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Antiherpes virus activity and effect on deoxyribonucleoside triphosphate pools of (E)-5-(2-bromovinyl)-2′-deoxycytidine in combination with deaminase inhibitors

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Summary

The antiviral activity and cytotoxicity of (E)-5-(2-bromovinyl)-2′-deoxycytidine (BrVdCyd) against herpes simplex virus type 1 (HSV-1), singly and in combination with deaminase inhibitors was determined using rabbit kidney (RK-13), HEP-2, BHK-21 and VERO cells. BrVdCyd was a potent inhibitor of HSV-1 replication with ED_{50} values of 0.30 to 1.20 μM depending on the cell line used. In the presence of tetrahydrouridine or tetrahydrodeoxyuridine (H_4dUrd), potency of BrVdCyd increased approximately two fold (ED_{50}: 0.54 μM) in HSV-infected VERO cells. The combination of BrVdCyd and H_4dUrd was also effective in decreasing virus yield. Dihydrodeoxyuridine (H_2dUrd) reversed the activity of BrVdCyd (ED_{50}: 6 to 7 μM).

The effect of (E)-5-(2-bromovinyl)-2′-deoxyuridine (BrVdUrd), BrVdCyd and BrVdCyd in combination with H_4dUrd on deoxyribonucleoside triphosphate (dNTP) pools was assessed in VERO cells infected with a high multiplicity of infection (10 PFU/cell). Significant differences in dNTP poll sizes (pmol/10^6 cell) were observed with different treatments. BrVdUrd and BrVdCyd treatment resulted in marked expansion of the dTTP pool (>1200 pmol) compared to HSV-infected VERO cells (303 pmol). Exposure to H_4dUrd resulted in a 12-fold expansion of the dCTP pool (326 pmol) and barely detectable levels of dTTP (<1.0 pmol). BrVdCyd plus H_4dUrd treatment resulted in a slight expansion of the dTTP pool

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(515 pmol). These results indicate: (i) H_4dUrd inhibits de novo dCyd/dCMP deaminase pathway and (ii) exposure to BrVdCyd plus H_4dUrd puts a strain on viral DNA synthesis to such an extent that even though dTTP is being formed from alternative pathways, its eventual utilization as a substrate is reduced and hence it builds up.

(E)-5-(2-bromovinyl)-2′-deoxycytidine; Tetrahydrodeoxyuridine (deaminase inhibitor); Antiherpes activity; dNTP pool; HSV-infected cell

Introduction

Halogenated analogs of deoxycytidine (dCyd)[1] are more selective inhibitors of herpes simplex virus (HSV) than the corresponding analogs of deoxyuridine (Greer et al., 1975; Schildkraut et al., 1975). This selective action was attributed to their preferential phosphorylation by a virus-induced deoxythymidine dThd/dCyd kinase (Doberson and Greer, 1978). Deoxycytidine incorporation was increased into DNA in virus-infected cells in the presence of tetrahydrouridine[2]. In contrast, H_4Urd did not increase dCyd incorporation into DNA of uninfected cells (North and Matthews, 1981). Of considerable interest are also the findings that 5-iodo-2′-deoxycytidine is incorporated as such into the DNA of HSV-infected cells as well as cells transformed with a restriction fragment containing the HSV dThd kinase gene in the presence of tetrahydrodeoxyuridine (Fox et al., 1983). These results are significant because they provide unequivocal proof that when deamination is prevented, anabolism of the deoxycytidine analog occurs predominantly through the virus-induced dCyd kinase-dCMP kinase pathway in HSV-infected cells (Fig. 1).

BrVdUrd is a potent and selective inhibitor of HSV type 1 (HSV-1) replication (De Clercq et al., 1979; De Clercq, 1986). The molecular basis for the selectivity of BrVdUrd is that it is preferentially phosphorylated by the virus-induced thymidine (deoxycytidine) kinase (Ayisi et al., 1985a,b; Fyfe, 1981; De Clercq, 1982) and BrVdUrd triphosphate inhibits viral DNA polymerase (Allaudeen et al., 1981; Ruth and Cheng, 1981). One major limitation with BrVdUrd for both therapeutic

[1]Abbreviations used are: dCyd (deoxycytidine), Cyd (cytidine), dCMP (deoxycytidylate), dThd (deoxythymidine), H_4Urd (tetrahydrouridine), H_4dUrd (tetrahydrodeoxyuridine), H_2dUrd (dihydrodeoxyuridine), BrVdUrd [(E)-5-(2-bromovinyl)-2′-deoxyuridine], BrVdCyd [(E)-5-(2′bromovinyl)-2-deoxycytidine], dCTP (deoxycytidine triphosphate), BrVdCTP [(E)-5-(2-bromovinyl)-2′-deoxycytidine triphosphate], BrVdUTP [(E)-5-(2-bromovinyl)-2′-deoxyuridine triphosphate], dGTP (deoxyguanosine triphosphate), dTTP (deoxythymidine triphosphate), GDP (guanosine diphosphate), CDP (cytidine diphosphate) and HSV-1 (herpes simplex virus type 1)

[2]Trivial name for 1-β-D-ribofuranosyl)-4-hydroxy-3,4,5,6-tetrahydropyrimidine-2(1H)-one).

