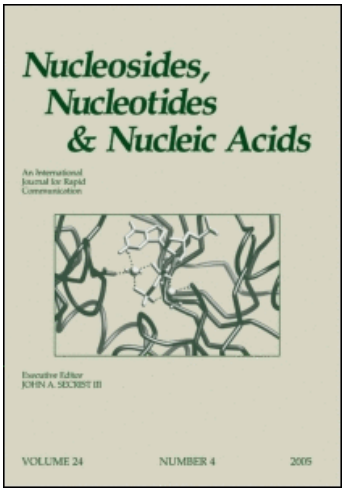

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STUDY OF ANALOGUES OF THYMIDINE-5'-MONOPHOSPHATE AND THYMIDINE AS SUBSTRATES OR INHIBITORS OF CHICK EMBRYO LIVER THYMIDYLATE KINASE

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Abstract: The phosphorylation of thymidine-5'-monophosphate (dTMP) by chick embryo liver thymidylate kinase (Km (dTMP) = 1.2 μM) was inhibited by the 5'-monophosphate derivatives of 5-bromo-2'-deoxyuridine (5-Br-dUMP), 5-iodo-2'-deoxyuridine (5-I-dUMP), 2',3'-dideoxythymidine (ddTMP), 3'-azido-3'-deoxythymidine (AZT-MP) and the methylene phosphonate analogue of AZT-MP with IC50 values of 8, 24, 14, 5 and 6 μM respectively. 5-Fluoro-2'-deoxyuridine (5-F-dUMP) and dUMP were poor inhibitors (IC50 values > 300 μM). 5-Br-dUMP and 5-I-dUMP were found to be significant substrates of thymidylate kinase with phosphorylation efficiencies (Vmax/Km) of 26 and 6% of that of dTMP, respectively. In contrast, AZT-MP and ddTMP were poor substrates, being phosphorylated 800-fold less efficiently than dTMP. Thymidylate kinase was also significantly inhibited by thymidine and AZT. Our data give a better insight into the topology of the dTMP binding site of this enzyme and show that the 3'-hydroxyl group of dTMP plays a critical role in catalysis.

Thymidylate kinase (dTMP kinase, E.C. 2.7.4.9) is an enzyme which catalyzes the phosphorylation of dTMP to form dTDP in the presence of ATP and a divalent cation such as Mg^{2+} (1-5). This enzymatic reaction is a mandatory step in the biosynthetic pathways from thymidine (salvage) or dUMP (*de novo*) to dTTP and is therefore essential for DNA synthesis (6). Thymidylate kinase activity, generally low or absent in non-proliferating tissues, is moderate to high in those growing rapidly. In this respect, it has been suggested that inhibitors of thymidylate kinase could prove effective in blocking cell proliferation and find application as anticancer agents (7-8). Also, thymidylate kinase plays an important role in antiviral chemotherapy by virtue of its ability to phosphorylate the nucleoside monophosphate derivatives of various thymidine (dThd) analogues. Thymidine analogues which inhibit human immunodeficiency virus (HIV) replication exert their antiviral effect after sequential activation by cellular kinases to their triphosphate derivatives which act as potent inhibitors of reverse transcriptase. This is the case for 3'-azido-3'-deoxythymidine (AZT) (9), 3'-deoxy-3'-fluorothymidine (10), 2',3'-didehydro-2',3'-dideoxythymidine

