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Effects of Ribonucleotide Reductase Inhibition on Pyrimidine Deoxynucleotide Metabolism in Acyclovir-Treated Cells Infected with Herpes Simplex Virus Type 1

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The pyrimidine metabolism of fibroblasts infected with herpes simplex virus type 1 was studied. Herpes simplex virus type 1 infection increased the dTTP pool and thymidylate synthetase activity but reduced thymidine excretion. Addition of acyclovir to infected cells increased thymidine excretion, the dTTP pool, and thymidylate synthetase activity. Addition of a virus-specific ribonucleotide reductase inhibitor (A723U) decreased all three. The synergy between the two compounds is discussed.

Herpes simplex virus type 1 (HSV-1) infections result in changes in enzyme activities involved in DNA synthesis (13). Acyclovir (ACV) is an antiherpetic agent selectively phosphorylated by HSV-1-encoded thymidine kinase (4, 6). Cellular enzymes (15, 16) catalyze the conversion of ACV monophosphate to ACV triphosphate, which inhibits viral DNA synthesis. Thymidine competes with ACV in the initial phosphorylation step (14), and dGTP competitively prevents the inhibition of HSV-1-induced DNA synthesis by ACV triphosphate (6).

Ribonucleotide reductase (RR) is a key enzyme for the de novo synthesis of deoxyribonucleotides (19). An HSV-1-encoded RR has been purified and characterized (1). 2-Acetylpyridine thiosemicarbazone (A723U) has previously been shown to inhibit HSV-1-encoded RR (18). The compound has been found to potentiate the antiviral activity of ACV (18). In the present study, we investigated the effects of A723U and ACV on DNA precursor metabolism in HSV-1-infected human lung fibroblast (HL) cells.

Confluent mycoplasma-free HL cells (10) were infected with HSV-1 strain F9004 (10) at a multiplicity of infection of 3 to 4. ACV (100 μM) or A723U (30 μM) was added at the time of infection. [6-^{3}H]deoxyuridine (UdR) and [5-^{3}H]cytidine (CR) were added to the dishes 4 h postinfection. After incubation for 120, 140, or 160 min, the medium and cell extracts were saved as previously described (17). The samples were subsequently analyzed with respect to dTTP and dCTP pools, nucleoside excretion, DNA synthesis, and thymidylate synthetase (TS) activity.

The dTTP and dCTP pools were assayed by a DNA polymerase assay (17). DNA synthesis was analyzed by measurement of [^{3}H]UdR incorporation into DNA (17). Excretion of nucleosides into the medium was determined by high-performance liquid chromatography (17).

TS activity was measured by the release of ^3H_2O into the medium from [5-^{3}H]CR-labeled cells (17).

The 50% virus-inhibiting concentrations of ACV, A723U, and phosphonoformic acid (PFA) were determined by using an enzyme-linked immunosorbent assay described previously (9, 20). In this experiment 0.7 μM ACV, 4 μM A723U, and 135 μM PFA inhibited the viral protein production 50% (Table 1). Concentrations of the three different compounds that alone did not decrease the viral protein production were selected for combination studies. The results are shown in Table 1.

With HSV-1 infection, both the size and the specific activity of the dTTP pool were increased in [^{3}H]UdR-labeled HL cells compared with uninfected cells (Fig. 1). In HSV-1-infected cells, ACV treatment increased the size and decreased the specific activity of the dTTP pool compared with that in untreated cells. A723U treatment, on the other hand, decreased the size and increased the specific activity of the dTTP pool compared with that in untreated HSV-1-infected cells. The combination of ACV and A723U resulted in approximately the same size and specific activity of the dTTP pool as in A723U-treated cells. dCTP increased twofold when HSV-1 infection was inhibited by ACV and decreased to very low values with A723U treatment (data not shown).

HSV-1 infection resulted in increased [^{3}H]UdR incorporation into DNA compared with that in uninfected HL cells (Fig. 2). Treatment of HSV-1-infected HL cells by A723U, ACV, and the combination of ACV and A723U equally inhibited [^{3}H]UdR incorporation into DNA. Similar results were obtained with [^{3}H]CR as the substrate (data not shown).

TS activity was quantified by using [^{3}H]CR as the substrate (Fig. 3). HSV-1 infection of HL cells increased TS activity compared with that in uninfected control cells. ACV treatment of HSV-1-infected HL cells further increased TS activity compared with that in untreated HSV-1-infected control cells. Treatment of HSV-1-infected HL cells with

TABLE 1. Mutual potentiation of antiherpetic activities of A723U, ACV, and PFA as measured by the enzyme-linked immunosorbent assay method

Agent and concn (μM)[a]	IC_{50}[b] (μM)		
	A723U	ACV	PFA
A723U (1)		0.08	90
ACV (0.125)	1.0		43
PFA (50)	4.9	0.2	
No addition	4.0	0.7	135

[a] None of the additives at the indicated concentrations elicited any reduction of viral protein production when tested individually.
[b] IC_{50}, 50% Virus-inhibiting concentration.

