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Inhibition of Uridine Kinase and the Salvage of Uridine by Modified Pyrimidine Nucleosides

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SUMMARY

Uridine kinase can play a crucial role in the provision of pyrimidine nucleotides for cellular nucleic acid synthesis, particularly when *de novo* synthesis is inhibited by chemotherapeutic agents. Therefore, uridine kinase is an attractive target for drug development. We examined a series of 29 analogs of uridine, most with modifications at the 5′-position, as inhibitors of uridine kinase *in vitro* and of uridine salvage by intact L1210 cells. Substitution at the 5′-position resulted in decreased efficacy as inhibitors of uridine kinase, particularly if the substituent was large. None of the analogs with 5′-position modifications effectively inhibited salvage of uridine by intact L1210 cells. Four carbocyclic pyrimidine nucleoside analogs (one series) were all effective competitive inhibitors of uridine kinase and of uridine salvage by intact L1210 cells. Cyclopentenyl uracil *19* shows promise for further development as it inhibits uridine salvage at nontoxic concentrations.

INTRODUCTION

Mammalian cells synthesize uracil nucleotides by two separate pathways: a *de novo* pathway utilizing glutamine, aspartate, and bicarbonate, and a salvage pathway utilizing the enzyme uridine kinase (EC 2.7.1.48). Inhibitors of *de novo* pyrimidine biosynthesis, such as PALA[2] and pyrazofurin, have had only limited success in the clinic as single agents (1–4). Since these agents inhibit only one of the pyrimidine biosynthetic pathways, their chemotherapeutic efficacy could be diminished by the utilization of exogenous uridine present in plasma. The *in vivo* toxicity and antitumor effectiveness of PALA and pyrazofurin have been reversed by uridine administration (1, 5). In addition, the concentration of uridine in the serum of oncology patients treated with PALA did not decrease substantially below serum levels found in healthy people (6). Recent studies (7) have shown that the concentration of uridine found in plasma is sufficient to reverse the growth-inhibitory effects of PALA on cultured L1210 leukemia cells.

A superior approach to the chemotherapeutic use of pyrimidine antimetabolites would, therefore, be a two-pronged approach in which both the *de novo* and salvage pathways of pyrimidine biosynthesis are blocked. To this end we are attempting to design an effective inhibitor of uridine kinase to be used in combination with an inhibitor of *de novo* biosynthesis. In this report we examine a series of compounds, mostly consisting of uridine modified in the 5′-position, as inhibitors of uridine kinase *in vitro* and of uridine salvage by intact L1210 cells.

MATERIALS AND METHODS

Chemicals

Dihydroazacytidine (NSC 264880), 5′-deoxy-5′-fluorouridine (NSC 524310), 5′-deoxy-5′-fluoro-5-bromouridine (NSC 354057), 5′-deoxy-5-fluorouridine (NSC 283905), and 5-aza-3-deazauridine (NSC 119854) were supplied by the Drug Synthesis Branch of the National Cancer Institute. 5′-*O*-Benzoyluridine, 5′-*O*-trityluridine, and uridine were purchased from Sigma Chemical Co. (St. Louis, MO). [U-^{14}C]Uridine (522 Ci/mol) was purchased from New England Nuclear (Boston, MA).

5′-*O*-Nitrouridine was a gift from Drs. J. Nemec and A. Welch of St. Judes Cancer Center (Memphis, TN). The carbocyclic cytidine and carbocyclic uridine (8, 9) were gifts from Dr. Y. F. Shealy of Southern Research Institute (Birmingham, AL). Cyclopentenyl cytosine and cyclopentenyl uracil were synthesized as described (10).

The synthesis of the following compounds was achieved using the methods outlined in the references indicated: 5′-deoxyuridine, *1* (11), 5′-chloro-5′-deoxyuridine, *3* (12), 5′-bromo-5′-deoxyuridine, *4* (12), 5′-deoxy-5′-iodouridine, *5* (13), 5′-azido-5′-deoxyuridine, *6* (14), 5′-amino-5′-deoxyuridine, *7* (14), 5′-*O*-methanesulfonyluridine, *8* (15), 5′-*N*-carboxymethyluridine, *9* (16), 5′-*O*-carboxymethyluridine, *13* (17), uridine 5′-methylphosphonate *14* (18), uridine 5′-(β-D-glucopyranosyl

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[2] The abbreviations used are: PALA, *N*-phosphonacetyl-L-aspartate; TLC, thin layer chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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phosphoric acid), *15* (19), and 1-(5-*O*-acetyl-β-D-erythropent-4-enofuranosyl uracil, *26* (20), 2-oxypyrimidine riboside (21).

Proton-decoupled ^{13}C-NMR spectra were recorded with an IBM NR80F instrument at 80 MHz or with a Bruker WM-300 instrument at 300 MHz. ^{13}C-NMR data for *3–9* and *13* were included in Table 1. Infrared spectra were recorded with a Beckman spectrophotometer (model IR-4210). Ultraviolet spectra were taken on a Beckman DU-8 spectrophotometer. Optical rotations were measured with a Perkin-Elmer model 243 polarimeter. Microanalyses were performed by Galbraith Analytical Laboratories (Knoxville, TN). TLC was performed on an aluminum support coated with silica gel 60 with fluorescent indicator. Spots were detected by a sulfuric acid-ethanol spray and heating. Preparative TLC was performed on precoated plates, silica gel GF (Analab, 20 × 20 cm, 100 μm thick).

