INTRODUCTION

Thymidylate synthase catalyzes the reductive methylation of dUMP to dTMP and is essential for the synthesis of DNA. Fluoropyrimidines, such as 5-fluorouracil (5-FU), are used extensively in cancer therapy. In the cell, 5-FU is metabolized to 5-fluoro-2'-deoxyuridine 5'-monophosphate, a tight binding covalent inhibitor of thymidylate synthase. Recent studies have identified 5-fluoro-2'-deoxyuridine (5-FdUR) and antifolate-resistant mutants of human thymidylate synthase (TS) that contain single residue substitutions within the highly conserved Arg50-loop, which binds the pyrimidine substrate (Y. Tong et al., J. Biol. Chem. 273: 11611–11618, 1998). We have used random sequence mutagenesis to gain structure-function information about the TS and to create novel drug-resistant mutants for gene therapy. A library of 1.5 million mutants of the Arg50-loop and the nearby residue Tyr 33 was selected to identify mutants of the human enzyme with the ability to complement a thymidylate synthase-deficient Escherichia coli strain and form colonies in the presence of 5-FdUR. E. coli-harboring plasmids that were encoding TS with single, double, and triple amino acid substitutions were identified that survive at dosages of 5-FdUR clearly lethal to E. coli harboring either wild-type thymidylate synthase or constructs encoding previously characterized drug resistant mutants. Four 5-FdUR-resistant mutants were purified to apparent homogeneity. Kinetic studies indicate that these enzymes are highly efficient. Inhibition constants (K_i) for the double mutant K47Q; D48E and the triple mutant D48E;T51S;G52C in the presence of 5-fluoro-2'-deoxyuridine 5'-monophosphate were determined to be 75 to 100 times higher, respectively, than that of the wild-type enzyme. These mutant TSs, or others similarly created and selected, could be used to protect bone marrow cells from the cytotoxic side effects of 5-FU chemotherapy.

ABSTRACT

Thymidylate synthase catalyzes the reductive methylation of dUMP to dTMP and is essential for the synthesis of DNA. Fluoropyrimidines, such as 5-fluorouracil (5-FU), are used extensively in cancer therapy. In the cell, 5-FU is metabolized to 5-fluoro-2'-deoxyuridine 5'-monophosphate, a tight binding covalent inhibitor of thymidylate synthase. Recent studies have identified 5-fluoro-2'-deoxyuridine (5-FdUR) and antifolate-resistant mutants of human thymidylate synthase (TS) that contain single residue substitutions within the highly conserved Arg50-loop, which binds the pyrimidine substrate (Y. Tong et al., J. Biol. Chem. 273: 11611–11618, 1998). We have used random sequence mutagenesis to gain structure-function information about the TS and to create novel drug-resistant mutants for gene therapy. A library of 1.5 million mutants of the Arg50-loop and the nearby residue Tyr 33 was selected to identify mutants of the human enzyme with the ability to complement a thymidylate synthase-deficient Escherichia coli strain and form colonies in the presence of 5-FdUR. E. coli-harboring plasmids that were encoding TS with single, double, and triple amino acid substitutions were identified that survive at dosages of 5-FdUR clearly lethal to E. coli harboring either wild-type thymidylate synthase or constructs encoding previously characterized drug resistant mutants. Four 5-FdUR-resistant mutants were purified to apparent homogeneity. Kinetic studies indicate that these enzymes are highly efficient. Inhibition constants (K_i) for the double mutant K47Q; D48E and the triple mutant D48E;T51S;G52C in the presence of 5-fluoro-2'-deoxyuridine 5'-monophosphate were determined to be 75 to 100 times higher, respectively, than that of the wild-type enzyme. These mutant TSs, or others similarly created and selected, could be used to protect bone marrow cells from the cytotoxic side effects of 5-FU chemotherapy.

