Differential Mechanism of Cytostatic Effect of (E)-5-(2-Bromovinyl)-2'-deoxyuridine, 9-(1,3-Dihydroxy-2-propoxymethyl)guanine, and Other Antiviral Drugs on Tumor Cells Transfected by the Thymidine Kinase Gene of Herpes Simplex Virus Type 1 or Type 2*

Jan Balzarini, Christina Bohman, and Erik De Clercq

From the Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

After they have been transfected with the herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) thymidine kinase (TK) gene murine mammary carcinoma (FM3A) cells become highly sensitive to the growth inhibitory properties of the antiviral agents (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU), 9-(1,3-dihydroxy-2-propoxymethyl)guanine (acyclovir, ACV), 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG, ganciclovir), and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU). BVDU was 100-fold more potent an inhibitor of HSV TK gene-transfected tumor cell growth (50% inhibition concentration (IC50), 0.002-0.047 μM) than FMAU or DHPG (IC50, 0.051-0.277 μM) and 1000-fold more potent than ACV (IC50, 0.42-4.9 μM). As a rule, the test compounds were more cytostatic to HSV-2 TK than HSV-1 TK gene-transfected FM3A cells. This may be ascribed to the higher phosphorylating capacity (Vmax/Km) of HSV-2 TK than HSV-1 TK and/or to the higher TK levels in HSV-2 TK gene-transfected FM3A cells than the HSV-1 TK gene-transfected FM3A cells. Thymidylate synthase of the HSV TK gene-transfected FM3A cells appears to be the target enzyme for the cytostatic action of BVDU, but not FMAU, DHPG, or ACV. Instead, the cytostatic activity of DHPG seems to be correlated with its conversion to the triphosphate form and subsequent incorporation into the DNA of HSV TK gene-transfected FM3A cells.

Several nucleoside analogues have been reported to selectively inhibit the replication of herpes simplex virus type 1 (HSV-1)* and type 2 (HSV-2) both in vitro and in vivo. Foremost among the antiviral compounds that have demonstrated efficacy in the treatment of herpesvirus infections in animals and/or humans are 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir, ACV), 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG, ganciclovir), and 5-(2-bromovinyl) deoxyuridine (BVDU), and 5-(2-bromovinyl)-2'-deoxyuridine (BVDU), and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU). BVDU was 100-fold more potent than ACV (1-11) (Fig. 1). These nucleoside analogues owe their selectivity to the fact that they are specifically recognized as substrate by the HSV-encoded thymidine kinase (TK), but not by its cellular counterpart or other cellular kinases (1). ACV, DHPG, BVDU, and FMAU display little, if any, cytotoxicity for noninfected cells. In 1985, we reported that BVDU, DHPG, FMAU, and ACV have increased cytostatic activity against murine mammary carcinoma cells transformed with the HSV-1 thymidine kinase gene (12-16). Also, other authors reported that DHPG (17), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (18), and acyclovir (18) are strongly inhibitory to the growth of HSV-1 TK gene-transfected cells either in vitro (cell cultures) or in mice inoculated with HSV-1 TK gene-transfected cells or in transgenic mice bearing the HSV-1 TK gene. The recent findings of Culver et al. (19) that DHPG is effective in the treatment of localized tumors after direct in situ introduction of the HSV-1 TK gene into the proliferating brain tumor cells of rats and the envisaged trials with DHPG in humans suffering from brain tumors (20) prompted us to assess the comparative cytostatic action of the antiherpetic compounds BVDU, DHPG, ACV, and FMAU against both HSV-1 TK or HSV-2 TK gene-transfected tumor (i.e. murine mammary carcinoma FM3A) cells. We found that (i) BVDU is 100-fold more potent a cytostatic agent for HSV TK gene-transfected tumor cells than DHPG (the drug chosen for the clinical trials), (ii) the antiviral compounds are more cytostatic for tumor cells transfected by HSV-2 TK gene than by HSV-1 TK gene due to a higher phosphorylating capacity of HSV-2 TK than HSV-1 TK and/or the higher TK levels in HSV-2 TK gene-transfected FM3A cells than HSV-1 TK gene-transfected FM3A cells, and (iii) the mechanism of cytostatic action of BVDU (which is targeted at the thymidylate synthase) is different from that of the other antiviral drugs (which seem to be targeted at the DNA polymerisation).

**MATERIALS AND METHODS**

Cells—FM3A cells (subline F98-7) were originally established from a spontaneous mammary carcinoma in a C3H/He mouse (21) and designated FM3A/0. The FM3A TK-/HSV-1 TK" and FM3A TK-/+HSV-2 TK" cells, which lack host cell TK activity but contain either the HSV-1 TK gene or HSV-2 TK gene, were derived from the FM3A/TK" cells as described previously (22, 23). FM3A TK-/HSV-1 TK"/TS" cells, which are deficient in thymidylate synthase, were isolated as previously described (24). To maintain this mutant cell line, 20 μM dThd was added to the growth medium, and this medium was renewed every 3-4 days. CEM/0 and CEM/TK" cells were kindly provided by Prof. S. Eriksson and Dr. A. Karlsson.

