Proc. Natl. Acad. Sci. USA
Vol. 75, No. 2, pp. 980–983, February 1978
Medical Sciences

Cytotoxicity of 5-fluoro-2′-deoxyuridine: Requirement for reduced folate cofactors and antagonism by methotrexate

(thymidylate synthase/irreversible inhibition/folinic acid)

BUDDY ULLMAN*†, MELINDA LEE*†, DAVID W. MARTIN, JR.*†, AND DANIEL V. SANTI*‡§

Departments of * Biochemistry and Biophysics, † Medicine, and ‡ Pharmaceutical Chemistry, University of California, San Francisco, California 94143

Communicated by Thomas C. Bruice, October 28, 1977

ABSTRACT **Potent *in vitro* inhibition of thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) by 5-fluoro-2′-deoxyuridylate requires 5,10-methylenetetrahydrofolate. The cytotoxicity of 5-fluoro-2′-deoxyuridine towards cultured L1210 mouse leukemia cells is reduced when intracellular reduced folates are depleted, either by limiting the source in media or by inhibition of dihydrofolate reductase with methotrexate. Likewise, the intracellular amount of 5-fluoro-2′-deoxyuridylate covalently bound to thymidylate synthase in L1210 cells treated with 5-fluoro-2′-deoxyuridine is greatly diminished when cells are depleted of folate cofactors. The folate requirement for optimal growth of L1210 cells is lower than that required for maximal cytotoxicity of 5-fluoro-2′-deoxyuridine. These findings provide a biochemical rationale that may be useful in designing clinical protocols that use 5-fluorinated uracil analogs.**

5-Fluorouracil (FUra) and methotrexate (MTX) have been used both separately and in combination to treat a variety of neoplasms. With few exceptions, clinical protocols for single and multiple drug cancer chemotherapy have of necessity been empirically derived, and often yield inconclusive data as to the efficacy of such agents. Indeed, while the combination of cyclophosphamide, MTX, and FUra has been acclaimed a significant breakthrough in adjuvant treatment of human breast cancer (1), studies on experimental systems have yielded conflicting reports as to whether MTX and FUra are additive, synergistic (2–5), or antagonistic (6, 7). It is reasonable to believe that a fundamental knowledge of the mechanism of action of such drugs, together with their effects on cellular metabolism and growth, might be used to predict and modify their effects and thus be useful in achieving a more rational approach for design of schedules and combinations. In this paper we demonstrate that some biochemical aspects of the actions of MTX and FUra are sufficiently understood to predict certain consequences of their use in cell culture models. This information could be of utility in formulating experimental protocols to increase the therapeutic efficacy of FUra and FUra–MTX combinations.

MATERIALS AND METHODS

Tissue Culture. Stock cultures of L1210 mouse leukemia cells were maintained in folate-free Fischer's medium (8) containing 10% heat-treated (56°, 30 min) dialyzed horse serum, 32 μM hypoxanthine and 5.6 μM thymidine. For growth studies, cells washed in folate-free Fischer's medium with 10% horse serum were suspended (6 to 8 × 10^4 cells per ml) in 1 ml of the same medium containing specified supplements or inhibitors and incubated at 37° for 48–72 hr; cell number was determined on a Coulter counter ZB_1 and maximally increased 10- to 20-fold over that innoculated. Under optimal growth conditions the doubling time was 12.7 ± 1.7 hr. EC_{50} values refer to the concentration of FdUrd necessary to inhibit cell growth by 50% compared to controls grown under identical conditions except that FdUrd was omitted.

Electrophoresis of Intracellular Macromolecule-Bound [^{3}H]FdUMP. Washed cell pellets were suspended in 1 ml of a solution containing 1% sodium dodecyl sulfate ($NaDodSO_4$), 10% (vol/vol) glycerol, and 20 mM 2-mercaptoethanol and sonicated (Biosonik III, 0.3 maximal output) for two 10-sec bursts. The suspensions were heated at 100° for 90 sec and 50-μl (*ca* 0.4 mg of protein) samples were electrophoresed on slab gels of 12% polyacrylamide containing 0.1% $NaDodSO_4$ (9, 10). The gel tracts were sliced into seventeen 0.5-cm fractions, each of which was incubated in 1 ml of NCS (Amersham/Searle Co.) at 60° overnight and assayed for radioactivity in a liquid scintillation system.

