0026-895X/89/020219-05$02.00/0

MOLECULAR PHARMACOLOGY, 36:219–223

Activity of Thymidylate Synthetase and its Inhibition by 5-Fluorouracil in Highly Enzyme-Overproducing Cells Resistant to 10-Propargyl-5,8-Dideazafolate

KATHLEEN D. DANENBERG and PETER V. DANENBERG

Department of Biochemistry and Comprehensive Cancer Center, University of Southern California School of Medicine, Los Angeles, California 90033

Received January 23, 1989; Accepted May 8, 1989

SUMMARY

Mouse FM3A mammary adenocarcinoma cells exposed to the specific thymidylate synthetase (TS) inhibitor 10-propargyl-5,8-dideazafolate (PDF) responded by overproducing TS up to 200-fold. In the absence of inhibitor, the elevation of TS levels decayed with a half-life of about 4 weeks. Southern blot analysis of restricted DNA from the PDF-resistant cells using a TS-specific probe showed that the TS gene was amplified to the same extent as enzyme levels. The PDF-resistant cells showed moderate cross-resistance to growth inhibition by 5-fluoro-2′-deoxyuridine, which increased with TS overproduction, but cross-resistance to 5-fluorouracil (FUra) was less (2- to 3-fold) and did not change with increased TS levels. TS activity, measured as release of tritium from [5-^{3}H]2′-deoxyuridine, was no higher in the intact PDF-resistant cells than in wild-type cells. Inhibition of TS activity by FUra in the wild-type cells was accompanied by a proportional decrease in the amount of free TS, presumably due to formation of the tight binding complex of TS with 5-fluoro-2′-deoxyuridylate and 5,10′-methylenetetrahydrofolate. However, in the PDF-resistant cells, most the TS was still in the free form even though TS activity was substantially (85–90%) inhibited. Addition of folinic acid did not change either the sensitivity of the cells to FUra or the rates of tritium release in the cells having overproduced TS. These results are consistent with compartmentalization of TS, possibly in a multienzyme complex.

The ability of cells to amplify genes permits them to develop a simple and effective mechanism of resistance to tight-binding inhibitors of essential enzymes. The resulting overproduction of the target enzyme "soaks up" the inhibitor and then provides enough extra enzyme activity to allow the cells to survive in normally lethal levels of the inhibitor. Gene amplification was first characterized in cells treated with the anticancer drug MTX (1), which is a tight-binding inhibitor of DHFR. The DHFR gene was found to be present at levels hundreds of times above normal in cultured cells exposed to the drug and, more recently, amplification of the DHFR gene has been observed in clinical specimens from patients treated with MTX (2, 3). Similarly, the levels of TS, an essential enzyme of DNA synthesis, become elevated in cells treated with the cytotoxic agent FdUrd (4, 5), which gives rise to the tight-binding TS inhibitor FdUMP (6), and with PDF, a folate analog that specifically and strongly inhibits TS (7).

We were interested in observing the effects of the pyrimidine base FUra on TS-overproducing cells, because this drug, in contrast to FdUrd, is thought to have multiple sites of action, including (i) inhibition of TS through the metabolite FdUMP, (ii) alteration of RNA processing because of extensive incorporation, and (iii) fragmentation of DNA due to incorporation and excision of FUra residues (6, 8). The idea was that specifically alterating one of the potential targets of FUra (i.e., increasing the amount of TS in the cells) would permit an assessment of the contribution of that particular pathway to the cytotoxicity of the drug. For example, it might be reasonable to assume that if TS were the major target of FUra action then cells which have elevated levels of TS ought to be proportionately more resistant to the drug, whereas if effects on other targets contribute significantly to the mechanism of action of FUra cells would be killed by FUra without appreciable inhibition of TS activity. Thus, we set out to develop highly TS-overproducing FM3A cells and to examine the effects of FUra on these cells with regard to the degree of cross-resistance, inhibition of TS activity, and free TS remaining after treatment with growth-inhibitory levels of FUra. The salient findings of this study were that, contrary to expectations, the apparent TS activity in intact cells is not proportional to TS amplification

This investigation was supported by American Cancer Society Grant CH-1.

