A Trojan Horse Approach for Silencing Thymidylate Synthase†

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ABSTRACT: In this paper we present a new and possibly more effective way of inhibiting thymidylate synthase (TS) in cells than through the use of substrate analogue inhibitors. An inactive double mutant of TS (DM), $Arg_{126}Glu/Cys_{146}Trp$, is shown to progressively impair the reactivation of native *Escherichia coli* TS when the two are denatured together in vitro. The individual single mutant proteins $Arg_{126}Glu$ and $Cys_{146}Trp$ showed little or no inhibition. When the DM is introduced into *E. coli* and induced from an expression plasmid, the mutant subunits act as a decoy in deceiving newly formed native TS subunits to fold with them to yield inactive heterodimers. As a consequence of the depletion of TS, the cells die a "thymineless" death when grown in medium devoid of thymine. Addition of thymine to the medium enables the cells to grow normally, although only very low levels of TS activity could be detected in those cells containing induced DM. The individual single-site mutations of the DM, $Arg_{126}Glu$ and Cys_{146}-Trp, did not inhibit growth, as might be expected from the in vitro studies. However, when a nontoxic level of 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) is added to growing DM-transformed cells, the combination is lethal to the cells. These experiments suggest that a similar dominant-negative response to the DM of TS could be affected in tumor cells, for which preliminary evidence is presented. This technique, either alone or combined with other modalities, suggest a new approach to targeting cells for chemotherapy.

Thymidylate (dTMP)[1] is an essential constituent of most cellular DNAs, and if it is prevented from being synthesized by inhibitors of thymidylate synthase (TS; EC 2.1.1.45), the cells die of what was referred to by Barner and Cohen (*1*) as "thymineless" death. As a consequence, the targeting of this enzyme for inactivation by substrate analogues has been and still is a major objective of those investigators interested in developing drugs for chemotherapy. Since 1957, however, when the first of these drugs appeared in the form of 5-fluorouracil (FU) (*2*), none more effective than this compound have been found to replace it in the clinic, despite a large synthetic effort. FU acts in the cell by being converted to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), whereupon it binds to TS (*3*−*5*) at its active-site cysteine with 5,10-methylenetetrahydrofolate ($CH_2H_4PteGlu$) (*6*, *7*), the second substrate of this enzyme (*8*), to form a tight inhibitory complex with a K_d of 10^{-13} M (*5*). By expanding the levels of folate within the cell (*9*−*11*), where folate and its derivatives are present as their corresponding polyglutamate intermediates (*12*, *13*), the transition-state analogue resulting from the interaction of FdUMP and $CH_2H_4Pte(Glu)_n$ at the active site of TS was shown to be even tighter than with CH_2H_4Glu (*14*, *15*). Since nucleotide analogues of TS, of which FU is the most potent, did not provide a satisfactory solution to the chemotherapy problem because of resistance and toxic side effects, an effort was made to develop folate analogues that might be more selective for TS. Although several of these have been reported (*16*), they suffer from defects similar to those of FU, although FU is still used clinically for want of a better product.

In this paper we describe a new means of silencing TS, one that employs a Trojan horse, or a dominant-negative approach where native TS subunits interact and fold with inactive mutant TS subunits, to yield an inactive heterodimer. This approach was suggested from our earlier studies, which demonstrated that specific inactive mutant dimers could exchange subunits in solution to yield a fully active enzyme, although only one of the enzyme's two subunits contained a functional active site (*17*, *18*). These findings are in agreement with earlier studies (*19*−*22*), which suggested that TS is a half-of-the-sites reactivity enzyme (*23*), a proposal that has received support from others more recently (*24*, *25*). We will describe how we have exploited this property to inactivate newly formed active TS subunits within a cell and how this strategy might be used to improve chemotherapeutic responses.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Competent *Escherichia coli* strains HMS174 and Tuner(DE3)pLysS, and the pET3d plasmid were obtained from Novagen (Madison, WI). The green fluorescent protein-containing plasmid pEGFP-C1 was purchased from BD Biosciences, Clontech, Palo Alto, CA.

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[1] Abbreviations: TS, thymidylate synthase; WT, wild-type; DM, double mutant of thymidylate synthase; FU, 5-fluorouracil; FUdR, 5-fluoro-2′-deoxyuridine; T, thymine; TdR, thymidine; dTMP, thymidine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; IPTG, isopropyl β-D-galactopyranoside; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; LB, Luria−Bertani broth; EGFP, enhanced green fluorescent protein; $CH_2H_4PteGlu$, 5,10-methylenetetrahydrofolate; $(Glu)_n$, polyglutamate; V, vector; h, human.

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Mutant Construction. Cloning of the *E. coli ThyA* gene into the plasmid Bluescript KS+ (*26*) (Stratagene, La Jolla, CA) and preparation of the $R_{126}E$ and $C_{146}W$ mutants was described earlier (*17*, *18*). The $R_{126}E/C_{146}W$ double mutant (DM) of TS was made by the QuikChange procedure (Stratagene, La Jolla, CA). The same procedure was used to create a *Nco*I cleavage site at the starting Met of the TS protein to facilitate cloning of the wild type (WT) and the various mutant *ThyA* genes into pET3d as an *Nco*I−*Bam*HI fragment. This construction changes the second amino acid in the enzyme sequence from Lys to Glu but does not alter the activity of the WT-TS**.** Cloning of the human TS gene into pEGFP-C1 to prepare its double mutant was performed in this laboratory by Dr. Agata Fillip (unpublished work). A full-length hTS PCR fragment with *Kpn*I and *Xma*I ends was amplified from a pET17xb-hTS template (*27*) and inserted into the corresponding restriction sites in the EGFP vector. This construction created a fusion protein with 238 amino acids of the EGFP at the N-terminus and the entire human TS at the C-terminus joined by an 18-amino acid linker when expressed from the resulting pEGFP-hTS(DM) vector. The $R_{175}E/C_{195}W$ human double mutant of TS corresponding to the DM of *E. coli* TS ($R_{126}E/C_{146}W$) was made from pEGFP-hTS by the QuikChange procedure. DH5α cells transformed with pEGFP-hTS plasmids were grown in LB with 50 μg/mL kanamycin. DNA minipreps were prepared and sequenced in the Molecular Genetics core facility of the Wadsworth Center to confirm mutagenesis.