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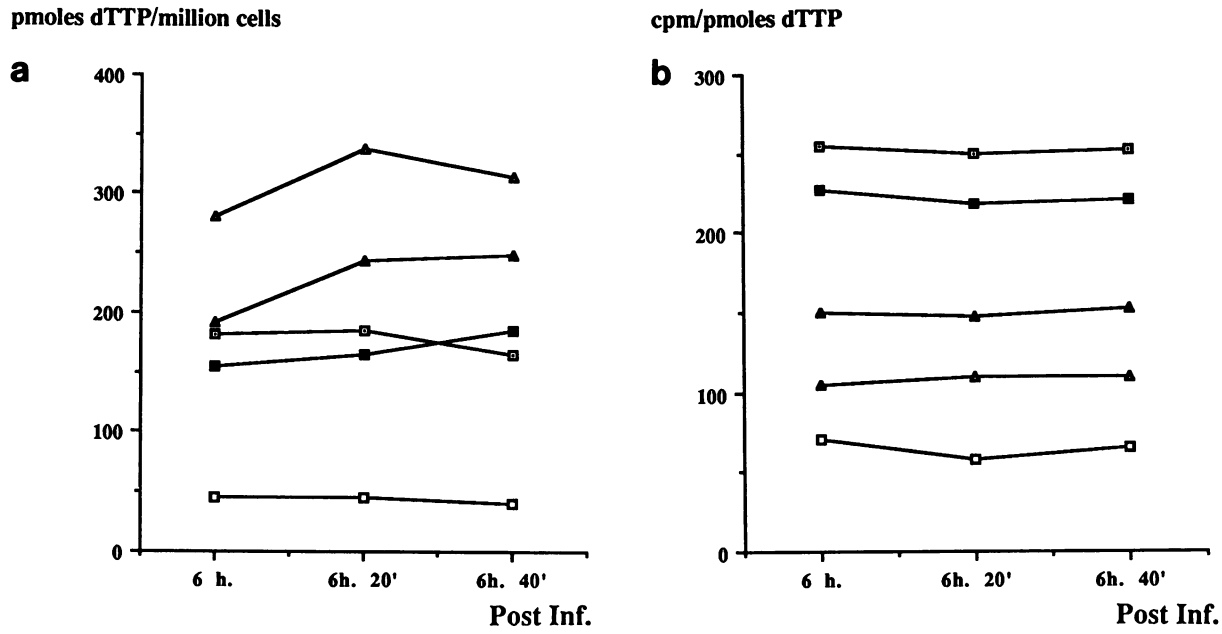

FIG. 1. (a) dTTP pools after HSV-1 infection (▲) and treatment with 100 μM ACV (△), 30 μM A723U (⊡), or the combination of 100 μM ACV and 30 μM A723U (■). The results are compared with that in uninfected control cells (□). (b) Specific activity in dTTP pools after addition of [^{3}H]UdR. Symbols are defined above.

A723U or the combination of A723U and ACV resulted in a nearly 10-fold reduction of TS activity.

HSV-1 infection decreased thymidine excretion compared with that in uninfected cells. In ACV-treated cells, thymidine excretion increased compared with that in untreated HSV-1-infected cells. This increase was not found in cells treated with A723U or the combination of A723U and ACV (Fig. 4).

We have previously shown that thymidine secretion into the medium and intracellular thymidine concentration are both decreased by HSV-1 infection (7, 8). Addition of ACV to HSV-1-infected cells has been found to increase the thymidine concentration in the medium and in the cells (8). The present investigation showed that addition of A723U alone or in combination with ACV decreases thymidine excretion into the medium. The synergistic action between A723U and ACV may be explained by the reduced thymidine pools found in A723U-treated HSV-1-infected cells. Since ACV is competitive with thymidine for phosphorylation by viral thymidine kinase, the combination of ACV and A723U could increase the phosphorylation and thereby the activation of ACV.

