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5-Nitro-2′-deoxyuridine and 5-Nitro-2′-deoxyuridine 5′-Monophosphate: Antiviral Activity and Inhibition of Thymidylate Synthetase *in Vivo*

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SUMMARY

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The antiviral activity of a series of nitrated uracil derivatives, including 5-nitrouracil, 1-methyl-5-nitrouracil, 1,3-dimethyl-5-nitrouracil, 5-nitro-2′-deoxyuridine (5-nitro-dU), 3′-*O*,5-dinitro-2′-deoxyuridine, 5-nitro-2′-deoxyuridine 5′-monophosphate (5-nitro-dUMP), and 3′-*O*,5-dinitro-2′-deoxyuridine 5′-monophosphate, was evaluated in primary rabbit kidney or human skin fibroblast cultures challenged with vaccinia, herpes simplex, or vesicular stomatitis virus. The most remarkable antiviral activity was shown by 5-nitro-dU and 5-nitro-dUMP, which inhibited the replication of vaccinia virus at concentrations as low as 0.1–0.4 μg/ml. Somewhat higher concentrations were required to inhibit the replication of herpes simplex virus (1–4 μg/ml for 5-nitro-dU and 40–100 μg/ml for 5-nitro-dUMP). Neither 5-nitro-dU nor its 5′-monophosphate was inhibitory to vesicular stomatitis virus at concentrations up to 100 μg/ml. Combination of 5-nitro-dU with 5-iodo-2′-deoxyuridine (5-iodo-dU) resulted in an increased antiviral activity over the activity of the compounds used individually, suggesting that 5-nitro-dU and 5-iodo-dU act at different steps in DNA biosynthesis. In fact, 5-nitro-dU and its 5′-monophosphate effectively blocked the incorporation of [^{14}C]2′-deoxyuridine into host cell DNA, but neither compound inhibited the incorporation of [^{3}H]2′-deoxythymidine, pointing to thymidylate synthetase as a specific target for the action of 5-nitro-dU (or 5-nitro-dUMP). Inhibition of thymidylate synthetase would account for the antiviral activity of 5-nitro-dU, since the inhibitory effect of 5-nitro-dU on vaccinia virus replication could be readily reversed by 2′-deoxythymidine, but not by 2′-deoxyuridine or 2′-deoxycytidine. Other deoxythymidine analogues that were found to inhibit deoxyuridine incorporation, but not deoxythymidine incorporation, and could therefore be assumed to block thymidylate synthetase selectively *in vivo*, include 5-fluoro-, 5-trifluoromethyl-, 5-cyano-, and 5-thiocyanato-2′-deoxyuridine.

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INTRODUCTION

C-5-substituted 2′-deoxyuridine derivatives, such as 5-iodo- and 5-trifluoromethyl-2′-deoxyuridine, exhibit distinct antiviral and antineoplastic activities (1, 2). Some of these 5-substituted 2′-deoxyuridines, viz. 5-iodo- and 5-bromo-dU,[1] are also well known for their mutagenic and oncogenic potentials (1). Recently developed dU derivatives include 5-methylamino-dU (3, 4), 5-ethyl-dU (5–7), 5-thiocyanato-dU (8, 9), 5-methoxymethyl-dU (10, 11), 5-mercaptomethyl-dU (12), 5-methylmercapto-dU (13), 5-allyl-dU (7), 5-vinyl-dU (7), 5-propyl-dU (7, 14), 5-cyano-dU (15, 16), and 5-propynyloxy-dU (17). Whereas most of the 5-substituted dU derivatives inhibited vaccinia and herpes simplex virus to the same extent, some compounds, viz. 5-methylamino-dU, 5-methoxymethyl-dU, 5-propyl-dU, and 5-propynyloxy-dU, proved efficacious against herpes simplex only (3, 11, 14, 17). Others (5-cyano-dU) were particularly inhibitory to vaccinia virus (16).

We describe here another specific anti-vaccinia agent, 5-nitro-dU; 5-nitro-dU and its 5′-monophosphate were found to inhibit the replication of vaccinia virus at a concentration 10–100-fold lower than that required to inhibit the replication of herpes simplex or vesicular stomatitis virus. The antiviral activity of 5-nitro-dU could be attributed to the inhibition of thymidylate synthetase, a key enzyme in the biosynthesis of DNA.

