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# Thymidylate Synthetase as Target Enzyme for the Inhibitory Activity of 5-Substituted 2′-Deoxyuridines on Mouse Leukemia L1210 Cell Growth

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SUMMARY

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A series of 26 5-substituted 2′-deoxyuridines (dUrd), including various dUrd analogues (viz. 5-(1-chlorovinyl)-dUrd, *E*-5-(2-bromovinyl)-dUrd, 5-azidomethyl-dUrd, 5-methylthiomethyl-dUrd, 5-propynyloxy-dUrd), which have never been the subject of extensive antitumor studies, were evaluated for their inhibitory effects on L1210 cell proliferation. The most effective inhibitors were (in order of decreasing activity): 5-fluoro-dUrd > 5-trifluoromethyl-dUrd > 5-nitro-dUrd (5′-monophosphate) > 5-ethynyl-dUrd > 5-formyl-dUrd > 5-(1-chlorovinyl)-dUrd. Their 50% inhibitory dose ($ID_{50}$) fell within the 0.5–0.001 μg/ml range. These and several other dUrd analogues (i.e., 5-cyano-dUrd, 5-thiocyano-dUrd via 5-mercapto-dUrd 5′-monophosphate, and the 5-oxime of 5-formyl-dUrd) have been recognized previously as potent and/or selective inhibitors of thymidylate synthetase. As could be expected from specific thymidylate synthetase inhibitors, all nine compounds were far more inhibitory to [2-$^{14}$C]dUrd incorporation into host cell DNA than to [methyl-$^{3}$H]dThd incorporation, and their inhibitory effects on L1210 cell proliferation were more readily reversed by dThd than by dUrd. The other 17 dUrd analogues, all of which had $ID_{50}$ values for L1210 cell growth that were greater than 1 μg/ml, did not discriminate between [2-$^{14}$C]dUrd and [methyl-$^{3}$H]dThd incorporation, and their inhibitory effects on L1210 cell growth were reversed equally well by dThd and dUrd, or not reversed at all. For the nine dUrd analogues which could be considered as thymidylate synthetase inhibitors, there was a strong correlation ($R = 0.904$) between their inhibitory effect on tumor cell growth, on the one hand, and their relatively greater inhibition of [2-$^{14}$C]dUrd incorporation and reversal of antitumor activity by dThd, on the other. This correlation points to thymidylate synthetase as the principal, if not the sole, intracellular target for the inhibitory activity of 5-substituted 2′-deoxyuridines on L1210 cell growth.

## INTRODUCTION

Among the best-known antiviral agents are the 2′-deoxyuridine (dUrd) derivatives, 5-iodo-dUrd (idoxuridine) and 5-trifluoromethyl-dUrd (trifluorothymidine) (1). In addition to 5-iodo-dUrd and 5-trifluoromethyl-dUrd, various other 5-substituted 2′-deoxyuridines have been developed, all of which are endowed with marked

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antiviral properties (2–11). Some of these compounds, i.e., 5-fluoro-dUrd, 5-nitro-dUrd, and 5-formyl-dUrd, were also found to inhibit dUrd incorporation into DNA (of primary rabbit kidney cells) to a significantly greater extent than dThd incorporation (6, 10). This was interpreted as a selective inhibition of thymidylate synthetase in cell culture (6). For most, if not all, 5-substituted 2′-deoxyuridines which were postulated to act as inhibitors of thymidylate synthetase in cell culture (6, 9, 10), inhibition of thymidylate synthetase has been confirmed with isolated enzyme systems, for example, with 5-fluoro-dUMP (12, 13), 5-trifluoromethyl-dUMP (12, 13), 5-nitro-dUMP (14), 5-formyl-dUMP (13, 15), 5-cyano-dUMP (16), and 5-thiocyano-dUMP [via 5-mercapto-dUMP (17)].

Thymidylate synthetase may be regarded as an attractive target enzyme in cancer chemotherapy, and some thymidylate synthetase inhibitors, such as 5-fluoro-dUrd (in its free base form), have for several years been employed in the treatment of patients with disseminated breast and colon cancers (18). The antitumor properties of 5-fluoro-dUrd and 5-trifluoromethyl-dUrd are well-documented (19). Other 5-substituted 2′-deoxyuridines have not been submitted to extensive antitumor studies. Some dUrd derivatives, viz., 5-ethyl-dUrd (20), 5-ethynyl-dUrd (21), 5-vinyl-dUrd (21), 5-mercapto-dUrd (17), 5-formyl-dUrd (22), 5-nitro-dUrd (23), and 5-hydroxymethyl-dUrd (24) were reported to inhibit the growth of either L1210 or other tumor (Ehrlich ascites carcinoma, $B_5$59 melanoma) cells in culture. However, it was not clearly established whether this antitumor activity was due to an inhibition of thymidylate synthetase or to some other mechanism(s).

To evaluate the role of thymidylate synthetase as a target for the antitumor activity of 5-substituted 2′-deoxyuridines, we have tested the potency of a large variety of 5-substituted dUrd analogues as inhibitors of L1210 cell growth, and have attempted to correlate their cytotoxic effects with two parameters which may be considered as valuable indices of thymidylate synthetase inhibition in cell culture: (a) the ability of dThd, relative to dUrd, to reverse the inhibitory effect of the 5-substituted dUrd on tumor cell growth and (b) the capacity of the 5-substituted dUrd to inhibit the incorporation of [2-$^{14}$C] dUrd, relative to [methyl-$^{3}$H]dThd, into cellular DNA. From the known metabolism of dThd and dUrd in the cell,

$$\begin{array}{l} \text{dThd} \rightarrow \text{dTMP} \rightarrow \text{dTDP} \rightarrow \text{dTTP} \rightarrow \text{DNA} \\ \qquad\qquad\quad \uparrow \\ \text{dUrd} \rightarrow \text{dUMP} \end{array}$$

one may postulate that (a) for those compounds that specifically act at the thymidylate synthetase level, the cytotoxic action should be reversed more efficiently upon addition of dThd than of dUrd. Also, (b) those compounds which owe their cytotoxic action to selective inhibition of the thymidylate synthetase reaction (dUMP → dTMP) should inhibit [2-$^{14}$C]dUrd incorporation into DNA to a significantly greater extent than [methyl-$^{3}$H] dThd incorporation.

## MATERIALS AND METHODS

*Cells.* Mouse leukemia L1210 cells were grown in 75 cm$^2$ tissue culture flasks (Falcon 3024F; Becton, Dickinson France S.A., Grenoble, France) in Eagle's minimal essential medium, supplemented with 10% (v/v) inactivated fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland), 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland), 0.075% (w/v) $NaHCO_3$ (Flow Laboratories) and 25 units/ml of nystatine (S. A. Labaz N.V., Brussels, Belgium). The L1210 cell line used was found to be *Mycoplasma*-free.

