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INHIBITION OF NUCLEOSIDE PHOSPHORYLASE CLEAVAGE OF 5-FLUORO-2′-DEOXYURIDINE BY 2,4-PYRIMIDINEDIONE DERIVATIVES*

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Abstract—Several 2,4-pyrimidinedione (uracil) derivatives were evaluated as inhibitors of the pyrimidine nucleoside phosphorylases that cleave 5-fluoro-2′-deoxyuridine (FUdR) to 5-fluorouracil. Pyrimidinediones substituted at either N-1 or C-5, or both, markedly inhibited the phosphorolysis of FUdR by the uridine–deoxyuridine phosphorylases of Ehrlich ascites and Novikoff hepatoma cells. The most potent inhibitors were 5-benzyluracil derivatives substituted with alkoxy groups on the *meta*-position of the benzyl moiety; the most active of these was 5-{[3-(phenylmethoxy)phenyl]methyl}uracil. The same derivatives, however, did not inhibit the phosphorolysis of FUdR by the thymidine phosphorylases of murine liver, human leukocytes and HeLa (S3) cells. 6-Anilino and 6-(1-naphthylmethylamino) derivatives of uracil, which have been shown by others to inhibit the cleavage of FUdR by the thymidine phosphorylase activity of *Escherichia coli*, did not inhibit any of the mammalian thymidine or uridine–deoxyuridine phosphorylase activities. By contrast, pyrimidinediones substituted with smaller, non-hydrophobic groups at either C-5 or C-6, or both, inhibited the cleavage of FUdR by both the mammalian thymidine and uridine–deoxyuridine phosphorylases. The most active of these, 6-aminothymine, was also the best inhibitor of thymidine phosphorylase. Our results demonstrate differences in the active sites of the various pyrimidine nucleoside phosphorylases, and should provide a basis for the design of more potent and specific inhibitors of the nucleoside phosphorylase(s) responsible for the cleavage of FUdR in man.

5-Fluorouracil (FU)¶ and 5-fluoro-2′-deoxyuridine (FUdR) inhibit the growth of many transplanted rodent tumors [1] and are used in the clinical therapy of advanced cancers of the breast and colon [2].

In mice, FUdR is more active than FU against several transplanted tumors [1] and is a better precursor of 5-fluoro-2′-deoxyuridine-5′-monophosphate, an active form of the drug [3–5]. In the clinic, however, FUdR is not much more effective than FU [2], partly because of the rapid degradation of FUdR to FU by pyrimidine nucleoside phosphorylases [6, 7]. Chemical modifications of FUdR designed to render the drug resistant to phosphorolytic cleavage have not been successful [6, 8], nor have attempts to inhibit nucleoside phosphorylase activity with other compounds [6, 9, 10].

Recently, we investigated the substrate specificities of several mammalian pyrimidine nucleoside phosphorylases and their cleavage of FUdR.** There was an apparent need to distinguish clearly between (a) uridine (UR) phosphorylases (EC 2.4.2.3; UR:orthophosphate ribosyltransferases) that cleave primarily UR [11–13] and are inhibited by 1-(2′-deoxy-β-D-glucopyranosyl)thymine (GPT, compound 5, Fig. 1) [14, 15], a specific inhibitor of UR phosphorylase that does not inhibit the activity of thymidine (TdR) phosphorylases (EC 2.4.2.4; TdR:orthophosphate deoxyribosyltransferases), which cleave only deoxynucleosides [11], and (b) phosphorylases that cleave both UR and TdR, as well as 2′-deoxyuridine (UdR) and other deoxynucleosides [16–18], and are inhibited by GPT. We therefore proposed a new definition for the term UR–UdR phosphorylase (EC 2.4.2.3.) [18, 19] to identify specifically the latter class of phosphorylases. By this definition, we concluded that FUdR was cleaved to FU by UR–UdR phosphorylase activity in Ehrlich ascites cells and Novikoff hepatoma cells and primarily by TdR phosphorylases in mouse liver, normal human leukocytes and HeLa (S3) cells.