* This investigation was supported by Krediet 3.0069.91 from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek, Krediet 7.0049.90 from the Belgian Nationaal Fonds voor Wetenschappelijke Onderzoek, and Krediet 91/94-2 from the Belgian Geconsoliderde Onderzoeksacties. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: HSV, herpes simplex virus; ACV, 9-[(2-hydroxyethoxy)methyl]guanine (acyclovir); DHPG, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (ganciclovir); BVDU, 5-(2-bromovinyl) deoxyuridine; FMAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)5-methyluracil; TK, thymidine kinase; TS, thymidylate synthase; IC50, 50% inhibitory concentration.
cells were thoroughly washed with dThd-free medium before they exogenous dThd. Before initiating the cell growth experiments, the TK-/HSV-1 TK+/TS- cells are triple mutant cells that are deficient in thymidylate synthase and are unable to proliferate. At the end of the incubation period, the cells were counted in a Coulter counter.

Inhibition of DNA and RNA Synthesis in FM3A Cells—The procedures to measure the incorporation of [methyl-3H]dThd, [1',2',3H]dUrd, and [5-3H]Urd into trichloroacetic acid-insoluble material have been described previously (26). Briefly, to each microplate well were added 10^6 FM3A/O, FM3A TK-/HSV-1 TK+ or FM3A TK-/HSV-2 TK+ cells, a given amount of test compound, and 0.25 μCi of [methyl-3H]dThd, [1',2',3H]dUrd, or [5-3H]Urd into trichloroacetic acid-insoluble material was assayed for radioactivity.

Determination of Tritium Release from [5-3H]dCyd—Activity of TS in intact FM3A cells was measured by estimation of tritium release from [5-3H]dCyd at 37°C by the method described previously (27) but was modified as follows. FM3A cells were collected by centrifugation at 200 × g for 8 min and resuspended in fresh medium; 300 μl of this cell suspension (0.75 × 10^6 cells) were added to 60 μl of medium containing an appropriate amount of test compound and 40 μl of 4 μCi of [5-3H]dCyd. At 0, 15, 30, and 60 min, 100 μl of the reaction mixture were withdrawn and mixed with 2 μl of a cold suspension of carbon black (160 mg/ml) in 5% trichloroacetic acid. After centrifugation at 1100 × g for 10 min, 200-μl samples of the supernatants were assayed for radioactivity.

Phosphorylation of [3H]dHPG in FM3A Cells—The metabolism of [3H]dHPG was monitored as for FM3A TK-/HSV-1 TK+ or FM3A TK-/HSV-2 TK+ cells. FM3A TK-/HSV-1 TK+ or FM3A TK-/HSV-2 TK+ cells were seeded at 10^5 to 10^6 cells/ml in 5-ml culture bottles and incubated with 0.05 μM [3H]dHPG (5 μCi/bottle). At 6 or 24 h, cells were centrifuged at 200 × g, washed twice with cold medium, and precipitated with cold methanol 66%. After centrifugation at 10,000 × g for 3 min, the supernatants were subjected to high performance liquid chromatography analysis using a Partisphere-SAX column. A linear gradient of 5 mM (NH4)2HPO4, pH 5.0 (Buffer A) to 500 mM (NH4)2HPO4, pH 5.0 (Buffer B) was used to separate the metabolites as follows: 5 min of 0% Buffer A, 15 min of linear gradient to 100% Buffer B, 15 min of linear gradient to 100% Buffer B, 10 min of 0% Buffer A, 5 min of linear gradient to 100% Buffer A, and 5 min of equilibration with Buffer A. The different fractions of the eluate were assayed for radioactivity in a toluene-based scintillator.

Phosphorylation of [3H]dHPG by Thymidine Kinase in FM3A TK-/HSV-1 TK+ and FM3A TK-/HSV-2 TK+ cells pellets were first washed with cold phosphate-buffered saline and then washed twice with suspension buffer (50 mM potassium phosphate, pH 7.6, containing 2 mM dithiothreitol, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine). The cell pellet could either be stored at -70°C or resuspended in ~4 ml of suspension buffer. The cell suspension was then sonicated three times for 10 s, supplemented with sucrose to a final sucrose concentration of 0.25 mM, and cleared by centrifugation at 43,000 × g for 20 min. The 70% (NH4)2SO4 precipitate of the cell homogenate was resuspended in ~5 ml of suspension buffer (50% sucrose, 0.5 M sucrose buffered at 4°C) was loaded on~1.4 ml of Partisphere-SAX columns. A linear gradient of 5 mM (NH4)2HPO4, pH 5.0 (Buffer A) to 500 mM (NH4)2HPO4, pH 5.0 (Buffer B) was used to separate the metabolites as follows: 5 min of 0% Buffer A, 15 min of linear gradient to 100% Buffer B, 15 min of linear gradient to 100% Buffer B, 10 min of 0% Buffer B, 5 min of linear gradient to 100% Buffer A, and 5 min of equilibration with Buffer A. The different fractions of the eluate were assayed for radioactivity in a toluene-based scintillator.