RESULTS AND DISCUSSION

Thymidylate synthase (EC 2.1.1.45) catalyzes the conversion of dUMP and 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to dTMP and dihydrofolate (H_2folate). The latter is reduced to H_4folate *via* dihydrofolate reductase and again acquires the appropriate one-carbon unit for continued dTMP synthesis. The active metabolite of FUra and FdUrd is generally believed to be FdUMP, which is a potent inhibitor of thymidylate synthase (11). In recent years the molecular mechanism of the interaction of FdUMP with thymidylate synthase has been established (12–16), and the salient point with regard to this paper is that CH_2-H_4folate is required for potent inhibition. In the absence of this cofactor, FdUMP binds poorly to the enzyme ($K \simeq 10$ μM) (12, 13, 17), whereas the presence of CH_2-H_4folate results in a ternary complex in which FdUMP is bound some 7 to 8 orders of magnitude more tightly (13). This dramatic increase in affinity results in part from two-ligand synergism in formation of reversible complexes (13, 17), but for the most part is due to the formation of covalent bonds that link the enzyme to FdUMP and CH_2-H_4folate as shown in Fig. 1 (12–16). Inhibition of dihydrofolate reductase, as by MTX, results in the accumulation of H_2folate as a product of dTMP synthesis and depletion of intracellular H_4folates required for one-carbon metabolism. From this, it can be surmised that intracellular levels of CH_2-H_4folate should be a critical determinant of FUra and FdUrd cytotoxicity and that depletion of this cofactor by MTX should antagonize the cytotoxic effects of these fluorinated pyrimidine analogs.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylate; H_4folate, tetrahydrofolate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; MTX, methotrexate; $NaDodSO_4$, sodium dodecyl sulfate; HAT, hypoxanthine/MTX/thymidine.

§ To whom reprint requests should be addressed.

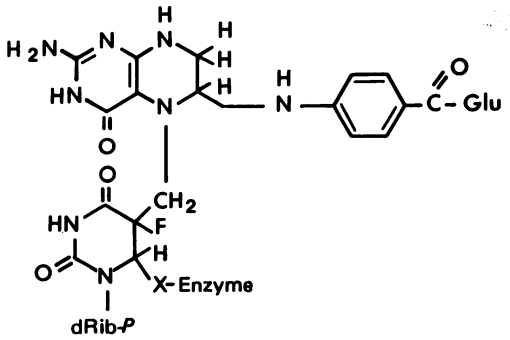

FIG. 1. Structure of the thymidylate synthase-FdUMP-CH_2-H_4folate complex.

To test these hypotheses, the ability of FdUrd to inhibit growth of L1210 leukemia cells in culture was examined under conditions that would result in various concentrations of intracellular H_4folates. A typical experiment using folinic acid (5-CHO-H_4folate) as the H_4folate source is shown in Fig. 2. In the absence of FdUrd, half-maximal growth is achieved at 4 nM folinic acid and maximal growth rate occurs at concentrations greater than 100 nM; the values shown are the means of three separate determinations and the range for any point was no greater than ±2.5% of control growth. The concentration of FdUrd necessary to inhibit growth by 50% (EC_{50}) is 0.3 nM at high folinic acid levels, but increases as the folinic acid is decreased below 500 nM; at 10 nM folinic acid, FdUrd has an EC_{50} of 1.25 nM. In a separate experiment, five determinations of the EC_{50} of FdUrd at 0.01 μM and 1 μM folinic acid gave values of 1.33 ± 0.01 nM (mean ± SEM) and 0.40 ± 0.03 nM, respectively. The significance of the difference between these EC_{50} values was shown by Student's t test ($P < 0.001$).