ABBREVIATIONS: MTX, methotrexate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; FdUrd, 5-fluoro-2′-deoxyuridine; PDF, 10-propargyl-5, 8-dideazafolic acid; FUra, 5-fluorouracil; 5,10-CH_3H_4folate, 5,10-methylenetetrahydrofolic acid; FdUMP, 5-fluoro-2′-deoxyuridylic acid; dUrd, 2′-deoxyuridine.

and most of this activity can be inhibited by FUra without binding up an appreciable percentage of the overproduced enzyme. These results suggest that the overproduced TS does not interact with exogenously supplied substrates and inhibitors, which presents a complication for the use of these cells to diagnose the mechanism of action of FUra.

Materials and Methods

FUra and FdUrd were obtained from Sigma Chemical Co. (St. Louis, MO). Radiolabeled [5-^{3}H]FdUMP (specific activity 21 Ci/mmol) and [5-^{3}H]dUrd (specific activity, 20 Ci/mmol) were obtained from Moravek Biochemicals (La Brea, CA). PDF was a gift of Dr. Terry Jones. Cell culture media and sera were purchased from GIBCO (Grand Island, NY). 5,10-CH_2H_4folate was prepared as described previously (9). Plasmid pMTS-3, which contains cDNA corresponding to mouse TS mRNA, was a gift of Dr. Lee F. Johnson.

Cell culture. Conditions for the growth of FM3A cells were similar to those described by Ayusawa *et al.* (10). RPMI medium supplemented with 10% dialyzed fetal calf serum (20 ml) in a plastic T-flask was inoculated with a 1-ml suspension of FM3A cells to a density of 5×10^4/ml. The cells were allowed to grow at 37° to a cell density of not greater than 10^6 cells/ml. At this point, an aliquot of the cell culture that contained about 5×10^5 cells was removed and placed into a fresh solution of medium and serum, so that the new culture would contain about 5×10^4 cells/ml (usually about 20-fold dilution). Using this growth procedure, cells were adapted to growth in stepwise increasing concentrations of PDF. Adaptation is defined as the point at which the growth rate of the cells in the presence of the drug became stabilized. The cell cultures designated as 0.3Q, 0.6Q, and 1.2Q correspond, respectively, to cultures adapted and maintained in 0.15, 0.3, and 0.6 mM PDF.

For determination of the growth inhibitory potency of the drugs, TS activity, and TS content, cells were first grown without any added PDF for at least three medium changes, resulting in a dilution of the original PDF concentration by about 8000-fold. Although we did not synchronize the cell cultures for these experiments, we used cells that were as nearly as possible at the same stage of mid-logarithmic growth (5×10^5 cells/ml).

Intracellular TS activity. The assay for TS activity in intact cells involved measuring the release of tritium from [5-^{3}H]dUrd, based on the method described by Yalowich and Kalman (11). About 10^6 cells were removed from a logarithmically growing culture, centrifuged at 800 rpm for 10 min, and resuspended in 5 ml of fresh medium. [5-^{3}H]dUrd (2.5 Ci/mmol) was added in volume of 10 μl to give a final concentration of 1 μM. Aliquots of 0.2 ml were removed from the reaction mixture at intervals, after it was verified that the cells were well suspended. Each aliquot was added to a 1.5-ml polypropylene Eppendorf micro-test tube containing 1 ml of 3% charcoal in 0.2 N hydrochloric acid. The mixtures were shaken on a vortex mixer for 10 sec and allowed to stand at room temperature for 10 min. The microtubes were then centrifuged in a model 235C Fisher microcentrifuge for 10 min and 0.6 ml of the supernate was removed for liquid scintillation counting.

Analysis of the TS gene. For Southern blot analysis (12) of TS gene sequence, 0.1–5 μg of DNA, isolated from the parent FM3A cells and the 0.6Q cells by the method of Pellicer *et al.* (13), were treated with *Eco*RI, fractionated through a horizontal 0.4% agarose gel, and transferred to a nylon membrane (Schleicher and Schuell). The 1.1-kilobase *Pst*I fragment of pMTS-3 (14) was used as the hybridization probe after it was labeled by the random priming method (15) with [^{32}P]dCTP (3000 Ci/mmol).

TS content of cells. The amount of TS present in cell-free supernatants was determined by binding the enzyme to excess [5-^{3}H]FdUMP in the presence of 5,10-CH_2H_4folate, essentially as described previously (16, 17).