MATERIALS AND METHODS

Cell Lines and Materials. CH3H4-folate was obtained as a racemic mixture from Schircks Labs (Jona, Switzerland). ABI Prism Dye Terminator Cycle Sequencing kits for fluorescent sequencing were the products of Perkin-Elmer (Branchburg, NJ). E. coli DNA Pol I was from New England Biolabs (Beverly, MA). Pfu DNA Polymerase was from Stratagene (La Jolla, CA). Plasmid DNA was isolated using the Maxiprep and Miniprep kits from Qiagen (Chatsworth, CA) or the PERFECTprep Plasmid DNA kit from 5 Prime-3 Prime, Inc. (Boulder, CO). 5-FdUr, 5-FdUMP, DUMP, N-irishydroxymethyl]methy1-2-aminoethane-sulfonic acid, and all of the other reagents were from Sigma Chemical Co. 6-3H]FdUMP (22.0 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Protein assay dye reagent concentrate was from Bio-Rad (Hercules, CA). E. coli NMS22 (Stratagene, La Jolla, CA) was used for cloning and library construction. E. coli χ2931recA (ΔthyA572, recA56), kindly provided by Dr. Daniel Santi (University of California, San Francisco, CA), is tetracycline resistant and was used in all of the complementation studies and in the purification of plasmid-encoded TS. Unless otherwise stated, all of the DNA oligodeoxycytidinenucleotides were from Operon Technologies (Alameda, CA).

Plasmids. Plasmid pGCHTS-TAA, from Dr. Daniel Santi, contains the wild-type TS cDNA in a pUC vector background and has been described previously (10, 15). A nonfunctional Arg50-TS stuffer vector that spans the residues targeted for randomization was created by replacing the TS open reading frame between nucleotides 94 (Ncol site) and 183 (SpeI site) with a 920-bp fragment derived from the pET34-LIC vector (Novagen, Madison, WI). The DNA insert was prepared by digestion of pET34-LIC with Ncol and SpeI, purified using the Quiaquick PCR spin kit (Qiagen, Chatsworth, CA), and ligated into the Ncol-SpeI-digested TS vector.
otides, both containing randomized nucleotide segments. Random oligomer TS-R50-Nco is a 67-mer that corresponds to the sense nucleotides 71–137 and contains a NcoI site (nucleotide 94) for cloning. It is 5'-d(GGCAGCCGCCCCATGTTGAACTGCAGTACCTGGGCAAGATCC-AAACACATCTCCGTGGCAGCTGTCAGG)-3', and it contains partially randomized nucleotides (underlined) corresponding to residue 33 of TS. Oligomer TS-R50-Sph is a 67-mer that corresponds to the antisense nucleotides 126–192 and contains a SphI site (nucleotide 183) for cloning. Its sequence is 5'-d(AACAGGGCCGCTAGCTAGCACTGCGGCGGAGATCCAC-AACACATCTCCGTGGCAGCTGTCAGG)-3', and it contains partially randomized nucleotides (underlined) corresponding to the Arg28-loop residues 47–52. All of the partially randomized nucleotides were designed to contain 80% of the wild-type base and 20% of the remaining bases.

The purified partially random oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Oligomers TS-R50-Nde and TS-R50-Sph were annealed in 50 μl of 200 mM Tris-HCl (pH 7.5), 100 mM MgCl₂, 250 mM NaCl by incubation at 80°C for 5 min, followed by 55°C for 15 min, at 37°C for 15 min, and at room temperature for 15 min. The partial oligonucleotide duplex was extended in a 40-μl reaction mixture that contained 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 7.5 mM DTT, 250 μM deoxynucleotide triphosphates, and 5 units Klenow fragment of E. coli DNA Pol I for 2 h at 37°C. The extended DNA was purified using the Qiagen Phusion Purification Kit, digested with NcoI (New England Biolabs) and SphI (New England Biolabs), and purified by phenol extraction and ethanol precipitation.