Similar experiments (not shown) were performed using folic acid as the source of intracellular H_4folate cofactors. Half-maximal growth rate was obtained with 0.3 μM folate, and optimal growth occurred at concentrations greater than 1.4 μM folate. The requirement for higher concentrations of folic acid than folinic acid is probably related to lower rates of folic acid transport (18) and its slow reduction by folate reductase (19). The EC_{50} of FdUrd decreased from 1.2 nM at 2.8 μM folate to 0.4 nM at folate concentrations greater than 23 μM. When cells were cultured in 2.8 μM folate, a concentration optimal for growth but insufficient for maximal inhibition by FdUrd (EC_{50} = 1.2 nM), the addition of 20 μM folinic acid decreased the EC_{50} of FdUrd 4-fold. This suggests that the effect of folate on the EC_{50} of FdUrd results from intracellular accumulation of reduced folates rather than folic acid itself. As will be discussed later, it may be quite significant that cells grow optimally at folate and folinic acid concentrations that are not sufficient for maximal cytotoxicity of FdUrd.

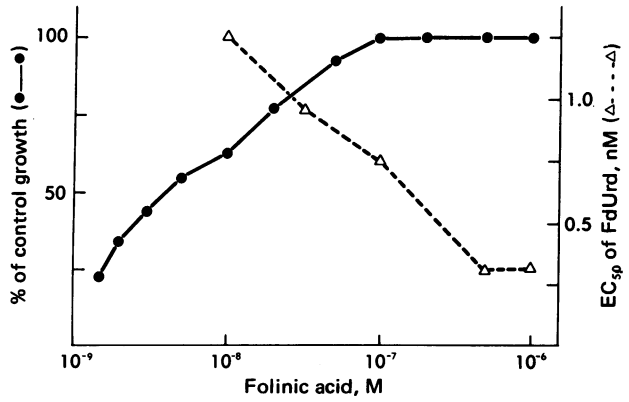

FIG. 2. Effect of folinic acid concentration in media on growth of L1210 cells and EC_{50} values of FdUrd. Cells were grown in Falcon multi-well tissue culture plates containing the specified amount of folinic acid and various concentrations of FdUrd (0, 0.2, 0.4, 1.6, 3.2, and 6.4 nM). Calculations of EC_{50} values were based on control cultures containing the specified amount of folinic acid.

Table 1. Effect of MTX on the EC_{50} of FdUrd in L1210 cells grown in culture*

MTX conc., nM	% control growth	EC_{50} of FdUrd, nM
0	100	0.4
5	74	0.7
10	26	0.9
13	21	1.2
20	12	1.6
20 + 10 μM folinic acid	100	0.4

* Cells were grown in the presence of 40 μM folic acid.

As shown in Table 1, growth of L1210 cells in increasing concentrations of MTX results in corresponding increases in the EC_{50} values of FdUrd, an effect that can be reversed by folinic acid. In view of the requirements of CH_2-H_4folate for potent inhibition of thymidylate synthase by FdUMP and of H_4folates for maximal inhibition of growth by FdUrd, the MTX antagonism of FdUrd cytotoxicity most likely results from depletion of intracellular H_4folate to levels insufficient for optimal formation of the covalent FdUMP-CH_2-H_4folate-thymidylate synthase complex. These results also support the previous suggestion that only intracellular reduced folates—not folate, dihydrofolate, or MTX at the intracellular concentrations achieved in these experiments—are responsible for optimizing the cytotoxicity of FdUrd.

