MOLECULAR PHARMACOLOGY, 25:303–309

Relationship of dUMP and Free FdUMP Pools to Inhibition of Thymidylate Synthase by 5-Fluorouracil

SONDRA H. BERGER[1] AND MAIRE T. HAKALA

Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, New York 14263

Received June 8, 1983; Accepted November 4, 1983

SUMMARY

The purpose of this study was to compare the pools of free FdUMP derived from 5-fluorouracil (FUra) and of dUMP synthesized *de novo* in Hep-2 and S-180 cells, their relationship to inhibition of thymidylate synthase (dTMP synthase; EC 2.1.1.45), and the effect of excess folinic acid (CF) on these parameters. These cells differ 50-fold in their sensitivity to FUra and, in the absence of thymidine, dTMP synthase is the growth-limiting site of action of FUra in S-180 cells, but in Hep-2 cells this site becomes growth-limiting only in the presence of excess folates. In both cells after a 3-hr incubation with varied concentrations of FUra, FdUMP comprised only 0.1–0.2% of the total acid-soluble pools derived from FUra. The changes in dUMP and FdUMP pools paralleled each other, dUMP being 1000–2000 times higher than FdUMP. The pools of dUMP increased only when dTMP synthase was significantly inhibited. This occurred in S-180 cells above 3 μM FUra and in Hep-2 cells above 30 μM, where the residual dTMP synthase was similar in both cells. Under these conditions, the dUMP and FdUMP pools in Hep-2 cells were 2 and 4 times higher, respectively, than in S-180 cells. After FUra removal, both pools continued to increase, dUMP and FdUMP pools in Hep-2 cells rising 6-fold and 10-fold higher, respectively, than in S-180 cells. The dTMP synthase inhibition and the high nucleotide pools in Hep-2 were short-lived, whereas in S-180 cells the inhibition and the pools were maintained longer. Excess CF retarded the recovery of dTMP synthase after FUra removal only in Hep-2 cells and led to a further increase in dUMP and FdUMP pools in these cells, while having no effect in S-180 cells. These data indicate that a high capacity of cells to accumulate free FdUMP does not alone guarantee that dTMP synthase inhibition will be growth-limiting. The relationship shown here between excess CF, dTMP synthase recovery, and the nucleotide pools suggests that some cell types, such as Hep-2, in spite of high levels of FdUMP, require in addition an excess of folates to retard dTMP synthase recovery and make it growth-limiting.

INTRODUCTION

FUra[2] has been utilized in the clinical management of carcinomas of the ovary, breast, pancreas, and gastrointestinal tract (1–3). This agent exerts its cytotoxic effects by at least two mechanisms of action: incorporation of FUra into RNA at the level of FUTP and inhibition of dTMP synthase (EC 2.1.1.45) by the FUra metabolite, FdUMP. Inhibition of cell growth has been observed after FUra action at either site, leading to the suggestion that the site of FUra action is dependent upon the cellular system under investigation (4–6).

dTMP synthase is a major target of cancer chemotherapy, since the reaction catalyzed by this enzyme provides the only *de novo* source of dTMP for DNA biosynthesis. The effect of FUra on dTMP synthase may be affected by several biochemical parameters, including the levels of the two substrates, dUMP and 5,10-CH_2-H_4PteGlu, and the inhibitor, FdUMP. The formation and level of FdUMP and/or duration of its retention have been suggested as critical determinants of FUra action (7–10); however, several investigations have demonstrated lack of correlation between these latter parameters and sensitivity to FUra (11–13). Instead, Myers et

This research was supported in part by Grants CA-13038 and CA-21071 from the National Institutes of Health and by Training Grant CA-09072 (to S. H. B.). This study represents research done by S. H. B. as partial requirement for a Ph.D. degree in pharmacology at Roswell Park Memorial Institute, State University of New York at Buffalo.

[1] Present address, Department of Molecular Biology, Roswell Park Memorial Institute, Buffalo, N. Y. 14263.

[2] The abbreviations used are: FUra, 5-fluorouracil; dTMP synthase, thymidylate synthase; S-180, mouse sarcoma 180; Hep-2, human epithelial carcinoma; CF, folinic acid, N^5-CHO-H_4PteGlu, N^5-formyltetrahydropteroylglutamic acid; H_4PteGlu, tetrahydropteroylglutamic acid; N^{10}-CHO-H_4PteGlu, N^{10}-formyltetrahydropteroylglutamic acid; FdUMP, 5-fluorodeoxyuridine-5′-monophosphate; 5,10-CH_2-H_4PteGlu, N^5,N^{10}-methylenetetrahydropteroylglutamic acid; 5-CH_3-H_4PteGlu, N^5-methyltetrahydropteroylglutamic acid; FdUrd, 5-fluorodeoxyuridine.

0026-895X/84/020303-07$02.00/0

al. (14) observed that it was the accumulation of dUMP which was associated with the resumption of DNA synthesis in P1534 ascites tumor during recovery from FUra. Also, several *in vitro* studies with dTMP synthase purified from *Lactobacillus casei* and from CCRF-CEM human lymphoblastic leukemic cells revealed that dUMP competes with FdUMP for binding to the enzyme (15–17). Moreover, such *in vitro* studies have shown that both the rate of association of FdUMP with dTMP synthase and the rate of its dissociation are dependent on 5,10-CH_2-H_4PteGlu concentration (15, 17).

