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INHIBITION OF MAMMALIAN TUMOUR THYMIDYLATE SYNTHETASE BY 5-ALKYLATED 2'-DEOXYURIDINE 5'-PHOSPHATES

W. RODE,* T. KULIKOWSKI,† B. KĘDZIERSKA,* M. JASTREBOFF* and D. SHUGAR†‡

* Nencki Institute of Experimental Biology, Polish Academy of Sciences, 02–093 Warszawa, Poland; and † Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02–532 Warszawa, Poland

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Abstract—Improved syntheses, based on Lewis acid–catalyzed nucleosidation, are described for the preparation of 5-alkyl-2'-deoxyuridines. These were converted to their 5'-phosphates with the use of wheat shoot phosphotransferase.

The dUMP analogues, 5-ethyl-dUMP and 5-propyl-dUMP, were competitive vs dUMP inhibitors of thymidylate synthetase purified from mouse L1210, Ehrlich ascites and HeLa cells, the former being the stronger inhibitor. Both analogues were shown to bind cooperatively to each of the mouse tumour enzymes, two molecules of inhibitor interacting with a single enzyme molecule, as reflected by the parabolic character of the replots of the slope vs inhibitor concentrations. dTMP was a stronger inhibitor of the mouse tumour enzymes than its higher alkyl homologues.

The pathway for *de novo* synthesis of dTMP proceeds *via* reductive methylation of dUMP by thymidylate synthetase with concomitant conversion of CH_2-H_4-folate to H_2-folate. Since this is the unique route for intracellular *de novo* synthesis of dTMP, it is to be anticipated that inhibitors of thymidylate synthetase would dramatically affect proliferating cells.

Following introduction of 5-fluorouracil in tumour chemotherapy, it was found that FdUMP is a potent inhibitor of dTMP synthetase *in vitro*, and considerable attention has been devoted to investigation of the mechanism of this inhibition, and the possibility of finding more effective inhibitors. The potent inhibitory properties of a number of 5-substituted analogues of dUMP have been extensively documented, and their mechanism of action is of widespread current interest.

For the most part, however, *in vitro* inhibition studies have focused on the enzymes from bacterial sources, because of their ready availability, as well as their higher stabilities. Furthermore, there are relatively few instances where comparisons have been made of the behaviour of a given inhibitor towards the enzyme from different sources.

The availability, in one of our laboratories, of purified dTMP synthetase from three tumour cell lines prompted us to undertake a study of the inhibitory properties towards these enzymes of two 5-substituted analogues of dUMP, namely 5-ethyl-dUMP and 5-propyl-dUMP. These two analogues are of particular interest, in relation to many other 5-substituted analogues of dUMP [1], in that the 5-alkyl substituents only minimally affect the properties of the pyrimidine ring and the nucleotide relative to those of dTMP (see below), the product of the dTMP synthetase reaction. Of additional interest is the fact that 5-ethyl-dUrd and 5-propyl-dUrd were originally synthesized as potential antiviral agents [2, 3]. Both are effective inhibitors of HSV types 1 and 2, but only 5-ethyl-dUrd also inhibits vaccinia virus [4, 5]. Furthermore, 5-ethyl-dUrd, but not 5-propyl-dUrd, exhibits cytotoxicity towards such tumour cells as Ehrlich ascites carcinoma [6], L1210 [1] and $B_5$59 clone derived from B16 mouse melanoma [7]. On the other hand, the corresponding nucleotides are virtually non-toxic towards primary rabbit kidney cells [8]. With a view to throwing further light on these differences in cytotoxicity, it appeared desirable to examine the activity of the active forms of the nucleosides, i.e. their 5'-phosphates, *vs* thymidylate synthetases from Ehrlich ascites, L1210, and HeLa cells.

MATERIALS AND METHODS

Ehrlich ascites carcinoma, and mouse leukemia L1210, cells were maintained intraperitoneally in female Swiss and DBA-2 mice, respectively [9]. The cells were harvested 5 days following transplantation of 10^6 Ehrlich ascites cells, or 10^5 L1210 cells, and stored as described earlier [9]. HeLa cells were maintained, harvested and stored as previously described [10].

(±)-L-Tetrahydrofolate was prepared according to Lorenson *et al.* [11], and other reagents as earlier described [9].

