was 41 mg (36%). Anal. ($C_{22}H_{28}N_{10}O_{22}P_5SNa_5 \cdot 3H_2O$) C, H, N, P, S.

Enzyme Kinetic Studies. Adenosine 5'-monophosphate, adenosine 5'-triphosphate, lactate dehydrogenase (type II, rabbit muscle), and phosphoenolpyruvate were from Sigma Chemical Co. The pyruvate kinase was purchased from Boehringer Mannheim, and the NADH was from PL Biochemicals. The AK II and AK III isozymes of adenylate kinase from rat liver and the adenylate kinase isozyme from rat muscle were obtained as described previously.[1]

The enzyme-catalyzed reactions were followed at 23 °C by measuring the rate of change of optical density at 340 nm for a period of 5 min in a Cary Model 15 spectrophotometer using 1-cm cells containing a final volume of 1 mL. Initial velocities were linear and proportional to the concentration of primary enzyme and independent of the level of secondary enzymes in the assay system. Each kinetic study of substrate activity of ATP derivatives employed five or more concentrations of substrate; the AMP level was 2 mM with rat muscle AK and 0.35 mM with AK III. AMP and ATP derivatives were tested initially for substrate activity of a level of 0.8–1.0 mM. Kinetic constants were determined from Lineweaver–Burk double-reciprocal plots of velocity vs. substrate level, all of which were linear. The systems for kinetic studies contained, in addition to the nucleotides, 0.1 M Tris-HCl (pH 7.6) containing $MgSO_4$ (2 mM), KCl (0.12 M), PEP cyclohexylammonium salt (0.3 mM), NADH (0.38 mM), pyruvate kinase (8.6 units), and lactate dehydrogenase (8.6 units). Stock solutions of ATP and ATP derivatives contained an equimolar amount of $MgSO_4$.

Inhibition studies used five concentrations of the variable substrate for each of two levels of inhibitor. Inhibitor levels were 1–6 times higher than the inhibition constant. With rat muscle AK the constant substrate concentration in inhibition studies was 2 mM and the varied substrate was 0.5–2.0 mM; higher levels of the fixed substrate were inhibitory. With AK II and AK III the constant substrate was 0.35 mM and the varied substrate was 0.1–0.4 mM. Inhibition constants (K_i values) were obtained from replots of inhibitor concentrations vs. slopes of the Lineweaver–Burk plots.

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Species- or Isozyme-Specific Enzyme Inhibitors. 5.[1] Differential Effects of Thymidine Substituents on Affinity for Rat Thymidine Kinase Isozymes

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Derivatives obtained by single replacements or substitutions of groups at eight positions of thymidine (TdR) have been examined as inhibitors of rat mitochondrial (M-TK) and cytoplasmic (C-TK) isozymes of thymidine kinase. A C-TK (pI = 7.5) and an M-TK (pI = 5.1) from rat spleen were purified to apparent isozymic homogeneity by isoelectric focusing. Affinities relative to that of TdR for the TdR sites of the isozymes were derived by dividing the Michaelis constants of TdR by the inhibition constants. Of the eight types of TdR derivatives, five had higher affinity for the M-TK site and two had higher affinity for the C-TK site. The most potent and/or selective inhibitors were 3'-*O*-benzyl-TdR (affinity for M-TK relative to TdR, 100%; differential affinity for M-TK vs. C-TK, 7.5), 5-amino-2'-deoxyuridine (relative affinity for M-TK, 11%; differential affinity for M-TK, 26), 5'-amino-5'-deoxy-TdR (relative affinity for C-TK, 67%; differential affinity for C-TK, 400), and 3-benzyl-TdR (relative affinity for C-TK, 5%; differential affinity for C-TK, >25). Effects of modifying certain of the substituents indicate that at least some of these TdR derivatives are potential progenitors of TK inhibitors of higher potency and selectivity.

A recent study of monosubstituted thymidine (TdR) derivatives as enzyme inhibitors showed that the introduction of substituents at any one of six positions of TdR produced species-selective effects on affinity for the TdR sites of *Escherichia coli* and hamster cytoplasmic thymidine kinases.[2] Later it was found that the introduction of certain substituents at two positions of ATP that were examined gave rise to both species- and isozyme-selective effects on affinity for the ATP sites of *E. coli* and rat isozymes of adenylate kinase.[3] These isozyme-selective effects were of interest to us because of the possibility, discussed previously,[4] that fetal isozyme-selective inhibitors might be useful starting points in the design of antineoplastic agents. In the present work, the tendency of single substituents attached to a substrate to influence affinity for the substrate site in an isozyme-selective manner has been further explored, using cytoplasmic and mitochondrial isozymes of rat thymidine kinase (TK). Eight types (1–8) of derivatives obtained by single replacements or substitutions of groups at various atoms of TdR have been analyzed kinetically as inhibitors in order to evaluate their affinity for the TdR sites of the mitochondrial (M-TK) and cytoplasmic (C-TK) isozymes.