Media and Growth Conditions. HMS174 cells transformed with the various pET3d constructs for the preparation of plasmid minipreps were grown in culture tubes containing 5 mL of LB supplemented with 100 μg/mL carbenicillin in a 37 °C shaking water bath overnight. The cells were collected by centrifugation at 1500*g* for 15 min in a Sorvall GLC-2B centrifuge at 4 °C, and the pellets were weighed before being stored at −20 °C. The DNA minipreps prepared from the frozen pellets were purified with a Qiagen kit (Valencia, CA) and used both for DNA sequencing to confirm mutant construction and for in vitro transcription−translation studies. Tuner(DE3)pLysS cells transformed with the pET3d constructs were grown in minimal medium (M9) supplemented with 0.2% casamino acids, 100 μg/mL carbenicillin, and 25 μg/mL chloramphenicol at 37 °C. When growth on a solid medium was undertaken, 1.5% Difco agar was added, and in experiments where thymine was required for growth, it was added at a final concentration of 50 μg/mL. IPTG was added at various times to induce expression of the TS enzymes encoded in the pET3d plasmid. Cell growth was measured by following absorbance at 600 nm in a Spectronic $20D^+$ spectrophotometer. Cells were collected at specific time points to be described and then frozen at −20 °C.

Measurement of TS Activity. Cell extracts were prepared by resuspension of the frozen pellets in a buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, and 10% ethylene glycol at 10 μL/mg of cells. After sonication 2−4 times for 20 s each in an ice/ethanol bath with the small probe of a Vibracel sonicator (Sonics and Materials Inc., Danbury, CT), 150 μL of these whole-cell extracts were centrifuged at 14 000 rpm for 10 min at 4 °C in an Eppendorf 5415 microfuge. The supernatant fractions were saved and assayed for TS activity either spectrophotometrically at 30 °C in the presence of 50 mM $MgCl_2$ as described previously (*28*) or for low levels of activity by the ^{3}H-release method (*29*). In the latter case the assay solutions were incubated at 37 °C for 20 min.

Eukaryotic Cell Studies. MCF-7 cells were grown at 37 °C, under 5% CO_2 in a humidified incubator in Iscove's modified Eagle's medium containing 10% fetal bovine serum, penicillin, streptomycin, and ciprofloxacin. Cells were transfected with pEGFP-hTS (DM) by use of FuGENE (Roche) transfection reagent as described in the protocol provided by the manufacturer. Briefly, 1 μg of plasmid DNA, 3 μL of FuGENE reagent, and 100 μL of serum-free medium were used to transfect MCF-7 cells in a single well of a 6-well tissue culture dish. Two hours after the transfection mixture was applied, 2 mL of the complete culture medium was added. The cells were incubated for 24 h, trypsinized, and plated onto 150-mm tissue culture dishes. After an additional 24 h, those transfected cells that were stable were selected for by use of G418 (Geneticin, Gibco, Carlsbad, CA) at 0.5 mg/mL. Individual G418-resistant colonies were subcultured and chosen for uniform expression of the EGFP-hTS(DM) fusion protein by fluorescence microscopy and flow cytometry. The mass of the EGFP-hTS(DM) fusion protein was also evaluated by Western blot, following SDS−PAGE, and comparison to known molecular weight markers.

The toxicity of FUdR to unmodified MCF-7 cells and to MCF-7 cells stably transfected with the EGFP-hTS(DM) plasmid was evaluated by a cell survival assay. Cells were trypsinized and resuspended at 5000 cells/mL in normal medium, with 100 μL of the cell suspension pipetted into each well of a 96-well tissue culture dish. After 24 h to allow reattachment, the medium was replaced with medium containing serial dilutions, in triplicate, of FUdR or fresh medium alone in the case of the control. The cells were incubated for 7 days, at which time they were fixed with 10% trichloroacetic acid and stained with 0.4% sulforhodamine-B in 1% acetic acid. The fixed, stained cells were dissolved, after being washed with 1% acetic acid, and the absorbance at 630 nm was subtracted from that at 540 nm. Staining intensity was compared to the untreated control and this ratio is presented as percent cell survival.

SDS−Polyacrylamide Gel Electrophoresis. The cell supernatant fractions and some whole-cell extracts from the sonicates were subjected to SDS−PAGE as described by Laemmli (*30*). A 5 μL aliquot was usually sufficient, but for some experiments, the absorbance at 280 nm of the samples was determined and volumes corresponding to 0.1 A_{280} unit were loaded onto a gel. Protein bands were detected by staining with GelCode blue stain reagent (Pierce, Rockford, IL).

In Vitro Transcription−Translation. The concentrations of DNA minipreps were determined by measuring absorbance at 260 nm (*31*). In vitro transcription−translation reactions with varying amounts of DNA were performed with either the PROTEINscript-PRO kit or the ActivePro kit, both from Ambion (Austin, TX). The latter is an improved version of the former, which is no longer commercially available. The extent of translation was measured by use of Easy Tag Express ^{35}S protein labeling mix (Perkin-Elmer Life Sciences Inc, Boston, MA) to label newly synthesized proteins.