We would predict that if cells were completely depleted of H_4folates, there would be no inhibition of thymidylate synthase by FdUMP, and FdUrd would show little or no cytotoxic effects. Unfortunately, this cannot be tested by cytotoxicity studies, because without a source of H_4folates cell growth requires exogenous purines and dThd, and the latter circumvents thymidylate synthase inhibition. As an alternate approach to test this hypothesis, we quantitated and compared the *in vivo* FdUMP-CH_2-H_4folate-enzyme complex formed in L1210 cells under conditions of H_4folate excess and deprivation. Thymidylate synthases from bacterial (20) and mammalian (15) sources are dimers with a subunit molecular weight of *ca* 35,000. The covalent bonds of the FdUMP-CH_2-H_4folate-enzyme complex are stable toward denaturants (12–16) and the 35,000-dalton complex can be readily identified by NaDodSO$_4$ gel electrophoresis (15, 16); in contrast, in the absence of CH_2-H_4folate, FdUMP is bound poorly to thymidylate synthase by noncovalent forces (12, 13, 17), and binary FdUMP–enzyme complexes are unstable towards denaturing conditions. L1210 cells were grown for 13 generations in folate-free medium containing HAT (32 μM hypoxanthine, 2 nM MTX, 5.6 μM thymidine) to deplete intracellular H_4folates. These cells were suspended (5×10^7 cells per ml) in folate- and serum-free Fischer's medium containing (*a*) 2 nM MTX and 0.5 μM [6-^{3}H]FdUrd (6 Ci/mmol) or (*b*) 2 nM MTX, 0.5 μM [6-^{3}H]-FdUrd, and 20 μM folinic acid. After 40 min at 37°, cells were washed with three 20-ml portions of cold phosphate-buffered saline, collected by centrifugation, and subjected to NaDodSO$_4$ electrophoresis. The only radioactivity recovered from the gels migrated in the fraction corresponding to proteins with mo-

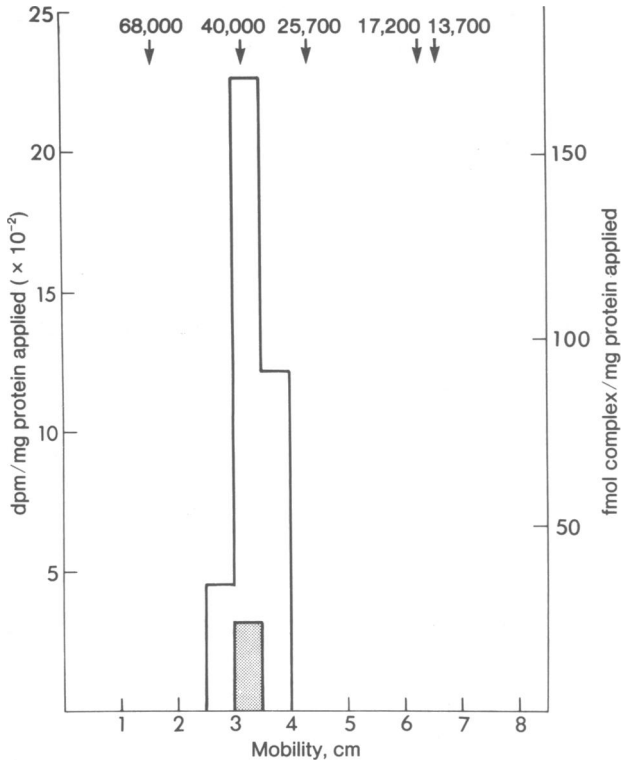

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of the [^{3}H]FdUMP-CH_2-H_4folate-thymidylate synthase complex formed *in vivo* upon incubation of cells with [^{3}H]FdUrd in the presence (open bars) and absence (shaded bar) of folinic acid; mobilities of molecular weight standards are indicated by arrows. Details are provided in the text and *Materials and Methods*.

lecular weight 35,000–40,000, the subunit molecular weight of the covalent [6-^{3}H]FdUMP-CH_2-H_4folate-enzyme complex.