Laskin *et al.* (18) observed that several mouse cell lines *in vitro* were more sensitive to growth inhibition by FUra than were several human cell lines and that the sensitivity was associated with a more rapid uptake and metabolism of FUra. In particular, mouse sarcoma S-180 cells were 50-fold more sensitive than the human carcinoma Hep-2 cells (6). In mouse and human cells, dTMP synthase was inhibited to a similar extent immediately after FUra. However, in mouse cells, such as S-180, the inhibition of dTMP synthase was the growth-limiting event while in human cells, such as Hep-2, it was the incorporation of FUra into RNA. In the presence of excess folates, the growth inhibitory effects of FUra were potentiated 3-fold, and inhibition of dTMP synthase now became the growth-limiting event in Hep-2 (19). It was also observed that after FUra treatment Hep-2 cells, in contrast to S-180, accumulated high levels of labeled dUMP when supplied with labeled dUrd (6). Also, the pools of the various folate cofactors in the two cell lines were vastly different (20). The data indicated that Hep-2 and S-180 cells differ in several parameters that affect the action of FUra on dTMP synthase.

In the present study, the levels of free FdUMP (acid-soluble) and of dUMP formed *de novo* as well as the effect of excess folinic acid on these pools and on the levels and recovery of free dTMP synthase have been examined in both cell lines to determine their relationship with the sensitivity to and the site of action of FUra. Some of these results have been reported briefly (21, 22).

EXPERIMENTAL PROCEDURES

Materials. [6-^{3}H]FdUMP (18 Ci/mmole) was purchased from Moravek Biochemicals (City of Industry, Calif.). [5-^{3}H]dUMP (14 Ci/mmole) was obtained from Schwarz-Mann (Orangeburg, N.Y.). [^{14}C]HCHO (56 mCi/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.). FUra, FdUMP, dUMP, dUrd, folic acid, and folinic acid were purchased from Sigma Chemical Company (St. Louis, Mo.). Horse serum and powdered RPMI 1640 medium were obtained from Grand Island Biochemical Company (Grand Island, N. Y.). Acid-washed activated charcoal, DEAE-cellulose microgranular anion exchanger, and phosphocellulose (fine mesh) were from Sigma Chemical Company. Dextran T-70 was obtained from Pharmacia Fine Chemicals (Piscataway, N. J.). (±)-H_4PteGlu was prepared from folic acid and purified by DEAE-cellulose chromatography by the method of Zakrzewski and Sansone (23).

dTMP synthase in crude extracts of dichloromethotrexate-resistant *Lactobacillus casei* (obtained from New England Enzyme Center, Boston, Mass.) was purified by phosphocellulose column chromatography by the procedure of Sharma and Kisliuk (24). The specific activity of the partially purified dTMP synthase was 1.1 units/mg of protein, as determined by the tritium-release procedure of Dunlap *et al.* (25). One unit is defined as the amount of enzyme catalyzing the formation of 1 μmole of dTMP per minute at 30°.

Cell culture. The origin and maintenance of mouse sarcoma 180 and human carcinoma Hep-2 have been described previously (26). The cells were maintained in monolayer cultures in RPMI 1640 medium supplemented with 5% horse serum. Further modifications of the medium are described in the legends to the figures.

Analysis of dUMP and free FdUMP. Monolayers of cells in T-75 flasks were treated as described for each experiment. At the end of the incubation period, the medium was removed and the cell layers were rinsed twice with cold, serum-free medium 1640. The flasks were placed on ice, and the cells were extracted with 5 ml of cold 5% trichloroacetic acid as described previously (18). After removal of trichloroacetic acid with ether and after lyophilization, the powder was dissolved in a small amount of water and applied to DEAE-cellulose (bicarbonate) columns; dUMP and free FdUMP were separated as described by Moran *et al.* (27). The fractions containing the nucleotides were lyophilized, and dUMP and free FdUMP were quantitated using assays developed by Moran *et al.* (27), using *L. casei* dTMP synthase.

Titration of free dTMP synthase. Cell extracts were prepared and the free dTMP synthase was determined by measuring the formation of [6-^{3}H]FdUMP-enzyme complex in the presence of excess cofactor (27). (±)-CH_2-H_4PteGlu was prepared from H_4PteGlu by the method of Dunlap *et al.* (25). The calculations of picomoles of dTMP synthase per milligram of protein are based on the assumption that 1.7 moles of FdUMP are bound per mole of enzyme (17, 22, 29).

Determination of molarities of cellular metabolites. The acid-extracted cell layers were dissolved in 5 ml of 0.2 N NaOH. Protein was determined by the method of Lowry *et al.* (30), using crystalline bovine serum albumin as the standard. Utilizing the values for the amount of intracellular water per milligram of total cell protein which had been determined previously (18), the molarities of dUMP and free FdUMP in cell water were calculated.

Determination of radioactivity. Aqueous samples were counted in 10 ml of Liquiscint-2 scintillation solution (National Diagnostics, Somerville, N.J.), using a Packard Tri-Carb Model 2450 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Characteristics of Hep-2 and S-180 cells. Some of the differences between S-180 and Hep-2 cells are listed in Table 1. The specific activity and the FdUMP-binding

TABLE 1

Some characteristics of Hep-2 and S-180 cells

Parameter	Hep-2 cells	S-180 cells
dTMP synthase		
Specific activity (pmoles/min/mg protein)[a]	109 ± 33 (12)	36 ± 9 (23)
FdUMP titration (pmoles/mg protein)[b]	2.1 ± 0.5 (27)	1.3 ± 0.2 (26)
Growth inhibition[c]		
ID_{50} in medium 1640 (μM FUra)	244 ± 109 (14)	4.7 ± 2.1 (19)
Incorporation of FUra into RNA[a]		
At ID_{50} (nmoles/mg RNA)	6.5	1.5
Folate cofactor pools[d]		
H_4PteGlu (μM)	3.5	7.3
5-CHO and 10-CHO-H_4PteGlu (μM)	1.3	6.3
5-CH_3-H_4PteGlu (μM)	5.3	1.4
Higher polyglutamyl derivatives (% of total)	32	91

[a] Data from Evans *et al.* (6).

