Metabolism of Uracil and 5-Fluorouracil in P388 Murine Leukemia Cells

DAVID KESSEL,[1] THOMAS C. HALL,[1] AND PHILIP REYES

Department of Pharmacology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14620, and Laboratories of Pharmacology, Children's Cancer Research Foundation, Boston, Massachusetts 02115

(Received May 24, 1969)

SUMMARY

The drug 5-fluorouracil shows a high degree of antitumor activity against both the P388 murine leukemia and the P388/38280 subline. Although previous studies had shown that the capacity of cell lines to convert 5-fluorouracil to its nucleotides *in vitro* was a major determinant of drug responsiveness, the P388/38280 cell line had a very low capacity for this conversion. Moreover, conversion of uracil to nucleotides could not be detected in P388/38280 cells. Enzymatic studies showed that P388/38280 cells have a very low level of uridine phosphorylase (EC 2.4.2.3) activity. In both P388 and P388/38280 cells, however, pyrimidine 5'-phosphoribosyltransferase activity could be demonstrated. The latter enzyme catalyzes the formation of 5-fluorouridylic acid from 5-fluorouracil and 5-phosphoribosyl 1-pyrophosphate. Uracil was a very poor substrate for the transferase. These data suggest that the total capacity of tumor cells for 5-fluorouracil nucleotide formation is not necessarily the major determinant of drug responsiveness.

INTRODUCTION

The conversion of 5-fluorouracil to nucleotide derivatives by tumor cells is a requisite for the antineoplastic action of the drug, and impairment of this conversion is associated with development of drug-resistant cell lines (1, 2). The most potent such derivative is 5-fluoro-2'-deoxyuridine 5'-monophosphate, which strongly inhibits thymidylate synthetase (3, 4). FU[2] is also converted into ribonucleotides and incorporated into RNA (2); the contribution of these transformations to the pharmacological action of FU is unknown, although there is evidence (5) that their major contribution is to host toxicity. FU was found to be anabolized by the enzymes responsible for the analogous biotransformations of uracil (2).

In previous studies on different murine leukemias, we found that the capacity of freshly isolated tumor cells to convert FU into nucleotides was correlated with responsiveness to FU (6). It was surprising, therefore, to find a subline of the P388 murine leukemia which showed a low capacity for nucleotide formation from FU (and almost no capacity for conversion of uracil to nucleotides), but which was as responsive to FU as the P388 parent cell line. Our studies on pyrimidine metabolism by P388 and the subline (P388/38280) are reported here.

This work was supported by Contracts PH 43-66-541 and NIH 69-39 with the Cancer Chemotherapy National Service Center, National Cancer Institute, and by Grant C6516 from the National Institutes of Health.

[1] To whom requests for reprints should be addressed, at the University of Rochester.

[2] The abbreviations used are: FU, 5-fluorouracil; UR, uridine; UdR, deoxyuridine; FUR, 5-fluorouridine; FUMP, 5-fluorouridylic acid; FUdR, 5-fluoro-2'-deoxyuridine; dFUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate.

MATERIALS AND METHODS

Studies on intact cells. The P388/38280 cell line was originally derived from the P388 murine leukemia by selection for resistance to a terephthalanilide (7). Sources of this subline and of the P388 murine leukemia have been described (8), as have methods for cell isolation (6, 9). P388 and P388/38280 cells were carried in DBA/2 or BDF_1 mice by intraperitoneal inoculation of 10^6 cells; ascitic tumor was collected 7 days after transplant. The original cell lines were provided by Mr. I. Wodinsky, Arthur D. Little Corporation, Cambridge, Massachusetts.

Cell suspensions (5% by weight) were prepared in a mixture of 3 parts minimal essential Eagle's medium (Grand Island Biological Company), 1 part horse serum, and 1 part 150 mM sodium phosphate buffer at pH 7.4. The suspensions were divided into 200-μl aliquots, which were incubated for 2, 5, 10, and 20 min at 37° with labeled substrates (usually 0.1 mM). Incubations were terminated by chilling the tubes, and the cells were collected by centrifugation at $500 \times g$ for 30 sec. The acid-soluble pool of nucleotides was extracted with three 0.5-ml portions of 10% $HClO_4$ at 0°. The $HClO_4$ extract was neutralized with KOH, the precipitate of $KClO_4$ was removed, and the supernatant fluid was taken to dryness under vacuum. The residue was taken up in 25 μl of water, and this solution was subjected to thin layer chromatography on cellulose sheets (Eastman No. 6064) to separate nucleotides from other labeled components (10). Regions of the chromatogram corresponding to the nucleotides were located under ultraviolet light, with the aid of chromatographic markers. These regions were cut out from the chromatograms, placed in vials, and moistened with an appropriate phosphor solution for determination of radioactivity by liquid scintillation counting (6).

