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ROLES OF THYMIDYLATE SYNTHETASE ACTIVITY IN HERPES SIMPLEX VIRUS-INFECTED HeLa CELLS

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Abstract—Thymidylate synthetase was not essential for herpes simplex virus replication. However, it appeared to contribute to the formation of a portion of thymidine nucleotides for DNA synthesis in virus-infected cells. Therefore, it is possible that the enzyme plays an important role in determining the potency of several selective antiviral thymidine analogs. Since synergistic effects of 5-fluoro-2′-deoxyuridine with other analogs on virus reproduction were observed, it is suggested that the effects of these analogs depend to a certain degree on the abilities of the monophosphate derivatives to inhibit thymidylate synthetase.

Thymidylate synthetase (5,10-methylene-tetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) is a key enzyme responsible for *de novo* synthesis of thymidine nucleotides, which are required for DNA synthesis in mammalian cells. In herpes simplex virus (HSV)-infected cells, the activity of thymidine kinase, a salvage enzyme for thymidine nucleotides, increased post infection [1–3], whereas the activity of thymidylate synthetase was found to be unchanged [4]. 5-Fluoro-2′-deoxyuridine (FdUrd), the monophosphate derivative of which is known as a potent inhibitor of thymidylate synthetase, has been found not to be potent in inhibiting virus replication [5]. This raises the possibilities that (1) FdUMP does not inhibit thymidylate synthetase in HSV-infected cells, (2) the intracellular thymidine nucleotide pool is enough to support viral DNA synthesis and, therefore, a continuous supply of the nucleotides is not required, or (3) HSV-infected cells have another source of thymidine nucleotides.

Furthermore, although the activity of thymidylate synthetase is not essential for virus replication, it may contribute to determining the potencies of several selective antiviral thymidine analogs.

MATERIALS AND METHODS

Cells and viruses. HeLa S_3 cells were grown at 37° in RPMI 1640 medium containing 100 μg/ml kanamycin, which was supplemented with 5% horse serum. Mycoplasma was checked by the 4,6-diamidino-2-phenylindole fluorescence method [6]. HSV-1 (strain KOS) and HSV-2 (strain 333) were obtained as described before [7], except that Vero cells were used instead of CV-1 cells.

Cell and virus growth inhibition studies. Cells were seeded in 25 cm^2 flasks and incubated for 24 hr before adding drugs. Cell number was determined with a hemocytometer. Seventy to eighty percent confluent cultures of HeLa cells were infected with 5–10 plaque forming units (PFU) of HSV. After 1 hr of incubation, monolayers were rinsed twice with phosphate buffered saline (PBS), followed by the addition of 5 ml of medium containing various drugs. The cells were incubated at 37° for 28 hr and were stored at −70° until titration [7].

Incorporation of radioactivity from [^{3}H]dUrd into acid-soluble and -insoluble fractions. Suspension cultures of HeLa cells were infected with 5–10 PFU of HSV-1. After 1 hr of incubation, the cells were collected by centrifugation and suspended in medium at a concentration of 2.5×10^6 cells/ml. FdUrd (0.1 μM) was added at this time. At indicated times, 0.2 ml of the cell suspension was removed and placed in a tube containing 4 μl (0.04 μCi) of [6-^{3}H]dUrd (20 mCi/μmole). After 10 min of incubation at 37°, 1 ml of ice-cold PBS was added, and the cells were collected by centrifugation and washed again. Then 0.2 ml of 0.5 N perchloric acid was added to the cell pellet. After 5 min at 0°, the supernatant fraction was collected by centrifugation. Radioactivity in the supernatant fraction was counted in ACS scintillant as the incorporation into the acid-soluble fraction. The residual pellet was washed with 0.2 ml of perchloric acid solution and counted in the scintillant as the incorporation into the acid-insoluble fraction.

Perchloric acid extracts from cells treated with or without FdUrd for 2.5 hr were neutralized with 4 M KOH, and aliquots were subjected to polyethyleneimine (PEI)-cellulose thin-layer chromatography in a solvent system of 5 M lithium chloride/formic acid/water (1/1/8). The chromatogram was cut into pieces, and radioactivity was measured in the scintillant.

dNTP pool size determination. Cells (10^7) were washed twice with ice-cold PBS and extracted with 100 μl of 0.5 N perchloric acid for 5 min at 0°. The precipitate was removed by centrifugation, and the supernatant fraction was neutralized by the addition of 12.5 μl of 4 M KOH solution. After addition of 12.5 μl of 1 M potassium phosphate buffer (pH 7.5), the precipitate formed was removed by centrifugation. The concentrations of dNTPs of the extract