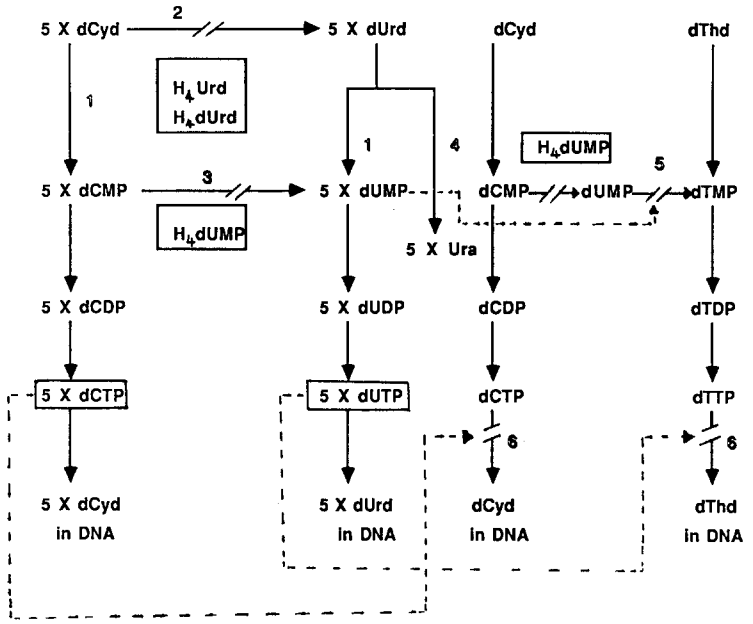

Fig. 1. Proposed pathways for metabolism of (E)-5-(2-bromovinyl)-2′-deoxycytidine in herpes simplex virus infected cells.

(Desgranges et al., 1983) and diagnostic (Gill et al., 1984) purposes is its susceptibility to phosphorolysis by dThd phosphorylase (Desgranges et al., 1983a; Veres et al., 1986). Formation of the inactive metabolite bromovinyluracil has been demonstrated in vivo (Desgranges et al., 1983) and in virus-infected cells in vitro (Ayisi et al., 1985a,b, 1987).

BrVdCyd is also a potent and selective inhibitor of HSV-1 (De Clercq et al., 1982). If deamination of BrVdCyd can be prevented it should be a better antiviral agent than its corresponding deoxyuridine (BrVdUrd). Rationale for this hypothesis are: (i) BrVdCyd, co-administered with H_4dUrd, will be anabolized exclusively by the dCyd kinase-dCMP kinase pathway in HSV-infected cells to BrVdCTP which may then be incorporated into viral DNA or inhibit HSV-induced DNA polymerase by competing with dCTP, and (ii) BrVdCyd in combination with H_4dUrd would be metabolically stable because dCyd and its analogs are not substrates of pyrimidine nucleoside phosphorylases. This paper reports the antiherpes activity and effect on dNTP pools of BrVdCyd in combination with deaminase inhibitors.

Materials and Methods

Cell cultures

The cell lines used in this study were: African green monkey kidney (VERO), human epidermoid carcinoma (Hep-2), baby hamster kidney (BHK-21) and rabbit kidney (RK-13). Stock cultures of VERO, Hep-2 and BHK-21 cell lines were initially provided by Dr L.A. Babiuk, Veterinary Microbiology, University of Saskatchewan. The RK-13 cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 2 mmol of L-glutamine, 4% $NaHCO_3$ containing phenol red, MEM non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone and 10% fetal bovine serum (FBS) for growth or 4% FBS for maintenance. Media and supplements were obtained from Flow Laboratories Inc. (Mississauga, Ontario). The cells were trypsinized and versenized by the standard procedure. For the antiviral assays, confluent monolayers of cells were prepared by seeding 5×10^4 cells into each well of a sterile 96-well microtiter tissue culture plate (Flow Laboratories, Mississauga, Ontario). The cultures were incubated at 37°C in a humidified CO_2 (5%) atmosphere and the cells were 100% confluent in 18–24 h.

