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Antiviral, Antimetabolic, and Cytotoxic Activities of 5-Substituted 2′-Deoxycytidines

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SUMMARY

Various 5-substituted 2′-deoxycytidines, including 5-bromo-dCyd, 5-iodo-dCyd, 5-nitro-dCyd, 5-ethynyl-dCyd, 5-propyl-dCyd, (*E*)-5-(2-bromovinyl)-dCyd, and (*E*)-5-(2-iodovinyl)-dCyd, were evaluated for their antiviral and antimetabolic properties in primary rabbit kidney (PRK) cell cultures and for their inhibitory effects on murine L1210 cell proliferation. All dCyd analogues proved to be selective inhibitors of herpes simplex virus (HSV) replication: 5-bromo-dCyd, 5-iodo-dCyd, 5-nitro-dCyd, and 5-ethynyl-dCyd were more selective in their anti-HSV activity than were the corresponding 5-substituted 2′-deoxyuridines, whereas 5-propyl-dCyd, (*E*)-5-(2-bromovinyl)-dCyd, and (*E*)-5-(2-iodovinyl)-dCyd were as selective as their dUrd counterparts. The dCyd analogues were also less cytotoxic (for both PRK and L1210 cells), as could be monitored by inhibition of either cell proliferation or host-cell DNA synthesis (incorporation of radiolabeled precursors). Of all 5-substituted 2′-deoxycytidines tested, the (*E*)-5-(2-halogenovinyl) derivatives emerged as the most potent and most selective inhibitors of HSV (Type 1) replication.

INTRODUCTION

Although numerous 5-substituted 2′-deoxyuridines (for a review, see ref. 1) have been shown to effect a selective inhibition of HSV[6] replication, 5-substituted 2′-deoxycytidines have attracted relatively little attention as potential antiviral agents. Two such dCyd analogues, 5-iodo-dCyd (2) and 5-bromo-dCyd, were found to be more selective inhibitors of HSV replication than were their dUrd counterparts (3), and this selective action was attributed to a virus-induced dThd/dCyd kinase converting 5-iodo-dCyd and 5-bromo-dCyd to their corresponding 5′-monophosphates. Apart from 5-iodo-dCyd and 5-bromo-dCyd, two other dCyd analogues have occasionally been accredited with antiviral properties. These are 5-ethyl-dCyd and 5-fluoro-dCyd. 5-Ethyl-dCyd was found to be effective against HSV, albeit to a much lower degree than was 5-ethyl-dUrd (4), and 5-fluoro-dCyd was found to be more efficacious as an anticytomegalovirus agent than were 5-iodo-dCyd and 5-bromo-dCyd (5).

5-Substituted 2′-deoxycytidines may be expected to exhibit a greater selectivity as antiherpes agents than 5-substituted 2′-deoxyuridines, since their processing in the virus-infected cell requires the assistance of at least two herpes virus-induced enzymes [dThd/dCyd kinase (6, 7) and dCMP deaminase (8, 9)], as compared with one (dThd/dCyd kinase) for the 5-substituted 2′-deoxyuridines, following the scheme: 5-X-dCyd → 5-X-dCMP → 5-X-dUMP. The resulting 5-X-dUMP may then be converted by cellular kinases to the 5′-di- and 5′-triphosphate, and, eventually be incorporated into DNA. Alternatively, 5-X-dCyd may first be deaminated to 5-X-dUrd and then be phosphorylated, according to the scheme: 5-X-dCyd → 5-X-dUrd → 5-X-dUMP. While the second step of this alternative pathway can be catalyzed by a herpesvirus-induced enzyme (dThd/dCyd kinase), there

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[6] The abbreviations used are: HSV, herpes simplex virus, Type 1 (HSV-1) or Type 2 (HSV-2); 5-X-dCyd, 5-substituted 2′-deoxycytidine; 5-X-dUrd, 5-substituted 2′-deoxyuridine; PRK, primary rabbit kidney; MIC, minimal inhibitory concentration; ID_{50}, inhibitory dose-50; $CCID_{50}$, cell culture-infecting dose-50; PFU, plaque-forming units.

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is no compelling evidence for the first step (deamination of 5-X-dCyd) being effected by a virus-induced enzyme. Indeed, original claims of the viral origin of dCyd deaminase (10) could not be substantiated.[7,8]

The hypothesis that 5-X-dCyd derivatives may acquire greater selectivity in their anti-herpes action than the corresponding 5-X-dUrd analogues has been verified with several newly synthesized dCyd derivatives, *viz.* 5-nitro-dCyd, 5-ethynyl-dCyd, 5-propyl-dCyd, (*E*)-5-(2-bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd. Three of these five dCyd analogues are derived from deoxyuridines that display considerable selectivity in their anti-herpes activity, i.e. 5-propyl-dUrd (11), (*E*)-5-(2-bromovinyl)-dUrd and (*E*)-5-(2-iodovinyl)-dUrd (12, 13). The other two dCyd analogues are derived from deoxyuridines which are not selective in their anti-herpes activity, i.e. 5-nitro-dUrd (14) and 5-ethynyl-dUrd (12, 13).