*Test compounds.* The 5-substituted 2′-deoxyuridines that were tested for their inhibitory effects on L1210 cells are depicted in Fig. 1. The sources of the compounds were as follows: 5-fluoro-dUrd (Aldrich Chemical Company, Milwaukee, Wisc.), 5-bromo-dUrd (Sigma Chemical Company, St. Louis, Mo.), 5-iodo-dUrd (Sigma Chem-

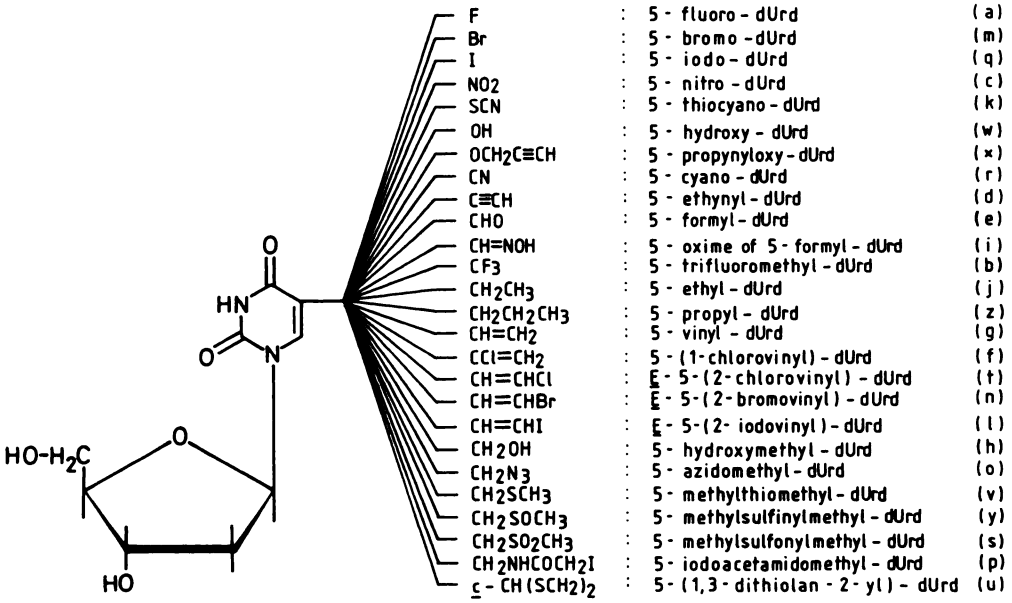


FIG. 1. *5-Substituted 2′-deoxyuridines*

ical Company), 5-nitro-dUrd (5′-monophosphate) (see ref. 6), 5-thiocyano-dUrd (see ref. 3), 5-hydroxy-dUrd (Sefochem Fine Chemicals, Emek Hayarden, Israel), 5-propynyloxy-dUrd (see ref. 8), 5-cyano-dUrd (see ref. 7), 5-ethynyl-dUrd (see ref. 9), 5-formyl-dUrd (see ref. 10), 5-oxime of 5-formyl-dUrd (see ref. 25), 5-trifluoromethyl-dUrd (P-L Biochemicals, Milwaukee, Wisc.), 5-ethyl-dUrd (see ref. 2), 5-propyl-dUrd (see ref. 4), 5-vinyl-dUrd (see ref. 9), 5-(1-chlorovinyl)-dUrd (see ref. 9), *E*-5-(2-chlorovinyl)-dUrd (see ref. 11), *E*-5-(2-bromovinyl)-dUrd (see ref. 9), *E*-5-(2-iodovinyl)-dUrd (see ref. 9), 5-hydroxymethyl-dUrd (Calbiochem-Behring Corporation, Lucerne, Switzerland), 5-azidomethyl-dUrd (see ref. 10), 5-methylthiomethyl-dUrd (see refs. 10, 26), 5-methylsulfinylmethyl-dUrd (see ref. 26), 5-methylsulfonymethyl-dUrd (see ref. 26), 5-iodoacetamidomethyl-dUrd (see ref. 10), and 5-(1,3-dithiolan-2-yl)-dUrd (see ref. 25).

*Radiochemicals.* The radiolabeled nucleosides [2-$^{14}$C]dUrd (specific radioactivity, 58 mCi/mmole), [6-$^{3}$H]dUrd (specific radioactivity 15 Ci/mmole), and [1′, 2′-$^{3}$H]dUrd (specific radioactivity, 42 Ci/mmole) were obtained from the Radiochemical Centre (Amersham, England), whereas [*methyl*-$^{3}$H]dThd (specific radioactivity, 38 Ci/mmole) was obtained from the Institute of Radio Elements (IRE, Fleurus, Belgium).

*Inhibition of tumor cell growth.* All assays were performed in Linbro microplates (model FB-48-TC, Linbro Chemical Company, New Haven, Conn.). To each well were added $5 \times 10^4$ L1210 cells and a given amount of the test compound. In those assays that were aimed at reversing the inhibitory effects of the test compounds, dUrd (125 μg/ml) or dThd (5 μg/ml) was added to the cells together with varying amounts of the test compound. The doses employed for dUrd and dThd correspond to the maximal concentrations of dUrd and dThd which were themselves not inhibitory to L1210 cell proliferation. The cells were allowed to proliferate for 48 hours at 37° in a humidified, $CO_2$-controlled atmosphere. The growth of the cells was linear during this 48 hour-incubation period. At the end of the incubation period, the cells were counted in a Coulter counter (Coulter Electronics Ltd, Harpenden Herts, England) and the number of dead cells was evaluated by staining with trypan blue. The $ID_{50}$ (50% inhibitory dose) was defined as the concentration of compound that reduced the number of living cells by 50%.

*Inhibition of [methyl-$^{3}$H]dThd and [2-$^{14}$C]dUrd incorporation.* The incorporation of [*methyl*-$^{3}$H]dThd and [2-$^{14}$C]dUrd into cellular DNA was also measured in Linbro microplates. To each well were added $10^5$ L1210 cells, 6.5 pmoles (0.25 μCi) of [*methyl*-$^{3}$H]dThd, or 4.31 nmoles (0.25 μCi) of [2-$^{14}$C]dUrd, and a given amount of the test compound. The cells were allowed to proliferate for 20 hr at 37° in a humidified, $CO_2$-controlled atmosphere. At the end of this incubation period, the contents of the wells (200 μl) were brought onto 25-mm glass fiber filters (type A/E, Gelman Instrument Company, Ann Arbor, Mich.), mounted on a Millipore 3025 sampling manifold apparatus. The filters were washed twice with cold NaCl/Pi (phosphate-buffered saline), twice with cold 10% trichloroacetic acid, twice with cold 5% trichloroacetic acid, once with cold ethanol, and once with cold ether. The filters were then allowed to dry for 10 min at 60° and assayed for radioactivity in a toluene-based scintillant.

