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Highly Selective Cytostatic Activity of (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine Derivatives for Murine Mammary Carcinoma (FM3A) Cells Transformed with the Herpes Simplex Virus Type 1 Thymidine Kinase Gene

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SUMMARY

(*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (BVDU) and various structurally related analogues thereof, i.e., (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (IVDU) and (*E*)-5-(2-bromovinyl)-2′-deoxycytidine (BVDC), and the carbocyclic analogues of BVDU, IVDU, and BVDC, were evaluated for their inhibitory effects on the growth of murine mammary carcinoma FM3A cells, deficient in thymidine kinase (TK) activity but transformed with the herpes simplex virus type 1 (HSV-1) TK gene (designated FM3A/TK$^-$/HSV-1 TK$^+$). BVDU and its congeners were much more inhibitory to the growth of FM3A/TK$^-$/HSV-1 TK$^+$ than to the growth of the wild type (FM3A/0) cells. For BVDU, for example, the 50% inhibitory dose for the FM3A/TK$^-$/HSV-1 TK$^+$ cells was 0.5 ng/ml, as compared to 11 μg/ml for the FM3A/0 cells. Evidently, BVDU and its congeners required phosphorylation by the HSV-1 TK to exert their cytostatic action. In attempts to evaluate further the mechanism of this cytostatic action, BVDU, IVDU, and their carbocyclic analogues were evaluated for their inhibitory effects on thymidylate synthetase (TS) and their incorporation into DNA. TS was identified as one, but not the sole, target in the cytostatic activity of BVDU and its derivatives. With [^{125}I]IVDU and its carbocyclic analogue C-[^{125}I]IVDU, clear evidence was obtained for the incorporation of these radiolabeled analogues into DNA of the FM3A/TK$^-$/HSV-1 TK$^+$ cell line and a TS-deficient mutant thereof, FM3A/TK$^-$/HSV-1 TK$^+$/TS$^-$. No incorporation was detected with [^{125}I]IVDU or C-[^{125}I]IVDU into DNA of FM3A/0 and FM3A/TS$^-$ cells. To what extent the incorporation of [^{125}I]IVDU and C-[^{125}I]IVDU contributed to their cytostatic action against FM3A/TK$^-$/HSV-1 TK$^+$ cells remains the subject of further study.

INTRODUCTION

BVDU[1] is a potent and selective inhibitor of the replication of several herpes viruses (i.e., HSV-1, varicella zoster virus, suid herpesvirus type 1, bovid herpesvirus type 1, and simian varicella virus) in both cell culture and animal systems (1–4). The activity of BVDU is specifically directed toward virus-infected cells; it does not affect the growth of uninfected cells (1). Its selectivity primarily depends on a preferential phosphorylation by the virus-induced thymidine (dThd) kinase (5). Also, BVDU 5′-triphosphate effects a greater inhibition of HSV-1 DNA polymerase than of the cellular DNA polymerases α, β, and γ (6). Furthermore, BVDU 5′-triphosphate and its iodo analogue, IVDU 5′-triphosphate, can substitute for dTTP as substrates for both viral and cellular DNA polymerases (7, 8). These substitutions have little, if any, effect on the primer-template activity of the resulting DNA (9). As demonstrated by Mancini *et al.* (10), the extent of virus yield reduction appears to be correlated with the amount of BVDU substituted for thymidine in HSV-1 DNA and the concomitant decrease in stability of this DNA (10). However, many of the

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[1] The abbreviations used are: BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; HSV-1, herpes simplex virus type 1; dThd, deoxythymidine; TS, thymidylate synthetase; TK, thymidine kinase; IVDU, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; BVDC, (*E*)-5-(2-bromovinyl)-2′-deoxycytidine; C-BVDU, C-IVDU, and C-BVDC, carbocyclic analogues of BVDU, IVDU, and BVDC, respectively; BVaraU, 1-β-D-arabinofuranosyluracil; BVRU, (*E*)-5-(2-bromovinyl)uridine; BVU, (*E*)-5-(2-bromovinyl)uracil; BVDDU, (*E*)-5-(2-bromovinyl)-2′,3′-dideoxyuridine; TCA, trichloroacetic acid.

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consequences resulting from the incorporation of BVDU into DNA of virus-infected cells still remain unclear.

Previously, we have described a TS-deficient mutant cell line (designated FM3A/TS$^-$) (11–15) and a dThd kinase (TK)-deficient mutant cell line (designated FM3A/TK$^-$) (16), both derived from mutagenized murine mammary carcinoma FM3A/0 cells. Recently, we constructed a TK-deficient FM3A cell line transformed with a fragment of HSV-1 DNA containing the TK gene but not including the gene coding for DNA polymerase or NDP reductase (designated FM3A/TK$^-$/HSV-1 TK$^+$) (17). Furthermore, a TS-deficient subline was selected from the FM3A/TK$^-$/HSV-1 TK$^+$ cell line and designated FM3A/TK$^-$/HSV-1 TK$^+$/TS$^-$ (17). We have also demonstrated that the FM3A/TS$^-$ cell line, which is auxotrophic for dThd, represents a very useful system for monitoring the incorporation of dThd analogues into cellular DNA (14, 15).

