Irreversible Enzyme Inhibitors. CLXIX.[1,2] Inhibition of FUDR Phosphorylase from Walker 256 Rat Tumor by 6-Substituted Uracils

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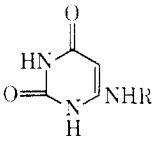
The Walker 256 rat tumor has been found to contain a uridine phosphorylase that can cleave FUDR to 5-fluorouracil. Inhibition of this enzyme by 35 6-substituted uracils was studied. The presence of a hydrophobic bonding region on the enzyme adjacent to the active site was detected, but hydrophobic interaction was weak. The best 6-substituted uracil found in this series was the phenylamylamino derivative (**35**), which complexed to the enzyme 33-fold better than uracil, but only 4-fold better than the substrate, FUDR.

Thymidine phosphorylase from *Escherichia coli* B[3] can phosphorylyze thymidine,[3] 2′-deoxyuridine,[3] and FUDR[4] (5-fluoro-2′-deoxyuridine) to the corresponding base and 2′-deoxy-α,D-ribofuranosyl 1-phosphate in a reversible reaction. In a series of 11 papers, systematic studies have led to highly potent reversible inhibitors of the *E. coli* B enzyme; these studies included the mode of pyrimidine binding,[5] the location of hydrophobic bonding areas with 1-substituted[6] or 6-substituted uracils,[7-10] the effect of the acidity of the substituted uracils on binding,[11,12] and irreversible inhibition.[13] Among the best inhibitors found were **14**,[9] **16**,[9] and **23**[10] which were complexed 450-, 1100-,



14, R = 2,3-$(CH_3)_2C_6H_3$
16, R = 2,3-$Cl_2C_6H_3$
23, R = α-$C_{10}H_7CH_2$

and 2000-fold better, respectively, than the substrate, FUDR; the most potent compound found was the 7-chloro derivative[10] of **23** which complexed to the enzyme 5000-fold better than FUDR.[14] These 6-substituents were complexed to a hydrophobic bonding region on the enzyme.

The next phase was to study the reversible inhibition of an enzyme in a rat tumor that could cleave FUDR to FU; such an enzyme has been found in the Walker 256 rat tumor.[16] However, in contrast to *E. coli*, there are two enzymes in mammalian sources that can cleave FUDR to FU. Mammalian uridine phosphorylase is less specific than *E. coli* uridine phosphorylase since the mammalian enzyme can also cleave thymidine and 2′-deoxyuridine,[16-21] but the *E. coli* enzyme cannot.[3,22] Both mammalian[16-21] and *E. coli*[3] thymidine phosphorylases are highly specific for 5-R-uracil-2′-deoxyribosides where R = H, CH_3, or halogen, but do not cleave uridine.

The Walker 256 enzyme has been shown to be a uridine–deoxyuridine phosphorylase (EC 2.4.2.3).[16,18] It is not surprising in retrospect that **14**, **16**, and **23** were relatively poor inhibitors of the Walker 256 uridine phosphorylase: they only complexed about as effectively as the substrate, FUDR. Therefore, it was necessary to completely reinvestigate what type of substituted uracils might give a strong hydrophobic interaction with the Walker 256 uridine phosphorylase cleaving FUDR to FU. Inhibition of the Walker 256 FUDR phosphorylase by 6-substituted uracils was investigated and the results are the subject of this paper; in the papers that follow, inhibition by 1-substituted[23] and 5-substituted uracils[24] are reported. By proper choice of substituents and position on uracil, inhibitors of the Walker 256 FUDR phosphorylase have been found that complex 800-fold better than the substrate FUDR.[24]

Enzyme Results.—The parent uracil (**1**) was complexed to the Walker 256 enzyme 7-fold less effectively than the substrate, FUDR (Table I). Introduction of a 6-amino group gave **2** which was slightly less ef-

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(2) For the previous paper of this series see B. R. Baker, N. M. J. Vermeulen, and A. J. Ryan, *J. Med. Chem.*, **13**, 280 (1970).

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(7) B. R. Baker and M. Kawazu, *ibid.*, **10**, 311 (1967), paper LXXVIII of this series.

(8) B. R. Baker and W. Rzeszotarski, *ibid.*, **10**, 1109 (1967), paper CIV of this series.

(9) B. R. Baker and W. Rzeszotarski, *ibid.*, **11**, 639 (1968), paper CXXI of this series.

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(11) B. R. Baker and M. Kawazu, *ibid.*, **10**, 316 (1967), paper LXXX of this series.