Several years ago, Kluepfel *et al.* (18) reported the enzymatic synthesis of 5-nitro-dU. This synthesis was based upon the conversion of 5-nitrouracil to its deoxyribonucleoside with the aid of *trans-N*-deoxyribosylase (from *Lactobacillus*). Although the product of the enzymatic reaction was not fully characterized, it exerted a potent inhibitory effect on vaccinia virus replication in cell culture (18). An unequivocal chemical synthesis of 5-nitro-dU and other 5-nitrouracil derivatives has been described recently (19), offering the opportunity to corroborate and extend the original observations of Kluepfel *et al.* (18).

[1] The abbreviations used are: dU, 2′-deoxyuridine; dT, 2′-deoxythymidine; dC, 2′-deoxycytidine; 5-nitro-dUMP, 5-nitro-2′-deoxyuridine 5′-monophosphate; ara-A, adenine arabinoside, 9-β-D-arabinofuranosyladenine; ara-C, cytosine arabinoside, 1-β-D-arabinofuranosylcytosine; ara-U, uracil arabinoside, 1-β-D-arabinofuranosyluracil; ara-T, thymine arabinoside, 1-β-D-arabinofuranosylthymine; PRK, primary rabbit kidney; HSF, human skin fibroblast; $CCID_{50}$, dose infecting 50% of the cell cultures; PFU, plaque-forming units.

MATERIALS AND METHODS

The synthesis and physicochemical characteristics of the nitrouracils, nitrouracil nucleosides, and nitrouracil nucleotides have been described previously (19). The radiolabeled DNA precursors [*methyl*-^{3}H] dT (specific radioactivity, 12 Ci/mmole) and [2-^{14}C]dU (specific radioactivity, 57 mCi/mmole) were obtained from the Institute of Radio-elements (IRE, Fleurus, Belgium) and the Radiochemical Centre (Amersham), respectively. 5-Iodo-dU was provided by Ludeco (Brussels), whereas dT, dU, and dC were purchased from Sigma Chemical Company.

The sources of the various other nucleosides were as follows: 5-bromo-2′-deoxyuridine, Sigma; 5-chloro-2′-deoxyuridine, P-L Biochemicals; 5-fluoro-2′-deoxyuridine, Aldrich Chemical Company; 5-cyano-2′-deoxyuridine, see ref. 16; 5-ethyl-2′-deoxyuridine, see refs. 5 and 6; 5-trifluoromethyl-2′-deoxyuridine, Sigma; 5-hydroxymethyl-2′-deoxyuridine, Calbiochem; 5-thiocyanato-2′-deoxyuridine, see refs. 8 and 9; 5-hydroxy-2′-deoxyuridine, Sefochem Fine Chemicals (Emek Hayarden, Israel); 5-allyloxy-, 5-propynyloxy-, and 5-carboxamidomethyloxy-2′-deoxyuridine, see ref. 17; cytosine arabinoside, Upjohn; uracil arabinoside, either Terra-Marine Bioresearch (La Jolla), Calbiochem, or Sefochem Fine Chemicals; thymine arabinoside, Terra-Marine Bioresearch; adenine arabinoside, Parke, Davis (courtesy of Dr. R. Wolfe, Parke, Davis Clinical Research Western Europe, München).

The technique for evaluating the effects of the nitrouracil derivatives on vaccinia, herpes simplex, and vesicular stomatitis virus-induced cytopathogenicity in primary rabbit kidney cells has been described previously (20). A similar technique was employed to determine inhibition of viral cytopathogenicity in human diploid cells (human skin fibroblasts, VGS strain). The

methodology for measuring vaccinia virus growth in PRK cell cultures also has been described (20).

The incorporation of [*methyl*-^{3}H]dT and [2-^{14}C]dU into cellular DNA was monitored in Linbro microplates (model FB-48-TC, Linbro Chemical Company, New Haven, Ct.). To each well were added 10^5 PRK cells, 0.01 nmole (0.12 μCi) of [*methyl*-^{3}H]-dT or 250 nmole (14 μCi) of [2-^{14}C]dU, and a given amount of the test compound. The cells were allowed to proliferate for 16 hr at 37° in a humidified, CO_2-controlled atmosphere. At the end of this incubation period, acid-insoluble radioactivity was measured as described previously (21). Briefly, the wells were treated with cold trichloroacetic acid (5′) for 30 min at 4°, washed 6 times with cold trichloroacetic acid (5%) and 4 times with cold ethanol, and allowed to dry for 1 hr at 37°. The bottoms of the wells were then cut out and assayed for radioactivity in a toluene-based scintillant. The total radioactivity obtained per well amounted to approximately 7000 cpm ([*methyl*-^{3}H]dT) and 4000 cpm ([2-^{14}C]dU).