As shown in Fig. 3, H_4folate-depleted cells that were deprived of folinic acid during incubation with [6-^{3}H]FdUrd possessed only 8% (24 fmol/mg of protein) of the covalent complex contained in cells treated identically except for the presence of folinic acid during treatment with [6-^{3}H]FdUrd (298 fmol/mg of protein). We attribute the residual covalent complex formed in cells deprived of H_4folates to accumulation of folates bound to serum present in the growth medium that were not removed by dialysis. A control experiment (not shown) in which cells grown in Fischer's complete medium were incubated with 0.5 μM [6-^{3}H]FdUrd and 20 μM folinic acid (37°, 40 min) and processed as described above gave a single radioactive peak on NaDodSO$_4$ gel electrophoresis (molecular weight 35,000–40,000) that had 84% of the radioactivity (250 fmol/mg of protein) present in cells grown on HAT medium and incubated with [6-^{3}H]FdUrd and folinic acid; this demonstrates there is no significant change in the amount of thymidylate synthase or in the ability to metabolize FdUrd to FdUMP when cells are grown in the folate-free HAT medium. The difference between the amounts of covalent complex formed in the absence and presence of folinic acid during incubation with [6-^{3}H]FdUrd clearly demonstrates that intracellular CH_2-H_4folate is necessary for potent FdUMP inhibition of thymidylate synthase *in vivo*.

Conclusions and Perspectives. Although it is generally accepted that the primary cytotoxic effect of FUra results from inhibition of thymidylate synthase by FdUMP, the biological activity of this drug very likely includes effects of its other metabolites and/or alterations of RNA metabolism and function (11). Nevertheless, the results described here demonstrate a definitive relationship between the cytotoxic effects of thymidylate synthase inhibition by FdUMP and the availability of intracellular H_4folates, and provide a biochemical basis for the design of experimental protocols that could lead to more efficacious use of this drug. In this regard, certain considerations and strategies appear especially worthy of mention and of experimental testing.

Because the cytotoxicity of low-dose MTX results from depletion of intracellular H_4folate cofactors, if MTX–FUra combinations are used, considerations of dosages, sequence, and intervals of administration of each drug should be of utmost importance in obtaining optimal therapeutic effects. For example, if during the time of FUra administration cells are deprived of the threshold amounts of CH_2-H_4folate required for FdUMP to inhibit thymidylate synthetase, folate-independent effects of the drug should predominate; whether this would be therapeutically beneficial or harmful is an important question that remains to be answered.

At sufficiently high concentrations a number of folate analogs synergize binding of FdUMP to thymidylate synthase, but covalent bonds are not formed and resultant ternary complexes are not as stable as those formed with CH_2-H_4folate (13, 17). For example, high concentrations of the noncytotoxic analog 10-CH_3-folic acid can partially reverse the MTX antagonism of FdUrd on L1210 cells in culture (unpublished data). Bertino *et al.* (5) have recently applied a similar rationale in designing an MTX–FUra schedule for treatment of the sarcoma 180 mouse tumor model. These workers surmised that although MTX would deplete H_4folates, sufficiently high intracellular concentrations of the drug might in addition synergize binding of FdUMP to thymidylate synthase; as it does *in vitro* (13), and override the antagonism observed at low MTX concentrations. Indeed, significant therapeutic enhancement was observed when tumor-bearing animals were treated with *high* doses of MTX 2 hr prior to FUra administration. While the basis for the success of this schedule remains unproven, a salient point is that its design was based on biochemical reasoning rather than the empiricism frequently used in developing such regimens.

Lastly, it may be quite relevant that, as shown here for L1210 cells, intracellular H_4folates can be sufficient for optimal growth but not for optimal inhibition of thymidylate synthase by FdUMP. This relationship may vary in different cells for a number of reasons; for example, cells with higher levels of thymidylate synthase would require higher concentrations of CH_2-H_4folate to achieve optimal inhibition of this enzyme by FdUMP. Indeed, thymidylate synthase increase has been identified as a progression-linked alteration that parallels the degree of malignancy or growth rate in a variety of rat hepatomas (21). Thus, the possibility exists that intracellular folate cofactors in tumors that are marginally responsive towards 5-FUra or FdUrd may be optimal for their growth, but not for inhibition of thymidylate synthase by FdUMP. Should this be the case, administration of a reduced folate, such as folinic acid, might increase the effectiveness of thymidylate synthase ihhibition by FdUMP while H_4folate-independent effects of FUra would remain unchanged; it is possible that such tumors could be made more responsive towards FUra or FdUrd with a resultant increase in the therapeutic index.