Replacement of the Wild-Type TS Sequence with the Random Library. The purified partially random oligonucleotides were used as inserts for construction of the human TS plasmid library. The nonfunctional “stuffer” insert was removed by digestion with SphI and NcoI, and the resulting 3-4-kb fragment was ligated with a 5.1 molar excess of the 122-bp restricted random insert using T4 DNA ligase (Life Technologies, Inc.). The ligation mixture was directly transformed (Bio-Rad GenePulser; 2 kV, 25 μF, 2200 ohm.) into fresh electrocompetent NM522 cells (Stratagene) in 20 separate transformations using 20 μl aliquots at 37°C. The extended DNA was purified using the Qiagen Phusion Purification Kit, digested with NcoI (New England Biolabs) and SphI (New England Biolabs), and purified by phenol extraction and ethanol precipitation.

Purification of Wild-type and Mutant TS. To construct a plasmid expressing the mutant TS enzymes linked to a 6X-His polypeptide, the TS mutants were digested with MroI and NcoI and ligated into a digested pHis-TS-WT construct. pHis-TS-WT was constructed via PCR amplification of TS, creation of a Sall restriction endonuclease site, digestion with Ndel and SalI, and ligation into the similarly digested vector pHis (a modified pUC12 vector provided by Amnon Hizi, Tel Aviv University, Tel Aviv, Israel). Details of its construction have been described previously (7). Cloning procedures were confirmed by restriction analysis and DNA sequencing. The TS-6X-His fusion proteins were purified by a one-step Ni²⁺ affinity chromatography procedure using resin and buffer (His-Bind resin and buffer kit; Novagen) according to a previously described protocol (6) modified from that of the supplier. An overnight culture of approximately 125 ml yielded from 400 to 600 μg of purified TS. After SDS polyacrylamide gel analysis and dialysis, the concentration of purified TS was determined by using the Bradford assay.

RESULTS

Construction of the Random Sequence Library. A library of 1.5 × 10⁹ human TS mutants was created by random sequence mutagenesis. The randomized oligonucleotides were designed with a bias such that each of the seven randomized codons contains on average 80% of the wild-type nucleotide at each nucleotide position and 20% of the remaining three nucleotides.
Sequence of Nonselected Clones to Determine Library Diversity. Before selection, plasmid DNA was isolated from 24 transformed clones and sequenced. The number of substitutions/clone is presented in Table 1, and the types of substitutions are tabulated in Fig. 1A. An average of 5.1 nucleotide changes and 2.8 amino acid changes/clone were detected in the nonselected library (Table 1). Amino acid substitutions in the nonselected clones were approximately evenly distributed among the seven residues encoded by the randomized nucleotides (Fig. 1A). The least number of substitutions were observed at G52. Four of the 24 nonselected clones analyzed (17%) contained frameshift mutations (all were deletions), and three contained termination codons. On the basis of the number and frequency of random substitutions, we calculated that the probability of obtaining a wild-type nucleotide sequence in the nonselected library is approximately 1%. The likelihood of obtaining a wild-type protein is higher because of the degeneracy of the nucleotide code and is approximately 4.5%. Therefore, it is not surprising that no wild-type molecules were detected among the 24 sequenced nonselected clones.

Selection of Active Mutant Thymidylate Synthase. We isolated active TS enzymes from the large plasmid libraries using a positive genetic complementation (7). The wild-type TS construct is able to rescue the TS− E. coli phenotype and form colonies on minimal medium (containing no thymidine), yet E. coli expressing an inactive TS (containing a “stuffer” insert) will not grow in the absence of thymidine. Approximately 1% of the members of the random library, or 15,000 clones, were able to complement the TS− phenotype and presumably encode and express active TS. Unlike the nonselected library, plasmid DNA from the active mutant library was without any nonsense or frameshift mutations. The average number of nucleotide and amino acid substitutions in the active library were 3.4 and 1.7, respectively (Table 1). Unlike the nonselected library, 7 of the 66 clones produced wild-type enzyme (10%). As statistically expected, each of the DNAs that encoded the wild-type enzymes was unique, as indicated by the presence of one or more silent nucleotide changes. Among protein sequences, the double mutant D48Y;G52C was detected twice. Most of the targeted residues retained a high level of mutability with the exception of Tyr 33, Arg 50, and, to a lesser degree, T51. Only two alterations at Y33 were observed, Y33C (in the context of the triple mutant Y33C;D48N;G52A), and Y33S (as Y33S; D48E). Arg 50 was able to be substituted by proline, asparagine, serine, and tryptophan. In each clone that harbored a mutation at Arg 50, at least one other amino acid substitution was detected within the Arg50-loop. Threonine 51 was altered in only seven clones, twice to alanine and five times to serine (Fig. 1B).