TK catalyzes phosphate transfer from ATP to TdR to form thymidine 5'-phosphate (TMP), which is also biosynthesized de novo from deoxyuridine 5'-phosphate. TK activity is low in nongrowing adult tissues but relatively high in rapidly proliferating or neoplastic cells in which it is believed to play a significant role in the biosynthesis of TMP.[5,6] Evidence indicates a direct correlation between TK content in rat tumor tissue and tumor growth rate.[7] M-TK and C-TK are two major forms of TK identified in mammalian tissue. M-TK is the predominant form in adult human liver,[8-11] spleen,[8,9] lung,[10] colon,[10] and fi-

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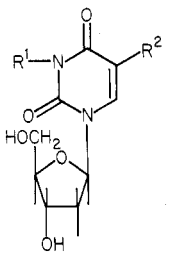
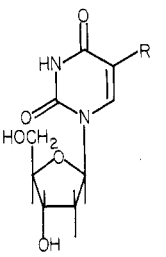
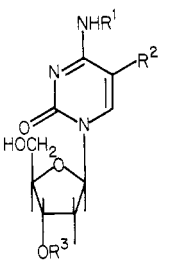


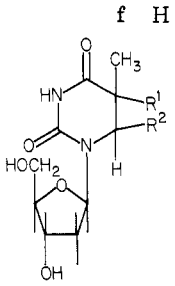


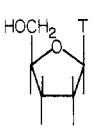
	R^1	R^2
1a	H	H
b	CH_3	H
c	CH_3	CH_3
d	n-C_5H_{11}	CH_3
e	CH_2Ph	CH_3

	R
2a	NH_2
b	$NHCOCH_3$
c	CH_2NH_2
d	SCH_3
e	H, CH_3, C=C, H



	R^1	R^2	R^3
3a	H	H	H
b	C_2H_5	H	H
c	H	CH_3	H
d	H	I	H
e	H	H	CH_3
f	H	H	COC_2H_5





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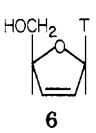
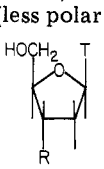
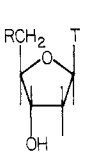


6

4a $R^1 = R^2 = H$
b $R^1 = Br$; $R^2 = OC_2H_5$ (more polar diastereomer)
c $R^1 = Br$; $R^2 = OC_2H_5$ (less polar diastereomer)





	R
7a	NH_2
b	$NHCOCH_3$
c	SC_2H_5
d	OC_2H_5
e	OCH_2Ph

	R
8a	NH_2
b	$NHCOCH_3$
c	$NHCH_2CH_3$
d	$NH(CH_2)_3CH_3$
e	$NH(CH_2)_5CH_3$

	R
8f	SCH_2CH_3
g	$S(O)CH_2CH_3$
h	SCH_2CH_2OH
i	$S(CH_2)_2CO_2H$
j	$S(CH_2)_3CO_2H$

T = thymine

broblasts[8,9] and constitutes ~40% of TK activity in the small intestine.[10] C-TK predominates in KB and HeLa human tumor cell lines,[9] in human fetal liver,[8,9] in Wilm's tumor,[8] rhabdomyosarcoma,[8] bladder adenocarcinoma,[8] and cervical carcinoma.[12] This suggests that effective antineoplastic chemotherapy might result from coadministration of a drug that blocks de novo TMP biosynthesis with a drug that selectively inhibits C-TK. Selective inhibitors of M-TK are also of chemotherapeutic interest because of their potential to increase the therapeutic index of known clinically useful TdR analogues by protecting nongrowing host tissues from the toxic effects of TMP analogues otherwise produced by the M-TK in those tissues.

Table I. Levels of Mitochondrial Thymidine Kinase (M-TK) and Cytoplasmic TK (C-TK) Activity in Rat Tissues

tissue	TK act. (cpm of [^{14}C]TMP/g of tissue)[a] M-TK	C-TK	M-TK + C-TK
adult liver	4750	11 900	16 650
adult spleen	8960	396 000	404 960
newborn liver	7200		
adult kidney			32 400
adult lung			30 100
adult skeletal muscle			0

[a] Enzyme preparations were obtained as described under Experimental Section and were assayed for 10 min in the described assay system containing 38 μM [^{14}C]-thymidine (27 mCi/mmol).

Syntheses. Most of the compounds studied as TK inhibitors were prepared by methods described in our earlier studies.[2,13] 3′-*O*-Benzylthymidine (**7e**) was prepared in 66% yield by the method of Griffin and Todd,[14] which involved treatment of 5′-*O*-tritylthymidine in benzene–dioxane with 6 equiv of benzyl chloride and 26 equiv of potassium hydroxide, followed by removal of the trityl group. Substitution of ethyl iodide for benzyl chloride under these conditions led to conversion of 30% of the starting material to the desired 3′-*O*-ethyl-5′-*O*-tritylthymidine and conversion of the remaining 70% to a less polar derivative, which from its UV spectrum was concluded to be 3-ethyl-3′-*O*-ethyl-5′-*O*-tritylthymidine. However, 5′-*O*-tritylthymidine, upon treatment with 2 equiv of ethyl iodide in benzene–dioxane in the presence of 2 equiv of potassium hydroxide, was converted to a 3:1 mixture of its 3′-*O*-ethyl and 3-ethyl-3′-*O*-ethyl derivatives. The trityl groups in the mixture were removed with aqueous 80% acetic acid, after which 3′-*O*-ethylthymidine (**7d**) was isolated by silica gel chromatography and obtained in crystalline form in 62% overall yield.

