Human thymidylate synthase (TS) contains three highly conserved residues Ile-108, Leu-221, and Phe-225 that have been suggested to be important for cofactor and antifolate binding. To elucidate the role of these residues and generate drug-resistant human TS mutants, 14 variants with multiple substitutions of these three hydrophobic residues were created by site-directed mutagenesis and transfected into mouse TS-negative cells for complementation assays and cytotoxicity studies, and the mutant proteins expressed and characterized. The I108A mutant confers resistance to raltitrexed and Thymitaq with respective IC_{50} values 54- and 80-fold greater than wild-type but less resistance to BW1843U89 (6-fold). The F225W mutant displays resistance to BW1843U89 (17-fold increase in IC_{50} values), but no resistance to raltitrexed and Thymitaq. It also confers 8-fold resistance to fluorodeoxyuridine. Both the kinetic characterization of the altered enzymes and formation of antifolate-resistant colonies in mouse bone marrow cells that express mutant TS are in accord with the IC_{50} values for cytotoxicity noted above. The human TS mutants (I108A and F225W), by virtue of their desirable properties, including good catalytic function and resistance to antifolate TS inhibitors, confirm the importance of amino acid residues Ile-108 and Phe-225 in the binding of folate and its analogues. These novel mutants may be useful for gene transfer experiments to protect hematopoietic progenitor cells from the toxic effects of these drugs.

Thymidylate synthase (TS), which catalyzes the conversion of dUMP to dTMP, is an attractive target for drug design (1-5). TS inhibitors, which occupy either the substrate or cofactor-binding site, have been designed based on the structure and properties of the enzyme. Fluoropyrimidines, such as 5-fluorouracil (5-FU) and fluorodeoxyuridine (FdUrd), are metabolized to 5-fluoro-2-deoxyuridylate (FdUMP) and compete subsequently with the substrate, dUMP, for its binding site and have been used in the clinic for over 40 years to treat breast and gastrointestinal cancers. However, fluoropyrimidines, due to their incorporation into DNA and RNA, are not pure TS inhibitors. Also, they are susceptible to metabolic degradation in vivo. In contrast, the cofactor CH_{2}H_{4}folate is a relatively large molecule and has a variety of binding sites that may be altered in drug design. In recent years folate analogues have been designed as highly specific and stable TS inhibitors (6). The inhibitor CB3717 was the first folate analogue inhibitor of TS tested in the clinic and although anti-tumor activity was demonstrated, its further development was abandoned due to renal and hepatic toxicity (7, 8). The information provided by the crystal structures of TS from bacterial and mammalian sources (9-18) has led to the design and synthesis of novel analogues of CH_{2}H_{4}folate, e.g. raltitrexed (Tomudex\textsuperscript{®}, ZD1694), BW1843U89, Thymitaq (AG337), and AG331 (19-27). These new and promising agents have entered clinical trials, and raltitrexed was recently approved for treatment of advanced colorectal cancer in the United Kingdom.

Raltitrexed and BW1843U89 contain a glutamate moiety and can be metabolized to nonexflusable polyglutamate forms within the cell, which bind to the enzyme more tightly than the corresponding monoglutamylated forms. Since the polyglutamates are necessary for cytotoxicity, the lack of polyglutamylation may lead to intrinsic drug resistance. The new generation of TS-directed inhibitors such as Thymitaq and AG331, lacking a negatively charged glutamate side chain, are based on computer-aided design using high-resolution x-ray crystallography results (24-27). These compounds do not utilize the reduced folate transporter for cell entry, and more importantly drug potency is independent of polyglutamylation.

In a previous study (28), we generated mutant forms of TS leading to resistance of antifolates and fluorodeoxyuridine by ethylmethanesulfonate mutagenesis and Thymitaq selection. We found two mutant forms of TS that displayed resistance to Thymitaq and FdUrd, but not to raltitrexed. Surprisingly, the mutations obtained were in the highly conserved Arg-50 loop, involved in dUMP binding to TS. In the present study, we mutated bases that code for three amino acids believed to be critical for binding of the folate coenzyme, methylene tetrahydrofolate, to TS. Human TS mutants that confer resistance to novel antifolates (raltitrexed, Thymitaq, and BW1843U89)
were obtained, with minimal to modest changes in the catalytic activity of the enzyme. Data presented herein indicates that two TS mutants (I108A and F225W) selectively confer resistance to antifolates and provide evidence that these TS inhibitors bind to TS in different ways. These mutant forms of TS are also of interest for their potential role in cancer gene therapy. For example, transfection of a mutant TS cDNA to human hematopoietic progenitors may allow dose-intense therapy in cancer patients by preventing dose-limiting myelotoxicity (29).