Selection of Mutants Resistant to Fluorodeoxyuridine. To isolate members from the random enzyme library that exhibit enhanced resistance to 5-FdUR, positive genetic selection was again used by plating on minimal medium agar plates that contained gradients of 5-FdUR. Previously, we reported (7–8) that the survival of the E. coli harboring wild-type TS is only modestly reduced to 90% at 75 nM 5-FdUR; however, it precipitously declines to 0.1% at 100 nM of the analogue. Consistent with these results, no clones harboring wild-type TS enzyme formed colonies on media that contained >100 nM 5-FdUR (Fig. 2). At 100 nM 5-FdUR, approximately 60% of the random library formed colonies. Library survival decreased linearly, to 40% at 150 nM 5-FdUR and 8% at 175 nM 5-FdUR.

The mutability of residues retained a trend similar to that observed in the active mutant library, with one exception. Thr 51, which was mutated only in 7 of the 66 active clones (11%), is altered in 30 (43%) of the 5-FdUR-selected clones. It has now become the third most commonly mutated residue. Serine is by far the most common substitution of this residue, present in 18 of the 30 mutations detected among the 5-FdUR-resistant clones.

Whereas wild-type TS represented 10% of the active mutant library, no wild-type enzymes were detected among the 70 5-FdUR survivors. Seven mutants were detected twice in the library (D48E, D48E;D49G, D48E;T51S, T51S;G52S, D48F;T51S;G52C, D48Y; T51S;G52A and K47E). One mutant, K47N, was detected three times. In all of the cases but two, the clones isolated were unique, as indicated by silent changes (nucleotide substitutions that did not alter the protein sequence) in other nucleotides within the random region. Thus these 70 mutants isolated from the 5-FdUR resistant clones, which harbored anywhere from one to three amino acid changes, were independently created and selected among 1.5 million original mutants.

It is apparent from Fig. 1C that many alterations lead to drug resistance. Mutants harboring a single amino acid change included: K47Q, K47E, D48E, G52S, and G52P. Interestingly, T51S was not detected as a single mutant despite being the single most common change seen in the library. However, the alteration T51S was often detected in concert with the mutation G52S. Examples include T51S; G52S, D48G;T51S;G52S, and D49E;T51S;G52S. T51S;G52S was detected twice, with unique silent nucleotide changes, in the library. As was observed among active mutants, changes at Arg 50 and Tyr 33 were rare and never occurred in the absence of other alterations. The only mutation at Y33 in the drug-selected clones was to tryptophan in the clone D48E;Y33W.

Several mutants were manually screened at increasing doses of 5-FdUR (Fig. 2). The previously identified drug-resistant mutant A197V;L198I;C199F was used as a positive control (7). This mutant, which was found to have a Kp for 5-FdUMP 20 times greater than that of wild type, formed colonies at dosages up to but not above 150 nM, consistent with previous observations (Fig. 2A; Ref. 7–8). The single mutants D48E and G52S formed colonies in media that contained as much as 200 nM 5-FdUR (Fig. 2A). The mutant G52C was identified in the active mutant pool. Because of the published importance of alterations at G52 in conferring drug resistance (5), this single mutant was manually screened and demonstrated drug resistance up to 175 nM 5-FdUR (Fig. 2A). Interestingly, with one exception, the multiple mutants demonstrated improved colony-forming ability compared with the single mutants. K47Q;D48E formed colonies at up to 250 nM 5-FdUR; D48E;T51S;G52C at up to 300 nM; and lastly T51S;G52S grew at up to 400 nM (Fig. 2B). The exception is the single mutant K47E, also identified by Tong et al. (5), which formed colonies at 400 nM.