We have now evaluated the inhibitory effects of BVDU and structurally related analogues thereof (i.e., IVDU, BVDC, C-BVDU, C-IVDU, C-BVDC, BVaraU, BVRU, BVU, BVDDU, and two methylated derivatives of BVDU (3-*N*-methyl-BVDU and 3′-*O*-methyl-BVDU)) on the growth of FM3A/0 and FM3A/TK$^-$/HSV-1 TK$^+$ cells. Two radiolabeled congeners of this series, i.e., [^{125}I]IVDU and C-[^{125}I]IVDU, were monitored for their incorporation into DNA of both FM3A/0 and FM3A/TK$^-$/HSV-1 TK$^+$ cells, and their TS-deficient counterparts FM3A/TS$^-$ and FM3A/TK$^-$/HSV-1 TK$^+$/TS$^-$. By using these single, double, and triple mutant cell lines, deeper insight should be obtained into the relative contributions of the viral TK, cellular TS, and incorporation into cellular DNA toward the cytostatic effects of BVDU and its derivatives on HSV-1-transformed cells.

MATERIALS AND METHODS

Cells. FM3A cells (subclone F28-7), originally established from a spontaneous mammary carcinoma in a C3H/He mouse (12, 16, 18), and designated FM3A/0, were grown in 75-cm^2 tissue culture flasks (Sterilin, Teddington, England) in Eagle's minimum essential medium supplemented with 10% (v/v) inactivated fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland), and 0.075% (w/v) $NaHCO_3$ (Flow Laboratories). FM3A/TS$^-$ cells were derived from FM3A/0 cells as described previously (11) and were maintained in the same culture medium but supplemented with 20 μM dThd (14, 15).

The FM3A/TK$^-$ cell line, which lacks host cell TK activity, was originally derived from FM3A/0 cells by selection in the presence of 5-bromo-2′-deoxyuridine (16). A subclone of FM3A/TK$^-$ cells with an additional deficiency for adenine phosphoribosyl transferase was used in our experiments.

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 by introduction of the HSV-1 TK gene using pBR322 plasmid containing the 3.6-kb *Bam*H 1 fragment of HSV-1 DNA at a *Bam*H1 site (17, 19). The cells were cultured in the same medium as the FM3A/0 cells.

From the FM3A/TK$^-$/HSV-1 TK$^+$ cells, a TS-deficient subclone was derived following a previously described procedure (11). The cells were cultured in the same medium as the FM3A/TS$^-$ cells; that is, in the presence of 20 μM dThd.

Compounds. The sources of the compounds were as follows. BVDU and IVDU were synthesized by R. Busson and H. Vanderhaeghe of the Rega Institute for Medical Research (Katholieke Universiteit Leuven, B-3000 Leuven, Belgium) following a modification of the method described by Jones *et al.* (20). BVDC, 3′-*O*-methyl-BVDU, and 3-*N*-methyl-BVDU were provided by R. T. Walker (University of Birmingham, Birmingham, U.K.) (see also Ref. 20). The carbocyclic analogues C-BVDU, C-IVDU, and C-BVDC were synthesized as described by Herdewijn *et al.* (21). BVDDU was obtained from W. H. Prusoff (Yale University, New Haven, CT); BVaraU was a gift from H. Machida (Yamasa Shoyu Co., Choshi, Japan) (see also Ref. 22). BVRU was a gift from L. Ötvös (Hungarian Academy of Sciences, Budapest, Hungary), and BVU was synthesized as described by De Clercq *et al.* (23).

Radiochemicals. [*methyl*-^{3}H]dThd (specific radioactivity 47 Ci/mmol), [1′,2′-^{3}H]dUrd (specific radioactivity 27 Ci/mmol), [5-^{3}H]Urd (specific radioactivity 25 Ci/mmol), and [4,5-^{3}H]leucine (specific radioactivity 120 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, U.K.). The synthesis of [^{125}I]IVDU (specific radioactivity 1.89 Ci/mmol) and C-[^{125}I]IVDU (specific radioactivity 2.4 Ci/mmol) has been described (24).

Inhibition of cell growth and DNA synthesis. All assays were performed as described previously (14, 15, 25, 26).