Denaturation−Renaturation of WT-TS with DM. The procedure used differed slightly from that described earlier

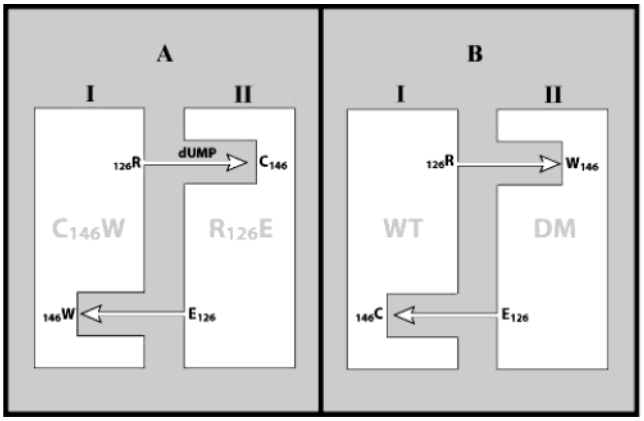

FIGURE 1: Diagrammatic presentation of how TS subunits interact or "swap" amino acids on incubating the homodimers $R_{126}E$ and $C_{146}W$ together in solution (*17*, *18*). As indicated, R_{126} exchanges between subunits I and II**,** either in its native form or as a mutant, $R_{126}E$. When exchange occurs between a subunit of the mutant $C_{146}W$ and a subunit of $R_{126}E$, a dimer results (panel A**)**, in which subunit II is fully active and I is inactive. The "active" active site is designated by dUMP on the arrow, since mutating Arg126 to a Glu severely affects dUMP binding (*35*), as does mutating Cys146 to Trp. When the exchange occurs between a subunit of WT-TS and a subunit of the double mutant, $R_{126}E-C_{146}W$, the resulting heterodimer (panel B) is completely inactive since each active site in I and II contains an inactivating amino acid, $R_{126}E$ in I and $C_{146}W$ in II.

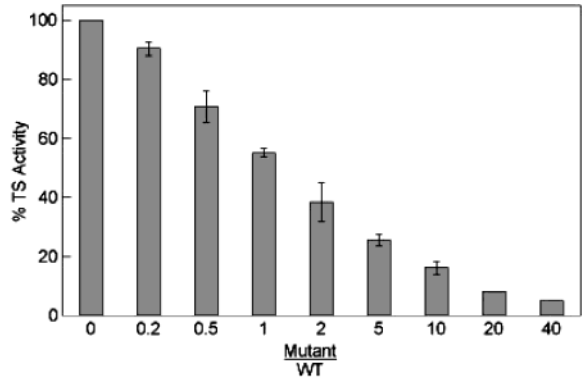

FIGURE 2: Denaturation−renaturation of WT-TS with the DM. The procedure employed to measure the extent of denaturation of WT-TS by the DM is presented under Materials and Methods. The error bars, where presented, represent the standard deviation of the mean in those cases where at least three assays were employed.

by Greene et al. (*32*). WT-TS (125 μg), in the presence of increasing amounts of $R_{126}E/C_{146}W$ (0, 25, 62.5, 125, 250, 625, 1250, 2500, and 5000 μg), was denatured in 750 μL (total volume) for 2 h on ice (0−4 °C) in a solution of 7.4 M urea, 50 mM KH_2PO_4, 0.1 mM EDTA, 1 mM DTT, and 0.5 M KCl, pH 7.5. Each reaction (400 μL) was diluted to 4 mL with 50 mM KH_2PO_4, 0.1 mM EDTA, 1 mM DTT, and 0.5 M KCl, pH 7.5. The proteins were allowed to refold overnight (∼20 h) on ice, after which 100 μL of the renatured solution was assayed for TS activity (*28*). The percentage of the TS activity recovered in each case, relative to the activity of WT-TS alone, was plotted against the ratio of mutant to WT-TS. The WT-TS activity recovered in the absence of the DM was about 90% of the activity prior to denaturation.

RESULTS

Inhibition of WT-TS by Subunit Exchange. The X-ray structures of a variety of TSs clearly show that two arginines from one subunit overlap with the other identical subunit, in what has been referred to as "domain swapping" (*33*). These arginines form part of the active-site region and contribute to the binding of dUMP through its phosphate residue. In the case of *E. coli* TS the arginines constitute amino acid residues 126 and 127 in one subunit and 126′ and 127′ in the other. When Arg_{126} is mutated to a Glu ($R_{126}E$) in one dimer and Cys_{146} to a Trp ($C_{146}W$) in the other and the two inactive mutants are mixed in solution, TS activity is gradually restored due to the restitution of one of the two active sites that results from the exchange of subunits between $R_{126}E$ and $C_{146}W$. This is shown in Figure 1 where $R_{126}E$ (E_{126}), is transferred or "swapped" from subunit AII to AI, resulting in the complete inactivation of the active site at subunit AI. However, the transfer of R_{126} from subunit AI to AII reconstitutes the active-site amino acids in the second subunit (AII) that are necessary for activity. The presence of dUMP at the AII active site indicates that the nucleotide substrate, whose binding is greatly impaired in both mutants, is restored in the AII site of the heterodimer. Since the specific activity of the resulting heterodimer is not unlike that of WT-TS, although one of the two subunits is nonfunctional, AI−II can be considered a half-of-the-sites reactivity enzyme (*23*).