In the synthesis of 3′-*O*-methyl-2′-deoxycytidine (**3e**), 5′-*O*-trityl-2′-deoxyuridine was treated at 45 °C for 6 h with 1.1–1.2 equiv each of methyl iodide and potassium hydroxide in benzene–dioxane. The major product, 3′-*O*-methyl-5′-*O*-trityl-2′-deoxyuridine, was isolated in 56% yield and thiated in P_2S_5-pyridine.[15] The resulting 3′-*O*-methyl-5′-*O*-trityl-2′-deoxy-4-thiouridine was treated without prior purification with ethanolic ammonia at 100 °C to furnish 3′-*O*-methyl-5′-*O*-trityl-2′-deoxycytidine. This was isolated by chromatography on silica gel and treated with aqueous 80% acetic acid to remove the trityl group to furnish **3e** in 19% overall yield.

5′-*O*-Trityl-2′-deoxycytidine could be converted quan-

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titatively to its N^4-(dimethylamino)methylene derivative[16] by the action of DMF dimethyl acetal at room temperature. This was readily converted to N^4-[(dimethylamino)methylene]-3′-*O*-propionyl-5′-*O*-trityl-2′-deoxycytidine with propionic anhydride in pyridine solution. Removal of the blocking groups at N^4 and O-5′ with aqueous 80% acetic acid furnished homogeneous 3′-*O*-propionyl-2′-deoxycytidine (**3f**) in 62% yield. A solution of **3f** in Tris buffer, pH 8, showed only 5% conversion to 2′-deoxycytidine as the sole decomposition product after 2 days at 25 °C, indicating that **3f** is essentially stable under the conditions (1 h, pH 8, 37 °C) used in the present studies of its interaction with thymidine kinases.

Isolation and Characterization of Rat Cytoplasmic and Mitochondrial Thymidine Kinases (C-TK and M-TK). Table I shows that of several major rat tissues examined the best source of the combined C-TK and M-TK was adult spleen. The level of M-TK activity was only 2.3% that of C-TK but was adequate, and spleen was therefore employed as the source of both isozymes. The level of M-TK in the livers of 3-day-old rats was determined in view of a report[7] that the level of TK (apparently C-TK) in this tissue can be 25 times higher than in adult liver. However, we found the level of M-TK in the newborn livers to be less than in adult rat spleen. Characteristics have been reported of rat C-TK from regenerating liver[17] and tumor tissue,[5] but little information is available on the properties of C-TK from rat spleen. In addition, no studies appear to have been made hitherto with rat M-TK, although a human M-TK has been purified to homogeneity,[18,19] and an M-TK from mouse cells in culture[20,21] and two types of M-TK from human cells in culture[22] have been identified.

The cytoplasmic fraction of rat spleen tissue showed a single zone of TK activity [R_M (relative mobility) 0.15] when analyzed by means by disc polyacrylamide gel electrophoresis. Similar R_M values have been reported for rat hepatoma TK,[23] for TK from regenerating rat liver,[17] and for human cytoplasmic TK.[19,22] The spleen C-TK was partially purified by Sephadex slab isoelectric focusing, which produced a single zone of TK activity. The resultant TK preparation was isozymically homogeneous as judged also by polyacrylamide gel electrophoresis. Table II shows that the rat spleen C-TK resembles human C-TK from neoplastic tissue in regard to several characteristic phosphate donor and inhibitor specificities, which have proved useful in distinguishing C-TK from M-TK from several biological sources. Thus, dATP is a more effective donor for C-TK,[18] and CTP is more effective for M-TK,[9,18,21] while 5-iodo-2′-deoxycytidine inhibits M-TK more strongly than C-TK.[18]

A crude M-TK preparation from rat spleen mitochondria showed with polyacrylamide gel electrophoresis a well-defined component with TK activity of R_M 0.24, together with a similar amount of unresolved TK activity in the R_M range 0.38–0.58. The enzyme could be purified by Sephadex slab isoelectric focusing with moderately good recoveries of activity, provided that MgATP was present. The purified preparation showed a single sharp band of TK activity of R_M 0.24 with the gel electrophoresis. That the electrophoretic mobility of rat M-TK is greater than that of rat C-TK accords with similar findings with human[19,22] and mouse[20,21] C-TK and M-TK. Table II shows that the substrate and inhibitor properties of the purified rat M-TK resemble those of human M-TK from leukemia blast cells. The isoelectric point (pI = 5.1) of the rat M-TK was the same as that of M-TK from human liver.[11] Uridine or cytidine inhibited conversion of TdR to TMP much less effectively than did 2′-deoxyuridine (**1a**) or 2′-deoxycytidine (**3a**) (Table III), confirming that the C-TK and M-TK activities were specific for 2′-deoxynucleosides.