To ensure that under our assay conditions [2-^{14}C]dU was actually incorporated into DNA (and not into RNA), we determined the sensitivity of the incorporated label to pancreatic ribonuclease A (Sigma). When cell homogenates prepared from PRK cells that had been exposed for 16 hr to either [2-^{14}C]dU or [5-^{3}H]uridine were treated with 100 μg of pancreatic ribonuclease A for 1 hr at 37°, a significant (more than 80%) decrease in acid-insoluble ^{3}H radioactivity but no decrease in acid-insoluble ^{14}C radioactivity was noted. These observations suggest that under our test conditions [2-^{14}C]dU was incorporated into DNA and not into RNA.

RESULTS AND DISCUSSION

Antiviral activity of 5-nitro-dU and 5-nitro-dUMP. Of the set of nitro compounds screened for inhibition of viral cytopathogenicity in PRK or HSF cell cultures, only 5-nitro-dU and 5-nitro-dUMP exhibited distinct antiviral activity (Table 1). This activity was most specifically directed against vaccinia virus. In both PRK and

TABLE 1

Effects of 5-nitro-dU, 5-nitro-dUMP, and other nitrated derivatives of uracil on virus-induced cytopathogenicity in PRK and HSF cell cultures

The compounds were added immediately after virus adsorption. Stock solutions of the compounds were prepared in either dimethyl sulfoxide (at 10 mg/ml) or distilled water (2 mg/ml). Since dimethyl sulfoxide itself showed cytotoxicity at ≧1% (v/v), viral cytopathogenicity could not be read at compound concentrations of ≧100 μg/ml.

Compound	Minimum inhibitory concentration[a]							
	Primary rabbit kidney cells				Human skin fibroblasts			
	Vaccinia virus	Herpes simplex-1 KOS	Herpes simplex-1 LYONS	Vesicular stomatitis virus	Vaccinia virus	Herpes simplex-1 KOS	Herpes simplex-1 LYONS	Vesicular stomatitis virus
	μg/ml	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*
5-Nitrouracil	>100	>100	>100	>100	>100	>100	>100	70
5-Nitrouridine	>100	>100	>100	>100	>100	>100	>100	>100
1-Methyl-5-nitrouracil	>100	>100	>100	>100	40	>100	100	40
1,3-Dimethyl-5-nitrouracil	>100	>100	>100	>100	40	>100	>100	70
5-Nitro-dU	0.1–0.4	1–4	1–4	>100	0.1	1	4	>100
5-Nitro-dUMP	0.4–4	40–100	40–100	>100	0.1–0.4	40	40–100	>100
3′-*O*,5-Dinitro-dU	100	100	>100	>100	20	>100	100	100
3′-*O*,5-Dinitro-dUMP	100	100	>100	>100	20	>100	100	100
5-Iodo-dU	0.1	0.2	1	>100	0.1	0.2	1	>100

[a] Required to reduce virus-induced cytopathogenicity by 50%. Viral cytopathogenicity was recorded as soon as it attained 100% in the untreated cell cultures, in general at 2 days for cell cultures that had been infected with herpes simplex or vesicular stomatitis, and at 3 days for cell cultures that had been infected with vaccinia virus. Virus input was 100 $CCID_{50}$ for both PRK cell cultures (in tubes) and HSF cell cultures (in microplates).

HSF cells 5-nitro-dU and 5-nitro-dUMP inhibited the cytopathic effects of vaccinia virus at concentrations as low as 0.1–0.4 μg/ml (Table 1). Neither 5-nitro-dU nor 5-nitro-dUMP proved effective against vesicular stomatitis virus at 100 μg/ml, the highest concentration tested. 5-Nitro-dU and 5-nitro-dUMP inhibited the cytopathogenicity of herpes simplex virus (type 1, strains KOS and LYONS) at concentrations of 1–4 μg/ml and 40–100 μg/ml, respectively (Table 1)—concentrations approximately 10–100-fold higher than those required to suppress the cytopathogenicity of vaccinia virus. The minimum dose at which 5-nitro-dU was found to inhibit vaccinia virus cytopathogenicity compared favorably with those obtained previously for 5-ethyl-dU (6) and 5-thiocyanato-dU (9) and closely corresponded to those recorded with ara-A (21) and 5-iodo-dU (Table 1).