MATERIALS AND METHODS

Test compounds. The 5-substituted 2′-deoxycytidines that were tested for their antiviral, antimetabolic and cytotoxic activity are depicted in Fig. 1. The sources of the compounds were as follows: 5-bromo-dCyd, Sigma Chemical Company (St. Louis, Missouri); 5-iodo-dCyd, Serva Feinbiochemica (Heidelberg, FRG); 5-nitro-dCyd, see ref. 15; 5-ethynyl-dCyd, see ref. 16; 5-propyl-dCyd, see ref. 17; (*E*)-5-(2-bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd, see ref. 18.

Radiochemicals. The radiolabeled nucleosides [1′,2′-^{3}H]dUrd (specific radioactivity, 31 or 42 Ci/mmole), [2-^{14}C]dUrd (specific radioactivity, 58 mCi/mmole), [5-^{3}H]dCyd (specific radioactivity, 22 Ci/mmole) were obtained from the Radiochemical Centre (Amersham, U.K.), whereas [*methyl*-^{3}H]dThd (specific radioactivity, 25, 38, 45, or 47 Ci/mmole) was obtained from the Institute of Radio-elements (IRE, Fleurus, Belgium).

Test procedures. The methods for evaluating antiviral activity, antimetabolic activity and antitumor cell activity have been described previously (13, 14, 19, 20).

Inhibition of virus-induced cytopathogenicity. Confluent PRK (primary rabbit kidney) cell cultures in Sterilin or Falcon microtiter trays were inoculated with 100 $CCID_{50}$ of virus, 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After 1 hr of virus adsorption, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations (400, 200, 100, . . . μg/ml) of the test compounds. Viral cytopathogenicity was recorded as soon as it reached completion in the control virus-infected cell cultures.

Inhibition of virus multiplication. Confluent PRK cell cultures in Falcon plastic Petri dishes were inoculated with $10^{4.5}$ PFU (plaque forming units) of virus per Petri dish. After 1 hr of virus adsorption, residual virus was removed and the cell cultures were incubated for varying times (as indicated) in the presence of 10 μg/ml of the test compounds. The cells were then freeze-thawed and the virus yields were determined by plaque formation in either Vero cells [for HSV-1(KOS)] or PRK cells (for vaccinia virus). Vero cells represent a continuous line of African green monkey kidney cells.

Inhibition of tumor cell growth. Murine leukemia L1210 cells were seeded in microtiter trays at 5×10^4 cells per well in the presence of varying concentrations (1000, 500, 250, . . . μg/ml) of the test compounds. The cells were allowed to proliferate for 48 hr at 37° in a humidified, CO_2-controlled atmosphere. The growth of the cells was linear during this 48-hr incubation period. At the end of the incubation period, the cells were counted in a Coulter counter, as described previously (20).

Inhibition of incorporation of radiolabeled precursors into cellular DNA. PRK or L1210 cells were seeded in microtiter trays at 10^5 cells per well in the presence of varying concentrations (1000, 500, 250, . . . or 200, 100, 50, . . . μg/ml) of the test compounds and a given amount of the radiolabeled precursor ([1′,2′-^{3}H]dUrd, [2-^{14}C]dUrd, [5-^{3}H]dCyd or [*methyl*-^{3}H]dThd, as indicated). The cells were allowed to proliferate for 16 hr (PRK) or 20 hr (L1210) at 37° in a humidified, CO_2-controlled atmosphere. At the end of this incubation period, the contents of the wells were brought onto 25-mm glass fiber filters and further processed for measurement of acid-insoluble radioactivity as described previously (20).

RESULTS AND DISCUSSION

Antiviral activity. All 5-substituted 2′-deoxycytidines were found to inhibit the cytopathogenicity of HSV-1, the (*E*)-5-(2-halogenovinyl)-2′-deoxycytidines being the most effective in this regard (Table 1). The latter com-

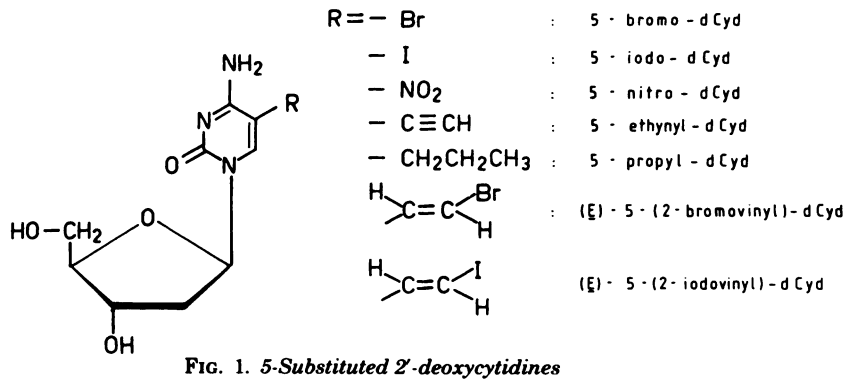

FIG. 1. *5-Substituted 2′-deoxycytidines*

[7] E. Krajewska, E. De Clercq, and D. Shugar, manuscript submitted for publication.