It was ascertained by CsCl density gradient analysis that, under our test conditions, both [*methyl*-$^{3}$H]dThd and [2-$^{14}$C]dUrd were incorporated into DNA. Therefore, L1210 cells were seeded in tissue culture Cluster 3524 Costar (Cambridge, Mass.) cups at $5 \times 10^5$ cells/ml/cup and incubated with 5 μCi of either [*methyl*-$^{3}$H]dThd or [2-$^{14}$C]dUrd (in later experiments [6-$^{3}$H]dUrd was used instead of [2-$^{14}$C]dUrd) for 20 hr at 37° in a humidified, $CO_2$-controlled incubator. The cells were then pelleted by centrifugation at 800 rpm for 5 min, washed with cold NaCl/Pi, pelleted again, and finally lysed by the addition of 100 μl of 2% sodium dodecyl sulfate in 0.15 M NaCl and 0.1 M EDTA (pH 8.4). The cell lysate was then brought on top of 3.9 ml of a CsCl solution in distilled water ($\rho = 1.7241$ g/ml, pH 8.0) and centrifuged for 72 hr at 19° and 100,000 × *g* in an MSE swinging-out rotor. Twenty-one fractions of ten drops each were collected from the bottom of the tubes and assayed for radioactivity; as can be seen in Fig. 2, the radioactivity profile of [6-$^{3}$H]dUrd coincided with the radioactivity profile of [*methyl*-$^{3}$H]dThd, both of which peaked at a density of approximately 1.755 g/ml; in other experiments, analogous to that presented in Fig. 2, peak radioactivity values were obtained at a density ranging from 1.730 to 1.755 g/ml. These values are somewhat higher than normally expected from mammalian DNA (density, ~1.70 g/ml), which may be related to the procedure used for preparing the samples (whole cell lysates without prior purification). However, they indicate that under our experimental conditions both dThd and dUrd were incorporated into DNA.

## RESULTS AND DISCUSSION

*Inhibition of thymidylate synthetase in cell-free systems.* From the $K_i/K_m$ values of the various 5-substituted dUMP derivatives tested for inhibition of thymidylate synthetase in isolated enzyme systems (Table 1), it would appear that most dUMP analogues possess $K_i/K_m$ values within the range 0.1–10. However, for some dUMP analogues, i.e., 5-fluoro-, 5-nitro-, 5-trifluoromethyl-, 5-formyl-, and 5-mercapto-dUMP, $K_i/K_m$ values are considerably lower than 1. The latter dUMP analogues may therefore be regarded as potent inhibitors of thymidylate synthetase. Their potency as thymidylate synthetase inhibitors is further reflected in the fact that in PRK cells[7] the corresponding nucleosides specifically inhibited [2-$^{14}$C]-dUrd incorporation into DNA; [*methyl*-$^{3}$H]dThd incorporation was not inhibited unless the nucleoside analogues were applied at concentrations that were 10- to 500-fold, and for 5-fluoro-dUrd even 200,000-fold, higher than the concentrations required to inhibit [2-$^{14}$C]dUrd incorporation (Table 1). Those 5-substituted dUrd derivatives that were relatively poor inhibitors of thymidylate synthetase in cell-free systems ($K_i/K_m \geq 1$) did not qualify as selective inhibitors of thymidylate synthetase in cell culture, as they inhibited the incorporation of [2-$^{14}$C]dUrd and [*methyl*-$^{3}$H]dThd to approximately the

[7] The abbreviation used is: PRK cells, primary rabbit kidney cells.

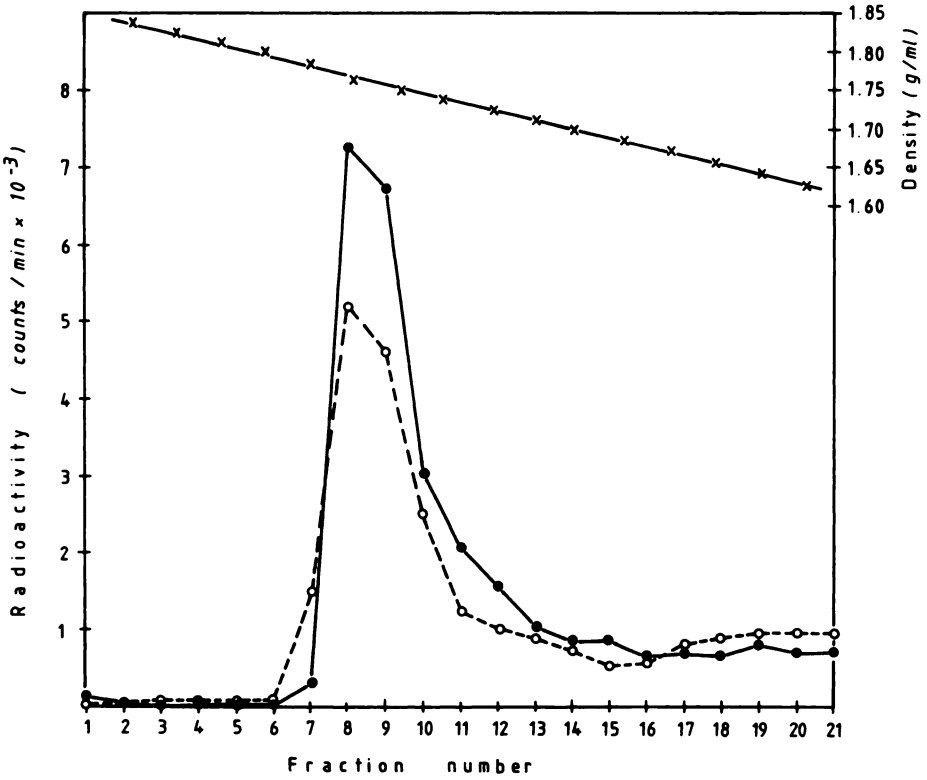


FIG. 2. *CsCl equilibrium density gradient profile of L1210 cell DNA labeled with either [methyl-$^3$H]dThd* (●——●) *or [6-$^3$H]dUrd* (○- - -○)

same extent (Table 1). For various dUMP analogues, viz., 5-ethynyl-dUMP, 5-(1-chlorovinyl)-dUMP, 5-vinyl-dUMP, and the *E*-5-(2-halogenovinyl)-dUMPs, $K_i/K_m$ values for inhibition of the isolated thymidylate synthetase remain to be determined, but, according to their differential effects on the incorporation of [2-$^{14}$C]dUrd and [*methyl*-$^3$H]dThd into PRK cell DNA, 5-ethynyl-dUMP and 5-(1-chlorovinyl)-dUMP may be predicted to be more potent inhibitors of thymidylate synthetase than 5-vinyl-dUMP and the *E*-5-(2-halogenovinyl)-dUMPs.

Inhibition of thymidylate synthetase is not a prerequisite for antiherpes activity, as the most selective antiherpes agents [compounds that inhibit herpes simplex virus replication at a significantly lower concentration than vaccinia virus replication (Table 1)] were found among those compounds that did not exhibit a specific inhibition of thymidylate synthetase either in cell culture or in cell-free systems. On the other hand, those compounds that proved specifically active against thymidylate synthetase did not display any selectivity toward herpes simplex virus in their antiviral action. In fact, some of the latter compounds, i.e., 5-nitro-dUrd, 5-formylp-Urd, and 5-cyano-dUrd, were even more inhibitory to vaccinia than to herpes simplex virus (Table 1). It would now seem of interest to examine whether inhibition of thymidylate synthetase, while not required for antiherpes activity, may determine the inhibitory effects of 5-substituted 2′-deoxyuridines on tumor cell growth.