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/TS$^-$, and FM3A/TK$^-$/HSV-1 TK$^+$ and FM3A/TK$^-$/HSV-1 TK$^+$/TS$^-$ cells was measured as follows. To a suspension of 2 ml of FM3A/0, FM3A/TS$^-$, FM3A/TK$^-$/HSV-1 TK$^+$, and FM3A/TK$^-$/HSV-1 TK$^+$/TS$^-$ cells, each containing 10^6 cells/ml, was added 20 μl of [*methyl*-^{3}H]dThd (1 μg, 2 μCi) or 10 μl of [^{125}I]IVDU (1 μg, 1.2 μCi). The cells were incubated at 37° in a shaking water bath. At 0, 2, 4, 8, 12, and 24 hours, 250 μl of the cell suspension (2.5×10^5 cells) was 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 [^{125}I]IVDU and C-[^{125}I]IVDU into cellular DNA. Incorporation of [^{125}I]IVDU and C-[^{125}I]IVDU into DNA of FM3A/0, FM3A/TS$^-$, FM3A/TK$^-$/HSV-1 TK$^+$, and FM3A/TK$^-$/HSV-1 TK$^+$/TS$^-$ cells was evaluated by CsCl density gradient analysis. Therefore, FM3A cells were seeded at 5×10^5 cells/0.5 ml of culture medium and incubated with either [^{125}I]IVDU or C-[^{125}I]IVDU at 1.8 μCi/0.5 μg/tube into a shaking water bath at 37°. After 24 hr, the cells were pelleted by centrifugation, washed twice with cold phosphate-buffered saline, and finally lysed by addition of 100 μl of 0.2% sodium dodecyl sulfate in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 0.5% sarcosyl. The cell lysate was then brought on top of a CsCl solution (ρ = 1.7089 g/ml) and centrifuged for 25 hr at 20° and 200,000 × *g*. Twenty to 24 fractions of 10 drops each were collected from the bottom of the tubes and assayed for radioactivity in a toluene-based scintillant.

Measurement of cell growth, DNA, RNA, and protein synthesis following preincubation of the cells with IVDU. FM3A/TK$^-$/HSV-1 TK$^+$/TS$^-$ cell suspensions containing 3×10^5 cells/ml were preincubated with IVDU at 1, 0.05, 0.0025, and 0.0001 μg/ml for 0, 2, 4, or 6 hr in a shaking water bath at 37°. After the indicated incubation period, the cells were washed extensively and seeded at 60,000 cells/200 μl of Eagle's minimum essential medium in 96-multiwell Microtest plates (Falcon, Becton Dickinson, Oxnard, CA). For the cell growth experiments the cells were incubated in the presence of 20 μM dThd and the cell number was determined every 12 hr. For the DNA, RNA, and protein synthesis measurements, the cells were incubated with [*methyl*-^{3}H]dThd, [5-^{3}H]Urd, or [4,5-^{3}H] leucine, each at 2 μCi/ml, for 2 hr in the presence of 2 μM dThd. The amount of DNA, RNA, and protein synthesized was determined by precipitating the cells with cold TCA (10%), washing the precipitate three times with TCA (5%), and assaying the TCA-insoluble material for radioactivity into a tuluene-based scintillant.

RESULTS

Inhibitory effects of BVDU and related compounds on the proliferation of FM3A/0 and FM3A/TK$^-$/HSV-1

TK⁺ cells. BVDU and its analogues were evaluated for their inhibitory effects on the growth of FM3A/0 and FM3A/TK⁻/HSV-1 TK⁺ cells (Table 1). All those compounds having MIC_{50} values for HSV-1 replication were far below 1 μg/ml proved much more inhibitory to the proliferation of FM3A/TK⁻/HSV-1 TK⁺ than of FM3A/0 cells (i.e., BVDU, 22,800-fold; BVDC, 20,250-fold; IVDU, 5,600-fold; C-BVDU, >2,336-fold; C-BVDC, >846-fold; and C-IVDU, >71-fold). The only exception to this rule was BVaraU, which showed an MIC_{50} value of 0.1 μg/ml for HSV-1 replication, yet did not inhibit the growth of FM3A/TK⁻/HSV-1 TK⁺ cells to a greater extent than the growth of FM3A/0 cells.

The methyl-substituted BVDU derivatives 3-*N*-methyl-BVDU and 3′-*O*-methyl-BVDU (MIC_{50} for HSV-1 replication: 20 and ≥400 μg/ml, respectively) were 70- and 14-fold more potent in inhibiting FM3A/TK⁻/HSV-1 TK⁺ than FM3A/0 cell growth (Table 1). However, the free pyrimidine base BVU and the riboside BVRU were, respectively, >37- and 56-fold more inhibitory to FM3A/TK⁻/HSV-1 TK⁺ than FM3A/0 cell growth. Finally, BVDDU was totally inactive in inhibiting the growth of both FM3A/0 and FM3A/TK⁻/HSV-1 TK⁺ cells.