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¶ Abbreviations used: FU, 5-fluorouracil; FUdR, 5-fluoro-2′-deoxyuridine; TdR, thymidine; UR, uridine, UdR, 2″-deoxyuridine; GPT, 1-(2′-deoxy-β-D-glucopyranosyl)thymine; S-MEM, minimum essential medium with Spinner's salts; Buffer 1, 20 mM sodium phosphate buffer at pH 8.0, containing 10 mM β-mercaptoethanol; and DMSO, dimethylsulfoxide.

** P. W. Woodman, A. M. Sarrif and C. Heidelberger, *Cancer Res.*, in press.

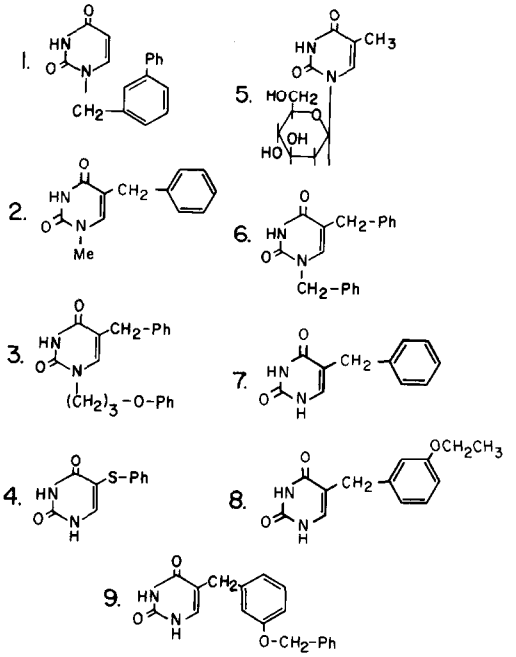

Fig. 1, Structures of inhibitors in Table 1.

In the present study, potent inhibitors of the TdR phosphorylase activity of *Escherichia coli* [20, 21] and the FUdR phosphorylase activity of Walker-256 carcinoma cells [19, 22, 23] have been tested, as have other 2,4-pyrimidinedione derivatives [10, 14, 24–26], for their ability to inhibit the phosphorolysis of FUdR by the five mammalian nucleoside phosphorylases mentioned previously.

MATERIALS AND METHODS

All chemicals were of analytical grade and were purchased from the Sigma Chemical Co., St. Louis, MO, unless otherwise stated. Some of the 2,4-pyrimidinedione derivatives were obtained from the Drug Development Branch, Division of Cancer Treatment, NCI, and these are designated by their NSC numbers. FUdR was provided by Hoffmann–LaRoche, Inc., Nutley, NJ.

Cell culture. HeLa (S3) cells and Novikoff hepatoma cells were grown in suspension culture in S-MEM medium (Eagle), supplemented with 10% heat-inactivated calf serum and 0.1% pluronic acid [27]. Media and sera were purchased from the Grand Island Biological Co., Grand Island, NY, and pluronic-F68 was a gift from the BASF Co., Wyandotte, MI. The cells were examined routinely for mycoplasma with standard methods [28–30], since many of these organisms have high nucleoside phosphorylase activity [29]; in each instance, the results were negative.

Animal tissues. Ehrlich ascites cells were grown in female Swiss/ICR mice (Sprague–Dawley, Madison, WI), as reported previously [6]. Mouse livers were obtained from non-tumor-bearing animals of the same sex and strain; before excision, blood was removed by perfusion of the portal vein with an ice-cold 0.9% NaCl solution.

Isolation of human leukocytes. Units of blood were drawn from healthy subjects and the erythrocytes were removed with 3% dextran in a 0.9% NaCl solution at 37°, followed by hypertonic shock [31]. The leukocytes, which were harvested by centrifugation at 800 *g* for 10 min at 4°, contained from 55 to 80 per cent mature granulocytes.