*Inhibition of L1210 cell growth.* The 5-substituted 2′-deoxyuridines were examined at a variety of concentrations (ranging from 1 ng/ml to 1 mg/ml) for their inhibitory effects on L1210 cell proliferation, and the dose-response curves (for the doses situated close to the $ID_{50}$) are presented in Fig. 3. There were marked differences in the inhibitory effects of the 5-substituted 2′-deoxyuridines on L1210 cell growth. With an $ID_{50}$ of 1 ng/ml, 5-fluoro-dUrd was the most potent, and with an $ID_{50}$ of >1 mg/ml; 5-propyl-, 5-propynyloxy-, 5-hydroxy- and 5-methylsulfinylmethyl-dUrd were the least effective agents (Fig. 3). The other compounds showed $ID_{50}$ values that were intermediate between these extremes. Clearly, the most potent inhibitors of L1210 cell growth (with an $ID_{50}$ <1 μg/ml) were among the specific thymidylate synthetase inhibitors [with $K_i/K_m \ll 1$ (Table 1)]. These compounds included (in order of decreasing antitumor activity): 5-fluoro-dUrd > 5-trifluoromethyl-dUrd > 5-nitro-dUMP > 5-ethynyl-dUrd 5-formyl-dUrd > 5-(1-chlorovinyl)-dUrd (Fig. 3).

Several 5-substituted 2′-deoxyuridines, i.e., 5-trifluoromethyl-dUrd, 5-hydroxymethyl-dUrd, 5-vinyl-dUrd and 5-ethyl-dUrd, were more inhibitory to L1210 cell proliferation than could perhaps be expected from their abilities as thymidylate synthetase inhibitors (Table 1). For these compounds, some other factors, in addition to inhibition of dTMP synthesis, may have contributed to the observed cytotoxic effects. It is noteworthy that 5-trifluoromethyl-dUrd, 5-hydroxymethyl-dUrd, 5-vinyl-dUrd, and 5-ethyl-dUrd are incorporated into DNA, and this incorporation may obviously disturb normal cell metabolism and growth.

For a limited number of 5-substituted 2′-deoxyuridines, inhibitory effects of L1210 cell growth have been evaluated in previous studies (21):for 5-fluoro-dUrd and 5-bromo-dUrd the reported $ID_{50}$ values (21) were $2 \times 10^{-9}$ M and $6 \times 10^{-5}$ M, respectively, thus similar to $ID_{50}$ values obtained here ($\sim 4 \times 10^{-9}$ M and $\sim 8 \times 10^{-5}$ M, respec-

TABLE 1

*5-Substituted 2′-deoxyuridines*

Correlation between inhibition of isolated thymidylate synthetase, inhibition of thymidylate synthetase in PRK cells and antiviral activity in PRK cells

| Compound | $K_i/K_m$ of 5′-monophosphate derivative for isolated thymidylate synthetase systems (ref.) | Ratio of $ID_{50}$ for (*methyl*-$^3$H)dThd incorporation to $ID_{50}$ for [2-$^{14}$C] dUrd incorporation in PRK cells (ref.) | Ratio of $ID_{50}$ for vaccinia virus replication to $ID_{50}$ for herpes simplex-1 virus replication in PRK cells (ref.) |
|---|---|---|---|
| 5-Fluoro-dUrd | 0.002, 0.003 (12, 13) | 200,000 (6) | 1 (5) |
| 5-Trifluoromethyl-dUrd | 0.007, 0.012 (12, 13) | 500 (6) | 0.5 (5) |
| 5-Nitro-dUrd | 0.015 (14) | >500 (6) | 0.1 (5, 6) |
| 5-Ethynyl-dUrd | NT[a] | 50 (9) | 2 (9) |
| 5-Formyl-dUrd | 0.003, 0.004, 0.01, 0.02, 0.05 (13, 15, 25) | 100 (10) | 0.1 (10) |
| 5-(1-Chlorovinyl)-dUrd | NT | 50 (9) | 1.7 (9) |
| 5-Oxime of 5-formyl-dUrd | 0.5 (25) | 40[b] (25) | 12 (25) |
| 5-Thiocyano-dUrd via 5-mercapto-dUMP | 0.008 (17) | >10 (6) | 1 (3, 5) |
| 5-Cyano-dUrd | 0.13 (16) | >5 (6) | 0.1 (5, 7) |
| 5-Hydroxymethyl-dUrd | 0.15, 0.16, 0.44, 1.6 (13, 15) | 0.7 (6) | 2 (5) |
| 5-Vinyl-dUrd | NT | 2 (9) | 10 (9) |
| *E*-5-(2-bromovinyl)-dUrd | NT | 1 (9) | 1000 (9) |
| *E*-5-(2-iodovinyl)-dUrd | NT | 1 (9) | 1000 (9) |
| *E*-5-(2-chlorovinyl)-dUrd | NT | 1[b, c] | 4000[c] (11) |
| 5-Bromo-dUrd | 0.3 (13) | 1 (6) | 1 (5) |
| 5-Iodo-dUrd | 0.3 (13) | 2 (6) | 1 (5) |
| 5-Azidomethyl-dUrd | 0.7, 1.2, 2.3 (15) | 2 (10) | 10 (10) |
| 5-Ethyl-dUrd | 4.4 (27) | 5 (6) | 1 (2, 5) |
| 5-Iodoacetamidomethyl-dUrd | 3.5, 15 (28) | 0.67 (10) | <0.5 (10) |
| 5-Methylsulfonylmethyl-dUrd | 0.58 (26) | <0.75 (26) | 50 (26) |
| 5-(1,3-Dithiolan-2-yl)-dUrd | 0.2 (25) | 1[b] (25) | 1 (25) |
| 5-Methylthiomethyl-dUrd | 2.4, 3.5, 5.8, 6 (26) | <0.25 (10, 26) | 50 (10, 26) |
| 5-Methylsulfinylmethyl-dUrd | 0.61 (26) | <1 (26) | <1 (26) |
| 5-Hydroxy-dUrd | NT | 3 (6) | 1 (5, 8) |
| 5-Propynyloxy-dUrd | NT | 1.5 (6) | 25 (5, 8) |
| 5-Propyl-dUrd | NT | 1 (4) | >100 (4, 5) |

[a] NT, Not tested.

[b] For 5-oxime of 5-formyl-dUrd, 5-(1,3-dithiolan-2-yl)-dUrd and *E*-5-(2-chlorovinyl)-dUrd, 1′,2′-$^3$H instead of 2-$^{14}$C served as the radiolabel for measuring incorporation of dUrd into DNA.

[c] E. De Clercq, J. Descamps, G. Verhelst, A. S. Jones, and R. T. Walker, unpublished observations.

tively). However, for 5-vinyl-dUrd, Bobek and Bloch (21) reported an $ID_{50}$ value (>$10^{-4}$ M) that was significantly higher and for 5-ethynl-dUrd they reported an $ID_{50}$ value ($2 \times 10^{-8}$ M) that was significantly lower than the $ID_{50}$ values we have obtained for 5-vinyl-dUrd and 5-ethynl-dUrd (~$10^{-5}$ M and ~$3 \times 10^{-7}$ M, respectively). The $ID_{50}$ value found here for 5-nitro-dUMP ($10^{-7}$ M) is in accord with the data reported by Washtien *et al.* (23) for the nucleoside ($ID_{50}$: $0.33 \times 10^{-7}$ M for L1210 cell growth). Interestingly, the latter authors postulated that the cytotoxic effect of 5-nitro-dUrd might be due solely to inhibition of dTMP synthetase (23).