(D4T) (11), 2',3'-dideoxythymidine (ddThd) (12), 3'-azido-5-chloro-2',3'-dideoxyuridine (13), 5-chloro-2',3'-dideoxy-3'-fluorouridine (13, 14), 3'-azido-5-bromo-2',3'-dideoxyuridine (15), 3'-azido-2',3'-dideoxy-5-iodouridine (15) and 4'-azidothymidine (16). The efficiency of conversion of these nucleoside analogues to their triphosphate derivatives by cellular kinases is a critical factor for antiviral activity since it determines the level of their active metabolites in cells. Whereas quite ample information is available on the substrate specificity of the nucleoside kinases responsible for the first phosphorylation step (9, 14-18), little is known concerning the interaction of the dTMP analogues with thymidylate kinase. In cells, thymidylate kinase is held responsible for the phosphorylation of the 5'-monophosphates of AZT (AZT-MP) (9) and 5-chloro-2',3'-dideoxy-3'-fluorouridine (14); the enzyme catalyzes the rate-limiting step in the anabolism of these nucleotide analogues to their triphosphate derivatives (9, 14). Recently, several phosphonate analogues of dTMP have been designed as antiviral agents (19-24) and some have been found to be effective inhibitors of HIV replication (21, 23). Again, the antiviral activity of these compounds is thought to be due to inhibition of reverse transcriptase by their diphosphate derivatives (nucleoside triphosphate analogues). After uptake by cells, the dTMP analogues interact directly with thymidylate kinase. In this respect, a better knowledge of the substrate specificity of this enzyme might help in the design of novel substrates. Some of these could also prove effective antiviral agents. Since chick embryo liver is known to be a quite rich and easily accessible source of thymidylate kinase (25), we used this tissue for purification of the enzyme. In this report, we describe the properties of several analogues of dTMP and thymidine as substrates or inhibitors of thymidylate kinase.

RESULTS AND DISCUSSION

Properties of chick embryo liver thymidylate kinase.

The high instability of dTMP kinase from animal origin is well known (1, 2, 4, 5, 25). In agreement with previous observations by Smith and Eakin (25), chick embryo liver dTMP kinase was stabilized by addition of 40 μM dTMP and 5 mM 2-mercaptoethanol in buffers throughout the purification process. The purified enzyme remained stable for several months at -20°C in 10 mM potassium phosphate buffer (pH 7.5) containing 5 mM 2-mercaptoethanol, 40 μM dTMP and 20 % glycerol. After dilution in ice-cold assay buffer, the enzyme was stable for several hours; it also appeared to be stable during incubations at 37°C, since the reaction remained linear with time for periods allowing the conversion of up to 30% dTMP, both at low (2 μM) and saturating (80 μM) concentrations of substrate.

The ATP regeneration system had no effect on the activity of the purified enzyme and was omitted. At 10 mM $MgCl_2$, activity was maximal at 1-2 mM ATP. The Km value of dTMP for dTMP kinase was 1.9 ± 0.5 μM (n = 10), i. e. significantly lower than that found for dTMP kinase from the same source (90 μM; ref. 25) but similar to that measured for dTMP kinases from various human cells in culture (range of Km values = 0.6 - 5 μM; ref. 9, 14-16) or human term placenta (Km = 4.9 μM, ref. 26).

Kinetic parameters of dTMP analogues for thymidylate kinase.

The nucleotide analogues ddTMP, AZT-MP, 5-Br-dUMP and 5-I-dUMP could be obtained as the tritium-labelled forms thus allowing their study as substrates of dTMP kinase. 5-Br-dUMP was phosphorylated with an efficiency of 26 % of that of dTMP as estimated from the Vmax/Km ratios (Table 1) and is therefore a good substrate of dTMP kinase. 5-I-dUMP had a Vmax comparable to that of 5-Br-dUMP. However, its Km was 2.8-fold greater than that of the bromo analogue (Table 1). Based on Vmax/Km ratios, 5-I-dUMP is a 4-fold less efficient substrate than 5-Br-dUMP. Our results on phosphorylation of 5-I-dUMP by chick embryo liver dTMP kinase are in agreement with previous reports using dTMP kinase from other sources. For 5-I-dUMP, a Ki value of 15 μM was reported (27) for Vero cells dTMP kinase for which dTMP had a Km value of 4.1 μM. 5-I-dUMP was phosphorylated at a rate of 57 % of that of dTMP by Syrian hamster melanoma cells dTMP kinase (28). AZT-MP was found to be a very poor substrate, being phosphorylated with an efficiency of 0.13 % of that of dTMP (Table 1). The Km of AZT-MP was only 2.5-fold higher than that of dTMP. However, a 313-fold difference in Vmax was observed. Quite similar results have been obtained with dTMP kinases purified from human H9 cells (9) and CD4+ CEM cells (14). In these studies, the phosphorylation efficiencies determined for AZT-MP were 0.14 and 0.87 % of that of dTMP, respectively. As AZT-MP, ddTMP was a very poor substrate of dTMP kinase. The efficiency of phosphorylation of ddTMP was 0.12 % of that of dTMP (Table 1). The Km of ddTMP was 5.7 times higher than that of dTMP whereas its Vmax was 143 times smaller. From these results, it follows that the 3'-hydroxyl group of dTMP appears to play a most important role in catalysis.