[8] G. A. Gentry, personal communication.

TABLE 1

Effects of 5-substituted 2'-deoxycytidines on virus-induced cytopathogenicity in PRK cell cultures

Compound	MIC[a] (μg/ml)						
	HSV-1 (KOS)	HSV-1 (F)	HSV-1 (McIntyre)	HSV-2 (Lyons)	HSV-2 (G)	HSV-2 (196)	Vaccinia virus
5-Bromo-dCyd	0.2	0.3	0.3	0.2	0.3	0.3	4
5-Iodo-dCyd	0.09	0.08	0.1	0.2	0.7	0.2	4
5-Nitro-dCyd	0.4	1	0.6	2	...	...	70
5-Ethynyl-dCyd	0.2	0.3	0.4	1	2	2	5
5-Propyl-dCyd	7	7	...	40	30	20	>400
(*E*)-5-(2-Bromovinyl)-dCyd	0.07	0.07	0.06	9	7	15	200
(*E*)-5-(2-Iodovinyl)-dCyd	0.07	0.09	0.07	7	8	8	150

[a] Minimum inhibitory concentration required to reduce virus-induced cytopathogenicity by 50%. Virus input: 100 $CCID_{50}$. Viral cytopathogenicity was recorded as soon as it attained 100% in the untreated virus-infected cultures (generally at 3 days after virus infection).

pounds inhibited HSV-1 cytopathogenicity at a concentration of 0.07 μg/ml, that is, about 5–10 fold higher than the concentration at which the (*E*)-5-(2-halogenovinyl)-2'-deoxyuridines were found to inhibit HSV-1 cytopathogenicity (12, 13). The minimum virus-inhibiting concentration (MIC) for 5-propyl-dCyd was also higher than the MIC previously noted for 5-propyl-dUrd (11). For the other 5-X-dCyd derivatives, the MIC was similar to that recorded previously for the corresponding 5-X-dUrd derivatives [5-bromo-dUrd (1), 5-iodo-dUrd (13), 5-nitro-dUrd (14), 5-ethynyl-dUrd (12, 13)].

All 5-X-dCyd derivatives, except the (*E*)-5-(2-halogenovinyl)derivatives, inhibited HSV-2 cytopathogenicity at a concentration which was similar to or only slightly higher than the concentration required to inhibit HSV-1 cytopathogenicity (Table 1). For (*E*)-5-(2-bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd the difference in the HSV-1-inhibiting and HSV-2-inhibiting concentration was about 100-fold; thus, these compounds, akin to their dUrd counterparts (13), could be advocated as useful markers to differentiate HSV-1 strains from HSV-2 strains.

The 5-X-dCyd derivatives inhibited vaccinia virus-induced cytopathogenicity at a concentration which was 15-fold (5-bromo-dCyd) to 3000-fold [(*E*)-5-(2-bromovinyl)-dCyd] higher than the concentration required to inhibit HSV-1 replication (Table 1). Vesicular stomatitis virus was totally resistant to the inhibitory effects of the 5-X-dCyd derivatives (data not shown). Based on these findings, the dCyd analogues could be considered as rather selective inhibitors of HSV-1 replication, the (*E*)-5-(2-halogenovinyl) derivatives being the most selective.

That the inhibitory effects of the dCyd analogues on viral cytopathogenicity actually reflected an inhibition of virus multiplication was ascertained by measuring virus growth in the presence of the compounds (Fig. 2). All 5-substituted 2'-deoxycytidines caused a significant reduction in HSV-1 yield; this reduction was most pronounced at 24 hr after virus infection (Fig. 2). Only three out of the seven dCyd analogues effected a significant reduction in vaccinia virus yield (Fig. 2B). These three compounds were 5-bromo-dCyd, 5-iodo-dCyd and 5-ethynyl-dCyd. The other four compounds, 5-nitro-dCyd, 5-propyl-dCyd, (*E*)-5-(2-bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd, did not markedly affect vaccinia virus growth. This lack of activity could be expected from the viral cytopathogenicity data (Table 1), since the concentration (10 μg/ml) at which the four compounds were evaluated for their inhibitory effects on vaccinia virus growth, fell far below their MIC for vaccinia virus-induced cytopathogenicity.