Enzyme isolation and partial purification. Cells or tissues were washed twice in ice-cold 0.9% NaCl solution and homogenized in 2 vol. of a 20 mM sodium phosphate buffer (pH 8.0) containing 10 mM β-mercaptoethanol (Buffer 1). The homogenates were centrifuged at 105,000 *g* for 1 hr at 4° and the cytosols were treated with ammonium sulfate (Schwarz Mann, Orangeburg, NY). The fractions obtained between 33 and 55% saturation were used for mouse liver, Ehrlich ascites cells and Novikoff hepatoma cells, while those obtained between 0 and 55% saturation were utilized with human leukocytes and HeLa (S3) cells; the precipitates were harvested by centrifugation at 16,000 *g* for 20 min at 4°. The pellets were resuspended in Buffer 1 and dialyzed against the same buffer for 4 hr with four changes of the buffer. Protein concentrations were determined by the method of Lowry *et al.* [32], and the dialyzed preparations were stored at −22° until used. Preparations stored in this manner showed no loss of activity over a month.

Inhibition studies. The phosphorolysis of FUdR by the extracts of mouse liver, Ehrlich ascites cells, Novikoff hepatoma cells, and HeLa (S3) cells were assayed in a 1-ml incubation mixture that contained 0.1 M sodium phosphate buffer (pH 6.4), 2.5 mM, β-mercaptoethanol, 1–2 mg protein, and 0.4 mM FUdR, while the inhibitor was dissolved in dimethyl sulfoxide (DMSO) added to a final concentration of either 5 or 10%. Incubations were at 37° for 20 min; the formation of FU and α-D-deoxyribofuranose was linear with time for up to 20 min for all preparations. The reaction was stopped with 1 ml of 10% trichloroacetic acid, and the precipitates were removed by centrifugation. The supernatant fractions were assayed for α-D-deoxyribofuranose with the thiobarbituric acid reaction [33].

The phosphorolytic cleavage of FUdR by extracts of human leukocytes was assayed with a radioisotopic technique. In 100 μl, the incubation mixture contained 0.1 M sodium phosphate buffer (pH 6.4), 2.5 mM, β-mercaptoethanol, 0.4 mM unlabeled nucleoside (final concentration), 20–200 μg protein, the inhibitor in DMSO to yield a final concentration of either 5 or 10%, and 1.5 μCi of [6-^{3}H]FUdR (2.5 Ci/mmole, Amersham Co., Arlington Heights, IL), which was shown by thin-layer chromatography to be greater than 97 per cent pure. The conditions of incubation were the same as those described above, and the reaction was terminated by boiling for 2 min. [6-^{3}H]FU (product) and [6-^{3}H]FUdR (substrate) were separated on silica gel UV_{254} Polygram plates (Brinkman, Westbury, NJ) with chloroform–methanol (9:1). Methanol eluates of the u.v.-absorbing spots were assayed for radioactivity in a liquid scintillation counter.

Table 1. Inhibition of FUdR phosphorolysis by N-1 or C-5 substituted 2,4-pyrimidinediones*

Inhibitor	Source of uridine–deoxyuridine phosphorylase activity	
	Ehrlich ascites cells	Novikoff hepatoma cells
	$[I]_{50}$ (μM)	
1. 1-[1,1'-Biphenyl)-3-yl-methyl]uracil (NSC 210775)	36	39
2. 1-Methyl-5-benzyluracil	26	36
3. 1-(3-Phenoxypropyl)-5-(phenylmethyl)uracil (NSC 210776)	26	22
4. 5-(Phenylthio)uracil (NSC 210778)	24	19
5. 1-(2'-Deoxy-β-D-glucopyranosyl)thymine (NSC 402666)	23	24
6. 1,5-Dibenzyluracil (NSC 210779)	10.3	10.5
7. 5-Benzyluracil	9	10
8. 5-(*m*-Ethoxybenzyl)uracil	2.8	4.8
9. 5-{[3-(Phenylmethoxy)phenyl]methyl}uracil (NSC 210777)	1.6	1

* FUdR (0.4 mM), the inhibitor in either 5 or 10% DMSO, and the enzyme were incubated in the 1-ml reaction mixture for 20 min. Phosphorolysis was measured by the liberation of α-D-deoxyribofuranose from FUdR, and the concentration of the inhibitor required for 50 per cent inhibition of phosphorolysis ($[I]_{50}$) was determined.