Results

Amplification of TS levels in cells grown in the presence of PDF. PDF inhibited the growth of the parent FM3A cells with an IC_{50} value of 2 μM. The cells were adapted to stepwise increases in drug concentration by propagating them in the presence of the drug until the doubling time of the cells did not decrease any further. Adaptation of the cells to 0.15 mM PDF required about 6 weeks of growth in culture, but subsequent steps of adaptation to the higher drug levels occurred more rapidly. The maximal growth rates of the cell cultures that were resistant to each successively higher drug concentration decreased from a doubling time of 12 hr for the parent cells to about 18 hr for the 1.2Q cells. As shown in Table 1, the amplification of TS was linearly proportional to the concentration of PDF to which the cells had become resistant. These values represent maximal plateau levels of TS in the PDF-resistant cells, which were attained concomitantly with stabilization of the growth rates of the cells in the presence of PDF. When the cells were grown in the absence of the inhibitor, the TS levels of the cell culture declined with a $t_{1/2}$ value of approximately 4 weeks (40–60 generations).

Analysis of the relative amount of the TS gene. In order to verify that the enhancement of TS levels in the PDF-resistant cells was due to gene amplification, we carried out a Southern blot analysis of the TS gene in *Eco*RI-restricted genomic DNA from wild-type FM3A and the 0.6Q cells, using mouse TS probe pMTS-3 developed by Geyer and Johnson (14) (Fig. 1). Densitometer scans showed that the bands were about 90-fold more intense than in the wild-type cells, which correlates well with the 100-fold TS elevation found in these cells (Table 1). A band at 5 kilobases did not appear to be amplified.

Cross-resistance of the TS-amplified cells to growth inhibition by FUra. Growth of the wild-type cells was inhibited by FUra with an IC_{50} value of 0.2 μM (Table 2). The IC_{50} values for growth inhibition of the PDF-resistant cells were only about 3-fold higher than this value even though these cells were substantially cross-resistant to FdUrd. The IC_{50} values of FUra against the TS-overproducing cells were similar, regardless of the degree of TS elevation, in contrast to those of FdUrd, which increased with increasing TS content of the cells.

TS activity in intact cells. The measurement of TS activity cells is based on the intracellular conversion of [5-^{3}H]dUrd by thymidine kinase to [5-^{3}H]dUMP, which, when converted to dTMP by the action of TS, will release 1 equivalent of tritium into the water (11, 18). Because the assay depends on a coupled enzyme system, it probably cannot be used to deter-

TABLE 1

TS content of PDF-resistant FM3A cells

FM3A cells were grown in the presence of incrementally increasing PDF concentrations, as described in Materials and Methods. The TS contents of the supernatants of the resistant cells were determined by the [^{3}H]FdUMP binding assay after the growth rate of the cells in the drug had become constant and represent plateau values that did not increase further upon prolonged maintenance of the cells in the concentrations of PDF indicated.

Cells resistant to concentration of PDF	Our designation	FdUMP binding	Ratio of TS content
μM		*pmol/10^6 cells*	
	Wild-type	0.12 ± 0.02	1
150	0.3Q	7.32 ± 1.20	61
300	0.6Q	12.2 ± 2.3	100
600	1.2Q	25.2 ± 3.2	210