The acid-insoluble material was washed twice each with 1 ml of ethanol, 1 ml of ethanol–ether (3:1, v/v), and 0.5 ml of ether. The defatted residue was taken up in 0.5 ml of 0.5 N KOH; after 24 hr at 24° this solution was neutralized with 2 M $HClO_4$, the precipitate of $KClO_4$ was removed by centrifugation, and the supernatant fluid was dried, redissolved, and subjected to chromatographic analysis as described above.

The data derived from studies on the acid-soluble extracts of cells were used to estimate the rate of conversion of labeled substrates to nucleotides. The product of alkaline hydrolysis of the acid-insoluble fraction was chromatographed with UMP, and taken to represent nucleotide released from cellular RNA. The rate of incorporation of label into this fraction was taken to indicate the corresponding rate of incorporation of substrate into cellular RNA.

Preparation of cell-free extracts. Freshly isolated P388 and P388/38280 cells were suspended in 1.5 volumes of 0.05 M Tris buffer at pH 8, containing 1 mM EDTA and 5 mM 2-mercaptoethanol. These suspensions were frozen rapidly, stored at —20° overnight, and then placed in a 10° water bath. The thawed suspensions were treated with a sonic oscillator for 10 sec to disperse the cellular material and then centrifuged at $100{,}000 \times g$ for 30 min at 5°. The supernatant fluid was retained; this could be stored at —20° for several weeks without loss of activity of any enzyme measured here.

Measurement of uridine phosphorylase (EC 2.4.2.3), uridine kinase (EC 2.7.1.48), and thymidine kinase (EC 2.7.1.21). These enzymes were measured simultaneously by a modification of the procedure of Skold (11). Cell-free extracts, obtained as described above, were used. Incubation mixtures contained 150–250 μg of enzyme protein, 5 mM ATP, 6 mM $MgCl_2$, 15 mM KH_2PO_4, and 60 mM Tris. The last two solutions were adjusted to pH 7.4. The total incubation volume was 50 μl. Labeled nucleoside (UR, UdR, FUR, or FUdR) was added to achieve a 1 mM level. Enzyme was omitted from control tubes. Incubations were carried out at 37° for 10 min, a time found to yield data during the linear period of enzymatic activity. To terminate the incubations, 5 μl of 0.1 N acetic acid were added. The acid solution contained, at 100

mM levels, nonlabeled materials which served as chromatographic markers, i.e., the free pyrimidine base, the nucleoside employed in the incubation mixture, and the phosphorylated product. (For example, incubations with labeled FUdR were terminated by addition of acid containing FU, FUdR, and dFUMP.) Protein was precipitated by warming the tubes to 100° briefly in a water bath and removed by centrifugation. From each incubation tube, a 25-μl aliquot was applied to a 5 × 30 mm region of a thin layer cellulose sheet (Eastman No. 6064), and the sheets were developed in appropriate solvents capable of separating the nucleoside from the pyrimidine base and the nucleotide. With the aid of the applied markers, the three labeled components of the mixtures were located under ultraviolet light; they were cut out from the sheets, and their radioactivity was measured by liquid scintillation techniques. The quantities of radioactively labeled pyrimidines present in the nucleoside substrates as impurities were determined from control, enzyme-free incubations.

Chromatographic solvents. To resolve mixtures of uracil, UR, and UMP, a water–ammonia–1-butanol solvent (2:1:18, by volume) was used. The mixture FU–FUR–FUMP was resolved in water–formic acid–1-butanol (13:10:77, by volume). Mixtures of uracil–UdR–dUMP or FU–FUdR–dFUMP were resolved in 1 M ammonium acetate at pH 9 saturated with sodium borate and containing 10 mM EDTA–90% ethanol (30:70 v/v). The crystalline precipitate was removed by decantation 1 hr after preparation of the last-mentioned solvent. The use of these and similar solvent mixtures in thin layer chromatography of pyrimidine derivatives has been described (10).