RESULTS AND DISCUSSION

The data presented in Tables 1 and 2 show that a reduction in the size and changes in the properties of substituents at N-1, C-5 or C-6 on the 2,4-pyrimidinedione ring strongly influenced the manner in which the activities of the pyrimidine nucleoside phosphorylases were inhibited.

Eight 2,4-pyrimidinedione derivatives, bearing hydrophobic or nonhydrophobic substituents at either N-1 or C-5, or both, which have been shown by Baker and Kelley [19, 22] to be potent inhibitors of the FUdR phosphorylase activity of extracts of Walker-256 rat carcinoma cells, markedly inhibited the phosphorolysis of FUdR by the UR–UdR phosphorylase activities of our Ehrlich ascites and Novikoff hepatoma preparations, as did GPT (Table 1). The most potent of these inhibitors were 5-benzyluracil derivatives substituted with alkoxy groups on the *meta*-position of the benzyl moiety; the most active inhibitor of both enzyme preparations was 5-{[3-(phenylmethoxy)phenyl]methyl}uracil (compound 9, Table 1, Fig. 1). 1-(3-Phenoxypropyl)-5-(phenylmethyl)-uracil (compound 3), however, which is a derivative of 5-benzyluracil with an alkoxy group at N-1, was approximately 24-fold less active than compound 9. Baker and Kelley [19, 22] made similar observations with these compounds, and suggested that C-5 and N-1 aralkyl groups cannot bind hydrophobically to the enzyme at the same time, because the pyrimidine ring rotates to give maximum hydrophobic interaction in only one area of the enzyme [22].

The similarity of the $[I]_{50}$ values for these inhibitors against the Novikoff hepatoma and Ehrlich ascites preparations (Table 1) indicates that the chemical and steric properties of the active sites of these UR–UdR phosphorylases are comparable. This proposal is supported by the competitive inhibition by 1,5-dibenzyluracil [22] of these two pyrimidine nucleo-

Table 2. Inhibition of FUdR phosphorolysis by C-5 or C-6 substituted 2,4 pyrimidinediones*

Inhibitor	Source of pyrimidine nucleoside phosphorylase activity				
	Mouse liver†	Ehrlich ascites cells‡	Novikoff hepatoma cells‡	HeLa (S3) cells†	Human leukocytes†
			$[I]_{50}$(μM)		
6-Aminouracil	280	§	§	350	468
5-Nitrouracil (NSC 9790)	200	70	75	200	278
6-Amino-5-bromouracil	130	350	290	46	88
6-Aminothymine	27	70	100	17	20

* See Table 1 for assay conditions. Phosphorolysis in the human leukocyte preparation was measured by the cleavage of [6-^{3}H] FUdR to [6-^{3}H]FU.

† FUdR cleaved by TdR phosphorylase activity.

‡ FUdR cleaved by UR–UdR phosphorylase activity.

§ Less than 10 per cent inhibition at 0.1 mM.

side phosphorylase activities and a similar ratio of K_m/K_i for each enzyme (P. W. Woodman, A. M. Sarrif and C. Heidelberger, unpublished data).

