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Comparative Metabolism of *E*-5-(2-Bromovinyl)-2′-deoxyuridine and 1-β-D-Arabinofuranosyl-*E*-5-(2-bromovinyl)uracil in Herpes Simplex Virus-Infected Cells

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SUMMARY

The antiviral activities and metabolic fates of *E*-5-(2-bromovinyl)-2′-deoxyuridine (BrVdUrd) and 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BrVaraUra) were compared in a dThd kinase-deficient human fibroblast cell line, infected with parental strains of herpes simplex virus, and other strains expressing no viral dThd kinase activity. Metabolic experiments were performed at concentrations well above the ID_{50} for each compound because radiolabeled agents were not available. BrVaraUra and its nucleotides qualitatively displayed chromatographic and anabolic characteristics which closely paralleled those of BrVdUrd and its nucleotides. Monophosphorylation of both drugs was dependent upon the presence of viral dThd kinase activity except in the case of one dThd kinase-negative type 1 mutant ($SC16R_5C_1$) which retained BrVdUrd/BrVaraUra kinase activity. Intracellular uptake of either parent compound was absent during mock-infection and minimal in the cases of infection with mutants unable to phosphorylate the parent compound. Parental type 1 strains were able to induce diphosphorylation and triphosphorylation of both compounds to a similar, dose-dependent degree. Extracts of type 2-infected cells contained greater quantities of BrVdUrd and its monophosphate compared with BrVaraUra and its monophosphate, after identical drug exposure and infection conditions. As previously observed for BrVdUrd, diphosphorylated and triphosphorylated nucleotides of BrVaraUra were not detected after type 2 infection. BrVdUrd and BrVaraUra metabolic breakdown pathways differed, however, as evidenced by the formation of *E*-5-(2-bromovinyl)uracil (BrVUra). Unlike BrVdUrd, BrVaraUra formed no BrVUra in infected cells, suggesting that replacement of 2′-deoxyribose with arabinose makes the compound biologically more stable, presumably because of resistance to enzymatic breakdown by pyrimidine nucleoside phosphorylases. In this dThd kinase-negative cell line, BrVdUrd and BrVaraUra displayed qualitatively similar susceptibility profiles in that activities were type 1 selective and dThd kinase dependent. Antiviral activities against dThd kinase-positive type 1 strains were similar with both compounds. These data would suggest that BrVdUrd and BrVaraUra have identical type-specific dThd-dTMP kinase-dependent mechanisms of cellular uptake and phosphorylation, but that the latter is not subjected to phosphorolysis and resultant formation of an inactive metabolite. Furthermore, the absence of presence of phosphorolysis of the parent nucleoside does not apparently adversely affect *in vitro* antiviral activity.

Nucleoside analogues have taken on an important role in the therapy of viral infections and cancers (1). Some of the difficulties in the search for new chemotherapeutic agents have included lack of specificity, DNA misreading and resultant tumor formation, or metabolic breakdown and production of inactive metabolites. BrVdUrd and its arabinose analogue, BrVaraUra, are similar antiviral nucleosides, characterized by their potent activities against HSV-1 HZV in cell culture and animal models (2–7). BrVdUrd shows better activity in certain non-human cell lines against HSV *in vitro* than does BrVaraUra (8, 9). Anti-HZV activities may be somewhat better with BrVaraUra (6). BrVdUrd is incorporated into DNA (10, 11), whereas BrVaraUra is not (12, 13). Both agents show low toxicity in cell culture and in animal models (5, 14).

dTK (EC 2.7.1.75) induced by HSV catalyzes the monophos-

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ABBREVIATIONS: BrVdUrd, *E*-5-(bromovinyl)-2′-deoxyuridine; BrVaraUra, 1-β-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil; HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; HZV, herpes zoster virus; dTK, thymidine kinase; HSV-2, herpes simplex virus type 2; BrVdUrdMP, BrVdUrdDP, and BrVdUrdTP, the 5′-monophosphate, 5′-diphosphate, and 5′-triphosphate, respectively, of BrVdUrd; dTK^-, does not express thymidine kinase activity; BrVUra, *E*-5-(2-bromovinyl)uracil; $HOSFTK^-$, thymidine kinase-deficient human osteosarcoma fibroblast cell line 143; FBS, fetal bovine serum; IVdUrd, *E*-5-(2-iodovinyl)-2′-deoxyuridine; BrVaraUraMP, BrVaraUraDP, and BrVaraUraTP, the 5′-monophosphate, 5′-diphosphate, and 5′-triphosphate, respectively, of BrVaraUra; HPLC, high performance liquid chromatography.

phorylation of BrVdUrd in HSV-1 and HSV-2. HSV-1 dTK possesses dTMP kinase activity which can effect the diphosphorylation of BrVdUrd (15). Accordingly, we observed that BrVdUrd was metabolized to its 5′-monophosphate (BrVdUrdMP), 5′-diphosphate (BrVdUrdDP), and 5′-triphosphate (BrVdUrdTP) derivatives in HSV-1-infected cells, whereas only BrVdUrd and BrVdUrdMP were detected in HSV-2-infected cells (16). BrVdUrd and BrVdUrdMP (but not BrVdUrdDP) were also detected in cells infected with a dTK-altered mutant of HSV-1 (SC16B3) and, surprisingly, those infected with a dTK-deficient (dTK^-) mutant of HSV-1 ($SC16R_6C_1$) (17). Thus, through mutation, while either retaining its dTK and BrVdUrd kinase activity (SC16B3) or retaining its BrVdUrd kinase activity ($SC16R_6C_1$) in the absence of dTK activity, the polypeptide expressed by the HSV-1 dTK gene can specifically lose its dTMP/BrVdUrdMP phosphorylating capacity. In addition, BrVdUrd was metabolized to BrVUra in HSV-infected human foreskin fibroblast cells and in HSV-infected dTK^- human osteosarcoma fibroblasts (line 143; $HOSFTK^-$) (17). BrVUra formation has also been reported in blood and tissue extracts (18, 19).