Uridine-5′-aldehyde 20. A mixture of 5′-deoxy-2′,3′-*O*-cyclohexylidene-5,5′-(*N,N*″-diphenylethylenediamino)uridine (22) (1 g, 1.94 mmol) and 2 g of dried Dowex 50 WX2 (H^+, 400 resin) in 24 ml of THF/water (1:1) was stirred at room temperature for 4 hr. The resin was removed by suction filtration and washed well with THF (six times in 25 ml). The THF was evaporated at reduced pressure and the aqueous solution was filtered of insolubles. Removal of water at reduced pressure gave a white solid, which was treated with a mixture of TFA/H_2O (4:1, 25 ml) and stored at room temperature for 4 hr. TLC (chloroform/methanol/water, 60:35:6) after this time showed the presence of one spot (R_f 0.55) as compared to that of uridine (R_f 0.65). Excess TFA and water were removed from the reaction mixture at reduced pressure; then, co-evaporation with methanol (twice in 5 ml) gave a white solid of uridine-5′-aldehyde (*20*), yield 0.535 g (71.3%); m.p. 125°; $[\alpha]_D^{25}$ −11.6° (c 0.5, water); $\lambda_{max}^{pH\ 6.0}$ 262 nm (ε mM, 10.4). ^{13}C-NMR (Table 1) indicated a downfield shift of the C-5′ resonance to 103.1 ppm.

$C_9H_{10}O_6N_2$

Calculated: C 44.63 H 4.13 N 11.57

Found: C 44.32 H 4.14 N 11.46

5′-Azido-5′-deoxy-6-azauridine 23. 5′-Azido-5′-deoxy-2′,3′-*O*-isopropylidene-6-azauridine (15) (0.564 g, 1.82 nmol) was dissolved in TFA (7 ml) and stored at room temperature for 17 hr. TLC (chloroform/methanol, 3:1) after this time showed one major spot (R_f 0.69). The reaction mixture was evaporated at 40° under reduced pressure and co-evaporated with toluene (twice in 5 ml) to a dry syrup. This syrup was adsorbed on 1 g of dry silica gel, packed in a dry silica gel (40 g) column (2 × 60 cm), and eluted with chloroform/methanol (4:1). The major component, *23*, was separated and crystallized from acetone in a yield of 0.25 g (44.3%), m.p. 145–147°, $[\alpha]_D^{25}$ + 0.4° (c 0.25, water), $\lambda_{max}^{pH\ 5.5}$ 263 nm (ε mM 6.78). The infrared spectrum showed a —N═N═N absorption band at 2080 cm^{-1}. The ^{13}C-NMR (Table 2) spectrum showed an upfield shift of 10 ppm for the C-5′ resonance due to the azido group.

$C_8H_{10}O_5N_6,0.3CH_3COCH_3$

Calculated: C 37.16 H 4.11 N 29.22

Found: C 37.07 H 3.93 N 28.77

5′-Amino-5′-deoxy-6-azauridine 24. 5′-Azido-5′-deoxy-6-azauridine (*23*, 0.145 g, 0.54 mmol) and triphenylphosphine (0.247 g, 0.94 mmol) were dissolved in pyridine (2 ml), and the solution was kept for 1 hr at room temperature. Concentrated ammonium hydroxide (0.5 ml) was then added and the solution was stirred for 2.5 hr at room temperature. The solution was then evaporated to dryness and washed with ether (twice in 30 ml). The residue was dissolved in 1 M ammonium hydroxide solution (9 ml) and extracted with benzene (twice in 10 ml) to remove residual triphenylphosphine, and then evaporated to dryness. TLC (chloroform/methanol, 3:1, R_f 0; 1-butanol-acetic acid/water, 4:1:1, R_f 0.28) indicated a single spot. The product was crystallized from methanol and dried to afford 0.072 g (49.7%) of an analytically pure product; m.p. 228–230° (decomposed), $[\alpha]_D^{25}$ −7.2° (c 0.25, water), $\lambda_{max}^{pH\ 6.0}$ 262 nm (ε mM 6.8). ^{13}C-NMR (Table 2) showed an upfield shift of C-5′ resonance by 20 ppm.