However, when the subunits of the DM ($R_{126}E/C_{146}W$) are permitted to exchange with those of WT-TS, one would expect the structure in panel B to be formed, where each subunit of the dimer now has an amino acid modified that is crucial for activity ($R_{126}E$ in I and $C_{146}W$ in II), resulting in a completely inactive enzyme. To demonstrate that the DM is capable of inactivating WT-TS, the latter was denatured with $R_{126}W/C_{146}W$ in 8M urea followed by renaturation, as described earlier (*32*). In contrast to the earlier study, where restoration of activity was the primary goal, it is shown in Figure 2 that addition of increasing amounts of the DM to the solution results in a progressive inhibition of a fixed amount of WT-TS, due to the formation of the inactive heterodimer (Figure 1B). When similar experiments were conducted with the inactive single mutants, $R_{126}E$ and $C_{146}W$, WT-TS was only slightly inhibited under the same conditions. Since the DM was capable of inactivating WT-TS in vitro, we considered whether a similar effect could be achieved within the cell. A more complete discussion of the mechanism of action of TS and the role of Arg126 is presented in Carreras and Santi (*34*) and Strop et al. (*35*).

Effect of the DM on the Growth of E. coli. To determine whether what was observed in Figure 2 could be transposed to the cell, the gene for the DM was inserted into pET3d for expression in *E. coli* BL21-Tuner cells. The latter cells, because of a mutation in the *lac* permease, permit a concentration-dependent induction of protein by IPTG. The transformed cells were examined for colony formation when grown in minimal medium (Figure 3). It is seen in those plates containing cells with plasmid minus a TS insert (plates A1−3) that there was no change in the extent of colony formation, even in the presence of IPTG. A similar response occurred when the plasmid insert was $R_{126}E$ plates (plates C1−3) or $C_{146}W$ (not shown). However, when cells containing the DM insert were induced by IPTG, no colonies were observed, indicating that the DM is toxic to the cells (plate

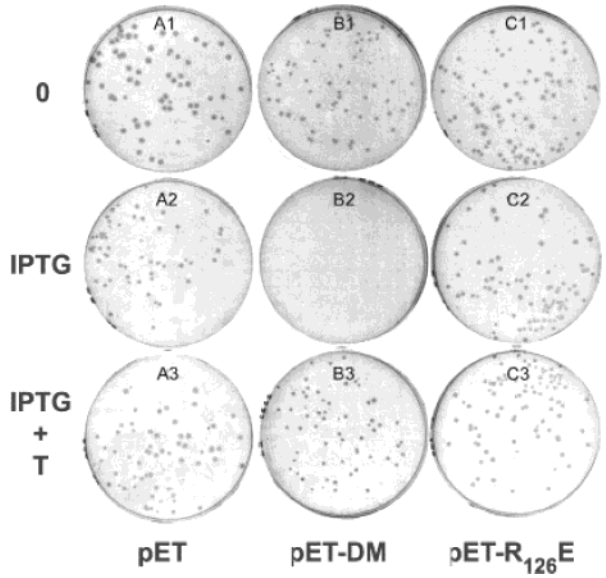

FIGURE 3: Demonstration that *E. coli* cell growth can be impaired by the DM. Single colonies of freshly transformed cells (with vector indicated on the horizontal axis) were used to inoculate 5 mL cultures of minimal medium containing casamino acids supplemented with 100 μg/mL carbenicillin, 25 μg/mL chloramphenicol, and 50 μg/mL thymine. The cultures were shaken at 37 °C for about 5 h. Aliquots of the cells were then diluted (2−5) × 10^5-fold into the same medium lacking thymine and plated onto agar plates of minimal medium with casamino acids and antibiotics. The plates were supplemented with 50 μM IPTG and/or thymine, as indicated on the vertical axis, and were incubated at 37 °C for about 24 h. Plates A1−3, Tuner(DE3)pLysS/pET3d; plates B1−3, Tuner(DE3)-pLysS/pET3d($R_{126}E-C_{146}W$); plates C1−3, Tuner(DE3)pLysS/pET3d($R_{126}E$).

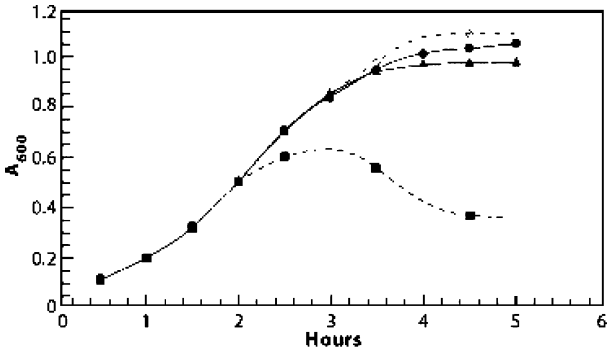

FIGURE 4: Effect of DM-TS and various constructs on the growth of cells in culture. Cells were grown overnight at 37 °C in M9 minimal medium supplemented with casamino acids, chloramphenicol, thymine, and carbenicillin (when pET3d vectors were present) as described under Materials and Methods. Cultures were diluted 50-fold into 5 mL of the same medium without thymine but with 50 μM IPTG and then shaken at 37 °C. Growth was measured as absorbance at 600 nm at 30 min intervals. (●) Tuner-(DE3)pLysS cells only; (▲) cells carrying pET3d; (□) cells carrying pET3d($R_{126}E$); (■) cells carrying pET3d(DM).

B2 in Figure 3). This effect is most likely due to the inhibition of endogenous WT-TS by the DM based on the fact that thymine (T) reversed the observed toxicity (Figure 3, plate B3).