The metabolic basis for BrVaraUra's type specificity of antiviral action has not been previously demonstrated. However, because of the striking similarities in antiviral spectra of BrVaraUra and BrVdUrd, we examined the metabolic fate of BrVaraUra and BrVdUrd in $HOSFTK^-$ cells infected with wild-type HSV-1 and HSV-2 strains. In addition, we investigated whether BrVaraUra, like BrVdUrd, will be monophosphorylated by $SC16R_6C_1$ (dTK^-), and whether this phenomenon extends to other dTK^- viruses.

Materials and Methods

Cells and viruses. Cell line 143 ($HOSFTK^-$) was derived as previously reported (20) and was provided by Dr. Silvia Bachetti, McMaster University (Hamilton, Ontario, Canada). The cells were grown in alpha Eagle's minimal essential medium supplemented with 30 μg/ml bromodeoxyuridine, 10% FBS, penicillin (100 IU/ml), streptomycin 100 μg/ml), Fungizone (2.5 μg/ml), glutamine (2 μM), and sodium bicarbonate (0.2%. Sera were purchased from British Drug Houses (Toronto, Ontario), and other supplements were obtained from Flow Laboratories, Inc. (McLean, VA). Strains SC16 and $SC16R_6C_1$ of HSV-1 were kindly provided by Dr. Hugh J. Field, Department of Veterinary Medicine, Cambridge University (Cambridge, England). $SC16R_6C_1$ is a dTK^- mutant which was derived by one passage of the parent strain in the presence of 5.0 μg/ml acyclovir (21). Strain KOS and its mutants, ACG′4 and ΔT143, were a gift from Dr. Donald M. Coen, Department of Pharmacology, Harvard Medical School (Boston, MA). ACG′4 was derived by exposure of KOS to acyclovir. This strain does not induce a detectable dTK polypeptide (22). ΔT143 is a deletion mutant, which demonstrates some alterations in DNA polymerase[1] and also possesses no dTK polypeptide. HSV-1, strain F, and HSV-2, strain G were obtained from Dr. Bernard Roizman, University of Chicago (Chicago, IL). HSV-2, strain 150 was a genital isolate obtained from a patient of the University of British Columbia Herpes Clinic which has been previously typed (23) and characterized (16).

Chemicals and equipment. BrVUra, BrVdUrd, BrVdUrdMP, BrVdUrDP, BrVdUrd TP, and IVdUrd were prepared by P. Herdewyn (BrVUra), R. Busson, L. Colla, and H. Vanderhaeghe (BrVdUrd and IVdUrd), and T. Fukui and L. Colla (BrVdUrd 5′-phosphates) at the Rega Institute (Katholieke Universiteit Leuven, Leuven, Belgium) by previously established procedures (24). BrVaraUra, BrVaraUraMP, and BrVaraUraTP were synthesized by S. Sakata of Research Laboratories, Yamasa Shoyu Co. (Choshi, Japan), as described previously (BrVaraUra and BrVaraUraMP, Refs. 4 and 25) and by the method of Ruth and Cheng (13) (BrVaraUraTP). Tetrabutylammonium bromide was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dihydrogen phosphate (NaH_2PO_4), HPLC grade methanol, and bromodeoxyuridine were purchased from Fisher Scientific Co. (Fairlawn, NJ). The HPLC apparatus was a Spectra Physics 8000B with ultraviolet detector 8400 (Spectra Physics Inc., San Jose, CA). Spherisorb ODS2 (Phase Separation Ltd., Queensberry, U.K.) was packed into 4.6 × 125 mm columns. A presaturation column (4.6 × 120 mm) packed with 20 μM Partisil was used in tandem.

Preparation of cell extracts. The method for the preparation of cell extracts and HPLC analyses has been previously reported (16, 26). Briefly, monolayers of $HOSFTK^-$ cells were mock-infected or HSV-infected at various multiplicities. After 1 hr, media were removed and replaced by alpha Eagle's minimal essential medium containing 4% FBS and various concentrations of BrVdUrd of BrVaraUra. The cultures were incubated for an additional 5, 7, or 19 hr at 34° in a 5% CO_2-humidified atmosphere. The medium was removed and the cells were washed twice with phosphate-buffered saline. Cell monolayers were then harvested with trypsin, collected in cold phosphate-buffered saline, and centrifuged at 800 × *g* for 10 min at 4°, resuspended in 2 ml of distilled water, and freeze-thawed. BrVdUrd or BrVaraUra and its acid-soluble metabolites were extracted by adding 0.2 ml of ice-cold perchloric acid (4 M) to the cell suspension, vortexing the mixture for 1 min, and letting it stand in an ice bath for 5 min. The samples were then centrifuged at 2000 × *g* for 10 min at 4°. The supernatant was then removed and mixed with 0.2 ml of IVdUrd (150 μg/ml) as the internal standard. The extract was neutralized with a mixture of 2 M K_2HPO_4 and centrifuged at 2000 × *g* for 10 min at 4°. The supernatants were freeze-dried and later redissolved in glass distilled water for HPLC analyses. Each set of biological conditions was established in duplicate.

