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Comparative Cytostatic Activity of Different Antiherpetic Drugs against Herpes Simplex Virus Thymidine Kinase Gene-Transfected Tumor Cells

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SUMMARY

A series of selective antiherpetic compounds were found to exert pronounced cytostatic activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) thymidine kinase (TK) gene-transfected mammary carcinoma FM3A cells. Based on their potency and mechanism of cytostatic action, the antiherpetic compounds could be divided into two different classes. The first class encompasses (*E*)-5-(2-bromovinyl)-2′-deoxyuridine and structurally related analogues thereof [i.e., the cytosine derivative (*E*)-5-(2-bromovinyl)-2′-deoxycytidine and the 4′-thio derivative (*E*)-5-(2-bromovinyl)-2′-deoxy-4′-thiouridine]. These compounds are exquisitely cytostatic against FM3A/TK$^-$/HSV-1 TK$^+$ and FM3A/TK$^-$/HSV-2 TK$^+$ cells (50% inhibitory concentrations ranging from 0.047 to 0.001 μM) and inhibit tumor cell proliferation by inhibiting cellular thymidylate synthase. The second class consists of the acyclic guanosine derivatives penciclovir, buciclovir, and ganciclovir. These compounds are also more inhibitory to the HSV-1 TK or HSV-2 TK gene-transfected FM3A cells than to FM3A/0 or FM3A/TK$^-$ cells, but at concentrations that are higher than the concentrations at which the (*E*)-5-(2-bromovinyl)-2′-deoxyuridine derivatives proved to be inhibitory. These acyclic guanosine analogues appear to be targeted at the cellular DNA polymerase. From this study, (*E*)-5-(2-bromovinyl)-2′-deoxy-4′-thiouridine emerged as a promising candidate compound for the treatment of HSV-1 TK gene-transfected tumors *in vivo*, due to its metabolic stability (i.e., resistance to hydrolysis by thymidine phosphorylase).

Retroviral vectors represent a useful strategy to transfect foreign genes into tumor cells. The HSV-1 TK gene has been introduced into rat glioma cells to increase their sensitivity to antiherpetic agents such as ganciclovir (1, 2). Ganciclovir proved to be able to completely suppress the development in rats of gliomas that were exposed to an HSV-1 TK gene-containing vector (2). Recently, a clinical trial has been initiated to investigate the usefulness of this combined gene/chemotherapy approach in the treatment of brain tumor cells (3). We previously reported that selective antiherpetic agents such as acyclovir, ganciclovir, BVDU (brivudine), and structurally related derivatives of BVDU exhibit increased cytostatic activity against murine mammary carcinoma cells transformed with the HSV-1 TK gene or HSV-2 TK gene (4–9). We also found that BVDU is a markedly more potent inhibitor of HSV TK gene-transfected tumor cells than is ganciclovir (4, 8, 9) and that the cytostatic activity of BVDU is based on inhibition of thymidylate synthase in the HSV TK gene-transfected cells, whereas the cytostatic activity of ganciclovir is due to inhibition of DNA polymerization and/or incorporation into cellular DNA (5–9).

We have now investigated seven selective antiherpetic drugs for their cytostatic activity against HSV-1 TK or HSV-2 TK gene-transfected mammary carcinoma FM3A tumor cells. In this study were included the 4′-thio derivative of BVDU (S-BVDU) and the acyclic guanosine derivatives penciclovir, buciclovir, and cyclobut-G, which had not been the subject of previous evaluations for their cytostatic potential. S-BVDU emerged as a highly potent and selective inhibitor of HSV TK gene-transfected tumor cell proliferation and may be considered as a promising candidate compound that should be further investigated for treatment of HSV TK gene-transfected tumor cells *in vivo*.

Materials and Methods

Cells. FM3A cells (designated FM3A/0), derived from a spontaneous murine mammary carcinoma in a C3H/He mouse, and FM3A/TK$^-$ cells, selected for resistance against 5-bromo-2′-dUrd and lacking host

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ABBREVIATIONS: HSV, herpes simplex virus; TK, thymidine kinase; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; BVDC, (*E*)-5-(2-bromovinyl)-2′-deoxycytidine; S-BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxy-4′-thiouridine; IVDU, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; dCyd, deoxycytidine; MIC_{50}, minimal inhibitory concentration; dThd, thymidine; dUrd, deoxyuridine; Urd, uridine; DTT, dithiothreitol; TCA, trichloroacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

cell TK activity, were maintained in RPMI 1640 culture medium containing 10% fetal calf serum and 2 mM L-glutamine, as described previously (10–12). The FM3A/TK⁻/HSV-1 TK⁺ and FM3A/TK⁻/HSV-2 TK⁺ cell lines, lacking cellular TK activity and containing the HSV-1 and HSV-2 TK genes, respectively, were derived from FM3A/TK⁻ cells as reported earlier (13, 14). The culture conditions for the cells were as described above.