To obtain a clearer view of how the DM-containing plasmid affects the rate of cell growth, we compared shake cultures of Tuner cells containing different plasmid constructs (Figure 4). It is clearly seen that cells containing empty vector or vectors with inserts of $R_{126}E$ or $C_{146}W$ do not exhibit

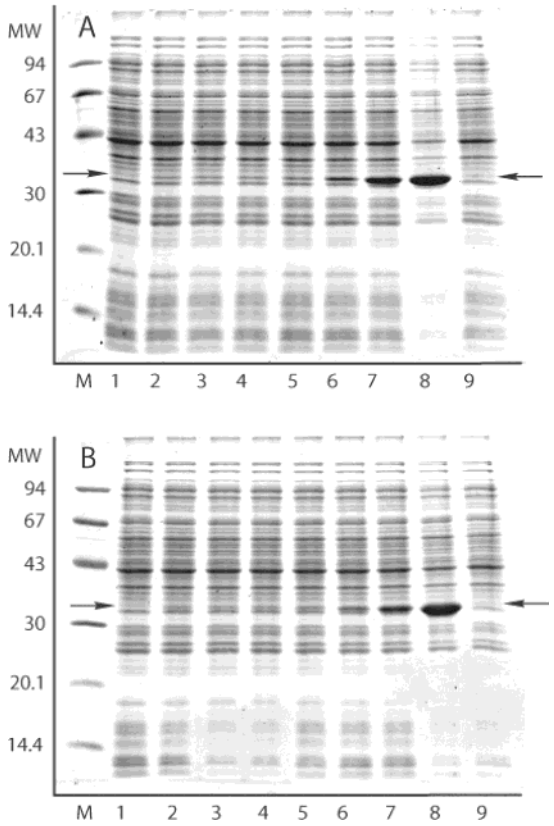

FIGURE 5: SDS−PAGE of WT-TS (A) and DM (B) before and after induction by varying concentrations of IPTG. Five-milliliter cultures of M9 medium supplemented with casamino acids, chloramphenicol, carbenicillin, and thymine were inoculated with Tuner (DE3)pLysS cells freshly transformed with pET3d, pET3d-(WT-TS), or pET3d(DM) and shaken overnight at 37 °C. These cells were then diluted 25-fold into fresh medium and 5-mL aliquots were distributed into culture tubes containing IPTG at the final concentrations indicated below. The tubes were shaken at 37 °C for 3.5 h, after which the cells were centrifuged and the pellets were dissolved in sample buffer. SDS−PAGE was performed on aliquots as described under Materials and Methods. The final IPTG concentrations in panels A and B were as follows: lanes 1 and 2, no IPTG; lane 3, 3 μM; lane 4, 6 μM; lane 5, 13 μM; lane 6, 25 μM; lane 7, 50 μM; lanes 8 and 9, 100 μM. The induced WT-TS (A) and DM (B) are designated by the arrow at about 32 kDa. Lane M represents the molecular weight markers, whose values are listed to the left.

impaired growth when induced by IPTG, while those cells containing the DM gradually slow in their growth and eventually die. To expand on this effect further, we then examined the relationship of DM expression and its effect on TS activity within the cell.

Comparison of WT-TS and DM Expression and the Effect of DM on TS Activity. Since the results in Figures 3 and 4 are qualitative in nature, we undertook a quantitative approach by examining the extent of DM induction in the cell at various concentrations of IPTG. Although DM has no measurable TS activity, this protein was assumed to be induced to the same extent as WT-TS. That this assumption is basically correct is shown in the SDS−PAGE gels in Figure 5, where WT-TS (Figure 5A) and DM (Figure 5B) appear to be expressed to the same extent at the higher levels (25−100 μM) of IPTG (lanes 6−8). If the DM is present to the same extent as the WT-TS, as appears to be the case, when the ratio of DM/V is 26 (same as WT/V in Table 1) due to the leakiness of expression in this system the

Table 1: Effect of DM on Expression of TS Activity in *E. coli*[a]

		relative TS activity		
lane	IPTG (μM)	V	WT/V	DM/V
1	0	1	−	−
2	0	−	26	0.13
3	3	−	27	0.12
4	6	−	34	0.11
5	13	−	44	0.10
6	25	−	108	0.06
7	50	−	281	0.04
8	100	−	370	0.02
9	100	1	−	−

[a] The extent of protein expression of WT-TS and of the DM of TS after induction by IPTG at the concentrations indicated is presented in panels A and B, respectively, of Figure 5 opposite the lanes designated by the arrows. The WT-TS activity in each of the supernatant fractions was assayed by the ^{3}H-release method (*29*) following a 20−100-fold dilution in the same buffer used for sonication of the cells (see Materials and Methods). V designates Tuner(DE3)pLysS cells carrying pET3d with no insert. WT represents V with the WT-TS insert, and DM represents the corresponding DM vector insert. Relative TS activity is expressed as the ratio of TS activity in cells containing WT or DM to the average TS activity in V-containing cells at 0 and 100 μM IPTG, representing 8331 ± 575 cpm/50 μL reaction mixture. See Materials and Methods for further details.

endogenous TS activity is reduced by 87%. In the presence of 25 μM IPTG, where a 100-fold increase in TS occurs, a comparable quantity of DM effects a 94% decrease in TS activity. It is of interest to note that another 2% decrease in TS activity at 50 μM IPTG is lethal to the cells, indicating how fine a line there is between life, death, and TS activity within a cell. It should be mentioned that the presence of thymine in the culture medium completely prevents the inhibition of growth associated with the DM (Figure 3).