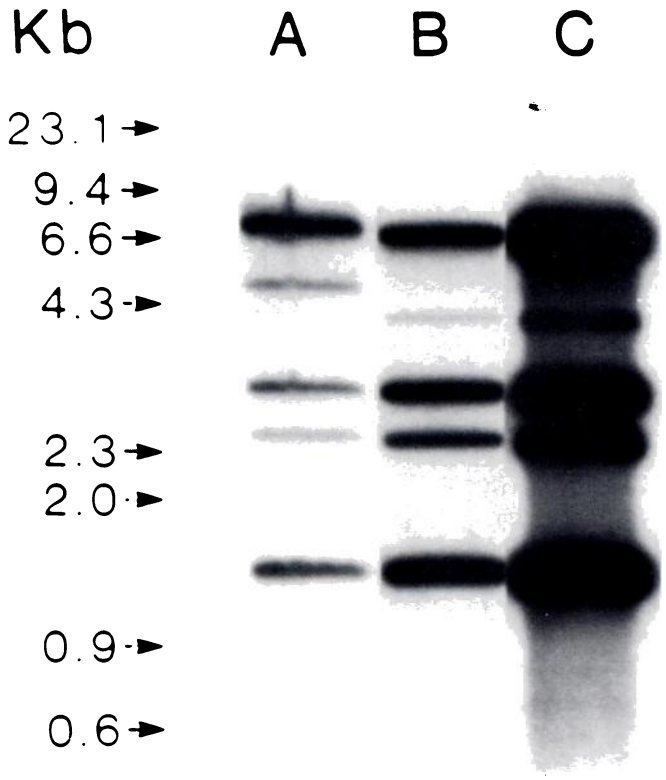

Fig. 1. Southern blot analysis of the TS gene from wild-type and PDF-resistant FM3A 0.6Q cells. DNAs were digested with *Eco*RI and fractionated on a 0.4% agarose gel. The products were transferred to nylon and hybridized to ^{32}P-labeled DNA from pMTS-3. *Lane A*, 5 μg of DNA from wild-type FM3A cells; *lane B*, 0.5 μg of DNA from 0.6Q cells; *lane C*, 5 μg of DNA from 0.6Q cells.

TABLE 2

Cross-resistance of PDF-resistant FM3A cells to cell growth inhibition by fluoropyrimidines

Cell type	IC_{50} values[a]	
	FdUrd	FUra
	nM	μM
FM3A wild-type	0.20 ± 0.05	2.0 ± 0.3
0.3Q	5.2 ± 0.5 (26)	5.1 ± 0.5 (2.5)
1.2Q	22 ± 5 (110)	5.5 ± 0.5 (2.7)
1.2Q + 0.1 mM folinic acid	22 ± 5 (110)	

[a] The concentration of inhibitor required to inhibit cell growth by 50%. Each value is an average of at least two determinations. The numbers in parentheses indicate the fold resistance.

mine V_{max} or K_m values for TS; however, it might be expected when comparing two or more cell lines that the initial rate of tritium release should be proportional to the respective amounts of TS in the cells, provided that the activity of thymidine kinase and other factors such as nucleotide transport, dUMP, and folate levels did not vary. Thus, we originally anticipated using this assay as a convenient method of following elevation of TS levels in the PDF-treated cells as they developed resistance to the drug. However, as seen in Fig. 2, the

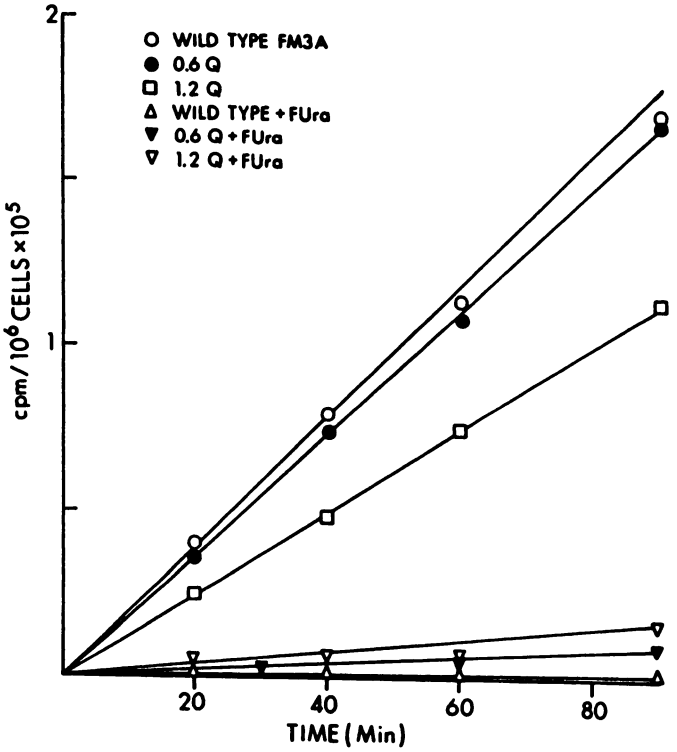

Fig. 2. TS activity in intact FM3A cells. TS activity was determined by the rate of tritium release form [5-^{3}H]dUrd in untreated cells and in cells after treatment with 0.1 mM FUra for 2 hr, as described in Materials and Methods.

rates of tritium release from [5-^{3}H]dUrd administered to the PDF-resistant cells were the same as or less than that of the wild-type control cells, regardless of the degree of TS amplification. When the concentration of [5-^{3}H]dUrd given to the cells was increased 10-fold, the absolute reaction velocities of tritium release increased proportionately, but the relative rates of tritium release were still the same as in Fig. 2 (data not shown).