To rule out that TS overexpression in the transfected E. coli

### Table 1 Library statistics

<table>
<thead>
<tr>
<th>Library</th>
<th>No. clones sequenced</th>
<th>Average no. nucleotide Δs</th>
<th>Average no. amino acid Δs</th>
<th>Frameshift mutations</th>
<th>Nonsense mutations</th>
<th>No. wild-type proteins*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unselected</td>
<td>24</td>
<td>5.1</td>
<td>2.8</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Functional clones</td>
<td>66</td>
<td>3.4</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>5-FdUR-selected*</td>
<td>70</td>
<td>4.4</td>
<td>2.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* No clones were wild-type at the nucleotide level.

The dose of 5-FdUR used is indicated in parentheses. Clones sequenced were from the 175 nM selection.
contributed to the observed drug resistance, multiple clones of the wild-type and mutant-transfected x2913 cells were analyzed using a [3H]-FdUMP binding assay (18). The wild-type and single mutant G52S demonstrated the highest level of expression (6.3 and 5.1 ng TS/μg total protein), four times that of the double and triple mutants (range 1.3–1.9 ng TS/μg total protein). However, these two proteins demonstrated the lowest survival against 5-FdUR in E. coli. These results suggest that the major reason for the observed improvement in survival at dosages of 5-FdUR clearly lethal to the wild-type TS can be attributed to the mutation in TS rather than to its level of expression.

Purification and Kinetic Analysis of Mutant Enzymes. Because augmented survival in E. coli could potentially result from mechanisms unrelated to altered enzymology, we purified the wild-type and four mutant enzymes as NH2-terminal fusions with a histidine tag. After purification, a single major component on SDS polyacrylamide gel migrating with an apparent molecular weight of human TS monomer (Mr 36,000) was observed for the wild-type and mutant enzymes. Purity was estimated to be >80%.

To obtain information about the catalytic and ligand-binding properties of these TS variants, the Vmax and Km values for the substrate and cofactor and Ki values for the inhibitor 5-FdUMP were determined (Table 2). Kinetic parameters of the single mutant G52S were consistent with the findings of Tong et al. (5). All of the mutants were catalytically efficient, with the lowest kcat measuring approximately half that of the wild-type enzyme.

As expected, the Ki values for FdUMP of all of the mutant enzymes were greater than that of the wild type, consistent with the E. coli survival data. The Ki value of the G52S single mutant was 18-fold higher than wild type, consistent with that observed by others (5). By comparison, the Ki values of the double mutant T51S;G52S, which demonstrated colony-forming ability in E. coli at much higher concentrations of 5-FdUR, were increased modestly to 26 times that of wild type. The mutant K47Q;D48E demonstrated 75-fold increase in Ki over wild type. The highest level of drug resistance was seen in the triple mutant D48E;T51S;G52C. This mutant demonstrated a high degree of drug resistance in E. coli (approximately 4-fold improvement in maximal tolerable dose), only a modestly increased Km for the normal substrate dUMP (6-fold), and an observed Ki for FdUMP 100-fold higher than that of wild-type TS.

