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Thymidylate Synthase Is the Principal Target Enzyme for the Cytostatic Activity of (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine Against Murine Mammary Carcinoma (FM3A) Cells Transformed with the Herpes Simplex Virus Type 1 or Type 2 Thymidine Kinase Gene

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SUMMARY

Murine mammary carcinoma FM3A cells, deficient in cytosol thymidine (dThd) kinase (TK) activity and transformed by the herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) TK gene (designated FM3A TK$^-$/HSV-1 TK$^+$ and FM3A TK$^-$/HSV-2 TK$^+$, respectively) proved extremely sensitive to the cytostatic action of the potent antiherpetic drugs (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) and (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (IVDU). The fact that FM3A TK$^-$/HSV-2 TK$^+$ cells were 5-fold more sensitive to the cytostatic action of BVDU and IVDU but incorporated [^{125}I] IVDU to a 20-fold lower extent into their DNA than did FM3A TK$^-$/HSV-1 TK$^+$ cells led us to conclude that incorporation of these compounds into DNA of HSV TK gene-transformed cell lines is not directly related to their cytostatic action. In attempts to unravel the mechanism of the cytostatic effects of BVDU and IVDU on HSV TK gene-transformed FM3A cells, both compounds were submitted to an intensive biochemical study. Thymidylate synthase was identified as the principal target enzyme for the cytostatic action of BVDU and IVDU since (i) both compounds were far more inhibitory to 2′-deoxyuridine (dUrd) than to dThd incorporation into HSV TK gene-transformed FM3A cell DNA, (ii) the cytostatic action of BVDU and IVDU was more readily reversed by dThd than by dUrd, (iii) both compounds strongly inhibited the metabolic pathway leading to the incorporation of 2′-deoxycytidine (dCyd) into DNA thymidylate, (iv) BVDU and IVDU strongly inhibited tritium release from [5-^{3}H]dCyd and [5-^{3}H] dUrd in intact HSV TK gene-transformed FM3A cells, and (v) [^{125}I]IVDU accumulated intracellularly as its 5′-monophosphate to concentration levels considerably higher than those required to inhibit partially purified thymidylate synthase. The inhibitory effects mentioned under (i) to (iv) were not observed with the parental FM3A/0 and FM3A/TK$^-$ cells; they were more pronounced for FM3A TK$^-$/HSV-2 TK$^+$ cells than for FM3A TK$^-$/HSV-1 TK$^+$ cells, which correlates with the differential cytostatic effects of BVDU and IVDU on these cells.

BVDU and IVDU are selective anti-herpes compounds with comparatively high potency in cell culture and efficacy in a variety of animal models (1–4). The selectivity of these compounds as inhibitors of HSV replication primarily depends upon a specific and preferential phosphorylation by the HSV-encoded thymidine (dThd) kinase (TK) (5).

In previous papers (6, 7), we described the construction of a TK-deficient FM3A cell line (FM3A/TK$^-$) transformed by a fragment of HSV-1 DNA containing the gene for TK (designated FM3A TK$^-$/HSV-1 TK$^+$) and reported that the transformation of this cell line with the HSV-1 TK gene made it extremely sensitive to the cytostatic action of BVDU and related compounds (7). Evidently, BVDU and IVDU required phosphorylation by the HSV-1 TK to exert their cytostatic action. Evidence was obtained for the incorporation of [^{125}I] IVDU into DNA of the FM3A TK$^-$/HSV-1 TK$^+$ cells, but not FM3A/0 and FM3A/TK$^-$ cells (8, 9). However, to what extent the incorporation of [^{125}I]IVDU into DNA contributed to its cytostatic action against FM3A TK$^-$/HSV-1 TK$^+$ cells remained unclear.

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ABBREVIATIONS: BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; IVDU, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; HSV-1, herpes simples virus type 1; HSV-2, herpes simplex virus type 2; dThd, 2′deoxythymidine; dUrd, 2′-deoxyuridine; dCyd, 2′-deoxycytidine; TK, thymidine kinase; TS, thymidylate synthase; TCA, trichloroacetic acid; IVDUTP, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine 5′-triphosphate; BVaraU, (*E*)-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil; IVDUMP, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine 5′-monophosphate; IVDUDP, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine 5′-diphosphate.

In this paper, we describe the cytostatic and antimetabolic effects of BVDU and IVDU on a novel HSV-2 TK gene-transformed FM3A/TK⁻ cell line (which has been constructed for this purpose), as compared to those obtained on FM3A/0, FM3A/TK⁻, and FM3A TK⁻/HSV-1 TK⁺ cells. [^{125}I]IVDU was also monitored for its metabolism and incorporation into DNA of the four FM3A cell lines. This approach allowed us to delineate the role and contribution of the cellular and HSV-specified TK and the incorporation of BVDU and IVDU into cellular DNA to the cytostatic effects of BVDU and IVDU against HSV-1 and HSV-2 TK gene-transformed cell lines, and led us to identify the cellular TS as the principal target for the cytostatic action of BVDU and IVDU against HSV TK gene-transformed cell lines.