Demonstration That the DM Inhibitory Effect Occurs in Vitro. Since the reduction of TS activity in the cell by the DM might be influenced by other factors, we sought to determine whether the same response could be obtained in a cell-free translation system. RNA transcripts of WT-TS DNA were measured in a cell-free protein synthesis system and were found to release tritium from [5-^{3}H]dUMP, as expected for newly formed TS. It is of interest to note that the transcripts from either $R_{126}E$ or $C_{146}W$ did not contribute any TS activity above background levels, but when both were translated together the TS activity obtained was equivalent to that of WT-TS (Figure 6). This result was comparable to that obtained upon mixing the proteins for $R_{126}E$ and $C_{146}W$ in vitro to restore TS activity (*17*, *18*). However, when the DM was synthesized at the same time as WT-TS from their respective mRNAs, WT-TS activity was greatly suppressed, in contrast to the lack of effect of $R_{126}E$ on the synthesis of WT-TS (Figure 7). These results are analogous to the denaturation−renaturation study in Figure 2, where the presence of the DM resulted in a marked impairment of TS activity, but that of $R_{126}E$ did not.

Synergy of FdUMP and DM on Growth Inhibition of E. coli. In view of the marked reduction of TS activity in cells containing uninduced DM (Table 1), we considered it feasible that an inhibitor of TS could bind the residual 10−15% of TS activity and would then be lethal to the cells at concentrations that are normally not toxic. We initially attempted to achieve this effect with FU and then FUdR, but in both cases the inhibition could not be reversed by

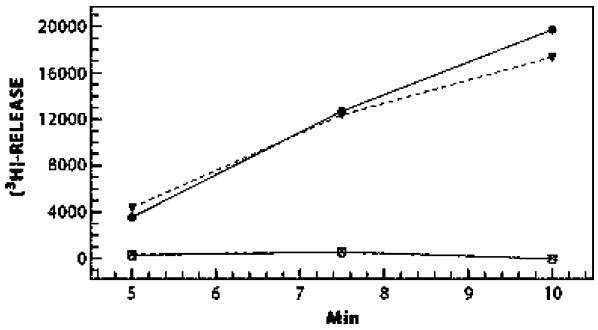

FIGURE 6: Measurement of TS activity following protein synthesis by use of an in vitro transcription−translation system (Ambion's Protein$_{script}$-PRO kit) with specific TS-containing genes. At the indicated times, 2 μL aliquots were removed and assayed for TS activity by ^{3}H-release (*29*). For background, a TS reaction was run minus DNA and the tritium released at each time point (4203−4727 cpm) was subtracted from those TS reactions containing the following DNA samples: (●) 50 ng of pET3d(WT-TS); (×) 50 ng of pET3d($R_{126}E$); (□) 50 ng of pET3d($C_{146}W$); (▼) 50 ng of pET3d($R_{126}E$) + 50 ng of pET3d($C_{146}W$).

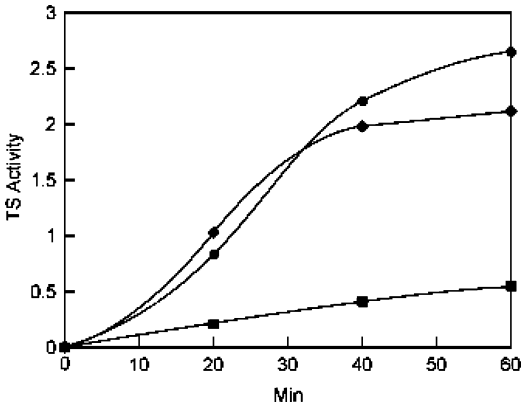

FIGURE 7: Effect of DM on the expression of WT-TS in vitro. Ambion's ActivePro kit was used in these translation studies. In vitro transcription−translation reactions (25 μL) were incubated at 37 °C. At the indicated times, 1-μL aliquots were removed and assayed by the ^{3}H-release method (*29*). A reaction was run minus DNA and the tritium released at each time point (4047−4288 cpm) was subtracted from TS reactions containing 10 ng of pET3d(WT-TS) plus the following additions: (●) none; (◆) 100 ng of pET3d-($R_{126}E$); (■) 100 ng of pET3d(DM).

thymine, indicating that this toxic response, in both cases, was due probably to the incorporation of FU into RNA (*36*). The observation of Breitman et al. (*37*) that dTMP is more effective than thymine or TdR in supporting growth of ThyA$^-$ mutants of *E. coli* suggested that FdUMP might be utilized more effectively than FU or FUdR. As shown in Figure 8, uninduced DM-containing cells grow, but not at their maximum rate. A lag in growth was observed routinely at 2.5−3.5 h, which could be reversed to optimal growth by addition of thymine. However, on inclusion of 1 μM FdUMP in the medium cell growth was completely inhibited, a behavior that was not observed in *E. coli* absent the DM vector. The involvement of TS in the inhibition is strongly suggested by the reversal of growth inhibition due to the presence of thymine in the medium.

