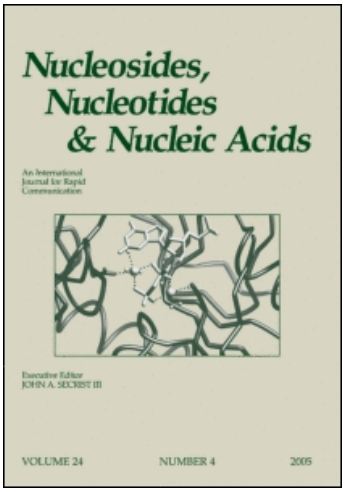

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Different Mechanisms of Inhibition of DNA Synthesis by (*E*)-5-(2-Bromovinyl)-2'-deoxyuridine in Cells Transfected with Gene for Thymidine Kinase of Herpes Simplex Virus Type 1 and in Cells Infected with the Virus

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DIFFERENT MECHANISMS OF INHIBITION OF DNA SYNTHESIS BY (*E*)-5-(2-BROMOVINYL)-2'-DEOXYURIDINE IN CELLS TRANSFECTED WITH GENE FOR THYMIDINE KINASE OF HERPES SIMPLEX VIRUS TYPE 1 AND IN CELLS INFECTED WITH THE VIRUS

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Abstract: The effect of (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) on deoxyribonucleoside 5'-triphosphate pools was studied in cells transfected with gene for thymidine kinase of herpes simplex virus type 1 and cells infected with the virus. When infected cells were treated with BVDU, the triphosphate form of the nucleoside analog was detected. When transfected cells were treated with BVDU, the triphosphate form was not detected and the pattern of changes in the pools was the same as after 5-fluoro-2'-deoxyuridine treatment. BVDU seems to inhibit DNA synthesis differently in the two cell lines and nucleotide metabolism in the transfected cells was not the same as in the infected cells.

(*E*)-5-(2-Bromovinyl)-2'-deoxyuridine (BVDU) was found by De Clercq *et al.* (1) to have a stronger antiherpetic effect than acyclovir, with little toxicity to cells. The compound is selectively phosphorylated by thymidine kinase (TK) of herpes simplex virus type 1 (HSV-1) to its mono- and diphosphate forms (2, 3), which are converted by cellular nucleotide kinase to the active triphosphate form (BVDU-TP) (2). The active form inhibits HSV-1 DNA replication and viral production by its inhibiting HSV-1 DNA polymerase (4). Two steps of the nucleotide metabolism pathway account for the selectivity of BVDU. One is the first phosphorylation by either HSV-1 or cellular TK, and the other is the incorporation of BVDU-TP into the DNA caused by either host or HSV-1 DNA polymerase. Phosphorylation by viral TK is important for the potency of BVDU (2).

Ayusawa *et al.* and Balzarini *et al.* have reported that transformation of TK-deficient murine mammary carcinoma FM3A cell line with the HSV-1 TK gene makes this cell

line extremely sensitive to the cytostatic action of BVDU and IVDU (5, 6). This transformed cell line (FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ cells) may be useful for studies of the conversion of BVDU to BVDU-TP. In this paper, we report the changes with BVDU treatment in the deoxyribonucleoside triphosphate (dNTP) pools in both cells transformed with the HSV-1 TK gene and HSV-1-infected cells.

MATERIALS AND MATHODS

Materials. BVDU were the gift of Yamasa Corporation (Japan). 1-β-D-arabino-furanosyl-5-(*E*-2-bromovinyl)uracil monophosphate (BVaraU-MP) was kindly provided by Dr. H. Machida (Yamasa Corporation, Japan). 1,1,2-Trichloro-trifluoroethane was purchased from Aldrich (USA) and tri-*n*-octylamine was purchased from Tokyo Kasei (Japan). (*E*)-5-(2-bromovinyl)-2'-deoxyuridine 5'-monophosphate (BVDU-MP) was synthesized as described previously (7) by nucleoside phosphotransferase obtained from carrots and purified by paper chromatography.

Cells. Human embryo lung (HEL) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and maintained in MEM supplemented with 5% FBS. FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ cells, which had been established by Ayusawa *et al.* from TK deficient FM3A cell line by introducing HSV-1 TK gene (6), were the gift of Prof. T. Seno (National Institute of Genetics, Japan). The cell line and FM3A cells were cultured in suspension in ES medium (Nissui Pharmaceutical Co.) with 2% FBS. These cells were incubated at 37 °C under a 5% CO_2 atmosphere. FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ cells were grown in a medium containing 1 x 10^{-4} M hypoxanthine, 1 x 10^{-6} M aminopterin, and 1 x 10^{-5} M thymidine for two weeks before the experiment.