The nucleosides presented in Fig. 1 and 3 represent β-anomers. Some congeners have also been evaluated as the α-anomers or the free bases. For the free bases, 5-fluorouracil, 5-ethynyluracil, 5-(1-chlorovinyl)uracil, *E*-5-(2-bromovinyl)uracil, and 5-vinyluracil, the $ID_{50}$ values (for inhibition of L1210 cell growth) amounted to 0.18, 50, >200, >77, and 62 μg/ml, respectively, thus significantly higher than the $ID_{50}$ values for the corresponding β-nucleosides (Table 2). Likewise, 5-nitrouracil failed to inhibit L1210 cell growth at 300 μM (47 μg/ml), whereas 5-nitro-dUrd was still effective at 0.033 μM (0.009 μg/ml) (23). The $ID_{50}$ values for the α-anomers of 5-ethynyl-dUrd (6.0 μg/ml), *E*-5-(2-bromovinyl)-dUrd (73 μg/ml) and 5-vinyl-dUrd (85 μg/ml) were also higher than those obtained for their β-counterparts (Table 2). It is noteworthy that the α-anomers of 5-ethynyl-dUrd and *E*-5-(2-bromovinyl)-dUrd inhibited L1210 cell proliferation at doses that were lower than those required for their free bases to inhibit L1210 cell growth. The relevance of these findings is not immediately clear.

The fact that established antiherpes compounds such as 5-propyl-dUrd (4) and 5-propynyloxy-dUrd (8) exhibited little, if any, inhibition of tumor cell growth, even when tested at a dose as high as 1 mg/ml (Fig. 3), points to their selectivity as antiherpes agents. The same reasoning obviously applies to several other 5-substituted 2′-deoxyuridines, viz., *E*-5-(2-bromovinyl)-dUrd (9), *E*-5-(2-iodovinyl)-dUrd (9), *E*-5-(2-chlorovinyl)-dUrd (11), 5-methylthiomethyl-dUrd (10, 26), and 5-methylsulfonylmethyl-dUrd (26), which were also found to inhibit L1210 cell proliferation at concentrations that were higher by several orders of magnitude than those required to inhibit herpes simplex virus replication.

*Inhibition of thymidylate synthetase in cultured L1210 cells.* One may assume that for these nucleoside analogues which owe their inhibitory effects on cell growth

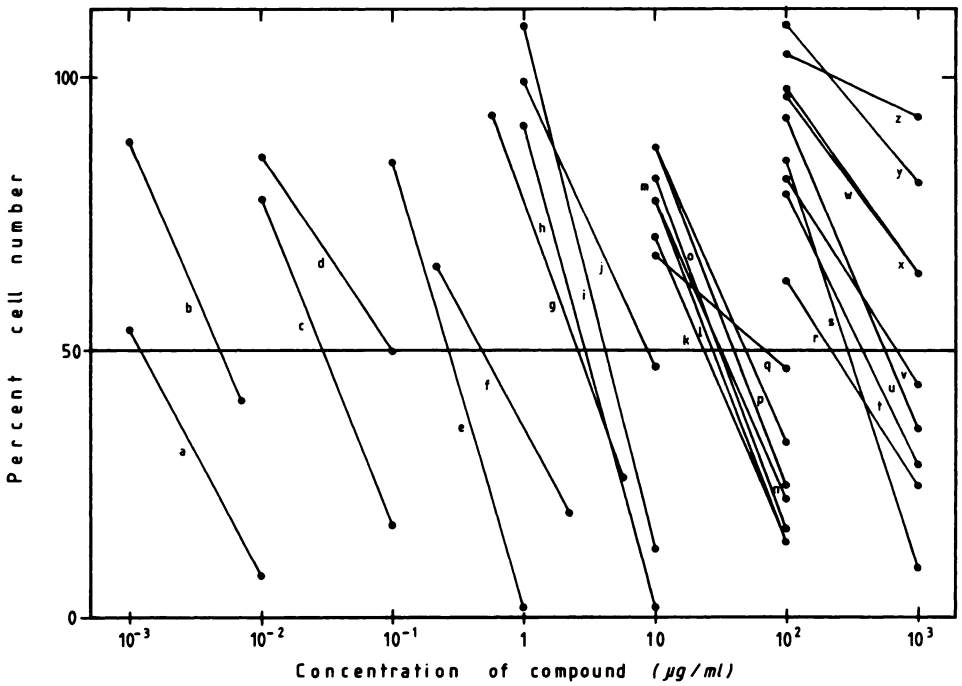


FIG. 3. *Dose-response curves for inhibition of L1210 cell proliferation by 5-substituted 2'-deoxyuridines*

Symbols for the 5-substituted 2'-deoxyuridines are indicated in Fig. 1. The cell counts were determined with varying concentrations ($10^3$, $10^2$, 10, 1, $10^{-1}$, ... μg/ml) of each compound, but in the graph the cell counts are presented for only two compound concentrations (viz., those that were closest to the 50% inhibitory dose).

TABLE 2

*Effects of 5-substituted 2'-deoxyuridines on the proliferation of L1210 cells*

| Compound | $ID_{50}$ | | | Ratio of $ID_{50}$ upon addition of dThd to $ID_{50}$ upon addition of dUrd |
|---|---|---|---|---|
| | As such | Upon addition of dUrd (125 μg/ml) | Upon addition of dThd (5 μg/ml) | |
| | | *μg/ml* | | |
| 5-Fluoro-dUrd | 0.001 (± 0.00096)[a] | 0.02 | 34 | 1700 |
| 5-Trifluoromethyl-dUrd | 0.007 (± 0.0025) | 0.13 | 29 | 223 |
| 5-Nitro-dUMP | 0.035 (± 0.004) | 1.04 | 932 | 896 |
| 5-Ethynyl-dUrd | 0.091 (± 0.025) | 0.81 | 46 | 57 |
| 5-Formyl-dUrd | 0.275 (± 0.018) | 4.3 | 43 | 10 |
| 5-(1-Chlorovinyl)-dUrd | 0.480 (± 0.012) | 2.6 | 102 | 39 |
| 5-Oxime of 5-formyl-dUrd | 4.20 (± 0.2) | 255 | >1000 | >3.92 |
| 5-Thiocyano-dUrd | 22.5 (± 5.3) | 66 | 279 | 4.23 |
| 5-Cyano-dUrd | 210 (± 10) | 840 | >1000 | >1.19 |
| 5-Hydroxymethyl-dUrd | 3.61 (± 0.45) | 250 | 291 | 1.16 |
| 5-Vinyl-dUrd | 2.37 (± 0.72) | >125 | >87 | ... |
| *E*-5-(2-bromovinyl)-dUrd | 26.9 (± 1.9) | 159 | 435 | 2.74 |
| *E*-5-(2-iodovinyl)-dUrd | 24.3 (± 3.6) | 96 | 304 | 3.16 |
| *E*-5-(2-chlorovinyl)-dUrd | 327 (± 28) | 408 | 417 | 1.02 |
| 5-Bromo-dUrd | 26.0 (± 4.7) | 20 | 18.0 | 0.90 |
| 5-Iodo-dUrd | 61.2 (± 6.2) | 61.2 | 125 | 2.04 |
| 5-Azidomethyl-dUrd | 35.0 (± 3.6) | 50.0 | 42.0 | 0.84 |
| 5-Ethyl-dUrd | 8.5 (± 2.9) | 140 | 100 | 0.71 |
| 5-Iodoacetamidomethyl-dUrd | 45.7 (± 2.0) | 40.0 | 39.5 | 0.99 |
| 5-Methylsulfonylmethyl-dUrd | 265 (± 42) | 344 | 430 | 1.25 |
| 5-(1,3-Dithiolan-2-yl)-dUrd | 625 (± 90) | 775 | 687 | 0.89 |
| 5-Methylthiomethyl-dUrd | 640 (± 72) | 400 | 590 | 1.48 |
| 5-Methylsulfinylmethyl-dUrd | >1000 | >1000 | >1000 | ... |
| 5-Hydroxy-dUrd | >1000 | >1000 | >1000 | ... |
| 5-Propynyloxy-dUrd | >1000 | >1000 | >1000 | ... |
| 5-Propyl-dUrd | >1000 | >1000 | >1000 | ... |