HPLC analyses of cell extracts. BrVUra and BrVdUrd concentrations were calibrated using IVdUrd as an internal standard. The same calibration factor was used for BrVdUrd, BrVaraUra, and their phosphorylated derivatives. The temperature of the HPLC oven was 48° and the detector was set at 292 nm. A linear gradient in methanol (25–50%, v/v in water) was used with solvents containing 0.0125 M tetrabutylammonium bromide and 0.05 M NaH_2PO_4, pH adjusted to 5 by addition of NaOH. Ten μl of each sample were injected and the flow rate was 1 ml/min. Identification of the compounds was based on their unique ratios of absorbance (292 nm/254 nm) and their retention times compared to chemically synthesized standards.

Antiviral assays. Monolayers of $HOSFTK^-$ cells were grown to confluence in flat-bottomed 96-well microtiter trays and infected in quadruplicate with 10–100 plaque-forming units of HSV in 0.1 ml of Eagle's minimal essential medium per well. After an adsorption period of 1 hr at 37°, the inoculum was removed and replaced with 2.0 ml of media containing serial 2-fold dilutions of either BrVdUrd or BrVaraUra ranging up to 20.0 μ/ml and 4% FBS. The trays were incubated for 48 hr at 37° under 5% CO_2. The ID_{50} represented the extrapolated drug concentration showing a 50% reduction in plaque number compared with the untreated control wells.

Results

The chromatographic characteristics of the bromovinyl compounds were not markedly altered by the substitution of the arabinose for deoxyribose. As shown in Fig. 1, BrVaraUra was metabolized to its 5′-mono-, -di-, and -triphosphorylated nucleotides in HSV-1 (strain F)-infected cells. Fig. 1C displays the chromatogram of HSV-2 (strain G)-infected cell extracts after exposure to BrVaraUra, where phosphorylation was halted after formation of the 5′-monophosphate. Qualitatively similar retention patterns have been described previously for BrVdUrd in HSV-1- and HSV-2-infected cells (16, 17). Drug susceptibilities and metabolite formations are quantitatively

[1] D. M. Coen, personal communication.

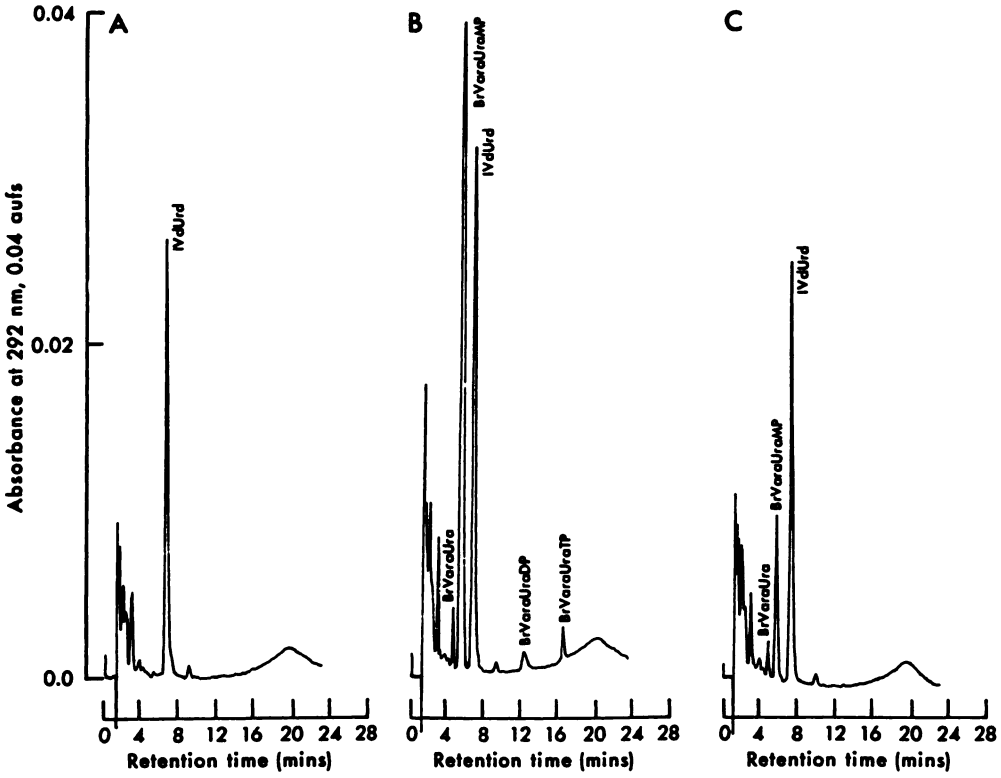

Fig. 1. HPLC analyses of extracts of 120 μM BrVaraUra-treated HOSFTK⁻ cells. Retention times are plotted by the UV detector as a function of absorbance at 292 nm, 0.04 absorbance unit, full scale. All samples were reconstituted in 0.2 ml of glass distilled water. A. Uninfected cells. B. HSV-1, strain F-infected cells exposed to drug for 5 hr. C. HSV-2 strain G-infected HOSFTK⁻ cells exposed to drug for 7 hr. Multiplicity of infection was 1.