[b] Determined as described under Experimental Procedures.

[c] These concentrations of FUra were applied for 3 hr and caused 50% inhibition of subsequent growth in FUra-free medium.

[d] Data from Yin *et al.* (20). The numbers indicate micromolar concentrations in cell water after folate-depleted cells were incubated for 24 hr with 10 μM folate.

capacity of dTMP synthase in Hep-2 cells is 2–3 times higher than in S-180 cells. At the same time, Hep-2 cells tolerate 50–60 times more FUra for growth inhibition. At the ID_{50} of FUra for Hep-2, the incorporation of FUra into RNA (6.5 nmoles/mg of RNA) is growth-limiting, reaching about 1% replacement of RNA-uracil (6). Therefore, thymidine can neither protect nor rescue Hep-2 cells. In contrast, thymidine protects as well as rescues S-180 cells up to a concentration of FUra (60 μM) at which the incorporation into RNA becomes growth-limiting even for these cells (6).

Addition of excess CF (1000-fold above normal) was found not only to potentiate FUra activity but, in Hep-2 cells, to make dTMP synthase inhibition growth-limiting (19). This was at least partially explained by the differences in pools of various folate cofactors in the two cell lines (20). Thus, those reduced folates which are easily converted to dTMP synthase cofactor were found to be much higher in S-180 cells than in Hep-2 cells. In contrast, the predominant form in Hep-2 cells was 5-CH_3-H_4-PteGlu, the cofactor for methionine synthase (EC 2.1.1.13). The conversion of the latter compound to H_4PteGlu requires methionine synthesis, which is limited in the presence of 100 μM methionine in the cell culture medium. The third striking difference between the two cells is the low content of higher polyglutamyl-folates in Hep-2. Work is presently in progress to quantitate in both cell lines the folate forms which promote the binding of FdUMP to dTMP synthase. Preliminary results indicate that the content of these folates is much higher in S-180 cells than in Hep-2 cells.

Pools of dUMP and FdUMP. The effect of 3-hr incubation with varied concentrations of FUra on these pools is shown in Fig. 1. In untreated cells, the dUMP pools were similar, 7.6 ± 3.8 μM in Hep-2, 10.8 ± 4.2 μM in S-180. At concentrations below 30 μM FUra, the pools of both dUMP and FdUMP were higher in S-180 cells than in Hep-2 cells. Since dUMP accumulation is at least partly the result of reduced substrate flux through dTMP synthase, the higher pools of dUMP in S-180 cells at these concentrations of FUra must be attributed to the lower residual activity and lower levels of free dTMP synthase in S-180 cells. Thus, after 3 hr with 1 μM FUra, the activity in S-180 cell extracts was only 20 pmoles/min/mg of protein whereas it was 5 times higher in Hep-2 cells (6). At the same time, the level of free dTMP synthase was 0.34 pmole/mg of protein in S-180 cells and 1.96 pmoles/mg of protein in Hep-2 cells (Fig. 2).

Only at concentrations above 30 μM FUra did Hep-2 cells accumulate more dUMP and FdUMP than S-180 cells. These pool changes coincide with maximal inhibition of dTMP synthase in Hep-2 (Fig. 2). At concentrations of FUra, where dTMP synthase is well blocked in both cells, the larger pools of both nucleotides in Hep-2 may reflect differences between the two cell lines in nucleotide breakdown and resynthesis. Namely, in Hep-2 cells the thymidine kinase activity is 6 times higher than in S-180 cells whereas thymidine phosphorylase activity is only half of that in S-180 (18). These circumstances would be expected to enhance the nucleoside salvage in Hep-2 over that in S-180.

It is also of interest that at those concentrations of FUra which inhibited dTMP synthase activity in both cells by 50%, the pools of both nucleotides were similar in the two cells: dUMP 23 μM and 28 μM, FdUMP 0.018 μM and 0.017 μM in Hep-2 and S-180, respectively. Considering the 2- to 3-fold higher dTMP synthase activity in Hep-2 cells under these conditions, the data on dUMP appear puzzling, but may again reflect the more efficient resynthesis of dUMP in Hep-2 as was discussed above.

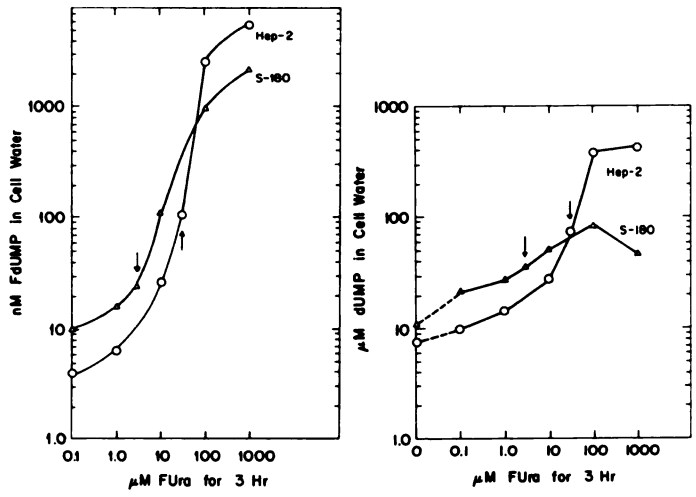

FIG. 1. *Pools of dUMP and free FdUMP after varied concentrations of FUra*

Confluent monolayers of cells in T-75 flasks were incubated for 3 hr with 30 ml of medium 1640 supplemented with 5% horse serum and varying concentrations of FUra. The medium was removed; the cell layers were rinsed with cold, serum-free medium; and dUMP and FdUMP were extracted and quantitated as described under Experimental Procedures. Each point represents an average of 2–13 separate determinations for FdUMP, 2–19 for dUMP. The *arrows* indicate the concentrations of FUra required to inhibit dTMP synthase to the same residual activity.