It should be pointed out that the inhibitory effects of BVDU and related compounds on the growth of FM3A/TS⁻ and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells could not be assessed, since TS-deficient cells fail to grow in normal culture medium. For TS⁻ mutant cell lines to grow, dThd (20 μM) must be added to the culture medium, and it has previously been established that BVDU, IVDU, and their congeners cannot substitute for dThd in sustaining the growth of FM3A/TS⁻ cells (14) and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells (25).

Studies aimed at evaluating the role of TS in the inhibitory effects of BVDU and related compounds on the proliferation of FM3A/0 and FM3A/TK⁻/HSV-1 TK⁺ cells. To evaluate the role of TS as a possible target for the cytostatic activity of BVDU and its analogues, the following parameters were used: 1) a relatively greater inhibition of incorporation of [1′,2′-^{3}H]dUrd than of [*methyl*-^{3}H]dThd into DNA and 2) a more pronounced reversal of cell growth inhibition upon addition of dThd than of dUrd. Parameter 1 has proven to be a more sensitive marker of TS inhibition than parameter 2 (26).

The cytostatic effects of BVDU and IVDU on FM3A/0 cells could to a large extent be attributed to an inhibition of TS. Addition of dUrd reversed the cytostatic effects of BVDU and IVDU by 16- and 14-fold, respectively, while addition of dThd did so by 52- and 137-fold (Table 2). Also, BVDU and IVDU inhibited the incorporation of [1′,2′-^{3}H]dUrd into DNA to a much greater extent than the incorporation of [*methyl*-^{3}H]dThd (differences in ID_{50} of BVDU and IVDU for dThd and dUrd incorporation: 52- and 79-fold, respectively). C-BVDU was not cytostatic for FM3A/0 cells (ID_{50} ≥ 1000 μg/ml) and did not affect [1′,2′-^{3}H]dUrd incorporation to a significantly greater extent than [*methyl*-^{3}H]dThd incorporation (Table 2).

TS could also be considered as one of the targets for the cytostatic effects of BVDU and IVDU on FM3A/TK⁻/HSV-1 TK⁺ cells: addition of dUrd reversed their cytostatic effects by 4- and 6-fold, respectively, while addition of dThd did so by 80- and 60-fold (Table 2). Also, the ratio of the ID_{50} for [*methyl*-^{3}H]dThd incorporation to the ID_{50} for [1′,2′-^{3}H]dUrd incorporation was as high as 2850 for BVDU and 1400 for IVDU (Table 2). However, TS may not be considered as the sole target for the cytostatic action of BVDU and IVDU against FM3A/TK⁻/HSV-1 TK⁺, since the addition of dThd did only partially reverse the inhibitory effects of BVDU and IVDU on cell growth. In fact, even in the presence of dThd, BVDU and IVDU inhibited the growth of FM3A/TK⁻/HSV-1 TK⁺ cells at a concentration of 0.04 and 0.03 μg/ml, respectively (Table 2). C-BVDU was significantly less inhibitory to the proliferation of FM3A/TK⁻/HSV-1 TK⁺ cells than were BVDU and IVDU, and

TABLE 1

Inhibitory effects of BVDU and related compounds on the proliferation of FM3A/0 and FM3A/TK⁻/HSV-1 TK⁺ cells

Compound	MIC_{50}[a]	ID_{50}[b]		Ratio
		FM3A/0	FM3A/TK⁻/HSV-1 TK⁺	ID_{50} (FM3A/0) / ID_{50} (FM3A/TK⁻/HSV-1 TK⁺)
	μg/ml		μg/ml	
BVDU	0.007[c]	11.4[d]	0.0005	22,800
C-BVDU	0.07[c]	>500	0.214	>2,336
IVDU	0.007[c]	2.8	0.0005	5,600
C-IVDU	0.07[c]	>100	1.4	>71
BVDC	0.07[c]	16.2	0.0008	20,250
C-BVDC	0.2[c]	≫500	0.775	846
BVaraU	0.1[e]	≥300	450	≥0.7
3-*N*-Methyl-BVDU	20	349	4.97	70
3′-*O*-Methyl-BVDU	≥400	528	38	14
BVRU	0.4 → > 400[d]	234	4.17	56
BVU	2 → > 400[d]	>300	8.16	>37
BVDDU	>400	>100	>100	

[a] Minimum inhibitory concentration required to reduce the cytopathogenicity of HSV-1 (KOS) by 50%.
[b] Fifty per cent inhibitory dose; mean value for 4–6 separate experiments.
[c] See Ref. 21.
[d] See Ref. 23; inhibitory effects of BVRU and BVU on HSV-1 cytopathogenicity varied markedly depending on the choice of cell system.
[e] See Ref. 27.