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(13) B. R. Baker and M. Kawazu, *ibid.*, **56**, 1086 (1967), paper C of this series.

(14) For the chemotherapeutic utility of a selective blockade of this enzyme see ref 4 and 15.

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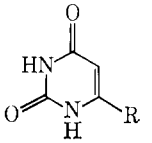
(23) B. R. Baker and J. L. Kelley, *J. Med. Chem.*, **13**, 458 (1970), paper CLXX of this series.

(24) B. R. Baker and J. L. Kelley, *ibid.*, **13**, 461 (1970), paper CLXXI of this series.

TABLE I

INHIBITION[a] OF WALKER 256 FUDR PHOSPHORYLASE BY

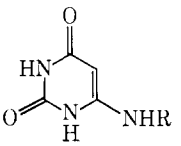


No.	R	I_{50}, μM[b,c]	No.	R	I_{50}, μM[b,c]
1	H	2900	20[l]	$NHCH_2C_6H_4$-2-Cl	240
2	NH_2	4000	21[l]	$NHCH_2C_6H_4$-3-Cl	330
3[d]	$CH_2C_6H_5$	4400	22[l]	$NHCH_2C_6H_4$-4-Cl	500
4[e]	COC_6H_5	>8000[f]	23[n]	$NHCH_2$-α-$C_{10}H_7$	240
5[e]	OC_6H_5	8100	24	$NHCH_2C_6H_5$-5-Br[i]	550
6[e]	SC_6H_5	>2000[f]	25[l]	$N(CH_3)CH_2C_6H_5$	1500[m]
7[e]	$SO_2C_6H_5$	2600	26[l]	$NHCH(CH_3)C_6H_5$	8000[m]
8[e]	NHC_6H_5	>2000[f]	27[l]	$NHCH(C_6H_5)_2$	380
9[g]	$CH_2C_6H_4NO_2$-*m*	>2000[f]	28[l]	$N(CH_2C_6H_5)_2$	410
10[h]	$CH_2C_6H_5$ (2-thio)[i]	>4000[f]	29[l]	$NHCH(C_6H_5)CH_2C_6H_5$	1500
11[i]	$CH_2C_6H_5$-5-Br[i]	330	30[e]	$NH(CH_2)_2C_6H_5$	1600
12[i]	$CH_2C_6H_4NO_2$-*m*-5-Br[i]	150	31	$NH(CH_2)_3C_6H_5$	850[m]
13[k]	*n*-C_5H_{11}-5-$C_6H_5N{=}N$[i]	570	32	$NH(CH_2)_4C_6H_5$	280
14[l]	NHC_6H_3-2,3-Me_2	600[m]	33	$NH(CH_2)_3OC_6H_5$	200
15[l]	NHC_6H_3-2,6-Me_2	3500	34	$NH(CH_2)_4OC_6H_5$	>280[f]
16[l]	NHC_6H_3-2,3-Cl_2	400[m]	35	$NH(CH_2)_5C_6H_5$	86
17[d]	$(CH_2)_2C_6H_5$	2700	36	NHC_4H_9-*n*	1900
18[d]	$(CH_2)_3C_6H_5$	1800	37	NHC_5H_{11}-*n*	390
19[l]	$NHCH_2C_6H_5$	400			

[a] The technical assistance of Maureen Baker and Julie Leseman is acknowledged. [b] I_{50} = concentration for 50% inhibition when assayed with 400 μM FUDR in pH 5.9 arsenate–succinate buffer containing 10% DMSO as previously described.[4] [c] See Experimental Section. [d] Synthesis: ref 7. [e] Synthesis: ref 8. [f] No inhibition at the maximum solubility which is one fourth of the concentration indicated. [g] Synthesis: ref 13. [h] Synthesis: ref 5. [i] Uracil substituent. [j] Synthesis: ref 11. [k] Synthesis: ref 12. [l] Synthesis: ref 9. [m] Estimated from the inhibition observed at the maximum solubility which is lower. [n] Synthesis: ref 10.