DISCUSSION

Alterations of Thymidylate Synthase. The three loop residues Asp 48, Asp 49, and Gly 52 tolerated many substitutions. In fact, by combining the substitutions found in the active library with the 5-FdUR resistant library, 14 of the possible 20 amino acids were tested at D48. D48E was the most commonly mutated residue, which is not surprising because of its exterior location. The number of mutations at Thr 51 has increased greatly from the active library (43% versus 10%). Drug-resistant mutants that encode single mutations include K47Q, K47E, D48E, G52S, and G52P. The only 5-FdUR-resistant clone that contained an alteration at residue 33 was the double mutant Y33W;D48E. Because the D48E single mutant leads to drug resistance in itself, it is impossible from this assay to determine whether this alteration was “hitchhiking” with D48E. Likewise, alterations at Arg 50 were rare and only occurred in the presence of other mutations in loop residues.
were detected at concentrations of 5-FdUR above the highest dose indicated, with the bonds with residues of the control. The highest level of 5-FdUR resistance are plotted with wild type (7). A197V;L198I;C199F is compared with A197V;L198I;C199F and wild-type TS (8). All of the three residues detected at Tyr 33 were to cysteine and serine in the active site in the drug-resistant clone. Although we did not found as a single substitution in the drug-resistant library, T51S was the most common substitution observed. It occurred synergistically with many changes, notably G52S. Also, survival data indicate that clones tested with the T51S alteration appear to demonstrate colony-forming ability in E. coli at the highest doses of 5-FdUR. After binding of the substrates, it has been observed that reorientation of the Arg-50 loop allows for the interaction of hydrophobic atoms of Thr 51 to contact the buried V313 side chain (10–12, 19, 27). Mutation to serine could alter the conformation of this absolutely required terminal valine or nearby residues.

Interestingly, whereas D48 and G52 are not conserved throughout evolution, and mutagenesis studies have indicated that G52 can tolerate any substitution without significant loss of activity (19), D49 is absolutely conserved. Yet in our studies, all of the three residues tolerated polar, charged, or hydrophobic alterations. The lack of conservation in D48 and G52 is likely a reflection of their exterior location in the loop. Although we would have expected alterations at D49 to be more restrictive, it has been a common finding that highly conserved residues of TS are often tolerant of amino acid mutations in evolution, and a near normal affinity for dUMP and the cosubstrate Lactobacillus Lactis, is located within the α-helix A and forms hydrogen bonds with residues of the α-helix J which forms a wall of the active site cavity (24–25). Our study did not confirm the Y33H alteration nor find other substitutions at this residue that were highly associated with drug resistance. Given the role of Arg 50 in maintaining the bound structure, we find it remarkable that even a modest breadth of residue substitutions were detected (Pro, Ser, Asn, Trp, Gly). None of these were single mutations, preventing us from ruling out the effects of complementary changes in the loop. However R50P, R50G, and R50S have been constructed as single mutants via site-directed mutagenesis and determined to be active via E. coli complementation (4, 26).

Although it was not found as a single substitution in the drug-resistant library, T51S was the most common substitution observed. It occurred synergistically with many changes, notably G52S. Also, survival data indicate that clones tested with the T51S alteration appear to demonstrate colony-forming ability in E. coli at the highest doses of 5-FdUR. After binding of the substrates, it has been observed that reorientation of the Arg-50 loop allows for the interaction of hydrophobic atoms of Thr 51 to contact the buried V313 side chain (10–12, 19, 27). Mutation to serine could alter the conformation of this absolutely required terminal valine or nearby residues.

Interestingly, whereas D48 and G52 are not conserved throughout evolution, and mutagenesis studies have indicated that G52 can tolerate any substitution without significant loss of activity (19), D49 is absolutely conserved. Yet in our studies, all of the three residues tolerated polar, charged, or hydrophobic alterations. The lack of conservation in D48 and G52 is likely a reflection of their exterior location in the loop. Although we would have expected alterations at D49 to be more restrictive, it has been a common finding that highly conserved residues of TS are often tolerant of amino acid mutations in evolution, and a near normal affinity for dUMP and the cosubstrate Lactobacillus Lactis, is located within the α-helix A and forms hydrogen bonds with residues of the α-helix J which forms a wall of the active site cavity (24–25). Our study did not confirm the Y33H alteration nor find other substitutions at this residue that were highly associated with drug resistance. Given the role of Arg 50 in maintaining the bound structure, we find it remarkable that even a modest breadth of residue substitutions were detected (Pro, Ser, Asn, Trp, Gly). None of these were single mutations, preventing us from ruling out the effects of complementary changes in the loop. However R50P, R50G, and R50S have been constructed as single mutants via site-directed mutagenesis and determined to be active via E. coli complementation (4, 26).