Affinity of Thymidine (TdR) Derivatives for C-TK and M-TK. Table III shows the inhibitory effect of 2 mM levels of some of the present series of TdR derivatives on the conversion of TdR to TMP catalyzed by C-TK or M-TK. The level of TdR in each test was similar in value

Table II. Substrate and Inhibitor Specificities of Rat Thymidine Kinase (TK) Isozymes

enzyme	potency of phosphate donors, rel % ATP	dATP	CTP	inhibn by 5-I-CdR,[a] %
C-TK (rat)	100[b]	82[b]	17[b]	0[c]
M-TK (rat)	100[b]	45[b]	71[b]	66[c]
C-TK (human)[d]	100[e]	108[e]	21[e]	0[c]
M-TK (human)[d]	100[e]	56[e]	79[e]	64[c]

[a] The assay systems contained 0.19 mM [^{14}C]thymidine. [b] The nucleoside triphosphate (5 mM) was employed in the assay system given under Experimental Section, which thereby initially contained 2.5 mM of the corresponding Mg^{2+} triphosphates; the [^{14}C]thymidine level was 14.4 μM. [c] 5-Iodo-2′-deoxycytidine was 0.19 mM in the assay system and Mg^{2+}-ATP was at a saturating level. [d] The values for these enzymes were reported in ref 18. [e] The assays were performed with 2 mM Mg^{2+} triphosphates and saturating thymidine levels.

Table III. Inhibition of Rat Thymidine Kinase (TK) Isozymes[a]

inhibitor	% inhibn M-TK	C-TK
uridine	4	4
1a	74	90
1b	4	62
1e	10[b]	
2b	11	0
2c	3	30
cytidine	13	1
3a	58	7
3b	5	7
3e	24	3
3f	19	8
4a	83	53
8b	19	20
8d	53	95
8f	22	11
8g	7	4
8h	22	49
8i	25	40
8j	36	48

[a] Inhibitor levels were 2 mM unless noted otherwise. The thymidine level was 7.2 μM for mitochondrial TK and 2.25 μM for the cytoplasmic TK. The remaining assay conditions are given under Experimental Section. [b] Solubility of the inhibitor limited its test level to 1 mM.

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Table IV. Inhibition Constants of Pyrimidine Nucleoside Derivatives with Rat Mitochondrial Thymidine Kinase (M-TK) and Rat Cytoplasmic TK (C-TK)

	M-TK		C-TK		affinity rel to TdR $[K_M (TdR)/K_i]$		differential affinity
no.	type of inhibn[a]	K_i, mM	type of inhibn	K_i, mM	M-TK	C-TK	(M-TK/C-TK)
1c	NC	28.0	C	0.25	<0.00014	0.00064	<0.022
1d	C	1.10	C	0.036	0.0073	0.044	0.17
1e		*b*	C-NC	0.032	>0.002[c]	0.05	>0.04
2a	C	0.072	C-NC	0.37	0.11	0.0043	26
2d	C	0.25	C	0.050	0.032	0.032	1.0
2e	NC	0.51	NC	15.5			
3a	C-NC	0.32			0.025		
3c	C-NC	1.30	C	2.1	0.006	0.00076	7.9
3d	NC	3.5	NC	11.4			
4a	C	0.077			0.10		
4b	C	0.011	C	0.0020	0.73	0.8	0.91
4c	C	0.011	C	0.0039	0.73	0.41	1.8
5	C	0.88	C-NC	0.40	0.0091	0.0040	2.3
6	C	3.25	C-NC	1.7	0.0025	0.00094	2.7
7a	C	2.6	C	0.38	0.0031	0.0042	0.74
7b	NC	1.8	C-NC	3.5	<0.0022	0.00046	<4.8
7c	C	0.062	C	0.024	0.13	0.067	1.9
7d	C	0.056	C	0.036	0.14	0.044	3.2
7e	C	0.008	C	0.012	1.0	0.133	7.5
8a	C	4.6	C	0.0024	0.0017	0.67	0.0025
8c	C	1.44	C	0.040	0.0056	0.040	0.14
8e	C	0.96	C	0.035	0.0083	0.046	0.18
8h			C	0.82		0.002	
8j	NC	8.5	NC	3.6			

[a] C = competitive, N = noncompetitive, and C-NC = mixed inhibition with respect to thymidine (TdR). [b] The K_i was not determined owing to poor solubility of 1e. [c] Calculated assuming that K_i = 4 mM. The data in Table III indicate that K_i is ~4 mM for competitive inhibition and ~10 mM for noncompetitive inhibition.

to its Michaelis constant (K_M) with the particular isozyme under study. Inhibition constants (Table IV) were determined for TdR derivatives which exhibited relatively marked inhibition or inhibition which might be isozyme selective. Because the K_M values of TdR with C-TK and M-TK are 5-fold different, K_M (TdR)/K_i was used to express the affinity of each compound relative to that of TdR; the values obtained are listed in Table IV. The differential affinity (M-TK/C-TK) was derived by dividing the relative affinity of each compound for M-TK by its relative affinity for C-TK.

