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# 5-SUBSTITUTED 2′-DEOXYURIDINES: CORRELATION BETWEEN INHIBITION OF TUMOR CELL GROWTH AND INHIBITION OF THYMIDINE KINASE AND THYMIDYLATE SYNTHETASE

JAN BALZARINI and ERIK DE CLERCQ*
Rega Institute for Medical Research, University of Leuven, B-3000 Leuven, Belgium

MATHIAS P. MERTES
Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, KS 66045, U.S.A.

DAVID SHUGAR
Institute of Biochemistry and Biophysics, Academy of Sciences, 02-532 Warszawa, Poland

and

PAUL F. TORRENCE
Laboratory of Chemistry, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, U.S. National Institutes of Health, Bethesda, MD 20205, U.S.A.

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**Abstract**—A large variety of 5-substituted 2′deoxyuridines (dUrds) and 2′-deoxyuridylates (dUMPs) have been evaluated for their inhibitory effects on the thymidine (dThd) kinase or thymidylate (dTMP) synthetase isolated from mouse leukemia L1210 cells. The most potent inhibitors of dThd kinase were 5-chloro-, 5-bromo- and 5-iodo-dUrd. Their $K_i/K_m$ values ranged from 0.57 to 0.82. All dUrd analogs tested showed competitive kinetics with respect to dThd. However, there was little, if any, correlation between the inhibitory effects of the compounds on L1210 cell growth and their inhibitory activities against dThd kinase ($r = 0.16$). The most potent inhibitors of dTMP synthetase were (in order of decreasing activity): 5-nitro-dUMP > 5-formyl-dUMP > 5-fluoro-dUMP > 5-oxime of 5-formyl-dUMP > 5-azidomethyl-dUMP > (*E*)-5-(2-bromovinyl)-dUMP. The $k_i/K_m$ values for these compounds ranged from 0.001 to 0.665. All dUMP analogs tested showed competitive kinetics with respect to dUMP (if not preincubated with the enzyme at 37°). There was a strong correlation ($r = 0.833$) between the inhibitory effects of these compounds on L1210 cell growth and their inhibitory activities against dTMP synthetase. Thus, the suppressive action of 5-substituted dUrd derivatives on tumor cell growth would involve prior conversion of the nucleoside analogs to the corresponding 5′-monophosphates followed by an inhibition of dTMP synthetase.

Thymidylate synthetase (EC 2.1.1.45) catalyses the reductive methylation of deoxyuridylate (dUMP) to deoxythymidylate (dTMP) with concomitant conversion of $N^5$,$N^{10}$-methylenetetrahydrofolic acid to 7,8-dihydrofolic acid [1]:

$$\text{dUMP} + \text{CH}_2\text{–H}_4 \text{ folate} \rightarrow \text{H}_2 \text{ folate} + \text{dTMP}.$$

Since dTMP synthetase represents the sole *de novo* pathway for dTMP synthesis, it is apparent that a blockade of its activity would have deleterious effects on proliferating cells. Indeed, when the dTMP supply is shut off, DNA synthesis is impaired, which may, in turn, lead to "thymineless death" of the cell [2].

The mechanism of action of dTMP synthetase and its role as a target enzyme in cancer chemotherapy have been extensively reviewed [3, 4]. Except for a weak product inhibition by dTMP, dTMP synthetase is not known to be regulated by any of the naturally occurring nucleotides; however, it can be inhibited by analogs of either folate [5] or dUMP [6].

The inhibition of dTMP synthetase by 5-substituted dUMP analogs is well documented (Table 1). The most potent inhibitors of dTMP synthetase are 5-fluoro-, 5-nitro-, 5-trifluoromethyl-, 5-formyl- and 5-mercapto-dUMP.

Deoxythymidine kinase (EC 2.7.1.21) is an enzyme of the pyrimidine salvage pathway which catalyses the phosphorylation of deoxythymidine (dThd) to dTMP in the presence of ATP and a divalent cation such as $Mg^{2+}$ [7]:

$$\text{dThd} + \text{ATP} \xrightarrow{Mg^{2+}} \text{dTMP} + \text{ADP}.$$

DThd kinase recognizes dThd and 5-substituted 2′-deoxyuridine (dUrd) analogs as substrates provided the 5-substituents of the latter are not too bulky. As a result, compounds like 5-fluoro-, 5-trifluoromethyl-, 5-nitro- and 5-bromo-dUrd are readily converted to their 5′-monophosphate form in cultured cells [1, 8, 9]. High levels of dThd kinase

* Author to whom all correspondence should be addressed: Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium.

Table 1. Inhibition of dTMP synthetases of various origins by 5-substituted dUMP analogs