[a] Standard deviations are indicated in parentheses.

to inhibition of dTMP synthetase, the inhibitory action would be more readily reversed by dThd than by dUrd. Indeed, dUrd, even if added in excess, may only be able to reverse partially the inhibitory action of the nucleoside analogue since it is itself blocked at the dTMP synthetase level. This dTMP synthetase step is circumvented in the pathway that leads to the incorporation of dThd, and, therefore, dThd may readily overcome a blockade at the dTMP synthetase level.

As is shown in Table 2, the inhibitory effects of most 5-substituted 2′-deoxyuridines on L1210 cell proliferation was markedly reduced when dUrd or dThd was added to the cell culture medium. However, the $ID_{50}$ of some compounds, i.e., 5-bromo-dUrd, 5-iodo-dUrd, 5-azidomethyl-dUrd, and 5-iodoacetamidomethyl-dUrd, was not significantly altered upon addition of dUrd or dThd (Table 2). Neither were compounds like 5-methylthiomethyl-dUrd and 5-(1,3-dithiolan-2-yl)-dUrd affected by the addition of dUrd or dThd, but it should be noted that these compounds were only inhibitory to cell growth at fairly high concentrations (approximately 500 μg/ml), thus much higher than the concentration at which dUrd or dThd was added.

Although dThd was added at a 25-fold lower concentration than dUrd (both compounds were used at the maximal doses that were not inhibitory to L1210 cell growth), it was more effective than dUrd in reversing the inhibitory action of those compounds that have been recognized as specific thymidylate synthetase inhibitors (5-fluoro-dUrd, 5-nitro-dUrd, 5-trifluoromethyl-dUrd, . . . ) (Table 2). For 5-fluoro-dUrd, the ratio of $ID_{50}$ upon addition of dThd to $ID_{50}$ upon addition of dUrd was as high as 1700 (Table 2). For 5-ethynyl-dUrd it was 57, a value that is comparable to the dThd/dUrd reversal ratio of 80 that has been proposed for 5-ethynyl-dUrd by Bobek and Bloch (21). For those compounds that were catalogued as nonspecific thymidylate synthetase inhibitors (see above), the ratio of $ID_{50}$ with dThd to $ID_{50}$ with dUrd was close to 1, although in some instances, e.g., 5-iodo-dUrd, *E*-5-(2-bromovinyl)-dUrd, and *E*-5-(2-iodovinyl)-dUrd, this ratio went up to 2–3 (Table 2).

It has been postulated that nucleoside analogues which inhibit the incorporation of (radiolabeled) dUrd into DNA and which do not inhibit the incorporation of dThd, or inhibit the latter to a significantly smaller extent than dUrd incorporation, selectively block thymidylate synthetase (6). This concept, derived from studies with primary rabbit kidney cells (in their exponential growth phase), was subsequently corroborated for a numer of compounds (9, 10, 25), and has now been applied in our studies with the L1210 cell system. For all 5-substituted 2′-deoxyuridines, the concentrations were determined at which either [*methyl*-$^3$H]dThd or [2-$^{14}$C]dUrd incorporation into cellular DNA was inhibited by 50%, and the ratio of $ID_{50}$ for [*methyl*-$^3$H]dThd incorporation to $ID_{50}$ for [2-$^{14}$C]dUrd incorporation was interpreted as a measure for the selective inhibition of thymidylate synthetase.

For most compounds, i.e., those that are assumed not to act specifically at the dTMP synthetase level, the ratio of $ID_{50}$ for [*methyl*-$^3$H]dThd incorporation to $ID_{50}$ for [2-$^{14}$C]dUrd incorporation varied from 0.5 (or lower) to 5, or at most 6.57, as proved to the be the case for *E*-5-(2-iodovinyl)-dUrd (Table 3). For those compounds that have been assumed to inhibit specifically dTMP synthetase, i.e., 5-fluoro-dUrd, 5-nitro-dUrd, 5-formyl-dUrd, and 5-ethynyl-dUrd, the ratio of $ID_{50}$ for [*methyl*-$^3$H]dThd incorporation to $ID_{50}$ for [2-$^{14}$C]dUrd incorporation was 10 or higher (Table 3); for 5-fluoro-dUrd it even attained a value of approximately 25,000.

Of all compounds tested, only a few (5-fluoro-, 5-trifluoromethyl-, 5-ethynyl-, 5-hydroxymethyl-, 5-bromo-, 5-iodo-, and 5-ethyl-dUrd) inhibited [*methyl*-$^3$H]dThd incorporation at a concentration <100 μg/ml (Table 3). Most of these compounds (viz., 5-trifluoromethyl-dUrd, 5-hydroxymethyl-dUrd, 5-bromo- and 5-iodo-dUrd, and 5-ethyl-dUrd) are known to be incorporated into DNA. One may speculate, therefore, that these compounds blocked the incorporation of dThd because they were themselves incorporated into DNA. If this is correct, one may also theorize that compounds which do not have a marked effect on [*methyl*-$^3$H]dThd incorporation are not incorporated into DNA, or are incorporated only to a minor extent, a prediction borne out for at least one nucleoside analogue, 5-cyano-dUrd (29).