TABLE II

PHYSICAL PROPERTIES OF



No.	R	Reaction time, hr[a]	Amine ratio[b]	% yield[c]	Mp, °C, dec	Formula[d]
31	$(CH_2)_3C_6H_5$	22	1	17[e]	276–280	$C_{13}H_{15}N_3O_2$
32	$(CH_2)_4C_6H_5$	16	4	15[e]	240–246	$C_{14}H_{17}N_3O_2$
33	$(CH_2)_3OC_6H_5$	43[f]	1.3	18[e]	239–247	$C_{13}H_{15}N_3O_3$
34	$(CH_2)_4OC_6H_5$	20[g]	2	27[h]	264–266	$C_{14}H_{17}N_3O_3$
35	$(CH_2)_5C_6H_5$	24[i]	1.5	5[j]	264–266	$C_{15}H_{19}N_3O_2$
36	C_4H_9-*n*	22	2	13[k]	273–274[l]	
37	C_5H_{11}-*n*	14	2	11[m]	267–268	$C_9H_{13}N_3O_2$

[a] All compounds were made using method B of ref 8. [b] Ratio of amine to 6-chlorouracil. [c] Yields are for analytically pure material and are minimum. [d] All compounds were analyzed for C, H, and N. [e] Recrystallized from MeOEtOH. [f] For starting amine see B. R. Baker and J. L. Kelley, *J. Med. Chem.*, **12**, 1039 (1969). [g] For starting amine see D. G. Doherty, R. Shapira, and W. T. Burnett, Jr., *J. Amer. Chem. Soc.*, **79**, 5667 (1957). [h] Recrystallized from DMF. [i] For starting amine see N. K. Kochetkov and N. V. Dudykina, *Zh. Obshch. Khim.*, **28**, 2399 (1958). [j] Recrystallized from MeOEtOH–H_2O. [k] Recrystallized from EtOH–MeOEtOH. [l] Mp 265° (dec) reported for this compound by a different method; S. Kuwada, T. Masuda, T. Kishi, and M. Asai, *Chem. Pharm. Bull.* (Tokyo), **8**, 798 (1960). [m] Recrystallized from H_2O–EtOH.

fective than **1**. Bridging of a Ph group to the 6 position of uracil by CH_2 (**3**), O (**5**), S (**6**), SO_2 (**7**), or NH (**8**) gave no increase in binding to the enzyme. Substitution of a *m*-NO_2 (**9**) on 6-benzyluracil (**3**) gave no appreciable increment in binding. However, substitution of 5-Br (**11**) on **3** gave a 13-fold increment in binding and similar results were seen with **12** *vs.* **9**; this enhanced binding is probably due to the increased acidity of the uracil ring caused by the 5-Br atom,[11,12] or hydrophobic bonding by Br, or both.

Substitution on the Ph group of 6-anilinouracil (**8**) by 2,3-Me_2 (**14**), or 2,3-Cl_2 (**16**) gave a >5-fold increment in binding, indicating a hydrophobic interaction with the enzyme; the 2,6-Me_2 substituents (**15**) were considerably less effective.

When the benzyl group of **3** was increased in size to phenethyl (**17**) or phenylpropyl (**18**) binding was enhanced only about 2-fold.

6-Benzylaminouracil (**19**) was 5-fold more effective than uracil (**1**). Substitution on the benzyl group by Cl (**20**–**22**) gave no enhancement in binding; a similar result occurred when the benzyl group of **19** was replaced with α-naphthyl (**23**). Introduction of 5-Br (**24**), or alkyl, aralkyl, or aryl on the α-C or the N

(**25–29**) also failed to enhance binding. The phenylamyl group of **35** was 5-fold more effective in binding than the benzyl group of **19**, but shorter alkyl bridges (**30–32**) or oxyalkyl bridges (**33**, **34**) were less effective than **35**. The *n*-AmNH substituent of **37** was just as effective as the benzylamino group of **19**, again indicating a hydrophobic interaction; the *n*-BuNH group (**36**) was 5-fold less effective.

6-Aminouracils substituted by hydrocarbon groups are excellent inhibitors of the *E. coli* B thymidine phosphorylase cleaving FUDR to FU that can bind to this enzyme 1100–5000 times more effectively[9,10] than the substrate. In contrast only weak hydrophobic interaction was seen with these compounds on the Walker 256 enzyme, a uridine phosphorylase that can cleave FUDR to FU. This weak interaction indicated that a hydrophobic bonding region was present on the Walker 256 enzyme, but that 6 substituents on the uracil could not properly orient for strong interaction. Therefore studies were turned to hydrophobic groups attached to the 1 or 5 positions;[23,24] excellent inhibitors of the Walker 256 enzyme emerged that could bind 100- to 1000-fold better than the substrate.

Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. All analytical samples had proper uv and ir spectra and moved as a single spot on tlc on Brinkmann silica gel GF; each gave combustion values for C, H, and N within 0.3% of theoretical.