Quantitation of Thymidine Kinase Activity in FM3A Cells and Determination of the Inhibitory Effects of BVDU, DHPG, and ACV on the Enzyme—FM3A TK-/HSV-1 TK+, FM3A TK-/HSV-2 TK+, FM3A TK-/HSV-1 TK+TS-, or FM3A TK-/HSV-2 TK+TS- cell pellets (~500 × 10^6 cells) were first washed with cold phosphate-buffered saline and then washed twice with suspension buffer (50 mM potassium phosphate, pH 7.6, containing 2 mM dithiothreitol, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine). The cell pellet could either be stored at -70°C or resuspended in ~4 ml of suspension buffer. The cell suspension was then sonicated three times for 10 s, supplemented with sucrose to a final sucrose concentration of 0.25 mM, and cleared by centrifugation at 43,000 × g for 20 min. The 70% (NH4)2SO4 precipitate of the cell homogenate was resuspended in ~5 ml of suspension buffer (50% sucrose, 0.5 M sucrose buffered at 4°C) was loaded on~1.4 ml of Partisphere-SAX columns. A linear gradient of 5 mM (NH4)2HPO4, pH 5.0 (Buffer A) to 500 mM (NH4)2HPO4, pH 5.0 (Buffer B) was used to separate the metabolites as follows: 5 min of 0% Buffer A, 15 min of linear gradient to 100% Buffer B, 15 min of linear gradient to 100% Buffer B, 10 min of 0% Buffer B, 5 min of linear gradient to 100% Buffer A, and 5 min of equilibration with Buffer A. The different fractions of the eluate were assayed for radioactivity in a toluene-based scintillator.
Cells—The assay procedure to measure the phosphorylation rate of [\textsuperscript{[H]}]dHPG by FM3A thymidine kinase was essentially as described above, except that the DE-31 discs were washed three times in 1.5 mM ammonium formate instead of ethanol. The concentrations used for [\textsuperscript{[H]}]dHPG were 20, 40, 80, 100, and 200 \(\mu\text{M}\) for the TK preparation from FM3A TK-/HSV-1 TK\(^{-}\) and FM3A TK-/HSV-2 TK\(^{-}\) cells, and 50, 100, 150, 200, and 250 \(\mu\text{M}\) for the TK preparation from FM3A/0 cells. Specific radioactivity of [\textsuperscript{[H]}]dHPG used was 600 cpm/pmols.

RESULTS

Cytostatic Activity of Antiviral Compounds against HSV TK gene-transfected FM3A Cells—BVDU, DHPG, ACV, and FMAU were evaluated for their inhibitory effects on the proliferation of a number of tumor cell lines, including the human T lymphocytes CEM/O, CEM/TK\(^{-}\), C8166, and Molt 4 (clone 8) cells, and the murine Li2120, FM3A/0, FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\), and FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\) cells. None of the human tumor cells and the murine leukemia Li2120 and mammary carcinoma FM3A cells were markedly inhibited by BVDU, DHPG, and ACV (IC\(_{50}\), 50 to >500 \(\mu\text{M}\)) (Table I). FMAU proved inhibitory to FM3A cells at ~20 \(\mu\text{M}\) (Tables I and II). In contrast, the growth of FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\) and FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\) cells was inhibited by BVDU at concentrations of 4.7 and 2.0 \(\text{pmol}\), that is at concentrations that are 10,000–25,000-fold lower than the concentrations required to inhibit the growth of the corresponding wild-type FM3A/0 cells. Also, DHPG and FMAU were inhibitory to the HSV-2 TK gene-transfected FM3A cells at 0.051 and 0.073 \(\mu\text{M}\) and to HSV-1 TK gene-transfected cells at 0.190 and 0.277 \(\mu\text{M}\), respectively. These concentrations were 750–3000-fold (DHPG) or 70–300-fold (FMAU), lower than those found inhibitory to the wild-type FM3A/0 cells (Tables I and II). Acyclovir inhibited the proliferation of HSV-TK gene-transfected FM3A cells at a concentration that was 50–60-fold lower than that required to inhibit the proliferation of the wild-type tumor cells. As a rule, the test compounds were lower than that required to inhibit the proliferation of the corresponding wild-type FM3A/0 cells at a concentration ranging from 0.19 to >200 \(\text{pmol}\). Addition of dThd reduced the cytostatic activity of DHPG, ACV, and FMAU by about 10-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\) or even >20,000-fold (FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\)) lower than those found inhibitory to the wild-type FM3A/0 cells (Tables I and II).