Materials and Methods

Cells. FM3A cells (subclone F28-7), originally established from a spontaneous mammary carcinoma in a C3H/He mouse (10–12) and designated FM3A/0, were cultured as previously described (7). FM3A/TK⁻ cells, which lack host cell TK activity, were originally derived from FM3A/0 cells and maintained in the same culture medium as FM3A/0 (8, 11). The FM3A TK⁻/HSV-1 TK⁺ cell line, which lacks host cell TK activity but contains the HSV-1 TK gene, was derived from the FM3A/TK⁻ cells as published earlier (6, 13). The cells were cultured in the same culture medium as the FM3A/0 cells but supplemented with 10 μM hypoxanthine, 0.2 μM amethopterine, and 16 μM dThd. The FM3A TK⁻/HSV-2 TK⁺ cell line, which lacks host cell TK activity but contains the HSV-2 TK gene, was derived from the FM3A/TK⁻ cells as recently described (14) and was maintained in the same culture medium as the FM3A TK⁻/HSV-1 TK⁺ cells. Neither HSV-1 nor HSV-2 TK gene-transformed FM3A cell lines contained the genes for viral DNA polymerase or ribonucleotide reductase.

Compounds. BVDU and IVDU were synthesized by R. Busson and H. Vanderhaeghe of the Rega Institute for Medical Research (Katholieke Universiteit Leuven, Leuven, Belgium) following a modification of the method described by Jones *et al.* (15). All other reagents used were of the highest quality obtainable.

Radiochemicals. [*methyl*-^{3}H]dThd (specific radioactivity 41 Ci/mmol), [1′,2′-^{3}H]dUrd (specific radioactivity 27 Ci/mmol), [5-^{3}H]dCyd (specific radioactivity 20 Ci/mmol), and [5-^{3}H]dUrd (specific radioactivity 23 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, UK). The synthesis of [^{125}I]IVDU (specific radioactivity 50.1 μCi/μg and 47.7 μCi/μg) has been described (16).

Inhibition of cell proliferation and DNA synthesis. The methods for evaluating the cytostatic effects of compounds against FM3A cells have been described previously (7, 17). Briefly, 5 × 10^4 cells were suspended in growth medium and added to microplate wells in the presence of varying concentrations of the test compounds. The cells were then allowed to proliferate for 48 hr at 37° in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The procedures to measure the incorporation of [*methyl*-^{3}H]dThd, [1′,2′-^{3}H]dUrd, and [5-^{3}H]dCyd into cellular DNA have been described previously (18). Briefly, to each microplate well were added 10^5 FM3A cells, a given amount of test compound, and 0.25 μCi of [*methyl*-^{3}H]dThd, [1′,2′-^{3}H]dUrd, or [5-^{3}H]dCyd. The cells were allowed to proliferate for 20 hr at 37°. At the end of this incubation period, TCA-insoluble material was assayed for radioactivity.

Tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd into FM3A cells. Activity of TS in the intact FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells was measured by estimation of tritium release from [5-^{3}H]deoxyuridylate that had been formed in the cells from [5-^{3}H]dUrd or [5-^{3}H]dCyd. The procedure for the determination of tritium release has been described previously (19).

Inhibition of [5-^{3}H]dCyd and [U-^{14}C]dCyd incorporation into DNA. The incorporation of [5-^{3}H]dCyd and [U-^{14}C]dCyd into cellular DNA was measured as described in Ref. 19. The amount of dCyd incorporation into DNA as dTMP was estimated by subtracting the amount of radioactivity incorporated into DNA with [5-^{3}H]dCyd as radiolabeled precursor from the total amount of radioactivity incorporated into DNA with [U-^{14}C]dCyd as radiolabeled precursor.

Incorporation of [*methyl*-^{3}H]dThd and [^{125}I]IVDU into TCA-insoluble material. Incorporation of [*methyl*-^{3}H]dThd and [^{125}I]IVDU into TCA-insoluble material of FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells was measured as follows. To a suspension of 4 ml of FM3A cells, each containing 10^6 cells/ml, was added 10 μl of [*methyl*-^{3}H]dThd (16 μg, 5 μCi) or 10 μl of [^{125}I]IVDU (2 μg, 10 μCi). The cells were incubated at 37° in a shaking water bath. At 0, 2, 4, 6, 16, and 24 hr, 250 μl of the cell suspension were collected; 1 ml of cold TCA (10%) was added and, upon centrifugation at 1500 × *g* for 10 min at 4°, the pellet was washed twice with 1 ml of cold TCA (10%) and assayed for radioactivity in a toluene-based scintillant.