Potassium nonaflate (Bayer AG) was a gift of Dr. H. Vorbrüggen. hexamethyldisilazane (HMDS) was from Serva (Heidelberg) and trimethylchlorosilane (TCS) from Merck (Darmstadt).

Melting points (uncorrected) were measured on a Boetius microscope hot stage. U.v. spectra were

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‡ To whom correspondence should be addressed.

recorded on a Pye-Unicam SP-500 instrument, using 10-mm pathlength cuvettes. Mass spectra were run on a Varian MAT Model 111 equipped with a combination field desorption-field ionization electron-impact ion source.

Preparative thin-layer chromatography was performed with 20 × 20 cm Merck PSC 60 F-254 silica gel plates. Preparative paper chromatography was carried out with Whatman 3 MM paper. Solvent systems (v/v) were: (A) benzene–ethyl acetate 7:3, (B) upper layer of mixture of sec-butanol and water at 20°. The superscript before each R_F value represents the number of developments.

Synthesis of 5-alkyl-2'-deoxyuridine-5'-phosphates (VII*a,b*).

1-(3,5-*Di*-O-*benzoyl-2-deoxy-β*-D-*ribofuranosyl*)-5-*ethyluracil* (III*a*) (Fig. 1). A mixture of 141 mg (1 mmole) 5-ethyluracil (Ia) [12] and 369 mg (1 mmole) 1-acetyl-3,5-di-*O*-benzoyl-2-deoxy-α-D-ribose (II) (m.p. 80°) [13, 14] in 14 ml anhydrous acetonitrile was treated with 50 μl (0.61 mmole) of HMDS, 390 μl (3.1 mmole) of TCS, and 812 mg (2.4 mmole) of the catalyst potassium nonaflate ($C_4F_9SO_3K$) with stirring under reflux for 16 hr. This was followed by addition of 20 ml CH_2Cl_2 and the solution shaken with 4 × 25 ml saturated $NaHCO_3$. The aqueous phase was once again extracted with CH_2Cl_2. The CH_2Cl_2 extracts were pooled, dried over anhydrous Na_2SO_4 and brought to dryness under reduced pressure. The residue was dissolved in 2.5 ml CH_3OH, deposited on five preparative Merck (Darmstadt, GFR) PSC 60 F-254 silica gel plates and developed with the solvent system A. This gave two barely separated bands with ^4R_f values of 0.50 and 0.57.

The higher mobility band was eluted with chloroform–methanol (1:1), the eluate brought to dryness under reduced pressure, and the residue taken up in 10 ml anhydrous ethanol and again brought to dryness. The residue was crystallized from methanol to yield 186 mg (43%) of the β-anomer IIIa, m.p. 143°. U.v.: $\lambda_{max_1}^{MeOH}$ 228 nm (ε_{max_1} 33.4 × 10^3), λ_{min}^{MeOH} 250 nm (ε_{min} 10.5 × 10^3), $\lambda_{max_2}^{MeOH}$ 266 nm (ε_{max_2} 13.7 × 10^3); m.s.: *m/e* 452 (M^+).

1-(3,5-*Di*-O-*benzoyl-2-deoxy-α*-D-*ribofuranosyl*)-5-*ethyluracil* (IV*a*). The lower band from the above chromatographic plates was eluted and crystallized as above to yield 182 mg (42%) of IVa, m.p. 175°. U.v.: $\lambda_{max_1}^{MeOH}$ 228 nm (ε_{max_1} 33.4 × 10^3), λ_{min}^{MeOH} 250 nm (ε_{min} 9.3 × 10^3), $\lambda_{max_2}^{MeOH}$ 268 nm (ε_{max_2} 13.4 × 10^3); m.s.: *m/e* 452 (M^+).

1-(3,5-*Di*-O-*benzoyl-2-deoxy-β*-D-*ribofuranosyl*)-5-*propyluracil* (III*b*). This was obtained from 5-propyluracil (Ib) [12] and II as described above for IIIa, in 35% yield, ^4R_f = 0.64 on PSC silica gel with solvent A, m.p. 166–167°. U.v.: $\lambda_{max_1}^{MeOH}$ 228 nM (ε_{max_1} 32.7 × 10^3), λ_{min}^{MeOH} 250 nm (ε_{min} 10.5 × 10^3), $\lambda_{max_2}^{MeOH}$ 268 nm (ε_{max_2} 13.7 × 10^3); m.s.: *m/e* 466 (M^+).

