Inhibition of Thymidine Kinase from Walker 256 Carcinoma by Thymidine Analogs†

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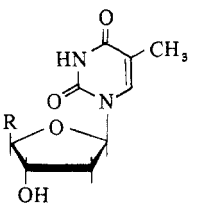
In a previous publication,[1] thymidine 5′-esters and 5′-carbamates were investigated as potential inhibitors of thymidine kinase from Walker 256 carcinoma. These compounds might be hydrolyzed *in vivo* by esterases before reaching their target enzyme.[2] Therefore, we examined the inhibitory effect of a number of thymidine derivatives which were substituted at the 5′ position with groups that would not be susceptible to esterases. The results of these studies are presented in Table I.

An appreciable loss in binding is observed when the 5′-hydroxyl group of thymidine (**1**) is replaced by a hydrophobic halogen (compounds **3–5**). As is evident from the poor binding of 5′-deoxythymidine (**2**), steric interference is not a likely cause for this loss in binding. Rather, it appears that in the enzyme-ligand complex, hydrogen bond formation between the enzyme and the 5′-hydroxyl of thymidine (**1**) increases the stability of the complex. This might provide an explanation for the notable inhibition of thymidine kinase by 5′-amino-5′-deoxythymidine (**6**); we note that **6** is one of the most potent inhibitors of thymidine kinase yet reported. Bromoacetylation of the 5′-amino group to give the irreversible inhibitor **9** is accompanied by a considerable decrease in binding which also occurs upon introduction of the polar azido and thiocyanate group (**7**, **8**) into the 5′ position. A similar decrease in binding is observed upon sp^2 hybridization of the 5′-carbon atom of thymidine (**1**) as observed with thymidine 5′-carboxylate (**10**) and its methyl ester **11**.

Kinetic data show that 5′-amino-5′-deoxythymidine (**6**) is a reversible, competitive inhibitor of thymidine kinase from Walker 256 carcinoma with K_i = 2.4 μM (Figure 1). Inhibition of thymidylate kinase from the same tissue by **6** occurs only at concentrations above 1 mM (I_{50} = 1.50 mM when assayed with 25 μM [^{14}C]thymidine 5′-monophosphate). In contrast, the thymidine analog 5′-deoxy-5′-fluorothymidine was reported to inhibit thymidylate kinase from Ehrlich ascites carcinoma, while there was only slight interference with the phosphorylation of thymidine.[3] Although 5′-amino-5′-deoxythymidine might be metabolized by thymidine phosphorylase from *Escherichia coli* B,[4] the compound is not susceptible to phosphorylytic cleavage by 5-fluoro-2′-deoxyuridine phosphorylase from Walker 256 carcinoma. From these results it appears that **6** is a potent inhibitor for selectively blocking the salvage pathway that leads to the formation of thymidylate *in vivo*.

Table I. Inhibition of Thymidine Kinase by



No.	R	I_{50},[a] μM
1[b]	CH_2OH	12
2[b]	CH_3	160
3	CH_2Cl	215
4[c]	CH_2Br	120
5[d]	CH_2I	240
6[e]	CH_2NH_2	21
7[e]	CH_2N_3	>500
8[f]	CH_2SCN	>500
9	$CH_2NHCOCH_2Br$	225
10[g]	CO_2H	250
11	CO_2CH_3	>500

[a] I_{50} = concentration of inhibitor giving 50% inhibition when assayed with 23 μM [^{14}C]thymidine; for experimental conditions see ref 1. [b] See ref 1. [c] For synthesis see A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 816 (1955). [d] For synthesis see J. P. H. Verheyden and J. G. Moffatt, *J. Org. Chem.*, **35**, 2319 (1970). [e] For synthesis see J. P. Horwitz, A. J. Tomson, J. A. Urbanski, and J. Chua, *ibid.*, **27**, 3045 (1962). [f] For synthesis see P. Langen and G. Kowollik, *J. Prakt. Chem.*, **37**, 311 (1968). [g] For synthesis see G. P. Moss C. B. Reese, K. Schofield, R. Shapiro, and A. R. Todd, *J. Chem. Soc.*, 1149 (1963).