Compounds. BVDU and BVDC were synthesized by P. Herdewijn and A. Van Aerschot at the Rega Institute for Medical Research. S-BVDU was obtained from the Wellcome Research Laboratories (Beckenham, UK). Ganciclovir was from Syntex (Palo Alto, CA), penciclovir was obtained from Dr. I. Winkler (Hoechst, Frankfurt, Germany), cyclobut-G (BMS-180194) was from Bristol Myers Squibb (Princeton, NJ), and buciclovir was from Astra Läkemedel (Sodertälje, Sweden). Unlabeled nucleosides and nucleotides were purchased from Serva (Heidelberg, Germany) and Sigma Chemical Co. (St. Louis, MO). The formulae of the test compounds are presented in Fig. 1.

Radiochemicals. [5-^{3}H]dCyd (specific radioactivity, 20.1 Ci/mmol), [1′,2′-^{3}H]dUrd (specific radioactivity, 27 Ci/mmol), [*methyl*-^{3}H]dThd (specific radioactivity, 45 Ci/mmol), and [5-^{3}H]Urd (specific radioactivity, 27 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, UK), and [2′-^{3}H]dUrd (specific radioactivity, 14 Ci/mmol) was provided by Moravek Biochemicals Inc. (Brea, CA).

TK preparation from FM3A/TK⁻/HSV-1 TK⁺, FM3A/TK⁻/HSV-2 TK⁺, and FM3A/0 cells. TK was purified as described previously, from about 7.5×10^9 FM3A/TK⁻/HSV-1 TK⁺ and FM3A/TK⁻/HSV-2 TK⁺ cells (9). Briefly, the supernatant from streptomycin sulfate-precipitated crude cell extract in suspension buffer (50 mM potassium phosphate, pH 7.6, containing 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 2 mM EDTA) was precipitated with ammonium sulfate, desalted by dialysis against buffer A (50 mM Tris·HCl, pH 7.6, 20% glycerol, 5 mM $MgCl_2$, 5 mM DTT), separated from cytosolic kinases by DEAE chromatography in buffer A, and chromatographed on hydroxyapatite-Sepharose in 10 mM potassium phosphate, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 15% glycerol, 0.5 mM CHAPS. The TK from FM3A/TK⁻/HSV-1 TK⁺ cells was further chromatographed on 3′-dTMP-Sepharose in 50 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol, 5 mM $MgCl_2$, 5 mM DTT, and 0.5 mM CHAPS. FM3A/0 cell extract was prepared by precipitation with ammonium sulfate, as described previously, and was then desalted by dialysis in the same buffers as indicated above (9).

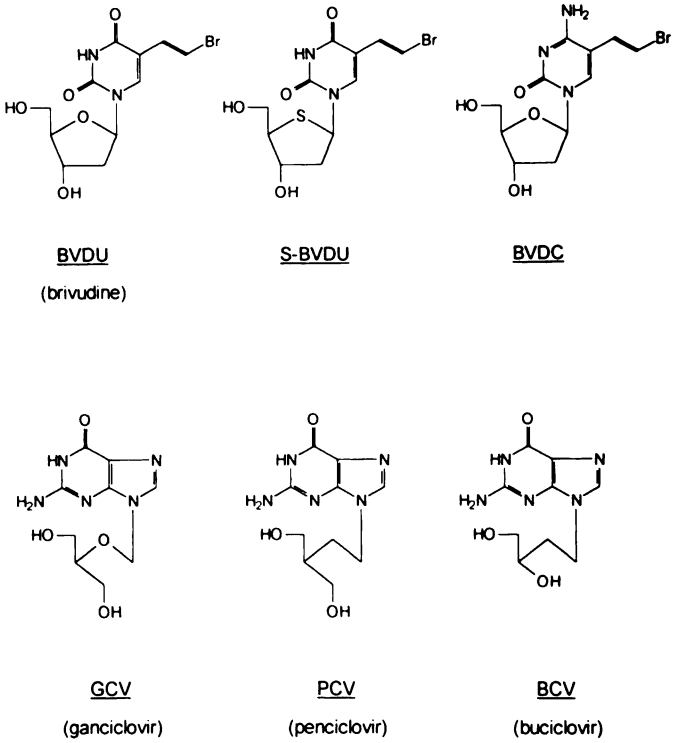

Fig. 1. Formulae of BVDU (brivudine), BVDC, S-BVDU, penciclovir [9-[4-hydroxy-3-(hydroxymethyl)butyl]guanine], buciclovir [(*R*)-9-[(3,4-dihydroxybutyl)guanine]], and ganciclovir [9-[(1,3-dihydroxy-2-propoxy)-methyl]guanine].