| Compound | $K_i(\mu M)$ | $K_i/K_m$ | Enzyme source | Reference |
|---|---|---|---|---|
| 5-Fluoro-dUMP | 0.014 | 0.0027 | *Lactobacillus casei* | [40] |
| | 0.005 | 0.002 | — | [42] |
| 5-Trifluoromethyl-dUMP | 0.039 | 0.0075 | *L. casei* | [40] |
| | 0.039 | 0.012 | Ehrlich ascites cells | [42] |
| 5-Chloro-dUMP | 0.19 | 0.036 | *L. casei* | [40] |
| 5-Bromo-dUMP | 1.4 | 0.269 | *L. casei* | [40] |
| 5-Iodo-dUMP | 1.6 | 0.308 | *L. casei* | [40] |
| 5-Nitro-dUMP | 0.027 | 0.010 | *L. casei* | [48] |
| | 0.029 | 0.019 | *L. casei* | [49] |
| | 0.023 | 0.009 | *L. casei* | [41] |
| | 0.21 | 0.07 | *L. casei* | [50] |
| 5-Formyl-dUMP | 0.17 | 0.050 | *L. casei* | [20] |
| | 0.017 | 0.0033 | *L. casei* | [40] |
| | 0.013 | — | *Escherichia coli* | [43, 51] |
| | 0.012 | — | *L. casei* | [43] |
| | 0.1 | 0.004 | *E. coli* | [37] |
| | 0.09 | 0.01 | Calf thymus | [37] |
| | 0.08 | 0.02 | Ehrlich ascites cells | [37] |
| 5-Hydroxymethyl-dUMP | 8.3 | 1.596 | *L. casei* | [40] |
| | 10.5 | — | — | [51] |
| | 4 | 0.16 | *E. coli* B | [37] |
| | 4 | 0.44 | Calf thymus | [37] |
| | 0.6 | 0.15 | Ehrlich ascites cells | [37] |
| 5-Oxime of 5-formyl-dUMP | 2 | 0.5 | *L. casei* | [20] |
| 5-(1,3-Dithiolan-2-yl)-dUMP | 0.64 | 0.2 | *L. casei* | [20] |
| 5-Azidomethyl-dUMP | 18 | 0.72 | *E. coli* | [37] |
| | 21 | 2.33 | Calf thymus | [37] |
| | 5 | 1.25 | Ehrlich ascites cells | [37] |
| 5-Methylthiomethyl-dUMP | 7.8 | 2.4 | *L. casei* | [34] |
| 5-Methylsulfinylmethyl-dUMP | 1.9 | 0.61 | *L. casei* | [34] |
| 5-Methylsulfonylmethyl-dUMP | 2.2 | 0.58 | *L. casei* | [34] |
| 5-Mercapto-dUMP | 0.048 | — | *L. casei* | [52] |
| | 0.04 | 0.0085 | *E. coli* B | [53] |
| 5-Hydroseleno-dUMP | 0.06 | — | *L. casei* | [52] |
| 5-Cyano-dUMP | 0.55 | 0.13 | *L. casei* | [54] |
| 5-Ethyl-dUMP | 22 | 4.4 | *E. coli* | [55] |
| 5-Acetyl-dUMP | 64 | 2.56 | *E. coli* | [37] |
| | 73 | 8.11 | Calf thymus | [37] |
| | 22 | 5.5 | Ehrlich ascites cells | [37] |
| 5-(α-Bromoacetyl)-dUMP | 4.1 | 0.82 | *L. casei* | [56] |
| 5-Dimethylaminomethyl-dUMP | 90 | 11.84 | *E. coli* | [57] |
| | 110 | 13.7 | Calf thymus | [57] |
| | 73 | 11.23 | Ehrlich ascites cells | [57] |
| 5-Dimethylaminoethylamino-methyl-dUMP | 6 | 0.79 | *E. coli* | [57] |
| | 3.1 | 0.39 | Calf thymus | [57] |
| | 14 | 2.15 | Ehrlich ascites cells | [57] |
| 5-Iodoacetamidomethyl-dUMP | 27 | 3.5 | Calf thymus | [58] |
| | 68 | 15 | Ehrlich ascites cells | [58] |
| (*E*)-5-(3,3,3-Trifluoro-1-propenyl)-dUMP | 8.6 | — | *L. casei* | [29] |
| 5-[(4-Methyl-1,2,3,4-tetra-hydroquinoxalyl)methyl]-dUMP | 0.75 | — | *L. casei* | [59] |

have been observed in proliferating normal and neoplastic tissues [10, 11]. The enzyme is inhibited by dTTP [12, 13], cAMP [14], dCTP [15] and D-glucosamine [16]. The kinetics of the interaction of dThd kinase with some 5-substituted dUrd analogs has been the subject of previous investigations (Table 2).

In a recent study [17], we have examined the inhibitory effects of a series of 5-substituted dUrd analogs on the growth of mouse leukemia L1210 cells. For the most potent inhibitors of L1210 cell growth, viz. 5-fluoro-dUrd, 5-trifluoromethyl-dUrd, 5-nitro-dUrd, 5-ethynyl-dUrd, 5-formyl-dUrd, 5-(1-chlorovinyl)-dUrd and 5-oxime of 5-formyl-dUrd, a close correlation was found between their inhibitory effect on tumor cell growth and their relatively greater inhibition of [2-$^{14}$C]dUrd incorporation than of [*methyl*-$^{3}$H]dThd incorporation. Also, the antitumor activity of these compounds was reversed to a greater extent by dThd than by dUrd. From these data we postulated that dTMP synthetase may act as the principal, if not the sole, intracellular target for the inhibitory activity of dUrd on L1210 cell growth. This hypothesis implied that the dUrd analogs would be readily converted to their nucleotide form by dThd kinase and has now been directly

Table 2. Inhibition of dThd kinases of various origins by 5-substituted dUrd analogs

| Compound | $K_i(\mu M)$ | $K_i/K_m$ | Enzyme source | Reference |
|---|---|---|---|---|
| 5-Fluoro-dUrd | 26 | 10 | Blast cells of acute myelocytic leukemia (cytoplasm) | [44] |
| 5-Bromo-dUrd | 1.8 | 0.69 | Blast cells of acute myelocytic leukemia (cytoplasm) | [44] |
| 5-Iodo-dUrd | 2.4 | 0.9 | Blast cells of acute myelocytic leukemia (cytoplasm) | [44] |
| | 3.1 | 1.24 | Ascites tumor cells | [46] |
| 5-Amino-dUrd | 450 | 1.36 | Hamster cytoplasm | [45] |
| 5-Cyano-dUrd | 220 | 66.6 | Hamster cytoplasm | [45] |
| 5-Aminomethyl-dUrd | 1700 | 515.15 | Hamster cytoplasm | [45] |
| 5-Vinyl-dUrd | 35 | 13.46 | Blast cells of acute myelocytic leukemia (cytoplasm) | [19] |
| 5-Ethyl-dUrd | 280 | 112 | Ascites tumor cells | [46] |
| | 900 | 42.9 | *Escherichia coli* | [46] |
| | 82 | 31.5 | Blast cells of acute myelocytic leukemia (cytoplasm) | [44] |
| 5-Allyl-dUrd | 315 | 121.1 | Blast cells of acute myelocytic leukemia (cytoplasm) | [44] |
| 5-Propyl-dUrd | 21 | 8.08 | Blast cells of acute myelocytic leukemia (cytoplasm) | [44] |
| | >500 | >200 | Ascites tumor cells | [46] |
| | >2000 | >95.2 | *E. coli* | [46] |
| 5-Isopropyl-dUrd | >500 | >200 | Ascites tumor cells | [46] |
| | >2000 | >95.2 | *E. coli* | [46] |
| 5-Mercapto-dUrd | 1600 | 80 | *E. coli* | [53] |
| 5-Methylmercapto-dUrd | 3200 | 160 | *E. coli* | [53] |
| 5-Ethylmercapto-dUrd | 3500 | 175 | *E. coli* | [53] |

verified by measuring the inhibitory effects of the dUrd analogs and their 5′-monophosphates on the dThd kinase and dTMP synthetase isolated from L1210 cells.