TABLE 1

Comparisons of susceptibilities, uptake, and metabolism of BrVdUrd and BrVaraUra in HSV-1- and HSV-2-infected and mock-infected human osteosarcoma fibroblast (HOSFTK⁻) cells

	Virus strain	ID_{50} of Parent Compound[a]	Quantities in cell extracts[b]					
			BrVUra	BrVdUrd	BrVdUrdMP	BrVdUrdDP	BrVdUrdTP	Total
		μg/ml			pmol/10^6 cells			
A.	Mock-infection		<80[c]	<20[d]	<20	<20	<20	
	F	0.04	1723	1380	7663	105	127	10998
	G	3.0	2087	975	6930	<20	<20	9992
	150	>3.0	6553	824	6801	<20	<20	14178
			BrVUra	BrVaraUra	BrVaraUraMP	BrVaraUraDP	BrVaraUraTP	Total
B.	Mock-infection		<80	<20	<20	<20	<20	
	F	0.16	<80	409	7789	240	162	8660
	G	>20	<80	276	1338	<20	<20	1614
	150	NT[e]	<80	146	293	<20	<20	439

[a] Tested by plaque reduction.
[b] Infected or mock-infected cells were exposed to 40 μg/ml BrVdUrd (A) or 41.8 μg/ml BrVaraUra (B) for 7 hr except for strain 150 (5 hr). Multiplicity of infection = 1.
[c] Reliable limit of detection for BrVUra was 80 pmol/10^6 cells.
[d] Reliable limit of detection for BrVdUrd or BrVaraUra and their derivatives was 20 pmol/10^6 cells.
[e] NT, not tested.

compared for each compound in Table 1. Both BrVdUrd and BrVaraUra show more activity against HSV-1 compared with HSV-2. In fact, no antiviral activity, whatsoever, was observed against HSV-2 with BrVaraUra at the highest concentration tested (20 μg/ml). Nevertheless, in HSV-2-infected cells, BrVaraUra was monophosphorylated, although to a lesser degree than BrVdUrd, under otherwise identical infection conditions. Monophosphorylations of BrVdUrd and BrVaraUra were essentially identical during HSV-1 infection. Further phosphorylations of BrVdUrd and BrVaraUra, after HSV-1 infection, to their 5′-di- and -triphosphates were qualitatively identical, although higher intracellular concentrations were observed for BrVaraUra nucleotides. Despite these slightly higher 5′-triphosphosphate levels, BrVaraUra displayed somewhat less antiviral activity against HSV-1 (strain F). Catabolism of these nucleosides differed markedly, however, as displayed by the phosphorolysis of BrVdUrd to BrVUra which occurred in virus-infected cells. No BrVUra was found in infected cells exposed to BrVaraUra.

Because of our previous observations of BrVdUrd phosphorylation by $SC16R_6C_1$-infected cells lacking dTK activity (17), additional dTK⁻ strains were selected for study. The results for BrVdUrd in Table 2 are compared with those for BrVaraUra in Table 3. Both BrVaraUra and BrVdUrd were taken up and monophosphorylated in $SC16R_6C_1$-infected cells. This virus showed detectable susceptibility to BrVdUrd, although its ID_{50} (17 μg/ml) was far greater than that of its parental strain, SC16 (0.19 μg/ml). BrVaraUra displayed no antiviral activity against

TABLE 2

Comparisons of susceptibilities, uptake, and metabolism of BrVdUrd in wild-type and drug-resistant HSV-infected and mock-infected human osteosarcoma fibroblast (HOSFTK⁻) cells

Time of drug exposure	Virus strain	ID_{50} of BrVdUrd[a]	MOI[b]	Quantities in cell extracts[c]					
				BrVUra	BrVdUrd	BrVdUrdMP	BrVdUrdDP	BrVdUrdTP	Total
		μg/ml		pmol/10^6 cells					
5 Hours	Mock-infection			<80[d]	<20[e]	<20	<20	<20	
	ACG′4(dTK⁻)	>20	2	<80	<20	<20	<20	<20	
	ΔT143(dTK⁻)	>20	2	<80	<20	<20	<20	<20	
	SC16(parent)	0.19	0.4	1103	384	1441	75	60	3063
	$SC16R_5C_1$(dTK⁻)	17.0	1	309	103	658	<20	<20	1070
19 Hours	Mock-infection			<80	<20	<20	<20	<20	
	ACG′4(dTK⁻)		10	<80[f]	38	<20	<20	<20	38
	ΔT143(dTK⁻)		10	153	36	<20	<20	<20	189

[a] Tested by plaque reduction. Drug exposure time does not apply to these results.
[b] MOI, multiplicity of infection.
[c] Infected or mock-infected cells were exposed to 40 μg/ml BrVdUrd.
[d] Reliable limit of detection for BrVUra was 80 pmol/10^6 cells.
[e] Reliable limit of detection for BrVdUrd and its derivatives was 20 pmol/10^6 cells.
[f] Small peak present but not quantitated.