1-(3,5-*di*-O-*benzoyl-2-deoxy-α*-D-*ribofuranosyl*)-5-*propyluracil* (IV*b*). This was obtained from IIIb as described for IVa, to yield 142 mg (34%), ^4R_f = 0.56 on PSC silica gel plates with solvent A, m.p. 174–175°. U.v.: $\lambda_{max_1}^{MeOH}$ 228 nm (ε_{max_1} 33.4 × 10^3), λ_{min}^{MeOh} 250 nm (ε_{min} 9.3 × 10^3), $\lambda_{max_2}^{MeOH}$ 268 nm (ε_{max_2} 13.4 × 10^3); m.s.: *m/e* 466 (M^+).

1-(2-*deoxy-β*-D-*ribofuranosyl*)-5-*ethyluracil* (V*a*). Compound IIIa (90 mg, 0.2 mmole) was dissolved in 5 ml anhydrous methanol in a glass tube and saturated with anydrous NH_3 at 0°. The tube was sealed and shaken overnight at 37°. The solution was concentrated to half-volume and filtered. The filtrate was deposited on a sheet of Whatman 3 MM and developed with solvent B (upper layer of a mixture

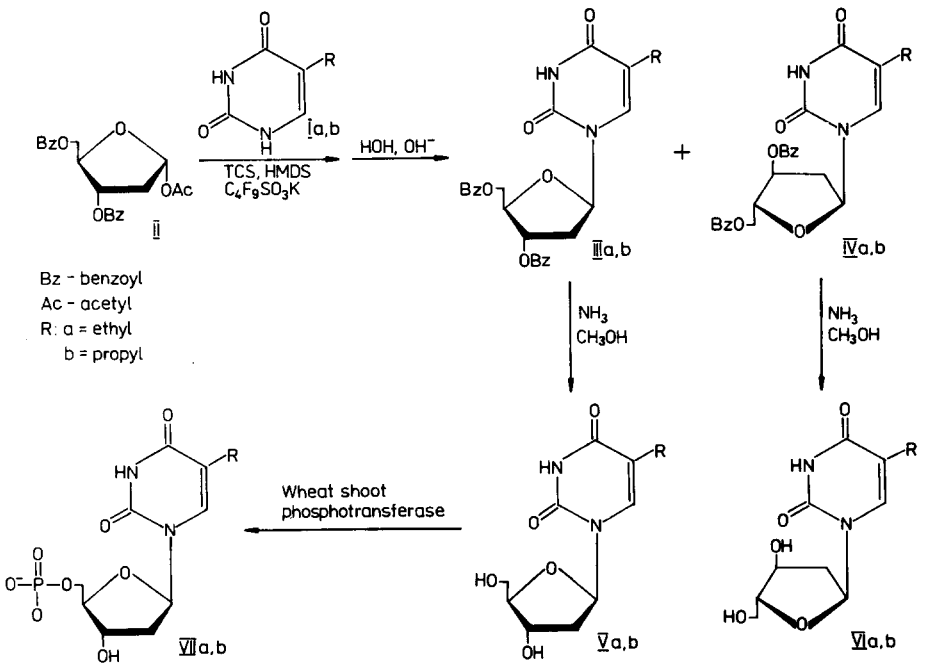

Fig. 1. Synthesis of 5-alkyl-2'-deoxyuridines and their 5'-monophosphates.

of sec.-butanol and water at 20°). The band with $R_f = 0.48$ was eluted with water, and the eluate brought to dryness. The residue was dissolved in 5 ml hot anhydrous ethanol, filtered while hot, and the filtrate brought to dryness. The residue was crystallized from anhydrous ethanol–ether to yield 43 mg (82%) of Va, with properties identical to those previously described [3, 15].

1-(2-*Deoxy*-α-D-*ribofuranosyl*)-5-*ethyluracil* (VI*a*). This was prepared from IVa as described for Va, yield 41 mg (80%), with properties identical to those previously described [3, 15].