Incorporation of [*methyl*-^{3}H]dThd and [^{125}I]IVDU into cellular DNA. Incorporation of [*methyl*-^{3}H]dThd and [^{125}I]IVDU into DNA of FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-1 TK⁺, and FM3ATK⁻/HSV-2 TK⁺ cells was evaluated by CsCl density gradient analysis as follows. FM3A cells were seeded at 5 × 10^5 cells/0.5 ml of culture medium and incubated with either [*methyl*-^{3}H]dThd or [^{125}I]IVDU at 10μCi/0.5 μg/tube into a shaking water bath at 37°. After 24 hr, the cells were pelleted, washed twice with cold phosphate-buffered saline, and finally lysed for 30 min at room temperature as described previously (8). The cell lysate was then brought on top of a CsCl solution (ρ = 1.71 g/ml) and centrifuged for 70 hr at 15–18° and 105,000 × *g*. Twenty to 24 fractions of eight drops each were collected from the bottom of the tubes and assayed for radioactivity in a toluene-based scintillant.

Metabolism of [^{125}I]IVDU into FM3A cells. The metabolic products, derived from [^{125}I]IVDU upon incubation with FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells were analyzed as follows. To an FM3A cell suspension at 4 × 10^5 cells/ml was added 2 μM [*methyl*-^{3}H]dThd (2 μCi/ml) or 1 μM [^{125}I]IVDU (2 μCi/ml). After 4 hr incubation at 37°, cells were centrifuged at 1,500 × *g* and washed twice with cold phosphate-buffered saline. To the cell pellet was then added 100 μl of cold TCA (10%), and TCA-soluble material was separated from TCA-insoluble material by centrifugation for 2 min at 10,000 × *g*. The supernatant was thoroughly shaken with a mixture of Freon and tri-*n*-octylamine (4:1) for 30 min at 10° and centrifuged again for 2 min at 10,000 × *g*. The upper phase, containing the metabolites of [*methyl*-^{3}H]dThd or [^{125}I]-IVDU, was then analyzed by thin layer chromatography on Silicagel M 5735 with a mixture of isopropanol/NH_3 (25%)/H_2O (6:3:1). R_f values were as follows: dThd, 0.86; dTMP, 0.34; dTDP, 0.14; dTTP, 0.04; IVDU, 0.86; IVDUMP, 0.43; IVDUDP, 0.25; IVDUTP, 0.11.

Results

Cytostatic and antimetabolic effects of BVDU and IVDU. BVDU and IVDU were evaluated for their inhibitory effects on the proliferation of FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells (Table 1). The growth of FM3A TK⁻/HSV-1 TK⁺ and FM3A TK⁻/HSV-2 TK⁺ cells was inhibited by BVDU and IVDU at concentrations of 0.6–6.0 nM, that is, at a concentration 1,000- to 50,000-fold lower than the concentration required to inhibit the growth of the corresponding wild-type FM3A/0 cells. Also note that FM3A/TK⁻ cells are in general 15- to 65-fold more sensitive to the cytostatic action of BVDU and IVDU than the parent FM3A/0 cells (9).

To explore the biochemical basis for the cytostatic activity of BVDU and IVDU against the HSV TK gene-transformed

TABLE 1
Inhibitory effect of BVDU and IVDU on the proliferation of FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells

Compound	MIC_{50}[a]		ID_{50}[b]			
	HSV-1	HSV-2	FM3A/0	FM3A/TK⁻	FM3A TK⁻/ HSV-1 TK⁺	FM3A TK⁻/ HSV-2 TK⁺
	μM		μM			
BVDU	0.021	6.0	34	0.51	0.006	0.001
IVDU	0.019	5.3	7.4	0.50	0.003	0.001

[a] Minimum inhibitory concentration required to reduce the cytopathogenicity of HSV-1 (KOS) or HSV-2 (G) by 50%. Data taken from Ref. 23.
[b] The 50% inhibitory dose. The data represent the mean values for at least six separate experiments. The range of the individual values was between 10 and 40% of the indicated values.

cell lines in more detail, we examined: (i) the effect of dUrd, dThd, and dCyd on the cytostatic effects of BVDU and IVDU against the FM3A TK⁻/HSV-1 TK⁺ and FM3A TK⁻/HSV-2 TK⁺ cells (Table 2); (ii) the effect of BVDU and IVDU on the incorporation of radiolabeled [1′,2′-^{3}H]dUrd, [*methyl*-^{3}H]dThd, and [5-^{3}H]dCyd into FM3A cell DNA (Table 2); (iii) the effect of BVDU and IVDU on tritium release from [5-^{3}H]dCyd and [5-^{3}H]dUrd in intact FM3A cells (Table 3); and (iv) the effect of BVDU and IVDU on the differential incorporation of dCyd into cell DNA as dCMP and dTMP nucleotide (Table 3).