TABLE 3

Comparisons of susceptibilities, uptake, and metabolism of BrVaraUra in wild-type and drug-resistant HSV-infected and mock-infected human osteosarcoma fibroblast (HOSFTK⁻) cells

Time of drug exposure	Virus strain	ID_{50} of BrVaraUra[a]	MOI[b]	Quantities in cell extracts[c]					
				BrVUra	BrVaraUra	BrVaraUraMP	BrVaraUraDP	BrVaraUraTP	Total
		μg/ml		pmol/10^6 cells					
5 Hours	Mock-infection			<80[d]	<20[e]	<20	<20	<20	
	SC16(parent)	0.17	0.4	<80	56	1320	111	132	1619
	$SC16R_5C_1$(TK⁻)	>20	1	<80	61	435	<20	<20	496
19 Hours	ACG′4(TK⁻)	>20	10	<80	30	<20	<20	<20	30
	ΔT143(TK⁻)	>20	10	<80	29	<20	<20	<20	29

[a] Tested by plaque reduction. Hours of exposure do not apply to these results.
[b] MOI, multiplicity of infection.
[c] Infected or mock-infected cells were exposed to 41.8 μg/ml BrVaraUra.
[d] Reliable limit of detection for BrVUra was 80 pmol/10^6 cells.
[e] Reliable limit of detection for BrVaraUra and its derivatives was 20 pmol/10^6 cells.

$SC16R_5C_1$ up to 20 μg/ml. Once again, greater quantities of BrVaraUraDP and BrVaraUraTP were formed in HSV-1 (strain SC16)-infected cells, although antiviral activities were similar. In ACG′4- and ΔT143-infected cells, BrVdUrd and its metabolites were not found (Table 2) after 5 hr of exposure of infected cells to the drug. The time of drug exposure was therefore extended to 7, 8, 9, or 19 hr. Small quantities of BrVaraUra or BrVdUrd and its metabolite, BrVUra (Fig. 2), were detected only after 19 hr of drug exposure. 5′-Monophosphates of either drug were still not detected after prolonged incubation. The resistance of BrVaraUra to intracellular phosphorolysis was observed with all strains tested.

Earlier work has shown that the uptake and phosphorylation rates for BrVdUrd is virus inoculum dependent (16). They are also dependent upon the timing of drug exposure (26). We have extended these studies to include the effects of extracellular drug concentration on the uptake and metabolism of BrVdUrd in SC16 (parent)- and $SC16R_5C_1$ (dTK⁻)-infected cells. The results are given in Fig. 3. Uptake of BrVdUrd and formations of both BrVdUrdMP and BrVUra in SC16-infected cells were proportional to the extracellular concentration of BrVdUrd. Small quantities of BrVdUrdDP and BrVdUrdTP were formed and these did not show any dependence on extracellular drug concentrations. Similar dose dependence was observed for BrVdUrd uptake and BrVdUrdMP and BrVUra formation with strain $SC16R_5C_1$. In this case, neither BrVdUrdDP nor BrVdUrd TP was detected, regardless of the extracellular drug concentration (Fig. 3B). Total intracellular uptake of BrVdUrd by SC16-infected cells was quantitatively greater than that observed in $SC16R_5C_1$-infected cells, at all extracellular drug concentrations.

Finally, the effects of varying the extracellular drug concentration on the uptake and metabolism of BrVdUrd and BrVaraUra were compared in HSV-1, strain F-infected cells (Fig. 4). Both BrVdUrd (Fig. 4A) and BrVaraUra (Fig. 4B) were monophosphorylated to the same extent, in a dose-dependent fashion. BrVUra production in cells exposed to BrVdUrd was dose dependent. BrVaraUra, in contrast, was biologically resistant to phosphorolysis at all concentrations tested (Fig. 4B). The total quantity of BrVdUrd taken up by infected cells was dose dependent and in all cases was more than the total amount of BrVaraUra taken up, which may be at least partly explained by the metabolism of BrVdUrd to BrVUra. With either compound, formation of the 5′-monophosphate was quantitatively greater and more dose dependent than any other component. Greater quantities of unaltered parent compound were seen with BrVdUrd than with BrVaraUra, and these intracellular quantities were more dose dependent. Formations of BrVdUrdDP and BrVdUrdTP appeared to be dose dependent, however. By contrast, uptake of BrVaraUra and formation of BrVaraUraDP and BrVaraUraTP appeared to be partly dose dependent.