Inhibition of TS activity by FUra and correlation with levels of total free TS in the cells. In the wild-type FM3A cells FUra inhibited almost all of the tritium-release TS activity within 30 min of exposure time, whereas in the TS-elevated cells FUra inhibited most, but not all, of the initial TS activity (Fig. 2). This residual TS activity, which was not eliminated by higher concentrations of FUra and was constant at least over the time period of this experiment (90 min), was higher (about 15% of original) in the 1.2Q cells than in the 0.6Q cells (about 10% of original). We wanted to see whether the decrease in TS activity after FUra treatment would be matched by a corresponding decrease in the total free TS present in the cells. Accordingly, free TS remaining after FUra treatment was measured by the [6-^{3}H]FdUMP binding assay (6, 17). The correlation between TS inhibition and free TS remaining was good in the wild-type cells. As shown in Table 2, there was little free TS remaining (10% of original) in the wild-type cells after 2 hr of FUra treatment. However, the levels of free TS in the 0.6Q and 1.2Q cells did not shown any significant decrease, in spite of the substantial inhibition of TS activity by FUra. The 1.2Q cells were exposed to FUra for longer time periods to see whether eventually more of the total TS in the cells might not become inhibited. After a 48-hr exposure time, the total free TS per surviving cell appeared to decrease by about 10%,

which corresponds to an amount of enzyme roughly 20 times that normally found in cells not adapted to the drugs.

Discussion

In this study, we have shown that resistance of mouse mammary carcinoma FM3A cells to the specific TS inhibitor PDF was accompanied by a linear elevation in intracellular levels of the target enzyme TS and a similar degree of amplification of the TS gene. Elevation of TS in response to TS inhibitors has been observed previously in cells resistant to FdUrd (4, 5) and PDF (7), but the reported increases were lower than obtained in the present study and, moreover, seemed to occur only to a limited extent. For example, Rossana *et al.* (4) found it difficult to obtain more than a 20-fold TS elevation in response to FdUrd unless MTX-resistant cells were used, in which case the TS elevation increased to 51-fold (4). Berger *et al.* (5) found that folinic acid was necessary to obtain appreciable TS elevation in response to FdUrd; they proposed that a limiting factor on TS overproduction was depletion of reduced folates in the cells due to the formation of large amounts of the ternary TS-FdUMP-5,10-CH_2H_4 folate complex. However, folate depletion would not be expected to limit TS overproduction in PDF-treated cells, because PDF can itself act as the folate component of the ternary complex (19). Jackman *et al.* (7) reported a 45-fold TS elevation in PDF-resistant L1210 cells, but it was not clear whether this was the maximal amount of enzyme elevation that could be attained with the drug. With the FM3A cells, we have not yet been able to determine that there is a maximal TS amplification. Further exposure of the 1.2Q cells to 1.0 mM PDF resulted in 400-fold overproduction of TS,[1] but at concentrations higher than this the inhibitor was insoluble. With more soluble inhibitors, it should be possible to push the TS elevation even higher. Because TS is easily purified by means of affinity chromatography (16), such highly TS-elevated cells may be useful as a source of large amounts of mammalian TS for enzymological, X-ray crystallographical, or sequencing studies.

The Southern blot of the DNA from the 0.6Q cells shows that the major portion of the bands hydridizing to the mouse TS probe were amplified to the same extent that the TS protein was overproduced, without any apparent rearrangements of the amplified gene. However, there was an unamplified band of about 5 kilobases, indicating the presence of pseudogenes or possibly another functional unamplified TS gene that is separate from the region of amplification. A similar unamplified band was observed previously in FdUrd-resistant mouse cells (5). The decline in TS levels after withdrawal of the inhibitor suggests that a substantial percentage of the amplified TS gene resides in unstable double minute chromosomes (20).