1-(2-*Deoxy*-β-D-*ribofuranosyl*-5-*propyluracil* (V*b*). This was obtained from IIIB as described for Va, in 75% yield, with properties identical to those previously reported [16].

1-(2-*Deoxy*-α-D-*ribofuranosyl*-5-*propyluracil* (VI*b*). This was obtained from IVb as described for Va, in 72% yield, and with properties as elsewhere reported [16].

5-*Ethyl*-2′-*deoxyuridine*-5′-*phosphate*·Na_2 (VII*a*). Prepared as previously described [8], from Va in 40% yield.

5-*Propyl*-2′-*deoxyuridine*-5′-*phosphate*·Na_2 (VII*b*). Prepared from Vb as previously described [8] in 35% yield.

Thymidylate synthetase purification. Electrophoretically homogeneous preparations of the enzyme from Ehrlich ascites, L1210 and HeLa cells were obtained, and checked for homogeneity, as elsewhere described [9, 10, 17].

Enzyme assay. A modification of the procedure of Roberts [18] was employed. The reaction mixture, in a total volume of 40 μl, contained: 0.05–0.25 nmole [5-^{3}H]dUMP (~6 × 10^7 cpm/μmole), 50 nmole (±)-L-tetrahydrofolate, 0.2 μmole formaldehyde, 4 μmole 2-mercaptoethanol, 2 μmole Tris–HCl buffer pH 7.5, 0.4 μmole ascorbate buffer pH 7.5, and enzyme (<0.5 pmole). Both 5-ethyl-dUMP and 5-propyl-dUMP were added as neutral aqueous solutions. The reaction was initiated, after 1 min preincubation at 37°, by addition of the enzyme, and incubation continued for 10 min at the same temperature. The reaction was terminated, and released tritium assayed, as elsewhere described [9]. All samples were done in triplicate.

Kinetics studies. Lineweaver–Burk plots were based on the use of values of the overall velocity in place of the initial velocity, and the mean substrate concentration during the period of the assay instead of the initial substrate concentration, as described by Lee and Wilson [19]. The Lineweaver–Burk plots, and the plots of $1/K_{i(\text{slope})}$ vs inhibitor concentration, were fitted, and results from these plots calculated, with the aid of the linear regression program of the "Sharp" EL-5100 calculator.

RESULTS

Results of syntheses are described in Materials and Methods.

Inhibition of mouse tumour enzyme by 5-ethyl-dUMP and 5-propyl-dUMP. Inhibition by each of the analogues was examined by varying the dUMP concentration at different concentrations of the inhibitor, and plotting the results as Lineweaver–Burk plots. The two analogues inhibited both L1210 and Ehrlich ascites thymidylate synthetases competitively with respect to dUMP, as manifested by the intersection at the ordinate of the linear 1/v vs 1/[dUMP] plots (Figs. 2–5). No time-dependent inactivation was observed.

To further characterize the nature of this inhibition, the slopes of the double reciprocal plots (slope 1/[dUMP]) from each experiment were replotted vs inhibitor concentration. All of these replots were found to be parabolas (Figs. 2–5B), indicative of pure competitive inhibition, with the inhibitor interacting with two sites on the enzyme molecule [20]. The reciprocal form of the velocity equation describing such inhibition is:

$$\frac{1}{v} = \frac{K_s}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)^2 \frac{1}{[S]} + \frac{1}{V_{max}}$$

where K_s is the dissociation constant of the enzyme–substrate complex, I is the inhibitor, K_i the inhibition