Nucleoside kinase assay. HSV-1 TK, HSV-2 TK, and cytosolic TK activity was assayed by a modification of the radiochemical method previously described by Ives and Wang (15). Standard assay conditions were 50 mM Tris·HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM DTT, 2.5 mM ATP, 1 mg/ml bovine serum albumin, 10 mM sodium fluoride, and 1 μM [*methyl*-^{3}H]dThd or 1 μM [5-^{3}H]dCyd. Assays were performed at 37°, and incubation time was 15 min. Aliquots of 25 μl of the reaction mixtures were spotted onto Whatman DEAE-81 filter paper disks. The filters were subsequently washed three times for 5 min with 1.5 mM ammonium formate and finally rinsed once with water and once with ethanol. The radioactivity was determined by scintillation counting. One enzyme unit is defined as the amount of enzyme catalyzing the formation of 1 nmol of dTMP or dCMP/min under the standard assay conditions described.

Anti-HSV-1 and anti-HSV-2 activity of antiherpetic drugs *in vitro*. The procedure for measuring antiviral activity in primary rabbit kidney and human skin fibroblast (E6SM) cells has been described previously (16). The assay was based on HSV-1 (strain KOS)- and HSV-2 (strain G)-induced cytopathicity in primary rabbit kidney or E6SM monolayer cells at day 3 after infection. The MIC_{50} was defined as the drug concentration required to reduce virus-induced cytopathicity by 50%.

Inhibition of cell proliferation by antiherpetic drugs. The methods for evaluating the cytostatic activity of the test compounds against FM3A cells have been reported previously (5, 7). Briefly, 5×10^4 cells suspended in growth medium were allowed to proliferate in 200-μl wells of microtiter plates in the presence of 5-fold dilutions (i.e., 500, 100, 20, 4, 0.8, 0.16, 0.032, 0.006, 0.012, and 0.00025 μM) of the test compounds, at 37° in a humidified CO_2-controlled atmosphere. When the cytostatic activity of the test compounds was assessed in the presence of natural nucleosides, subtoxic concentrations of dThd (5 μg/ml), dUrd (125 μg/ml), or dCyd (500 μg/ml) were added to the varying dilutions of the antiherpetic drugs. After 48 hr, the number of cells was counted in a Coulter counter (Harpenden, Hertz, UK). The IC_{50} was defined as the drug concentration required to inhibit FM3A cell proliferation by 50%.

Inhibition of DNA and RNA synthesis. The procedures to measure the incorporation of [*methyl*-^{3}H]dThd and [1′,2′-^{3}H]dUrd into DNA, and of [5-^{3}H]Urd into RNA, have been described previously (17). Briefly, 10^5 FM3A cells were added to 200-μl wells of microtiter plates, together with a given amount of test compound and 0.25 μCi of the radiolabeled nucleosides. The cells were allowed to proliferate for 20 hr at 37°, and TCA-insoluble radioactivity was determined.

Tritium release from [5-^{3}H]dCyd in FM3A cells. Thymidylate synthase activity was measured in intact FM3A/0 and FM3A/TK⁻/HSV-1 TK⁺ cells by estimating tritium release from [5-^{3}H]dUrd, which itself had been formed intracellularly from exogenous [5-^{3}H]dCyd. This procedure has been previously described in detail (18).

Phosphorolysis of nucleosides by human dThd phosphorylase. Phosphorolysis of BVDU, BVDC, and S-BVDU by dThd phosphorylase prepared from human blood platelets was measured by high performance liquid chromatographic analysis as described before (9). One milliliter of the reaction mixture contained 10 mM Tris·HCl, pH 7.6, 1 mM EDTA, 2 mM potassium phosphate, 150 mM NaCl, 0.1 mM concentrations of the test compounds, and 0.55 mg of crude blood platelet protein extract. Reaction mixtures were incubated at 37°, and at different times 200-μl fractions were taken, rapidly cooled on ice, and centrifuged for 5 min at 4000 × *g*, at 4°. The supernatants were then precipitated with 400 μl of ice-cold methanol and analyzed by high performance liquid chromatography at the wavelength of 292 nm, at which the difference between the absorbance values of the free base and the intact nucleoside was optimal.

Results

Inhibitory effects of antiherpetic drugs on HSV-1 and HSV-2 replication and HSV-1, HSV-2, and cytosolic TK activity. The pyrimidine 2′-deoxyribonucleoside analogues BVDU, BVDC, and S-BVDU were markedly more inhibitory to HSV-1 than HSV-2 replication in E6SM cells. The MIC_{50} values of BVDU, BVDC, and S-BVDU for HSV-1 were 0.01, 0.2, and 0.01 μM, respectively, whereas the MIC_{50} values of these compounds for HSV-2 ranged from 0.8 (for S-BVDU) to >300 μM (for BVDU) (Table 1). In contrast, the acyclic guanosine derivatives ganciclovir, buciclovir, penciclovir, and acyclovir were virtually equally active against HSV-1 and HSV-2 (MIC_{50} ranging from 0.03 to 0.09 μM for HSV-1 and from 0.04 to 0.31 μM for HSV-2).