The low tritium-release activity in the PDF-resistant cells (Fig. 1) was contrary to our initial expectation that the rate of this reaction would be greater in cells having higher TS levels. In fact, the apparent TS activity in the 1.2Q cells was 30% lower than in wild-type cells, roughly in proportion to the reduced growth rate of these cells. These results show that in the drug-resistant cells, which contain TS levels hundreds of times greater than normal, not more than a small fraction of the potentially available enzyme activity is utilized. The most likely explanation for this phenomenon is that enzyme activity is being limited by substrate availability. It seems reasonable to suppose that, if the cellular metabolism is adapted to furnish a sufficient quantity of substrates for wild-type levels of enzyme, abnormally high levels of activity from overproduced enzyme could only be sustained for a short time until the available substrate pool is used up. When this happens, the enzyme activity would be limited by the rate of production of its substrates by other enzymes. In the case of TS, these enzymes would most likely be either dihydrofolate reductase or ribonucleotide reductase, which furnish tetrahydrofolate and dUMP, respectively.

Folinic acid has been shown to expand the 5,10-C_2H_4folate pool in mouse cells by about 6-fold (21), but addition of this precursor to the cell cultures produced no change in tritium release rates, suggesting that reduced folate levels are not the limiting factor on the TS activity. The previous observation that hydroxyurea, an inhibitor of ribonucleotide reductase, also causes concomitant inhibition of TS activity (18) shows that the turnover rate of TS could be dependent on the rate of dUMP synthesis. If the amount of dUMP produced really is limiting for the activity of TS, the rate of tritium release may be mostly determined by the activity of thymidine kinase, that is, by the rate of conversion of labeled dUrd to dUMP.

Even though the above data show that the tritium-release activity cannot be used to determine absolute TS levels in cells, the assay should still be valid for evaluating inhibition of TS, because this involves measuring a relative decrease in enzyme activity. Fig. 2 shows that, in wild-type FM3A cells treated with a growth-inhibitory dose of FUra, almost complete inhibition of tritium release activity was observed within 2 hr of exposure. Although in some experiments about 10% of the total TS still appeared to be in the free form when there was little detectable tritium release activity, in general the loss of enzyme activity corresponded fairly closely to formation of the ternary TS-FdUMP-5,10-CH_2H_4folate complex (Table 3). With the TS-overproducing cells, there were several unexpected results. It was reasonable to suppose that the FdUMP produced from FUra would be soaked up by the excess enzyme present in these cells just as PDF is and, therefore, inhibition of TS activity would require much higher drug levels and/or longer exposure times. However, maximal inhibition of TS activity occurred just as rapidly (i.e., within 2 hr) at the same concentration of FUra that inhibited TS in wild-type cells. The most surprising aspect of the behavior of the PDF-resistant cells was that inhibition of most of the TS activity (85–90%) occurred without any apparent depletion of the total free TS in these cells. This observation does not appear to be consistent with the basic principle of enzyme inhibition theory that loss of enzyme

TABLE 3

Effect of FUra treatment on the intracellular TS levels

The wild-type and PDF-resistant FM3A cells were treated with 0.1 mM FUra for the indicated times, after which total intracellular TS content was determined by the [5-^{3}H]FdUMP binding assay, as described in Materials and Methods. Each value is an average of at least three determinations.

Cells	FUra treatment	TS content	
		Before FUra	After FUra
	hr	*cpm of [5-^{3}H]FdUMP bound/10^6 cells*	
FM3A wild-type	2	5400 ± 500	600 ± 250
0.6Q	2	5.20 ± 0.04 × 10^5	5.10 ± 0.05 × 10^5
1.2Q	2	1.08 ± 0.05 × 10^6	1.10 ± 0.06 × 10^6
	48		0.98 ± 0.05 × 10^6

[1] P. V. Danenberg, unpublished results.

activity is due to the formation of a corresponding amount of an enzyme-inhibitor complex. One possible explanation is that the decrease in TS activity is an indirect effect due to depletion of 5,10-CH_2H_4folate as the ternary complex is formed. This mechanism was proposed by Jackman *et al.* (7) to account for the fact that their PDF-resistant L1210 cells with highly elevated TS were not initially cross-resistant to FdUrd but did become cross-resistant when folinic acid was added. However, the FM3A cells that we used in these studies appear to have higher endogenous levels of reduced folates because (i) the cells had some cross-resistance to FdUrd and (ii) addition of folinic acid altered neither sensitivity to FdUrd nor the effect of FUra on intracellular TS activity.