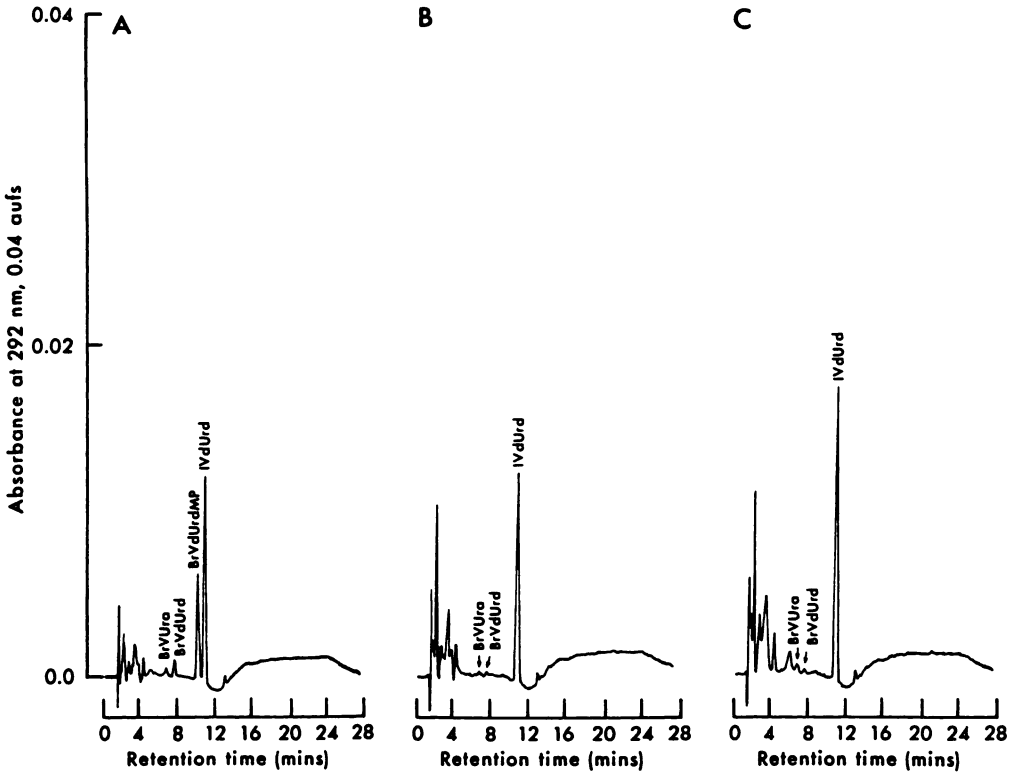

Fig. 2. HPLC analysis of extracts of HSV-infected HOSFTK$^-$ cells treated with BrVdUrd for 19 hr. Retention times are plotted by the UV detector as a function of absorbance at 292 nm, 0.04 absorbance unit, full scale. All samples were reconstituted in 0.4 ml of glass distilled water. A. HSV-1, strain SC16R$_6$C$_1$-infected cells. B. HSV-1, strain ACG^{r}4-infected cells. C. HSV-1, strain ΔT143-infected cells. Multiplicity of infection was 10.

Discussion

The molecular basis for the preferential activity of BrVdUrd against HSV-1 has been the subject of previous publications (16, 17). BrVdUrd is phosphorylated to BrVdUrdMP, BrVdUrdDP, and BrVdUrdTP in HSV-1-infected Vero cells. The present study was performed exclusively in a dTK-deficient cell line (HOSFTK$^-$) in order to eliminate the possibility of effects from cellular dTK, and they confirm that phosphorylation and drug uptake are independent of cellular dTK.

Monophosphorylation of both compounds by SC16R$_6$C$_1$ was observed, however, and must, therefore, result from the actions of a virus-specified enzyme, which is lost when the dTK gene is deleted (ΔT143) or not expressed (ACG^{r}4). Specifically, viral-induced alterations in permeability were not sufficient for drug phosphorylation, although a minor increase in uptake of the parent compound did occur after prolonged infection at high multiplicities of infection with those dTK$^-$ isolates which do not induce drug phosphorylation. Presumably, the BVaraUra-BrVdUrd kinase activity retained in SC16R$_6$C$_1$-infected cells must reside with a polypeptide altered only enough to result in the loss of dTK and dTMP kinase activity (21). This remains to be proven through isolation of the protein and testing of its activity *in vitro*. Subsequent amino acid analysis of this BrVdUrd/BrVaraUrd kinase of SC16R$_6$C$_1$ may shed light on the active site of BrVaraUra-BrVdUrd phosphorylation. Furthermore, the qualitative similarities of phosphorylation of BrVdUrd and BrVaraUra across the barriers of mutant strains adds to the strong evidence that the monophophorylation and type-specific diphosphorylation of BrVdUrd are not affected by the presence of arabinose. The absence of drug uptake in mock-infected cells and the low quantities of parent drug uptake in ΔT143$^-$- and ACG^{r}4-infected cells, only seen after prolonged infection and drug exposure, suggest that uptake is partly dependent on permeability factors and/or exposure time, but mainly dependent on monophosphorylation. Pulsed exposure to nucleosides after prolonged incubation will help to determine which factor predominates when dTK is absent. Pyrimidine nucleoside phosphorylase activity may have partially driven the greater degree of intracellular uptake seen in all cases of BrVdUrd treatment, since BrVdUrdMP formation is quantitatively linked to BrVUra formation. Uptake is the critical component to BrVUra formation, however, and this could, therefore, only be the result of increased availability of the BrVdUrd substrate in cells capable of monophosphorylating this compound. The potential reversibilities of these processes have not been evaluated with these agents *in situ*. However, others have described marked differences between agents in the reversibility of antiviral activity after drug withdrawal (27, 28). One difficulty with BrVdUrd for both therapeutic (19) and diagnostic (29) purposes, has been its susceptibility to phosphorolysis by dThd phosphorylase (30), with resultant breakdown to an inactive metabolite, BrVUra. While this metabolite is specifically found in our experiments in infected cells, others have demonstrated its formation in the circulation after *in vivo* administration in the absence of HSV infection (19). The absence of BrVUra in uninfected cells is likely due to a relative lack of parent drug availability inside the cell. Even in the presence of large quantities of drug uptake, however, we could not demonstrate the formation of BrVUra from BrVaraUra. Thus, this compound is resistant *in situ* to dThd phosphorylase. This phenomenon was also observed *in vivo* by Machida *et al.* (31), who could not detect BrVUra in the plasma or urine of mice after systemic administration of BrVaraUra. Loss of *in vivo* activity of BrVdUrd or potential increase in toxicity because of BrVUra formation may make BrVaraUra a more suitable candidate for further testing as a chemotherapeutic