Addition of dUrd did not significantly reverse the cytostatic effects of BVDU and IVDU on HS-1-transformed FM3A cells but required 10 and 25 times as much BVDU and IVDU to obtain 50% inhibition of HSV-2-transformed cell growth, respectively (Table 2). In contrast, addition of dThd required 4,000–9,000 times as much BVDU and IVDU to obtain 50% inhibition of HSV-1 TK gene-transformed FM3A cell growth and 300,000–1,000,000 times as much BVDU and IVDU to obtain 50% inhibition of HSV-2 TK gene-transformed FM3A cell growth (Table 2). Also, BVDU and IVDU inhibited the incorporation of [1′,2′-^{3}H]dUrd into cellular DNA to a much greater extent than the incorporation of [*methyl*-^{3}H]dThd (difference: 1,000- to 4,000-fold and 120,000- to 400,000-fold for HSV-1 TK- and HSV-2 TK gene-transformed FM3A cells, respectively). Remarkable differences between FM3A TK⁻/HSV-1 TK⁺ and FM3A TK⁻/HSV-2 TK⁺ cells were observed concerning the effect of dCyd on the cytostatic activity of BVDU and IVDU, and the influence of these compounds on the incorporation of [5-^{3}H]dCyd into FM3A cell DNA. Although addition of dCyd had no substantial effect on the inhibitory activity of BVDU and IVDU against FM3A TK⁻/HSV-1 TK⁺ cell proliferation, it dramatically decreased the cytostatic effects of these compounds by 40- to 250-fold in FM3A TK⁻/HSV-2 TK⁺ cells. Moreover, BVDU and IVDU inhibited [5-^{3}H]dCyd incorporation into DNA at a 1,000- to 7,000-fold higher concentration that [1′,2′-^{3}H]dUrd incorporation into DNA of HSV-1 TK gene-transformed FM3A cells, and at a 14,000- to 1,000,000-fold higher concentration than [1′,2′-^{3}H]dUrd incorporation into DNA of HSV-2 TK gene-transformed FM3A cells.

When evaluated for their inhibitory effects on the tritium release from [5-^{3}H]dCyd and [5-^{3}H]dUrd in intact FM3A cells, BVDU and IVDU inhibited ^{3}H release to a much greater extent in the HSV TK gene-transformed cell lines than in the parental FM3A/0 and FM3A/TK⁻ cells (difference: 1,000-fold) (Table 3). None of the cell lines in which inhibition of tritium release was evaluated showed significant differences in tritium release from [5-^{3}H]dUrd versus [5-^{3}H]dCyd.

For FM3A/0 and FM3A/TK⁻ cells, no substantial differences were noted in the inhibitory effects of BVDU and IVDU on the dCyd → dCTP pathway and the dCyd → dTTP pathway. In contrast, dramatic differences were observed for the HSV TK gene-transformed cell lines. In FM3A TK⁻/HSV-1 TK⁺ cells, incorporation of dCyd into DNA through the dCyd → dTTP pathway was inhibited to a 1,000-fold greater extent by BVDU than the incorporation of dCyd into DNA through the dCyd → dCTP pathway. For the FM3A TK⁻/HSV-2 TK⁺ cells, this difference went up to even 100,000-fold.

Incorporation of [*methyl*-^{3}H]dThd and [^{125}I]IVDU into TCA-insoluble material and DNA of FM3A cells. The incorporation of [*methyl*-^{3}H]dThd and [^{125}I]IVDU into TCA-insoluble material of FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-

TABLE 2
Inhibitory effects of BVDU and IVDU on the proliferation and DNA synthesis of FM3A TK⁻/HSV-1 TK⁺ and FM3A TK⁻/HSV-2 TK⁺ cells

Compound	ID_{50} (μM)[a]						
	Cell proliferation				DNA synthesis		
	As such	Upon addition of[b]			As monitored by the incorporation of		
		dUrd	dThd	dCyd	[1′,2′-^{3}H] dUrd	[*methyl*-^{3}H] dThd	[5-^{3}H] dCyd
	FM3A TK⁻/HSV-1 TK⁺ cells						
BVDU	0.006	0.003	15.8	0.003	0.006	5.4	7.1
IVDU	0.003	0.003	27.1	0.001	0.002	7.1	12.4
	FM3A TK⁻/HSV-2 TK⁺ cells						
BVDU	0.001	0.012	835	0.128	0.001	162	180
IVDU	0.001	0.015	652	0.029	0.001	634	1144

[a] The 50% inhibitory dose. Mean values for three to five separate experiments. The range of the individual values was between 5 and 30% of the indicated values.
[b] dUrd, dThd, and dCyd were added at subtoxic concentrations (i.e., 550 μM, 20 μM, and 2200 μM, respectively).