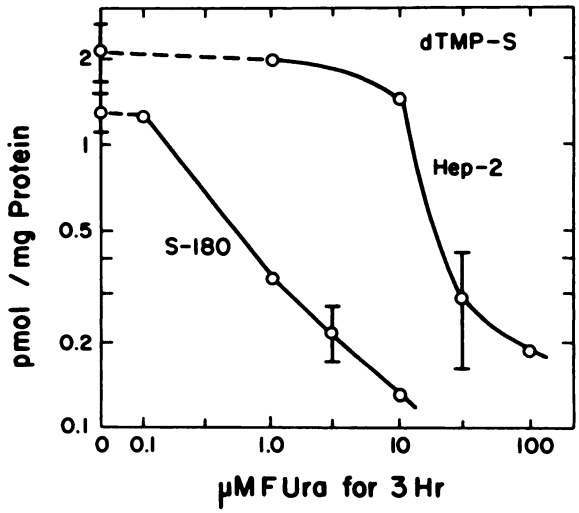

FIG. 2. *Effect of 3-hr incubation with varied concentrations of FUra on the remaining levels of free dTMP synthase (dTMP-S)*

The cell extracts were prepared and the enzyme content was titrated with [6-^{3}H]FdUMP as described under Experimental Procedures. The *bars* indicate the standard deviation from 26 and 27 determinations for untreated cells and from 9 (S-180) and 8 (Hep-2) for cells treated with 3 μM and 30 μM FUra, respectively.

The parallel nature of dUMP and FdUMP pools is most likely a reflection of the same pathways of breakdown of the two nucleotides. Thus, one may envision the increase in dUMP pools as being the primary phenomenon, which only secondarily causes an increase in free FdUMP by protecting it from breakdown. This is a reasonable assumption in view of the 1000- to 2000-fold excess of dUMP over FdUMP. In both cells the 1000- to 2000-fold excess of dUMP over FdUMP was independent of the actual concentrations. Being the substrate of dTMP synthase, one would expect dUMP to compete with FdUMP for the enzyme. However, since the ratio of the respective K_m and K_i values is 1000 (31), the 1000- to 2000-fold excess of dUMP over FdUMP appears adequate for maintaining only an equilibrium or maybe a slow recovery of enzyme activity. It appears that dUMP has to be present at much higher than 1000- to 2000-fold excess to interfere efficiently with inhibition of dTMP synthase by FdUMP. Preformed dUrd, when provided at a concentration of 1 mM, was able to rescue S-180 cells after FUra treatment as effectively as thymidine. Hep-2 cells were rescued in this manner after treatment with FdUrd (19). Such dUrd concentrations, when maintained continuously, are likely to create very high dUMP pools, considering that even after 60 min with 1 mM dUrd these pools already were 4.4 mM in Hep-2 cells and 100 μM in S-180 cells (21).

In our earlier studies using [2-^{14}C]FUra, no FdUMP was detected by radiochromatography (6, 18, 19). The detection limit was estimated to be 2–4% of the total acid-soluble radioactivity. Indeed, FdUMP became detectable by radiochromatography only when [2-^{14}C]FUra was applied together with 1 mM deoxyinosine (19). This raised the FdUMP content to 5–13% of the total pools in both cells. Table 2 lists the molarities in cell water of the total acid-soluble pools derived from FUra and of free FdUMP after varied concentrations of FUra. Generally, FdUMP comprised only 0.1–0.2% of the total pools, except at 100 to 1000 μM FUra in Hep-2 cells, where FdUMP rose to 0.3–1% of the total.

Effect of 5 μM extracellular dUrd on free FdUMP and on total dUMP pools. We had observed earlier (6) that in the less sensitive Hep-2, HeLa, and KB cells, but not in the more sensitive S-180, LM, or U-cells, the pools of [2-^{14}C]dUMP were very high when these cells were incubated for 60 min with 5 μM [2-^{14}C]dUrd after a 3-hr exposure to growth-inhibitory concentrations of FUra. At that time, the total dUMP pools were not determined. In order to assess the *de novo* contribution of dUMP to these pools in the presence of extracellular 5 μM dUrd, the total dUMP pools were determined after 60 min in the presence and absence of 5 μM dUrd after a 3-hr exposure of Hep-2 cells to 0.1–1000 μM FUra. Unlike 1 mM dUrd (see above), this low extracellular level of dUrd had no detectable effect on the total dUMP pools (data not shown). It appears that the cells utilize preferably the preformed dUrd and that this may limit the *de novo* synthesis, possibly through feedback inhibition, at aspartate transcarbamylase (32). In contrast, 5 μM dUrd reduced the pools of free FdUMP in Hep-2 cells by approximately 60% in 60 min at all concentrations of FUra (data not shown). This observation is in accord with our earlier studies showing that the rate of disappearance of free FdUMP from Hep-2 cells was greatly increased in the presence of 1 mM dUrd (21).

TABLE 2

Relationship of FdUMP pools to total acid-soluble FUra metabolites

μM FUra for 3 hr	Hep-2 cells		S-180 cells	
	Total acid-soluble[a]	FdUMP	Total acid-soluble[a]	FdUMP
	μM in cell water		*μM in cell water*	
1	—	0.006 ± 0.003	10	0.017 ± 0.004
3	—	—	32	0.025 ± 0.004
10	25.5	0.027 ± 0.012	96	0.112 ± 0.03
30	82	0.108 ± 0.009	296	—
100	228	2.6 ± 0.5	980	0.96 ± 0.37
300	640	—	2660	—
1000	2100	5.5 ± 1.1	—	2.2 ± 0.5

[a] Data from Evans *et al.* (6) converted to molarities in cell water.