Effect of Thymidine, BVDU, DHPG, and ACV on the Growth of FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)/TS\(^{-}\) Cells—FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)/TS\(^{-}\) cells were incubated in the presence of different concentrations of thymidine (dThd), BVDU, DHPG, and ACV for 48 h, and then the cell number was determined (Table I). For BVDU, however, the IC\(_{50}\) required to inhibit [\textsuperscript{[H]}]dThd incorporation into DNA (Table III) was the same as that required to inhibit the proliferation of the corresponding wild-type FM3A/0 cells. In contrast, dThd was able to sustain cell growth at concentrations ranging from 1 to 1000 \(\mu\text{M}\) (data not shown). Cell number was equal if not lower after the 48-h incubation period than the initial cell number. In contrast, dThd was able to sustain cell growth at all concentrations tested (1–1000 \(\mu\text{M}\)); 20–100 \(\mu\text{M}\) was the optimal. At these thymidine concentrations, FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)/TS\(^{-}\) cells did not differ in their growth rate, as compared with the wild-type FM3A/0 cells cultured in the absence of dThd (generation time, 12–14 h) (data not shown).

TABLE I

| Cell line                  | IC\(_{50}\) \(\mu\text{M}\) |  |
|---------------------------|-----------------------------|---|
| CEM/O                     | >500                        |  |
| CEM/TK\(^{-}\)            | >500                        |  |
| C8166                     | 265 ± 13                    | >500 |
| Molt 4 (clone 8)          | 180 ± 3                     | >500 |
| Li2120/0                  | 50.2 ± 0.3                  | >500 |
| FM3A/0                    | 68.1 ± 17.9                 | >500 |
| FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\) | 0.0047 ± 0.0022         |  |
| FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\) | 0.002 ± 0.0009          |  |

* 50% inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.

Additive Effects of Antiviral Compounds in FM3A Cells—To explore the biochemical basis for the cytostatic activity of the antiviral compounds against the HSV TK gene-transformed cell lines, we examined (i) the reversing effect of dUrd and dThd on the cytostatic activity of the test compounds against the different FM3A cell lines (Table II), (ii) the effect of the test compounds on the incorporation of radiolabeled [\textsuperscript{[H]}]dUrd, [methyl\textsuperscript{[3H]}]dThd, and [\textsuperscript{[5H]}]Urd into FM3A cell DNA and RNA (Table III), and (iii) the effect of the test compounds on tritium release from [\textsuperscript{[5H]}]dCyd into the intact cells (Table IV). The cytostatic activity of the test compounds against HSV-TK gene-transfected cells was reduced by 4–35-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\) or 25–100-fold (FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\)) upon addition of dUrd and ACV (Table II). Addition of dThd reduced the cytostatic activity of DHPG, ACV, and FMAU by about 10-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)) to 100-fold (FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\)). In contrast, the cytostatic activity of BVDU against FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\) was reduced by 1000-fold, and its cytostatic activity against FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\) was totally annihilated (>150,000-fold reduced) following addition of dThd (Table II).

DHPG, ACV, and FMAU inhibited the incorporation of [\textsuperscript{[1H]}]dUrd into DNA of FM3A TK\(^{-}\)/HSV TK\(^{-}\) cells at a 5–10-fold lower concentration than that required for the inhibition of [methyl\textsuperscript{[3H]}]dThd incorporation into DNA (Table III). For BVDU, however, the IC\(_{50}\) required to inhibit [\textsuperscript{[1H]}]dUrd incorporation was 1000-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)) or even >20,000-fold (FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\)) lower than the IC\(_{50}\) required to inhibit [methyl\textsuperscript{[3H]}]dThd incorporation into DNA. Thus, in contrast with the other antiviral drugs, BVDU markedly discriminated between dUrd and dThd incorporation into DNA of HSV TK gene-transformed FM3A cells. None of the test compounds affected the incorporation of [\textsuperscript{[5H]}]Urd into RNA of FM3A/0, FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\), or FM3A TK\(^{-}\)/HSV-2 TK\(^{-}\) cells (Table III).