Interestingly, BVDU and S-BVDU had similar affinities for purified HSV-1 TK (IC_{50}, 0.58–0.63 μM), but S-BVDU proved to be 4–5-fold less inhibitory to HSV-2 TK than was BVDU. The K_i values of BVDU and S-BVDU for HSV-1 TK were 0.09 μM and 0.11 μM, respectively (data not shown). Inhibition was competitive with respect to the natural substrate dThd. BVDC was a much less potent substrate for HSV-1 TK than were BVDU and S-BVDU, but it should be mentioned that in these experiments [*methyl*-^{3}H]dThd was used as the radiolabeled substrate. When [5-^{3}H]dCyd was used as substrate, BVDC proved to be inhibitory to [5-^{3}H]dCyd phosphorylation by both HSV-1 and HSV-2 TK (IC_{50}, 11 and 25 μM, respectively). As a rule, the guanosine nucleoside analogues showed a much lower affinity for HSV TK than did BVDU and S-BVDU (Table 1). The K_i values for penciclovir and buciclovir were 6.3 μM and 5.3 μM, respectively (data not shown). Inhibition was competitive with respect to the natural substrate dThd. None of the test compounds had a significant affinity for cytosolic TK, pointing to their selectivity as antiherpetic drugs. This is evidenced by the ratio of the cytosolic TK IC_{50} to the HSV-1 or HSV-2 TK IC_{50} (Table 1).

Cytostatic activity of antiherpetic drugs against different mutant FM3A tumor cells. The (*E*)-5-(2-bromovinyl)-substituted 2′-deoxypyrimidine nucleoside analogues showed only weak cytostatic activity against wild-type FM3A/0 cells. BVDU, BVDC, and S-BVDU were 70-, 30-, and 12-fold more cytostatic against FM3A cells that were deficient in cytosolic TK (FM3A/TK$^-$). However, the test compounds showed a dramatic increase in cytostatic activity against HSV-1 TK gene-transfected FM3A/TK$^-$ cells. The cytostatic activity of S-BVDU against FM3A/TK$^-$/HSV-1 TK$^+$ cells was increased by 650-fold, compared with FM3A/TK$^-$ cells, and even 8000-fold, compared with wild-type FM3A/0 cells. Under the same experimental conditions, BVDU and BVDC had 50- and 250-fold higher cytostatic activities, respectively, against FM3A/TK$^-$/HSV-1 TK$^+$ cells than FM3A/TK$^-$ cells and 3000- and 8000-fold higher cytostatic activities, respectively, against FM3A/TK$^-$/HSV-1 TK$^+$ cells than FM3A/0 cells. Whereas BVDU and BVDC were 2–4-fold more inhibitory to FM3A/TK$^-$/HSV-2 TK$^+$ cells than FM3A/TK$^-$/HSV-1 TK$^+$ cells, S-BVDU proved to be 7-fold less inhibitory to FM3A/TK$^-$/HSV-2 TK$^+$ cells than FM3A/TK$^-$/HSV-1 TK$^+$ cells (Table 2).

It was ascertained that the inhibitory effects of BVDU, S-BVDU, ganciclovir, and penciclovir on FM3A/0 and FM3A/TK$^-$/HSV-2 TK$^+$ cell proliferation was due to a cytostatic effect rather than a direct cytotoxic effect of the test compounds, because no marked amounts of blue-stained cells were found in the compound-treated FM3A cell cultures, using the trypan blue dye exclusion assay, at compound concentrations in the range of their IC_{50} values (data not shown). Also IC_{50} values obtained for these compounds by cell counting in the Coulter counter assay and the trypan blue dye exclusion assay did not differ markedly from one another, i.e., the IC_{50} values of BVDU, S-BVDU, ganciclovir, and penciclovir for FM3A/0 cells were 56, ≥100, 747, and 600 μM, respectively, in the Coulter counter assay and 37, >100, 526, and 525 μM, respectively, in the trypan blue dye exclusion assay.

The acyclic guanosine analogues buciclovir, penciclovir, and ganciclovir showed equally poor cytostatic activity against FM3A/TK$^-$ and FM3A/0 cells. Their IC_{50} values were decreased by 130–320-fold against FM3A/TK$^-$/HSV-1 TK$^+$ cells and by 500–1250-fold against FM3A/TK$^-$/HSV-2 TK$^+$ cells. The other acyclic guanosine derivatives (acyclovir and cyclobut-G) showed increased cytostatic activity, particularly against the HSV-2 TK gene-transfected tumor cells (Table 2).