Inhibition of thymidylate kinase by dTMP analogues.

To better define some of the essential requirements for binding of dTMP to dTMP kinase, the nucleotide analogues shown in Table 2 were tested as inhibitors. The inhibitory potency (IC_{50}) of these compounds was determined at a concentration of substrate (dTMP) of 2 μM i.e. equal to the Km of dTMP. Since the nucleotide analogues tested share significant structural similarities with dTMP, one could expect these compounds to be

TABLE 1. Kinetic parameters of dTMP analogues for thymidylate kinase

Compound	Km (μM)	Vmax [a] (% rel. to dTMP)	Vmax/Km [b] (% rel. to dTMP)
dTMP	1.2 (± 0.3)	100	100
ddTMP	6.8 (± 1.2)	0.70	0.12
AZT-MP	3.0 (± 1.2)	0.32	0.13
5-Br-dUMP	2.7 (± 0.2)	58.5	26.1
5-I-dUMP	7.6 (± 0.3)	40.0	6.4

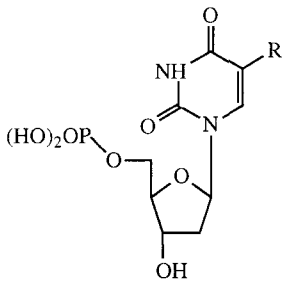
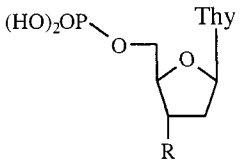
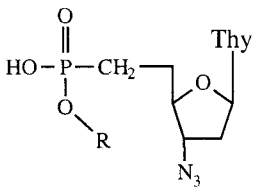
Data are means of 2 (ddTMP, 5-Br-dUMP, 5-I-dUMP), 3 (AZT-MP) and 9 (TMP) independent determinations. For ddTMP and AZT-MP, the kinetic parameters were determined at a concentration of enzyme 8-fold higher than that used for dTMP, 5-Br-dUMP and 5-I-dUMP. For dTMP, 5-Br-dUMP, 5-I-dUMP, ddTMP and AZT-MP, reaction times were 2, 5, 10, 60 and 60 min, respectively.

[a] Vmax of dTMP = 6 μmol. hour^{-1}. (mg protein)$^{-1}$

[b] Efficiency of phosphorylation of nucleotide analogue relative to dTMP = $[(Vmax/Km)_{compound} / (Vmax/Km)_{dTMP}] \times 100$

competitive inhibitors with respect to dTMP. If so, the IC50 value determined under the above described conditions corresponds to about twice the value of the inhibition constant (Ki) and can be used to compare the relative ability of the compounds to interact with the dTMP binding site. dUMP and 5-F-dUMP inhibited dTMP kinase only at concentrations around 500 μM, i.e. about 250-fold higher than the Km value of dTMP (Table 2). dCMP did not inhibit significantly dTMP kinase at 300 μM. This clearly shows that the thymine base is essential for binding of dTMP to dTMP kinase. In previous works, dUMP was also found not to inhibit significantly dTMP kinases of human (5, 26) and animal (25) origins although it was reported to be a substrate of the mouse hepatoma enzyme (4). 5-I-dUMP and 5-Br-dUMP were found to be 21- and 63-fold more effective inhibitors of dTMP kinase than dUMP, respectively (Table 2). The binding of these compounds to dTMP kinase appears to be related to the bulkiness of the substituents at C-5 of the pyrimidine ring. Indeed, the maximal distance between C-5 and the Van der Waals surface of the hydrogen atoms of the methyl group of dTMP is 3.8 Angströms, i. e. identical to the distance between C-5 and the Van der Waals surface of the bromine atom of 5-Br-