Changes in dUMP and FdUMP pools with time. In order to reduce the dTMP synthase in both cells to similar residual levels of activity (16–22 pmoles/min/mg of protein), Hep-2 and S-180 cells were treated for 3 hr with 30 μM and 3 μM FUra, respectively. Under these conditions the residual levels of free dTMP synthase were also similar: 0.29 ± 0.13 pmole/mg of protein in Hep-2 cells and 0.22 ± 0.05 in S-180 cells (Fig. 2). These concentrations of FUra had no effect on the subsequent growth of either cell line when transferred into drug-free medium (6, 19).

Figure 3 shows the changes in dUMP and FdUMP pools with time in both cell lines. The levels of both nucleotides were much higher in Hep-2 cells than in S-180 cells until 24 hr after FUra removal, where the relationship was reversed. Immediately after FUra removal, the dUMP pools in Hep-2 were twice as high as in S-180, namely 76 ± 15 μM versus 37 ± 8 μM. These pools rose to 470 ± 190 μM in Hep-2 at 6 hr but only to 75 ± 8 μM in S-180 at 9 hr. Thereafter the pools declined very rapidly in Hep-2, much more slowly in S-180.

Immediately after FUra removal, the total acid-soluble pools of FUra were 82 μM in Hep-2 and 32 μM in S-180 (Table 2). The major metabolite (70–80%) was FUTP (6). In contrast, FdUMP comprised only a minor fraction of the total (0.1%), being 0.11 ± 0.01 μM in Hep-2 and 0.025 ± 0.004 μM in S-180. After FUra removal, the pools of FdUMP continued to increase in parallel with dUMP pools, reaching 0.4 ± 0.1 μM in Hep-2 at 6 hr and 0.04 μM in S-180 at 9 hr. Thus, the acid-soluble FUra metabolites, which were present at the time of FUra removal, continued to be converted to FdUMP. As with dUMP, FdUMP was retained in S-180 much longer than in Hep-2. Thus, at 24 hr, FdUMP in S-180 (25 nM) was more than 3 times higher than in Hep-2 (7.4 nM). This long retention of dUMP and FdUMP in S-180 cells and their rapid disappearance in Hep-2 cells correlate with the continued maintenance of dTMP synthase inhibition in S-180 in contrast to its spontaneous recovery in Hep-2 (Fig. 5). Thus, the high pools of FdUMP in Hep-2 initially do not alone guarantee the role of dTMP synthase inhibition in limiting growth.

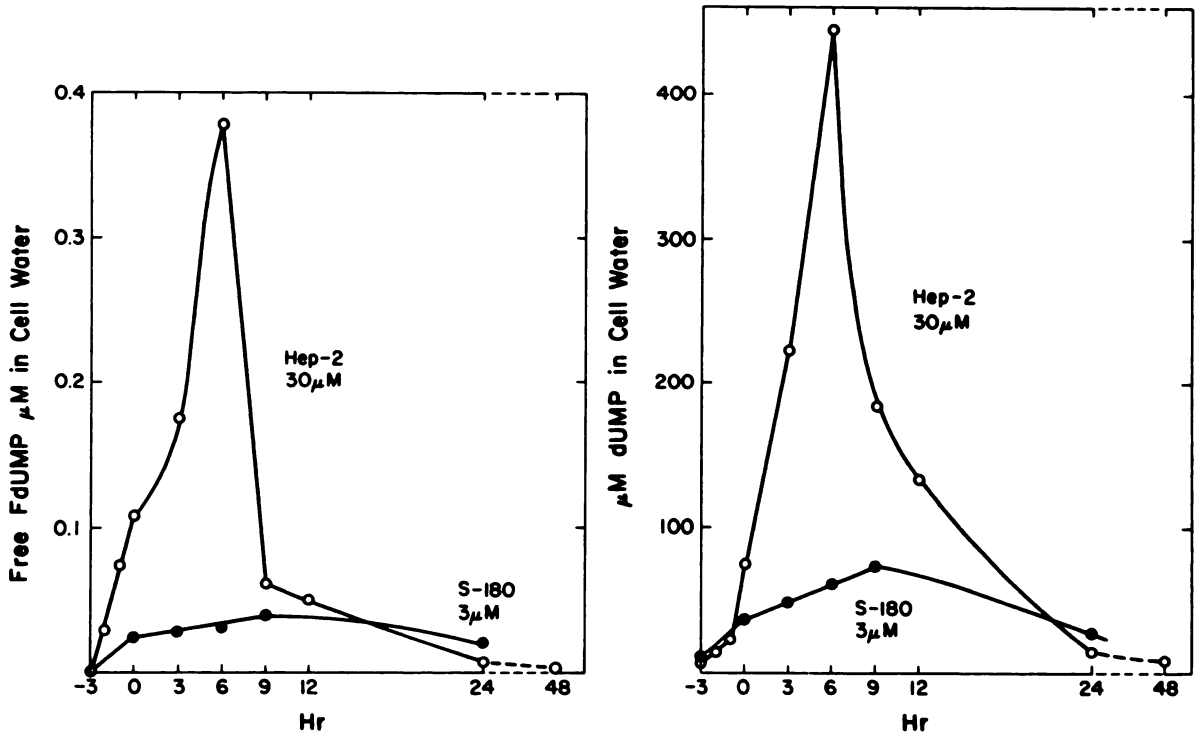

FIG. 3. *Progress of dUMP and free FdUMP pools during and after exposure to FUra*

Monolayers of cells in T-75 flasks were incubated for 3 hr with 30 ml of medium 1640 supplemented with 5% horse serum and either 30 μM (Hep-2) or 3 μM (S-180) FUra. The medium was removed and the cell layers were rinsed with warm, drug-free medium. The incubation with drug-free medium was continued up to 48 hr. At various times during and after treatment, flasks were removed; the cell layers were rinsed with cold, serum-free medium; and dUMP and FdUMP were extracted and quantitated as described under Experimental Procedures. The points represent the mean values of 2–19 separate determinations for FdUMP and of 2–9 for dUMP.