TABLE 2

Inhibitory effects of BVDU, IVDU, and C-BVDU on the growth and DNA synthesis of FM3A/0 and FM3A/TK⁻/HSV-1 TK⁺ cells

Compound	ID_{50}[a] (μg/ml)				
	Cell growth			DNA synthesis	
	Alone	Upon addition of dUrd (125 μg/ml)	Upon addition of dThd (5 μg/ml)	[1′,2′-^{3}H]dUrd incorporation into DNA	[*methyl*-^{3}H]dThd incorporation into DNA
			FM3A/0 cells		
BVDU	11.4	181	599	6.41	331
IVDU	2.8	38.6	384	4.91	387
C-BVDU	≥1000	≥1000	≥1000	271	407
			FM3A/TK⁻/HSV-1 TK⁺ cells		
BVDU	0.0005	0.002	0.040	0.0002	0.570
IVDU	0.0005	0.003	0.030	0.0004	0.560
C-BVDU	0.267	4.39	7.26	0.021	0.540

[a] The 50% inhibitory dose; mean value for 3–5 separate experiments.

according to the dUrd/dThd reversal and [1′,2′-^{3}H]dUrd/[*methyl*-^{3}H]dThd incorporation experiments (Table 2), only part of the cytostatic action of C-BVDU can be attributed to an inhibitory effect on TS.

Incorporation of [methyl-^{3}H]dThd and [^{125}I]IVDU into TCA-insoluble material of FM3A/0, FM3A/TS⁻, FM3A/TK⁻/HSV-1 TK⁺, and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells. The incorporation of [*methyl*-^{3}H]dThd and [^{125}I]IVDU into TCA-insoluble material of FM3A/0 and its mutant sublines was measured as a function of incubation time. The TS-deficient FM3A/TS⁻ and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cell lines were included in these experiments, since, as we demonstrated previously (14, 15), TS⁻ mutant cell lines readily incorporate dThd and its analogues into TCA-insoluble material (DNA) *via* the salvage pathway (14, 15). As shown in Fig. 1, [*methyl*-^{3}H]dThd was incorporated about 2-fold more efficiently into the two TS⁻ cell lines than their corresponding TS⁺ cell lines. Incorporation of [*methyl*-^{3}H]dThd was linear for up to 12 hr for the FM3A/0 and FM3A/TK⁻/HSV-1 TK⁺ cells, and for up to 4 and 8 hr for the FM3A/TS⁻ and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells, respectively. As has been pointed out (17), the HSV-1 TK⁺ cell line contains about 37% of the dThd kinase activity of the parental cell line, and this explains why [*methyl*-^{3}H]dThd was incorporated about 2-fold more efficiently into FM3A/0 than into FM3A/TK⁻/HSV-1 TK⁺ cells (Fig. 1).

With [^{125}I]IVDU no incorporation was found into TCA-insoluble material of either FM3A/0 or FM3A/TS⁻ cells (Fig. 1). However, [^{125}I]IVDU was clearly incorporated into TCA-insoluble material of both HSV-1 TK⁺ cell lines. The incorporation was linear for at least 24 hr and was 2- to 3-fold more efficient for FM3A/TK⁻/HSV-1 TK⁺/TS⁻ than for FM3A/TK⁻/HSV-1 TK⁺ cells. Thus, the presence of HSV-1 TK activity appeared to be an absolute prerequisite for the incorporation of [^{125}I]IVDU into TCA-insoluble material of the cells.

Under our experimental conditions (initial concentration of dThd and IVDU: 1 μg/10^6 cells/ml), [^{125}I]IVDU was incorporated into FM3A/TK⁻/HSV-1 TK⁺ and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells at about 1/10 the efficiency rate of [*methyl*-^{3}H]dThd.

Furthermore, we observed that the growth of FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells which had been preincubated with IVDU at a concentration of 1 μg/ml (or lower) for 2, 4, or 6 hr, recovered completely following washing out the drug and subsequent addition of 20 μM dThd (data not shown). Macromolecule (DNA, RNA, and protein) synthesis in these cells did not significantly differ from macromolecule synthesis in cells which had not been the subject of preincubation with IVDU (data not shown).