TABLE 2. IC50 values of various dTMP analogues for thymidylate kinase

Compound	R =	name	IC_{50} (μM)
	—H	dUMP	500
	—F	5-F-dUMP	31% inhibition at 300 μM
	—Br	5-Br-dUMP	8
	—I	5-I-dUMP	24
	—H	ddTMP	14 (± 2)
	—N_3	AZT-MP	5 (± 1)
	—H		6
	—CH_3		56

IC50 is defined as the concentration of inhibitor producing a 50% reduction of the initial rate of the enzyme reaction
IC50 values were determined at a concentration of 2 μM dTMP, i.e. at a concentration equal to the Km value of dTMP. Except for IC50 values of AZT-MP and ddTMP which are means of 10 and 4 independent experiments (± SD) and 5-F-dUMP (1 determination), data are means of 2 independent experiments, the values of which do not exceed 19% of the mean value.

dUMP, but different from those calculated for the other substituents (2.3, 2.7 and 4.2 Angströms, for H, F and I, respectively). For ddTMP, an IC50 value of 14 μM was determined. In agreement with this result, kinetic studies revealed that ddTMP is a competitive inhibitor with respect to dTMP with a Ki value of 6 μM, i.e. only 3-fold higher than the Km value of dTMP. This indicates that the 3'-hydroxyl group of dTMP is not critical for binding to the enzyme. This conclusion is consistent with the fact that AZT-MP which bears an azido group in place of the 3'-OH group of dTMP was also a significant inhibitor of dTMP kinase (Table 2). The methylene phosphonate analogue of AZT-MP had an IC50 value similar to that of AZT-MP (Table 2). Kinetic studies showed that this phosphonate is a competitive inhibitor with respect to dTMP with a Ki value of 4 μM. Thus, substitution of the oxygen atom of the phosphoester bond by a methylene bridge does not affect binding. Finally, a 9-fold difference was observed between the IC50 value of the methylene phosphonate analogue of AZT-MP and that of its methyl ester. This difference could be due to the loss of one negative charge. This would indicate that dTMP kinase binds dianionic species preferentially. Yet, a steric effect due to the methyl group could also account for the weaker inhibition observed with the methyl ester.

Inhibition of thymidylate kinase by thymidine analogues.

It is known that 5'-deoxy-5'-halogenothymidines are inhibitors of dTMP kinase (8, 29, 30). Thymidine itself was found to be an inhibitor of dTMP kinases from mouse ascites sarcoma 180 (30), chick embryo liver (25) and yeast (31). The mode of inhibition of 5'-deoxy-5'-fluorothymidine (8) and thymidine (25, 30) was found to be competitive with respect to dTMP. As shown in Table 3, we found that thymidine and AZT are inhibitors of chick embryo liver dTMP kinase with IC50 values of 23 μM. Interestingly, ddThd and D4T which have no 3'-hydroxyl group were 3.5-and 3-fold less potent than thymidine and AZT. The mode of inhibition of these nucleoside analogues has not been investigated in this work. Yet, based on previous works with 5'-deoxy-5'-fluorothymidine and thymidine (8, 25, 30), one could expect these inhibitors to be competitive with respect to dTMP. If so, our results would suggest that the hydroxyl and azido groups of thymidine and AZT interact with the same site as that involved in the binding of the hydroxyl and azido groups of dTMP and AZT-MP.

In conclusion, this report provides additional knowledge on the topology of the dTMP binding site of dTMP kinase and shows the critical role of the 3'-hydroxyl group of dTMP in catalysis. A better knowledge of the structure of the dTMP binding site and factors important for catalysis should help in the design of novel inhibitors (i.e. potential anti-cancer agents) and/or substrates (potential anti-cancer/antiviral agents) of this enzyme.