The cytostatic activity of the test compounds against HSV-TK gene-transfected cells was reduced by 4–35-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)) by the test compounds against HSV-TK gene-transfected cells was reduced by 4–35-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)) by the test compounds against HSV-TK gene-transfected cells was reduced by 4–35-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)) by the test compounds against HSV-TK gene-transfected cells was reduced by 4–35-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)) by the test compounds against HSV-TK gene-transfected cells was reduced by 4–35-fold (FM3A TK\(^{-}\)/HSV-1 TK\(^{-}\)).
Sensitivity of Tumor Cells to Antivireric Drugs

**TABLE II**

Effect of the addition of dThd and dUrd on the cytostatic activity of BVDU, DHPG, ACV, and FMAU against FM3A cells

| Compound | FM3A/O upon addition of | FM3A TK+/-HSV-1 TK+ upon addition of | FM3A TK+/-HSV-2 TK+ upon addition of |
|----------|-------------------------|--------------------------------------|--------------------------------------|
|          | dThd (5 µg/ml) | dUrd (125 µg/ml) | dThd (5 µg/ml) | dUrd (125 µg/ml) | dThd (5 µg/ml) | dUrd (125 µg/ml) |
| BVDU     | >100          | >100         | 0.0015        | 2              | 0.0047        | 0.0007        | >100           | 0.067 |
| DHPG     | 150           | 180          | 195           | 4.9            | 68            | 42            | 0.42           | >20  |
| ACV      | 214           | 270          | 265           | 4.9            | 68            | 42            | 0.42           | >20  |
| FMAU     | ≥20           | ≥20          | ≥20           | 0.277          | 1.6           | 4.1           | 0.073          | 5.23 |

* 50% inhibitory concentration or compound concentration required to inhibit FM3A cell proliferation by 50%.

**TABLE III**

Inhibitory effect of BVDU, DHPG, ACV, and FMAU on the incorporation of radiolabeled DNA and RNA precursors into trichloroacetic acid-insoluble material of FM3A cells

| Compound | FM3A/O | FM3A TK+/-HSV-1 TK+ | FM3A TK+/-HSV-2 TK+ |
|----------|--------|---------------------|---------------------|
|          | [methyl-3H]dThd | [1',2'-3H]dUrd | [5'-3H]Urd | [methyl-3H]dThd | [1',2'-3H]dUrd | [5'-3H]Urd |
| BVDU     | >100   | 25                  | >100               | 1.7              | 0.0014         | >10         | 0.0005        | >10   |
| DHPG     | >100   | 83                  | >100               | 0.88             | 0.16           | >100        | 1.8            | 0.017  | >100 |
| ACV      | ≥100   | 45                  | >100               | 19               | 2.2            | >100        | 18             | 1.1    | >100 |
| FMAU     | 19     | 2.9                 | ≥20                | 0.19             | 0.16           | ≥20         | 0.50           | 0.098  | >20  |

* 50% inhibitory concentration or compound concentration required to inhibit radiolabeled DNA or RNA precursors into trichloroacetic acid-insoluble cell material by 50%.

**TABLE IV**

Inhibitory effect of BVDU, DHPG, ACV, and FMAU on the release of tritium from [5'-3H]dCyd in FM3A cells

| Compound | FM3A/O | FM3A TK+/-HSV-1 TK+ | FM3A TK+/-HSV-2 TK+ |
|----------|--------|---------------------|---------------------|
|          |        | [methyl-3H]dCyd     | [1',2'-3H]dCyd     | [5'-3H]dCyd     |
| BVDU     | 63     | 0.004              | 0.007              |
| DHPG     | >100   | >100                | >100               |
| ACV      | >100   | >100                | >100               |
| FMAU     | ≥20    | >20                 | >20                |

* 50% inhibitory concentration or compound concentration required to inhibit tritium release from [5'-3H]dCyd in intact FM3A cells.

HSV-1 TK+, and FM3A TK+/-HSV-2 TK+ Cells—Phosphorylation of [3H]DHPG was examined in FM3A/O, FM3A TK+/-HSV-1 TK+, and FM3A TK+/-HSV-2 TK+ cells, and intracellular [3H]DHPG metabolites were quantified after 6- and 24-h incubations of the cells with the radiolabeled drug. As shown in Fig. 2, conversion of [3H]DHPG to its 5'-monophosphate, 5'-diphosphate, and 5'-triphosphate derivatives was markedly more pronounced in HSV TK gene-transfected FM3A cells than wild-type FM3A/O cells. In fact, phosphorylated metabolites of DHPG appeared at a 30-50-fold higher extent in FM3A TK+/-HSV-1 TK+ cells than FM3A/O cells after 6- or 24-h incubation of the cells with 0.05 µM of the radiolabeled drug. FM3A TK+/-HSV-2 TK+ cells converted [3H]DHPG to its phosphorylated metabolites at a 3-4-fold higher extent than FM3A TK+/-HSV-1 TK+ cells (Fig. 2). Interestingly, [3H]DHPG was also 2-3-fold more incorporated into methanol-insoluble material of FM3A TK+/-HSV-2 TK+ cells than FM3A TK+/-HSV-1 TK+ cells, and the differences in incorporation rates closely correspond to the differences in the DHPG triphosphate levels in the HSV-1 and HSV-2 TK gene-transfected cells (Table V). Thus, FM3A TK+/-HSV-2 TK+ cells proved superior to FM3A TK+/-HSV-1 TK+ cells in anabolizing [3H]DHPG to its triphosphate form. From Fig. 2, it is also evident that the amount of phosphorylated [3H]DHPG metabolites in FM3A cells was virtually constant per cell number between 6-24 h of incubation of exponentially growing cells with radiolabeled drug.