While relatively effective against herpes simplex-1 strain KOS, 5-nitro-dU was inactive against herpes simplex-2 strain 333, even at 100 μg/ml, the highest concentration tested. Similar results have been obtained previously for other deoxythymidine analogues, e.g., 5-iodo-dU, 5-ethyl-dU, and 5-thiocyanato-dU (22). Since herpes simplex-1 strain KOS increases the dT kinase activity of PRK and HSF cells, whereas herpes simplex-2 strain 333 fails to do so (22), our observations suggest that the anti-herpes activity of 5-nitro-dU and the other dU derivatives may depend on a specific virus-induced dT kinase.

Additive effect in antiviral activities of 5-nitro-dU and 5-iodo-dU. At 100 μg/ml, 5-nitro-dU completely arrested vaccinia virus growth; at 10 μg/ml, it caused a 3 $\log_{10}$ reduction in virus yield; and at 1 μg/ml, it effected a 1 $\log_{10}$ drop in virus yield (Fig. 1, left panel). Again, these data compare favorably with those obtained previously for the inhibitory effects of 5-ethyl-dU and 5-thiocyanato-dU (6, 9) on vaccinia virus growth. As noted before (6), 5-iodo-dU completely suppressed vaccinia virus multiplication when added to the cells at 10 or 100 μg/ml; at 1 μg/ml it caused a partial reduction of virus multiplication (Fig. 1, middle panel).

Combination of 5-nitro-dU (1 μg/ml) and 5-iodo-dU (1 μg/ml) exerted a markedly greater antiviral effect than did either compound alone. Such combination resulted in the complete arrest of virus multiplication (Fig. 1, right panel). When used individually at 1 μg/ml, the drugs achieved only a partial reduction in virus titer (Fig. 1, left and middle panels). Similar additive effects have been observed previously with the combinations 5-methoxymethyl-dU + 5-iodo-dU, 5-methoxymethyl-dU + ara-A, 5-methoxymethyl-dU + ara-C, and 5-iodo-dU + ara-C (11), although other reports (23) have mentioned an antagonistic effect for the combination ara-C + 5-iodo-dU. The

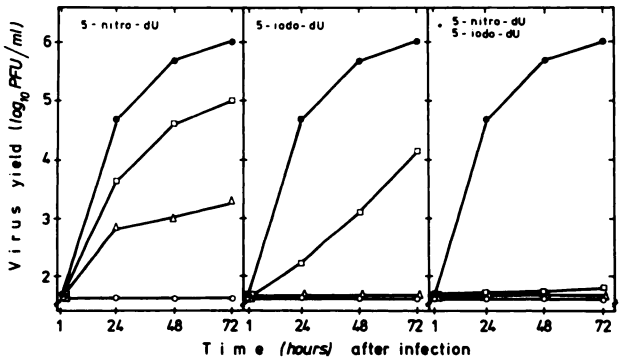

FIG. 1. *Effects of 5-nitro-dU and 5-iodo-dU on vaccinia virus growth in PRK cells*

Virus input was 4.5 $\log_{10}$ PFU/Petri dish. Compounds were added immediately after virus adsorption. Left: 5-Nitro-dU added at 1 μg/ml (□), 10 μg/ml (△), or 100 μg/ml (○). Middle: 5-Iodo-dU added at 1 μg/ml (□), 10 μg/ml (△), or 100 μg/ml (○). Right: Both 5-nitro-dU and 5-iodo-dU added at 1 μg/ml (□), 10 μg/ml (△), or 100 μg/ml (○). ●, control.

observation that combination of 5-nitro-dU with 5-iodo-dU produced enhanced antiviral activity may be interpreted to mean that these nucleoside analogues interfere at different stages of DNA biosynthesis.