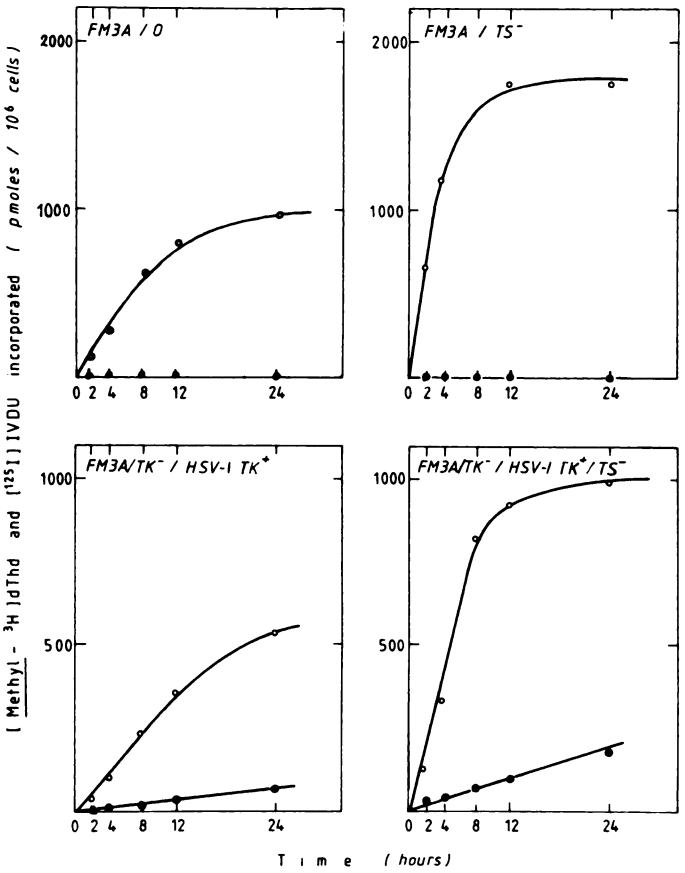

FIG. 1. *Incorporation of [methyl-^{3}H]dThd (○) and [^{125}I]IVDU (●) into TCA-insoluble material of FM3A/0, FM3A/TS⁻, FM3A/TK⁻/HSV-1 TK⁺, and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells as a function of incubation time*

For further details, see "Materials and Methods."

Incorporation of [^{125}I]IVDU and C-[^{125}I]IVDU into DNA of FM3A/0, FM3A/TS⁻, FM3A/TK⁻/HSV-1 TK⁺, and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells. By CsCl gradient analysis it was ascertained that the TCA-insoluble material of FM3A/TK⁻/HSV-1 TK⁺ and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells in which [^{125}I]IVDU had been incorporated corresponded to DNA (Fig. 2). No [^{125}I]IVDU incorporation at all could be detected into DNA of FM3A/0 and FM3A/TS⁻ cells (Fig. 2). However, FM3A/TK⁻/HSV-1 TK⁺ cell DNA could be nicely labeled with [^{125}I]IVDU, and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cell DNA contained even as much [^{125}I]IVDU as FM3A/TK⁻/HSV-1 TK⁺ DNA. Thus, the amounts of radiolabel incorporated into DNA correlated well with the amounts found in the TCA-insoluble material (compare the *lower two panels* of Fig. 1 with those of Fig. 2), indicating that [^{125}I]IVDU was consumed only for incorporation into DNA. The [^{125}I]IVDU-labeled DNA of FM3A/TK⁻/HSV-1 TK⁺ and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells peaked at a density of about 1.75 g/ml. It has been established in previous studies (24) that the ^{125}I label incorporated into DNA of HSV-1-infected cells corresponds to [^{125}I]IVDU and not to a derivative thereof or a (hypothetically) transiodinated product.

We also evaluated the incorporation of C-[^{125}I]IVDU into DNA of FM3A/0, FM3A/TS⁻, FM3A/TK⁻/HSV-1 TK⁺, and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells (Fig. 3). C-IVDU, in which the sugar moiety is replaced by a cyclopentane ring, is, like BVDU and IVDU, a potent and selective inhibitor of HSV-1 replication (21). Recently, De Clercq *et al.* (24) demonstrated that C-[^{125}I]IVDU is incorporated into DNA of HSV-1-infected Vero cells. This is the first evidence for the incorporation of a cyclopentyl pyrimidine into DNA. We have now been able to extend these findings to FM3A cells transformed with the HSV-1 TK gene. In FM3A cells which had not been transformed with the HSV-1 TK gene, namely, FM3A/0 and FM3A/TS⁻, no incorporation of C-[^{125}I]IVDU into DNA could be detected (Fig. 3). However, substantial amounts of C-[^{125}I]IVDU were incorporated into DNA of both FM3A/TK⁻/HSV-1 TK⁺ and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells (Fig. 3), and, like the [^{125}I]IVDU-labeled DNA, the C-[^{125}I]IVDU-labeled DNA peaked at a density of 1.75 g/ml. The amounts of C-[^{125}I]IVDU incorporated into DNA of FM3A/TK⁻/HSV-1 TK⁺ and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells were about 20-fold lower than those found for [^{125}I]IVDU (compare *lower panels* of Figs. 2 and 3).