TABLE 3. IC50 values of various thymidine analogues for thymidylate kinase

Compound	IC_{50} (μM)
Thymidine	23 (± 9)
AZT	23 (± 3)
ddThd	81 (± 9)
D4T	69 (± 15)

IC_{50} were determined at 2 μM TMP (i.e. the Km of TMP)
Data are mean (± SD) of 3 to 5 independent experiments.

EXPERIMENTAL

Compounds and enzymes.

[^{3}H] dTMP (40 Ci/mmole) and [^{3}H] dTTP (30 Ci/mmole) were obtained from Amersham. [^{3}H] ddTMP, [^{3}H] AZT-MP, [^{3}H] 5-Br-dUMP and [^{3}H] 5-I-dUMP (5, 18, 20, and 18 Ci/mmole) were from Moravek Biochemicals (Brea, USA). Thymidine, ddThd, AZT, dTMP, dTDP, dTTP, dUMP, 5-F-dUMP, 5-Br-dUMP, 5-I-dUMP, dCMP, ATP, phosphocreatine, creatine phosphokinase (rabbit muscle) and nucleoside diphosphate kinase (NDPK) (bovine liver) were from Sigma. ddTMP was purchased from Pharmacia. AZT-MP was a kind gift of Dr. R. Snyder (Marion Merrell Dow, Cincinnati, USA). D4T was prepared by the Chemistry Department, Marion Merrell Dow Research Institute, Strasbourg Center, according to Mansuri et al. (32). The phosphonate analogue of AZT-MP and its methyl ester derivative were prepared according to Tanaka et al. (33).

Purification of thymidylate kinase.

Homogenization of 18-day chick embryo livers and preparation of a 32000xg supernatant were performed as described previously (25). The supernatant was frozen before use. All purification procedures were carried out at 4°C.

Ammonium sulfate fractionation and dialysis

The supernatant (150 ml obtained from 32 g of liver) was thawed and the dTMP concentration brought to 40 μM. Ammonium sulfate was dissolved slowly up to 40 % saturation and the pH maintained with ammonium hydroxide. After stirring for 30 min, the suspension was centrifuged for 10 min at 9500xg. The supernatant was brought to 70 % saturation, stirred for 30 min and centrifuged at 9500xg for 15 min. The pellet was dissolved in 10 mM potassium phosphate buffer (pH 7.5) containing 5 mM 2-mercaptoethanol and 40 μM dTMP (buffer A) to have a final volume of 20 ml. The sample was dialysed for 4 hours against buffer A (2 x 500 ml), centrifuged for 10 min at 9500 x g and frozen at -20°C.

DEAE sepharose Fast Flow chromatography
The sample (28 ml) was diluted with water containing 40 µM dTMP until the conductivity of the solution was 4 milliSiemens. The pH was adjusted at 7.5 and the sample was loaded on a 2.6 x 17 cm DEAE sepharose Fast Flow column equilibrated with buffer A. After washing with 1.5 column volume of buffer A, the column was eluted (3 ml/min) with a 300 ml linear gradient of 10 to 600 mM potassium phosphate buffer (7.5) containing 5 mM 2-mercaptoethanol and 40 µM dTMP. The fractions containing the activity (40 ml) were pooled and frozen.

Butyl sepharose 4B chromatography
To the unfrozen enzyme solution, a 4 M ammonium sulfate solution in buffer A was added until a conductivity of 110-130 milliSiemens was reached, which corresponded to that of the equilibration buffer of the butyl sepharose 4B (Pharmacia) column (buffer B: 2 M ammonium sulfate in buffer A; pH adjusted at 7.5). The preparation was then centrifuged at 9500xg for 10 min and the supernatant (70 ml) loaded on the column (1.6 x 10 cm). After washing with 40 ml buffer B, the proteins were eluted with a 140 ml linear gradient (buffer B / buffer A) at 1 ml/min. The active fractions (25 ml) were concentrated 10-fold in an Amicon cell (PM 10 membrane). After addition of glycerol (20 %, final concentration), the enzyme preparation was frozen.