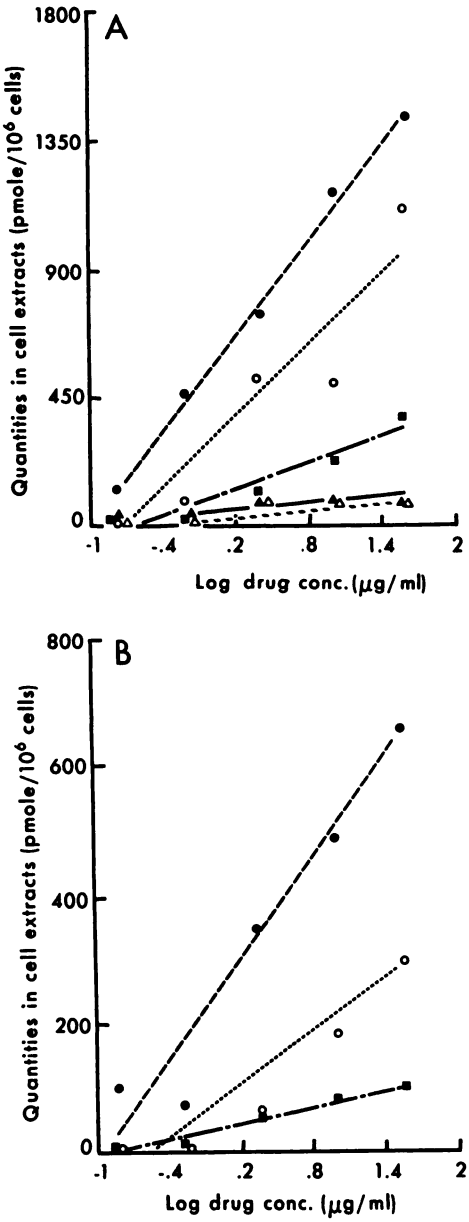

Fig. 3. Effect of extracellular drug concentration, after 5 hr exposure, on the intracellular uptake and metabolism of BrVdUrd. Multiplicity of infection was 0.4 (SC16) and 1 ($SC16R_5C_1$). A. SC16 (parent, dTK^+)-infected $HOSFTK^-$ cell extracts. B. $SC16R_5C_1$ (dTK^-)-infected $HOSFTK^-$ cell extracts. ○, BrVUra; ■, BrVdUrd; ●, BrVdUrdMP; ▲, BrVdUrdDP; △, BrVdUrdTP.

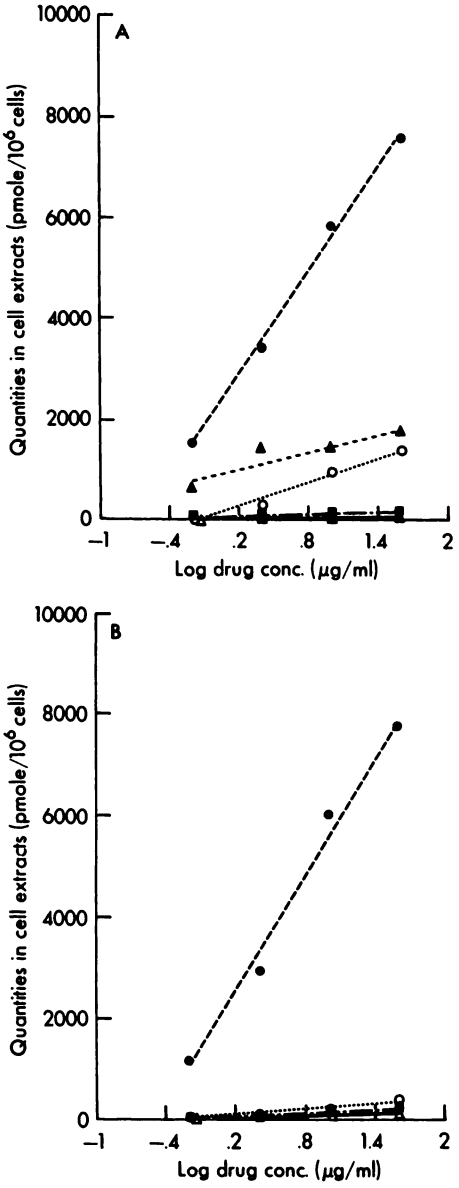

Fig. 4. BrVdUrd versus BrVaraUra: comparison of effects of extracellular drug concentrations, after 7 hr exposure, on intracellular drug uptake and metabolism in HSV-1, strain F-infected $HOSFTK^-$ cells. Multiplicity of infection was 1.0. A. Uptake and metabolism after BrVdUrd exposure. ▲, BrVUra; ○, BrVdUrd; ●, BrVdUrdMP; ■, BrVdUrdDP; △, BrVdUrdTP. B. Uptake and metabolism after BrVaraUra exposure. ○, BrVaraUra; ●, BrVaraUraMP; ■, BrVaraUraDP; △, BrVaraUraTP.