## MATERIALS AND METHODS

*Cells.* Murine 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, U.K.) and 2 mM L-glutamine (Flow Laboratories, Irvine, U.K.).

*Chemicals.* The following materials were obtained from E. Merck (Darmstadt, West Germany): formaldehyde, Tris, NaCl, KF, $MgCl_2$ and $(NH_4)_2SO_4$. Pyruvate kinase, phosphoenolpyruvate and ATP were obtained from Sigma Chemical Co. (St. Louis, MO). 2-Mercaptoethanol was obtained from Fluka AG (Buchs, Switzerland). Carbon black GSX was obtained from Union Chimique Belge (Brussels, Belgium) and tetrahydrofolic acid from Koch Light Laboratories (Colnbrook, U.K.). The sources of the test compounds were as follows: 2′-dThd (Sigma Chemical Co.), 2′-deoxythymidine-5′-monophosphate (dTMP) (Sigma Chemical Co.), 2′-deoxythymidine-5′-triphosphate (dTTP) (Serva Feinbiochemical GmbH & Co., Heidelberg, West Germany), 5-fluoro-dUrd (Aldrich Chemical Co., Milwaukee, WI), 5-chloro-dUrd (Calbiochem-Behring Corp., Lucerne, Switzerland), 5-bromo-dUrd (Sigma Chemical Co.), 5-iodo-dUrd (Sigma Chemical Co.), 5-trifluoromethyl-dUrd (P-L Biochemicals, Milwaukee. WI), 5-amino-dUrd (Sigma Chemical Co.), 5-formyl-dUrd (see Refs 18 and 19), 5-oxime of 5-formyl-dUrd (see Ref. 20), 5-hydroxymethyl-dUrd (Calbiochem-Behring Corp.), 5-azidomethyl-dUrd (see Refs 18 and 19), 5-thiocyano-dUrd (see Ref. 21), 5-cyano-dUrd (see Ref. 22), 5-ethynyl-dUrd (see Refs 23 and 24), 5-vinyl-dUrd (see Ref. 24), 5-(1-chlorovinyl)-dUrd (see Ref. 24), (*E*)-5-(2-chlorovinyl)-dUrd (see Ref. 25), (*E*)-5-(2-bromovinyl)-dUrd (see Refs 24 and 26), (*E*)-5-(2-iodovinyl)-dUrd (see Ref. 24), (*E*)-5-(2-cyanovinyl)-dUrd (see Ref. 27), (*E*)-5-(1-propenyl)-dUrd (see Ref. 28), (*E*)-5-(3,3,3-trifluoro-1-propenyl-dUrd (see Ref. 29), 5-ethyl-dUrd (see Refs 30 and 31), 5-propyl-dUrd (see Ref. 32), 5-propynyloxy-dUrd (see Ref. 33), 5-methylthiomethyl-dUrd (see Refs 18 and 34), 5-methylsulfinylmethyl-dUrd (see Ref. 34), 5-methylsulfonylmethyl-dUrd (see Ref. 34), 5-(1,3-dithiolan-2-yl)-dUrd (see Ref. 20), 5-fluoro-dUMP (Sigma Chemical Co.), 5-bromo-dUMP (Sigma Chemical Co.) 5-iodo-dUMP (Sigma Chemical Co.), 5-nitro-dUMP (see Refs 35 and 36), 5-azidomethyl-dUMP (see Ref. 37), 5-formyl-dUMP (see Refs 20 and 37), 5-oxime of 5-formyl-dUMP (see Ref. 20), 5-(1,3-dithiolan-2-yl)-dUMP (see Ref. 20), 5-ethyl-dUMP (see Ref. 38), 5-propyl-dUMP (see Ref. 38), 5-methylthiomethyl-dUMP (see Ref. 34), 5-methylsulfinylmethyl-dUMP (see Ref. 34), 5-methylsulfonylmethyl-dUMP (see Ref. 34); (*E*)-5-(2-bromovinyl)-dUMP was synthesized from (*E*)-5-(2-bromovinyl)-dUrd according to the procedure described by Yoshikawa *et al.* [39].

*Radiochemicals.* The radiolabelled nucleoside [*methyl*-$^3$H]dThd (spec. act. 47 Ci/mmole) was obtained from the Institute of Radio-Elements (IRE, Fleurus, Belgium). [5-$^3$H]dUMP (spec. act. 10 Ci/

Table 3. Inhibition of L1210 dTMP synthetase by 5-substituted dUMP analogs

| Symbol | Compound | $K_i/K_m$* |
|---|---|---|
| 1 | dTMP | 8.512 |
| 2 | 5-Fluoro-dUMP | |
| 3 | 5-Bromo-dUMP | 2.486 |
| 4 | 5-Iodo-dUMP | 3.270 |
| 5 | 5-Nitro-dUMP | 0.0014 |
| 6 | 5-Formyl-dUMP | 0.0088 |
| 7 | 5-Oxime of 5-formyl-dUMP | 0.082 |
| 8 | 5-Azidomethyl-dUMP | 0.561 |
| 9 | (*E*)-5-(2-Bromovinyl)-dUMP | 0.665 |
| 10 | 5-Ethyl-dUMP | 6.404 |
| 11 | 5-Propyl-dUMP | 6.002 |
| 12 | 5-(1,3-Dithiolan-2-yl)-dUMP | 2.220 |
| 13 | 5-Methylthiomethyl-dUMP | 4.080 |
| 14 | 5-Methylsulfinylmethyl-dUMP | 2.203 |
| 15 | 5-Methylsulfonylmethyl-dUMP | 6.290 |

* $K_m$-Values for the individual experiments ranged from 1.27 to 3.12 μM. The average $K_m$-value was 1.84 μM. The type of inhibition was competitive with respect to dUMP for all dUMP analogs tested.

mmole) was obtained from the Radiochemical Centre (Amersham, U. K.).