Measurement of pyrimidine 5′-phosphoribosyltransferase activity. Cell extracts (150–250 μg of enzyme protein) were incubated with 2 mM 5-phosphoribosyl 1-pyrophosphate, 2 mM $MgCl_2$, 60 mM Tris buffer (pH 7.5), and 0.5 mM labeled uracil or FU in a total volume of 50 μl. After incubation for 20 min at 37°, the mixture was chilled, and a 25-μl aliquot was immediately applied to a 2-cm circle of DEAE-paper (H. Reeve Angel & Company). Non-ionized radioactive substrate was removed by washing the discs as described by Breitman (12), except that 0.01 M citric acid was used for washing. The discs were finally rinsed in water and acetone and dried in air. Alternatively, 25-μl aliquots were applied to the origin of sheets of DEAE-paper (5 × 100 cm) which had been equilibrated for 24 hr over formic acid (13). The strips were developed with water, and a 2-cm circle was cut from the origin. The dried DEAE-paper discs from either procedure were placed on the bottoms of vials and covered with 5 ml of a phosphor solution (9) for determination of radioactivity by liquid scintillation counting. The product of this reaction has been characterized as the nucleoside 5′-monophosphate of the pyrimidine employed (13).

Substrates. Uracil-2-^{14}C (20 mCi/mmole), uridine-2-^{14}C (25 mCi/mmole), and generally labeled deoxyuridine-^{3}H (5 Ci/mmole) were purchased from New England Nuclear Corporation. The labeled UdR was purified by chromatography on Eastman No. 6064 thin layer cellulose sheets to remove uracil, which gradually formed during storage. The solvent system was ethyl acetate–formic acid–water (60:5:35, by volume; upper layer). 5-Fluorouracil-2-^{14}C (10 mCi/mmole), 5-fluorouridine-2-^{14}C (10 mCi/mmole), and generally labeled 5-fluoro-2′-deoxyuridine-^{3}H (2 Ci/mmole) were purchased from Calbiochem. Nonlabeled FU, FUR, and FUdR were supplied by Hoffmann-La Roche, and dFUMP by Dr. Charles Heidelberger. Other chemicals were purchased from Mann Research Laboratories, Sigma Chemical Company, and Calbiochem.

RESULTS

Nucleotide formation by intact cells. When intact P388 or P388/38280 cells were incubated with labeled UR or FUR, these nucleosides were readily converted to nucleotides and incorporated into RNA (Table 1). Studies on FU showed that the P388/38280 cells had only a limited capac-

TABLE 1

Conversion of pyrimidines to ribonucleotides and RNA by intact cells

Cells were incubated with 0.1 mM labeled substrate, and the initial rate of incorporation of label into ribonucleotides and RNA fractions was measured. Data represent the means of five experiments, which varied by no more than ±10%.

Substrate	P388 cells		P388/38280 cells	
	Nucleotides	RNA	Nucleotides	RNA
	mμmoles incorporated/g cells/hr			
Uracil	2100	600	12	0
Uridine	3000	900	1500	480
5-Fluorouracil	2700	780	540	180
5-Fluorouridine	2400	660	2400	720

ity for nucleotide formation from this compound; almost no conversion of uracil into nucleotides by this subline could be found (Table 1). These data suggest that conversion of uracil to nucleosides is blocked in P388/38280 cells, and that conversion of FU to nucleosides might be partly blocked. The finding that the P388/38280 cell line could convert FU, but not uracil, to nucleotides required further explanation.

In another experiment, using intact P388 cells, we found that addition of a 10-fold excess of nonlabeled FU or FUR significantly decreased the extent of conversion of labeled FU to labeled nucleotides (Table 2). This suggests a rapid interconversion of FU and FUR in P388 cells. In contrast, addition of nonlabeled FUR did not affect the conversion of labeled FU to nucleotides in P388/38280 cells, although nonlabeled FU did decrease the extent of this conversion. This experiment provides additional evidence that the FU-FUR interconversion is blocked in the P388/38280 subline, and suggests that conversion of FU to nucleotides in P388/38280 cells might not involve intermediate formation of the nucleoside, FUR.

TABLE 2

Effect of FUR on FU incorporation into nucleotides

Cells were incubated with 0.1 mM FU-^{14}C[a] and, where indicated, 1 mM nonlabeled FU or FUR for 10 min at 37°. Total incorporation of label into the acid-soluble nucleotide pool was measured. Results are shown for a typical experiment.

Addition	Total radioactivity in nucleotide fraction	
	P388 cells	P388/38280 cells
	cpm	*cpm*
None	15,100	3,100
FU	1,750	290
FUR	1,850	2,900

[a] Under the conditions used, 1 mμmole of FU-^{14}C = 4500 cpm.

Studies on cell-free extracts. To estimate relative levels of the enzymes responsible for conversion of uracil and FU to nucleotides, cell extracts were incubated with labeled nucleosides, ATP, inorganic phosphate, and magnesium ions. The simultaneous formation of the pyrimidine base and the nucleotide was measured. In preliminary studies, we found that omission of ATP prevented nucleotide formation, and that omission of inorganic phosphate inhibited degradation of the nucleoside to the pyrimidine.