Viruses

HSV-1 strains KOS and 76 were kindly provided by Dr Misra and Dr Babiuk, Veterinary Microbiology, University of Saskatchewan. HSV-1 strain 76 was originally isolated from a human labial lesion by Dr L.A. Babiuk. The virus stocks were propagated by low multiplicity of infection of confluent VERO cell monolayers in sterile 250 ml tissue culture flasks (Miles Scientific, Naperville, IL). After 48–72 h incubation at 37°C, there was complete cytopathic effect. The cells were brought into suspension by shaking the flasks. The detached cells were pelleted by centrifugation ($800 \times g$) for 15 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in fresh MEM. Intracellular virus particles were released by 3 pulses of 30-s sonar boom; the cell debris was separated by centrifugation and the supernatant was collected. Virus titer was determined on confluent VERO or RK-13 monolayers in 96-well microtiter plates as previously described (Ayisi et al., 1980; Babiuk et al., 1975). Antibodies to HSV-1 were prepared according to literature procedures (Ayisi et al., 1980).

Antiviral assays

Plaque reduction assay Antiviral activity was determined according to procedures described previously (Ayisi et al., 1980; Babiuk et al., 1975). Briefly, confluent cell monolayers of RK-13, BHK-21, VERO and Hep-2 were infected with 50 PFU of virus per well. Virus dilutions were made using serum-free MEM. The infected cells were incubated for 1 h in a humidified CO_2 (5%) atmosphere after

which the unadsorbed virus was removed by washing with MEM. Each compound dissolved in maintenance medium was added at the appropriate concentration along with one neutralizing unit of HSV-1 antibody. Plaques were allowed to develop for 72 h prior to fixation, staining and enumeration. In each experiment toxicity controls (containing the test compound and medium only), cell controls (containing medium only), and virus controls were run concurrently. The assays for each drug and combination were carried out in quadruplicate.

Virus yield studies The effect of BrVdCyd and BrVdCyd in combination with deaminase inhibitors on virus titer was investigated according to the procedure described earlier (Ayisi et al., 1985a,b). Briefly, monolayers of VERO cells were infected with HSV-1 at 50 PFU per well and incubated as described for the plaque reduction assay. Neutralizing antibody was not included in the overlay. At the end of the incubation period, the plates were frozen at −70°C. The total virus yield was determined by two cycles of freeze-thawing, the culture fluid from each treatment group was pooled and titrated to determine the amount of virus present. The assays for each treatment were carried out in quadruplicate.

Deoxyribonucleoside triphosphate (dNTP) pools

Extraction of dNTP pools Confluent VERO cells seeded in 100-mm Petri dishes were either mock-infected (MEM, 1 ml) or infected with HSV-1 KOS at 10 PFU/cell and incubated for 1 h at 37°C. Culture fluid was removed, cells were washed with MEM to remove unadsorbed virus. At this time, each compound dissolved in maintenance medium was overlaid on cell monolayers. Uninfected and virus-infected controls were overlaid with maintenance medium only. All dishes were incubated for a further period of 7 h. After 8 h post infection, the medium was removed by aspiration and the cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (0.15 M, pH 7.2). Two ml of cold 60% methanol was added to each dish, cells dislodged with a sterile rubber policeman and transferred into tubes stored in an ice-bath. The dishes were rinsed again with 2 ml of cold 60% methanol, washings combined with the first suspension; and stored at −20°C for 18–24 h. The suspension was centrifuged for 30 min (40000 × *g*) at 4°C in an Ultracentrifuge (Model L8055M Beckman Instruments, Palo Alto, CA). The methanol soluble fraction was evaporated under reduced pressure at 37°C. The residue was dissolved in water and the aqueous fraction was freeze-dried and processed for dNTP pools using the following procedure. To each sample representing extract from two dishes was added 200 μl of cold 0.5 M perchloric acid, vortexed and stored for 30 min at 4°C. The precipitate (acid-insoluble) was collected by centrifugation at 12000 × *g* for 10 min. Supernatant (acid-soluble extract) was neutralized with 25 μl 4 M KOH and buffered with 1 M potassium phosphate buffer, pH 7.2, centrifuged and the supernatant containing the nucleotides was stored at −20°C until assays were done.

Enzymatic assay of dNTP pools dNTP was determined by the method of Solter and Handschumacher (1969) with minor modifications. The reaction was carried in 0.5 ml microcapped tubes. The final reaction mixture contained the following: 1 nmol of each of the three non-limiting dNTP (one radiolabelled), limiting dNTP standards 3.9 to 125 pmol or 2.5 μl to 20 μl of cell extract, 10 μmol TRIS-HCl pH 7.5, 1 μmol $MgCl_2$, 10 μg activated calf thymus DNA, 100 μg/ml BSA and one unit of *E. coli* DNA polymerase I in a total volume of 0.2 ml. The mixture was incubated for 60 min at 37°C in a water bath. The reaction was stopped by adding 1 ml of cold 5% TCA and the reaction mixture was kept in ice for 1 h. The acid-insoluble fraction was collected by filtration on Whatman GF/F filters, premoistened with 5% TCA. The filters were washed twice with 5% TCA (to remove any residual soluble material), followed with 95% ethanol and air-dried for 1 h. Radioactive counts were determined using a Beckman LS 3800 counter. The lowest amount of dNTP detectable by the enzyme assay was 0.5 pmol. The bacterial DNA polymerase preferentially utilizes natural dNTPs (dTTP, dCTP) and therefore the presence of BrVdCTP or BrVdUTP does not interfere with dNTP determinations.