Introduction of a methyl at N-3 of TdR, giving **1c**, diminished affinity for the TdR site to 0.64% that of TdR in the case of C-TK and less than 0.014% with M-TK. Replacement of the 3-methyl by a 3-amyl group (**1d**) increased affinity for M-TK 50-fold and affinity for C-TK 7-fold, and introduction of a 3-benzyl group (**1e**) increased affinity for M-TK by no more than 15-fold and affinity for C-TK by 8-fold. Compounds **1c-e** appear to bind preferentially to the TdR site of C-TK by factors that are, respectively, >44, 6, and >25.

5-Amino-2′-deoxyuridine (**2a**) bound to M-TK one-ninth as avidly as TdR and showed 26 times more affinity for M-TK than for C-TK. Acetylation of the amino group (**2b**) drastically reduced affinity for both isozymes, as did interposition of a methylene between the amino group and the uracil ring (**2c**) (Table III). 5-(Methylthio)-2′-deoxyuridine (**2d**) showed relatively weak and nondifferential affinity for the isozymes. *trans*-5-(1-Propenyl)-2′-deoxyuridine (**2e**) was a weak noncompetitive inhibitor of M-TK and C-TK.

2′-Deoxycytidine (**3a**) showed evidence (Tables III and IV) of tighter binding to M-TK than to C-TK; the extent of this effect was not determined. Attachment of an ethyl to N^4 (**3b**) or of a methyl or propionyl group to O-3′ (**3e,f**) reduced affinity for either isozyme to a relatively low level (Table III). 5-Methyl-2′-deoxycytidine (**3c**) bound weakly to both isozymes and exhibited an 8-fold preference for M-TK. 5-Iodo-2′-deoxycytidine (**3d**) was a weak noncompetitive inhibitor of the two TK isozymes. It is of interest to note that human M-TK activity and mitochondrial 2′-deoxycytidine kinase activity appear to be mediated by the same protein[24-26] and, hence, that this might be found to be true also for rat M-TK and rat mitochondrial CdR kinases.

The two diastereomers of *trans*-5-bromo-6-ethoxy-5,6-dihydrothymidine (**4b,c**) were relatively effective inhibitors of rat M-TK and C-TK but exhibited little, if any, selective affinity. A lesser inhibitory potency was exhibited by 5,6-dihydrothymidine (**4a**), showing that the good affinity of **4b,c** is not attributable solely to their 5,6-dihydro structure. Affinity of **4b,c** for hamster C-TK[2] was approximately 100-fold less than for rat C-TK, possibly due to species differences in the enzymes or to enzymatic attack on **4b,c** by the relatively crude hamster C-TK preparation. TLC analysis indicated that no detectable decomposition (<2.5%) of **4b,c** occurred during measurement of their K_i values with rat C-TK and M-TK.

3′-Deoxythymidine (**5**), 2′,3′-anhydrothymidine (**6**), 3′-deoxy-3′-aminothymidine (**7a**), and its *N*-acetyl derivative **7b** were weak inhibitors with little differential affinity. Attachment to C-3′ of 3′-deoxythymidine of ethylthio or ethoxy groups (**7c** and **7d**, respectively) produced moderate affinities and, in the case of the 3′-ethoxy compound, a significant (>2-fold) preferential affinity for M-TK. Replacement of the ethoxy group of **7d** by a benzyloxy group (**7e**) elevated the affinity for M-AK to that of TdR itself and produced an enhanced (7.5-fold) preferential affinity for M-TK.

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5′-Amino-5′-deoxythymidine (**8a**) exhibited an affinity for C-TK which was 67% that of TdR and 400 times greater than its affinity for M-TK. TK of Walker 256 rat carcinoma, which is presumably principally a C-TK, is also efficiently inhibited by **8a** (K_i = 2.4 μM).[27] It is of interest that **8a** has been shown to reduce the toxicity of 5-iodo-2′-deoxyuridine to mammalian cells in culture, possibly as a result of inhibition of TK.[28] Attachment of ethyl or butyl groups to the amino group of **8a** diminished affinity for C-TK 15-fold and reduced the preferential affinity for C-TK from 400-fold to 7-fold. The five 5′-deoxy-5′-(alkylthio) derivatives of TdR that were examined (**8f-j**) inhibited both isozymes more weakly than the 5′-deoxy-5′-(alkylamino)-TdR derivatives **8c–e** (Table III). The most inhibitory of the 5′-(alkylthio) derivatives, **8h** and **8j**, had weak affinity for the TdR site of C-TK (Table IV).

Overall Conclusions. Of the eight types of TdR derivatives or analogues (**1–8**) that were examined as inhibitors, seven showed a significant degree of differential affinity (>2 or <0.5) for the TdR sites of M-TK and C-TK. Compounds **1c–e** and **8a,c,e** preferentially inhibited C-TK, and compounds **2a**, **3c**, **5**, **6**, and **7d–e** preferentially inhibited M-TK. The most potent and selective inhibitors were **2a** (relative affinity, 11% of TdR; differential affinity, 26), **7e** (relative affinity, 100%; differential affinity, 7.5), and **8a** (relative affinity, 67%; differential affinity, 400). Additional modifications of the substituents at these positions, in particular the O-3′ substituent, might further enhance inhibitory potency and/or selectivity. Also of interest as potential progenitors of potent and selective inhibitors are the 3-substituted TdR derivatives, such as **1e** (relative affinity, 5%; differential affinity, >25).