Evaluation of thymidylate synthase as a potential target for cytostatic activity of the test compounds. To investigate whether thymidylate synthase acted as a target enzyme for the cytostatic activity of BVDU and its congeners, three metabolic parameters were considered to be relevant for thymidylate synthase inhibition. The first parameter concerns the markedly greater inhibitory effect of dTMP synthase inhibitors on the incorporation of dUrd and dCyd, compared with dThd, into DNA of HSV TK gene-transfected FM3A cells. Indeed, to be incorporated into DNA, dUrd and dCyd have to be channeled through the dTMP synthase step, whereas dThd does not have to, and thus dUrd and dCyd incorporation can be blocked by dTMP synthase inhibitors. We found that the BVDU derivatives were more inhibitory to [^{3}H]dUrd incorporation (IC_{50}, 6.1–35 μM) than [*methyl*-^{3}H]dThd or [5-^{3}H]Urd

TABLE 1

Inhibitory effects of antiherpetic drugs on the replication of HSV-1 and HSV-2 and the activity of HSV-1, HSV-2, or cytosolic TK

Compound	MIC_{50}[a]		IC_{50}[b]			IC_{50} cytosolic TK/IC_{50} HSV-1 TK	IC_{50} cytosolic TK/IC_{50} HSV-2 TK
	HSV-1	HSV-2	HSV-1 TK	HSV-2 TK	Cytosolic TK		
	μM		μM				
BVDU	0.01	300	0.58 ± 0.1	3.2 ± 0.25	>500	>862	>154
BVDC	0.2	24	11 ± 4.8	>250	>500	>46	>2
S-BVDU	0.01	0.8	0.63 ± 0.3	14 ± 7.2	>500	>793	>35
Buciclovir	0.03	0.04	11 ± 6.8	37 ± 2.5	>500	>44	>13
Penciclovir	0.04	0.04	18 ± 3.5	180 ± 25	>500	>27	>2.7
Ganciclovir	0.02	0.16	49 ± 4.0	405 ± 40	>500	>10	>1.2
Acyclovir	0.09	0.31	>500	410 ± 45	>500	>1	>1.2

[a] Concentration required to reduce virus-induced cytopathicity in E6SM cell cultures by 50%. MIC_{50} data for BVDC against HSV-1 and HSV-2 were obtained in PRK cells (see Ref. 7).

[b] Concentration required to inhibit TK activity by 50%.

TABLE 2

Cytostatic activity or antiherpetic drugs against different mutant FM3A tumor cells

Compound	IC_{50}[a]			
	FM3A/0	FM3A/TK$^-$	FM3A/TK$^-$/ HSV-1 TK$^+$	FM3A/TK$^-$/ HSV-2 TK$^+$
			μM	
BVDU	13 ± 1.1	0.19 ± 0.15	0.004 ± 0.0004	0.002 ± 0.0006
BVDC	33 ± 8.5	1.08 ± 0.23	0.004 ± 0.002	0.001 ± 0.0005
S-BVDU	55 ± 7.4	4.6 ± 0.85	0.007 ± 0.003	0.047 ± 0.019
Buciclovir	515 ± 114	304 ± 15	4.0 ± 1.5	1.1 ± 0.18
Penciclovir	694 ± 167	315 ± 12	5.1 ± 1.3	0.88 ± 0.42
Ganciclovir	321 ± 99	188 ± 45	1.0 ± 0.25	0.26 ± 0.08
Acyclovir	199 ± 49	148 ± 28	62 ± 28	4.5 ± 2.6
Cyclobut-G	98 ± 44	72 ± 3	34 ± 6	2.5 ± 0.3

[a] Concentration required to inhibit cell proliferation by 50%.

TABLE 3

Inhibitory effects of antiherpetic drugs on the incorporation of radiolabeled dUrd, dThd, or Urd into TCA-insoluble FM3A/0 cell material

Compound	IC_{50}[a]			
	[^{3}H]dUrd[b]	[*methyl*-^{3}H]dThd	[5-^{3}H]Urd	[*methyl*-^{3}H]dThd/ [^{3}H]dUrd ratio
		μM		
BVDU	6.1 ± 2.7	>100	>100	>16
BVDC	10 ± 5.3	>100	>100	>10
S-BVDU	35 ± 13	>100	>100	>2.8
Buciclovir	>400	>400	>400	><1
Penciclovir	>400	>400	>400	><1
Ganciclovir	427 ± 63	>500	>500	>1.1

[a] Concentration required to inhibit incorporation of the radiolabeled precursors by 50%.
[b] Either [1′,2′-^{3}H]dUrd or [2′-^{3}H]dUrd.