Desalting and Mono Q column
The enzyme solution was desalted using a Fast Desalting HR 10/10 column (Pharmacia) equilibrated in buffer A. The active fractions (11 ml) were immediately loaded on a Mono Q HR 5/5 (Pharmacia) column equilibrated in buffer A. After a 5 ml wash, the column was eluted with a 20 ml linear gradient of 10 to 100 mM potassium phosphate buffer (7.5) containing 5 mM 2-mercaptoethanol and 40 µM dTMP. The active fractions (3.6 ml) were supplemented with glycerol (20 %, final concentration) and frozen.

Desalting and concentration
The enzyme solution was desalted as described above, concentrated to 1.8 ml (PM 10 membrane) and stored at -20°C in the presence of glycerol (20 %, final concentration).

The purification procedure is summarized in Table 4. A 149-fold purification was achieved. The specific activity of the purified enzyme preparation was 6100 nmoles dTMP phosphorylated per hour per mg protein under the standard assay conditions. The preparation was not contaminated by NDPK.

Thymidylate kinase assay.
The standard assay contained 50 mM Tris-HCl (pH 7.5), 0.1% BSA, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 2 mM ATP, 3 mM phosphocreatine, 0.54 unit of creatine phosphokinase, 25 mM NaF, 0.2 unit of NDPK, 0.4 µCi [^{3}H] dTMP, 80 µM dTMP and the enzyme in a final volume of 100 µl. The ATP regeneration system was used only in assays performed during enzyme purification. The reaction was started by adding 20 µl of enzyme after a 4-min preincubation at 37°C and was stopped after 10 min by spotting 10 µl of the reaction mixture at the bottom of a polyethyleneimine (PEI) cellulose plastic sheet for t.l.c. (Merck) on which dTMP, dTDP, dTTP (10 µg each) had been previously spotted. After ascending chromatography with a mixture of 1 M LiCl / 4 M acetic acid (1/1, v/v), the carriers were localized with a U.V. lamp. The Rf of dTMP, dTDP and dTTP were 0.72, 0.36 and 0.08. For studies with inhibitors, NDPK was included in the assay. Under various experimental conditions, it was checked that all dTDP formed from dTMP was immediately converted into dTTP. In this case, the band containing dTTP was cut out and extracted in a scintillation vial for 10 min with a 2 ml mixture of 16 M formic acid / 2 M ammonium formate (1/1, v/v) to which 10 ml aquasol-2 (Dupont) were added before counting. For determination of the kinetic parameters of

TABLE 4. Purification of thymidylate kinase

Fraction	Total activity (units)	Specific activity (units / mg prot.)	Yield (%)	Purification factor
32000xg supernatant	44700	41	100	1
Amm. sulfate precip. + dialysis	50708	79	113	1.9
DEAE seph. F	40640	706	91	17
Butyl seph.4B	31800	1421	71	35
Concentration	22694	1528	51	37
Desalt. + Mono Q	15233	5960	34	145
Desalt. + concent.	9280	6105	21	149

1 unit = 1nmol. hour $^{-1}$

dTMP analogues (substrates), NDPK was omitted and the radioactivity in the band corresponding to the nucleoside diphosphate was measured. In these studies, the assay contained 0.4 μCi of labelled and 0.5 to 100 μM unlabelled substrate. The Rf values of ddTDP, AZT-DP, 5-Br-dUDP, 5-I-dUDP on t.l.c. were 0.32, 0.31, 0.21, 0.14 and those of the corresponding monophosphates 0.75, 0.69, 0.63, 0.59, respectively. All kinetics were of the Michaelis-Menten type. Stock solutions of nucleotides and nucleosides were prepared in 5 mM Tris-HCl buffer (pH 7.5) except for D4T (in DMSO). At concentations of D4T tested, DMSO (1.5%) had no inhibitory effect on dTMP kinase activity.

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