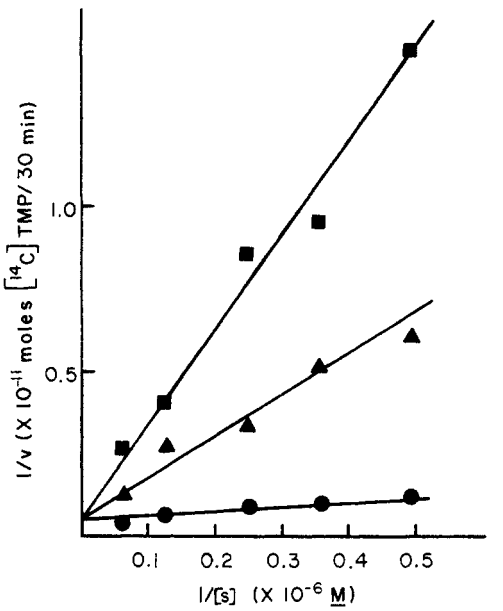


Figure 1. Effect of 5′-amino-5′-deoxythymidine on the phosphorylation of [^{14}C]thymidine: ■, 30 μM inhibitor; ▲, 10 μM inhibitor; ●, no inhibitor; K_m = 3.2 μM. The reaction mixture contained 50 mM Tris·HCl (pH 8.0), 5 mM ATP, 5 mM $MgCl_2$, 50 μg of protein (0–45% ammonium sulfate fraction; see ref 1), 2–16 μM [^{14}C]thymidine (62 mCi/mmol), and 10 vol % of DMSO in a final volume of 0.1 ml. Data are plotted according to Lineweaver and Burk.

Experimental Section

Melting points were determined on a Mel-Temp block and are uncorrected. Spectroscopic data of the compounds were compatible with the assigned structures.

5′-Chloro-5′-deoxythymidine (3). A solution of 400 mg (1 mmol) of 5′-tosylthymidine[5] and 175 mg (3 mmol) of NaCl in 10 ml of DMF was heated for 2 hr at 120° with stirring. The solvent was removed *in vacuo*, the residue dissolved in acetone and filtered, and the product precipitated by adding anhydrous ether. Recrystallization from ethanol gave 65 mg (25%) of **3**, mp 195–197° (lit.[6] mp 156–159°, recrystallized from water). *Anal.* ($C_{10}H_{13}ClN_2O_4$) C, H, N.

5′-Bromoacetamido-5′-deoxythymidine (9). To a solution of 241 mg (1 mmol) of **6** in 1 ml of DMF was added 8 ml of a dioxane solution containing 130 μmol/ml of the bromoacetic acid ester of *N*-hydroxysuccinimide,[7] and the mixture was kept at room temperature for 12 hr. The product was precipitated by the addition of ether and recrystallized from dioxane–ether to yield 135 mg (37%) of colorless needles, mp 197–198°. *Anal.* ($C_{12}H_{16}BrN_3O_5$) C, H, N.

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Thymidine-5'-carboxylic Acid Methyl Ester (11). A solution of 1.85 g (7.2 mmol) of **10** in 10 ml of DMF was titrated with ethereal diazomethane (prepared from 3 g of *N*-methyl-*N*-nitrosourea in 40 ml of ether) until the yellow color persisted (after *ca.* 2 min). Excess CH_2N_2 was destroyed with 0.5 ml of AcOH, and the precipitate was washed with Et_2O to give pure methyl ester: 1.78 g (91%); mp 247° dec. *Anal.* ($C_{11}H_{14}N_2O_6$) C, H, N.

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Catechol *O*-Methyltransferase. 4. *In Vitro* Inhibition by 3-Hydroxy-4-pyrones, 3-Hydroxy-2-pyridones, and 3-Hydroxy-4-pyridones

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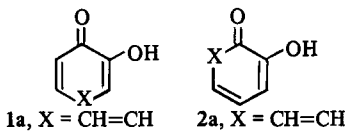
Tropolones[1,2] have been shown to be reversible dead-end inhibitors of the enzyme catechol *O*-methyltransferase (COMT) (E.C. 2.1.1.6).† Tropolone appears to bind to a similar site as the catechol substrate, but it itself is not O-methylated. If one looks at the tropolone molecule as shown in structure **1a** or **2a**, it becomes readily apparent that any functional group or atom (X) capable of maintaining these keto tautomeric structures should show similar inhibitory properties toward COMT. Possible examples include 3-hydroxy-4-pyridone (**1b**), 3-hydroxy-4-pyrone (**1c**), 3-hydroxy-2-pyridone (**2b**), and 3-hydroxy-2-pyrone (**2c**). All have been shown to exist predominatly in these tautomeric forms[3-5] and thus would be isosteric to tropolone and



1a, X = CH=CH **2a**, X = CH=CH
b, X = NH **b**, X = NH
c, X = O **c**, X = O

catechol. This communication reports the preliminary results of a study on the *in vitro* inhibition of COMT by these heterocyclic systems.