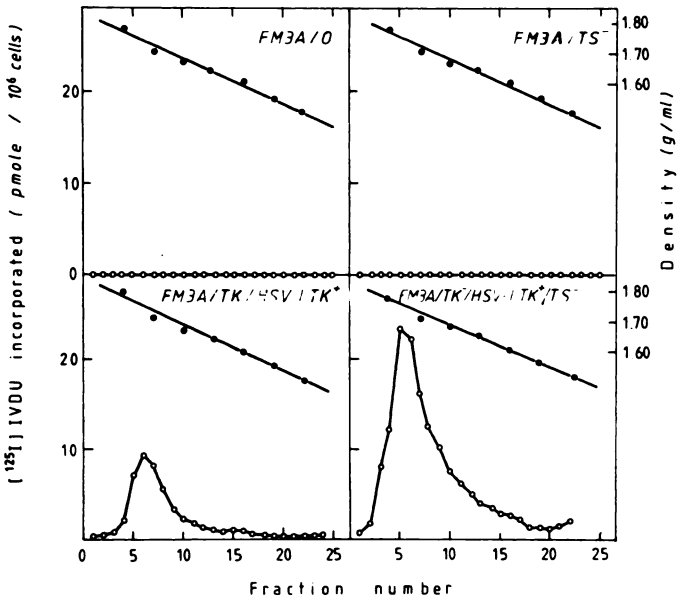

FIG. 2. *CsCl equilibrium density gradient profile of DNA from FM3A/0, FM3A/TS⁻, FM3A/TK⁻/HSV-1 TK⁺, and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells incubated with [^{125}I]IVDU*
For further details, see "Materials and Methods."

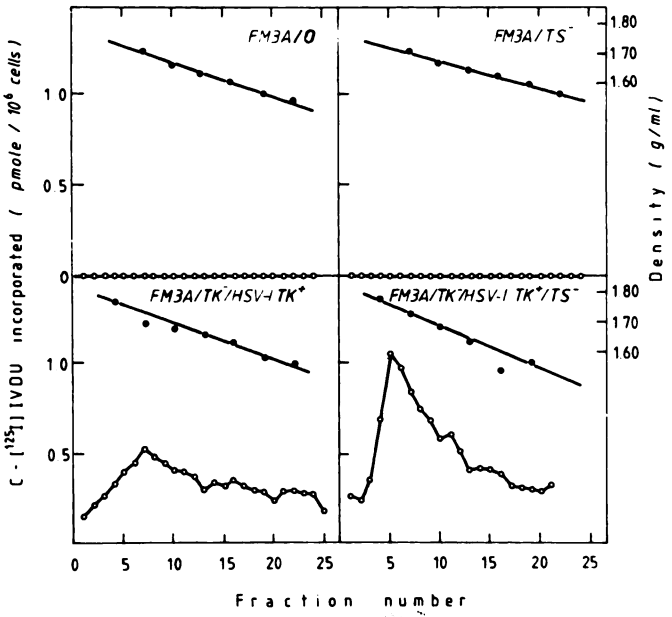

FIG. 3. *CsCl equilibrium density gradient profile of DNA from FM3A/0, FM3A/TS⁻, FM3A/TK⁻/HSV-1 TK⁺, and FM3A/TK⁻/HSV-1 TK⁺/TS⁻ cells incubated with C-[^{125}I]IVDU*
For further details, see "Materials and Methods."

DISCUSSION

Murine mammary carcinoma FM3A/TK⁻/HSV-1 TK⁺ cells, which are dThd kinase deficient, but have been transformed with the HSV-1 TK gene, are inhibited in their growth by BVDU and some of its derivatives (i.e., BVDC, IVDU) at a concentration which is 5,000- to 20,000-fold lower than that required to inhibit the growth of the parental FM3A/0 cells. Also, the carbocyclic analogues of BVDU, BVDC, and IVDU proved considerably more inhibitory to the growth of FM3A/TK⁻/HSV-1 TK⁺ than to FM3A/0 cells. Clearly, phosphorylation by the viral TK is required for these compounds to achieve their inhibitory effects on the growth of FM3A/TK⁻/HSV-1 TK⁺ cells. Although obligatory, this phosphorylation may not be sufficient to ensure a cytostatic effect on HSV-1-transformed cells, since BVaraU, the antiherpetic activity of which also depends on a specific phosphorylation by the viral TK (28), did not significantly affect the growth of FM3A/TK⁻/HSV-1 TK⁺ cells. That BVaraU, in contrast with BVDU, did not prove inhibitory to the growth of FM3A/TK⁻/HSV-1 TK⁺ cells, could be ascribed to differences in the extent of phosphorylation within the transformed cells and/or

to the site of incorporation into DNA. Indeed, BVDU and IVDU are incorporated in the interior of the DNA chain, whereas BVaraU is incorporated at the 3′-terminal (27, 29), and this incorporation may be reversible, since 3′→5′ exonucleases could easily remove such terminal nucleotides.