Inhibitory Effect of BVDU, DHPG, and ACV on Thymidine Kinase from FM3A/O, FM3A TK+/-HSV-1 TK+, and FM3A TK+/-HSV-2 TK+ Cells—When BVDU, DHPG, and ACV were evaluated for their inhibitory effect on thymidine kinase from FM3A cells, none of the compounds proved inhibitory to TK from the wild-type FM3A/O cells at 100-500 µM. In contrast, BVDU proved markedly inhibitory to thymidine phosphorylation by TK from FM3A TK+/-HSV-1 TK+ and FM3A TK+/-HSV-2 TK+ cells, HSV-1 TK being 15-fold more strongly inhibited than HSV-2 TK (Table VI). DHPG and ACV did not markedly affect HSV-1 or HSV-2 TK (IC50 53 to >500 µM).

Phosphorylation of [3H]DHPG by Thymidine Kinase from FM3A/O, FM3A TK+/-HSV-1 TK+, and FM3A TK+/-HSV-2 TK+ Cells—[3H]DHPG did not exhibit pronounced affinity for the TK from FM3A/O cells (Km 689 µM). However, DHPG had a Km value of 45 and 162 µM for the TK from FM3A TK+/-HSV-1 TK+ and FM3A TK+/-HSV-2 TK+ cells, respectively, and the Vmax values for the HSV-1 and HSV-2 TK, derived from the HSV TK gene-transfected FM3A cells, were as high as 133 and 964 pmol/min (Table VII). Consequently, the phosphorylative capacity of HSV-2 TK for DHPG was 2-fold higher than that of HSV-1 TK.

**DISCUSSION**

BVDU is a remarkably efficient substrate for HSV-1 TK (Km 0.24-0.40 µM) and HSV-2 TK (Km 3.42 µM) (28, 29). DHPG and ACV are much poorer substrates for HSV-1 TK (Km 11-66 and 375-426 µM, respectively) (5, 30) and HSV-2 TK (Km 16 and 305 µM, respectively) (30). These data are in agreement with our experimental data obtained for the thymidine kinases from HSV TK gene-transfected FM3A cell lines. These characteristics may explain, at least in part, why...
Sensitivity of Tumor Cells to Antitherpetic Drugs

**Fig. 2. Phosphorylation of [³H]DHPG in FM3A/O, FM3A TK-/+ HSV-1 TK*, and FM3A TK-/+ HSV-2 TK* cells upon 6- or 24-h incubation. DHPG-MP, DHPG-DP, and DHPG-TP are the monophosphate, diphosphate, and triphosphate derivatives, respectively, of DHPG.**

**TABLE V**

| Time of incubation with 0.05 mM [³H]DHPG | pmoles/10⁶ cells* |
|-----------------------------------------|-------------------|
| 6 h                                     | 29                |
| 24 h                                    | 64                |

| TK source* | IC₅₀ µM | IC₅₀ pmoles/mg/min |
|------------|--------|-------------------|
| FM3A/O     | >100   | >500              |
| FM3A TK-/+ HSV-1 TK* | 0.38   | 55                |
| FM3A TK-/+ HSV-2 TK* | 6.86   | 405               |

* Specific radioactivity of [³H]DHPG; 0.089 pmoles/10⁶ cpm.

**TABLE VI**

Inhibitory effect of BVDU, DHPG, and ACV on TK activity from FM3A/O, FM3A TK-/+ HSV-1 TK*, and FM3A TK-/+ HSV-2 TK* cells

| TK source* | IC₅₀ µM | IC₅₀ pmoles/mg/min |
|------------|--------|-------------------|
| FM3A/O     | 689    | 238               |
| FM3A TK-/+ HSV-1 TK* | 45.2   | 133               |
| FM3A TK-/+ HSV-2 TK* | 162    | 964               |

* Specific TK activity was 0.4, 0.08, and 0.33 nmol [methyl-³H]dThd converted/mg of protein/min for FM3A/O, FM3A TK-/+ HSV-1 TK*, and FM3A TK-/+ HSV-2 TK* cell-derived TK, respectively.

**TABLE VII**

Kinetic properties of [³H]DHPG for cytosol, HSV-1, and HSV-2 TK derived from FM3A cells*

| TK source* | Kₐ µM | Vₘₐₓ pmoles/mg/min | Vₘₐₓ/Kₐ |
|------------|-------|--------------------|---------|
| FM3A/O     | 689   | 238                | 0.35    |
| FM3A TK-/+ HSV-1 TK* | 45.2   | 133               | 2.95    |
| FM3A TK-/+ HSV-2 TK* | 162    | 964               | 5.95    |

* Kinetic parameters were calculated by linear regression from Lineweaver-Burk plots.