Response of DM-Containing MCF-7 Cells to FUdR. To determine whether the DM inhibitory response shown in Figure 4 could be demonstrated in eukaryotic cells, we prepared a human pEGFP-TS equivalent of the *E. coli* TS-DM containing $R_{175}E/C_{195}W$ in place of $R_{126}E/C_{146}W$. The

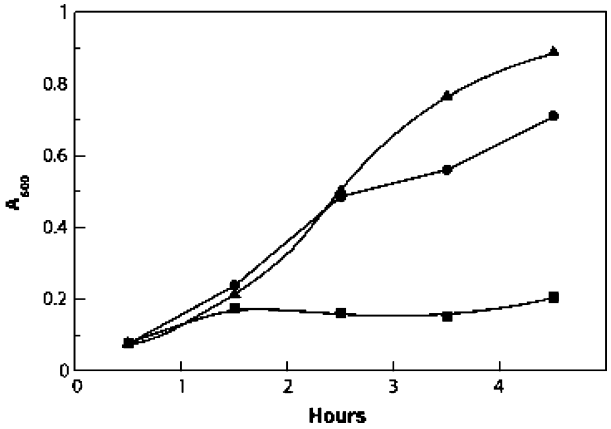

FIGURE 8: Demonstration of a synergistic response between those cells containing the DM and FdUMP. A 5-mL culture of M9 medium containing casamino acids, chloramphenicol, carbenicillin, and thymine was inoculated with Tuner(DE3)pLysS cells transformed with pET3d(DM) and shaken at 37 °C overnight. The cells were diluted 50-fold into the same medium, without thymine but containing the additions indicated below, and then shaken at 37 °C. Growth was measured as absorbance at 600 nm at hourly intervals. Additions: (●) none; (■) 1 μM FdUMP; (▲) 1 μM FdUMP + 50 μg/mL thymine. See Materials and Methods for additional details.

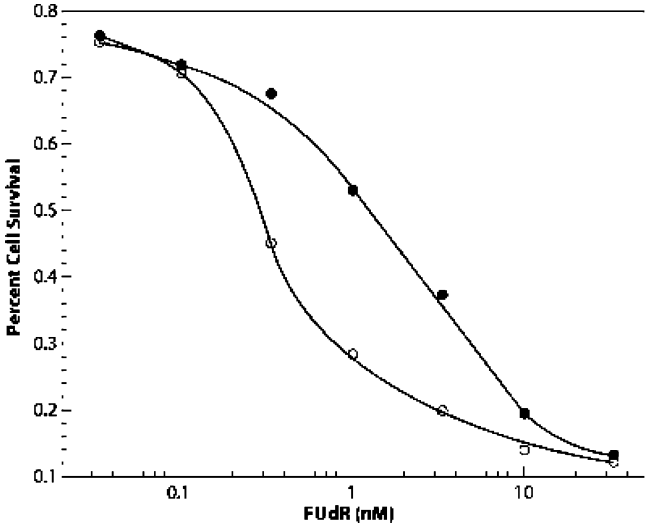

FIGURE 9: Enhancement of sensitivity of MCF-7 cells to FUdR by the DM. MCF-7 (●) and MCF-7 containing pEGFP-hTS(DM) (○) cells were plated onto 96 well tissue culture dishes at a density of 500 cells/well. FUdR was added after 24 h to each well at the concentrations indicated. After 7 days, the cells were fixed and the percent cell survival of treated cells compared to untreated cells was determined by staining with 0.4% sulforhodamine-B. For additional details, see Materials and Methods.

pEGFP-hTS and hTS(DM) plasmids were transfected into MCF-7 cells and specific clones were isolated containing the fluorescent EGFP marker attached at the amino terminus of the hTS- and hTS(DM)-encoding genes. pEGFP-hTS-transfected MCF-7 cells expressed TS activity about 100-fold greater than that in nontransfected MCF-7 cells, and it is assumed that the same extent of expression occurred with the hTS(DM) vector. pEGFP-hTS(DM) expression did not appear to affect normal MCF-7 cell growth, but a comparison of pEGFP-hTS(DM)-transfected MCF-7 cells and nontransfected MCF-7 cells for their sensitivity to FUdR told another story (Figure 9). The data in this figure clearly show that the cells containing the EGFP-hTS(DM) vector are about an order of magnitude more sensitive to FUdR. It should be emphasized that these are preliminary experiments, and conditions to optimize the toxic response to this drug or others in eukaryotic cells containing the DM of TS are currently under investigation.

DISCUSSION

In this paper we describe an entirely new means of suppressing TS, a key enzyme involved in cell division. As a result of providing an essential substrate for DNA synthesis, TS has been an important chemotherapeutic target for decades, but unfortunately it has proved to be a very elusive target to drugs because of problems associated with resistance and attendant side effects. As a consequence, we undertook a new route for impairing TS, the basis of which was to exploit the natural ability of a cell to translate proteins from mRNAs rather indiscriminately. In this instance we introduced the DNA from an inactive double mutant of TS, which when transcribed and translated to the mutant protein uniquely folds with the subunits of the natural host TS to form an inactive dimer (Figure 1B). Alternatively, the inactivation could occur due to the exchange of subunits between WT-TS dimer and the DM dimer, a reaction that occurs even more slowly (not shown) than that between the inactive mutants $R_{126}E$ and $C_{146}W$, one which, in contrast to the former, results in the restoration of TS activity (*17*, *18*). It is not possible at this time to state with certainty which process predominates in the inactivation since so little is known about oligomer formation in the cell during the course of protein synthesis. We tend, however, to favor the folding of newly formed single subunits following or during the course of de novo protein synthesis (Figure 10) as being primarily responsible for the inactivation of WT-TS. Whether folding occurs between the subunits as they are released from the ribosome or as they are being formed on the ribosome has not been resolved (*38*). Regardless, the cells are seen to die slowly as the concentration of DM escalates within them, following induction by IPTG (Figure 4). The lethal effect is gradual since it takes time for the active TS molecules resident in the cells to turn over and to be replaced with the newly formed inactive heterodimer. If the two subunits are present to begin with, as occurs during the course of in vitro protein synthesis (Figure 7), the interaction of WT-TS and DM mutant subunits occurs almost immediately as proposed in Figure 10. As a consequence, TS would be trapped as an inactive heterodimer and would never exist in the cell as an active enzyme. Evidence in support of this proposal is seen in Figure 7 where a 10-fold excess of the DM mRNA over that of the WT-TS mRNA results in little if any TS activity. The fact that the heterodimer $R_{126}E-$WT-TS is as active as the WT-TS homodimer is based on our earlier work (*17*, *18*), which demonstrated that WT-TS is a half-of-the-sites reactivity enzyme wherein only one functional active site in the dimer is required to provide a fully active enzyme and Glu126 does not affect this site (Figure 1, AII).