For growth-inhibition studies, FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ and FM3A cells were seeded at a density of 5 x 10^4 cells/well in 24-well plates, and were immediately treated with various concentrations of BVDU. The compound was present in the medium throughout the culture. After 48 h of incubation, the number of cell was counted using Coulter Multisizer II (Coulter Electronics, England). The EC_{50} value is the concentration of BVDU necessary to inhibit the increase in cell density by 50% compared with control.

Virus. F strain of HSV-1 was used for all experiments. The virus stock solution was prepared in Vero cell monolayers and stored at -80 °C. In all experiments examining the nucleotide pool, the virus was adsorbed at a multiplicity of infection of 15 plaque-forming units/cell for 2 h at 37 °C, and the cells were washed once with phosphate-buffered saline (PBS) and maintained in minimally supplemented medium.

Preparation of cell extracts. Cells infected with HSV-1 were detached by trypsin treatment and suspended in PBS at 4 °C. After the cells were counted, the suspension

was transferred to a 1.5-ml Microtest tube 3810 (Eppendorf) and ice-cold 100% trichloroacetic acid was added to the suspension to a final concentration of 3 x 10^{-1} M. The mixture was vortexed and kept for 30 min at 4 °C. After centrifugation (15600 x *g*, 4 °C, 30 s) of the mixture, the supernatant was taken, to which 1.1 volumes of a cold solution of 5 x 10^{-1} M tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane was added. The mixture was kept for 5 min at 4 °C, during which vortexing was repeated 5 to 6 times. After centrifugation (15600 x *g*, 4 °C, 30 s), the aqueous upper layer containing nucleotides was separated off and analyzed with high-performance liquid chromatography (HPLC) after the periodate oxidation procedure for measurement of dNTPs (8). An extract from FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ cells was prepared by the same method without trypsin treatment, because the cells were grown in suspension.

Measurement of dNTPs in cell extracts. To 80 µl of a cell extract in a 1.5-ml Microtest tube, 20 µl of 2 x 10^{-2} M 2'-deoxyguanosine and 20 µl of 2 x 10^{-1} M $NaIO_4$ were added. After vortexing and centrifugation (15600 x *g*, 4 °C, 30 s), the solution was incubated at 37 °C for 2 min. The tube was placed on ice, and then 2 µl of 1 M rhamnose and 30 µl of 4 M CH_3NH_2, neutralized to pH 6.5 with H_3PO_4, were added to the reaction mixture. The suspension was mixed well and centrifuged at 15600 x *g* at 4 °C for 10 s. After incubation at 37 °C for 30 min, the sample was cooled on ice. Chromatography on Partisil-10 SAX (4.6 x 250 mm, Whatman) was done as described previously (8). HPLC analyses were performed using a Waters 6000A pump with a Waters 440 absorbance detector set at 254 nm, and a Hewlett-Packard 3390A integrator. Measurement of ribonucleoside triphosphate (rNTP) was done as described elsewhere (8).

Measurement of BVDU-MP in cell extracts. BVDU was added at a concentration of 6 x 10^{-7} M. After 20 h, the cell extract was prepared as described above. The cell extract was analysed with an HPLC system consisting of a Waters 600 multisolvent delivery system with a 481 LC spectrophotometer and a Hewlett-Packard 3390A integrator. The amount of extract injected into the apparatus was equivalent to 1.6 x 10^6 cells. For the analytical column, we used TSK gel DEAE-2SW (4.6 x 250 mm, Tosoh) with a mobile phase of 2.5 x 10^{-2} M $NH_4H_2PO_4$ (adjusted to pH 4.5 with H_3PO_4) containing 20% CH_3CN. The flow rate was 1 ml/min, and absorbance was monitored at 294 nm.