Antimetabolic activity. The effects of 5-substituted 2'-deoxycytidines on normal cell metabolism were monitored by three parameters: inhibition of incorporation of [*methyl*-^{3}H]dThd, [1',2'-^{3}H]dUrd (or [2-^{14}C]dUrd) and [5-^{3}H]dCyd into DNA. A greater inhibitory effect of 5-X-dUrd derivatives on dUrd than on dThd incorporation has previously been interpreted as evidence for a selective inhibition of the thymidylate synthetase reaction (14). Based on this criterion, several dUrd analogues, *viz.* 5-nitro-dUrd (14), 5-ethynyl-dUrd (12), 5-cyano-dUrd (14), 5-thiocyano-dUrd (14), 5-formyl-dUrd (21) and the 5-oxime of 5-formyl-dUrd (22), have been recognized as potent and/or specific inhibitors of thymidylate synthetase. This premise has been substantiated by our studies with cell-free dTMP synthetase. The 5'-monophosphates of those dUrd-analogues that were recognized as selective inhibitors of dTMP synthetase in intact cells, i.e., PRK cells (14) or L1210 cells (20) were also potent inhibitors of the cell-free dTMP synthetase. Moreover, the K_m/K_i values for the cell-free dTMP synthetase (isolated from L1210 cells) closely correlated with the extent of dTMP synthesis inhibition (23).[9]

Extending our hypothesis to the 5-X-dCyd derivatives, we may consider 5-nitro-dCyd and 5-ethynyl-dCyd as specific inhibitors of thymidylate synthetase, since both compounds were found to inhibit [1',2'-^{3}H]dUrd (or [2-^{14}C]dUrd) incorporation to a significantly greater extent than [*methyl*-^{3}H]dThd incorporation (Table 2). However, the ID_{50} of 5-nitro-dCyd and 5-ethynyl-dCyd for [1',2'-^{3}H] (or [2-^{14}C]dUrd) incorporation was markedly higher than the ID_{50} previously obtained with 5-nitro-dUrd (14) and 5-ethynyl-dUrd (12). This indicates that 5-nitro-dUrd and 5-ethynyl-dUrd are more potent inhibitors of thymidylate synthetase than their dCyd counterparts, which is not surprising since the latter must first be deaminated before they can act as inhibitors of the thymidylate synthetase reaction [dUMP → dTMP (Fig. 3)]. Theoretically, this deamination may occur at both the nucleoside and nucleotide level. However, additional investigations[7] have indicated that PRK cell cultures,

[9] J. Balzarini, E. De Clercq, M. P. Mertes, D. Shugar, and P. F. Torrence, submitted for publication.

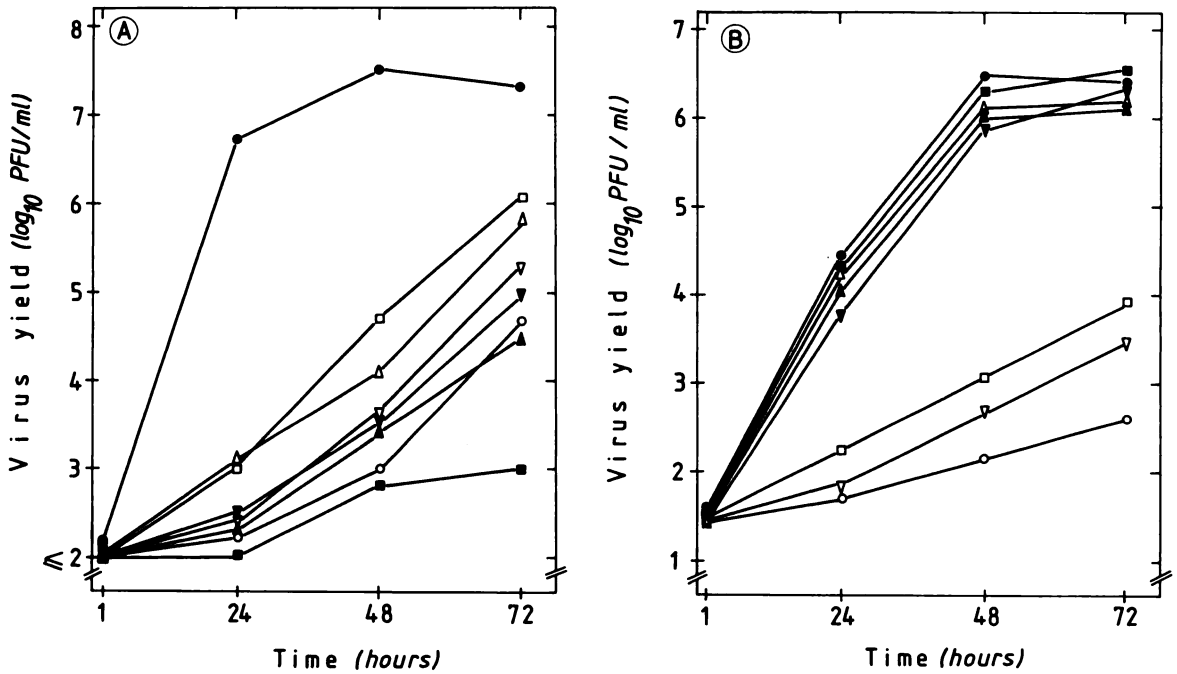

FIG. 2. *Effect of 5-substituted 2′-deoxycytidines on the multiplication of either HSV-1 (KOS) (A) or vaccinia virus (B) in PRK cell cultures*
Virus input: 4.5 $\log_{10}$ PFU per Petri dish. The compounds were added at 10 μg/ml immediately after virus adsorption. All data represent average values for two or three separate determinations. ●, control; ○, 5-bromo-dCyd; □, 5-iodo-dCyd; △, 5-nitro-dCyd; ▽, 5-ethynyl-dCyd; ■, 5-propyl-dCyd; ▲, (E)-5-(2-bromovinyl)-dCyd; ▼, (E)-5-(2-iodovinyl)-dCyd.

