washed with water or ammonium formate (1 mM), rinsed with absolute ethanol, dried, and assayed for radioactivity. The radioactivity counts are presented as counts per minute per milligram of protein [determined by the method of Lowry *et al.* (22)] per 1-min incubation. These data were obtained after subtracting the zero-time values. The zero-time samples were treated in the same way as the other samples, except that the radiolabeled substrates were added after the boiled mixtures had been chilled to 0°. A full description of the technique used to measure nucleoside kinase levels in primary rabbit kidney and human skin fibroblast cells will be described elsewhere.[5]

kinase to the respective 5'-monophosphates. Previous findings (23) have indicated that the antitumor activity of some deoxythymidine analogues (e.g., BrUdR) also depends on deoxythymidine kinase activity of the target cell.

Previous reports (3, 17) have already alluded to an apparent correlation between the sensitivity of herpes simplex virus strains toward inhibition by nucleoside analogues and the ability of these virus strains to induce deoxythymidine-deoxycytidine kinase activities. However, the experimental design on which our conclusions are based diverges from that of Jamieson *et al.* (17) and Cheng *et al.* (3), in that our results were obtained in normal primary (rabbit and human) cell cultures,

both of which possess significant base levels of deoxythymidine and deoxycytidine kinase activity. Previous experiments (3, 17) were carried out with highly selected mutant cell lines, e.g., PyY/TG/CAR/BUdR cells (17), which have lost their own deoxythymidine kinase activity. As our results were also extended to a wide variety of nucleoside analogues, they bear directly on the therapeutic use of these analogues in herpes simplex infections.

It is noteworthy that the same virus HSV-2 strain (333), which we found unable to raise the deoxythymidine-deoxycytidine kinase activities of primary rabbit kidney and human skin fibroblasts cells, was shown to induce deoxythymidine kinase activity in deoxythymidine kinase-deficient HeLa mutant cells (19). Whether our HSV-2 strain 333 is identical with or a variant from the HSV-2 (333) strain used by Cheng *et al.* (3, 19) remains to be established.

If the anti-herpes activity of nucleosides such as IUdR, BrUdR, and ara-C indeed depends on their conversion to the respective 5′-monophosphates by a virus-coded pyrimidine deoxyribonucleoside kinase, one may expect nucleosides which do not require this virus-induced deoxythymidine kinase activity to be equally effective against inducer and noninducer HSV strains. This proved to be the case for ara-A, which inhibited HSV-1 (KOS) and HSV-2 (333) to approximately the same extent (Table 1). We have recently shown that the levels of adenosine kinase activity and deoxyadenosine kinase activity in primary rabbit kidney and human skin fibroblast cell cultures are not markedly altered upon infection of the cells with either of the two HSV strains studied.[7]

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[7] E. Krajewska, E. De Clercq, and D. Shugar, manuscript in preparation.

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