Assay of thymidylate synthetase (TS, EC 2.1.1.45) in the presence of BVDU-MP. TS was purified from *Lactobacillus casai* as previously described (9). Its activity was assayed in a reaction mixture that contained 5 x 10^{-2} M Tris (pH 7.4), 2.5 x 10^{-2} M $MgCl_2$, 1 x 10^{-3} M EDTA, 6.5 x 10^{-3} M formaldehyde, 7.5 x 10^{-2} M 2-mercaptoethanol, 1.42 x 10^{-4} M N^5, N^{10}-methylenetetrahydrofolic acid, 3.25 x 10^{-6} to 21.6 x 10^{-6} M dUMP, 0 to 8.6 x 10^{-6} M BVDU-MP, and the enzyme. Incubation was at 25 °C and the initial velocity of absorbance at 340 nm, indicating the conversion of N^5, N^{10}-methylenetetrahydrofolic acid to dihydrofolic acid, were measured starting when enzyme was added (10).

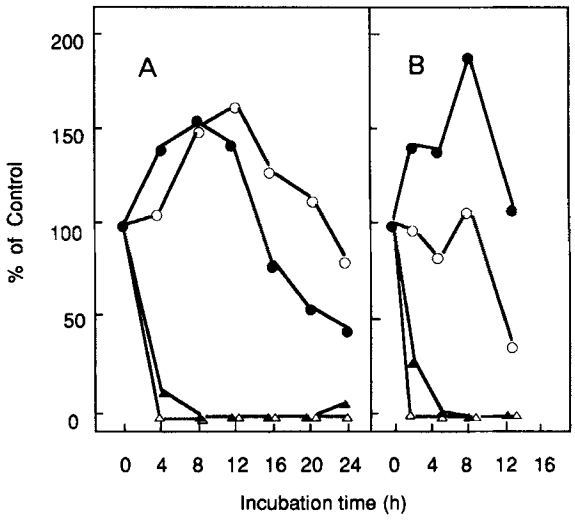

FIG. 1. dNTP pool changes in FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ cells treated with 6.0 x 10^{-8} M BVDU (A) and in FM3A cells treated with 1.0 x 10^{-6} M FdUrd (B). The compound was added at 0 h. The absolute values of dNTP pools at the start were as follows: in FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ cells, dATP (●), 45 pmol/10^6 cells; dCTP (○), 18 pmol/10^6 cells; dGTP (△), 8 pmol/10^6 cells; and dTTP (▲), 45 pmol/10^6 cells; and in FM3A cells, dATP (●), 29 pmol/10^6 cells; dCTP (○), 26 pmol/10^6 cells; dGTP (△), 7 pmol/10^6 cells; dTTP (▲), 70 pmol/10^6 cells . Retention times for dNTPs and BVDU-TP were as follows: dCTP, 8.0 min; dTTP, 9.3 min; dATP, 12.1 min; dGTP, 19.5 min; BVDU-TP, 10.4 min.

RESULTS

The inhibition by BVDU of the growth of the transformed cells and FM3A (parent) cells was examined. The EC_{50} values were 4.0 x 10^{-10} and 3.6 x 10^{-6} M, respectively; so the transformed cells were 9000-fold more sensitive than the parent cells.

The amounts of both dNTP and rNTP pools in the transformed cells were assayed every 4 h after BVDU was added at the final concentration of 6 x 10^{-8} M, which was the minimum concentration inhibiting cell growth completely. Both dGTP and dTTP pools were depleted for the first 8 h after BVDU was added (FIG. 1A). Pools of dATP and dCTP increased and then decreased, but rNTP pools were unchanged by BVDU treatment (data not shown). Unexpectedly, BVDU-TP was not detected at all throughout this experiment. This pattern of nucleotide pool alteration was the same as that of FM3A cells treated with 5-fluoro-2'-deoxyuridine (FdUrd) as shown in FIG. 1. FdUrd is phosphorylated to 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) by TK, and inhibits thymidylate synthetase (TS) (12, 13). Thus FdUrd causes depletion of the