$C_8H_{12}O_5N_4,0.5H_2O$

Calculated: C 37.94 H 5.14 N 22.13

Found: C 37.91 H 5.29 N 21.73

5′-Deoxy-5′-iodo-6-azauridine 25. A solution of 2′,3′-*O*-isopropylidene-6-azauridine (23) (0.286 g, 1 mmol) and *methyl*-triphenoxyphosphonium iodide (1 g, 2 mmol) in *N,N*′-dimethylformamide (5 ml) was stored at room temperature for 30 min. After this time, TLC (chloroform/methanol, 10:1) indicated complete disappearance of the starting material (R_f 0.47) and the appearance of a major fast-moving spot (R_f 0.71). Methanol (2 ml) was added to the reaction mixture, and after 15 min at room temperature the solvent was evaporated under reduced pressure at 40° yielding a straw-colored syrup. The residue was dis-

TABLE 1

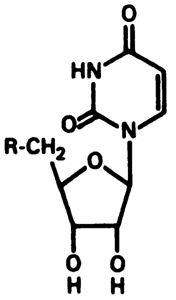
^{13}C-NMR chemical shifts of 5′-substituted uridine derivatives

	C-4	C-2	C-6	C-5	C-1′	C-4′	C-2′	C-3′	C-5′
Uridine (D_2O) (31)	167.3	152.8	143.0	103.4	90.6	85.5	74.8	70.6	62.0
5′-Chloro-5′-deoxyuridine *3* (DMSO-d_6)	162.95	150.65	140.81	102.18	88.27	82.89	72.31	70.7	44.84
5′-Bromo-5′-deoxyuridine *4* (DMSO-d_6)	162.6	150.35	140.64	101.93	88.29	82.5	72.12	71.43	33.61
5′-Deoxy-5′-iodouridine *5* (DMSO-d_6)	162.48	150.33	140.82	101.93	88.36	82.72	72.58	72.12	7.39
5′-Azido-5′-deoxyuridine *6* (D_2O)	166.3	151.8	142.3	102.8	90.3	82.4	73.2	70.3	51.7
5′-Amino-5′-deoxyuridine *7* (D_2O)	166.3	151.6	143.4	102.5	92.8	79.6	72.8	70.98	41.4
5′-*O*-Mesyl-uridine *8* (DMSO-d_6)	163.07	150.68	140.83	102.16	88.71	81.06	72.33	69.59	69.51 (39.91) (CH_3)
5′-*N*-Carboxy-methyluridine *9* (D_2O)	166.29	151.47	142.61	102.35	91.96	77.52	72.63	71.28	56.83 (57.35) (170.53) (N—CH_2) (—COOH)
5′-*O*-Carboxy-methyluridine *13* (D_2O)	166.79	152.49	142.79	103.25	90.34	83.89	74.55	70.83	69.17 (31.07) (175.18) (O—CH_2) (—COOH)
Uridine-5′-aldehyde *20* (D_2O)	166.2	152.2	142.3	102.1	89.3	86.2	73.6	69.5	103.1

TABLE 2

^{13}C-NMR chemical shifts of 5′-substituted 6-azauridine derivatives

	C-4	C-2	C-5	C-1′	C-4′	C-2′	C-3′	C-5′
6-Azauridine (D_2O)	158.1	149.52	137.32	90.38	84.66	73.25	70.74	62.11
5′-Azido-5′-deoxy-6-azauridine *23* (D_2O)	158.41	149.6	137.31	90.78	83.06	73.31	71.35	52.05
5′-Amino-5′-deoxy-6-azauridine *24* (D_2O)	158.81	149.1	137.06	91.87	80.12	73.68	71.98	42.01
5′-Deoxy-5′-iodo-6-azauridine *25* (methanol-d_4)	160.2	151.5	137.3	91.89	84.47	75.2	74.44	6.23



	R =		R =
1	—H	**8**	$-OSO_2CH_3$
2	—F	**9**	$-NHCH_2CO_2H$
3	—Cl	**10**	—O-Benzoyl
4	—Br	**11**	—O-Trityl
5	—I	**12**	$-ONO_2$
6	$-N_3$	**13**	$-OCH_2CO_2H$
7	$-NH_2$	**14**	$-OPO(OH)CH_3$
		15	β-D-glucopyranosyl monophosphate

FIG. 1. *Uridine analogs modified at the 5′-position.*

solved in ethyl acetate (50 ml) and extracted with aqueous sodium thiosulfate solution (twice in 30 ml) followed by water (four times in 25 ml). The aqueous layer was reextracted with ethyl acetate (30 ml), and the organic layers were combined, dried over anhydrous sodium sulfate, and evaporated to a syrup. The dried residue was purified by preparative TLC using chloroform/methanol (10:1). Elution of the major band with methanol gave 0.17 g (43%) of crystalline 5′-deoxy-5′-iodo-2′,3′-*O*-isopropylidene-6-azauridine with m.p. 179–181°, literature m.p. 178–181° (15). The isopropylidene group was removed with TFA (3 ml) at room temperature for 1 hr. TLC (chloroform/methanol, 10:1) showed the expected major slow-moving spot (R_f 0.32) with minor fast-moving spots. The TFA was removed from the reaction mixture under reduced pressure at 40° followed by co-evaporation with toluene (5 ml). The syrupy product was applied to four preparative TLC plates and developed in chloroform/methanol, 10:1. The major band was separated, eluted with methanol, filtered over celite, and evaporated to give crystalline 5′-deoxy-5′-iodo-6-azauridine *25*, yield 0.13 g (85%), m.p. 157–159°, $[\alpha]_D^{25}$ −6.70 (c 0.3, methanol), $\lambda_{max}^{pH\ 5.5}$ 262 nm (ϵ mM 7.72). ^{13}C-NMR (Table 2) showed an upfield shift of 56 ppm for the C-5′ resonance.