The findings from these experiments are summarized in Table 3. Nucleotide formation from UR (via uridine kinase) and from UdR and FUdR (via thymidine kinase) could readily be demonstrated in both P388

TABLE 3

Nucleoside conversion to pyrimidines and nucleotides

Cell extracts were incubated with labeled substrates at 1 mM, together with $MgCl_2$, ATP, inorganic phosphate, and Tris buffer as described in the text. Initial rates of product formation were calculated from chromatographic analysis of the reaction mixtures. Results of a typical series of experiments are shown.

Labeled substrate	Pyrimidine formation		Nucleotide formation	
	P388 cells	P388/38280 cells	P388 cells	P388/38280 cells
	μmoles/g protein/hr			
UR	102	6	66	66
UdR	33	1.2	14.5	8.2
FUR	105	6	105	81
FUdR	39	1.2	9	5.4

and P388/38280 cells. Phosphorolytic cleavage of these nucleosides was markedly impaired in the P388/38280 cell line.

Extracts of both P388 and P388/38280 cells were found to be capable of catalyzing a reaction between uracil or FU and 5-phosphoribosyl 1-pyrophosphate to form the nucleoside 5'-monophosphate (Table 4). The reaction rates with the two different substrates were not altered when the source of enzyme was changed (P388 vs. P388/38280), but the rate was about 25 times more rapid with FU than with uracil. This enzymatic pathway is described in detail elsewhere (13).

TABLE 4

Pyrimidine 5'-pyrophosphorylase activity in cell extracts

Cell extracts were incubated with labeled FU or uracil, 5-phosphoribosyl 1-pyrophosphate, $MgCl_2$, and Tris buffer as described in the text, and the rate of FUMP or UMP formation was measured. The results shown are the means of five experiments.

Substrate	P388 cells	P388/38280 cells
	μmoles product/g protein/hr	
FU	7.5	7.8
Uracil	0.3	0.36

Drug responsiveness in vivo. The therapeutic effectiveness of FU and FUdR against P388 and P388/38280 cells was evaluated by treatment of tumor-bearing animals with these drugs. Both tumor and agent were inoculated intraperitoneally. The drug was administered from day 1 to day 10 following transplant of 10^6 tumor cells. The life span of P388-bearing animals was increased 122% by treatment with 25 mg/kg of FU, and 104% by 80 mg/kg of FUdR. The life span of animals bearing the P388/38280 leukemia was increased 96% by FU and 59% by FUdR.[3]

[3] Data on drug-promoted survival of tumor-bearing animals were provided by Mr. I. Wodinsky, Arthur D. Little Corporation, Cambridge, Massachusetts.

DISCUSSION

The data presented here, together with those available elsewhere (1, 2, 13, 14), indicate that conversion of FU to dFUMP can occur by at least the following three routes.

$$\text{FU} + \text{ribose-1-P} \xleftrightarrow{\text{uridine phosphorylase}} \text{FUR} \xrightarrow{\text{uridine kinase}} \text{FUMP} \rightarrow\rightarrow \text{dFUMP} \quad (1)$$

$$\text{FU} + \text{deoxyribose-1-P} \xrightarrow{\text{uridine phosphorylase}} \text{FUdR} \xrightarrow{\text{thymidine kinase}} \text{dFUMP} \quad (2)$$

$$\text{FU} + \text{phosphoribosyl-PP} \xrightarrow{\text{pyrimidine 5'-phosphoribosyltransferase}} \text{FUMP} \rightarrow\rightarrow \text{dFUMP} \quad (3)$$

Our previous studies (6) had shown that the capacity of different murine leukemias to convert FU to nucleotides was an important determinant of drug responsiveness. We would therefore have predicted that the P388/38280 cell line should be relatively unresponsive to FU. But direct experiments have shown the line to be as drug-sensitive as P388. Moreover, the conversion of uracil to nucleotides was almost undetectable in P388/38280, although the conversion of FU was clearly demonstrable. This was surprising, since previous investigations on FU (summarized in ref. 2) suggested that enzymes responsible for uracil anabolism carry out the analogous reactions of FU. We discuss this point further below, however.

In a related series of experiments, Reyes and Hall (15) found that the activity of a pyrimidine 5'-pyrophosphorylase, which catalyzes reaction 3 above, was correlated with responsiveness to FU in different murine leukemias. Impairment of this enzyme in a FU-resistant cell line was initially reported by Kasbekar and Greenberg (14). Both the P388 and P388/38280 cell lines contained equivalent levels of phosphoribosyltransferase.