*Correlation between inhibition of L1210 cell growth and inhibition of thymidylate synthetase in cultured L1210 cells.* From the data presented in Tables 2 and 3 it is clear that there is a correlation among (a) the $ID_{50}$ for L1210 cell growth; (b) the ratio of the $ID_{50}$ for cell growth upon addition of dThd to the $ID_{50}$ for cell growth upon addition of dUrd, henceforth referred to as A; and (c) the ratio of the $ID_{50}$ for [*methyl*-$^3$H]dThd incorporation to the $ID_{50}$ for [2-$^{14}$C]dUrd incorporation, henceforth

TABLE 3

*Effects of 5-substituted 2′-deoxyuridines on the incorporation of [methyl-$^3$H]dThd and [2-$^{14}$C]dUrd into DNA of L1210 cells*

| Compound | $ID_{50}$ | | Ratio of $ID_{50}$ for [*methyl*-$^3$H] dThd incorporation to $ID_{50}$ for [2-$^{14}$C]dUrd incorporation |
|---|---|---|---|
| | [*methyl*-$^3$H] dThd incorporation | [2-$^{14}$C] dUrd incorporation | |
| | μg/ml | | |
| 5-Fluoro-dUrd | 80.0 | 0.003 | 24,742 |
| 5-Trifluoromethyl-dUrd | 4.92 | 0.02 | 247 |
| 5-Nitro-dUMP | 900 | 0.187 | 4,800 |
| 5-Ethynyl-dUrd | 28.5 | 0.162 | 176 |
| 5-Formyl-dUrd | 535 | 21.9 | 24.5 |
| 5-(1-Chlorovinyl)-dUrd | >94.2 | 0.89 | >106 |
| 5-Oxime of 5-formyl-dUrd | >1000 | 72.2 | >14 |
| 5-Thiocyano-dUrd | 286 | 20.5 | 14 |
| 5-Cyano-dUrd | 925 | 76.7 | 12 |
| 5-Hydroxymethyl-dUrd | 24.5 | 29.5 | 0.83 |
| 5-Vinyl-dUrd | 163 | 43.7 | 3.73 |
| *E*-5-(2-bromovinyl)-dUrd | 315 | 62.7 | 5.03 |
| *E*-5-(2-iodovinyl)-dUrd | 613 | 93.3 | 6.57 |
| *E*-5-(2-chlorovinyl)-dUrd | 422 | 445 | 0.95 |
| 5-Bromo-dUrd | 2.18 | 1.23 | 1.77 |
| 5-Iodo-dUrd | 4.27 | 0.82 | 5.23 |
| 5-Azidomethyl-dUrd | 516 | 272 | 1.90 |
| 5-Ethyl-dUrd | 96.7 | 46.7 | 2.07 |
| 5-Iodoacetamidomethyl-dUrd | 200 | 100 | 2.00 |
| 5-Methylsulfonylmethyl-dUrd | >1000 | 514 | >1.95 |
| 5-(1,3-Dithiolan-2-yl)-dUrd | 338 | >1000 | <0.34 |
| 5-Methylthiomethyl-dUrd | 406 | >1000 | <0.41 |
| 5-Methylsulfinylmethyl-dUrd | >1000 | >1000 | . . . |
| 5-Hydroxy-dUrd | 103 | 31.5 | 3.27 |
| 5-Propynyloxy-dUrd | >1000 | 487 | >2.05 |
| 5-Propyl-dUrd | 595 | >1000 | <0.59 |

referred to as B. This correlation has been further assessed by statistical means, at least for those compounds (5-fluoro-, 5-trifluoromethyl-, 5-nitro-, 5-ethynyl-, 5-formyl-, 5-(1-chlorovinyl)-, 5-thiocyano, 5-cyano-dUrd, and 5-oxime of 5-formyl-dUrd) that according to the data presented in Tables 1, 2, and 3 could be considered as specific thymidylate synthetase inhibitors. The selection of these nine compounds was based upon the following criteria: $K_i/K_m \leq 0.5$ (Table 1), B (PRK cells) > 5 (Table 1), A > 5 (Table 2), and B (L1210 cells) > 10 (Table 3). The log $ID_{50}$ for L1210 cell growth was plotted as a function of either log A or log B (Fig. 4), and log A was plotted as a function of log B (Fig. 5).

There appeared to be a negative linear correlation between log $ID_{50}$ for cell growth and log A or log B; the estimating equations for these correlations are indicated on Fig. 4. The coefficient of determination ($r^2$) was 0.797 for the correlation between log $ID_{50}$ for cell growth and log A, and 0.696 for the correlation between log $ID_{50}$ for cell growth and log B. Multiple correlation between the three variables, log $ID_{50}$ for cell growth, log A and log B, gave an estimating equation of log $ID_{50}$ = 4.234 − 2.100 log A + 0.705 log B and a coefficient of multiple determination ($R^2$) of 0.818. Thus, the inhibition of tumor cell growth showed a strong correlation with either parameter A or B, and even more so, with both parameters taken together ($R$ = 0.904). There was also a strong linear correlation ($r$ = 0.934) between parameters A and B (Fig. 5).

The data presented in Figs. 4 and 5 represent the first attempt to assess by statistical methods the role of dTMP synthetase as target for the antineoplastic activity of 5-substituted 2′-deoxyuridines. That dTMP synthetase may be an important target enzyme for various cytotoxic nucleoside analogues such as 5-formyl-dUrd, 5-mercapto-dUrd, and 5-nitro-dUrd has also been suggested in previous studies (17, 18, 23). As demonstrated here with a series of 5-substituted 2′-deoxyuridines [5-fluoro-, 5-trifluoromethyl-, 5-nitro-, 5-ethynyl-, 5-formyl-, 5-(1-chlorovinyl)-, 5-thiocyano-, 5-cyano-dUrd, and the 5-oxime of 5-formyl-dUrd], the extent of antitumor activity closely correlated with a selective inhibition of thymidylate synthetase, irrespective of the parameter (A or B) that was chosen as a measure for inhibition of dTMP synthetase. In fact, parameters A and B strongly correlated with one another, indicating that both served as equally valuable indexes for thymidylate synthetase inhibition. For the above-mentioned set of nine nucleoside analogues, our data point to dTMP synthetase as the principal, if not the sole, intracellular target for cytotoxic activity.

## CONCLUSIONS

As pointed out previously (6), there are a number of conditions to be met before a nucleoside analogue that