Chemicals and biologicals

The origin of BrVdCyd and BrVdUrd has been described previously (De Clercq et al., 1979, 1982). H_4Urd was purchased from Calbiochem (La Jolla, CA), H_4dUrd was obtained by custom synthesis from Terochem Laboratories (Edmonton, Alberta) and H_2dUrd was synthesized as described by Hanze (1967). *E. coli* DNA polymerase I, activated calf thymus DNA, and cold deoxyribonucleoside 5′-triphosphates (dTTP, dCTP, dATP, and dGTP) were purchased from Sigma Chemical Co., St. Louis, MO. Radiolabelled deoxynucleotides, [methyl-^{3}H]thymidine 5′-triphosphate ([^{3}H]dTTP, specific activity 46 Ci/mmol) and deoxy[5-^{3}H]cytidine 5′-triphosphate ([^{3}H]dCTP, specific activity 24 Ci/mmol) were purchased from Amersham Corporation, Oakville, Ontario. BrVdCyd, H_4Urd, H_4dUrd and H_2dUrd were dissolved in MEM and stored at −20°C.

TABLE 1

Antiviral activity of BrVdCyd in different cell lines

Cell line	ED_{50}[a] (μM)
VERO	1.08
Hep-2	1.20
BHK-21	0.45
RK-13	0.30

[a]ED_{50}, concentration required to reduce viral plaque formation by 50%.

Results

Antiviral studies

Inhibition of HSV-1 replication by BrVdCyd and BrVdCyd in combination with deaminase inhibitors The antiviral activity against HSV-1 was determined using RK-13, BHK-21, Hep-2 and VERO cells, by plaque and virus yield reduction assays. Representative data from these experiments are shown in Table 1 and Figs. 2 and 3. The concentration required to inhibit cytopathogenicity of HSV-1 by 50% (ED_{50}) ranged from 0.30 μM to 1.20 μM in different cell lines. The antiviral potency was higher in RK-13 and BHK-21 cells with ED_{50} values of 0.30 and 0.45 μM, respectively, which are in agreement with values reported in primary rabbit kidney cells (De Clercq et al., 1982). In contrast, the antiviral potency was approximately four-fold lower in Hep-2 and VERO cells. These results indicate that activity of BrVdCyd was cell-dependent and was most likely being influenced by the Cyd/dCyd deaminase (E.C.3.5.4.5) and dCMP deaminase (E.C.3.5.4.12) content of the cell lines used for the antiviral assays. In order to understand the significance of deaminases in relation to antiherpes activity, antiviral activity was determined in the presence of dihydrodeoxyuridine (H_2dUrd), tetrahydrouridine (H_4Urd) and tetrahydrodeoxyuridine (H_4dUrd) by plaque reduction assay, and the results are shown in Fig. 2. Potency of BrVdCyd was increased approximately two-fold against HSV-1 (ED_{50}: 0.54 μM) in the presence of either H_4Urd or H_4dUrd. In contrast, the activity of BrVdCyd decreased when combined with H_2dUrd (ED_{50}: 6 to 7 μM).

The effect of BrVdCyd and the combination of BrVdCyd plus H_4dUrd or HSV-1 replication was assessed by the virus yield reduction assay of total infectious vi-

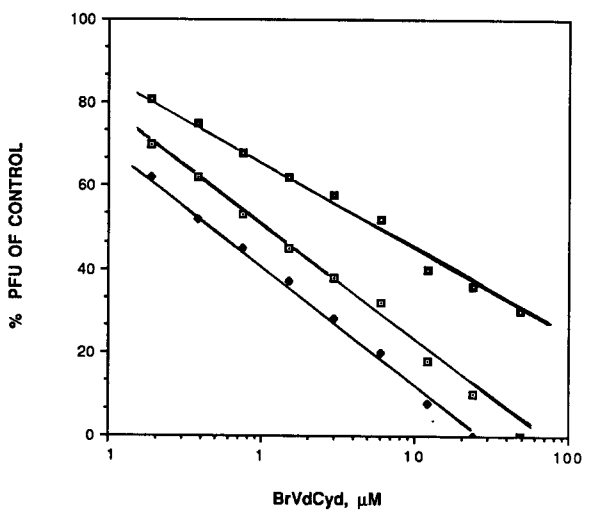

Fig. 2. Dose-response curves. BrVdCyd, (-■-■-); BrVdCyd + H_4Urd/H_4dUrd, (-■-■-); BrVdCyd + H_2dUrd, (-■-■-). Antiviral assays were carried out using HSV-1 strain KOS in VERO cells. Virus input was 50 PFU per well.