Previous studies with TK from *E. coli* and C-TK from hamster showed that when substituents were attached to any of six different atoms of TdR, species-selective effects on affinity for the TdR sites were encountered in all cases.[2] Later studies showed that groups attached to either N^6 or C-8 of ATP produced both species- and isozyme-selective effects on affinity for the ATP sites of *E. coli* and rat adenylate kinases.[3] These and the present findings indicate that the attachment of simple substituents to various atoms in turn of a substrate is of interest as a potentially useful initial step in the attempted design of species- or isozyme-selective enzyme inhibitors, although it is not yet possible to gauge the general utility of the approach. In our studies to date, the isozyme-selective affinity produced by a substituent has usually been accompanied by a loss in affinity for the substrate site of the preferentially inhibited isozyme. One method of compensating for this effect would be to modify the substituent in the hope of obtaining increased affinity and possibly increased selectivity via interactions of the substituent with structurally dissimilar areas of the isozymes adjacent to the substrate site. For example, the loss in binding to M-TK resulting from introduction of an ethyl group at O-3′ of TdR (**7d**) is fully regained by substitution of a benzyl group (**7e**) for the ethyl group, and there is a concomitant 2.3-fold increase in differential affinity. Similarly, substitution of benzyl for methyl at N-3 of TdR (**1e**) provides an 8-fold increase in affinity with maintenance of selectivity for C-TK. An alternative approach to increasing the affinity of such isozyme-selective inhibitors was illustrated recently by studies which showed that the isozyme-selective inhibition of a monosubstituted substrate could be transferred to a multisubstrate inhibitor of the same isozyme, giving rise to a large enhancement in inhibitory potency.[1]

Experimental Section

Chemical Synthesis. General. Thin-layer chromatograms were obtained with Merck F-254 silica gel plates in the following solvent systems: (A) chloroform–methanol, 19:1; (B) chloroform–methanol, 4:1; or (C) chloroform–methanol, 9:1. Preparative layer chromatography was conducted with 2-mm layers of silica gel on glass (Merck). Spots on chromatograms were detected by their ultraviolet absorption and by spraying with the Molisch reagent. Melting points (uncorrected) were determined on a Fisher-Johns melting point apparatus. Ultraviolet spectra were determined with a Cary 15 spectrophotometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and unless otherwise indicated are within ±0.4% of the theoretical values.

3-Methyl-2′-deoxyuridine,[29] 3-methylthymidine,[30] and 3-benzylthymidine[31] were prepared by alkylation of the appropriate nucleoside by the procedure of Baker et al.[32] 5,6-Dihydrothymidine,[33] 3′-deoxythymidine,[34] and 2′,3′-anhydrothymidine[35] were obtained by described methods. 5-Methyl- and 5-iodo-2′-deoxycytidine were obtained from Sigma Chemical Co.

3′-*O*-Ethylthymidine (7d). Ethyl iodide (0.3 mL, 3.72 mmol) and powdered potassium hydroxide (208 mg, 3.72 mmol) were added to a solution of 5′-*O*-tritylthymidine (900 mg, 1.86 mmol) in benzene (9 mL) and dioxane (3 mL). The mixture was stirred in a bath at 70 °C for 5 h. TLC analysis in solvent A indicated complete conversion of starting material to a major product (R_f 0.46) and a minor product (R_f 0.70). The reaction mixture was evaporated to dryness. A solution of the residue in methanol (1 mL) was poured into water (25 mL), and the white suspension was extracted with chloroform (3 × 10 mL). The extracts were combined and evaporated, and the residue was dissolved in aqueous 80% acetic acid (10 mL) and heated on a steam bath for 1 h. The acetic acid was removed by azeotropic distillation with benzene under reduced pressure. The residue was chromatographed in solvent A over silica gel. The major band (R_f 0.32) was eluted with ethanol. The product was crystallized from methanol to yield 310 mg (62%) of white needles: mp 182–183 °C; UV λ_{max} 267 nm at pH 1 (ε 9600), 267 nm at pH 12 (ε 7400). Anal. ($C_{12}H_{18}N_2O_5$) C, H, N.