while containing significant dCMP aminohydrolase activity, do not possess detectable dCyd aminohydrolase activity. From these findings we could infer that the dCyd analogues are converted to the corresponding dUrd analogues at the nucleotide (5′-monophosphate) rather than the nucleoside level.

5-Bromo-dCyd, 5-iodo-dCyd and the (*E*)-5-(2-halogenovinyl)-2′-deoxycytidines were also more inhibitory for [1′,2′-^{3}H]dUrd incorporation than for [*methyl*-^{3}H]dThd incorporation (Table 2). However, these differences were not as dramatic as for 5-ethynyl-dCyd. While 5-propyl-dCyd did not prove inhibitory to either dThd, dUrd or dCyd incorporation, the other dCyd analogues (5-bromo-dCyd excepted) inhibited [5-^{3}H]dCyd incorporation to a greater degree than [*methyl*-^{3}H]dThd incorporation. This may also be explained by an inhibitory effect at the

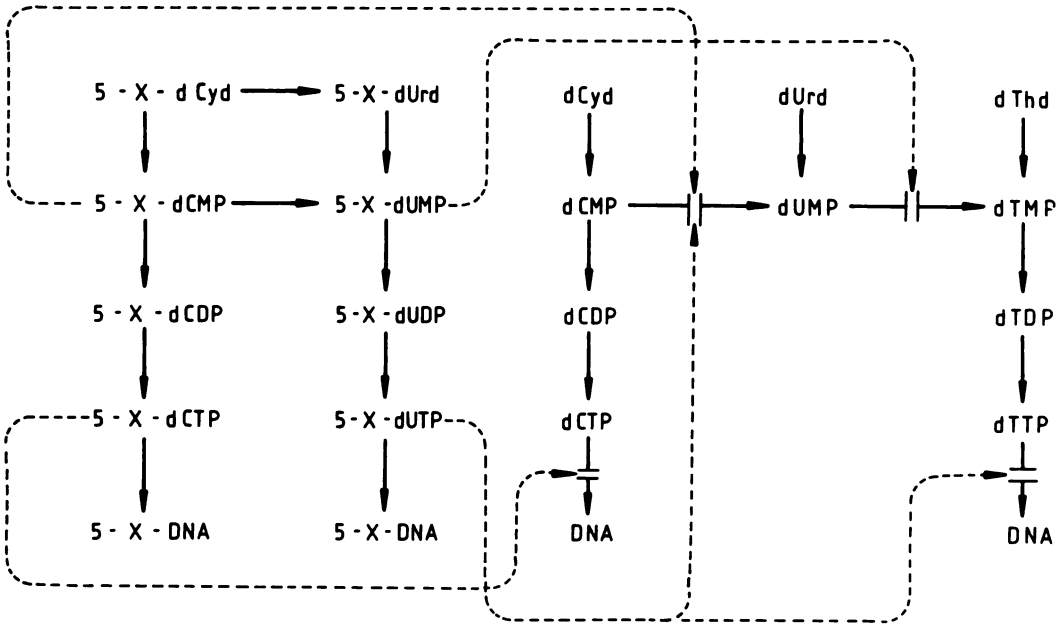

FIG. 3. *Pathways for incorporation of dThd, dUrd and dCyd into DNA, and inhibition of this incorporation by 5-substituted 2′-deoxycytidines (5-X-dCyd)*
As has been demonstrated with different cell lines, i.e., Novikoff hepatoma cells (24) and Syrian hamster melanoma cells (25), dCyd is primarily processed according to the route dCyd → dCMP → dUMP → dTMP → dTDP → dTTP.

TABLE 2

Effects of 5-substituted 2′-deoxycytidines on the incorporation of (methyl-^{3}H)dThd, (1′,2′-^{3}H)dUrd (or (2-^{14}C)dUrd) and (5-^{3}H)dCyd into DNA of PRK cell cultures

Input of the radiolabeled precursors (per 10^5 PRK cells): 10 pmoles (0.38 μCi) of [*methyl*-^{3}H]dThd; 6 pmoles (0.25 μCi) of [1′,2′-^{3}H]dUrd; 0.25 μmole (14 μCi) of (2-^{14}C)dUrd; and 11 pmoles (0.25 μCi) of [5-^{3}H]dCyd.