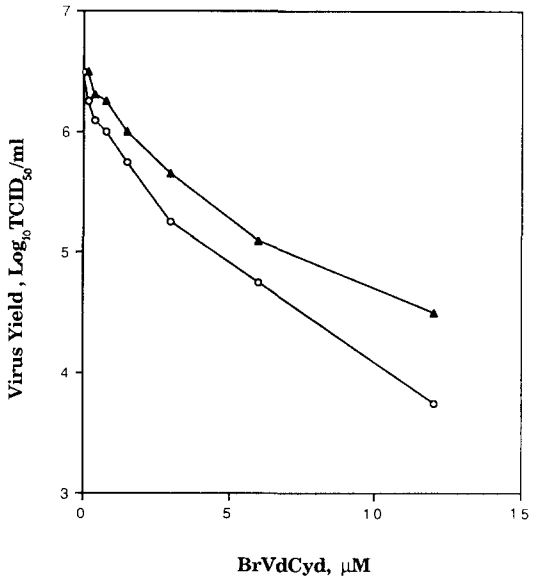

Fig. 3. Effect on production of infectious virus particles (virus yield). BrVdCyd, (-o-o-); BrVdCyd + H_4dUrd, (-▲-▲-). Antiviral assays were carried out using HSV-1 strain KOS in VERO cells. Virus input was 50 PFU per well.

rus particles produced after 72 h incubation. Decrease in virus yield was observed at a concentration of 0.38 to 0.75 μM or higher when BrVdCyd was used alone. However, in combination with H_4dUrd, a decrease in virus yield was observed at a concentration of 0.19 μM or higher (Fig. 3).

In both monolayers and rapidly dividing VERO cells, BrVdCyd was devoid of cytotoxicity up to 600 μM (the highest concentration tested). It has a wide margin of safety as shown by the high antiviral index (AI >1300), which is further enhanced (AI >2500) in the presence of H_4Urd or H_4dUrd (Table 2). However, H_2dUrd, by reversing its activity, reduces its safety margin by approximately 15-fold.

TABLE 2

Antiviral indices

Compound	MTC[a] (μM)	ED_{50}[b] (μM)	AI[c]
BrVdCyd	> 600	0.45	> 1,333
BrVdCyd + H_4Urd	> 600	0.24	> 2,500
BrVdCyd + H_4dUrd	> 600	0.20	> 3,000
BrVdCyd + H_2dUrd	> 600	6.6	> 90

[a]MTC, minimum toxic concentration determined using confluent monolayer VERO cells.
[b]ED_{50}, concentration required to inhibit production of infectious virus particles by 50% (data taken from Fig. 2).
[c]AI, antiviral index determined by dividing MTC by ED_{50}.

Effects on DNA precursor levels

After virus infection, there is a rapid expansion of dNTP pools (Table 3). In HSV-1 infected VERO cells the total dNTP content of cells increased approximately ten-fold in comparison to that found before infection. In uninfected cells, the amount of dTTP is 40 pmol, dGTP 19 pmol, dCTP and dATP, 15–16 pmol. By 8 h post infection, there was a very dramatic rise in the dTTP pool (303 pmol) which represents an approximately 7.5-fold increase over the uninfected cell levels. The dCTP (26 pmol) and dGTP (68 pmol) pool size is also increased in HSV-1 infected cells. These values are two- to four-fold higher than the dCTP pool and dGTP pool in uninfected cells. In contrast, the dATP pool size is reduced after HSV-1 infection. These results are in general agreement with findings of other investigators (Cheng et al., 1975; Jamieson and Bjursell, 1976; North, 1984).