We are grateful to Dr. Florence R. White of the National Cancer Institute for her encouragement and aid. This work was supported by U.S. Public Health Service Grant CA-14394 and Contract NO1-CP-43239 from the National Cancer Institute. D.V.S. is the recipient of a U.S. Public Health Service Career Development Award from the

National Cancer Institute, and D.W.M. is an investigator of the Howard Hughes Medical Institute. B.U. is supported by U.S. Public Health Service Training Grant GM 01791, and M.L. was the recipient of a Medical Student Research Fellowship from the University of California.

1. Bonadonna, G., Brusamoline, E., Valagussa, P., Rossi, A., Brugnatelli, L., Brambilla, C., De Lena, M., Tancini, G., Bajetta, E., Musumeci, R. & Veronesi, U. (1976) *N. Engl. J. Med.* **294,** 405–410.
2. Kline, I., Vendetti, J. M., Mead, J. A. R., Tyrer, D. D. & Goldin, A. (1966) *Cancer Res.* **26,** 848–852.
3. Bareham, C. R., Griswold, D. E. & Calabresi, P. (1974) *Cancer Res.* **34,** 571–575.
4. Martin, D. S., Hayworth, P., Davin, E., Stolfi, R. & Fugmann, R. (1976) *Proc. Am. Assoc. Cancer Res. 17,* **130,** Abstr. no. 518.
5. Bertino, J. R., Sawicki, W. L., Lindquist, C. A. & Gupta, V. S. (1977) *Cancer Res.* **37,** 327–328.
6. Tattersal, M. H. N., Jackson, R. C., Connors, T. A. & Harrup, K. R. (1973) *Eur. J. Cancer* **9,** 733–739.
7. Waxman, S., Bruckner, H., Rubinoff, M. & Greenspan, E. M. (1976) *Proc. Am. Assoc. Cancer Res. 17,* **157,** Abstr. no. 628.
8. Fischer, G. A. & Sartorelli, A. S. (1964) *Methods Med. Res.* **10,** 247–262.
9. Studier, F. W. (1973) *J. Mol. Biol.* **79,** 237–248.
10. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121,** 404–427.
11. Heidelberger, C. (1975) in *Antineoplastic and Immunosuppressive Agents,* eds. Sartorelli, A. C. & Johns, A. G. (Springer-Verlag, New York), Part II, pp. 193–231.
12. Santi, D. V. & McHenry, C. S. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1855–1857.
13. Santi, D. V., McHenry, C. S. & Sommer, H. (1974) *Biochemistry* **13,** 471–480.
14. Sommer, H. & Santi, D. V. (1974) *Biochem. Biophys. Res. Commun.* **57,** 689–695.
15. Langenbach, R. J., Danenberg, P. V. & Heidelberger, C. (1972) *Biochem. Biophys. Res. Commun.* **48,** 1565–1571.
16. Danenberg, P. V., Langenbach, R. J. & Heidelberger, C. (1974) *Biochemistry* **13,** 926–933.
17. Galivan, J. H., Maley, G. F. & Maley, F. (1976) *Biochemistry* **15,** 356–362.
18. Goldman, I. D. (1971) *Ann. N.Y. Acad. Sci.* **186,** 400–422.
19. Bertino, J. R., Booth, V. A., Beiber, A. L., Cashmore, A. & Sartorelli, A. C. (1964) *J. Biol. Chem.* **239,** 479–485.
20. Dunlap, R. B., Harding, N. G. L. & Huennekens, F. M. (1971) *Biochemistry* **10,** 88–97.
21. Weber, G. (1977) *N. Engl. J. Med.* **296,** 486–551.