$C_8H_{12}O_5N_3I$

Calculated: C 27.0 H 2.8 I 35.8 N 11.8

Found: C 26.95 H 2.68 I 35.4 N 11.55

Assay of Uridine Kinase

Uridine kinase was prepared from L1210 murine leukemia cells by a modification of the procedure of Ahmed and Baker (24). L1210 ascites cells were harvested from the peritoneal cavity of CDF_1 mice inoculated 7 days before with 10^6 cells/mouse. Erythrocytes in the L1210 cell preparation were lysed by a 30-sec incubation in 3 vol of H_2O followed by the immediate addition of 1 vol of a 3.6% NaCl solution, centrifugation at 200 × *g* for 10 min, and decanting the supernatant. All remaining steps were performed at 0–4°. The L1210 cells were disrupted by freeze-thawing three times, and the crude homogenate was diluted 5-fold with a 0.2 M Tris buffer, pH 7.5, containing 0.1% mercaptoethanol. Saturated ammonium sulfate solution was added dropwise to the supernatant fraction of the homogenate, obtained by centrifugation at 100,000 × *g* for 60 min. The fraction that precipitated between 30 and 50% saturation of ammonium sulfate was dialyzed against two changes of the homogenizing buffer. This fraction was the source of uridine kinase used in this study.

Uridine kinase activity was measured by a modification of the procedure of Ives *et al.* (25) using a reaction mixture composed of 25 μl of [U-^{14}C]uridine at 200 μM, 25 μl of a buffered ATP-generating system (containing 200 mM Tris (pH 7.5), 30 mM ATP, 15 mM $MgCl_2$, 25 mM creatine phosphate, and 40 mg of bovine serum albumin and 18 units of creatine kinase/ml), 25 μl of water or inhibitor, and 50 μl of the enzyme solution. Reaction mixtures were incubated in duplicate or triplicate at 37° for 10 min at which time 50-μl aliquots were removed from each mixture and spotted on DE81 filter disks (Whatman, Inc., Clifton, NJ). The disks were immediately placed in 95% ethanol for 45 min, then washed twice with H_2O for 20 min each, rinsed with acetone, and allowed to dry. The radioactivity adsorbed onto the disks was counted. Under these conditions, examination of incorporation at 0, 3, 6, 9, and 12 min indicated that the label incorporated was directly proportional to time of incubation for at least 12 min.

For more detailed kinetic studies the assay was performed with final [^{14}C]uridine concentrations of 200, 40, and 10 μM and inhibitor concentrations between 0.3 and 1 mM. Examination of the data as Dixon plots indicated that inhibition was competitive in each instance. K_i values were determined by examination of the Dixon plots or by fitting all data to the Michaelis-Menten equation for competitive inhibition by use of a linear least squares fit program (MLAB, Ref. 26).

Salvage of [^{14}C]uridine by intact L1210 cells. Exponentially dividing L1210 cells in Fisher's medium containing 10% horse serum at 2 to 6 × 10^5 cells/ml were distributed into 15-ml culture tubes (5 ml/tube) and incubated for 2 hr with 0.1 mM potential inhibitor at 37° in a shaking water bath. [^{14}C]Uridine (0.125 μCi, 2.8 μM) was added to each tube and incubation was continued for 1 hr. The cells were then collected by centrifugation, lysed in 2 ml of H_2O, and filtered through Whatman DE81 filter disks. The disks were washed with H_2O and then with acetone, and the radioactivity adsorbed onto the disks was counted. In some instances the scale of the incubation was reduced to a 1-ml suspension of cells. Typical uridine salvage by untreated (control) cells was 7450 cpm/10^6 cells. The contribution of uridine from 10% horse serum to the total uridine present during uptake would be <10%, based on a uridine concentration of 2.3 μM in horse serum (27).

Inhibition of L1210 cell replication. L1210 cells were grown in Fisher's medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% horse serum. The cultures were initiated at 20,000 cells/ml and incubated at 37° with constant exposure to inhibitor

TABLE 3

Uridine analogs modified at the 5′-position

Each compound was tested as an inhibitor of uridine kinase *in vitro*, of uridine salvage by intact L1210 cells, and of L1210 cell replication as described under Materials and Methods. Each value is the mean of determinations from two or three independent experiments.