Treatment of uninfected VERO cells with BrVdUrd and BrVdCyd resulted in a moderate expansion of dNTP pool size (Table 3). Both compounds caused doubling of the dATP and dGTP pools compared to the control cells. The dCTP pool remained constant after treatment with BrVdCyd, whereas in the presence of BrVdUrd the dCTP pool size increased to 22 pmol (1.5 times higher) compared to basal dCTP levels in VERO cells. In contrast, treatment with BrVdUrd and BrVdCyd resulted in a marked expansion of dNTP pools in HSV-infected VERO cells. The total dNTP content (1285–1330 pmol) of cells increased more than three-fold compared to that found before exposure to the halogenovinyl compounds. The effect of both nucleoside analogs was most pronounced on dTTP pool size (1240–1250 pmol), with an approximately four-fold increase over the untreated cell

TABLE 3

Effect of BrVdUrd and BrVdCyd on deoxyribonucleoside triphosphate pools of uninfected and HSV-infected VERO cells

Treatment	Deoxyribonucleoside triphosphate (dNTP) (pmol/10^6 cells ± SE)[a]			
	dCTP	dTTP	dATP	dGTP
Uninfected VERO cells[b]				
Control	15.6 ± 1.17	40 ± 1.23	16.2 ± 0.9	19.3 ± 0.96
BrVdUrd	22.1 ± 1.5	47.8 ± 1.15	28.7 ± 0.54	44.1 ± 1.54
BrVdCyd	17.8 ± 1.27	50.8 ± 2.0	31 ± 2.2	42.4 ± 1.06
HSV-infected VERO cells[c]				
Control	26 ± 1.18	303 ± 12.0	10.2 ± 0.34	67.8 ± 1.76
BrVdUrd	8.6 ± 0.9	1249 ± 7.8	31 ± 1.05	40.9 ± 0.36
BrVdCyd	2.53 ± 1.05	1238 ± 8.0	23.8 ± 1.28	21.1 ± 1.55

[a]Values are means of at least six determinations. dNTP pools were determined at 8 hours post infection. Standard error (± SE).

[b]Cells were mock-infected for 1 h.

[c]Cells were infected with HSV-1 strain KOS at an MOI of 10 PFU/cell for 1 h.

levels. BrVdUrd and BrVdCyd decreased the intracellular pools of dCTP to 33 and 9% of control, respectively.

Results of dNTP pool sizes following treatment with BrVdCyd, H_4dUrd and BrVdCyd in combination with H_4dUrd are summarized in Table 4. In HSV-infected VERO cells, intracellular pool size of dCTP, dATP and dGTP increased by approximately 12-, 9- and 1.5-fold respectively and dTTP levels decreased by >99% as compared to untreated cells in the presence of H_4dUrd. Treatment with BrVdCyd plus H_4dUrd also resulted in marked perturbations of dNTP pools in HSV-infected Vero cells. Combination chemotherapy diminished dCTP, dATP and dGTP pools to 1.4%, 22% and 19% of values obtained after incubation with H_4dUrd respectively. The dTTP pool size was 41% of that observed after treatment with BrVdCyd alone. However, it is interesting to note that the total dNTP pool size was essentially similar after treatment with H_4dUrd (522 pmol) or with BrVdCyd plus H_4dUrd (560 pmol). These dNTP levels are approximately 40% lower than the one found after treatment with BrVdCyd (1285 pmol).

In uninfected VERO cells, treatment with H_4dUrd resulted in doubling of the dCTP and dGTP pools, no effect on dATP pool and reduction of dTTP pool to 69% of control. Exposure to BrVdCyd in combination with H_4dUrd resulted in a slight increase of dNTP pool (180 pmol) as compared to H_4dUrd (108 pmol) and BrVdCyd (140 pmol) alone. The only significant difference was an increase in dCTP pool size noted following treatment with H_4dUrd alone or combined with BrVdCyd alone.

TABLE 4

Effect of BrVdCyd, H_4Urd and the combination of BrVdCyd with H_4Urd on deoxyribonucleoside triphosphate pools of uninfected and HSV-infected VERO cells

Treatment	Deoxyribonucleoside triphosphate (dNTP) (pmol/10^6 cells ± SE)[a]			
	dCTP	dTTP	dATP	dGTP
Uninfected VERO cells[b]				
BrVdCyd	17.8 ± 1.27	50.8 ± 2.0	31 ± 2.2	42.4 ± 1.06
H_4dUrd[c]	30.8 ± 1.2	27.5 ± 1.25	17.6 ± 1.28	32 ± 1.3
BrVdCyd + H_4dUrd	53.6 ± 2.34	41.4 ± 0.93	34.7 ± 0.60	51 ± 2.65
HSV-infected VERO cells[d]				
BrVdCyd	2.53 ± 1.05	1238 ± 8.0	23.8 ± 1.28	21.1 ± 1.55
H_4dUrd[c]	326 ± 12.6	0.5 ± 0.34	94.7 ± 2.06	101.3 ± 2.28
BrVdCyd + H_4dUrd	4.5 ± 0.3	515 ± 5.15	21.2 ± 1.93	19.2 ± 2.09

[a]Values are means of at least six determinations. dNTP pools were determined at 8 hours post infection. Standard error (± SE).

[b]Cells were mock-infected for 1 h.