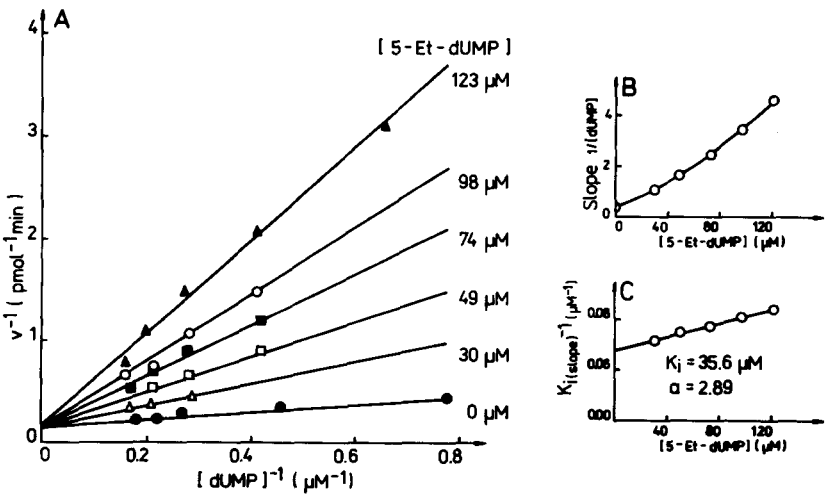

Fig. 2. Inhibition of L1210 thymidylate synthetase by 5-ethyl-dUMP.

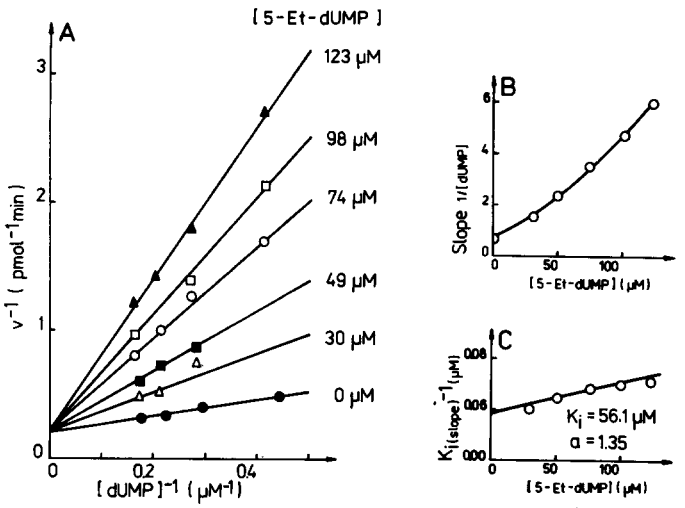

Fig. 3. Inhibition of Ehrlich ascites carcinoma thymidylate synthetase by 5-ethyl-dUMP.

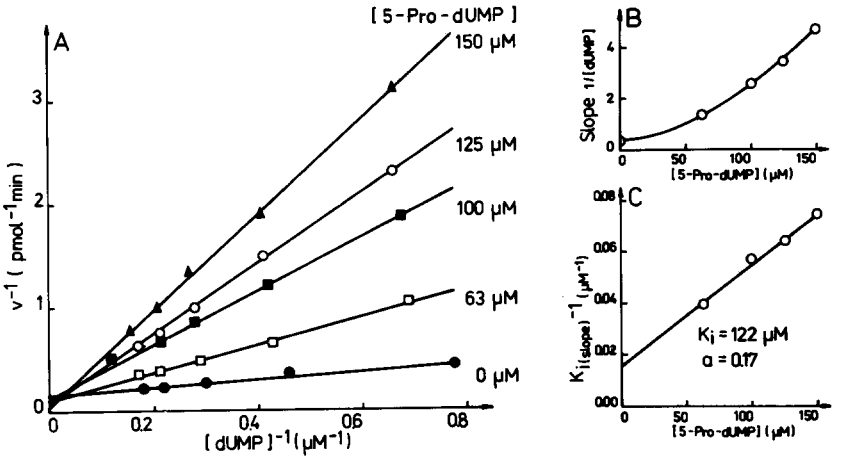

Fig. 4. Inhibition of L1210 thymidylate synthetase by 5-propyl-dUMP.

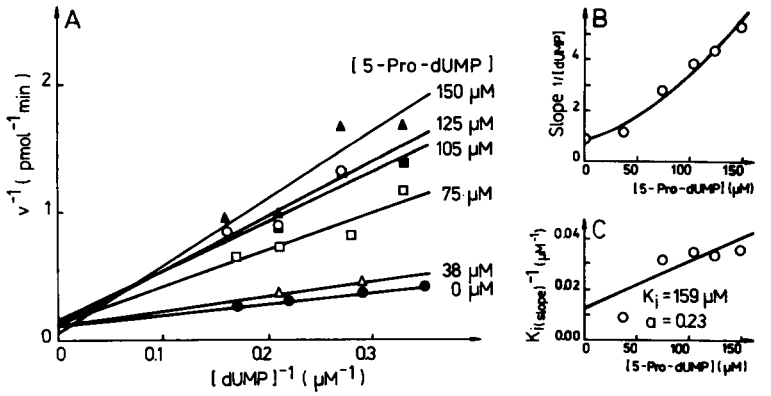

Fig. 5. Inhibition of Ehrlich ascites carcinoma thymidylate synthetase by 5-propyl-dUMP.