BVDU has a markedly higher cytostatic activity against FM3A TK-/+HSV TK* cells than the other antitherpetic compounds.

The fact that DHPG exhibits a stronger cytostatic activity than acyclovir may also be ascribed to the greater affinity of DHPG than of ACV for HSV-1 TK and HSV-2 TK; Vₘₐₓ/Kₐ of HSV-1 TK for DHPG is 30-244-fold higher than for ACV (5, 30) and Vₘₐₓ/Kₐ of HSV-2 TK for DHPG is 60-fold higher than for ACV (30). Also, DHPG monophosphate has a greater affinity for GMP kinase than ACV monophosphate; Vₘₐₓ/Kₐ of GMP kinase for DHPG monophosphate is 56-492-fold higher than for ACV monophosphate (5, 29). A factor that may play a role in the higher cytostatic effects of DHPG and ACV in HSV-2 TK gene-transfected cells than in HSV-1 gene-transfected cells are the higher levels of HSV TK activity expressed in FM3A TK-/+HSV-2 TK* cells, as compared with FM3A TK-/+HSV-1 TK* cells (Table VI). In addition, HSV-2 TK has a higher phosphorylating capacity for DHPG than HSV-1 TK (Table VII).

Our antimetabolic data indicate that BVDU, but not the other antitherpetic drugs, inhibits [¹',²'-³H]dUrd incorporation into DNA at concentrations that are 10,000- to >150,000-fold lower than the concentrations required to inhibit [methyl-³H]dThd incorporation (Table III). This discriminative behavior is compatible with an antimetabolic action targeted at thymidylate synthase. Also, the ability of dThd, but not dUrd, to almost completely reverse the cytostatic activity of BVDU against the HSV TK gene-transfected cells (Table II), as well as the extremely potent inhibitory effect of BVDU on tritium release from [5-³H]dCyd in HSV-TK gene-transfected FM3A cells (4-7 nM) (Table IV), are in full agreement with thymidylate synthase being the molecular target for the antiproliferative effect of BVDU. In fact, BVDU 5'-monophosphate is a potent inhibitor of cell-free thymidylate synthase (Kₐ/Kₐ = 0.66) (31).

The lack of marked cytostatic activity of BVDU against FM3A/O (and other cell lines) may obviously be explained by the inability of the cellular TK of normal cells to phosphorylate BVDU to its 5'-monophosphate. It is well established that BVDU is not a substrate for cytosol TK (31).
In contrast with the cytostatic activity of BVDU, the cytostatic activity of the other antitherpetic drugs DHPG, ACV, and FMAU is almost equally well reversed by dUrd and dThd. Furthermore, BVDU, ACV, and FMAU do not inhibit tritium release from [5-3H]dThd at relatively high concentrations (20–100 μM). This means that, whereas the cytostatic activity of BVDU against FM3A TK-/HSV TK' cells can be readily explained by an action targeted at thymidylate synthase, the other antitherpetic drugs must exert their cytostatic activity by an entirely different mechanism of action.

DHPG is 30–50-fold better phosphorylated by FM3A TK-/HSV-1 TK' and 100–200-fold better phosphorylated by FM3A TK-/HSV-2 TK' cells than by wild-type FM3A/0 cells to its 5'-triphosphate form. Also, DHPG is incorporated to a 4- and 10-fold greater extent into nucleic acids of FM3A TK-/HSV-1 TK' and FM3A TK-/HSV-2 TK' cells (as compared with FM3A/0 cells). These findings point to the necessity of DHPG (i) to be phosphorylated to its 5'-triphosphate and (ii) to be subsequently incorporated into DNA to achieve its cytostatic effect on HSV TK gene-transfected FM3A cells. Moreover, DHPG is unable to sustain cell growth upon incorporation into FM3A TK-/HSV-1 TK'/TS- cells, which means that the incorporation of DHPG into the cells' nucleic acids will have a deleterious effect on cell growth. This alternate mode of cytostatic action of DHPG (and ACV) versus BVDU in HSV TK gene-transfected FM3A cells corresponds well with the inhibitory effects of the triphosphate derivatives of the test compounds against DNA polymerase α. Indeed, the triphosphate derivatives of ACV and DHPG were reported to show a much greater binding affinity for, and preferential inhibition of, DNA polymerase α (Kₐ 0.996 and 0.146 μM, respectively) (32) than did the triphosphate derivative of BVDU (Kₐ 3.6 μM) (33). In fact, the observations of Mac et al. (32) that the triphosphate derivative of DHPG is able to (i) substitute for dGTP, (ii) act as a substrate for DNA polymerases, and (iii) incorporate into DNA, resulting in a drastic suppression of DNA chain elongation, are in agreement with our findings that DHPG is incorporated into cellular DNA (Table V), does not allow FM3A TK-/HSV-1 TK'/TS- cell growth, and principally acts at the level of DNA polymerization.