Effect of CF on dUMP and FdUMP pools. It had been shown earlier (19) that, although an excess of folates (10 μM CF, 10 μM 5-CH_3-H_4PteGlu, or 300 μM PteGlu) potentiated FUra action and made dTMP synthase inhibition growth-limiting in Hep-2, it had no effect on the total acid-soluble pools, on FUTP, or on the incorporation of FUTP into RNA. Figure 4 shows that 10 μM CF increased both dUMP and free FdUMP pools in Hep-2 cells in a parallel fashion after 30 μM FUra. Thus, immediately after FUra removal, CF had increased the pools of dUMP from 76 μM to 197 μM and the maximal level (at 6 hr) from 470 μM to 860 μM. Similarly, the

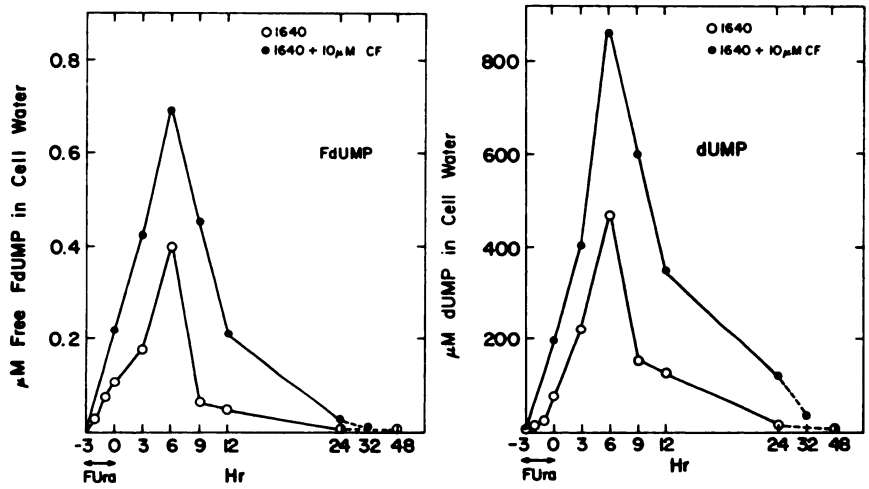

FIG. 4. *Effect of excess CF in combination with FUra on dUMP and free FdUMP pools in Hep-2 cells*

Monolayers of Hep-2 cells in T-75 flasks were preincubated for 24 hr with 30 ml of medium 1640 supplemented with 5% horse serum with (●) or without (O) 10 μM CF. The medium was replaced with 30 ml of the same medium containing 30 μM FUra; the cells were incubated for 3 hr; and the cell layers were then rinsed with warm, drug-free medium. The incubation of both groups of cells was continued up to 48 hr with 30 ml of medium 1640 supplemented with 5% horse serum. At various times during and after FUra treatment, flasks were removed; the cell layers were rinsed with cold, serum-free medium; and dUMP and FdUMP were extracted and quantitated as described under Experimental Procedures. The points represent mean values of 2–9 separate determinations with CF and of 2–19 without.

pools of FdUMP after FUra removal were increased from 0.11 μM to 0.22 μM and the maximum from 0.4 μM to 0.7 μM. Even though the decline of both pools was rapid thereafter, the pools of FdUMP at 24 hr were still 0.027 μM, equal to the pools in S-180 cells at that time whether or not CF was present (Fig. 3). Indeed, it is of interest that CF had no effect on either dUMP or free FdUMP pools in S-180 cells (data not shown). It thus appears that only in Hep-2 cells did the presence of high CF potentiate the inhibition of dTMP synthase leading to increased accumulation of dUMP and secondarily to that of free FdUMP.

Effect of CF on recovery of free dTMP synthase. In our earlier studies we had shown that 10 μM CF, when applied together with FUra, had no effect on the degree of dTMP synthase inhibition if measured immediately after FUra removal (19). However, the recovery of dTMP synthase activity, measured indirectly by [2-^{14}C]dUrd incorporation into DNA, was slowed down by CF at varied concentrations of FUra. Also, the presence of CF limited the rescue with 1 mM dUrd, which in the absence of CF was as complete as with dThd (19). All of these observations had led to the conclusion that excess CF increased the stability of the dTMP synthase-FdUMP complex.

In the present study, a direct titration of free dTMP synthase was undertaken in both cells to determine whether CF indeed had an effect on the rate of appearance of the free enzyme (Fig. 5). The concentrations of FUra (30 μM for Hep-2, 3 μM for S-180) were those chosen to result in similar residual activity immediately after FUra removal. CF did not increase the extent of dTMP synthase inhibition when measured immediately after FUra removal. However, the rate of appearance of free dTMP synthase was clearly affected in Hep-2 cells, the differences in dTMP synthase levels being significant at 9, 12, 24, and 45 hr (data not shown) after treatment, with p values of <0.05, < 0.02, 0.005, and <0.02, respectively. In S-180 cells no effect by CF was apparent. These findings explain the effect, described above, which CF had in increasing the dUMP (and FdUMP) pools in Hep-2, but not in S-180. These results, in conjunction with the observation that CF made dTMP synthase inhibition by FUra growth-limiting in Hep-2, indicate that enzyme recovery is an important determinant of FUra response.