constant, and S the substrate. The K_i must be determined from its dependence on $K_{i(\text{slope})}$, i.e. K_i^{app} calculated from the slope of the double reciprocal plot, which is a function of K_i that varies with inhibitor concentration, as follows:

$$\frac{1}{K_{i(\text{slope})}} = \frac{1}{K_i^2}[I] + \frac{2}{K_i}$$

where the $K_{i(\text{slope})}$ may be calculated from the expression:

$$\text{slope}_{1/s} = \frac{K_s}{V_{max}}\left(1 + \frac{[I]}{K_{i(\text{slope})}}\right)$$

The replot of $1/K_{i(\text{slope})}$ vs the corresponding value of $[I]$ is a straight line with a slope of $1/K_i^2$, an ordinate

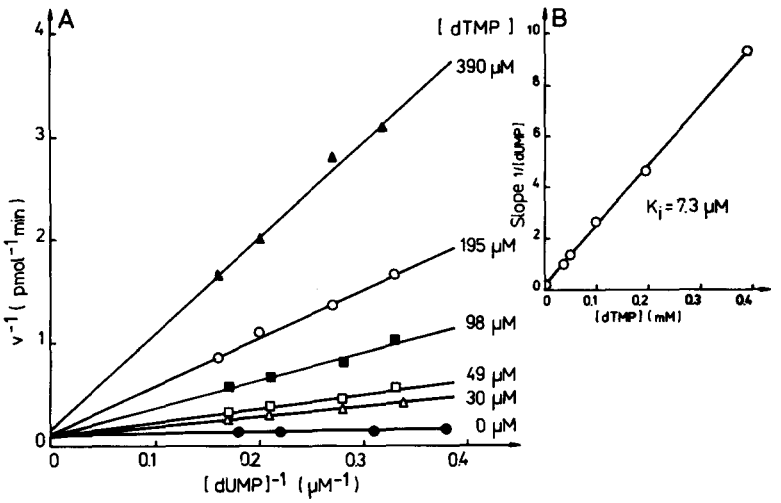

Fig. 6. Inhibition of L1210 thymidylate synthetase by dTMP.

intercept of $2/K_i$, and an abscissa intercept of $2K_i$. If If there is cooperative inhibitor binding, the slope of the replot is $1/aK_i^2$, the ordinate intercept is still $2/K_i$, and the abscissa intercept is $-2aK_i$, where a is an interaction factor (aK_i = dissociation constant describing binding of second molecule of an inhibitor to an enzyme molecule containing one molecule of the inhibitor already bound) [20].

The two analogue inhibitors were found to bind both mouse tumour enzymes cooperatively. For 5-ethyl-dUMP, $K_i = 35.6\ \mu M$, and $a = 2.89$ with the L1210 enzyme (Fig. 2C), and $K_i = 56.1\ \mu M$, $a = 1.35$ with the Ehrlich ascites enzyme (Fig. 3C). For 5-propyl-dUMP, $K_i = 122\ \mu M$ and $a = 0.17$ with the L1210 enzyme (Fig. 4C), and $K_i = 159\ \mu M$ and $a = 0.23$ with the Ehrlich ascites enzyme (Fig. 5C). Duplicate series of experiments yielded K_i values within 8%, and a values within 15% of the foregoing. The apparent K_m values (±S.E.) for dUMP were $1.96 \pm 0.25\ \mu M$ for the L1210 enzyme, and $1.3 \pm 0.4\ \mu M$ for the Ehrlich ascites enzyme.