It is of interest to note in Figure 8 that uninduced cells containing the DM plasmid do not grow as well as control cells, in that there is a lag in growth between 2.5 and 3.5 h. This effect is undoubtedly due to the "leaky" nature of plasmid expression, even in the absence of IPTG. That this

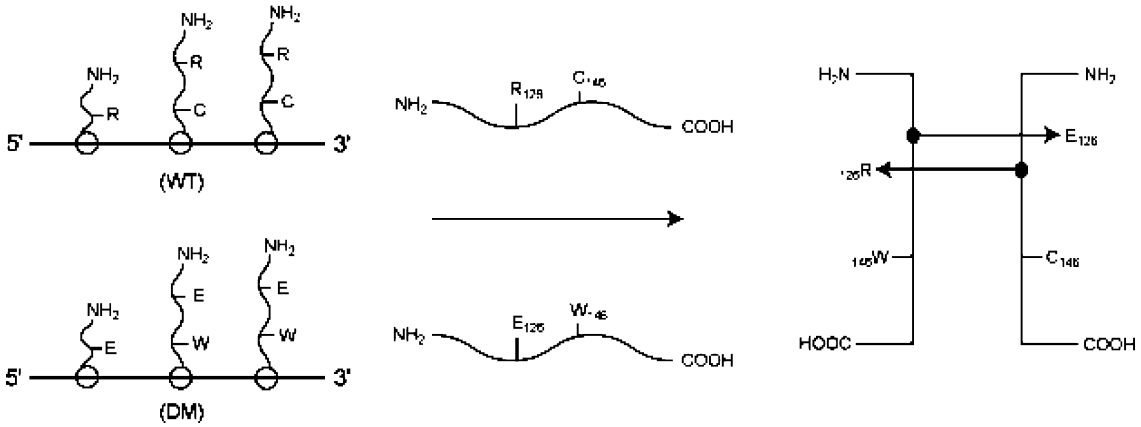

FIGURE 10: Diagrammatic presentation of the ribosomal translation of WT-TS and DM-TS from their respective mRNAs. When completed single-subunit peptides are released, they combine as shown to form an inactive heterodimer due to the excess of DM subunits.

is the case is seen from the data in Table 1, where it is shown that in such cells the TS activity is reduced by 85−90%. However, when the DM is induced by 25 μM IPTG, the TS activity is reduced to about 94% with growth still evident. On increasing the level of IPTG to 50 μM, the induced DM reduces TS activity by another 2% and cell growth ceases (Figure 4). In an attempt to determine whether the addition of another TS inhibitor to DM-containing cells would promote a synergistic response, FdUMP was added to the growth medium of uninduced DM-containing cells. The rationale for the use of FdUMP as opposed to FUdR or FU is based on the earlier studies of Breitman et al. (*37*), who showed that dTMP is, surprisingly, a better supporter of growth in *E. coli* BU$^-$ than TdR or thymine. As shown in Figure 8, a concentration of 10^{-6} M FdUMP under these conditions is lethal to the cells, a concentration of FdUMP that normally has no effect on cells not containing the DM. It will be of interest to determine whether cells induced to increase their DM levels would be even more susceptible to FdUMP. In the same vein, folate analogue inhibitors of TS, such as D1694 or GW1843, might show a similar if not more effective impairment of growth.

It should be emphasized that these experiments can be complicated by the fact that bacteria can overcome introduced toxic genes due to plasmid instability, in addition to the gradual increase in solution of β-lactamase produced by the plasmids, which destroys the ampicillin that was added for selectivity. The net result of these effects is that the untransformed bacteria eventually overgrow the culture (*39*) and interfere with the toxicity initially observed in these studies. Thus, the lag period seen at 3.5−4.5 h in Figure 8 might be even greater if overgrowth was not a problem.

To determine if an inhibitory response similar to that observed in *E. coli* (Figure 8) could be obtained in eukaryotic cells, we introduced pEGFP-hTS(DM) into MCF-7 cells and on exposing them to FUdR found that they were about 10-fold more susceptible to this drug than the MCF-7 cells alone. Conditions are currently being refined to improve the synergy of this response to drug exposure in the presence of the DM or related mutants of TS.

Additional Considerations for Improving the Effectiveness of the TS-DM. In several instances it has been demonstrated that proteins associated with critical pathways for the synthesis of purines and pyrimidines share active-site amino acids similar to those described for TS. Thus, introduction of inactivating mutations into such proteins as aspartate transcarbamylase (*40*), ornithine decarboxylase (*41*), arginosuccinate lyase (*42*), adenylsuccinate synthetase (*43*), or orotate phosphoribosyl transferase (*44*) to yield double mutants analogous to those described for TS could enhance the potency of the latter when combined with one or more of the DMs for the other proteins. By reduction of the levels of these critical enzymes in the cell, in combination with hTS(DM) or with various combinations of the other DMs, the effective therapeutic response might be greatly enhanced, particularly in the presence of potentiating drugs. Alternatively, peptides related to the TS(DM) could be even more inhibitory than the DM itself. These suggested newer venues to impairing a well-traversed pathway, such as described in this paper, may provide a window of opportunity for the development of a fresh approach to chemotherapy.

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