TABLE 4

Inhibitory effects of antiherpetic drugs on the incorporation of radiolabeled dUrd, dThd, or Urd into TCA-insoluble FM3A/TK$^-$/HSV-1 TK$^+$ cell material

Compound	IC_{50}[a]			
	[^{3}H]dUrd[b]	[*methyl*-^{3}H]dThd	[5-^{3}H]Urd	[*methyl*-^{3}H]dThd/ [^{3}H]dUrd ratio
		μM		
BVDU	0.0007 ± 0.0001	1.3 ± 0.24	57 ± 38	1857
BVDC	0.002 ± 0.0004	3.8 ± 0.31	67 ± 38	1900
S-BVDU	0.001 ± 0.0007	0.48 ± 0.08	37 ± 6.7	480
Buciclovir	6.1 ± 2.6	13 ± 1.1	>400	2.1
Penciclovir	0.28 ± 0.07	6.8 ± 2.8	>400	24
Ganciclovir	0.092 ± 0.003	2.5 ± 0.95	>100	27

[a] Concentration required to inhibit incorporation of the radiolabeled precursors by 50%.
[b] Either [1′,2′-^{3}H]dUrd or [2′-^{3}H]dUrd.

incorporation (IC_{50}, >100 μM) into TCA-insoluble FM3A/0 cell material. The acyclic guanosine derivatives were virtually not inhibitory to [^{3}H]dUrd, [*methyl*-^{3}H]dThd, or [5-^{3}H]Urd incorporation (IC_{50}, >400–500 μM) (Table 3). However, the BVDU derivatives proved to be exquisitely inhibitory to [^{3}H]dUrd incorporation into TCA-insoluble FM3A/TK$^-$/HSV-1 TK$^+$ cell material (IC_{50}, 0.0007–0.001 μM) (see Table 5). Also, the BVDU derivatives were markedly more inhibitory to [^{3}H]dUrd than [*methyl*-^{3}H]dThd incorporation into FM3A/TK$^-$/HSV-1 TK$^+$ cells (ratio ranging from 480 for S-BVDU to 1900 for BVDC) (Table 4). The corresponding ratios (IC_{50} for [*methyl*-^{3}H]dThd incorporation to IC_{50} for [^{3}H]dUrd incorporation) for the guanosine derivatives were much less pronounced, being at most 27. [5-^{3}H]Urd incorporation into TCA-insoluble material was only marginally affected by all test compounds (Table 4).

A second parameter of dTMP synthase inhibition is reflected by a more readily reversed cytostatic activity of the test compounds by dThd than by dUrd or dCyd. Indeed, dUrd and dCyd may be less able than dThd to reverse the cytostatic activity of the dTMP synthase inhibitors because they are themselves blocked at the dTMP synthase level by these inhibitors. We found that addition of dThd, but not dUrd or dCyd, markedly reversed the cytostatic activity of the BVDU derivatives (Table 5). Indeed, whereas dThd reversed the cytostatic activity of BVDU, BVDC, and S-BVDU by 300–1000-fold, dUrd and dCyd (at 25–100-fold higher concentrations) did so by only 2–15-fold (Table 5). Under our experimental conditions, the natural nucleosides dUrd, dThd, and dCyd were administered at subtoxic concentrations. Addition of dUrd at 25-fold higher concentrations than dThd most likely results in equivalent rates of dUrd/dThd phosphorylation and dTTP levels, because (i) dUrd has a 20-fold lower affinity for cytosolic TK than does dThd (19), (ii) dUrd proved to be ~20-fold less toxic to FM3A cells than was dThd, and (iii) the toxicity of both dUrd and dThd is due to dTTP formation and subsequent decrease of intracellular dCTP levels after inhibition of CDP reductase by dTTP. Therefore, the substantially higher degree of cytostatic activity of BVDU and related compounds in the presence of dUrd (125 μg/ml), compared with dThd (5 μg/ml), most likely does not reflect a different competitive potential of dUrd versus dThd against TK-catalyzed phosphorylation of BVDU and related analogues. Rather, it results from the lack of eventual conversion of dUrd to dTMP, because of the blockage of thymidylate synthase by BVDU and related compounds. Interestingly, the cytostatic activity of the acyclic guanosine derivatives was much less markedly influenced by dUrd, dThd, or dCyd and there was virtually no difference when dThd was used instead of dUrd or dCyd (Table 5).