6-Benzylamino-5-bromouracil (24).—To a stirred mixture of 0.217 g (1.0 mmol) of **19**[8] and 2 ml of AcOH at ~70° was added 0.351 g (1.1 mmol) of pyridinium hydrobromide perbromide. A clear solution formed which soon deposited white crystals. After 0.5 hr the mixture was cooled and diluted with 10 ml of H_2O. The product was collected, washed with H_2O, recrystallized twice from MeOEtOH, and then from AcOH; yield, 0.121 g (41%) of off-white clusters which had no definite melting point but moved as a single spot on tlc in 1:5 AcOH–C_6H_6 or 1:3 EtOH–C_6H_6. *Anal.* ($C_{11}H_{10}BrN_3O_2$) C, H, N.

FUDR Phosphorylase.—This enzyme was present in the 0–45% $(NH_4)_2SO_4$ fraction of extracts of Walker 256. The enzyme in this fraction was stable for at least several months at −15° when stored in 1.8-ml aliquots sufficient for 1 day of use. The assay was performed with pH 5.9 arsenate–succinate as previously described,[14] sufficient enzyme and time being used to give about 0.15 O.D. change in the controls.

Irreversible Enzyme Inhibitors. CLXX.[1,2] Inhibition of FUDR Phosphorylase from Walker 256 Rat Tumor by 1-Substituted Uracils

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1-Substituted uracils (44) were investigated as inhibitors of Walker 256 uridine-deoxyuridine phosphorylase (EC 2.4.2.3) which can also cleave FUDR to 5-fluorouracil. A good hydrocarbon interaction was seen with benzyl (**10**), phenylbutyl (**13**), or phenoxybutyl (**18**) substituents. Further enhancement of binding of the benzyl group was achieved with *m*-OR substituents, the best binding being observed by *m*-OC_2H_5 (**32**) and *m*-$C_6H_5(CH_2)_nO$ (**33–37**) groups; **32–37** were complexed to the enzyme about 300-fold better than the parent uracil and about 40-fold better than the substrate, FUDR.

6-Arylamino-[3] and 6-arylmethylaminouracils[3,4] are excellent inhibitors of FUDR phosphorylase from *Escherichia coli* B[5] due to a hydrocarbon interaction of the aryl or aralkyl group with the enzyme; these compounds were much less effective on the FUDR phosphorylase from Walker 256 rat tumor, although weak hydrocarbon interaction was seen.[2] Since it appeared that a hydrocarbon group bridged to the 6 position of uracil could not orient for maximum hydrophobic bonding,[2] attention was turned to possible hydrocarbon interaction from 1-substituted and 5-substituted uracils. Some excellent inhibitors have emerged in both areas; the inhibition of the uridine–deoxyuridine phosphorylase (EC 2.4.2.3) from Walker 256 rat tumor with 1-substituted uracils is the subject of this paper.[6]

Enzyme Assays.—In Table I uracil (**1**) has I_{50} = 2900 μM.[2] Introduction of a 1-Me (**2**) substituent gave no loss in binding on this Walker 256 uridine phosphorylase; this result should be contrasted to the result with *E. coli* B thymidine phosphorylase where **2** was 33-fold less effective than uracil (**1**), indicating that the 1-H was a binding point to the *E. coli* enzyme, but not the Walker 256 enzyme.

Hydrophobic bonding was seen with higher alkyl groups (**4–7**), the maximum increment being about 15-fold compared to 1-methyluracil (**2**). Ring substituents were detrimental to binding; about a 2-fold loss in binding compared with **2** occurred with cyclopentyl (**8**) and a >6-fold loss with Ph (**9**).

Hydrocarbon interaction by aralkyl groups was then studied. 1-Benzyl (**10**) gave a 24-fold increment in binding compared with **2**, but phenethyl (**11**) and phenylpropyl (**12**) were considerably less effective. Activity maximized again at phenylbutyl (**13**), which was 80-fold more effective than **2**; phenylamyl (**14**) and phenylhexyl (**15**) were about 2-fold less effective than **13**. With the phenoxyalkyl group (**16-19**),

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series see B. R. Baker and J. L. Kelley, *J. Med. Chem.*, **13**, 456 (1970).

(3) B. R. Baker and W. Rzeszotarski, *ibid.*, **11**, 639 (1968), paper CXXI of this series.

(4) B. R. Baker and S. E. Hopkins, *ibid.*, **13**, 87 (1970), paper CLXVII of this series.

(5) B. R. Baker, *ibid.*, **10**, 297 (1967), paper LXXV of this series.

(6) For the chemotherapeutic utility of selective inhibitors of this enzyme see (a) ref 5, and (b) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," John Wiley & Sons, New York, N. Y., 1967, pp 79–81.