Results and Discussion

Table I shows the degree of COMT inhibition produced by derivatives of 4-pyrone and 4-pyridone, as well as by 3-hydroxy-2-pyridone (**2b**) and tropolone (**1a**). The inhibitory activity of 3-hydroxy-4-pyridone (**1b**), 3-hydroxy-4-pyrone (**1c**), *N*-methyl-3-hydroxy-4-pyridone (**4**), meconic acid (**7**), kojic acid (**8**), and 3-hydroxy-2-pyridone (**2b**) clearly illustrates that these heterocyclic systems are biochemically isosteric to tropolone (**1a**) with respect to their ability to inhibit COMT. Complete absence of inhibitory activity for the 3-methoxylated compounds **3**, **5**, and **6** illustrates the importance of the free hydroxyl group for inhibition. The lower inhibitory activity of the 3-hydroxy-4-pyrones **1c**, **7**, and **8** probably reflects the fact that these systems can also exist in a diketo tautomeric structure,[5] which would be expected to be inactive as a COMT inhibitor.

Since these heterocyclic systems are isosteric to catechol, they could conceivably serve as substrates for COMT. However, incubation of **1b**, **1c**, or **2b** in the presence of the enzyme under conditions which were optimal for the O-methylation of *l*-NE resulted in no detectable methylation products being formed.

Using reciprocal velocity *vs.* reciprocal substrate plots, the kinetics patterns for 3-hydroxy-4-pyridone (**1b**), 3-hydroxy-2-pyridone (**2b**), and kojic acid (**8**) inhibition of the COMT-catalyzed O-methylation of DHB were determined. As shown in Table II linear competitive patterns of inhibition were observed when DHB was the variable substrate and either **1b**, **2b**, or **8** was the inhibitor. When SAM was the variable substrate, uncompetitive patterns of inhibition were observed as summarized in Table II. Except for the competitive rather than noncompetitive pattern observed with varying DHB, the inhibitory kinetics of these heterocyclic systems (**1b**, **2b**, and **8**) are quite similar to those observed for tropolone (**1a**).[2]

In an attempt to determine if tropolone and these heterocyclic systems are truly isosteric with respect to COMT, a study of the kinetics of multiple inhibition of COMT by 3-hydroxy-4-pyridone (**1b**) and tropolone (**1a**) was conducted using the procedures of Yonetani and Theorell.[6] These studies were carried out at pH 7.24 where both tropolone (**1a**)[2] and 3-hydroxy-4-pyridone (**1b**) show pure competitive kinetics with respect to DHB. As shown in Figure 1, a series of parallel straight lines were obtained when reciprocal velocities were plotted *vs.* tropolone (**1a**) concentrations at varying concentrations of 3-hydroxy-4-pyridone (**1b**). The slope of the lines remained constant; however, the intercepts were a linear function of the concentration of **1b**. These data, as well as the inhibitory kinetic data, provide strong evidence that tropolone and

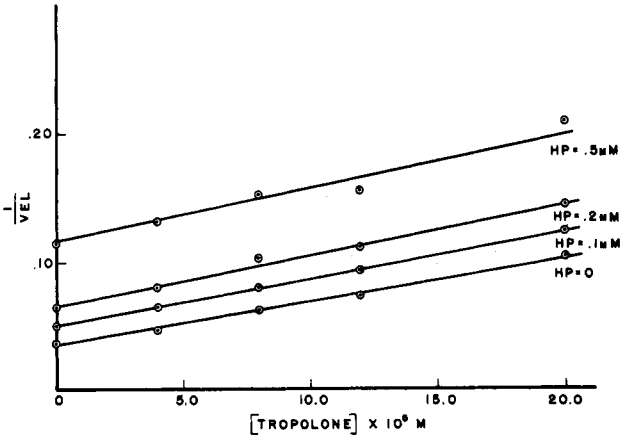


Figure 1. Reciprocal velocity *vs.* tropolone concentration with varying 3-hydroxy-4-pyridone (HP). Assay conditions are the same as in Table II except pH 7.24. Vel = nmol of product/mg of N Kjeldahl/min.

†Abbreviations used are: SAM, *S*-adenosyl-L-methionine; DHB, 3,4-dihydroxybenzoic acid; *l*-NE, *l*-norepinephrine; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.