Analogue inhibition of human tumour (HeLa) enzyme. Preliminary experiments revealed inhibition by both 5-ethyl-dUMP and 5-propyl-dUMP to be competitive with respect to dUMP, but did not permit of an unequivocal conclusion regarding the mechanism of inhibitor binding. On the assumption that this mechanism is similar to that for the two mouse tumour enzymes, the K_i values for 5-ethyl-dUMP and 5-propyl-dUMP were calculated as 40 and 74 μM, and the apparent $K_m = 4.05 \pm 0.85\ \mu M$.

Inhibition of mouse tumour enzymes by dTMP. The reaction product, dTMP, inhibited both the L1210 and Erhlich ascites enzymes competitively with respect to dUMP (Figs. 6A and 7A). Replots of the slopes of the double-reciprocal plots vs inhibitor concentration were, in both instances, linear rather than parabolic (Figs. 6B and 7B). Linear regression fits of both replots gave correlation coefficients of 1.00, and the inhibition constants calculated from these replots (abscissae intercepts of $-K_i$) were 7.3 and 9.6 μM for the L1210 and Ehrlich ascites

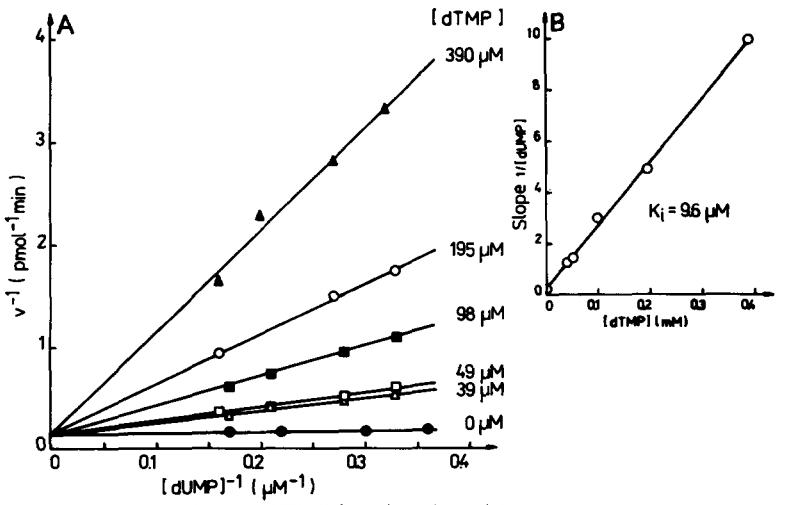

Fig. 7. Inhibition of Ehrlich ascites thymidylate synthetase by dTMP.

enzymes, respectively. However, since it appeared likely that dTMP should interact with the enzyme in an analogous manner, the inhibition constants were also calculated from replots of $1/K_{i(slope)}$ vs [dTMP] (up to 98 μM), to yield values of $K_i = 12.5$ μM and $a = 15$ for the L1210 enzyme, and 11.1 μM and 38 for the Ehrlich ascites enzyme.

DISCUSSION

Synthetic procedures. The initial compounds required for the synthesis of the 5′-phosphates of 5-ethyl-dUrd and 5-propyl-dUrd (Fig. 1) were obtained by condensation procedures. The current method for such "one-pot" synthesis is based on the use of the 1-*O*-methyl derivative of 2-deoxy-3,5-di-*O*-*p*-toluoyl-D-ribofuranose, which is a mixture of the furanose and pyranose forms [21], and gives a yield of anomeric nucleosides in about 40% overall yield.

With a view to improving the yield, we have employed the stable, crystalline, single anomeric form of 1-*O*-acetyl-3,5-di-*O*-benzoyl-2-deoxy-α-D-ribose [13, 14]. This reacts with the appropriate *in situ* generated *O*-trimethylsilyl-5-alkyluracil, in the presence of potassium nonaflate in acetonitrile, to give a 1:1 mixture of β and α anomers of 1-(3,5-di-*O*-benzoyl-D-ribofuranosyl)-5-alkyluracil with an overall yield of about 80%. The anomeric pairs IIIa and IVa, and IIIb and IVb, were fractionated by preparative TLC on silica gel, protecting groups removed in methanolic ammonia under mild conditions, and the free nucleosides, 5-ethyl-dUrd (Va) and 5-propyl-dUrd (Vb), and their α-anomers (IVa and IVb) obtained in almost 80% yield.