TABLE 3

Inhibitory activity of BVDU and IVDU on tritium release from [5-^{3}H]dUrd and [5-^{3}H]dCyd

Compound	ID_{50}[a] for ^{3}H release from		ID_{50}[a]	
	[5-^{3}H] dUrd	[5-^{3}H] dCyd	dCyd→dCTP pathway[b]	dCyd→dTTP pathway[c]
	μM		μM	
FM3A/0				
BVDU	3.1	6.2	0.050[d]	0.313[d]
IVDU	9.9	8.4	0.091[d]	0.294[d]
FM3A/TK⁻				
BVDU		7.8	0.063[d]	0.040[d]
IVDU		8.8	0.143[d]	0.046[d]
FM3A TK⁻/HSV-1 TK⁺				
BVDU	0.004	0.008	3.1	0.004
IVDU	0.009	0.006		
FM3A TK⁻/HSV-2 TK⁺				
BVDU	0.007	0.007	45	0.001
IVDU	0.010	0.011		

[a] The 50% inhibitory dose. Mean value for three to five separate experiments. The range of the individual values was between 10 and 40% of the indicated values.
[b] As monitored by incorporation of radiolabel into DNA with [5-^{3}H]dCyd as precursor.
[c] As monitored by subtracting the amount of radiolabeled material incorporated into DNA with [5-^{3}H]dCyd as precursor from the amount of radiolabeled material incorporated into DNA with [U-^{14}C]dCyd as precursor.
[d] Values taken from Ref. 19.

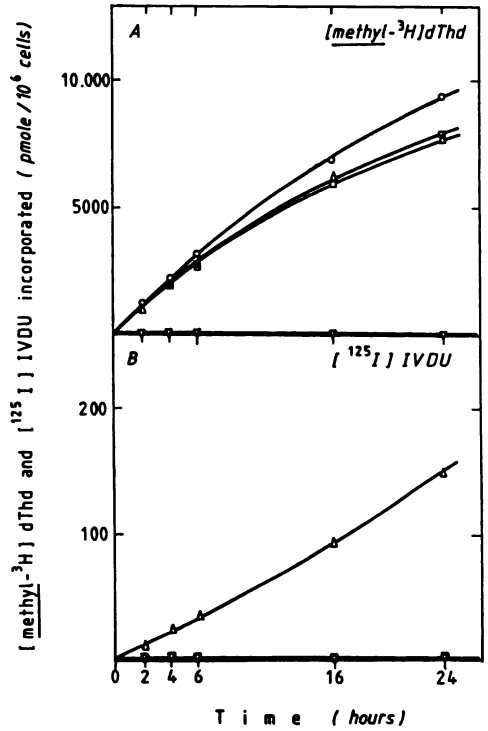


Fig. 1. Incorporation of [*methyl*-^{3}H]dThd (A) and [^{125}I]IVDU (B) into TCA-insoluble material of FM3A/0 (○), FM3A/TK⁻ (▽), FM3A TK⁻/HSV-1 TK⁺ (△), and FM3A TK⁻/HSV-2 TK⁺ (□) cells as a function of incubation time.

1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells was measured. As shown in Fig. 1, [*methyl*-^{3}H]dThd incorporation into TCA-insoluble material was linear for up to 12 hr for the FM3A/0, FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells. [*methyl*-^{3}H]dThd was incorporated into TCA-insoluble material of both HSV-1 TK- and HSV-2 TK gene-transformed FM3A cell lines to an equal extent. The TK-deficient FM3A/TK⁻ cells did not incorporate [*methyl*-^{3}H]dThd to a marked extent (Fig. 1). With [^{125}I]IVDU, no incorporation could be detected into TCA-insoluble material of either FM3A/0 and FM3A/TK⁻ cells. In contrast, [^{125}I]IVDU was clearly incorporated into TCA-insoluble material of FM3A TK⁻/HSV-1 TK⁺ cells. Under our experimental conditions, the incorporation was linear for at least 24 hr, although [^{125}I]IVDU was incorporated ~50-fold less efficiently into this cell line than was [*methyl*-^{3}H]dThd. The amount of [^{125}I]IVDU incorporated into TCA-insoluble material of FM3A TK⁻/HSV-2 TK⁺ cells, following a 24-hr incubation period of the radiolabeled compound with the cells, was ~20-fold lower than for the FM3A TK⁻/HSV-1 TK⁺ cells (Fig. 1). Thus, the presence of HSV-1 TK activity, but not HSV-2 TK activity, appeared to be an absolute prerequisite for the extensive incorporation of [^{125}I]IVDU into acid-insoluble material of the HSV TK gene-transformed cells.

By CsCl gradient analysis, it was ascertained that [*methyl*-^{3}H]dThd was incorporated to a comparable extent into DNA of FM3A/0 (9), FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells (Fig. 2). No [*methyl*-^{3}H]dThd was incorporated into DNA of the TK-deficient FM3A/TK⁻ cell line (9). With [^{125}I]IVDU, FM3A TK⁻/HSV-1 TK⁺ cell DNA could be nicely labeled, showing a density of 1.73 g/ml (Fig. 2). No [^{125}I]IVDU was incorporated into DNA of FM3A/0 and FM3A/TK⁻ cells (9). Little [^{125}I]IVDU was incorporated into DNA of FM3A TK⁻/HSV-2 TK⁺ cells. The amount of [^{125}I]IVDU incorporated into DNA of these cells was only 1/20 of the amount incorporated into FM3A TK⁻/HSV-1 TK⁺ cell DNA. These data are in full agreement with the data obtained from the TCA-insoluble radioactivity analyses.