Compound	ID_{50}[a] (μg/ml)			
	[*methyl*-^{3}H]dThd incorporation	[1′,2′-^{3}H]dUrd incorporation		[5-^{3}H]dCyd incorporation
5-Bromo-dCyd	10	5	(50[b])	120
5-Iodo-dCyd	60	9		35
5-Nitro-dCyd	>200	...	(10[b])	...
5-Ethynyl-dCyd	120	3	(25[b])	3
5-Propyl-dCyd	>200	>200		⩾200
(*E*)-5-Bromovinyl)-dCyd	>200	100	(>200[b])	15
(*E*)-5-(2-Iodovinyl)-dCyd	>200	50	(>200[b])	40

[a] Dose inhibiting the incorporation of [*methyl*-^{3}H]dThd, [1′,2′-^{3}H]dUrd or [5-^{3}H]dCyd by 50%.

[b] ID_{50} for [2-^{14}C]dUrd incorporation.

thymidylate synthetase level, at least to the extent that [5-^{3}H]dCyd is processed to [5-^{3}H]dUMP (Fig. 3). The fact that some compounds, *viz.* (*E*)-5-(2-bromovinyl)-dCyd, were more inhibitory for [5-^{3}H]dCyd than for [1′,2′-^{3}H] (or [2-^{14}C])dUrd incorporation could, hypothetically, be attributed to an inhibitory effect on the deamination of dCMP to dUMP (or phosphorylation of dCyd to dCMP).

Of all dCyd analogues tested, only 5-bromo-dCyd inhibited [5-^{3}H]dCyd to a lesser degree than [*methyl*-^{3}H]dThd incorporation (Table 2). Perhaps [5-^{3}H]dCyd avoided the inhibitory action of 5-bromo-dCyd by following the dCyd → dCMP → dCDP → dCTP rather than the dCyd → dCMP → dUMP → dTMP → dTDP → dTTP pathway (Fig. 3). This may mean that within the dosage range of 5–120 μg 5-bromo-dCyd per ml (Table 2), [5-^{3}H]dCyd was incorporated into DNA as dCMP (and not as dTMP). A similar situation may occur in L1210 cells where the discrepancy in the ID_{50} of 5-bromo-dCyd for [5-^{3}H]dCyd incorporation and [*methyl*-^{3}H]dThd incorporation was even larger than in PRK cells (Tables 2 and 5).

Selectivity. All 5-substituted 2′-deoxycytidines displayed considerable selectivity as inhibitors of HSV-1 replication, irrespective of the index upon which this selectivity was based (Table 3). Those derivatives that were highly specific anti-herpes agents in their dUrd form, i.e., (*E*)-5-(2-bromovinyl)-dUrd and (*E*)-5-(2-iodovinyl)-dUrd, retained their selectivity when converted to their dCyd form. For example, (*E*)-5-(2-bromovinyl)-dUrd and (*E*)-5-(2-iodovinyl)-dUrd have been accredited with vaccinia virus/HSV-1 ratios of 875 and 833, respectively (13). For (*E*)-5-(2-bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd, this ratio reached 3000 and 1950, respectively (Table 3). Similarly, 5-propyl-dUrd maintained its state of specific anti-herpes agent (11, 13) when aminated to the corresponding dCyd analogue (Table 3).

5-Nitro-dUrd and 5-ethynyl-dUrd are not selective or even negatively selective inhibitors of HSV-1 replication in that they inhibit vaccinia virus replication or host-cell DNA metabolism at concentrations that are below those required for inhibition of HSV-1 replication (13, 14). However, these compounds acquired a significant increase in their specificity toward HSV when converted to their dCyd form. This increase in specificity was particularly marked for 5-nitro-dCyd which proved 100 times more inhibitory to HSV-1 than to vaccinia virus (Table 3), while its dUrd counterpart was 10 times more active against vaccinia virus (14). A similar increase in specificity was apparent if the dUrd or dCyd incorporation/HSV-1 ratios were compared: this ratio increased from 0.07–0.1 for 5-ethynyl-dUrd and 5-nitro-dUrd (13, 14) to 10–15 for 5-ethynyl-dCyd and 5-nitro-dCyd (Table 3).

For 5-iodo-dUrd the selectivity indexes, vaccinia virus/HSV-1 and dUrd incorporation/HSV-1, amounted to only 2–2.3 (ref. 13), thus pointing to little specificity in the anti-HSV activity of 5-iodo-dUrd. For 5-iodo-dCyd, these indexes attained 44–100 (Table 3), thereby confirming the contention of Greer and his colleagues that 5-halogenated analogues of dCyd are indeed more selective inhibitors of HSV replication than the 5-halogenated dUrd analogues (3, 26).