Effects of 5-nitro-dU and 5-nitro-dUMP on [methyl-^{3}H]dT and [2-^{14}C]dU incorporation into cellular DNA. Both 5-nitro-dU and 5-nitro-dUMP effectively blocked the incorporation of [2-^{14}C]dU into DNA of PRK cells (Fig. 2, lower panel). The concentrations of 5-nitro-dU and 5-nitro-dUMP required to effect 50% inhibition of [2-^{14}C]dU incorporation amounted to 0.2–0.3 μg/ml (Table 2). Two related compounds, 3′-*O*,5-dinitro-dU and 3′-*O*,5-dinitro-dUMP, were also found to inhibit [2-^{14}C]dU incorporation, but only at a 100-fold higher concentration (25–50 μg/ml) (Table 2). Neither 5-nitro-dU nor 5-nitro-dUMP inhibited incorporation of [*methyl*-^{3}H]dT into PRK cell DNA (Fig. 2, upper panel). In fact, both nitro compounds stimulated [*methyl*-^{3}H]dT incorporation when applied to the cells at concentrations higher than 5 μg/ml. A similar increase in [*methyl*-^{3}H]dT incorporation has been observed in PRK cells exposed to relatively high concentrations (more than 5 μg/ml) of 5-ethyl-dU and 5-thiocyanato-dU (6, 9).

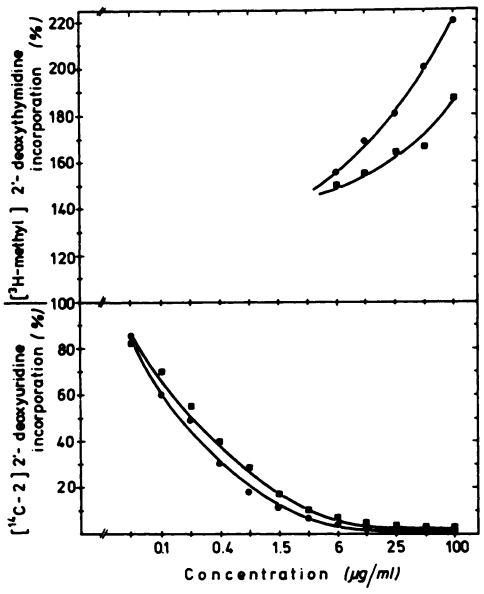

FIG. 2. *Effects of 5-nitro-dU (●) and 5-nitro-dUMP (■) on [methyl-^{3}H]dT and [2-^{14}C]dU incorporation into DNA of PRK cells*

TABLE 2

Effects of 5-nitro-dU, 5-nitro-dUMP, and other nitrated derivatives of uracil on incorporation of [methyl-^{3}H]dT and [2-^{14}C]dU into DNA of PRK cells

Compound	ID_{50}[a]	
	[*methyl*-^{3}H] dT incorporation	[2-^{14}C]dU incorporation
	μg/ml	μg/ml
5-Nitrouracil	50	40
1-Methyl-5-nitrouracil	>100	>100
1,3-Dimethyl-5-nitrouracil	>100	>100
5-Nitro-dU	>100	0.2
5-Nitro-dUMP	>100	0.3
3′-*O*,5-Dinitro-dU	>100	25
3′-*O*,5-Dinitro-dUMP	>100	50
5-Iodo-dU	2.5	1.2

[a] Dose inhibiting the incorporation of [*methyl*-^{3}H] dT or [2-^{14}C]dU by 50%.

Effects of various deoxythymidine analogues on [methyl-^{3}H]dT and [2-^{14}C]dU incorporation into cellular DNA. In addition to 5-nitro-dU, several other nucleoside analogues, i.e., 5-fluoro-, 5-cyano-, 5-trifluoromethyl-, and 5-thiocyanato-2′-deoxyuridine, were found to inhibit the incorporation of dU into DNA at doses considerably lower than those required to inhibit dT incorporation (Table 3).

Nucleoside analogues that block the incorporation of dU but not that of dT may be assumed to act specifically at the thymidylate synthetase level. Thymidylate synthetase, which catalyzes the conversion of dUMP to dTMP, is the only metabolic step that distinguishes the pathways of dU and dT incorporation into DNA dTMP (Fig. 3).

That 5-fluoro- and 5-trifluoromethyl-dU would specifically block thymidylate synthetase in intact cells is not surprising, since both 5-fluoro-dUMP and 5-trifluoromethyl-dUMP are known to be potent inhibitors of thymidylate synthetase *in vitro* (25). 5-Fluoro-dUMP was originally described as an inhibitor of phage-induced thymidylate synthetase (26), but since this discovery 5-fluoro-dUMP has been shown to inhibit thymidylate synthetase from various other

TABLE 3

Effects of various deoxythymidine analogues on incorporation of [methyl-^{3}H]dT and [2-^{14}C]dU into DNA of PRK cells