Metabolism of [*methyl*-^{3}H]dThd and [^{125}I]IVDU in FM3A cells. The metabolic fate of [*methyl*-^{3}H]dThd and [^{125}I]IVDU in FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells was examined analyzing the TCA-soluble extracts of drug-treated cells by thin layer chromatography (Table 4). After a 4-hr incubation period of the cells with 2 μM [*methyl*-^{3}H]dThd, the predominant phosphorylated [*methyl*-^{3}H]dThd metabolite detected was dTTP; the intracellular concentration of dTTP was 4- to 6-fold higher than the dTMP and dTDP levels in FM3A/0 cells, and at least 20- to 80-fold higher than the dTMP and dTDP levels in the HSV-1 (or HSV-2) TK gene-transformed FM3A cell lines. In FM3A/TK⁻ cells, only low dTTP levels were detected. These

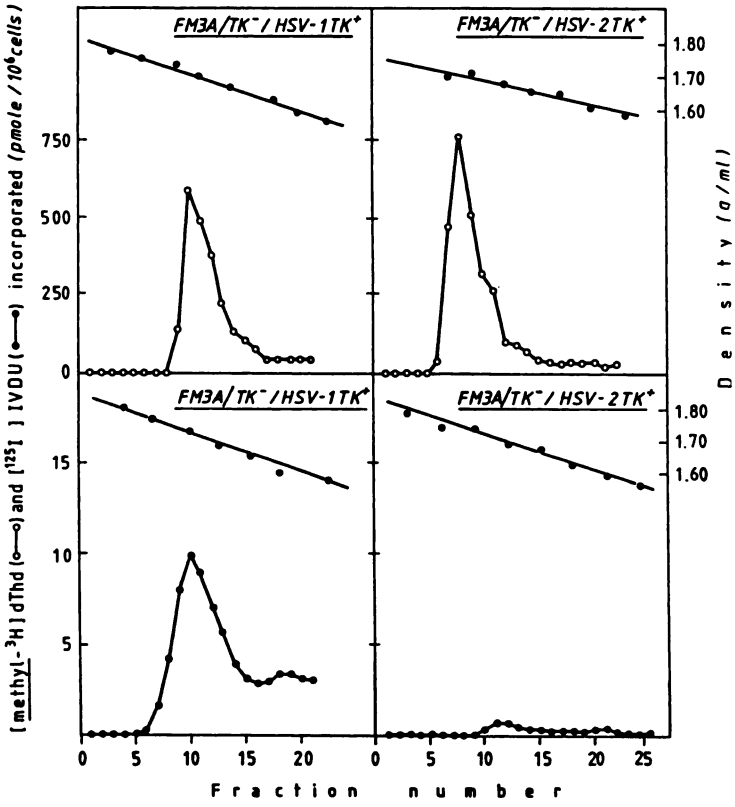


Fig. 2. CsCl equilibrium density gradient profile of DNA from FM3A TK⁻/HSV-1 TK⁺ and FM3A TK⁻/HSV-2 TK⁺ cells incubated with [*methyl*-^{3}H]dThd (○) or [^{125}I]IVDU (●).

TABLE 4

Intracellular concentration of phosphorylated metabolites of [*methyl*-^{3}H]dThd and [^{125}I]IVDU in FM3A/0, FM3A/TK⁻, FM3A TK⁻/HSV-1 TK⁺, and FM3A TK⁻/HSV-2 TK⁺ cells after a 4-hr incubation period

Compound[a]	FM3A/0	FM3A/TK⁻	FM3A TK⁻/HSV-1 TK⁺	FM3A TK⁻/HSV-2 TK⁺
	pmol/10⁸ cells (μM)			
dTMP	2.46	0.01	0.14	0.59
dTDP	3.51	0.01	0.71	1.63
dTTP	15.77	0.27	11.94	43.93
IVDUMP	0.05	0.13	9.03	5.57
IVDUDP	0.03	0.09	0.17	0.06
IVDUTP	0.02	0.02	0.41	0.19

[a] The initial extracellular concentrations of [*methyl*-^{3}H]dThd and [^{125}I]IVDU were 2 μM and 1 μM, respectively. The data represent the mean values for at least three to five separate experiments. The range of the individual values was between 10 and 35% of the indicated values.

did not exceed ~2% of the amounts recorded with the parental FM3A/0 cell line.