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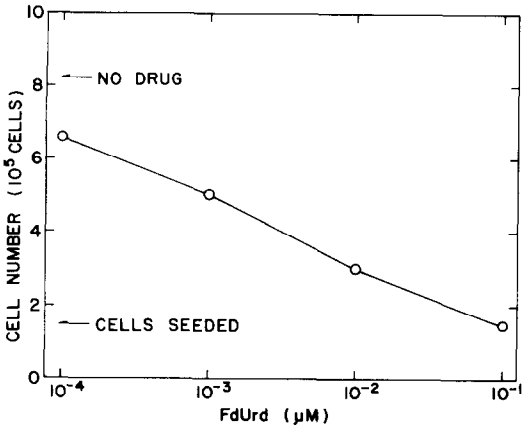

Fig. 1. Effects of FdUrd on HeLa S_3 cell growth. For details, see Materials and Methods.

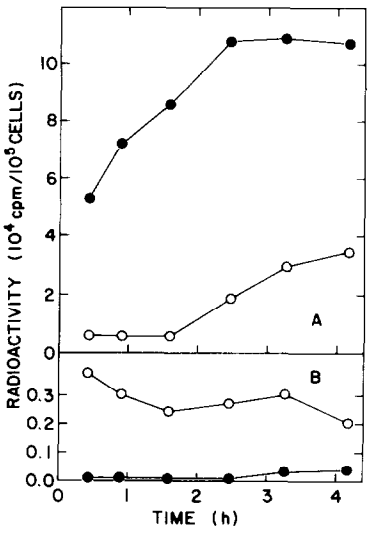

Fig. 3. Effects of FdUrd on the incorporation of radioactivity from [^{3}H]dUrd into acid-soluble (A) and -insoluble (B) fractions in HSV-infected cells. HSV-1-infected HeLa cells were incubated with (●) or without (○) 0.1 μM FdUrd. For details, see Materials and Methods.

were determined by the DNA polymerase method [8].

RESULTS AND DISCUSSION

Activity of FdUrd in inhibiting HeLa S_3 cell growth and HSV-1 and -2 reproduction in HeLa cells. The concentration of FdUrd required to inhibit HeLa cell growth by 50% was 1 nM (Fig. 1), whereas no inhibition of HSV-1 or -2 replication was observed at this concentration (Fig. 2). More than 5 μM FdUrd in the culture medium was required to inhibit virus growth by 90% (Fig. 2). Therefore, it is suggested that the target of FdUrd in exerting its cytotoxicity is not essential for virus reproduction. The major target of FdUrd in cells has been suggested to be thymidylate synthetase, which can be inhibited by FdUMP [9–11].

Inhibition of thymidylate synthetase by FdUrd in virus-infected cells. The possibility that thymidylate synthetase activity in infected cells is not inhibited was examined by determining the intracellular activity of thymidylate synthetase in the presence of 0.1 μM FdUrd, which did not inhibit the growth of either type of HSV, utilizing the incorporation of radioactivity from [6-^{3}H]dUrd into DNA as an indicator of the activity. The results are shown in Fig. 3. The uptake of [^{3}H]dUrd into the acid-soluble fraction of infected cells was increased in FdUrd-treated cells, suggesting that interference with the transport or phosphorylation of dUrd was not important in the estimation of intracellular thymidylate synthetase activity. The incorporation of [^{3}H]dUrd into the acid-insoluble fraction of FdUrd-treated cells was inhibited as shown in Fig. 3B. Acid-soluble fractions, which were treated with or without FdUrd for 2.5 hr, were subjected to PEI-cellulose thin-layer chromatography. Radioactivities associated with nucleoside mono-, di-, and triphosphates were 97.2, 0.6, and 2.2%, respectively, in FdUrd-treated cells, in contrast with 54.7, 3.6, and 41.7% in untreated cells. These results indicate that thymidylate synthetase was inhibited by FdUrd in virus-infected cells. Thus, it can be concluded that

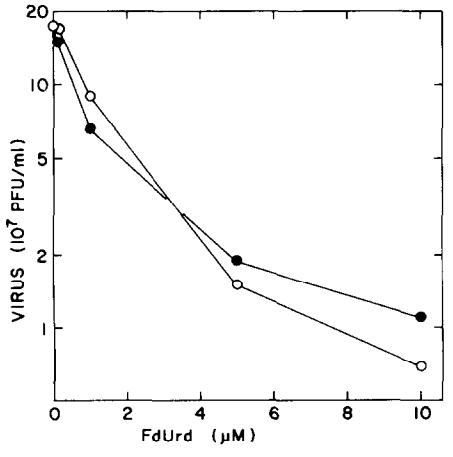

Fig. 2. Effects of FdUrd on herpes simplex virus reproduction. Key: (○) HSV-1, and (●) HSV-2. For details, see Materials and Methods.