agent. The same logic might apply to its use as a diagnostic agent. Gill *et al.* (29) have shown the potential utility of radiolabeled IVdUrd in this regard, but studies have been hampered by the formation of *E*-5-(2-iodovinyl)uracil (IVUra) and the resultant increase in nonspecific background (32). We have shown that BrVaraUra would not likely suffer from that problem. All of our metabolic assays were performed at concentrations well above the ID_{50}, because of lack of availability of radiolabeled agents.

Despite a striking difference in metabolic breakdown, BrVdUrd and BrVaraUra were qualitatively identical with regard to their formation of 5′-phosphorylated products. Thus, replacement of the 2′-deoxyribose moiety by its arabinose analogue did not significantly alter the phosphorylation pathways. However, formation of the nucleoside triphosphate may not fully explain the antiviral potency of these compounds. Both triphosphates are potent inhibitors of HSV-specified DNA polymerase. However, BrVdUrd is incorporated into DNA (10, 11), whereas BrVaraUra is either not incorporated, or incorporated only at the 3′-end (12, 13). This may partially account for differences in antiviral potency observed against some herpesvirus strains. The antiviral activity of BrVaraUra observed in this study closely paralleled that of BrVdUrd, except in the case of HSV-2, strain G, where BrVaraUra displayed no detectable antiviral activity. Others have found markedly reduced susceptibilities of HSV to BrVaraUra when compared to BrVdUrd in primary rabbit kidney cells (9) and green monkey kidney cells (8). Ayisi *et al.* (17), Reefschlager *et*

al. (8, 14), and De Clercq (9) have found no significant differences in anti-HSV potencies between these compounds when tested in human embryonic lung fibroblasts. Our susceptibility results would suggest that these agents have similar activities in another human cell line. It remains to be seen whether these cell-dependent susceptibility differences will be explained by differences in the formation of phosphorylated derivatives by different cell lines infected with the same virus. However, others have reported cell-to-cell quantitative differences in HSV-specified dTK activity (33) and naturally available intracellular dThd pools (34), all of which could markedly alter susceptibility results. Furthermore, the clinical significance of these quantitative differences is not known. Indeed, Reefschlager *et al.* (14) have recently demonstrated excellent protection of mice from encephalitis due to intracerebral infection with HSV-1 after intraperitoneal treatment with BrVaraUra. Only small differences in *in vitro* susceptibilities have been demonstrated for HZV (6, 7), where only human cell lines were used for the assay. Both BrVdUrd and BrVaraUra have also shown excellent *in vitro* and *in vivo* activity against simian varicella (35, 36).

As others have shown (4, 7, 8), BrVaraUra was less potent than BrVdUrd against HSV-2 isolates in this study. We found the same drug susceptibility differences with respect to the HSV-1 dTK$^-$ mutant, SC16R$_6$C$_1$. Reduced susceptibility to BrVaraUra was paralleled by reduced quantities of its 5′-monophosphate compared to BrVdUrd. Nevertheless, the observed antiviral activities of BrVdUrd against HSV-2 and SC16R$_6$C$_1$ are poorly understood. One possibility is the inhibition of cellular dTMP synthase (EC 2.1.1.45) which has been demonstrated to occur *in vitro* with BrVdUrdMP (37). In fact, BrVdUrdMP is also an alternate substrate for dTMP synthase. This enzyme has not been tested against BrVaraUraMP, however. Since BrVdUrdMP is only found in infected cells, inhibition of cellular dTMP synthase would be a virus-specific event, despite the fact that it is a cellular enzyme. The specific antiviral role for this enzyme has not been assessed.

Since we found no intracellular uptake of either compound in uninfected cells, little cytotoxicity would be expected, as has been previously demonstrated for both compounds (5, 14). It is possible that phosphorylation or metabolism of BrVdUrd is occurring at levels below our limits of detection. We have not compared cytotoxicities in HOSFTK$^-$ cells.

In conclusion, we have shown that, at high exposure concentrations, the phosphorylation of BrVaraUra is qualitatively similar to that of BrVdUrd in HSV-infected cells. BrVdUrd, however, is more effectively monophosphorylated by HSV-2-infected cells than BrVaraUra and is more active *in vitro* in this setting. The stability of the arabinose analogue of BrVdUrd to pyrimidine phosphorylases has also been demonstrated *in situ*, but this stability did not reflect on the antiviral activity of this compound. Furthermore, the monophosphorylation of BrVdUrd and BrVaraUra in SC16R$_6$C$_1$ (dTK$^-$)-infected cells and the slight antiviral activity associated with monophosphorylation of BrVdUrd parallels the situation in HSV-2 infection and does not occur in cells infected with viruses lacking the dTK gene.

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