Analog	5′-Substituent	Inhibition of uridine kinase		K_i	Inhibition of uridine salvage (0.1 mM)	Inhibition of cell replication		
		0.1 mM	1.0 mM			0.1 m	0.5 mM	1.0 mM
		%		μM	%		%	
1	—H	16	57	510	<10	<10	<10	<10
2	—F	<10	55	840	<10	<10	<10	<10
3	—Cl	29	84	140	<10	<10	13	12
4	—Br	16	64	470	<10	<10	<10	<10
5	—I	<10	39	-[a]	<10	<10	<10	<10
6	—N_3	19	67	380	31	<10	<10	<10
7	—NH_2	24	68	330	<10	22	84	89
8	—OSO_2CH_3	0	18	-	20	12	18	<10
9	—$NHCH_2CO_2H$	<10	<10	-	10	<10	<10	24
10	—O-Benzoyl	<10	<10	-	95	-	36	40
11	—O-Trityl	-	<10	-	<10	-	-	-
12	—O-NO_2	42	88	80	<10	<10	<10	<10
13	—OCH_2CO_2H	-	<10	-	<10	<10	<10	<10
14	Methyl phosphonate	<10	<10	-	26	<10	<10	<10
15	β-D-Glucopyranosyl monophosphate	<10	<10	-	34	<10	<10	<10

[a] -, not determined

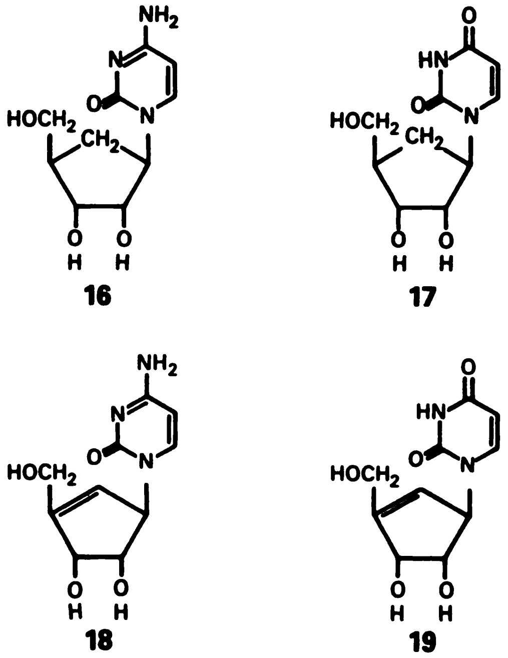


FIG. 2. *The structures of some carbocyclic derivatives of uridine and cytidine.*

for 72 hr. Cell number was determined with a Coulter counter. Inhibition is expressed as percentage of controls which typically grew to 600,000 cells/ml. The value given is the mean of three or four independent determinations.

TABLE 4

Carbocyclic derivatives of uridine and cytidine

Each compound was tested as an inhibitor of uridine kinase *in vitro* and as an inhibitor of uridine salvage by intact L1210 cells as described under Materials and Methods.

Compound	Inhibition of uridine kinase		K_i	Inhibition of uridine salvage (0.1 mM)
	0.1 mM	1.0 mM		
	%		μM	%
16	26	73	250	83
17	9	41	930	51
18	57	89	60	93
19	35	78	230	58

RESULTS AND DISCUSSION

Ahmed and coworkers (24, 28) examined 39 compounds as inhibitors of uridine kinase, and of these, the compounds substituted at the 5′-position of the ribose moiety were the most potent inhibitors. We therefore synthesized or obtained series of 5′-modified uridine analogs as shown in Fig. 1. These compounds were tested as inhibitors of uridine kinase *in vitro* and as inhibitors of the salvage of uridine by intact L1210 cells (Table 3).

Uridine analogs in which the 5′-hydroxyl group was replaced with hydride, halogens, amino, or azido were competitive inhibitors of uridine kinase. However, in comparison to the K_m for uridine, determined as 47 ± 7 μM (mean ± SD, $n = 14$) in our studies, these compounds were not very potent inhibitors. The most potent inhibitors in this series were 5′-chloro-5′-deoxyuridine and the 5′-*O*-nitrouridine examined previously by Ahmed and Baker (24), and analogs with bulky substituents at the 5′-position such as benzoyl, trityl, or glucopyranosyl were inactive as inhibitors, as were compounds with anionic substituents at the 5′-position such as compounds *9*, *14*, *13*, or *15*.

In addition to the studies with cell-free uridine kinase preparations, we examined the ability of these nucleosides to decrease salvage of uridine by intact L1210 cells. This assay, described in Materials and Methods, is a