Compound	ID_{50}[a]	
	[*methyl*-^{3}H]dT incorporation	[2-^{14}C]dU incorporation
	μg/ml	*μg/ml*
5-Iodo-dU	2.5	1.2
5-Bromo-dU	0.3	0.3
5-Chloro-dU	0.3	0.2
5-Fluoro-dU	100	0.0005
5-Cyano-dU	>400	75
5-Ethyl-dU	150	30
5-Trifluoromethyl-dU	25	0.05
5-Hydroxymethyl-dU	10	15
5-Thiocyanato-dU	>400	40
5-Hydroxy-dU	25	8
5-Allyloxy-dU	50	45
5-Propynyloxy-dU	75	50
5-Carboxamidomethyloxy-dU	300	150
Ara-C	0.1	0.05
Ara-U	300	>400
Ara-T	300	300
Ara-A	25	20

[a] Dose inhibiting the incorporation of [*methyl*-^{3}H]dT or [2-^{14}C]dU by 50%.

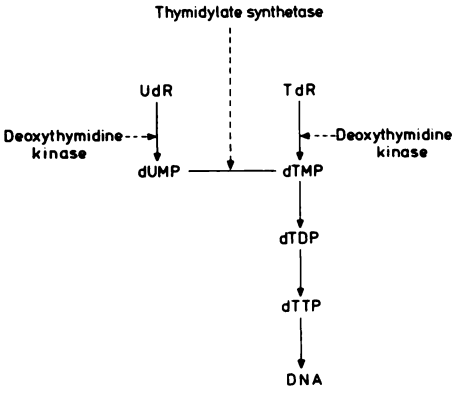

FIG. 3. *Metabolic pathways leading to incorporation of dU and dT into DNA*

The first reaction is the conversion of dU and dT to dUMP and dTMP, respectively. This reaction is catalyzed by deoxythymidine kinase, an enzyme that accepts as substrates both dU and dT and various 5-substituted derivatives of dU (e.g., 5-fluoro-, 5-chloro-, 5-bromo-, and 5-iodo-dU), as demonstrated *in vitro* with the deoxythymidine kinase of *Escherichia coli* (24).

sources, including Ehrlich ascites carcinoma cells (27). In addition to 5-fluoro-dUMP and 5-trifluoromethyl-dUMP, various other 2′-deoxyuridylate derivatives, such as 5-mercapto-dUMP (28), 5-formyl-dUMP (29, 30), 5-hydroxymethyl-dUMP (29, 30), 5-ethyl-dUMP (31), and 5-methoxymethyl-, 5-acetyl-, 5-allyl-, 5-(2,3-oxypropyl)-, 5-azidomethyl-, and 5-iodoacetamidomethyl-dUMP (30, 32), have been reported to inhibit thymidylate synthetase of either bacterial or mammalian origin. However, the K_i values calculated for most of these dUMP analogues were considerably higher than that originally determined for 5-fluoro-dUMP.

The results presented in Table 3 indicate that the inhibitory activity of 5-fluoro- and 5-trifluoromethyl-dUMP on thymidylate synthetase in cell-free systems *in vitro* (25–27) may be extended to conditions *in vivo*. Even the concentrations at which 5-fluoro-dU and 5-trifluoromethyl-dU inhibited dTMP synthesis *in vivo* [ID_{50}, 1.7 and 170 nM, respectively (Table 3)] corresponded well to the K_i values recorded with 5-fluoro-dUMP and 5-trifluoromethyl-dTMP *in vitro* [K_i, 4.9 and 60 nM, respectively (as obtained in a standard thymidylate synthetase assay at pH 7.4, with prior incubation; see Table 5 of ref. 25)]. That 5-thiocyanato-dU may also act as an inhibitor of thymidylate synthetase *in vivo* (Table 3) is not surprising, if one considers the possibility (8) that within the cell the thiocyanato nucleoside of its 5′-monophosphate is reduced to yield 5-mercapto-dUMP, itself a powerful inhibitor of thymidylate synthetase (28).

Nucleoside analogues such as ara-C and ara-A, which are assumed to inhibit DNA synthesis by a competitive action of their 5′-triphosphates at the DNA polymerization step (33–36), appeared to inhibit [2-^{14}C]dU and [*methyl*-^{3}H]dT incorporation to the same extent (Table 3). 5-Iodo-dU, which has been found to inhibit various enzymes involved in DNA synthesis [either competitively or allosterically (1), although it is considered to act primarily after its incorporation into DNA (1)], also inhibited [2-^{14}C]dU and [*methyl*-^{3}H]dT incorporation at nearly identical concentrations (Ta-

ble 3). Ara-C, ara-A, 5-iodo-dU, and all other nucleoside analogues for which the doses required to inhibit dU incorporation corresponded closely to the doses required to inhibit dT incorporation (Table 3) may be assumed not to interfere with the thymidylate synthetase reaction. However, a specific interaction at the thymidylate synthetase level may be postulated for 5-nitro-dU and all other compounds (5-fluoro-, 5-trifluoromethyl-, 5-cyano-, and 5-thiocyanato-dU) that suppressed dU incorporation, but not dT incorporation, into DNA dTMP.