By contrast, none of the compounds listed in Table 1 inhibited by more than 10 per cent the cleavage of FUdR by the TdR phosphorylase activities of the extracts of mouse liver, HeLa (S3) cells and human leukocytes, even when tested at concentrations of up to 0.1 mM. These findings indicate large differences between the bulk tolerances and hydrophobic bonding regions of the active sites of these UR–UdR and TdR phosphorylases and, in particular, those regions proximal to the N-1 and C-5 positions of the pyrimidinedione ring.

The introduction of bulky, hydrophobic substituents at C-6 of uracil disclosed differences between the chemical and steric properties of pyrimidine nucleoside phosphorylases from prokaryotes and eukaryotes. 6-Anilino and 6-(1-naphthylmethylamino) derivatives of uracil, which were shown by Baker *et al.* [20, 21] to be highly potent inhibitors of the TdR phosphorylase activity of *E. coli*, failed to inhibit by more than 10 per cent (at 0.1 mM) the phosphorolysis of FUdR by either the TdR or UR–UdR phosphorylase activities of our five mammalian preparations (data not shown). The compounds studied were: 6-[(2,3-dimethylphenyl)amino]uracil (NSC 210500), 6-[2,4-dimethylphenylamino]uracil (NSC 210521), 6-[(2,3-dichlorophenyl)amino]uracil (NSC 210497), 6-[(6,7-dichloro-1-naphthalenyl)-methyl]uracil (NSC 210774) and 6-{[6,7-dimethyl-1-naphthalenyl)methyl]amino}uracil (NSC 210773). Our findings are comparable to those of Baker and Kelly [25], in which these inhibitors were bound up to 900 times less effectively by the TdR phosphorylase activity of rabbit liver than by the *E. coli* enzyme. Moreover, these results support the hypothesis [25, 26] that the hydrophobic bonding regions adjacent to the active sites of these enzymes have undergone marked evolutionary changes between species. The importance of using mammalian pyrimidine nucleoside phosphorylases, ideally human, to evaluate potential inhibitors of these enzymes for clinical use is emphasized by these findings.

The introduction of smaller and less hydrophobic substituents on C-6 or C-5, or both, of uracil produced the inhibition of both TdR and UR–UdR phosphorylase activities in our mammalian preparations (Table 2). 6-Aminouracil [25, 26] was a weak inhibitor of TdR phosphorylase activity, but this inhibition was increased up to 8-fold by the introduction of bromine on C-5; moreover, the latter compound (6-amino-5-bromouracil) [10] produced some inhibition of UR–UdR phosphorylase activity (Table 2). The introduction of 6-NH_2 on thymine produced an even more potent inhibitor (6-aminothymine) of TdR phosphorylase activity that could also inhibit UR–UdR phosphorylase activity, but to a lesser degree (Table 2). Similar results for these derivatives have been reported previously by Langen *et al.* [10] for the inhibition of TdR phosphorylase activity of horse liver. By contrast, 5-nitrouracil [25] inhibited UR–UdR phosphorylase activity to approximately the same degree as 6-aminothymine, but was considerably less active against TdR phosphorylase (Table 2). These findings suggest that substitution on both C-5 and C-6 with either small electron-withdrawing groups or those capable of hydrophobic bonding or both could be used in the design of a more potent inhibitor of TdR phosphorylase activity.

To inhibit the phosphorolysis of FUdR and enhance the chemotherapeutic efficacy of the drug in man, it will be important to determine which of the pyrimidine nucleoside phosphorylases are primarily responsible for the cleavage of FUdR. Of those normal and neoplastic human tissues studied so far, the activity found is predominantly that of TdR phosphorylase [34, 35]. If further investigations extend this observation, the present data will be used to guide the design of more potent and more specific inhibitors of this phosphorylase.