*dThd kinase assay.* One-milliter cell pellets were first washed with cold 0.9% NaCl–0.01 M Tris–HCl buffer, pH 8.0; then 1 ml of 0.05 M Tris-HCl, pH 8.0, containing 0.02 M β-mercaptoethanol was added. The cell suspension was sonicated twice for 10 sec, cleared by centrifugation at 100,000 *g* for 45 min and stored in aliquots at −70°.

The cell extracts were assayed for dThd kinase activity in a standard reaction mixture containing 5 mM ATP, 5 mM $MgCl_2.6\ H_2O$, 9 mM KF, 5 mM phosphoenolpyruvate, 5 μg pyruvate kinase, 10 mM β-mercaptoethanol, 0.2 mM (0.1 μCi) [*methyl*-$^3$H]dThd, an appropriate amount of inhibitor (dUrd analog), and 10 μl of cell extract in a total volume of 40 μl of 0.05 M Tris–HCl, pH 8.0. The reaction mixture was incubated at 37° for 15 min and the

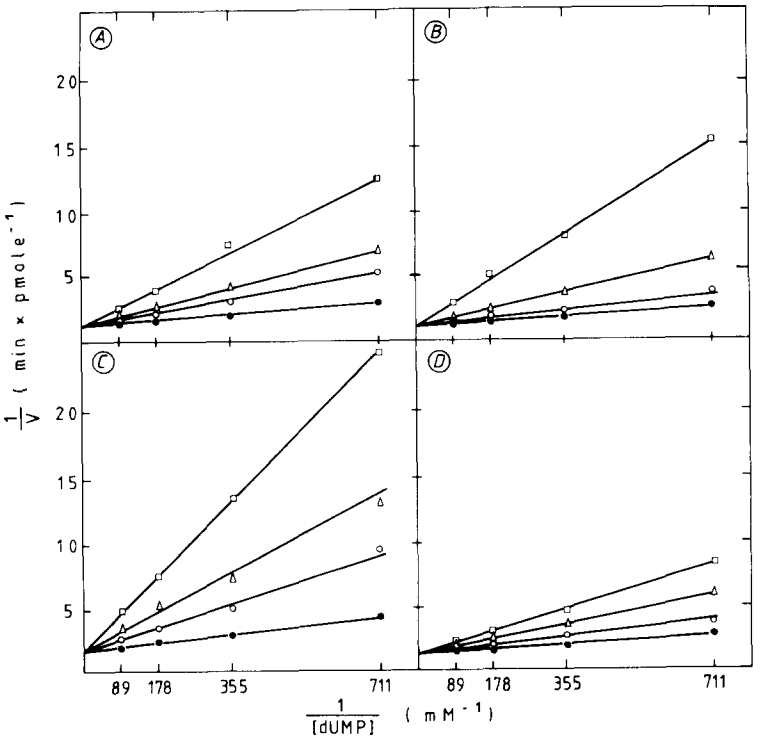


Fig. 1. Double-reciprocal plots for inhibition of L1210 dTMP synthetase by 5-fluoro-dUMP (panel A), 5-oxime of 5-formyl-dUMP (panel B), 5-bromo-dUMP (panel C) and 5-propyl-dUMP (panel D). Inhibitor concentrations: none (●), 0.090 μM (□), 0.045 μM (△) and 0.022 μM (○) for 5-fluoro-dUMP; none (●), 0.90 μM (□), 0.45 (△) and 0.22 μM (○) for 5-oxime of 5-formyl-dUMP; none (●), 45 μM (□), 22.5 μM (△) and 11.25 μM (○) for 5-bromo-dUMP; and none (●), 45 μM (□), 22.5 μM (△) and 11.25 μM (○) for 5-propyl-dUMP.

Table 4. Kinetics of L1210 dTMP synthetase inhibition after preincubation of the enzyme with 5-substituted dUMP analogs

| Compound | Preincubation for 20 min at 37° | | Preincubation for 20 min at 0° | |
|---|---|---|---|---|
| | $K_i/K_m$ | Type of inhibition | $K_i/K_m$ | Type of inhibition |
| 5-Fluoro-dUMP | — | Non-competitive | 0.0104 | Competitive |
| 5-Bromo-dUMP | 15.96 | Competitive | 3.873 | Competitive |
| 5-Iodo-dUMP | 18.65 | Competitive | 5.530 | Competitive |
| 5-Nitro-dUMP | — | Non-competitive | 0.0025 | Competitive |
| 5-Formyl-dUMP | 0.015 | Competitive | 0.012 | Competitive |
| 5-Oxime of 5-formyl-dUMP | 0.096 | Competitive | 0.067 | Competitive |
| 5-Azidomethyl-dUMP | 2.343 | Competitive | 1.109 | Competitive |

reaction terminated by addition of 75 μl of ice-cold 0.05 M Tris–HCl buffer, pH 8.0. After boiling for 2 min, the mixture was applied onto DE81 discs and washed with 1 mM $NH_4OOCH$, pH 8.2, ethanol and ether. The filters were then assayed for radioactivity in a toluene-based scintillant.

*dTMP synthetase assay.* One-milliter cell pellets were first washed with cold PBS (phosphate-buffered saline); then 3 ml of suspension buffer (10 mM potassium phosphate buffer, pH 7.5, containing 0.01 M β-mercaptoethanol and 0.1 M KCl) was added. The cell suspension was sonicated three times for 10 sec and cleared by centrifugation at 25,000 *g* for 30 min. The 30–70% $(NH_4)_2SO_4$ precipitate of the cell homogenate was resuspended in 1 ml of suspension buffer and dialysed against the same buffer for 2–3 hr at 4° and stored in aliquots at −20°.