Assay in vivo for inhibition of thymidylate synthetase. In animal cells dT is specifically incorporated into DNA dTMP; it does not contribute to either DNA deoxycytidylate or pyrimidine ribonucleotides (37). Normally dU is incorporated into DNA as dTMP, although part of the dU may undergo phosphorolysis, leading to the release of free uracil, which will eventually enter the pyrimidine ribonucleotide pool (37). There is little doubt, however, that during the exponential growth phase (as in our assay) cells preferentially utilize deoxyuridine for the synthesis of dTMP *de novo*. In such proliferating cells, dT kinase activity is particularly high (38, 39) and DNA synthesis can be monitored by the incorporation of either dT or dU. The incorporation of dU will be suppressed by any compound that interacts at the thymidylate synthetase level, whether the compound is a dU analogue (Table 3) or a folic acid antagonist (40).

In view of the differential effects that a nucleoside analogue exerts on the incorporation of dU and dT into the DNA of proliferating cells, one can readily distinguish between analogues that selectively inhibit thymidylate synthetase and analogues that act primarily at another stage of DNA biosynthesis. Thus the method described here can be considered a rapid screen for inhibitors of thymidylate synthetase *in vivo*. This concept seems to be validated by some previous observations. Nelson and Carter (41) found that when 4-*N*-hydroxy-2′-deoxycytidine, an inhibitor of thymidylate synthetase *in vitro*, was incubated with L5178Y leukemic cells, dU incorporation into DNA was markedly suppressed whereas dT incorporation was not decreased but, rather, elevated.

Obviously, some conditions have to be fulfilled before a nucleoside analogue that blocks dU incorporation, but not dT incorporation, can be identified as a specific inhibitor of thymidylate synthetase *in vivo*: (a) the compound should not impair the uptake of dU or dT by the cells, or at least not preferentially inhibit the uptake of dU; (b) the compound should be phosphorylated by cellular dT kinase to its 5′-monophosphate; (c) the compound should not interfere with the conversion of dU to dUMP or with the conversion of dT to dTMP, or, if it does, it should inhibit these conversions to the same extent; and (d) the compound should not differentially affect the endogenous pools of dUMP or dTMP. Finally, one should envisage the possibility that the nucleoside analogue may act at more than one metabolic step.

Antiviral activity of 5-nitro-dU may be attributed to inhibition of thymidylate synthetase. In the uninfected cell, 5-nitro-dU appears to inhibit thymidylate synthetase. Could this inhibitory effect account for the antiviral activity of 5-nitro-dU? If the inhibition of thymidylate synthetase is responsible for the antiviral action of 5-nitro-dU, one may expect the antiviral activity of 5-nitro-dU to be reversed by dT, but not by dU or dC. As shown in Fig. 4, dT readily reversed the inhibitory effect of 5-nitro-dU on vaccinia virus growth. At a concentration 100-fold lower than that of 5-nitro-dU, dT virtually abolished the anti-vaccinia activity of 5-nitro-dU (Fig. 4, upper panel). We have previously demonstrated that the anti-vaccinia effect of 5-thiocyanato-dU was also suppressed upon addition of dT at a concentration 100-fold lower than the concentration at which the thiocyanato compound was employed (9). However, the inhibitory effect of 5-iodo-dU on vaccinia virus replication could not be reversed by dT, unless the concentration of dT equaled or surpassed that of 5-iodo-dU (Fig. 4, upper panel). Thus the antiviral activity of 5-iodo-dU proved approximately 100 times less sensitive to reversal by thymidine than did the antiviral activity of 5-nitro-dU, corroborating the hypothesis that 5-iodo-dU