When the phosphorylated metabolites of [^{125}I]IVDU were determined in the FM3A cell lines under identical experimental conditions where [*methyl*-^{3}H]dThd anabolism was determined (except for the initial concentration of [^{125}I]IVDU, which was 1 μM instead of 2 μM), IVDU 5′-monophosphate was the predominant species found in both HSV TK gene-transformed FM3A cell lines; it was 20- to 30-fold more abundant than IVDU 5′-triphosphate and even 50- to 100-fold more abundant than IVDUDP. The concentration of [^{125}I]IVDUTP found in FM3A TK⁻/HSV-1 TK⁺ cells was 30-fold lower than the [*methyl*-^{3}H]dTTP levels (as recorded in the previous experiment), whereas the [^{125}I]IVDUTP levels in FM3A TK⁻/HSV-2 TK⁺ cells were more than 200-fold lower than the intracellular [*methyl*-^{3}H]dTTP levels.

Discussion

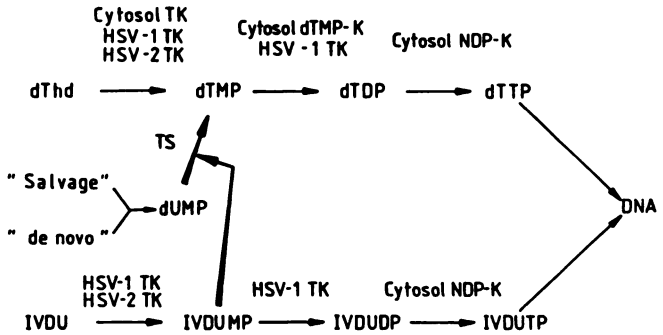
BVDU and IVDU inhibit the proliferation of HSV-1 TK and HSV-2 TK gene-transformed FM3A cell lines at a 2,000- to 40,000-fold lower concentration than that required to inhibit the growth of the parental FM3A/0 cells. FM3A TK⁻/HSV-2 TK⁺ cells were even 5-fold more susceptible to the cytostatic effects of BVDU and IVDU than FM3A TK⁻/HSV-1 TK⁺ cells. Evidently, BVDU and IVDU require phosphorylation by the HSV TK to exert their inhibitory effect on the growth of the HSV-1 TK gene- and HSV-2 TK gene-transformed FM3A cell lines.

We have previously pointed out that this phosphorylation may be necessary but not sufficient to ensure a cytostatic effect of BVDU and IVDU in HSV-1-transformed cells, since BVaraU, a close analogue of BVDU whose anti-herpetic activity also depends on a specific phosphorylation by the viral TK (20), does not significantly affect the growth of HSV-1 TK gene-transformed cells (ID_{50} > 300 μg/ml and 450 μg/ml for FM3A/0 and FM3A TK⁻/HSV-1 TK⁺ cells, respectively) (8). The lack of cytostatic activity of BVaraU against the HSV-1 TK gene-transformed cell line was tentatively attributed to an insufficient phosphorylation of the compound within the transformed cells and/or peculiarities in the interaction of BVaraU 5′-triphosphate at the DNA polymerase level (8). Indeed, BVDU and IVDU are incorporated in the interior of the DNA chain, whereas BVaraU is incorporated at the 3′-terminal, and this incorporation may be reversible since 3′ → 5′ exonucleases could easily remove such terminal nucleotides (21, 22).

Now we have obtained clear evidence that incorporation of

IVDU into cellular DNA is not obligatory to achieve its cytostatic effect on HSV-2 TK gene-transformed cells. IVDU is clearly more cytostatic for FM3A TK⁻/HSV-2 TK⁺ than for FM3A TK⁻/HSV-1 TK⁺ cells, whereas the incorporation of IVDU into FM3A TK⁻/HSV-2 TK⁺ cell DNA is at least 20-fold less pronounced than for FM3A TK⁻/HSV-1 TK⁺ cell DNA. Also, DNA, RNA, and protein synthesis in IVDU-treated FM3A TK⁻/HSV-1 TK⁺ cells is completely restored after the drug has been washed out from the cells, indicating that under our experimental conditions, incorporation of IVDU into cell DNA has no severe irreversible inhibitory effects on crucial cell functions (i.e., replication, transcription, translation) (8). That [^{125}I]IVDU is incorporated more extensively into FM3A TK⁻/HSV-1 TK⁺ cell DNA than FM3A TK⁻/HSV-2 TK⁺ cell DNA is most likely due to a more intensive flow of IVDU to IVDUTP in the FM3A TK⁻/HSV-1 TK⁺ cells than in FM3A TK⁻/HSV-2 TK⁺ cells.

Such differential phosphorylation rates can be rationalized in view of the differences that exist in the properties of the HSV-1 and HSV-2 TKs. IVDU (and BVDU) not only have a higher affinity for the HSV-1-encoded TK than for the HSV-2-encoded TK (5), but, also, the HSV-1-specified dTMP kinase activity (23) is able to further convert the 5′-monophosphates of IVDU (and BVDU) to their 5′-diphosphates, whereas the HSV-2-specified TK is endowed with only ~2% of the dTMP kinase activity of the HSV-1 enzyme (24). These data suggest a possible partial block in the metabolism of IVDU (and BVDU) in HSV-2-infected cells at the 5′-monophosphate level. Thus, in HSV-1-infected cells IVDU may be readily converted via IVDUMP and IVDUDP to IVDUTP, whereas in HSV-2-infected cells IVDU metabolism may be blocked at the 5′-monophosphate level (25). The same assumption can obviously be made for the metabolism of BVDU in HSV-infected cells. This rationale has been extended for the HSV-1 and HSV-2 TK gene-transformed FM3A cells in Scheme 1.