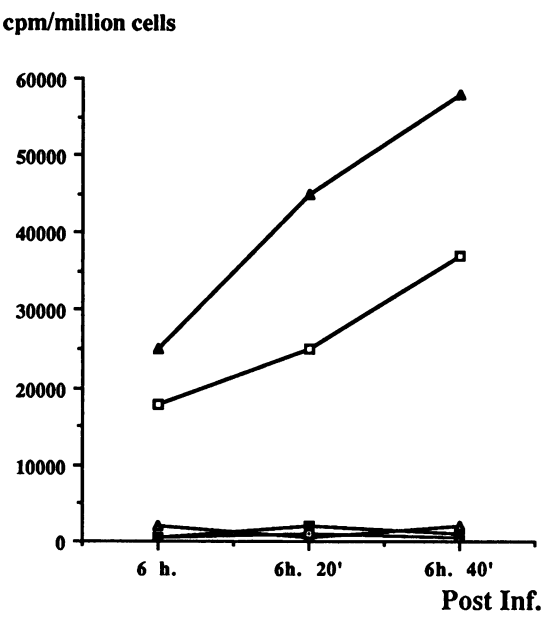

FIG. 2. DNA synthesis measured as [^{3}H]UdR incorporation into DNA. Symbols are defined in the legend to Fig. 1.

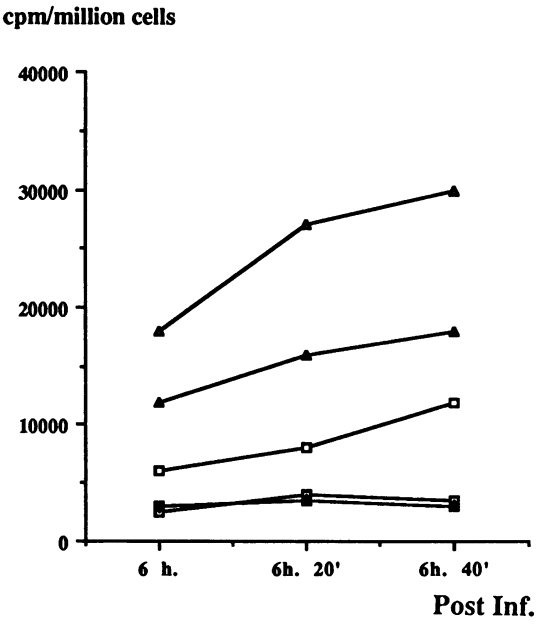

FIG. 3. In situ TS activity measured as ^3H_2O excretion after addition of [^{3}H]CR. Symbols are defined in the legend to Fig. 1.

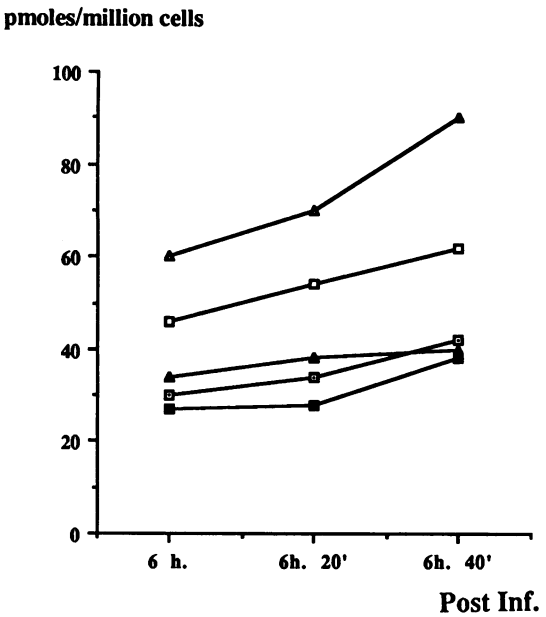

FIG. 4. Thymidine excretion measured as secretion of [^{3}H]thymidine after addition of [^{3}H]UdR. Symbols are defined in the legend to Fig. 1.

A723U has been shown by Spector and co-workers to be a specific inhibitor of purified HSV-1 RR in vitro (18). The object of the present investigation was to clarify the mechanism of action of A723U in vivo. The compound also showed synergy with ACV in cell culture experiments. In agreement with previous reports (18), we found that subinhibitory concentrations of either ACV or A723U could potentiate the activity of the other compound.

HSV-1 infection has previously been shown to increase all deoxynucleoside triphosphate pools (2, 5, 12), with the possible exception of dATP (11). Addition of an HSV-1-specific DNA polymerase inhibitor (like ACV) has been shown to further increase the dCTP, dTTP, and dGTP pools (3, 5, 12, 18). The present results are in accordance with these reports. In contrast to one previous report (18), the present investigation showed that treatment with the combination of ACV and A723U decreased the dTTP pools in HSV-1-infected cells compared with untreated cells. The analysis of the specific radioactivity of the dTTP pools after the addition of [^{3}H]UdR showed decreased utilization of the de novo pathway for dTMP synthesis after treatment of HSV-1-infected cells with A723U or the combination of ACV and A723U. The result could be explained by the inhibition of viral RR by A723U.