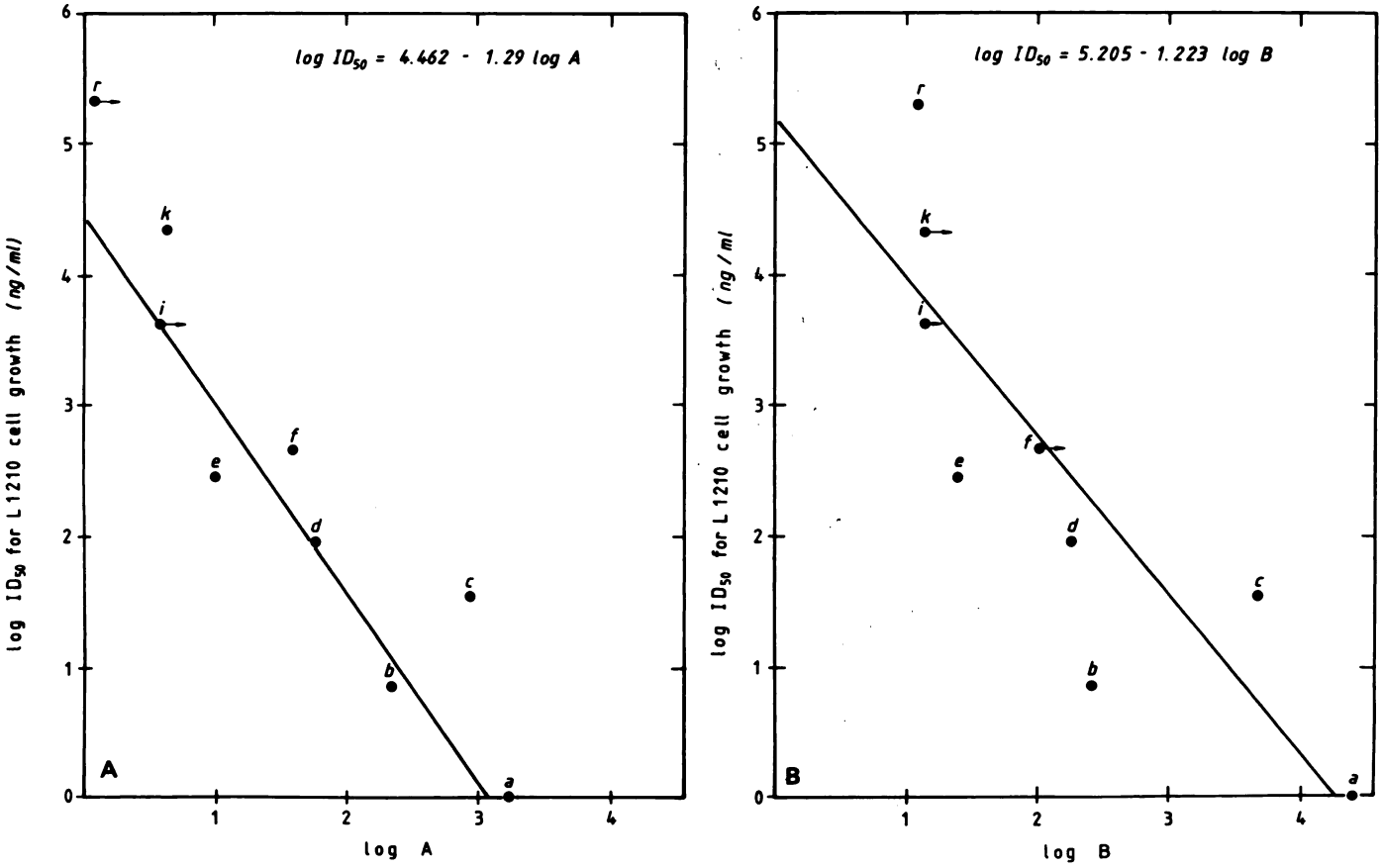


FIG. 4. *Linear regression lines for the $ID_{50}$ for inhibition of L1210 cell growth as a function of either A or B*

A = $ID_{50}$ for L1210 cell growth upon addition of dThd/$ID_{50}$ for L1210 cell growth upon addition of dUrd (see Table 2). B = $ID_{50}$ for [*methyl*-$^3$H]dThd incorporation/$ID_{50}$ for (2-$^{14}$C)dUrd incorporation (see Table 3). Symbols for the 5-substituted 2′-deoxyuridines are indicated in Fig. 1.

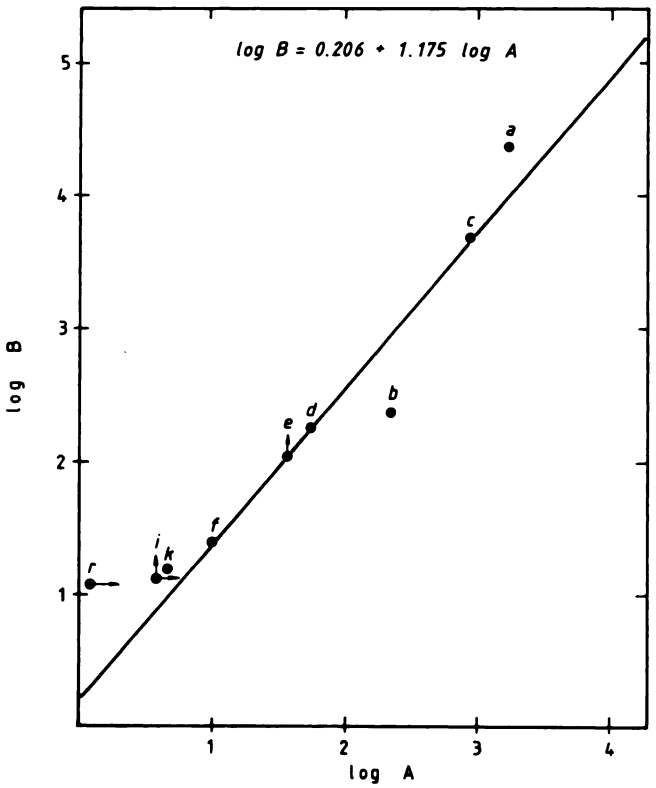


FIG. 5. *Linear regression line for B as a function of A*

A and B are explained in legend to Fig. 4. Symbols for the 5-substituted 2′-deoxyuridines are indicated in Fig. 1.

blocks the incorporation of dUrd into DNA to a significantly greater extent than dThd incorporation can unequivocally be identified as a selective and/or potent inhibitor of thymidylate synthetase in intact cells: (a) the compound should not impair the uptake of dUrd or dThd by the cells, or at least not preferentially inhibit the uptake of dUrd; (b) the compound should be phosphorylated by cellular dThd kinase to its 5′-monophosphate; (c) the compound should not interfere with the conversion of dUrd to dUMP or with the conversion of dThd to dTMP, or, if it does, it should affect these conversions in the same manner; and (d) the compound should not differentially affect the endogenous pools of dUMP and dTMP. Finally, (e) if a given compound were to be considered as a selective and/or potent inhibitor of dTMP synthetase in intact cells, it should be strongly inhibitory to the isolated enzyme. Our recent investigations[8] have indicated that this proves to be the case: the 5′-monophosphates of those dUrd analogues that were recognized as selective inhibitors of dTMP synthetase in intact L1210 were also potent inhibitors of the isolated L1210 enzyme. Moreover, the $K_i/K_m$ values for the isolated dTMP synthetase closely correlated with the extent of dTMP synthesis recorded *in vivo*. That the *in vivo* activity obtained with the dUrd analogues could be ascribed to an inhibition of dTMP synthetase, and not to an inhibition of dThd kinase, was ascertained by measuring the direct inhibitory effects of the compounds on dThd kinase isolated from L1210 cells. These inhibitory effects were much weaker than the inhibitory effects noted for the isolated dTMP synthetase, and did not correlate with the inhibitory activity on dTMP synthesis in intact L1210 cells.[8]

Thus, our data strongly suggest that in cultured cells (viz., L1210 cells), 5-substituted dUrds, such as 5-fluoro-, 5-nitro-, 5-formyl- and 5-ethynyl-dUrd, achieve their cytotoxic activity primarily by inhibition of dTMP synthetase. To serve in this quality, they require only phosphorylation by a single enzyme, dThd kinase. It remains to be established, however, whether 5-substituted dUrds such as 5-nitro-, 5-formyl-, and 5-ethynyl-dUrd would be useful chemotherapeutic agents in the treatment of human or animal neoplasia. Indeed, in the whole organism, 2′-deoxyuridines may be hydrolyzed to the free pyrimidine bases by dThd phosphorylase before they can be converted to the nucleotide form by dThd kinase (30). Only if methods could be designed that would prevent this catabolism could 5-substituted 2′-deoxyuridines reach their full potential as chemotherapeutic agents (30).

[8] J. Balzarini, E. De Clercq, P. F. Torrence, D. Shugar, C. L. Schmidt, and M. P. Mertes, in preparation.

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