Reyes (13) has presented evidence to suggest that a single phosphoribosyltransferase, found in murine leukemia cells, can utilize uracil, FU, or orotic acid as substrate. This enzyme is probably different from the orotidylate phosphoribosyltransferase (EC 2.4.2.10) described by others (16, 17).

The basis for the impaired capacity of P388/38280 cells to form nucleotides from uracil and FU was found to be the very low level of uridine phosphorylase activity. This enzyme was described in Ehrlich ascites tumor cells by Krenitsky *et al.* (18, 19) and is responsible for the degradation of UR, UdR, FUR, FUdR, and thymidine (20). In certain tissues, both "uridine phosphorylase" and "thymidine phosphorylase" activities are found (18, 19, 21–24). The former enzyme shows broad activity toward pyrimidine nucleosides; the latter was found to be specific for the deoxynucleoside linkage. Thymidine phosphorylase activity was not found in the Ehrlich carcinoma (18, 19), and is apparently not found in P388. Therefore, the deletion of uridine phosphorylase in P388/38280 accounts for the inability of this cell line to carry out interconversions of uracil and FU to ribonucleosides and deoxyribonucleosides. In other studies, we have found that P388/38280 cells cannot convert thymidine to thymine.

The present data suggest that the total capacity of tumor cells for FU nucleotide formation is not necessarily a major determinant of drug responsiveness in murine leukemias. Although dFUMP is the most potent known antimetabolic derivative of FU (2), it must be admitted that the mode of action of the drug is not yet entirely understood, nor have all pathways of dFUMP formation been elucidated. Studies on murine leukemias lacking different enzymes involved in FU metabolism may shed more light on this problem. Because of the block between FU and FUdR in P388/38280, this cell line might be useful in delineating different biochemical modes of action of the two drugs, which have recently been described (25).

REFERENCES

1. R. W. Brockman, *Advan. Cancer Res.* **7**, 129 (1963).
2. C. Heidelberger, *Progr. Nucleic Acids Mol. Biol.* **4**, 1 (1965).
3. S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb and J. Lichtenstein, *Proc. Nat. Acad. Sci. U. S. A.* **44**, 1004 (1958).
4. N. K. Chaudhuri, B. J. Montag and C. Heidelberger, *Cancer Res.* **18**, 318 (1958).
5. D. Kessel and T. C. Hall, *Cancer Res.* In press.
6. D. Kessel, T. C. Hall and I. Wodinsky, *Science* **154**, 911 (1966).
7. J. H. Burchenal, M. S. Lyman, J. R. Purple, V. Coley, S. Smith and E. Bucholz, *Cancer Chemother. Rep.* **19**, 19 (1962).
8. D. Roberts, T. C. Hall and I. Wodinsky, *Cancer Res.* **25**, 1899 (1965).
9. D. Kessel, T. C. Hall, D. Roberts and I. Wodinsky, *Science* **150**, 752 (1965).
10. J. E. Ciardi and E. P. Anderson, *Anal. Biochem.* **22**, 398 (1968).
11. O. Sköld, *Biochim. Biophys. Acta* **44**, 1 (1960).
12. T. R. Breitman, *Biochim. Biophys. Acta* **67**, 153 (1963).
13. P. Reyes, *Biochemistry* **8**, 2057 (1969).
14. D. K. Kasbekar and D. M. Greenberg, *Cancer Res.* **23**, 818 (1963).
15. P. Reyes and T. C. Hall, *Biochem. Pharmacol.* In press.
16. I. Lieberman, A. Kornberg and E. S. Simms, *J. Biol. Chem.* **215**, 403 (1955).
17. I. Crawford, A. Kornberg and E. S. Simms, *J. Biol. Chem.* **226**, 1093 (1957).
18. T. A. Krenitsky, M. Barclay and J. A. Jacquez, *J. Biol. Chem.* **239**, 805 (1964).
19. T. A. Krenitsky, J. W. Mellors and R. K. Barclay, *J. Biol. Chem.* **240**, 1281 (1965).
20. G. D. Birnie, H. Kroeger and C. Heidelberger, *Biochemistry* **2**, 566 (1963).
21. E. W. Yamada, *J. Biol. Chem.* **243**, 1649 (1968).
22. H. Pontis, G. Degerstedt and P. Reichard, *Biochim. Biophys. Acta* **51**, 138 (1961).
23. M. Friedkin and D. Roberts, *J. Biol. Chem.* **207**, 245 (1954).
24. P. Reichard and O. Sköld, *Biochim. Biophys. Acta* **28**, 376 (1958).
25. H. Madoc-Jones and W. R. Bruce, *Cancer Res.* **28**, 1976 (1968).