3′-*O*-Methyl-2′-deoxycytidine (3e). To a solution of 5′-*O*-trityl-2′-deoxyuridine (1.88 g, 4 mmol) in a mixture of benzene (12 mL) and dioxane (4 mL) was added powdered potassium hydroxide (0.5 g, 4.5 mmol) and methyl iodide (0.3 mL, 4.8 mmol). The mixture was stirred at 40–45 °C for 6 h. TLC in solvent system A showed ~90% conversion to a major UV-absorbing product (R_f 0.40) and a minor product (R_f 0.68). The residue obtained from evaporation of solvents was dissolved in methanol (10 mL) and poured into water (200 mL). The white suspension was extracted with ethyl acetate (3 × 50 mL). The extracts were combined, dried ($MgSO_4$), and evaporated. The residue was chromatographed in solvent system A over silica gel. The major band was eluted with ethanol, which on evaporation yielded 1.1 g (56%) of the intermediate 5′-*O*-trityl-3′-*O*-methyl-2′-deoxyuridine as an off-white powder of UV λ_{max} 262 nm at pH 2 and 12. At pH 12, the absorbancy was 30% less than at pH 2.

To a solution of 1.1 g (2.2 mmol) of the above intermediate in pyridine (25 mL) was added phosphorus pentasulfide (2.0 g, 12 mmol), and the mixture was refluxed in an oil bath for 16 h. The mixture was concentrated to approximately two-thirds volume and poured into ice-cold water (250 mL). The solution was extracted with ethyl acetate (3 × 50 mL). The extracts were combined, washed with water, dried ($MgSO_4$), and evaporated.

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A solution of the residue in ethanol (10 mL) was saturated with anhydrous ammonia at 0 °C, and the mixture was heated at 100 °C in a stainless-steel bomb for 16 h. After evaporation of ammonia, the solution was concentrated to ~1 mL and chromatographed in solvent system A over silica gel. The major band (R_f 0.35) was eluted with ethanol. The solvent was evaporated, and the residue was dissolved in aqueous 80% acetic acid (5 mL) and heated on a steam bath for 1 h. The acetic acid was removed by azeotropic distillation with benzene under reduced pressure. The residue was chromatographed in solvent system B over silica gel. The major band, R_f 0.30, was eluted with chloroform–methanol (8:2), and the solution was concentrated to 2 mL. Addition of an excess of anhydrous ether yielded **3e** (180 mg, 19% overall yield) as an off-white powder: mp 184–185 °C; UV λ_{max} 271 nm at pH 6 (ϵ 9300) and 280 nm at pH 1 (ϵ 13100). Anal. ($C_{10}H_{15}N_3O_4 \cdot 0.5CH_3OH$) C, H, N.

2′-Deoxy-3′-*O*-propionylcytidine (3f). To a solution of 900 mg (2 mmol) of 5′-*O*-trityl-2′-deoxycytidine in anhydrous *N*,*N*-dimethylformamide (5 mL) was added *N*,*N*-dimethylformamide dimethyl acetal (1 mL), and the solution was stored at room temperature for 16 h. TLC in solvent system B showed that conversion to the N^4-dimethylaminomethylene derivative was then complete. The residue obtained upon removal of volatiles in vacuo was dissolved in anhydrous pyridine (10 mL), and to this solution was added propionic anhydride (1 mL). TLC in solvent system C indicated that acylation was complete after 24 h at 22 °C. The residue obtained upon evaporation of volatiles under reduced pressure was dissolved in aqueous 80% acetic acid (5 mL), and the solution was heated on a steam bath for 1 h. The acetic acid was removed by azeotropic distillation with benzene under reduced pressure. The residue was dissolved in methanol and chromatographed on 2-mm thick silica gel plates. The plates were developed first in chloroform and then in solvent system C. The major band (R_f 0.15) was eluted with ethanol–$CHCl_3$ (1:1), then dissolved in methanol (2 mL), and precipitated by addition to a 1:1 mixture of ether and light petroleum. This yielded 350 mg (62%) of an off-white powder: mp 112–114 °C; UV λ_{max} (H_2O) 271 nm (ϵ 8200) at pH 11, 280 nm (ϵ 12500) at pH 2. Anal. ($C_{12}H_{17}O_5N_3$) C, H, N.

Enzyme Kinetic Studies. Adenosine 5′-monophosphate, adenosine 5′-triphosphate, cytidine 5′-triphosphate, 2′-deoxyadenosine 5′-triphosphate, and dithiothreitol were obtained from Sigma Chemical Co. [2-^{14}C]Thymidine (57 mCi/mmol) was obtained from New England Nuclear, Inc. The protein assay reagent was obtained from Bio-Rad Laboratories. Protein assays were carried out by Bradford's method.[36] Disc polyacrylamide gel electrophoresis was done as described by Kit et al.[22] The thymidine kinase assay mixture contained in a 0.1 mL final volume, Tris-HCl buffer (0.05 M) at pH 8.0, ATP (5 mM), $MgCl_2$ (2.5 mM), [^{14}C]thymidine (3.6–18 μM, 57 mCi/mmol, for the mitochondrial isozyme and 0.9–7.2 μM for the cytoplasmic isozyme), and thymidine kinase (5 or 10 μL). The mixture was rocked in a water bath at 37 °C for 60–90 min and then immersed in boiling water for 2 min and cooled in ice. Denatured protein was removed by centrifugation, and 0.02 mL of the supernatant was applied to a 16-mm DEAE-cellulose disk. Each disk was washed three times with 1 mM ammonium formate and once with ethanol, dried under an IR lamp, immersed in a toluene–phosphor mixture, and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 2425. The radioactivity was corrected for the blank value found for unincubated reaction mixtures: in all reactions, the [^{14}C]TMP (thymidine 5′-phosphate) present in the 20-μL aliquot generated more than 500 cpm. Reaction rates remained linear for 90 min and were proportional to the enzyme concentration.