[c]Cells were infected with HSV-1 strain KOS at an MOI of 10 PFU/cell for 1 h.

Discussion

BrVdCyd is a selective inhibitor of HSV-1 with ED_{50} values ranging from 0.3 to 1.20 μM depending on the cell line used. Tetrahydrouridine inhibits only Cyd/dCyd deaminase and H_4dUrd inhibits both Cyd/dCyd deaminase and dCMP deaminase (Cohen and Wolfenden, 1971; Maley and Maley, 1971). Based on the observation that antiviral activity of BrVdCyd was potentiated to the same degree by H_4dUrd and H_4Urd, the following conclusions can be drawn: (i) both H_4dUrd and H_4Urd do not interfere with the transport or phosphorylation of BrVdCyd in HSV-infected VERO cells; (ii) BrVdCyd is deaminated in HSV-infected VERO cells and (iii) BrVdCMP once formed is rapidly converted to its diphosphate by HSV dThd/dCyd kinase which also has dTMP kinase activity without leaving the enzyme, and thus BrVdCMP is not accessible to dCMP deaminase. Thus, based on results of deaminase inhibitors and information available on the levels of deaminases in different cell lines (De Clercq et al., 1982; Fox et al., 1983), it appears that in HSV-infected cells, BrVdCyd is most likely metabolized by the following pathway when deamination is prevented: BrVdCyd → BrVdCMP → BrVdCDP → BrVdCTP → DNA.

HSV virus infection causes perturbation of nucleotide pools partly due to induction of its own enzymes which either compete with corresponding host enzymes or cause a shut-off of host cell metabolism (Cheng et al., 1975; Jamieson and Bjursell, 1976; Karlsson and Harmenberg, 1988; North, 1984). The viral dCMP deaminase is resistant to allosteric regulation by dTTP (Rolton and Keir, 1974) and therefore deamination can occur even in the presence of high dTTP levels. Thus in HSV-infected VERO cells increase in dTTP levels ensues primarily from deamination of dCMP. The significance of dCMP deaminase pathway for generation of dTTP was further corroborated by the observation that treatment of HSV-infected cells with H_4dUrd resulted in a build up of the dCTP pool, whereas dTTP levels were barely detectable (Table 4). Averett et al. (1983) have reported that of the four ribonucleoside diphosphates, the K_m values for the viral ribonucleotide reductase are lowest for GDP and CDP reduction, and this is attributed to the fact that approximately 70% of the herpes virus DNA is comprised of cytosine and guanine nucleotides. This indeed may explain the 1.7-fold and 3.5-fold increase in dCTP and dGTP levels, respectively. Thus, a substantial increase in dGTP concentration seems to be consistent with higher Vmax (2-fold greater) for GDP reduction by the viral enzyme.

BrVdUrd is preferentially phosphorylated by virus-induced dThd/dCyd kinase and subsequent phosphorylation takes it to the triphosphate (BrVdUTP), which not only acts as a competitive inhibitor of, but also as an alternate substrate for the viral DNA polymerase (Allaudeen et al., 1981; Fyfe, 1981; De Clercq, 1986). The utilization of BrVdUTP by the enzyme in place of the natural metabolite allows for a build up of dTTP levels (Table 3). BrVdUTP has also been shown to have a greater inhibitory effect on the viral ribonucleotide reductase than on cellular enzyme (Nakayama et al., 1982). This is probably the reason for the decrease in the dCTP and dGTP levels.

In the presence of BrVdCyd, the pattern of alteration of the dNTP pools was essentially similar to that of BrVdUrd. BrVdCyd can be expected to be deaminated, at least to some extent at the nucleoside and possibly the nucleotide level. Therefore, perturbations in dNTP pools in HSV-infected cells on exposure to BrVdCyd are consistent with expected results: for example, a massive increase in dTTP in a manner similar to that observed following exposure to BrVdUrd. BrVdCyd that is not deaminated would be channelled to its triphosphate level by the dCyd kinase/dCMP-kinase pathway (Fig. 1). If BrVdCTP acts as an alternate substrate or competitively inhibits viral DNA polymerase, as has been shown for BrVdUTP (Allaudeen et al., 1981; Fyfe, 1981), then dCTP levels should rise. This does not appear to be the case (Table 3). In fact, the fall in dCTP concentration is greater than that observed with BrVdUrd. In order to explain this phenomenon, one should refer to the earlier work of Jamieson and Subak-Sharpe (1974) on the regulatory effects of pyrimidine nucleoside triphosphates on the viral enzyme. In this study, it was shown that while the HSV-induced dThd and dCyd phosphorylating activities reside in the same protein, there are marked differences in the regulation of the two activities. For example, the virus-induced dThd kinase is resistant to inhibition by dTTP, whereas the dCyd kinase activity is inhibited by both dTTP and dCTP. Therefore, one possible explanation is that the build-up of high dTTP levels probably acts on dCyd kinase site and inhibits phosphorylation of dCyd. In addition, the activity of dCyd deaminase ensures a low supply of dCyd.