Antitumor cell activity. The selectivity of the 5-substituted 2′-cytidines as anti-herpes agents is further attested by their relatively weak inhibitory effects on tumor cell proliferation (Table 4). The most effective inhibitors were 5-nitro-dCyd and 5-ethynyl-dCyd but their ID_{50} for mouse leukemia L1210 cell growth were significantly higher than those reported previously for 5-nitro-dUrd (5-nitro-dUrd: 0.035 μg/ml) and 5-ethynyl-dUrd (0.091 μg/ml) (20). (*E*)-5-(2-Bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd gave ID_{50} values that were similar to the ID_{50} values of (*E*)-5-(2-bromovinyl)-dUrd and (*E*)-5-(2-iodovinyl)-dUrd (20), whereas 5-propyl-, 5-iodo- and 5-

TABLE 3

Selectivity indexes of 5-substituted 2′-deoxycytidines as anti-HSV-1 agents in PRK cell cultures

	$\left(\frac{\text{HSV-2}}{\text{HSV-1}}\right)$[a]	$\left(\frac{\text{Vaccinia virus}}{\text{HSV-1}}\right)$[b]	$\left(\frac{\text{dUrd or dCyd incorporation}}{\text{HSV-1}}\right)$
5-Bromo-dCyd	1	15	18.5
5-Iodo-dCyd	4	44	100
5-Nitro-dCyd	3	105	15
5-Ethynyl-dCyd	5.7	17	10
5-Propyl-dCyd	4.3	>57	⩾29
(*E*)-5-(2-Bromovinyl)-dCyd	154	3000	225
(*E*)-5-(2-Iodovinyl)-dCyd	100	1950	520

[a] Ratio of the average MIC for all three HSV-2 strains to the average MIC for all three HSV-1 strains (calculated from the data presented in Table 1).

[b] Ratio of the MIC for vaccinia virus to the average MIC for all three HSV-1 strains (calculated from the data presented in Table 1).

[c] Ratio of the ID_{50} for either dUrd or dCyd incorporation, whatever was lowest, to the average MIC for all three HSV-1 strains (calculated from the data presented in Tables 1 and 2).

TABLE 4

Effects of 5-substitutes 2′-deoxycytidines on the proliferation of L1210 cells

Compound	ID_{50}[a] (μg/ml)			
	as such	upon addition of		
		dThd (5 μg/ml)	dUrd (125 μg/ml)	dCyd (500 μg/ml)
5-Bromo-dCyd	>1000	>1000	>1000	>1000
5-Iodo-dCyd	>1000	>1000	>1000	>1000
5-Nitro-dCyd	2.7	>500	30.5	50
5-Ethynyl-dCyd	4.4	1000	64.5	260
5-Propyl-dCyd	>1000	>1000	>1000	>1000
(*E*)-5-(2-Bromovinyl)-dCyd	30	519	127	129
(*E*)-5-(2-Iodovinyl)-dCyd	21	1000	56	103

[a] Dose inhibiting cell proliferation by 50%. The doses employed for dThd, dUrd and dCyd correspond to the maximum concentrations of dThd, dUrd and dCyd which were themselves not inhibitory to L1210 cell growth. The cells were seeded at 5×10^4 per microplate well and allowed to proliferate for 48 hours.

bromo-dCyd were devoid of any L1210 cell growth-inhibiting properties.

It has been postulated that the cytotoxic activity of those dUrd analogues that specifically inhibit the thymidylate synthetase reaction is reversed more efficiently upon addition of dThd than of dUrd (20). To the extent that 5-X-dCyd derivatives are deaminated intracellularly [at either the nucleoside or nucleotide level (Fig. 3)], this premise may also hold for dCyd analogues; and, indeed, the inhibitory effects of 5-nitro-dCyd and 5-ethynyl-dCyd on L1210 cell growth were overcome more efficiently by dThd than by dUrd (or dCyd) (Table 4). Likewise, the cytotoxic activity of (*E*)-5-(2-bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd was reversed more readily by dThd than by dUrd or dCyd. Thus, akin to 5-ethynyl-dCyd and 5-nitro-dCyd, (*E*)-5-(2-bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd may owe their cytotoxic properties for L1210 cells to an inhibition of thymidylate synthetase.

This assumption was further supported by [*methyl*-^{3}H]dThd, [1′,2′-^{3}H]dUrd and [5-^{3}H]dCyd incorporation studies. As could be expected from compounds that specifically act at the thymidylate synthetase level, 5-nitro-dCyd, 5-ethynyl-dCyd, (*E*)-5-(2-bromovinyl)-dCyd and (*E*)-5-(2-iodovinyl)-dCyd inhibited [1′,2′-^{3}H]dUrd (and [5-^{3}H]dCyd) incorporation to a significantly greater degree than [*methyl*-^{3}H]dThd incorporation (Table 5). The fact that these four dCyd analogues inhibited [5-^{3}H]dCyd incorporation at a concentration that was quite similar to the inhibitory concentration for [1′,2′-^{3}H]dUrd incorporation may suggest that, under the test conditions, [5-^{3}H]dCyd followed the dCyd → dCMP → dUMP → dTMP rather than the dCyd → dCMP → dCDP → dCTP route (Fig. 3).