The cell extracts were assayed for dTMP synthetase activity in a standard reaction mixture containing 0.26 mM tetrahydrofolate, 5.0 mM formaldehyde, 15 mM β-mercaptoethanol, 0.1 M NaF, 45 μM (0.025 μCi) [5-$^3$H]dUMP and an appropriate amount of inhibitor (dUMP analog) in a total volume of 30 μl of 0.05 M potassium phosphate buffer, pH 7.5. The reaction was initiated by addition of 10 μl of enzyme. The reaction mixture was incubated at 37° for 30 min and the reaction terminated by addition of 160 μl of a charcoal suspension (at 100 mg/ml in 2% trichloroacetic acid). After centrifugation for 10 min at 1000 *g*, 100 μl of supernatant was assayed for radioactivity in a toluene-based scintillant. For the preincubation studies, the reaction mixtures contained all components except dUMP. After 20 min preincubation at 37° or 0°, the reaction was initiated by the addition of the appropriate amount of dUMP.

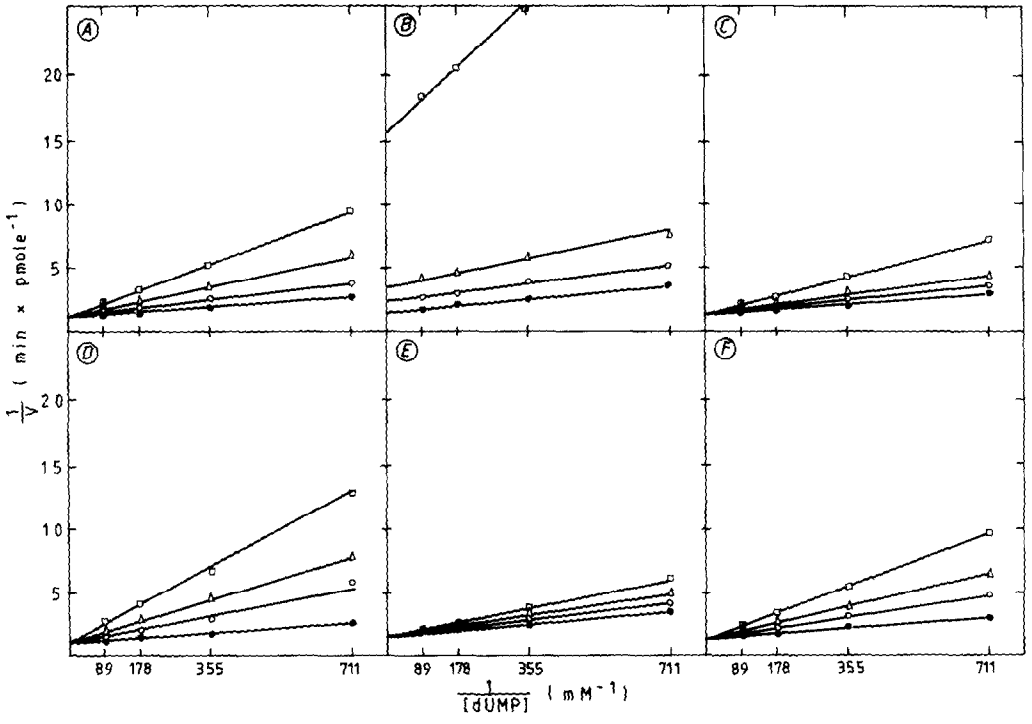


Fig. 2. Double-reciprocal plots for inhibition of L1210 dTMP synthetase by 5-nitro-dUMP (panels A, B and C) and 5-iodo-dUMP (panels D, E and F) without preincubation (panels A and D), with preincubation at 37° (panels B and E) and with preincubation at 0° (panels C and F). Inhibitor concentrations: none (●), 0.0090 μM (□), 0.0045 μM (△) and 0.0022 μM (○) for 5-nitro-dUMP; none (●), 45 μM (□), 22.5 μM (△) and 11.25 μM (○) for 5-iodo-dUMP.

## RESULTS

*Inhibition of L1210 dTMP synthetase by 5-substituted dUMP analogs*

The $K_i/K_m$ values of L1210 dTMP synthetase for a series of 15 5-substituted dUMP analogs are presented in Table 3. The $K_m$-value of the enzyme for dUMP was 1.84 $\mu$M and the $K_i/K_m$ values for most dUMP analogs were between 0.5 and 10. However, for some dUMP analogs, i.e. 5-fluoro-dUMP, 5-nitro-dUMP, 5-formyl-dUMP and 5-oxime of 5-formyl-dUMP, the $K_i/K_m$ values were far below 0.1. The latter dUMP analogs may therefore be regarded as potent inhibitors of dTMP synthetase.

For most compounds (i.e. 5-fluoro-, 5-nitro-, 5-ethyl- and 5-methylthiomethyl-dUMP) the $K_i/K_m$ values of the L1210 dTMP synthetase (Table 3) corresponded quite well with the $K_i/K_m$ values reported previously with dTMP synthetases from other sources (Table 1). However, for 5-bromo- and 5-iodo-dUMP we found $K_i/K_m$ values that were 10 times higher than those found for the dTMP synthetase from *Lactobacillus casei* (Table 1). Also for 5-formyl-dUMP the 5-oxime and the 5-dithiolane derivative of 5-formyl-dUMP, we found $K_i/K_m$ values which were 7–10 times higher than the values obtained with the *L. casei* enzyme (Table 1). Obviously, the $K_i/K_m$ values may vary considerably depending on the source and purity of the enzyme. For example, the $K_i/K_m$ of the *L. casei* dTMP synthetase for 5-formyl-dUMP was 0.0033 in one study [40] and 0.05 in another [20]. The value obtained with the L1210 enzyme was intermediate between these two values (Table 3).