The third parameter that was evaluated concerned the inhibitory effect of the test compounds on tritium release from [5-^{3}H]dCyd in the intact FM3A cells. Indeed, the tritium atom at the C-5 position of the cytosine ring is, upon deamination of dCyd (or dCMP) to dUrd (or dUMP), replaced by a methyl group in the thymidylate synthase reaction (dUMP → dTMP + ^{3}H2O) and thus inhibition of dTMP synthase can be monitored, and is matched, by inhibition of ^{3}H release. Whereas none of the test compounds proved to be markedly inhibitory to tritium release from [5-^{3}H]dCyd in FM3A/0 cells, tritium release from [5-^{3}H]dCyd was markedly inhibited in the HSV TK gene-transfected FM3A tumor cells by the BVDU derivatives but not by the acyclic guanosine analogues (Table 6). In

TABLE 5

Effect of dUrd, dThd, or dCyd on the cytostatic activity of antiherpetic drugs against FM3A/TK$^-$/HSV-1 TK$^+$ cells

Compound	IC_{50}[a]			
	As such	Upon addition of		
		dUrd (125 μg/ml)[b]	dThd (5 μg/ml)[b]	dCyd (500 μg/ml)[b]
		μM		
BVDU	0.007 ± 0.0034	0.037 ± 0.019	2.7 ± 0.93	0.014 ± 0.004
BVDC	0.005 ± 0.0031	0.058 ± 0.020	5.6 ± 0.97	0.054 ± 0.011
S-BVDU	0.008 ± 0.0039	0.12 ± 0.09	2.2 ± 0.86	0.038 ± 0.015
Buciclovir	4.2 ± 0.04	29 ± 1.7	14 ± 4.8	12 ± 3.3
Penciclovir	12 ± 2.7	179 ± 38	74 ± 15.5	84 ± 55.2
Ganciclovir	2.7 ± 0.8	47 ± 3.3	17 ± 3.6	20 ± 12

[a] Concentration required to inhibit cell proliferation by 50%.
[b] The chosen concentrations of the nucleosides were subtoxic to the FM3A cells and did not cause any inhibition of cell proliferation or cell death.

TABLE 6

Inhibitory effects of antiherpetic drugs on tritium release from [5-^{3}H]dCyd in different mutant FM3A tumor cells

Compound	IC_{50}[a]			
	FM3A/0	FM3A/TK$^-$	FM3A/TK$^-$/HSV-1 TK$^+$	FM3A/TK$^-$/HSV-2 TK$^+$
			μM	
BVDU	3.0	3.6 ± 3.19	0.008 ± 0.0005	0.008 ± 0.001
BVDC	11	7.6 ± 0.55	0.066 ± 0.006	0.018 ± 0.014
S-BVDU	63	68 ± 21.8	0.084 ± 0.022	0.033 ± 0.022
Buciclovir	>40		>40	
Penciclovir	>40		>40	
Ganciclovir	>100		>100	>100

[a] Concentration required to inhibit tritium release by 50%.

fact, whereas penciclovir, buciclovir, and ganciclovir were without effect, BVDU, BVDC, and S-BVDU inhibited tritium release from [5-^{3}H]dCyd in FM3A/TK$^-$/HSV-1 TK$^+$ and FM3A/TK$^-$/HSV-2 TK$^+$ cells with IC_{50} values of 0.008 μM (BVDU), 0.018–0.066 μM (BVDC), and 0.033–0.084 μM (S-BVDU), that is, 100- to >2000-fold lower than the concentration required to inhibit tritium release from FM3A/TK$^-$ and FM3A/0 cells (Table 6).

Substrate activity of BVDU, BVDC, and S-BVDU for human dThd phosphorylase. It is well known that BVDU and other 5-substituted dUrd derivatives act as good substrates for human dThd phosphorylase (20). In this enzymatic reaction, BVDU is converted to (*E*)-5-(2-bromovinyl)uracil, which is inactive against HSV-1 replication. Therefore, we wanted to know to what extent S-BVDU may also act as a substrate for human dThd phosphorylase. In the presence of human blood platelet extracts that contain high levels of dThd phosphorylase activity, there was no evidence of any marked hydrolysis of 100 μM BVDC or S-BVDU after 6 hr of incubation, whereas 54% of BVDU was converted to its free base, (*E*)-5-(2-bromovinyl)uracil, within 15 min of incubation (data not shown).

Discussion

The selectivity of the anti-herpes virus drugs as inhibitors of HSV-1 and HSV-2 replication depends primarily upon specific and preferential phosphorylation by the HSV-encoded TK. This enzyme is also endowed with dCyd kinase activity, has an associated thymidylate kinase activity (at least for the HSV-1-encoded TK), and shows a much higher affinity for several nucleoside analogues, including BVDU (21), acyclovir (22, 23), ganciclovir (23, 24), buciclovir (23, 25, 26), and penciclovir (23, 25), than does cytosolic TK. The high selectivity of these antiherpetic drugs is further substantiated by their low cytostatic activity against murine and human cell lines and their relatively low toxic side effects at antivirally effective doses in animal studies and in patients. Most importantly, the antiherpetic drugs, which depend on selective phosphorylation by the herpetic TK for their antiviral activity, acquire marked cytostatic activity against tumor cell lines that have been transfected with the HSV-1 or HSV-2 TK gene.