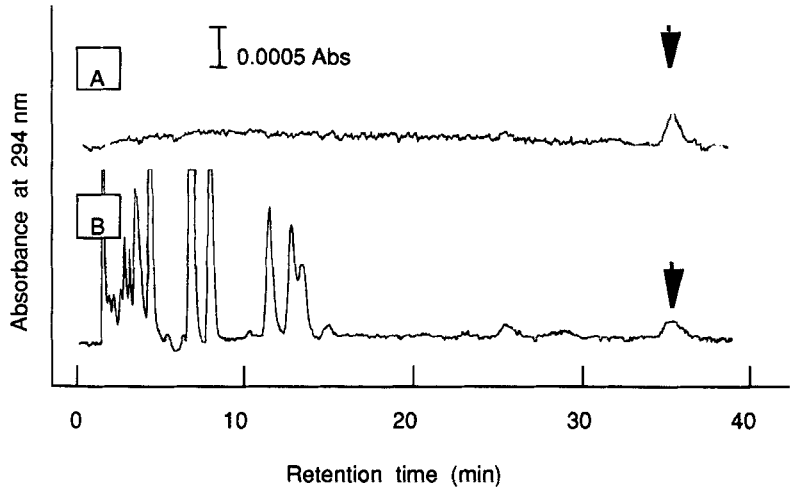

FIG. 2. Detection of BVDU-MP in FStk⁻10/aprt⁻3/HSV-1 tk⁺ cells.
A: Standard (BVDU-MP: 25.9 pmol). B: Treated with BVDU. Arrows, BVDU-MP.
Abs, Absorbance.

dTTP pool and changes in other dNTP pools (11). We therefore examined whether BVDU-MP was produced by this cell line. Twenty hours after the addition of 6 x 10^{-7} M BVDU, BVDU-MP was detected at a concentration of 2.7 x 10^{-5} M (FIG. 2). dGTP and dTTP pools were depleted and BVDU-TP was not detected in this condition, too (data not shown). In FM3A cells treated with the same condition, BVDU-MP was not detected (data not shown).

BVDU-MP inhibited TS activity competitively, inhibition constant (K_i) being 2.2 x 10^{-6} M (FIG. 3). In contrast, the *Ki* of BVaraU-MP, which is the monophosphate form of 1-β-D-arabinofuranosyl-5-(*E*-2-bromovinyl)uracil (BVaraU), was 5.3 x 10^{-4} M (Hirota *et al.*, unpublished data). BVaraU lacks the cytostatic activity against the transformed cells (6).

To ascertain whether these phenomena are the same as those in HSV-1-infected cells, we measured dNTP and rNTP pools in HSV-1-infected HEL cells (TABLES 1 and 2). The dose of BVDU used was 7.0 x 10^{-7} M, the concentration that completely prevented plaque formation (data not shown). The dNTP pools in HEL cells were very small, and the dCTP pool could not be detected. The dNTP pools changed after virus infection, but rNTP pools did not change. Virus infection caused dNTP pools, particularly the dTTP pool, tend to increase. By treatment with BVDU, the dNTP pools, but not the dCTP pool which could not be detected, were not affected, and BVDU-TP was detected

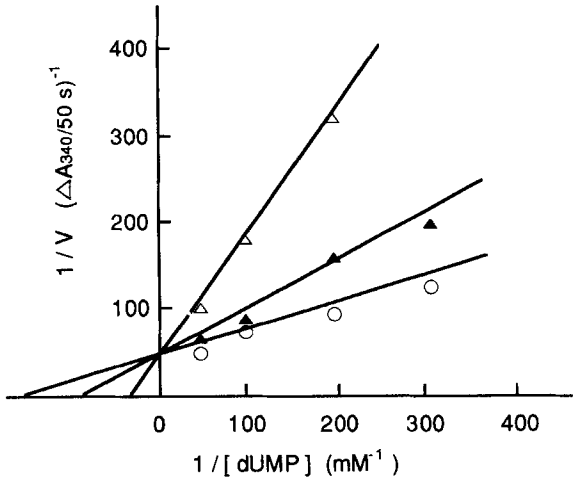

FIG. 3. Double reciprocal plots for inhibition of *L. casei*. TS, showing the effects on the reaction catalyzed by TS in the presence of different concentrations of dUMP. ○, No inhibitor; ▲, 1.7 x 10^{-6} M BVDU-MP; and △, 8.6 x 10^{-6} M BVDU-MP. The *Ki* value of BVDU-MP was 2.2 x 10^{-6} M, and the *Km* value of dUMP was 5.9 x 10^{-6} M.