Table 2 Rate and inhibition constants for the wild-type and mutant forms of TS

<table>
<thead>
<tr>
<th>Wild-type</th>
<th>K_m dUMP</th>
<th>K_m CH_2H_4-folate</th>
<th>K_cat</th>
<th>K_i FdUMP</th>
<th>Ratio K_i/FdUMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>D48E</td>
<td>25 μM</td>
<td>11 μM</td>
<td>1.2/sec</td>
<td>6.4 nM</td>
<td>75%</td>
</tr>
<tr>
<td>D48E</td>
<td>45 μM</td>
<td>300 μM</td>
<td>1.1/sec</td>
<td>480 nM</td>
<td>26%</td>
</tr>
<tr>
<td>T51S,G52S</td>
<td>30 μM</td>
<td>120 μM</td>
<td>0.54/sec</td>
<td>170 nM</td>
<td>100×</td>
</tr>
<tr>
<td>G52S</td>
<td>16 μM</td>
<td>25 μM</td>
<td>1.2/sec</td>
<td>120 nM</td>
<td>18×</td>
</tr>
</tbody>
</table>

a Determined at 500 μM dUMP.
b Determined at 600 μM CH_2H_4-folate.
c Determined at 1200 μM CH_2H_4-folate.
Kinetic Parameters of Mutant Thymidylate Synthases. On the basis of both robust survival in *E. coli* and the substitutions encoded, we selected three mutants (T51S;G52S, D48E:T51S;G52S, and K47Q:D48E) alongside the wild type and the previously identified G52S, for purification and kinetic studies. Each of these mutant TSs were catalytically active, with no mutant displaying more than a 50% decrease in $k_{cat}$. $K_m$ values for dUMP for all of the mutants were approximately five times the wild type, with the exception of K47Q;D48E ($K_m \sim 20$ times wild type). CH$_3$H$_4$-folate affinity appeared to follow the same pattern as dUMP affinity. G52S demonstrated a $K_m$ for CH$_3$H$_4$-folate not significantly different from wild type, consistent with that observed by Tong *et al.* (5). However, the same alteration in combination with T51S (T51S;G52S) had a 10-fold increase in $K_m$ for the cosubstrate. The triple mutant, with alterations at these two positions alongside D48E (D48E:T51S;G52C), demonstrated a $K_m$ of almost 20-fold wild type. Lastly, K47Q:D48E demonstrated a 30-fold increase in $K_m$ for CH$_3$H$_4$-folate (Table 2).

Kinetic inhibition studies confirmed the *E. coli* genetic selection studies in that all of the enzymes were 5-FdUR resistant compared with the wild-type TS. In accord with the *E. coli* data but to a larger degree, K47Q:D48E demonstrated a 75-fold increase in $K_i$, and the triple mutant D48E:T51S;G52C displayed the greatest resistance with a $K_i$ for 5-FdUMP 100-fold greater than that of the wild type. In some cases, $K_i$ has been shown to not fully correlate with IC$_{50}$ (5–6). However, as our enzymes have been selected in a biological system, we expect the observed survival advantage to extend to mammalian cells. Work is currently underway to determine survival in a mammalian system.

For many years, the only known drug-resistant human TS enzyme was the mutant Y33H, discussed previously (20). In the last 2 years, other drug-resistant TSs that contain amino acid substitutions in many different regions of the protein have been identified. We have previously identified several 5-FdUR-resistant TSs by creating random substitutions in a stretch of 13 residues near the active site (7–8). By subjecting highly conserved residues important in cofactor binding to site-directed mutagenesis, I108A was found to be resistant to the antifolates Raltitrexed (Tomudex; ZD1694) and Thymitaq, and F225W was found resistant to the antifolate BW1843U89 and 5-FdUR (6). Recently, a cell line adapted to increasing concentrations of 5-FdUR was determined to encode a P303L mutant that, although metabolically unstable, was nonetheless able to confer resistance to transfected cells againstFdUR, Raltitrexed, Thymitaq, and BW1843U89 (9).