All antitherpetic compounds proved more inhibitory to FM3A TK-/HSV-1 TK' than FM3A TK-/HSV-2 TK' cells. The greater inhibitory effect of BVDU for FM3A TK-/HSV-2 TK' cells can be expected from an action targeted at thymidylate synthase. In HSV-2 TK gene-transfected FM3A cells, BVDU is readily metabolized to its 5'-monophosphate but not further onto the 5'-triphosphate, whereas in FM3A TK-/HSV-1 TK' cells, high levels of BVDU 5'-triphosphate are found (14, 15). The reason for this differential phosphorylation pattern is that HSV-1 TK is able to convert dTdf (and dThd analogues such as BVDU) to their 5'-diphosphate form (which is then further phosphorylated to the 5'-triphosphate forms by cellular enzymes), whereas HSV-2 TK converts dTdf (and related analogues) to the 5'-monophosphate but not further onto the 5'-diphosphate (34).

Other antitherpetic compounds (i.e., DHPG) are more efficiently phosphorylated in FM3A TK-/HSV-1 TK' cells than in FM3A TK-/HSV-1 TK' cells (Fig. 2); the principal reason is most likely the higher TK levels achieved in FM3A TK-/HSV-2 TK' cells than in FM3A TK-/HSV-1 TK' cells (Table VI), rather than a higher affinity of DHPG for HSV-2 TK than for HSV-1 TK. Indeed, the Vₐₘₙ/Kₐₚ ratios of HSV-1 TK and HSV-2 TK for DHPG differ only 2-fold (Table VI and Ref. 29). For acyclovir, greater capacity to inhibit FM3A TK-/HSV-2 TK' cells than FM3A TK-/HSV-1 TK' cells may be related to both higher TK levels in FM3A TK-/HSV-1 TK' cells and better phosphorylating capacity (3–4 fold) of HSV-1 TK for ACV (Table VI and Ref. 30).

In conclusion, we have demonstrated that BVDU is by far more effective than DHPG, ACV, or FMAU in inhibiting the proliferation of HSV-1 TK and HSV-2 TK gene-transformed FM3A tumor cells. All compounds exerted a relatively greater cytostatic activity against HSV-2 TK gene-transfected than HSV-1 TK gene-transfected cells due to higher TK levels in HSV-2 TK-/HSV-2 TK' cells than FM3A TK-/HSV-1 TK' cells and/or the higher phosphorylating capacity of HSV-2 TK-1 TK for these compounds. The mechanism of cytostatic activity of BVDU is clearly different from that of DHPG and other antitherpetic drugs. If HSV TK gene therapy would become an amenable modality for the selective treatment of tumors, HSV-2 TK gene-transfected cells should be preferred over HSV-1 TK gene-transfected cells, and BVDU should be considered as the prime candidate drug for the treatment of tumors transfected with the HSV-2 TK gene.

Acknowledgments—We thank Lizette van Berckelaer and Ria van Berwaer for excellent technical assistance and Christiane Callebat and Laurent Palmans for dedicated editorial help.

REFERENCES
1. De Clercq, E. (1984) Biochem. Pharmacol. 33, 2159-2169
2. Elion, G. B., Furman P. A., Fye, J. A., de Miranda, P., Beauchamp, L., and Schaeffer, H. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5716-5720
3. Smeets, F. M., Martin, J. C., Verbruggen, J. P. H., and Mathews, T. R. (1985) Antimicrob. Agents Chemother. 23, 676-692
4. Chen, Y.-C., Huang, E.-S., Lin, J.-C., Mar, E.-C., Pagano, J. S., Dutschman, G. E., and Grill, S. P. (1985) Proc. Natl. Acad. Sci. U. S. A. 80, 2767-2771
5. Fujiwara, K., Davies, M. E., De Witt, C., Perry, H. C., Liu, R., Germer-Hausen, J., Karkas, J. D., Ashton, W. T., Johnston, D. B. R., and Tolman, R. L. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4139-4143
6. De Clercq, E., Descomps, E., Seno, T., Barr, P. J., Jones, A. S., and Walker, R. T. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2947-2951
7. De Clercq, E., Descomps, E., Verhelst, G., Walker, R. T., Jones, A. S., Torrence, P. F., and Shugars, D. (1980) J. Infect. Dis. 141, 635-637
8. Lopez, C., Watanabe, K. A., and Fox, J. J. (1980) Antimicrob. Agents Chemother. 17, 803-806
9. Machida, H., Kunimaka, A., and Yoshino, H. (1982) Antimicrob. Agents Chemother. 21, 358-361

Table V

| Compound | Sensitivity ratios of HSV-1 TK/HSV-2 TK |
|----------|---------------------------------------|
| ACV      | 3.6 | 10.0 |
| DHPG     | 3.3 | 10.0 |
| ACV      | 3.6 | 10.0 |
| DHPG     | 3.3 | 10.0 |

(continued)