In conclusion, it appears that in cells such as Hep-2, which are less sensitive to FUra, the capacity to accumulate large pools of FdUMP is by itself not sufficient for ensuring the significance of dTMP synthase inhibition in inhibition of growth. These types of cells may require additional folates to slow down the spontaneous recovery of dTMP synthase activity for a period necessary for inhibition of DNA synthesis and thereby of growth. Combination of FUra with deoxyinosine increased FdUMP pools by 100-fold, and a further addition of CF resulted in a 10-fold potentiation of FUra against both S-180 and Hep-2 (19). These results suggest that

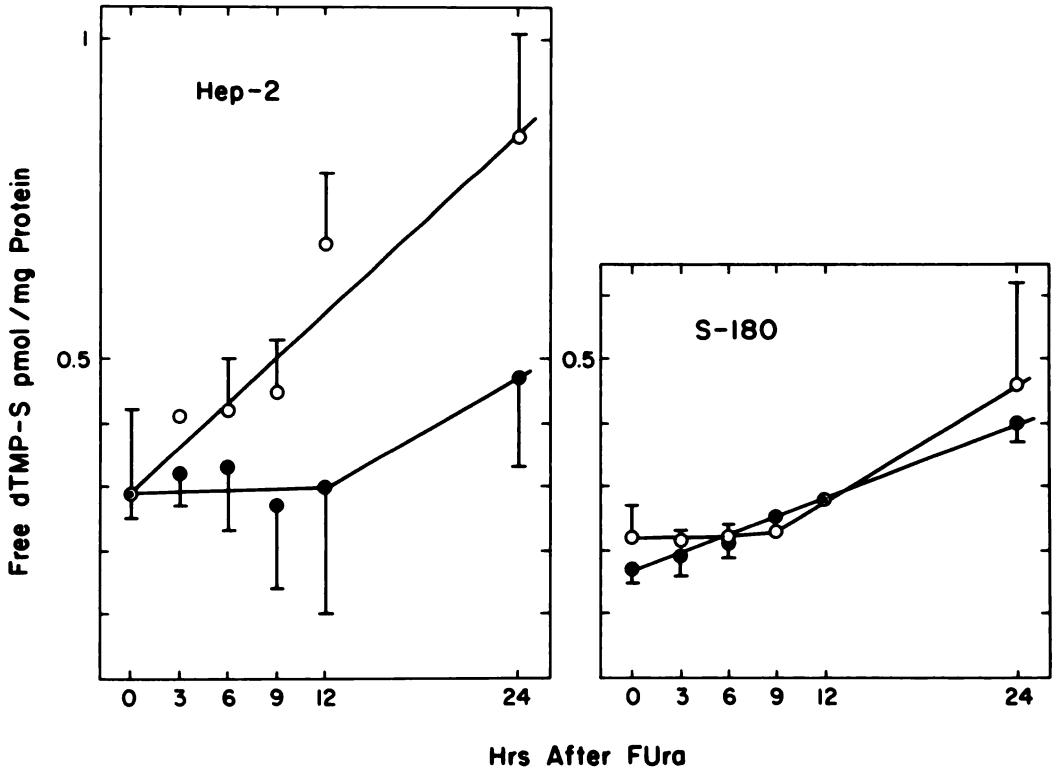

FIG. 5. *Effect of excess CF in combination with FUra on the spontaneous recovery of free dTMP synthase (dTMP-S) in Hep-2 and S-180 cells*
Monolayers of cells were preincubated for 24 hr in medium 1640 supplemented with 5% horse serum with (●) or without (○) 10 μM CF. The medium was replaced with the same medium containing 30 μM (Hep-2) or 3 μM (S-180) FUra, and the incubation was continued for 3 hr. The cell layers were rinsed with warm, drug-free medium, and the incubation was continued up to 24 hr with medium 1640 supplemented with 5% horse serum. At various times, flasks were removed and rinsed, and cell extracts were prepared and analyzed for free dTMP synthase as described under Experimental Procedures. Each point represents an average of four or five determinations; the bars indicate ± standard deviation.

both CF and deoxyinosine should be tested for their potential usefulness in improving the anticancer effect of FUra.