For determinations of inhibition constants, four or more levels of thymidine in the above ranges were used for each of two levels of inhibitor (used at 1–4 times its K_i value) and also for a control which lacked inhibitor. All the enzyme-catalyzed reaction mixtures were made up in duplicate. Inhibition constants (K_i values) were obtained from replots of inhibitor concentrations vs. slopes of double-reciprocal plots of velocity vs. substrate level (all the latter were linear).

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To determine the thymidine kinase activity levels of various tissues (Table I), the tissues other than muscle were homogenized in 2 mL/g of buffer A (see next section) using a Teflon pestle; muscle tissue was homogenized in a blender. The homogenates were sonicated as described below for mitochondrial suspensions, and, after centrifugation at 33000*g* for 30 min, were assayed as described in Table I.

Rat Cytoplasmic Thymidine Kinase. All operations were carried out a 0–2 °C unless noted otherwise. Frozen adult rat spleens (22 g) obtained from Pel-Freez Biologicals Inc. were thawed in 50 mL of buffer A [0.01 M Tris-HCl at pH 8.0, 0.25 M sucrose, 0.05 M ϵ-aminocaproic acid, 3 mM dithiothreitol (DTT), and 0.2 mM thymidine] for 20 min and then homogenized using a motor-driven Teflon pestle until no unbroken cells were left. The homogenate was centrifuged at 1000*g* for 20 min to remove nuclei. The resulting supernate was centrifuged at 10000*g* for 10 min. The mitochondrial pellet so obtained was twice suspended in buffer A (2 mL), collected by centrifugation, and finally stored at −20 °C. The supernate from the mitochondria was centrifuged at 33000*g* for 30 min, and the resulting supernate was mixed with an equal volume of glycerol and stored at −20 °C. A portion (2.5 mL) of this fraction was subjected to Sephadex slab electrofocusing at 8 °C with an LKB Model 2117-501 apparatus, using ampholine of pH 3.5–10 in the presence of 20% glycerol. The homogenate was applied to the anode end of the gel. A single band of activity of p*I* = 7.4–7.5 was obtained. This was centrifuged through glass-wool and then dialyzed twice (16 h, 4 h) against 100 mL of a buffer containing Tris-HCl (0.05 M) at pH 8.0, ATP (5 mM), $MgCl_2$ (2.5 mM), DTT (5 mM), and glycerol (50% v/v). The solution (~4 mL) was then concentrated in Dextran (type 200C from Sigma Chemical Co.) at 5 °C to ~2 mL and stored at −20 °C. The activity was constant for more than 3 months. The recovery of activity from electrofocusing was then 20–23%. The specific activity of the preparation was 0.00019 μmol min^{-1} (mg of protein)$^{-1}$, and the K_M of thymidine was 1.6 μM. Values of 3.7 and 5.6 μM have been reported for rat Walker 256 carcinoma TK[5] and rat regenerating liver TK,[17] respectively.

Rat Mitochondrial Thymidine Kinase. The mitochondria obtained as described above were suspended in 2 mL of an aqueous solution of 0.01 M Tris-HCl at pH 8.0, 0.15 M KCl, 3 mM dithiothreitol, 0.05 M ϵ-aminocaproic acid, 0.2 mM thymidine, and Nonidet P-40 from Sigma Chemical Co. (0.5%, v/v) at 2 °C and disrupted by ultrasonic vibration (60 W for 3 × 10 s). Freezing and thawing the suspension prior to sonication did not increase the recovery of M-TK. The use of mitochondria from spleens kept frozen for longer than 5 days after sacrifice of the rats led to diminished levels of TK activity at this stage. The supernate (2.5 mL) obtained after centrifugation at 33000*g* for 30 min was mixed with glycerol (0.6 mL), ampholine (40%, 0.15 mL) of pH 4–6, ATP (0.10 mL of 50 mM), and $MgCl_2$ (0.10 mL of 25 mM) and applied to the anode end of a gel containing ampholine of pH 4–6 in which a pH gradient had been established in the preceding 12 h. Electrofocusing was continued for an additional 4 h at 8 °C. A solution (1 mL) containing ATP (10 mM) and $MgCl_2$ (5 mM) was applied across the gel slab ~2 cm to the cathodic side of the enzyme preparation at the outset of electrofocusing and every 30 min thereafter. A single band of activity of p*I* = 5.1 was obtained. This was centrifuged through glass-wool, then dialyzed against the same buffer used with the cytoplasmic isozyme, and stored at −20 °C. Activity was constant for more than 5 weeks. The recovery of activity from electrofocusing was then 47–53%. The specific activity of the preparation was 0.00022 μmol min^{-1} (mg of protein)$^{-1}$, and the K_M of thymidine was 8.0 μM.

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