The inhibition of all dUMP derivatives tested was competitive with respect to dUMP. Lineweaver–Burk plots are shown for four representative compounds: 5-fluoro-dUMP, 5-oxime of 5-formyl-dUMP, 5-bromo-dUMP and 5-propyl-dUMP (Fig. 1). Preincubation of the enzyme with the compounds at 37° in the presence of 5,10 $CH_2H_4$ folate did not produce an alteration in the inhibition kinetics of either 5-bromo-dUMP, 5-iodo-dUMP, 5-formyl-dUMP, 5-oxime of 5-formyl-dUMP or 5-azidomethyl-dUMP. However, for two compounds, 5-fluoro- and 5-nitro-dUMP, the type of inhibition changed from competitive to non-competitive following preincubation of the compounds with the enzyme at 37° (Table 4). The effects of preincubation on the inhibition kinetics of 5-iodo- and 5-nitro-dUMP are graphically presented in Fig. 2. For 5-nitro- and 5-fluoro-dUMP a similar change in inhibition pattern has been described following preincubation of 5-fluoro-dUMP with dTMP synthetases from other sources [41, 42]. For 5-formyl-dUMP we could not establish a transition from competitive to non-competitive inhibition upon preincubation. This finding contrasts with that of Santi and Sakai [43]. While the $K_m$-value (1.84 $\mu$M) was not altered by preincubation at 37°, the $K_i$-values for 5-bromo-, 5-iodo- and 5-azidomethyl-dUMP were increased by 6.4-, 5.7- and 4.2-fold, respectively. For 5-formyl-dUMP and 5-oxime of 5-formyl-dUMP, the $K_i$-values were only slightly increased. In contrast with the 37° preincubation, preincubation at 0° did not cause any change in inhibition kinetics (Table 4).

Table 5. Inhibition of L1210 dThd kinase by 5-substituted dUrd analogs

| Symbol | Compound | $K_i/K_m$* |
|---|---|---|
| 2 | 5-Fluoro-dUrd | 8.84 |
| 16 | 5-Chloro-dUrd | 0.74 |
| 3 | 5-Bromo-dUrd | 0.57 |
| 4 | 5-Iodo-dUrd | 0.82 |
| 17 | 5-Trifluoromethyl-dUrd | 5.37 |
| 6 | 5-Formyl-dUrd | 51.24 |
| 18 | 5-Hydroxymethyl-dUrd | 179.3 |
| 8 | 5-Azidomethyl-dUrd | >57.74 |
| 19 | 5-Ethynyl-dUrd | 6.51 |
| 20 | 5-Vinyl-dUrd | 47.50 |
| 21 | 5-(1-Chlorovinyl)-dUrd | 6.09 |
| 22 | (*E*)-5-(2-Chlorovinyl)-dUrd | >46.5 |
| 9 | (*E*)-5-(2-Bromovinyl)-dUrd | 223.0 |
| 23 | (*E*)-5-(2-Iodovinyl)-dUrd | >46.5 |
| 24 | (*E*)-5-(2-Cyanovinyl)-dUrd | >46.5 |
| 25 | (*E*)-5-(1-Propenyl)-dUrd | >46.5 |
| 26 | (*E*)-5-(3,3,3-Trifluoro-1-propenyl)-dUrd | 120.22 |
| 10 | 5-Ethyl-dUrd | 35.60 |
| 11 | 5-Propyl-dUrd | >115.48 |
| 27 | 5-Cyano-dUrd | 103.7 |
| 28 | 5-Thiocyano-dUrd | 36.22 |
| 12 | 5-(1,3-Dithiolan-2-yl)-dUrd | >46.19 |
| 13 | 5-Methylthiomethyl-dUrd | >115.48 |
| 14 | 5-Methylsulfinylmethyl-dUrd | >57.74 |
| 15 | 5-Methylsulfonylmethyl-dUrd | >46.19 |
| 29 | 5-Amino-dUrd | 28.31 |
| 30 | 5-Propynyloxy-dUrd | >57.74 |

* $K_m$-Values for the individual experiments ranged from 11.7 to 23.5 $\mu$M. The average $K_m$-value was 17.32 $\mu$M. The type of inhibition was competitive with respect to dThd for all dUrd analogs tested.

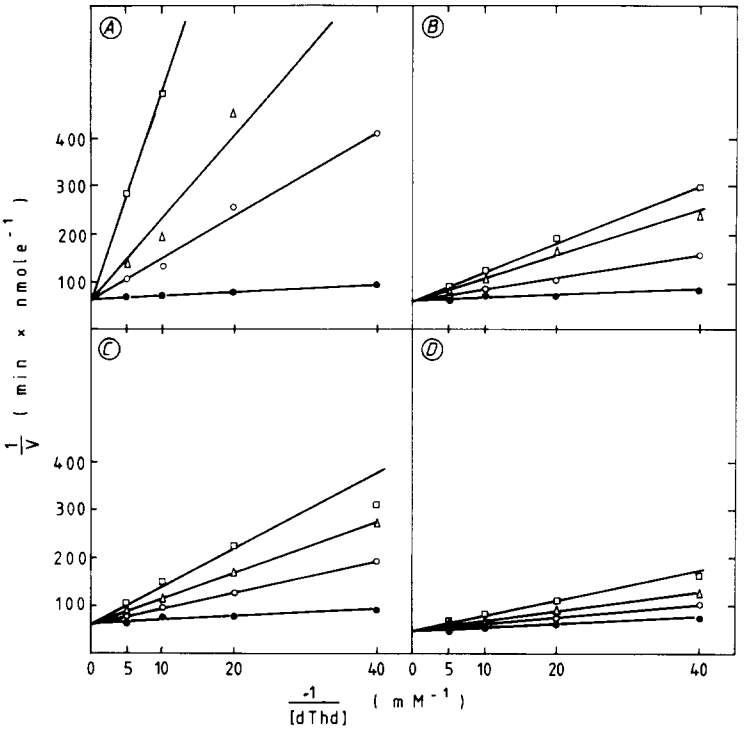


Fig. 3. Double-reciprocal plots for inhibition of L1210 dThd kinase by 5-iodo-dUrd (panel A), 5-fluoro-dUrd (panel B), 5-ethynyl-dUrd (panel C) and 5-ethyl-dUrd (panel D). Inhibitor concentrations: none (●), 400 μM (□), 200 μM (△) and 100 μM (○) for 5-iodo-dUrd; none (●), 2000 μM (□), 1000 μM (△) and 500 μM (○) for 5-fluoro-dUrd; none (●), 1000 μM (□), 500 μM (△) and 200 μM (○) for 5-ethynyl-dUrd; and none (●), 1000 μM (□), 500 μM (△) and 200 μM (○) for 5-ethyl-dUrd.