Studies that used EMS mutagenesis followed by selection in human HT1080 cells with the antifolate Thymitaq have identified three mutants that demonstrate resistance to Thymitaq and 5-FdUR (5). All three of these identified mutants contain substitutions in the essential and conserved Arg$^{50}$-loop. These mutants, all of which harbor a single amino acid alteration (K47E, D49G, or G52S), confer a high degree of drug resistance to mammalian cells in culture (5). The mutant G52S conferred almost a 100-fold increase in IC$_{50}$ against 5-FdUR, whereas D49G demonstrated a 40-fold increase in IC$_{50}$ for Thymitaq while still retaining resistance to 5-FdUR. Inhibition studies with 5-FdUMP and three antifolate inhibitors indicated that D49G and G52S demonstrated an increase in the $K_i$ for FDUMP of 5.4- and 20-fold, respectively, while retaining a $K_i$ for Thymitaq approximately 6-fold that of wild type. Interestingly, despite a 5-fold increase in IC$_{50}$ for Thymitaq and 5-FdUR, K47E did not demonstrate variant kinetics for any of the inhibitors tested (5).

Because EMS mutagenesis is statistically unlikely to test synergistic effects of multiple mutations in one polypeptide or to allow multiple nucleotide substitutions within a particular codon, we view the mutants identified by Tong *et al.* (5) as prototype, or first-generation, drug-resistant TSs that can guide the discovery of mutants with yet greater resistance. Because 5-FdUMP is structurally similar to the natural substrate dUMP, it is difficult to predict how single amino acid substitutions or multiple substitutions could restrict the binding of 5-FdUMP without affecting binding of dUMP. Random oligonucleotide mutagenesis provides a combinatorial alternative that can examine a large amount of sequence space and create altered enzymes without requiring detailed knowledge about amino acid interactions or effects of specific alterations. Reselection of the single mutants discovered by Tong *et al.* (Ref. 5; G52S and K47E) demonstrates the validity of our *E. coli* assay in several ways. Firstly, this rapid *E. coli* assay was effective in identifying previously detected 5-FdUR-resistant TSs among an extremely large plasmid library. Secondly, the types of alterations seen as a whole in the resistant mutants appear to mimic those seen via selection in human cells. The fact that the identical mutants (G52S, K47E) could be selected via either 5-FdUR or a folate-based inhibitor (Thymitaq) can be explained likely by the central role of the Arg$^{50}$-loop in coordination with both of the corresponding substrates.

The decrease in affinity to 5-FdUMP in mutants with near normal catalytic activity suggests these mutants may be suitable for use as drug-resistant genes in gene therapy applications. Although effective, the use of 5-FU as a chemotherapeutic agent has been limited by toxicity to bone marrow, gastrointestinal, and other tissues (28). The introduction and expression of mutants of human TS that can function in the presence of systemic 5-FU chemotherapeutic treatment could protect normal cells from cytotoxicity or allow augmentation of the maximally tolerated dose of 5-FU. Additionally, a better understanding of the interactions of thymidylate synthase with 5-FdUMP will be useful in the targeted drug design of more clinically effective pyrimidine or antifolate inhibitors.

**ACKNOWLEDGMENTS**

We thank Dr. Joseph Bertino and members of his laboratory for guidance, detailed kinetic information, and stimulating discussion.

**REFERENCES**


Creation and Characterization of 5-Fluorodeoxyuridine-resistant Arg⁵⁰ Loop Mutants of Human Thymidylate Synthase

Daniel M. Landis, Cory C. Heindel and Lawrence A. Loeb

Cancer Res 2001;61:666-672.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/2/666

Cited articles
This article cites 27 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/2/666.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/2/666.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.