The observation that BVU and BVRU inhibited the growth of FM3A/TK$^-$/HSV-1 TK$^+$ cells to a significantly greater extent than the growth of FM3A/0 cells is, at first glance, surprising. As pyrimidine base and pyrimidine ribonucleoside, BVU and BVRU are, as such, supposed not to function as a substrate for the viral dThd kinase. However, it was found that, depending on the choice of the cell system (i.e., HeLa, E_6SM), BVU and BVRU may exhibit a marked activity against HSV-1 *in vitro* (23). It has also been suggested that, in these cell systems, BVU and BVRU could be enzymatically converted to BVDU or a phosphorylated product thereof (23). To account for the increased inhibitory effects toward FM3A/TK$^-$/HSV-1 TK$^+$ cell growth, one may postulate that BVU is converted to either BVRU or BVDU by pyrimidine nucleoside (i.e., uridine and 2′-deoxythymidine) phosphorylases and that BVRU, as such, could serve as substrate for the HSV-1-encoded dThd kinase. Upon phosphorylation to its 5′-diphosphate, BVRU 5′-diphosphate may then be converted to the corresponding BVDU 5′-diphosphate by the cellular ribonucleotide reductase. Whether BVRU is actually recognized as substrate by the HSV-1 TK is now under investigation.

To elucidate the mechanism of cytostatic action of BVDU and its analogues against FM3A/TK$^-$/HSV-1 TK$^+$ cells, we evaluated the effects of BVDU, IVDU, and C-BVDU on [*methyl*-^{3}H]dThd and [1′,2′-^{3}H]dUrd incorporation into DNA and also followed the incorporation of [^{125}I]IVDU and C-[^{125}I]IVDU into DNA. TS emerged as one of the targets for the cytostatic action of BVDU, IVDU, and C-BVDU against FM3A/TK$^-$/HSV-1 TK$^+$ cells. C-BVDU was clearly less effective than BVDU and IVDU as an inhibitor of TS as reflected by its higher ID_{50} value for the incorporation of [1′,2′-^{3}H]dUrd into DNA (relative to the incorporation of [*methyl*-^{3}H]dThd). This observation is in agreement with our earlier findings that carbocyclic pyrimidine nucleotide analogues (e.g., 5-nitro-dUMP) have a much weaker affinity for TS than the parent nucleotides (30). The fact that BVDU, IVDU, and C-BVDU proved more inhibitory to TS in FM3A/TK$^-$/HSV-1 TK$^+$ than in FM3A/0 cells (Table 2) can be regarded as the consequence of the phosphorylation of the compounds to their 5′-monophosphates by the HSV-1 TK.

When [^{125}I]IVDU and C-[^{125}I]IVDU were examined for their incorporation into cell DNA, no significant incorporation could be detected with either compound into DNA of FM3A/0 and FM3A/TS$^-$ cells. This is most likely due to an inadequate phosphorylation of IVDU and C-IVDU by the cellular kinase. Consequently, insufficient amounts of IVDU triphosphate and C-IVDU triphosphate are formed to enable their eventual incorporation into DNA of the FM3A/0 and FM3A/TS$^-$ cells. In contrast, [^{125}I]IVDU and C-[^{125}I]IVDU were extensively incorporated into DNA of FM3A/TK$^-$/HSV-1 TK$^+$ and FM3A/TK$^-$/HSV-1 TK$^+$/TS$^-$ cells. Since the latter cells have been transformed with part of the HSV-1 genome, not containing the gene for HSV-1 DNA polymerase, our data thus indicate that IVDU and C-IVDU, after conversion to their corresponding 5′-triphosphates, are recognized as substrates by the cellular DNA polymerase(s). This is the first evidence for the incorporation of IVDU and C-IVDU into DNA of an intact cell system that has to proceed *via* DNA polymerase of cellular, and not viral, origin. In other studies (8, 24) it has been ascertained that [^{125}I]IVDU and C-[^{125}I]IVDU are incorporated into DNA of HSV-1-infected Vero cells as [^{125}I]IVDU monophosphate or C-[^{125}I]IVDU monophosphate.

Although IVDU and C-IVDU are extensively incorporated into DNA of FM3A/TK$^-$/HSV-1 TK$^+$ cells, it is, at present, unclear to what extent this incorporation accounts for the inhibitory effects of these compounds on the growth of the cells. The observation that cell growth, as well as DNA, RNA, and protein synthesis of the FM3A/TK$^-$/HSV-1 TK$^+$ cells, was completely restored after the IVDU had been washed out from cells which had been preincubated with the drug at 1 μg/ml, led us to conclude that IVDU-containing DNA, under our experimental conditions, is still able to maintain its normal metabolic functions (i.e., replication, transcription) or is subject to a prompt repair process.

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