The experiments presented in this paper all indicate that A723U functions as an RR inhibitor in vivo as well as in vitro. Combination therapy with A723U and ACV is a promising clinical prospect.

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LITERATURE CITED

1. **Averett, D. R., C. Lubbers, G. B. Elion, and T. Spector.** 1983. Ribonucleotide reductase induced by herpes simplex type 1 virus. Characterization of a distinct enzyme. J. Biol. Chem. **258:** 9831–9838.
2. **Cheng, Y.-C., B. Goz, and W. H. Prusoff.** 1975. Deoxyribonucleotide metabolism in herpes simplex virus infected HeLa cells. Biochim. Biophys. Acta **390:**253–263.
3. **Cheng, Y.-C., S. P. Grill, G. E. Dutschman, K. B. Frank, J.-F. Chiou, K. F. Bastow, and K. Nakayama.** 1984. Effects of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new antiherpesvirus compound, on synthesis of macromolecules in herpes simplex virus-infected cells. Antimicrob. Agents Chemother. **26:**283–288.
4. **Elion, G. B., P. A. Furman, J. A. Fyfe, P. de Miranda, L. Beauchamp, and H. J. Schaeffer.** 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA **74:**5716–5720.
5. **Furman, P. A., C. U. Lambe, and D. J. Nelson.** 1982. Effect of acyclovir on the deoxyribonucleoside triphosphate pool levels in Vero cells infected with herpes simplex virus type 1. Am. J. Med. **73:**14–17.
6. **Fyfe, J. A., P. M. Keller, P. A. Furman, R. L. Miller, and G. B. Elion.** 1978. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. J. Biol. Chem. **253:**8721–8727.
7. **Harmenberg, J.** 1983. Intracellular pools of thymidine reduce the antiviral action of acyclovir. Intervirology **20:**48–51.
8. **Harmenberg, J., G. Abele, and B. Wahren.** 1985. Nucleoside pools of acyclovir-treated herpes simplex type 1 infected cells. Antiviral Res. **5:**75–81.
9. **Harmenberg, J., V.-A. Sundqvist, H. Gadler, B. Levén, G. Brännström, and B. Wahren.** 1986. Comparative methods for detection of thymidine kinase-deficient herpes simplex virus type 1 strains. Antimicrob. Agents Chemother. **30:**570–573.
10. **Harmenberg, J., and B. Wahren.** 1980. Influence of cells and virus multiplicity on the inhibition of herpesviruses with acycloguanosine. Intervirology **14:**239–244.
11. **Jamieson, A. T., and G. Bjursell.** 1976. Deoxyribonucleoside triphosphate pools in herpes simplex type 1 infected cells. J. Gen. Virol. **31:**101–113.
12. **Karlsson, A. H. J., J. G. Harmenberg, and B. E. Wahren.** 1986. Influence of acyclovir and bucyclovir on nucleotide pools in cells infected with herpes simplex virus type 1. Antimicrob. Agents Chemother. **29:**821–824.
13. **Kit, S.** 1979. Viral-associated and induced enzymes. Pharmacol. Ther. **4:**501–585.
14. **Larsson, A., G. Brännström, and B. Öberg.** 1983. Kinetic analysis in cell culture of the reversal of antiherpes activity of nucleoside analogs by thymidine. Antimicrob. Agents Chemother. **24:**819–822.
15. **Miller, W. H., and R. L. Miller.** 1980. Phosphorylation of acyclovir (acycloguanosine) monophosphate by GMP kinase. J. Biol. Chem. **255:**7204–7207.
16. **Miller, W. H., and R. L. Miller.** 1982. Phosphorylation of acyclovir diphosphate by cellular enzymes. Biochem. Pharmacol. **31:**3879–3884.
17. **Nicander, B., and P. Reichard.** 1985. Relation between synthesis of deoxyribonucleotides and DNA replication in 3T6 fibroblasts. J. Biol. Chem. **260:**5376–5381.
18. **Spector, T., D. R. Averett, D. J. Nelson, C. U. Lambe, R. W. Morrison, Jr., M. H. St. Clair, and P. A. Furman.** 1985. Potentiation of antiherpetic activity of acyclovir by ribonucleotide reductase inhibition. Proc. Natl. Acad. Sci. USA **82:**4254–4257.
19. **Thelander, L., and P. Reichard.** 1979. Reduction of ribonucleotides. Annu. Rev. Biochem. **48:**133–158.
20. **Wahren, B., J. Harmenberg, V.-A. Sundqvist, B. Levén, and B. Sköldenberg.** 1983. A novel method for determining the sensitivity of herpes simplex virus to antiviral compounds. J. Virol. Methods **6:**141–149.