*Inhibition of L1210 dThd kinase by 5-substituted dUrd analogs*

The $K_i/K_m$ values of L1210 dThd kinase for a series of 27 5-substituted dUrd analogs are presented in Table 5. The $K_m$-value of the enzyme for dThd was 17.32 μM. The most potent inhibitors of dThd kinase were 5-chloro-, 5-bromo- and 5-iodo-dUrd. Their $K_i/K_m$ values were between 0.5 and 0.9. 5-Fluoro-, 5-trifluoromethyl-, 5-ethynyl- and 5-(1-chlorovinyl)-dUrd showed a relatively low affinity for the enzyme ($K_i/K_m$ between 5 and 9), whereas 5-amino-, 5-vinyl-, 5-ethyl-, 5-formyl, 5-cyano-, 5-thiocyano-, 5-hydroxymethyl-, (*E*)-5-(3,3,3-trifluoro-1-propenyl)- and (*E*)-5-(2-bromovinyl)-dUrd exerted an even lower inhibitory effect on dThd kinase ($K_i/K_m$ between 28 and 223). For the other compounds that were examined (i.e. 5-azidomethyl-dUrd, 5-propyl-dUrd, 5-methylthiomethyl-dUrd, (*E*)-5-(2-chlorovinyl)-dUrd, (*E*)-5-(1-propenyl)-dUrd etc.), no inhibitory activity against dThd kinase could be detected (if tested at a concentration as high as 1 mM).

The $K_i/K_m$ values observed for 5-fluoro-, 5-bromo-, 5-iodo- and 5-ethyl-dUrd (Table 5) corresponded well with the values reported by Lee and Cheng (44) for the dThd kinase, derived from myelocytic leukemia blast cells (Table 2). 5-Vinyl- and 5-cyano-dUrd, however, were slightly more inhibitory for the dThd kinase from myelocytic leukemia blast cells [44] or hamster cells [45] than for the enzyme from L1210 cells. Also, 5-propyl-dUrd was reported to possess a $K_i/K_m$ of 8.08 for the dThd kinase from myelocytic leukemia blast cells [44], although it proved inactive against the dThd kinase from either ascites tumor cells [46], *Escherichia coli* [46] or L1210 cells (Table 5).

For all dUrd analogs listed in Table 5, the type of inhibition appeared to be competitive with respect to dThd. Lineweaver–Burk plots are shown for four representative compounds: 5-iodo-, 5-fluoro-, 5-ethynyl- and 5-ethyl-dUrd (Fig. 3).

## DISCUSSION

None of the 5-substituted dUrd analogs tested proved to be a potent inhibitor of L1210 dThd kinase. In fact, the most active inhibitor, 5-bromo-dUrd, showed a $K_i/K_m$ value of 0.57 (Table 5), which is still higher than the $K_i/K_m$ value (0.154) noted for dTTP, an allosteric inhibitor of dThd kinase. It is unlikely that the inhibitory activity of the dUrd analogs on dThd kinase bears on their suppressive effects for tumor cell proliferation, since: (i) for most compounds, except for 5-chloro-, 5-bromo- and 5-iodo-dUrd, the doses required to inhibit L1210 cell growth were lower by several orders of magnitude than the $K_i$-values of L1210 dThd kinase for these analogs; and (ii) there was no correlation ($r = 0.16$) between the log $ID_{50}$ of the dUrd analogs for L1210 cell growth and the log $K_i/K_m$ of L1210 dThd kinase (Fig. 4). Indeed, 5-chloro-, 5-bromo- and 5-iodo-dUrd, which showed an average $K_i$-value of 8.4 μM for L1210

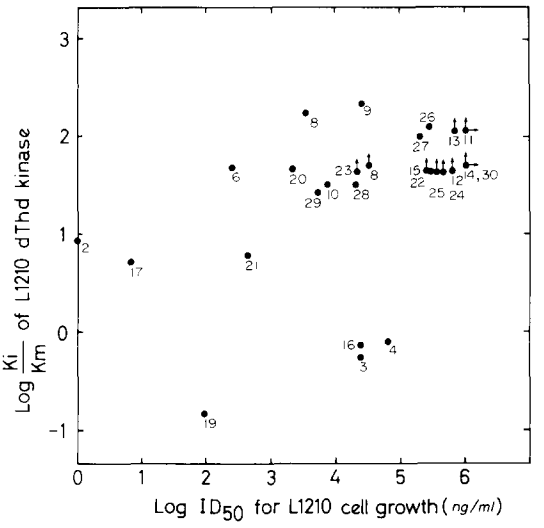


Fig. 4. Log $K_i/K_m$ of L1210 dThd kinase for various 5-substituted dUrd analogs as a function of log $ID_{50}$ of these nucleoside analogs for L1210 cell growth. The $K_i/K_m$ values are taken from Table 5. The $ID_{50}$ values correspond to the 50% inhibitory doses for L1210 cell proliferation and are taken from Refs 17 and 60. The symbols for the dUrd analogs are indicated in Table 5.

dThd kinase, inhibited L1210 cell proliferation at a concentration which was about 10 times higher than their $K_i$-value. However, 5-fluoro-, 5-trifluoromethyl- and 5-ethynyl-dUrd, which had an average $K_i$-value of 98 $\mu$M, inhibited L1210 cell proliferation at a concentration of about 40–340 nM, approximately 300–2500 times lower than their $K_i$-value.

Some compounds, i.e. 5-propyl-dUrd, 5-propynyloxy-dUrd, 5-methylthiomethyl-dUrd, (*E*)-5-(2-cyanovinyl)-dUrd and the dithiolane derivative of 5-formyl-dUrd, which were barely inhibitory for L1210 cell proliferation, did not display any affinity towards L1210 dThd kinase (Table 5). The inactivity of these compounds as L1210 cell growth inhibitors may be related to an insufficient phosphorylation by the cellular dThd kinase. Such phosphorylation would be necessary if the corresponding dUMP analogs were to interfere at the dTMP synthetase level. That the antitumor cell activity of 5-substituted dUrd derivatives is to a large extent dependent on prior conversion to the 5′-monophosphate by dThd kinase is further attested in the findings that these derivatives were much less inhibitory to mutant cell lines which had become deficient in dThd kinase activity [47].