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REFERENCES

1. C. Heidelberger, L. Griesbach, O. Cruz, R. J. Schnitzer and E. Grunberg, *Proc. Soc. exp. Biol. Med.* **97**, 470 (1958).
2. C. Heidelberger, in *Cancer Medicine* (Eds. J. F. Holland and E. Frei, III), p. 768. Lea & Febiger, Philadelphia (1973).
3. L. Bosch, E. Harbers and C. Heidelberger, *Cancer Res.* **18**, 335 (1958).
4. N. K. Chaudhuri, B. J. Montag and C. Heidelberger, *Cancer Res.* **18**, 318 (1958).
5. K. U. Hartmann and C. Heidelberger, *J. biol. Chem.* **236**, 3006 (1961).
6. G. D. Birnie, H. Kroeger and C. Heidelberger, *Biochemistry* **2**, 566 (1962).
7. K. L. Mukherjee, J. Boohar, D. Wentland, F. J. Ansfield and C. Heidelberger, *Cancer Res.* **23**, 49 (1963).
8. R. J. Kent, T. A. Khwaja and C. Heidelberger, *J. med. Chem.* **13**, 70 (1970),
9. B. R. Baker, in *Design of Active-Site Directed Irreversible Enzyme Inhibitors*, p. 70. R. E. Kreiger, New York (1975).
10. P. Langen, G. Etzold, D. Barwolff and B. Preussel, *Biochem. Pharmac.* **16**, 1833 (1967).
11. M. Friedkin and H. Kalckar, in *The Enzymes* (Eds. P. E. Boyer, H. Lardy and D. Myrback), Vol. 5, 2nd Edn., p. 237. Academic Press, New York (1961).
12. J. C. Leer, K. Hammer-Jesperson and M. Schwartz, *Eur. J. Biochem.* **75**, 217 (1977).
13. R. Bose and E. W. Yamada, *Can. J. Biochem.* **55**, 528 (1976).
14. P. Langen and G. Etzold, *Biochem. Z.* **339**, 190 (1963).
15. M. Zimmerman, *Biochem. biophys. Res. Commun.* **16**, 600 (1964).
16. P. P. Saunders, B. A. Wilson and G. F. Saunders, *J. biol. Chem.* **244**, 3691 (1969).
17. T. A. Krenitsky, M. Barclay and J. A. Jacquez, *J. biol. Chem.* **239**, 805 (1964).
18. H. Pontis, G. Degerstedt and P. Reichard, *Biochem. biophys. Acta* **51**, 138 (1961).
19. B. R. Baker and J. L. Kelley, *J. med. Chem.* **13**, 458 (1970).
20. B. R. Baker and S. E. Hopkins, *J. med. Chem.* **13**, 87 (1970).
21. B. R. Baker and W. Rzeszotarski, *J. med. Chem.* **11**, 639 (1968).
22. B. R. Baker and J. L. Kelley, *J. med. Chem.* **13**, 461 (1970).

23. B. R. Baker and J. L. Kelley, *J. med. Chem.* **13**, 456 (1970).
24. J. A. Baker and P. V. Chatfield, *J. chem. Soc.* (*C*) No. 18, 2478 (1970).
25. B. R. Baker and J. L. Kelley, *J. med. Chem.* **14**, 812 (1971).
26. B. R. Baker and W. Rzeszotarski, *J. med. Chem.* **10**, 1109 (1967).
27. M. Umeda and C. Heidelberger, *Cancer Res.* **28**, 2529 (1968).
28. L. Hayflick, *Tex. Rep. Biol. Med.* **23**, (suppl. 1), 285 (1965).
29. E. M. Levine, *Meth. Cell Biol.* **8**, 229 (1974).
30. G. J. Todaro, S. A. Aaronson and E. Rands, *Expl Cell Res.* **65**, 265 (1971).
31. J. H. Cutts, in *Methods in Cell Separation Used in Hematology*. Academic Press, New York (1970).
32. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
33. V. S. Waravdekar and L. D. Saslaw, *J. biol. Chem.* **234**, 1945 (1959).
34. M. Zimmerman, *Biochem. biophys. Res. Commun.* **8**, 169 (1962).
35. M. Zimmerman and J. Seidenberg, *J. biol. Chem.* **239**, 2618 (1964).