In the presence of BrVdCyd in combination with H_4dUrd, dCTP levels remained essentially unchanged but dTTP levels increased in HSV-infected VERO cells (Table 4). The increase in dTTP levels indicates that thymine nucleotides are being formed *via* pathways other than the dCyd deaminase-dCMP deaminase pathway. It is possible that the HSV-induced dThd kinase, which is not normally required for productive infection of mammalian cells, is now being used to a greater extent to provide dTTP for viral DNA synthesis. BrVdCTP in a manner similar to dCTP may also exert a stimulatory effect on virus-induced thymidine kinase thereby enhancing the phosphorylation of preformed thymidine. In our opinion, exposure to BrVdCyd plus H_4dUrd puts a strain on viral DNA synthesis to such an extent that even though dTTP is being formed, its eventual utilization as a substrate is reduced and hence it builds up. The (greater than 2-log) reduction in infectious virus production on exposure to this combination provides further support for this hypothesis. Why dCTP levels remain low after combination of BrVdCyd with H_4dUrd is intriguing. Possible explanations are: (i) BrVdCyd and dCyd compete for phosphorylation and BrVdCyd is preferentially phosphorylated by the viral dThd/dCyd kinase; (ii) dNTP levels under these conditions reflect balance and interplay of competition at the single nucleoside diphosphate binding site because the reduction of all four ribonucleoside diphosphates occurs at a common site and each diphosphate is a competitive inhibitor with respect to each other; (iii) BrVdCTP (or its 5′-diphosphate precursor) act at other sites. One such site may be the viral ribonucleotide reductase, as has been reported for BrVdUTP (Nakayama et al., 1982). Failure of CDP and GDP reduction would account for the low dCTP and dGTP levels; (iv) BrVdCTP is neither substrate nor inhibitor of the viral DNA

polymerase. However, this assumption is unlikely, considering the fact that BrVdUTP is both a substrate and inhibitor of the DNA polymerase (Allaudeen et al., 1981; Fyfe, 1981; Ruth and Cheng, 1981; De Clercq and Walker, 1984). Experiments to elucidate the nature of the viral DNA formed after different treatment regimens are in progress. Further studies on the mechanism of action of BrVdCyd and its metabolism in infected cells are also planned.

BrVdUrd, at higher concentrations (70 μg/ml and above), has been shown to interfere with the incorporation of DNA precursors in primary rabbit kidney cells (De Clercq et al., 1979). BrVdUTP is an inhibitor of purified DNA polymerase α (Allaudeen et al., 1981). However, its initial phosphorylation by cytosolic dThd kinase is less than 5% of that of the natural substrate dThd, while human mitochondrial dThd kinase is more efficient in phosphorylating BrVdUrd (Cheng et al., 1981). Thus, metabolic processing of BrVdUrd at higher concentrations (100 μM, as used in this study) by cellular kinases in VERO cells is likely responsible for the marginal increase observed in dTTP and dCTP pools. High levels of dTTP, by feedback inhibition of mammalian dCMP deaminase, would divert BrVdCyd to the dCMP → dCDP → dCTP pathway.

VERO cells contain high levels of dCyd deaminase and dCMP deaminase. The increase in dTTP when these cells are treated with BrVdCyd reflects its deamination to the corresponding deoxyuridine derivatives. Treatment with H_4dUrd alone caused a fall in dTTP and a 2-fold increase in dCTP. These results are consistent with the effect of H_4dUrd and H_4dUMP as inhibitors of dCyd deaminase and dCMP deaminase, respectively. The observation that dTTP levels fall only slightly indicates that, in uninfected cells, inhibition of these enzymes does not completely deprive the cell of thymine nucleotides because there is still some dTMP available derived from the dUTP → dUDP → dUMP → dTMP pathway. When VERO cells were treated with the combination of BrVdCyd and H_4dUrd, dTTP levels were restored to control values, while dCTP levels were elevated three-fold. These results indicate that when deamination is prevented, BrVdCyd has no effect on dTTP. However, its phosphorylation to BrVdCTP and subsequent competition with dCTP for cellular DNA polymerases, allows for the build-up of dCTP levels. The present findings are therefore consistent with an earlier report that at higher concentrations BrVdCyd has an effect on labelled dCyd incorporation (De Clercq et al., 1982). The changes observed in the purine dNTP pools are most likely a reflection of the perturbation in the pyrimidine nucleotide pool levels.

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