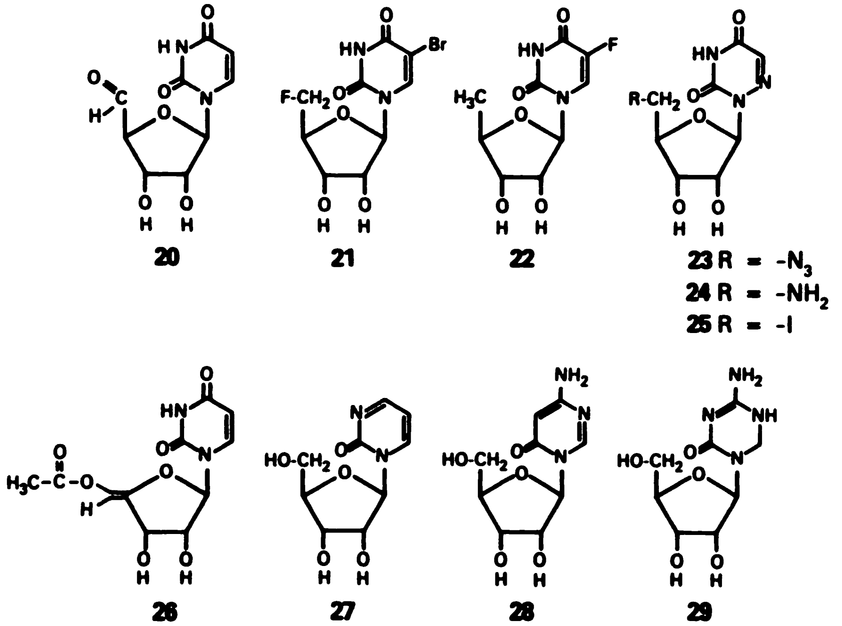


FIG. 3. *The structures of uridine analogs.*

TABLE 5

Uridine analogs modified in the pyrimidine ring or at multiple sites

Each compound was tested as an inhibitor of uridine kinase *in vitro*, of uridine salvage by intact L1210 cells, and of L1210 cell replication as described under Materials and Methods.

Compound	Inhibition of uridine kinase		K_i	Inhibition of uridine salvage (0.1 mM)	Inhibition of cell replication		
	0.1 mM	1.0 mM			0.1 mM	0.5 mM	1.0 mM
	%		*μM*	%	%		
20 Uridine-5′-aldehyde	20	69	140	12	<10	<10	50
21 5′-Deoxy-5′-fluoro-5-bromouridine	<10	<10	–[a]	<10	<10	<10	33
22 5′-Deoxy-5-fluorouridine	22	68	350	<10	17	70	88
23 5′-Azido-5′-deoxy-6-azauridine	<10	<10	–	<10	<10	<10	21
24 5′-Amino-5′-deoxy-6-azauridine	40	86	68	<10	<10	<10	<10
25 5′-Deoxy-5′-iodo-6-azauridine	<10	29	–	<10	<10	38	59
26 1-(5-*O*-acetyl-β-D-erythro-pent-4-enofuranosyl)uracil	<10	<10	–	<10	–	–	–
27 2-Oxypyrimidine riboside	–	<10	–	<10	42	98	–
28 5-Aza-3-deazacytidine	<10	<10	–	<10	89	96	96
29 5,6-Dihydro-5-azacytidine	<10	<10	–	30	86	92	96

[a] –, not determined.

functional measure of uridine uptake but cannot distinguish between inhibitors of nucleoside transport or of uridine kinase. Of the 5′-modified nucleosides listed in Table 3, only 5′-*O*-benzoyluridine produced a ≥40% inhibition of salvage of [^{14}C]uridine by intact L1210 cells when tested at 0.1 mM (Table 1). The two most potent inhibitors of the kinase, compounds *3* and *12*, inhibited uridine salvage by intact L1210 cells 52% and 40%, respectively, at 1 mM when tested as described in Materials and Methods. Although 5′-*O*-benzoyluridine *10* produced an apparent 95% inhibition of uridine salvage, further studies revealed that serum contains an activity that hydrolyzes the benzoyl ester, releasing free uridine and benzoic acid. This release of unesterified uridine was most likely responsible for the apparent inhibition of uridine salvage. Barrie *et al.* (29) have recently reported that a series of thymidine derivatives esterified at the 5′-position was subject to hydrolysis in aqueous solution. 5′-*O*-Benzoyluridine *10*, by contrast, appeared stable in Fisher's medium (<10% hydrolysis in 6 hr at 37°) but was 84% hydrolyzed in 1 hr when 10% horse serum was added. The inability of the remainder of the nucleosides listed in Table 3 to inhibit nucleoside salvage at 0.1 mM may reflect their relatively high K_i values but, alternatively, these compounds may not easily enter the cells. Further studies with radiolabeled analogs would distinguish between these two possibilities.

The second class of nucleoside analogs examined was the carbocyclic derivatives of uridine and cytidine shown in Fig. 2. These analogs are apparent competitive inhibitors of uridine kinase (Table 4) and also inhibit salvage of uridine by intact L1210 cells. The unsaturated compounds *18* and *19* are approximately 4-fold more potent than the saturated derivatives *16* and *17*, and the cytidine analogs are about 4-fold more potent than the corresponding uridine analogs. Previously published results have indicated that both compounds *16* and *18* are cytotoxic (9, 10), but compound *19* only slightly inhibited replication of L1210 cells even at a concentration of 1 mM. The inhibition of salvage of uridine is specific; salvage of thymidine or deoxycytidine was not affected (10).

A number of additional uridine analogs (shown in Fig. 3) were evaluated by the test systems described above, to gain further insight concerning structure-activity relationships. The uridine 5′-aldehyde *20* was an effective inhibitor of uridine kinase with a K_i of 140 μM, but it failed to significantly reduce salvage of uridine by cultured L1210 cells (Table 5). Substitution at the 5-position of the pyrimidine ring reduced the affinity of the inhibitors (compare compounds *21* and *2* or *22* and *1*).