In order to avoid possible formation of contaminating 3′-phosphates during chemical phosphorylation [22], enzymatic phosphorylation was employed, with the aid of the wheat shoot phosphotransferase system, as elsewhere described [8].

Inhibition studies. 5-Ethyl-dUrd and 5-propyl-dUrd have been reported to exhibit appreciably different inhibitory activities towards growth of L1210 cells, with ID_{50} values of 8.5 μg/ml and >1000 μg/ml, respectively [23]. The results of the present *in vitro* inhibition studies show that 5-ethyl-dUMP is a distinctly stronger inhibitor (Figs. 1 and 2) than 5-propyl-dUMP (Figs. 3 and 4) of mammalian tumour thymidylate synthetase. Such a difference might have been inferred from the fact that 5-ethyl-dUrd exhibited a higher ratio of ID_{50} for (methyl-^{3}H)dThd incorporation to ID_{50} for (2-^{14}C)dUrd incorporation in both primary rabbit kidney [24, 25] and L1210 [23] cells than 5-propyl-dUrd.

Subsequently it was reported that the K_i values for inhibition of a crude preparation of L1210 thymidylate synthetase by 5-ethyl-dUMP and 5-propyl-dUMP were closely similar, 11.8 and 11.0 μM, respectively [1]. Assuming this result to be valid, the lack of inhibition of L1210 cell proliferation by 5-propyl-dUrd is explicable only in terms of its low intracellular phosphorylation, due to poor substrate properties of the analogue towards L1210 thymidine kinase [1]. Our results suggest that the differential inhibition of thymidylate synthetase by 5-ethyl-dUMP and 5-propyl-dUMP may equally be involved in the mechanism of the differential response of the cells to the parent nucleosides.

5-Ethyl-dUMP seems to inhibit mammalian thymidylate synthetases slightly less than the enzyme from *Escherichia coli* ($K_i = 22$ μM) [26]. The K_i values describing inhibition of the mouse tumour thymidylate synthetases by dTMP are comparable with those found for the enzymes from human leukemic cells (13 μM) [27], *Lactobacillus casei* (15.5, 2.4 μM) [28, 29] and *Bacillus subtilis* (15 μM for thymidylate synthetase A) [30]. On the other hand, they are distinctly lower than those reported for thymidylate synthetases from calf thymus (59 μM) [31], chick embryo (140 μM) [11], *Saccharomyces cerevisiae* (80 μM) [32] and *Bacillus subtilis* (130 μM for thymidylate synthetase B) [30]. The Ehrlich ascites enzyme K_i for dTMP presented here is also much lower than two estimates published earlier [33, 34].

Each of the L1210 and Ehrlich ascites enzymes appears to interact with two molar equivalents of the inhibitor analogues. This is consistent with the fact that both enzymes, as well as that from HeLa cells are dimers which bind two molecules of FdUMP per enzyme molecule [10, 17, 34]. On the other hand, the linear slope of the 1/[dUMP] vs [dTMP] replots indicate that dTMP interacts with only a single site on the enzyme molecule. One possible interpretation of this result is the existence of a strong negative cooperativity of dTMP binding, so that its interaction with the second site is very weak. Such an interpretation is supported by the high interaction factors calculated for dTMP binding on the assumption of the existence of two interaction sites (see Results). Furthermore, both mouse tumour enzymes exhibit weak negative cooperativity for interaction with 5-ethyl-dUMP ($a > 1$, Figs. 2C and 3C), and positive cooperativity for interaction with 5-propyl-dUMP ($a < 1$, Figs. 4C and 5C).

The cooperativity of interaction of the 5-alkyl dUMP analogues with thymidylate synthetase is of some interest in relation to our recent observation of a cooperative effect for dUMP binding by the Ehrlich ascites enzyme, as reflected in a biphasic Hill plot with a slope of 1.0 for [dUMP] $\geq$ 0.5 μM, which increases to 1.9 for [dUMP] $\leq$ 0.5 μM [35]. We have since noted a similar biphasic Hill plot with the L1210 enzyme, for which the slope changes from 1.0 to 1.4 at [dUMP] ~ 1.3 μM (Rode and Kędzierska, unpublished). These results imply that the active centers of the thymidylate synthetase molecule are not independent.

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