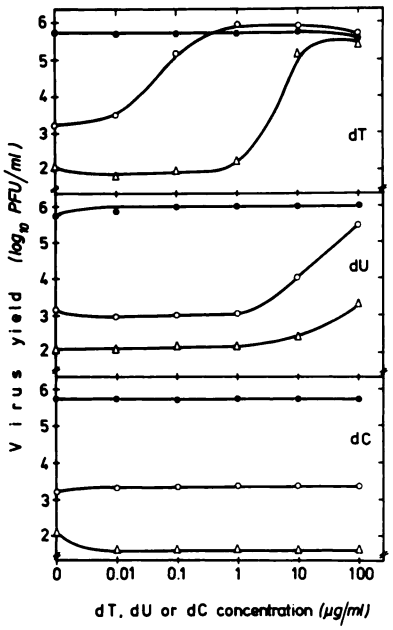

FIG. 4. *Reversal of antiviral activity of 5-nitro-dU and 5-iodo-dU by dT, dU, and dC*

The test system was vaccinia virus growth in PRK cells. Virus input was 4.5 log_{10} PFU/Petri dish. Compounds were added immediately after virus adsorption. Virus yield was determined 24 hr after virus infection. Upper: dT added at the concentrations indicated on the abscissa. ●, dT alone; ○, dT plus 10 μg/ml of 5-nitro-dU; △, dT plus 10 μg/ml of 5-iodo-dU. Middle: dU added at the concentrations indicated on the abscissa. ●, dU alone; ○, dU plus 10 μg/ml of 5-nitro-dU, △, dU plus 10 μg/ml of 5-iodo-dU. Lower: dC added at the concentrations indicated on the abscissa. ●, dC alone; ○, dC plus 10 μg/ml of 5-nitro-dU; △, dC plus 10 μg/ml of 5-iodo-dU.

and 5-nitro-dU interfere at different stages of (viral) DNA synthesis.

The inhibitory effect of 5-nitro-dU on vaccinia virus growth was not reversed upon the addition of dU, unless dU was added at rather high concentrations, 10^3-fold higher than those required for dT to reverse the antiviral effect of 5-nitro-dU (Fig. 4, upper and middle panels). Addition of dC did not influence the antiviral effects of 5-nitro-dU or 5-iodo-dU, even if dC was added at 10 times the concentrations at which 5-nitro-dU and 5-iodo-dU were applied (Fig. 4, lower panel).

The observation that the inhibitory activity of 5-nitro-dU on vaccinia virus replication is much more easily reversed by thymidine than by other deoxyribonucleosides (dU, dC) supports the concept that the antiviral activity, or at least the anti-vaccinia activity, of 5-nitro-dU could indeed be mediated by inhibition of the thymidylate synthetase reaction. It is noteworthy that the minimum dose at which 5-nitro-dU inhibited thymidylate synthetase ([2-^{14}C]dU incorporation; see Table 2) closely corresponded to the minimum dose that was found inhibitory to vaccinia virus (Table 1). Although our data point to thymidylate synthetase as the target for the antiviral activity (and, possibly, other biological activities) of 5-nitro-dU, they do not offer an explanation for any selectivity demonstrated in the antiviral activities of 5-nitro-dU.

CONCLUSION

As postulated by Prusoff and Ward (42), the ideal antiviral drug should conform to a number of requirements, including solubility, stability, low cost of preparation, and lack of immunosuppressive, teratogenic, mutagenic, and carcinogenic properties. It should not activate oncogenes and, most important, it should not be incorporated into the DNA of the normal uninfected cell. Evidently these premises do not hold for 5-iodo-dU, which is incorporated into both viral and host cell DNA. As a corollary, nucleoside analogues with equal antiviral potency but considerably less toxicity should be sought. Among the 2′-deoxyuridine derivatives, possible candidates are (a) 5-ethyl-dU (5, 6), which does not appear to be mutagenic and does not activate oncornavirus expression (43), although it is incorporated into DNA, (b) 5-iodo-5′-amino-2′,5′-dideoxyuridine (44), which is incorporated into both viral and host cell DNA of virus-infected cells, but not into DNA of uninfected cells (45, 46), (c) 5-cyano-dU (15, 16), which is not incorporated at all, and which may specifically interact at the thymidylate synthetase level (Table 3, this report),(d) 5-thiocyanato-dU (8, 9) and (e) 5-nitro-dU, which would, as reported here, also specifically inhibit thymidylate synthetase, and, finally, (f) 5-methylamino-dU (3, 4), 5-methoxymethyl-dU (10, 11), 5-propyl-dU (7, 14), and 5-propynyloxy-dU (17), which could be considered as selective anti-herpes agents.

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