Three analogs of 6-azauridine with substitutions at the 5′-position (*23*, *24*, and *25*) were also examined. Compounds *23* and *25* were less effective than the corresponding uridine analog as listed in Table 3. This is consistent with the lower activity of 6-azauridine in comparison with uridine as substrates for uridine kinase (30). In contrast, 5′-amino-5′-deoxy-6-azauridine *24* was a highly effective inhibitor (K_i = 68 μM). None of the 6-azauridine analogs effectively inhibited salvage of uridine by cultured L1210 cells.

Compound *27*, 2-oxypyrimidine riboside, was ineffective as an inhibior of both uridine kinase *in vitro* and salvage of uridine by intact L1210 cells. The growth-inhibitory properties of this compound, reported previously (21), were confirmed. Thus, although either an amino or a carbonyl group at the 4-position of the pyrimidine ring (cytidine or uridine) yields a good substrate, lack of substitution at this point virtually eliminates affinity for the kinase.

In summary, previously published studies (24) have indicated that substitution of halogens or other substituents at the 5- or 6-position of the pyrimidine ring markedly reduce the ability of uridine to serve as an inhibitor or substrate for uridine kinase. More recent results indicate that substitution of halogens at the 2′- or 3′-position of uridine also greatly reduce affinity for uridine kinase.[3] Of 39 compounds examined by Ahmed and Baker (24), the most active inhibitors were analogs modified at the 5′-position, We have extended the series of 5′-modified uridine analogs and find that several inhibit uridine kinase at a concentration of 100 μM (Table 3). Of the compounds tested in this study, three had K_i values similar to the K_m values of uridine: 5′-*O*-nitrouridine (K_i = 80 mM), 5′-amino-5′-deoxy-6-azauridine (K_i = 68 μM), and cyclopentenyl cytosine (K_i = 60 μM). The tolerance of uridine kinase for large substituents at the 5′-position appears limited, and compounds with large substituents at this position were ineffective inhibitors. Carbocyclic analogs appear to offer the best opportunities for further development, as they were effective inhibitors of uridine kinase and of uridine salvage by intact cells. Most promising is cyclopentenyl uracil *19*, as this nucleoside has a number of characteristics required of a useful uridine kinase inhibitor: it is relatively non-toxic and is not cleaved by uridine phosphorylase (10). Further studies will determine the usefulness of cyclopentenyl uracil as an inhibitor of uridine salvage *in vivo* and in combination with inhibitors of pyrimidine synthesis *de novo*.