However, with the 5-halogenated analogues of dCyd, no inhibition of [5-^{3}H]dCyd incorporation could be witnessed, although these analogues effectively inhibited [1′,2′-^{3}H]dUrd incorporation (Table 5). The reason(s) for the anomalous behavior of 5-bromo-dCyd and 5-iodo-dCyd are not immediately clear, but, as pointed out above (see section on "Antimetabolic activity") could be related to a salvage of [5-^{3}H]dCyd through the dCyd → dCMP → dCDP → dCTP pathway (Fig. 3). This pathway may be followed if the deamination of dCMP to dUMP were blocked. Such a block could be envisaged if 5-bromo-dCyd and 5-iodo-dCyd, upon intracellular conversion to their 5′-monophosphates, acted as competitive inhibitors of the dCMP deaminase reaction, or if 5-bromo-dCTP and 5-iodo-dCTP, like dTTP (24), acted as allosteric inhibitors of this reaction (Fig. 3).

A striking parallelism was noted between the ID_{50} values for L1210 cell proliferation (Table 4, first column) and the ID_{50} values for [5-^{3}H]dCyd incorporation into L1210 cell DNA (Table 5, last column). Thus, the cytotoxicity of the 5-X-dCyd derivatives is more accurately reflected by an inhibition of [5-^{3}H]dCyd, rather than [*methyl*-^{3}H]dThd or [1′,2′-^{3}H]dUrd, incorporation. This implies that the cytotoxicity of dCyd analogues could be adequately monitored by measuring their inhibitory effects on [5-^{3}H]dCyd incorporation.

CONCLUSION

The salient features emerging from this study could be summarized as follows: 5-X-dUrd derivatives such as 5-nitro-dUrd and 5-ethynyl-dUrd which are not selective as anti-herpes agents become specific inhibitors of HSV replication after conversion to their dCyd counterparts. 5-X-dUrd derivatives such as 5-propyl-dUrd, (*E*)-5-(2-bromovinyl)-dUrd and (*E*)-5-(2-iodovinyl)-dUrd which are highly selective in their anti-herpes activity retain their specificity upon amination to the corresponding dCyd analogues. The selectivity of the 5-X-dCyd derivatives as anti-herpes agents may at least partly be related to their capacity to serve as substrate for the herpesvirus-induced dThd/dCyd kinase (6, 7) as has been demonstrated for several 5-X-dUrd derivatives, *viz.* 5-propyl-dUrd and the (*E*)-5-(2-halogenovinyl)-2′-deoxyuridines (27, 28). Deamination of the dCMP analogues to the corresponding dUMP analogues by a virus-induced

TABLE 5

Effects of 5-substituted 2′-deoxycytidines on the incorporation of [methyl-^{3}H]dThd, [1′,2′-^{3}H]dUrd, and [5-^{3}H]dCyd into DNA of L1210 cells

Compound	ID_{50}[a] (μg/ml)		
	[*methyl*-^{3}H]dThd incorporation	[1′,2′-^{3}H]dUrd incorporation	[5-^{3}H]dCyd incorporation
5-Bromo-dCyd	6.5	2.1	>1000
5-Iodo-dCyd	140	3.7	>1000
5-Nitro-dCyd	>250	4.6	3.0
5-Ethynyl-dCyd	>1000	1.9	4.7
5-Propyl-dCyd	>1000	>1000	>1000
(*E*)-5-(2-Bromovinyl)-dCyd	>1000	21	27
(*E*)-5-(2-Iodovinyl)-dCyd	>1000	17	29

[a] Dose inhibiting the incorporation of [*methyl*-^{3}H]dThd, [1′,2′-^{3}H]dUrd, or [5-^{3}H]-dCyd by 50%. Input of the radiolabelled precursors (per 10^5 L1210 cells): 6.5 pmoles (0.25 μCi) of [*methyl*-^{3}H]dThd; 8 pmoles (0.25 μCi) of [1′,2′-^{3}H]dUrd; and 11 pmoles (0.25 μCi) of [5-^{3}H]dCyd.

dCMP deaminase (8, 9) may further contribute to the selective anti-herpes activity of the 5-X-dCyd derivatives. Although the target for the antiviral action of the 5-X-dCyd derivatives has not yet been identified, one may envisage the possibility that, upon conversion of 5-X-dCyd, through 5-X-dCMP, to 5-X-dUMP, 5-X-dUDP, 5-X-dUTP, the latter could inhibit the DNA polymerase reaction and/or be incorporated into DNA (Fig. 3). As has been demonstrated for the 5'-triphosphate of (*E*)-5-(2-bromovinyl)-dUrd, 5-X-dUTP derivatives may inhibit the HSV DNA polymerase to a significantly greater extent than the cellular DNA polymerases α and β (29) and be specifically incorporated into HSV DNA (30, 31). 5-X-dCyd derivatives are less cytotoxic than the corresponding dUrd analogues. Their cytotoxicity may at least partially be attributed to an inhibitory activity at the thymidylate synthetase reaction. To achieve this effect, the dCyd analogue should first be deaminated, either at the nucleoside or at the nucleotide level (Fig. 3).

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