REFERENCES

1. Carter, S. K. Single and combination nonhormonal chemotherapy in breast cancer. *Cancer* **30**:1543–1555 (1972).
2. Moertel, C. G. Chemotherapy of colorectal cancer, in *Carcinoma of the Colon and Rectum* (W. E. Enker, ed.). Yearbook Medical Publishers, Chicago, 172–186 (1978).
3. Young, R. C., S. P. Hubbard, and V. T. DeVita. The chemotherapy of ovarian carcinoma. *Cancer Treat. Rev.* **1**:99–110 (1974).
4. Santelli, G., and F. Valeriote. *In vivo* potentiation of 5-fluorouracil cytotoxicity against AKR leukemia by purines, pyrimidines, and their nucleosides and deoxynucleosides. *J. Natl. Cancer Inst.* **64**:69–72 (1980).
5. Umeda, M., and C. Heidelberger. Comparative studies of fluorinated pyrimidines with various cell lines. *Cancer Res.* **28**: 2529–2538 (1968).
6. Evans, R. M., J. D. Laskin, and M. T. Hakala. Assessment of growth-limiting events caused by 5-fluorouracil in mouse cells and in human cells. *Cancer Res.* **40**:4113–4122 (1980).
7. Ardalan, B., D. A. Cooney, H. N. Jayaram, C. K. Carrico, R. I. Glazer, J. MacDonald, and P. S. Schein. Mechanisms of sensitivity and resistance of murine tumors to 5-fluorouracil. *Cancer Res.* **40**:1431–1437 (1980).
8. Rustum, Y. M., L. Danhauser, and G. Wang. Selectivity of action of 5-FU: biochemical basis. *Cancer* **66**:44–47 (1979).
9. Ardalan, B., M. D. Buscaglia, and P. S. Shein. Tumor 5-fluorodeoxyuridylate concentration as a determinant of 5-fluorouracil response. *Biochem. Pharmacol.* **27**:2009–2013 (1978).
10. Klubes, P., K. Connelly, I. Cerna, and H. G. Mandel. Effects of 5-fluorouracil on 5-fluorodeoxyuridine 5′-monophosphate and 2-deoxyuridine 5′-monophosphate pools, and DNA synthesis in solid mouse L1210 and rat Walker 256 tumors. *Cancer Res.* **38**:2325–2331 (1978).
11. Houghton, J. A., and P. J. Houghton. On the mechanism of cytotoxicity of fluorinated pyrimidines in four human colon adenocarcinoma xenografts maintained in immune-deprived mice. *Cancer* **45**:1159–1167 (1980).
12. Spears, C. P., A. H. Shahinian, R. G. Moran, C. Heidelberger, and T. H. Corbett. *In vivo* kinetics of thymidylate synthetase inhibition in 5-fluorouracil-sensitive and -resistant murine colon adenocarcinomas. *Cancer Res.* **42**:450–456 (1982).
13. Houghton, J. A., S. J. Maroda, Jr., J. O. Phillips, and P. J. Houghton. Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinomas in mice. *Cancer Res.* **41**:144–149 (1981).
14. Myers, C. E., R. C. Young, D. G. Johns, and B. A. Chabner. Assay of 5-fluorodeoxyuridine 5′-monophosphate and deoxyuridine 5′-monophosphate pools following 5-fluorouracil. *Cancer Res.* **34**:2682–2688 (1974).
15. Danenberg, P. V., and K. D. Danenberg. Effect of 5,10-methylenetetrahydrofolate and the dissociation of 5-fluoro-2′-deoxyuridylate from thymidylate synthetase: evidence for an ordered mechanism. *Biochemistry* **17**:4018–4024 (1978).
16. Gallivan, J. H., G. F. Maley, and F. Maley. Factors affecting substrate binding in *Lactobacillus casei* thymidylate synthetase as studied by equilibrium dialysis. *Biochemistry* **15**:356–362 (1976).
17. Lockshin, A., and P. V. Danenberg. Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding to human thymidylate synthetase. *Biochem. Pharmacol.* **30**:247–257 (1981).
18. Laskin, J. D., R. M. Evans, H. K. Slocum, D. Burke, and M. T. Hakala. Basis for natural variation in sensitivity to 5-fluorouracil in mouse and human cells in culture. *Cancer Res.* **39**:383–390 (1979).
19. Evans, R. M., J. D. Laskin, and M. T. Hakala. Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. *Cancer Res.* **41**:3288–3295 (1981).
20. Yin, M.-B., S. F. Zakrzewski, and M. T. Hakala. Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. *Mol. Pharmacol.* **23**:190–197 (1983).
21. Berger, S. H., M. B. Yin, J. Whalen, and M. T. Hakala. Role of folate (FA) and dUMP pools in response to 5-fluoropyrimidines. *Proc. Am. Assoc. Cancer Res.* **22**:215 (1981).
22. Berger, S. H., and M. T. Hakala. Role of cellular dUMP and FdUMP pools and of excess folinic acid in recovery of thymidylate synthetase activity after 5-fluorouracil treatment. *Proc. Am. Assoc. Cancer Res.* **23**:217 (1982).
23. Zakrzewski, S. F., and A. M. Sansone. A new preparation of tetrahydrofolic acid. *Methods Enzymol.* **18B**:728–731 (1971).
24. Sharma, R. K., and R. L. Kisliuk. Quenching of thymidylate-synthetase fluorescence by substrate analogs. *Biochem. Biophys. Res. Commun.* **64**:648–655 (1975).
25. Dunlap, R. B., N. G. L. Harding, and F. M. Huennekens. Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*. *Biochemistry* **10**:88–97 (1971).
26. Slocum, H. K., and M. T. Hakala. Mechanism of natural resistance to N^6-(Δ^2-isopentenyl)adenosine in cultured cells. *Cancer Res.* **35**:423–428 (1975).
27. Moran, R. G., C. P. Spears, and C. Heidelberger. Biochemical determinants of tumor sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2′-deoxyuridylate, 2′-deoxyuridylate, and thymidylate synthetase. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1456–1460 (1979).
28. Danenberg, P. V., and A. Lockshin. Tight-binding complexes of thymidylate synthetase, folate analogs and deoxyribonucleotides. *Adv. Enzyme Regul.* **20**:99–110 (1982).
29. Aull, J. L., J. A. Lyon, and R. B. Dunlap. Gel electrophoresis as a means of detecting ternary complex formation of thymidylate synthetase. *Microchem. J.* **19**:210–218 (1974).
30. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
31. Dolnick, B. J., and Y.-C. Cheng. Human thymidylate synthetase derived from blast cells of patients with acute myelocytic leukemia. *J. Biol. Chem.* **252**:7697–7703 (1977).
32. Bresnick, E. Feedback inhibition of aspartate transcarbamylase in liver and in hepatoma. *Cancer Res.* **22**:1246–1251 (1962).

Send reprint requests to: Dr. Maire T. Hakala, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, N. Y. 14263.