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REFERENCES

1. Cadman, E. C., D. E. Dix, and R. E. Handschumacher. Clinical, biological, and biochemical effects of pyrazofurin. *Cancer Res.* **38**:682–688 (1978).
2. Casper, E. S., R. J. Gralla, D. P. Kelsen, A. Houghton, R. B. Golbey, and C. W. Young. Phase II evaluation of *N*-(phosphonacetyl)-L-aspartic acid (PALA) in patients with non-small carcinoma of the lung. *Cancer Treat. Rep.* **64**:705–707 (1980).
3. Ervin, T. J., R. H. Blum, M. W. Meshad, D. W. Kufe, R. K. Johnson, and G. P. Cannellos. Phase I trial of *N*-(phosphonacetyl)-L-aspartic acid (PALA). *Cancer Treat. Rep.* **64**:1067–1071 (1980).
4. Hart, R. D., T. Ohnuma, and J. F. Holland. Initial clinical study with *N*-(phosphonacetyl)-L-aspartic acid (PALA) in patients with advanced cancer. *Cancer Treat. Rep.* **64**:617–624 (1980).
5. Johnson, R. K. Reversal of toxicity and antitumor activity of *N*-(phosphonacetyl)L-aspartate by uridine or carbamyl-DL-aspartate *in vivo*. *Biochem. Pharmacol.* **26**:81–84 (1977).
6. Karle, J. M., L. W. Anderson, C. Erlichman, and R. L. Cysyk. Serum uridine levels in patients receiving *N*-(phosphonacetyl)-L-aspartate. *Cancer Res.* **40**:2938–2940 (1980).
7. Karle, J. M., L. W. Anderson, and R. L. Cysyk. Effect of plasma concentrations of uridine on pyrimidine biosynthesis in cultured L1210 cells. *J. Biol. Chem.* **259**:67–72 (1984).
8. Shealy, Y. F., and C. A. O'Dell. Synthesis of the carbocyclic analogs of uracil nucleosides. *J. Heterocycl. Chem.* **13**:1015–1020 (1976).
9. Shealy, Y. F., and C. A. O'Dell. The carbocyclic analog of cytidine, synthesis and antineoplastic activity. *J. Heterocycl. Chem.* **13**:1353–1354 (1976).
10. Lim, M.-I., J. D. Moyer, R. L. Cysyk, and V. E. Marquez. Cyclopentenyluridine and cyclopentenylcytidine analogues as inhibitors of uridine-cytidine kinase. *J. Med. Chem.* **27**:1536–1538 (1984).
11. Codington, J. F., R. Fecher, and J. J. Fox. Pyrimidine nucleosides. VII. Reactions of 2′,3′,5′-trimesyloxyuridine. *J. Am. Chem. Soc.* **82**:2794–2803 (1980).
12. Dods, R. F., and J. S. Roth. Halogenation of nucleosides by (halomethylene)dimethylene ammonium halides. *Tetrahedron Lett.* No. 3. 165–168 (1969).
13. Verheyden, J. P. H., and J. G. Moffatt. Halo sugar nucleosides. I. Iodination of the primary hydroxyl groups of nucleosides with methyltriphenoxyphosphonium iodide. *J. Org. Chem.* **35**:2319–2326 (1970).
14. Horwitz, J. P., A. J. Tomson, J. A. Urbanski, and J. Chua. Nucleosides. I. 5′-Amino-5′-deoxyuridine and 5′-amino-5′-deoxythymidine. *J. Org. Chem.* **27**:3045–3048 (1962).
15. Zemlicka, J., and F. Sorm. Nucleic acid components and their analogues. LXXXIX. Synthesis of 2′,3′-*O*-isopropylidene-O^2,5′-cyclo-6-azauridine and 2-(β-D-ribofuranosyl)-3-amino-4,5-dihydro-1,2,4-triazine-3-one(6-azaisocytidine). *Collect. Czech. Chem. Commun.* **32**:576–589 (1967).
16. Delaney, E. J., P. W. Morris, T. Masushita, and D. L. Venton. Synthesis of 5′-substituted EDTA-like nucleosides as analogs of nucleoside phosphates. *J. Carbohydr. Nucleosides Nucleotides* **8**:445–459 (1981).
17. Holy, A., and H. Pischel. Preparation of 5′-*O*-carboxymethyluridine and its 5-halo derivatives. *Collect. Czech. Chem. Commun.* **42**:2261–2265 (1977).
18. Wigler, P. W., and C. B. Lozzio. Cytotoxic nucleotide analogs as potential anticancer agents, 5-bromodeoxyuridine 5′-methylphosphonate. *J. Med. Chem.* **15**:1020–1024 (1972).
19. Salam, M. A., and E. J. Behrman. Synthesis of nucleoside 5′-(β-D-glucopyranosyl monophosphates) by the sugar ortho ester route. *Carbohydr. Res.* **102**:139–146 (1982).
20. Cook, S. L., and J. A. Secrist, III. Enol acetates of nucleoside-5′-aldehydes. *Carbohydr. Res.* **52**:C3–C6 (1976).
21. McCormack, J. J., V. E. Marquez, P. S. Liu, D. T. Vistica, and J. S. Driscoll. Inhibition of cytidine deaminase by 2-oxopyrimidine riboside and related compounds. *Biochem. Pharmacol.* **29**:830–832 (1980).
22. Jones, G. H., and J. G. Moffatt. Oxidation of carbohydrates by the sulfoxide-carbodiimide and related methods. *Methods Carbohydr. Chem.* **6**:315–322 (1972).
23. Smrt, J., J. Beranek, and F. Sorm. Nucleic acid components and their analogues. IV. Synthesis of β-D-ribofuranoxyl-6-azauracil-5′-phosphate and pyrophosphate. *Collect. Czech. Chem. Commun.* **25**:130–137 (1960).

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[3] W. P. Gati, personal communication.

24. Ahmed, N. K., and D. R. Baker. Properties of uridine-cytidine kinase derived from L1210 leukemia cells. *Cancer Res.* **40**:3559–3563, (1980).
25. Ives, D. H., J. P. Durham, and V. S. Tucker. Rapid determination of nucleoside kinase and nucleotidase activities with tritium labeled substrates. *Anal. Biochem.* **28**:192–205 (1969).
26. Knott, G. D. MLAB—a mathematical modeling tool. *Comput. Programs Biomed.* **10**:271–280 (1979).
27. Moyer, J. D., J. T. Oliver, and R. E. Handschumacher. Salvage of circulating pyrimidine nucleosides in the rat. *Cancer Res.* **41**:3010–3017 (1981).
28. Ahmed, N. K., and A. D. Welch. Some properties of uridine-cytidine kinase from a human malignant lymphoma. *Cancer Res.* **39**:3102–3106 (1979).
29. Barrie, S. E., L. C. Davies, J. A. Stock, and K. R. Harrap. A reappraisal of the effect upon thymidine kinase of thymidine derivatives carrying large groups at the 5′-position. *J. Med. Chem.* **27**:1044–1047 (1984).
30. Liacouras, A. S., and E. P. Anderson. Uridine-cytidine kinase. Purification from a murine neoplasm and characterization of the enzyme. *Arch. Biochem. Biophys.* **168**:66–73 (1975).
31. Mantsch, H. H., and I. C. P. Smith. A study of solvent effects on the ^{13}C nuclear magnetic resonance spectra of cholesterol, pyrimidine, and uridine. *Can. J. Chem.* **51**:1384–1391 (1973).