The inhibition data are difficult to interpret in terms of a one-compartment model for intracellular TS. Instead, it appears as if only a small portion of the TS in the overproducing cells comes into contact with substrates or inhibitors, possibly because of compartmentalized localization of the enzyme combined with channeling of nucleotide precursors directly to this pool of TS. There is considerable evidence, although mostly indirect, that DNA synthesis enzymes, including TS, are assembled in a multi-enzyme complex during replication (22, 23, 24). One of the main functions of such complexes presumably is to channel substrates from one enzyme to the next in the metabolic pathway, thereby achieving greater metabolic efficiency. If such an apparatus did exist in these cells, both the inhibition data and the enzyme activity data could be explained by a channeling of dUMP and FdUMP directly to the active TS in the replication complex. In this way, the TS activity could be inhibited by FUra without the need for complexing of the bulk of the overproduced enzyme, which would be inactive because of lack of access to substrates. However, this inactive enzyme is of survival value to cells exposed to PDF because it acts as a sponge for the drug, which enters the cell as the active inhibitory form. Fernandes and Cranford (25), noting that CCRF cells exposed to FdUrd contained substantial amounts of free TS even though DNA synthesis was inhibited, also suggested that the accumulated free TS might be enzyme that had not become integrated into a multi-enzyme complex.

It is interesting to note that 10–15% of the TS activity was not inhibitable by FUra in the PDF-resistant cells. In the context of the above hypothesis, this residual activity may be due to leakage of [5-^{3}H]dUMP into the larger pool of TS. The effect of FdUrd against the PDF-resistant cells may be explained in similar terms. The cells do show cross-resistance to FdUrd, indicating that the amplified TS pool does sequester a portion of the FdUMP generated from FdUrd. However, the cells are much more sensitive to growth inhibition by FdUrd than would have been expected considering the amount of extra TS in these cells. In the previous study of Rossana *et al.* (4), 3T6 cells that were selected for resistance to FdUrd had 13-fold elevated TS and were 250-fold resistant to FdUrd; by contrast, our 1.2Q cells contain 200-fold more TS than normal but are only about 100-fold resistant to FdUrd. Thus, it is likely that there is still considerable free TS in the PDF-resistant FM3A cells when they are being growth-inhibited by FdUrd.

These results have shown that there is not necessarily a close association between enzyme activity and the total amount of enzyme in intact cells. Thus, the assumption made in many studies that the ratio of "free" TS to FdUMP-bound TS accurately reflects the percentage of TS enzyme activity remaining after fluoropyrimidine treatment may not be correct in all cases. The compartmentalization model that we have suggested can be considered as a working hypothesis at this time, which should be further tested. If true, it will probably have novel implications for many studies of drug mechanisms.