Table 1. Effects of FdUrd on dNTP pool sizes of HSV-infected HeLa cells*

dNTP	Mock-infected	HSV-1-infected FdUrd (10^{-6} M) −	HSV-1-infected FdUrd (10^{-6} M) +
dATP	0.20	0.27	0.18
dGTP	0.06	0.24	0.12
dCTP	0.05	0.05	0.02
dTTP	0.46	1.02	0.82

* Values are expressed in nmoles dNTP/10^7 cells. At least two determinations were made for each value.

Table 2. Synergistic effects of selective antiviral nucleoside analogs with FdUrd on HSV replication*

Additives	Concn (μM)	HSV-1 (PFU/ml) FdUrd (0.1 μM) −	HSV-1 (PFU/ml) FdUrd (0.1 μM) +	HSV-2 (PFU/ml) FdUrd (0.1 μM) −	HSV-2 (PFU/ml) FdUrd (0.1 μM) +
None		$2.0 + 10^8$	1.5×10^8	2.0×10^8	1.9×10^8
5-Propyl-dUrd	10	4.2×10^6	7.8×10^5	9.7×10^6	7.0×10^5
E-5-(2-Bromovinyl)-dUrd	10	6.9×10^5	4.0×10^5	ND†	ND
E-5-(2-Bromovinyl)-araUrd	5	7.8×10^6	1.6×10^5	1.5×10^7	4.0×10^5
2′-Fluoro-araThd	2.5	2.2×10^7	5.5×10^6	2.5×10^7	1.2×10^6
Acyclovirin	2.5	7.0×10^7	2.2×10^6	ND	ND

* At least two determinations were made for each value.
† Not determined.

thymidylate synthetase activity was not essential for virus replication, since the concentration of FdUrd (0.1 μM) did not inhibit the reproduction.

Effects of FdUrd on dNTP pool sizes. Although thymidylate synthetase activity was not essential for virus reproduction, it could still have played a role in supplying some of the thymidine nucleotide for the DNA synthesis. The intracellular pool of dTTP in FdUrd-treated infected cells was found to be decreased, but not depleted (Table 1). dGTP and dATP pool sizes were also decreased in the cells. Since dTTP and dGTP may be required for the activation of ribonucleotide reductase for GDP and ADP reduction, respectively, it is conceivable that the decrease of dGTP and dATP pool sizes was related to the decrease in dTTP pool size.

Synergistic effects of FdUrd with other nucleoside analogs. FdUrd caused a decrease in the pool sizes of intracellular dTTP and dGTP, which could compete with the triphosphate derivatives of several antiviral nucleoside analogs [4, 12–15] for DNA polymerase [6, 13, 16]. FdUrd may potentiate the antiviral actions of these selective nucleoside analogs. As shown in Table 2, synergistic effects were observed in all the cases, although the magnitude of the effect of FdUrd varied among the thymidine analogs. The differential effects by FdUrd in potentiation of the action by these analogs might be ascribed to the degrees to which the monophosphate derivatives of the analogs have inhibitory effects on thymidylate synthetase. Thus, the analog which has less effect on the enzyme might be able to show stronger synergistic effect with FdUrd.

The concept of "self potentiation", as we suggested previously for several other nucleoside analogs as anticancer agents [17], may also apply to these analogs. The anti-viral actions of these analogs appear to be mediated through DNA polymerase [13, 16, 18]. If they also inhibited thymidylate synthetase, which would result in a decrease of the intracellular concentration of thymidine nucleotides, it would lead to less competition of these analogs with the thymidine nucleotides for phosphorylation and for DNA polymerase. Further exploration of this concept is in progress. The action of acyclovirin, a guanosine analog, was also potentiated by FdUrd, which may be related to the decrease in dGTP pool size.

In uninfected cells, the main *de novo* source of thymidine nucleotide is through the pathway of thymidylate synthetase. Our results suggest that, in infected cells, there may be additional pathways which provide thymidine nucleotides for viral DNA synthesis. The induction of DNase in virus-infected cells, which could degrade DNA to deoxynucleotide 5′-monophosphates, may be one of the alternative sources, as suggested previously by us [19]. Another possibility is that the intracellular concentration of dTTP in virus-infected cells is sufficient for viral DNA synthesis, in spite of the inhibition of thymidylate synthetase by FdUMP.

In conclusion, thymidylate synthetase activity appears not to play an essential role in virus replication. Utilizing thymidylate synthetase in HSV-infected cells as a target, when developing selective anti-HSV compounds, may not be a viable approach. However, antiviral thymidine analogs which act on DNA polymerase as well as on thymidylate synthetase would have more advantages than those which act only on DNA polymerase.

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