Scheme 1. Metabolism of dThd and IVDU in HSV TK gene-transformed FM3A cells. *dTMP-K*, thymidylate kinase; *NDP-K*, nucleoside 5′-diphosphate kinase.

The different properties of the HSV-1 and HSV-2 dThd kinases may not only account for the higher activity of BVDU and IVDU against HSV-1 than HSV-2 (2, 26), but also may explain the more extensive incorporation of [^{125}I]IVDU into DNA of FM3A TK⁻/HSV-1 TK⁺ cells than of FM3A TK⁻/HSV-2 TK⁺ cells (Fig. 2). Thus, the fact that IVDU is incorporated into FM3A TK⁻/HSV-1 TK⁺ cell DNA to a 20-fold greater extent than into FM3A TK⁻/HSV-2 TK⁺ cell DNA—but is 5-fold less cytostatic against FM3A TK⁻/HSV-1 TK⁺ than FM3A TK⁻/HSV-2 TK⁺ cells—argues against incorporation of the compound into DNA of the HSV TK gene-transformed FM3A cell lines as the principal reason for its cytostatic action. However, we were able to identify the crucial role of thymidylate synthase (TS) in the cytostatic action of BVDU and IVDU against HSV TK gene-transformed FM3A cells. It has been postulated previously (17) that those dUrd analogues that are more inhibitory to dUrd than to dThd incorporation into cell DNA, and whose cytostatic effects are more readily reversed by dThd than by dUrd, may owe their cytostatic activity to a selective inhibition of dTMP synthase (see Table 2). Furthermore, the differential effects of BVDU and IVDU on the incorporation of dCyd into DNA via the dCyd → dTTP or dCyd → dCTP pathway (see Table 3) and their potent inhibitory effects on the release of tritium from [5-^{3}H]dUrd and [5-^{3}H]dCyd within the cells (see Table 3) strongly point to TS as the principal, if not the sole, target enzyme in the marked cytostatic activity of BVDU and IVDU against HSV TK gene-transformed FM3A cell lines.

Additional evidence that TS might be envisaged as the principal target for the cytostatic action of BVDU and IVDU against the HSV TK gene-transformed FM3A cells was obtained by determining the phosphorylated metabolites of [*methyl*-^{3}H]dThd and [^{125}I]IVDU in the different FM3A cell lines. Upon incubation with [*methyl*-^{3}H]dThd, all four FM3A cell lines acquired [*methyl*-^{3}H]dTTP levels which were significantly higher than the 5′-mono- or 5′-diphosphate levels. In contrast, upon incubation of the HSV TK gene-transformed FM3A cells with [^{125}I]IVDU, a marked accumulation of the 5′-monophosphate (IVDUMP) ensued. The intracellular levels of IVDUMP (5–10 μM) were 20- to 30-fold higher than those of the corresponding 5′-triphosphate. The levels of IVDUMP and BVDU 5′-monophosphate attained intracellularly exceeded the concentrations required to inhibit TS activity in cell-free conditions as determined earlier (K_i, 1.2 μM; K_i/K_m, 0.6) (27).

The fact that [^{125}I]IVDU was incorporated into FM3A TK⁻/HSV-1 TK⁺ cell DNA to a 20-fold greater extent than into FM3A TK⁻/HSV-2 TK⁺ cell DNA (measured after a 24-hr incubation period) does not appear to correlate with the only 2-fold higher IVDUTP levels in FM3A TK⁻/HSV-1 TK⁺ cells than in FM3A TK⁻/HSV-2 TK⁺ cells (measured after a 4-hr incubation period). After a 20-hr incubation period, the IVDUTP levels in FM3A TK⁻/HSV-1 TK⁺ cells were 6-fold higher than those in FM3A TK⁻/HSV-2 TK⁺ cells (data not shown), the discrepancy with the differential amounts of IVDU incorporated into DNA thus becoming less meaningful.

In conclusion, our data point to TS as the principal target enzyme for the inhibitory effects of BVDU and IVDU on the growth of HSV (type 1 and 2) TK gene-transformed FM3A cell lines. The HSV-specified TK is required for the activation of BVDU and IVDU, whereas abundant incorporation of the compounds into cell DNA does not appear to be a prerequisite for their cytostatic action. However, the possibility would remain that inhibition of virus-coded DNA polymerase by IVDU- or BVDU 5′-triphosphate, or incorporation of IVDU or BVDU 5′-monophosphate into viral DNA might contribute to the antiherpetic activity of these compounds.

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