References

1. Alt, F. W., R. E. Kellems, J. R. Bertino, and R. T. Schimke. Multiplication of dihydrofolate genes in methotrexate-resistant variants of cultured murine cells. *J. Biol. Chem.* **251**:3063–3074 (1978).
2. Trent, J. M., R. M. Buick, S. Olson, R. C. Horns, Jr., and R. T. Schimke. Cytologic evidence for gene amplification in methotrexate-resistant cells obtained from a patient with ovarian adenocarcinoma. *J. Clin. Oncol.* **1**:8–15 (1984).
3. Carman, M. D., J. H. Schornagel, R. S. Rivest, S. Srimatkandada, C. S. Portlock, T. Duffy, and J. R. Bertino. Resistance to methotrexate due to gene amplification in a patient with acute leukemia. *J. Clin. Oncol.* **1**:16–20 (1984).
4. Rossana, C., L. G. Rao, and L. F. Johnson. Thymidylate synthase overproduction in 5-fluorodeoxyuridylate-resistant mouse fibroblasts. *Mol. Cell. Biol.* **2**:1118–1125 (1982).
5. Berger, S. H., C.-H. Jenh, L. F. Johnson, and F. Berger. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol. Pharmacol.* **28**:461–467 (1985).
6. Heidelberger, C., P. V. Danenberg, and R. G. Moran. Fluorinated pyrimidines and their nucleosides. *Adv. Enzymol. Relat. Areas Mol. Biol* **54**:58–119 (1983).
7. Jackman, A. L., D. L. Alison, A. H. Calvert, and K. R. Harrap. Increased thymidylate synthase in L1210 cells possessing acquired resistance to N^{10}-propargyl-5,8-dideazafolic acid (CB3717): development, characterization, and cross-resistance studies. *Cancer Res.* **46**:2810–2615 (1986).
8. Schuetz, J. D., H. J. Wallace, and R. B. Diasio. 5-Fluorouracil incorporation into DNA of CF-1 mouse bone marrow cells as a possible mechanism of toxicity. *Cancer Res.* **44**:1358–1363 (1984).
9. Danenberg, P. V., and K. D. Danenberg. The effect of 5,10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2′-deoxyuridylate from thymidylate synthetase: evidence for an ordered mechanism. *Biochemistry* **17**:4018–4024 (1978).
10. Ayusawa, D., H. Koyama, K. Iwata, and T. Seno. Selection of mammalian thymidine auxotrophic cell mutants defective in thymidylate synthase by their reduced sensitivity to methotrexate. *Somat. Cell Genet.***7**:523–534 (1981).
11. Yalowich, J. C., and T. I. Kalman. Rapid determination of thymidylate synthase activity and its inhibition in intact L1210 mouse leukemia cells *in vitro*. *Biochem. Pharmacol.* **34**:2319–2324 (1985).
12. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517 (1975).
13. Pellicer, A., M. Wigler, R. Axel, and S. Silverstein. *Cell* **14**:133–141 (1978).
14. Geyer, P. K., and L. F. Johnson. Molecular cloning of DNA sequences complementary to mouse thymidylate synthase mRNA. *J. Biol. Chem.* **259**:7206–7211 (1984).
15. Feinberg, A. P., and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137**:266–267 (1984).
16. Lockshin, L., R. G. Moran, and P. V. Danenberg. Thymidylate synthetase purified to homogeneity from human leukemic cells. *Proc. Natl. Acad. Sci. USA* **76**:750–754 (1979).
17. Moran, R. G., C. P. Spears, and C. Heidelberger. Biochemical determinants of sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2′-deoxyuridylate, 2′-deoxyuridylate, and thymidylate synthase. *Proc. Natl. Acad. Sci. USA* **76**:1456–1460 (1979).
18. Rode, W., K. J. Scanlon, B. A. Moroson, and J. R. Bertino. Regulation of thymidylate synthase activity in mouse leukemia cells. *J. Biol. Chem.* **255**:1305–1311 (1980).
19. Pogolotti, A. L., P. V. Danenberg, and D. V. Santi. Kinetics and mechanism of interaction of 10-propargyl-5,8-dideazafolate with thymidylate synthase. *J. Med. Chem.* **29**:478–482 (1986).
20. Schimke, R. T., P. C. Brown, R. J. Kaufman, M. McGrogan, and D. L. Slate. Chromosomal and extrachromosomal localization of amplified dihydrofolate reductase genes in cultured mammalian cells. *Cold Spring Harbor Symp. Quant. Biol.* **55**:785–797 (1981).
21. Keyomarsi, K., and R. G. Moran. Folinic acid augmentation of the effects of fluoropyrimidines on murine and human leukemic cells. *Cancer Res.* **41**:3288–3295 (1986).
22. Srere, P. A. Complexes of sequential metabolic enzymes. *Annu. Rev. Biochem.* **56**:89–124 (1987).
23. Reddy, G. P. V., and A. B. Pardee. Multienzyme complexes for metabolic channeling in mammalian DNA replication. *Proc. Natl. Acad. Sci. USA* **77**:3312–3316 (1980).
24. Reddy, G. P. V. Catalytic function of thymidylate synthase is confined to the S phase due to its association with replitase. *Biochem. Biophys. Res. Commun.* **109**:908–915 (1982).
25. Fernandes, D. J., and S. K. Cranford. Dissociation of thymidylate biosynthesis from DNA biosynthesis by 5-fluoro-2′-deoxyuridine and 5,8-dideazaisofolic acid. *Cancer Res.* **46**:1741–1747 (1986).

Send reprint requests to: Peter V. Danenberg, USC Cancer Center, 1303 N. Mission Rd., Los Angeles, CA 90033.