The cytostatic activities of BVDU and its congeners S-BVDU and BVDC appear to be based upon inhibition of cellular thymidylate synthase, whereas that of the acyclic guanosine derivatives ganciclovir, penciclovir, and buciclovir can be attributed to inhibition of cellular DNA polymerases and/or incorporation of the drug into DNA, as recently shown for ganciclovir (8).

Interestingly, the antiherpetic drugs that show structural analogy with BVDU (i.e., BVDC and S-BVDU) (27–31) proved to be markedly more cytostatic to the HSV TK gene-transfected cells than were the acyclic guanosine derivatives acyclovir, ganciclovir, penciclovir, buciclovir, and cyclobut-G. Thus, thymidylate synthase may represent a more efficient target for antitumor chemotherapy in HSV-1 TK gene-transfected tumor cells, compared with the cellular DNA polymerase(s) (or incorporation of the drugs into cellular DNA).

It is noteworthy that S-BVDU proved to be 10-fold more cytostatic to FM3A/TK$^-$ cells than FM3A/0 cells. This phenomenon has also been observed previously for BVDU and BVDC in FM3A/TK$^-$ cells, as well as in murine leukemia L1210/TK$^-$ and murine embryo fibroblast LM/TK$^-$ cells, compared with the corresponding wild-type cells (32, 33). The biochemical basis of this phenomenon has not yet been resolved. However, it was found that neither FM3A/0 and LM/0 cells, nor FM3A/TK$^-$ and LM/TK$^-$ cells, expressed a detectable phosphorylating activity for [^{125}I]IVDU, a closely related analogue of BVDU (32, 33). However, BVDU (and IVDU) specifically inhibited the incorporation of [^{14}C]mannose and [^{14}C]glucose into glycoproteins of FM3A/TK$^-$ and L1210/TK$^-$ cells but not wild-type cells (32). To what extent the inhibition of the incorporation of these monosaccharides into glycoproteins may contribute to the increased cytostatic effects of BVDU and IVDU on TK$^-$ cells remains to be determined.

Because BVDU is a relatively good substrate for mitochondrial TK (i.e., K_m of 1–3 μM for mitochondrial TK from L1210 and L1210/TK$^-$ cells), BVDU and related compounds may be postulated to be more selective for HSV TK than cytosolic TK in dividing cells but might be more toxic to nondividing cells (in which cytosolic TK activity is virtually absent and only mitochondrial TK is present). However, this explanation does not fully support the differential cytostatic activities of BVDU and related compounds in wild-type and TK$^-$ cells, because (i) in both cell lines mitochondrial TK is most likely equally expressed and (ii) there is no increased toxicity of BVDU and related compounds against nondividing confluent monolayer cell cultures of human E6SM, primary rabbit kidney cells, HeLa cells, or Vero cells. Moreover, we found BVDU (and BVDC and S-BVDU) not to be more cytostatic to TK-deficient human T lymphoblast CEM, B lymphoblast Raji, and cervix carcinoma HeLa cells than to the corresponding wild-type cells (IC_{50} of BVDU for CEM/0 and CEM/TK$^-$, 213 and 217 μM; for HeLa/0 and HeLa/TK$^-$, ≥200 and >200 μM; for Raji/0 and Raji/TK$^-$, 90 and 300 μM, respectively). Therefore, our observations for the three different murine TK$^-$ cells may be a peculiar property of murine TK$^-$ cells and not human TK$^-$ cells.

A potential drawback of BVDU is its susceptibility to hydrolysis by pyrimidine nucleoside phosphorylases (i.e., dThd phosphorylase and Urd phosphorylase), which are able to convert BVDU to its free base (*E*)-5-(2-bromovinyl)uracil (9, 20). This may hamper the *in vivo* activity of BVDU as a cytostatic agent against HSV TK gene-transfected tumors. In the present study, we found that BVDC and S-BVDU are, like BVDU, exquisitely inhibitory to HSV TK gene-transfected FM3A tumor cells. In contrast to BVDU, however, BVDC and S-BVDU are not hydrolyzed by human dThd phosphorylase. Whereas BVDC can still be deaminated to BVDU and thus may become vulnerable to hydrolysis by dThd phosphorylase, S-BVDU is not catabolized by such a mechanism. S-BVDU could therefore be considered a promising candidate compound for further *in vivo* studies as a cytostatic agent in the treatment of HSV-1 TK gene-transfected tumors.

Only ganciclovir has so far been investigated in experimental animal models and humans for its cytostatic activity against HSV-1 TK gene-transfected tumors (i.e., glioma). We have now demonstrated that related acyclic nucleoside analogues such as penciclovir and buciclovir show comparable cytostatic effects *in vitro* and that other antiherpetic agents such as BVDU, BVDC, and S-BVDU are far superior to ganciclovir in their cytostatic potential against HSV TK gene-transfected tumor cells.

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