TABLE 1. Concentrations of nucleotide pools in HEL cells after HSV-1 infection.

dNTP or NTP	Concentrations of nucleotide pools (pmol/10^6 cells)			
	Time after HSV-1 infection (h)			
	0	4	10	20
dTTP	N.D.	125	300	300
dATP	1.3	23	17	8.5
dGTP	8.8	13	13	15
ATP	6500	6800	6200	4500
GTP	2100	2600	3100	2300
UTP	1600	1900	2000	1500
CTP	500	500	570	520

Nucleotide pool levels were measured as described in MATERIALS AND METHODS. The values (pmol/10^6 cells) are means of duplicate measurements. dCTP was not detected at any time. Retention times for dNTP and BVDU-TP were the same as in FIG. 1 and those for rNTPs were as follows: CTP, 7.2 min; UTP, 8.3 min; ATP, 10.3 min; and GTP, 17.6 min.
N.D.: Not detected.

TABLE 2. Concentrations of nucleotide pools in HEL cells after HSV-1 infection and BVDU treatment.

dNTP or NTP	Concentrations of nucleotide pools (pmol/10^6 cells) Time after HSV-1 infection (h) 0	4	10	20
dTTP	N.D.	80	115	240
dATP	1.3	29	56	34
dGTP	8.8	17	29	19
BVDU-TP	N.D.	56	72	130
ATP	6500	6200	7100	7700
GTP	2100	2600	2800	4400
UTP	1600	1700	2400	2900
CTP	500	450	530	940

BVDU was added at a final concentration of 7.0×10^{-7} M at 0 h.
See footnotes of TABLE 1 for other details.

at the level as other dNTPs. This finding supports those of others (2-4) that BVDU inhibits viral DNA polymerase in its BVDU-TP form in HSV-1-infected cells. The effects of BVDU treatment on dNTP pools in HSV-1-infected cells differed from those in the transformed cells.

DISCUSSION

The transformed cells we used did not convert BVDU to BVDU-TP but HSV-1-infected cells phosphorylated BVDU to BVDU-TP. Treatment with BVDU changed the dNTP pools in the transformed cells, as occurred when cells were treated with FdUrd. These results suggested that BVDU inhibits the growth of the transformed cells and HSV-1 replication by different mechanisms. In the transformed cells, the growth inhibition is mediated by the inhibition of TS by BVDU-MP, as when FdUrd is added. In HSV-1-infected cells, inhibition of HSV-1 replication is mediated by the inhibition of HSV-1 DNApolymerase by BVDU-TP. The K_i/K_m of BVDU-MP toward TS was 0.37, although this value is 140 times that of FdUMP (0.0027) (13). Moreover, we could detect BVDU-MP in the transformed cells, although the concentration of BVDU added was 10 times the minimum amount for inhibition of cell growth. This cell line resists purine nucleoside analogs having potent anti-HSV-1 activity like acyclovir but is sensitive to most of pyrimidine nucleoside analogs (6). This partial sensitivity also supports our hypothesis that BVDU-MP inhibits TS and thus prevents cell growth. On the other hand, in HSV-1-

infected cells, BVDU-TP was produced at the same level as other dNTPs. Therefore, BVDU-TP can inhibit HSV-1 DNA polymerase effectively (4).

BVDU is phosphorylated to (*E*)-5-(2-bromovinyl)-2'-deoxyuridine 5'-diphosphate (BVDU-DP) by HSV-1 TK (2, 3), and then further phosphorylated to BVDU-TP by cellular nucleotide kinase (2). FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ cells have HSV-1 TK instead of cellular TK. Accordingly, we expected that this cell line had the same metabolic pathway as HSV-1-infected cells for conversion from BVDU to BVDU-TP. But we did not, however, detect BVDU-TP in this cell line, while we did in HSV-1-infected cells. This inconsistency suggests that HSV-1 may encode an unknown enzyme to phosphorylate BVDU-DP, or a transactivated protein that can express such unknown enzyme. BVDU is less active against HSV-2 than HSV-1. In HSV-2-infected cells, BVDU and BVDU-MP have been detected, but BVDU-DP and BVDU-TP have not (3). HSV-2 TK phosphorylates BVDU to BVDU-MP, but not to BVDU-DP. The anti-HSV-2 mechanism of BVDU may involve inhibition of TS, as it dose in transformed cells. The anti-HSV-2 activity of BVDU is much weaker than inhibition of the growth of transformed cells, because it is likely that HSV-2 infection may stimulate *de novo* synthesis of dTTP, as HSV-1 infections do. The metabolism of nucleotides in FStk$^-$10/aprt$^-$3/HSV-1 tk$^+$ cells is different from that in HSV-1-infected cells, and BVDU has two different mechanisms that disturb DNA synthesis in the two kinds of cells.

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