Although the $K_m$-value of the dThd kinase was about 10 times higher than the $K_m$-value of dTMP synthetase for dUMP, the $K_i$-values of the dThd kinase for the dUrd analogs (except for 5-bromo- and 5-iodo-dUrd) were 100–1000 times higher than the $K_i$-values of dTMP synthetase for the corresponding dUMP analogs. This means that the 5-substituted dUMP analogs have, in general, a much higher affinity for dTMP synthetase than their dUrd counterparts for dThd kinase. In addition, there was only a modest correlation between the log $K_i/K_m$ of dThd kinase and the log $K_i/K_m$ of dTMP synthetase ($r = 0.42$) (Fig. 5).

For several dUMP analogs, viz. 5-fluoro-, 5-nitro- and 5-formyl-dUMP, the $K_i/K_m$ values of dTMP synthetase were considerably lower than 0.1. The latter dUMP analogs may therefore be regarded as potent inhibitors of dTMP synthetase. As we have demonstrated previously [17], their potency as dTMP synthetase inhibitors is reflected by the fact that in intact cells these nucleosides are far more inhibitory to [2-$^{14}$C]dUrd incorporation than to [*methyl*-$^{3}$H]dThd incorporation into L1210 cell DNA, and their inhibitory effects on L1210 cell proliferation

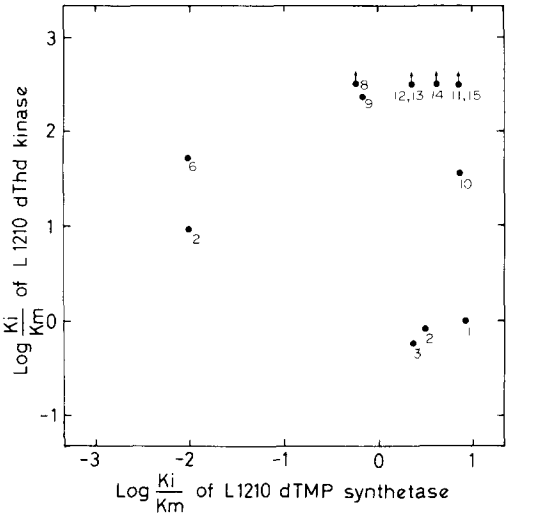


Fig. 5. Log $K_i/K_m$ of L1210 dThd kinase for various 5-substituted dUrd analogs as a function of log $K_i/K_m$ of L1210 dTMP synthetase for the corresponding dUMP analogs. The $K_i/K_m$ values for dThd kinase and dTMP synthetase are taken from Tables 5 and 3 respectively. The symbols for the dUrd analogs and the corresponding 5′-monophosphates are indicated in Tables 3 and 5.

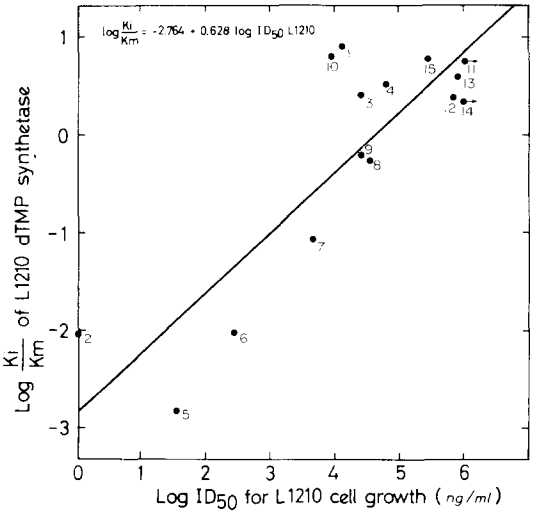


Fig. 6. Linear regression line for log $K_i/K_m$ of L1210 dTMP synthetase for various 5-substituted dUMP analogs as a function of log $ID_{50}$ of the corresponding dUrd analogs for L1210 cell growth. The $K_i/K_m$ values are taken from Table 3. The $ID_{50}$ values correspond to the 50% inhibitory doses for L1210 cell proliferation and are taken from Refs 17 and 60. The symbols for the dUMP analogs are indicated in Table 3.

are more readily reversed by dThd than by dUrd. *Vice versa*, those 5-substituted dUMP derivatives that emerge as poor inhibitors of dTMP synthetase from the present study (viz. 5-bromo-, 5-iodo-, 5-ethyl-, 5-propyl-, 5-methylthiomethyl- and 5-(1,3-dithiolan-2-yl)-dUMP: see Table 3), have previously been recognized as non-specific inhibitors of dTMP synthetase, since they inhibited the incorporation of [2-$^{14}$C]dUrd and [*methyl*-$^{3}$H]dThd into DNA of intact cells to approximately the same extent [17]. Moreover, there appeared to be a strong correlation ($r = 0.833$) between the log $K_i/K_m$ of dTMP synthetase for the dUMP analogs and the log $ID_{50}$ values of the corresponding nucleosides for L1210 cell proliferation (Fig. 6).

In conclusion, the inhibitory activity of 5-substituted dUrds on mouse leukemia L1210 cells can be attributed to an inhibitory effect on dTMP synthetase, rather than on dThd kinase.

Indeed, the inhibitory effects of the dUMP analogs on dTMP synthetase were much stronger than were the inhibitory effects of the corresponding dUrd analogs on dThd kinase, as directly measured with cell-free enzymes isolated from L1210 cells. Furthermore, there was no correlation between the $K_i/K_m$ values of dThd kinase and the $ID_{50}$ values for L1210 cell proliferation. In contrast, there appeared to be a strong correlation between the $K_i/K_m$ values of dTMP synthetase and the $ID_{50}$ values for L1210 cell proliferation. While dTMP synthetase may serve as a target for the antitumor cell action of the 5-substituted dUrds, dThd kinase would be required to convert the dUrd analogs to their active (5'-monophosphate) form.

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