EXPERIMENTAL PROCEDURE

Site-directed Mutagenesis—Human TS cDNA in the pET17b vector was inserted into the mammalian expression vector pcDNA3, named as pcDNA3hTS (28, 30). For construction of the 14 human TS mutants, the plasmid pcDNA3hTS was used as a template for site-directed mutagenesis (Transformer™ site-directed mutagenesis kit, CLONTECH Laboratories). The mutagenic primer in each reaction was a 22 to 26-base long oligonucleotide complementary to the human TS coding domain with corresponding mutant codons. The selective primers were the same as described previously (28). The correct constructs with human TS mutants were verified further by restriction mapping and DNA sequencing.

Expression of Constructs in TS-negative Cells—Mouse TS-negative FSth21 cells were used as the host for expression of human TS mutants (31). Cells were transfected with mammalian expression plasmids using DOTAP (Boehringer Mannheim, Indianapolis, IN). Three days after transfection, cells were switched to selective medium lacking thymidine. Surviving cells, able to grow in the absence of thymidine, were cloned in soft agarose.

Growth Inhibition Assay for Transfected Cells—Growth inhibition assays for cloned mouse TS-negative FSth21 cells transfected with wild-type or variant human TS cDNA were conducted with raltitrexed, Thymitaq, BW1843U89, andFdUMP (28). Cell viability was measured after transfection, cells were switched to selective medium lacking thymidine. Surviving cells, able to grow in the absence of thymidine, were cloned in soft agarose.

Enzyme Kinetics—To generate human TS variant (I108A or F225W) proteins for the analysis of enzyme kinetics, the mutant TS cDNA was inserted into NdeI and XhoI sites of the protein expression vector pET-17b (Novagen, Madison, WI). After transformation with pET-17b-hTS (I108A or F225W), expression of wild-type or mutant TS was induced with isopropyl-β-D-thiogalactoside in Escherichia coli strain BL21 (DE3). The purification procedures of wild-type and mutant TSs were carried out as described previously (30, 32). TS activity was monitored by a spectrophotometric assay as described previously (33, 34). Methods for measurement of \( K_m \) values, the Michaelis constants, and \( IC_{50} \) values for raltitrexed, Thymitaq, BW1843U89, and FdUMP were as described previously (28).

RESULTS

Substitutions of amino acid residues located in binding regions of enzymes may cause dramatic shifts of binding affinities to substrates and inhibitors. For example, the amino acids at positions 22 and 31 of dihydrofolate reductase that interact with the PABA ring of H2folate have been found to be altered in methotrexate-resistant cell lines, suggesting that these positions are mutational hot spots in the dihydrofolate reductase coding regions (38, 39). X-ray crystallographic studies of liganded E. coli TS have revealed that the binding site for the PABA ring of cofactor is associated with a hydrophobic region that includes Trp-109, Asn-112, Met-311, Ile-108, Leu-221, and Phe-225 (9, 10) (see Fig. 1). The residue Met-311 is not as highly conserved as others, while positions 109 and 112 are quite sensitive to mutagenesis, as shown by previous mutation studies in both Lactobacillus casei and E. coli TS (4).
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Table I

| Transfected TS-negative cells and its clone | Raltitrexed (ZD1694) | Thymitaq (AG337) | BW1843U89 | FdUrd |
|--------------------------------------------|----------------------|------------------|-----------|-------|
| IC₅₀ values (×10⁻⁹ M)                       | IC₅₀ values (×10⁻⁹ M) | IC₅₀ values (×10⁻¹¹ M) | IC₅₀ values (×10⁻¹¹ M) |
| Wild-type/clone (1)                         | 1.80 ± 0.47          | 3.83 ± 0.69      | 3.68 ± 0.14 | 8.96 ± 0.62 |
| I108A/clone (1)                             | 97.9 ± 17.1          | 305 ± 37         | 22.8 ± 4.2  | 140 ± 19  |
| I108F/clone (2)                             | 1.52 ± 0.84          | 11.4 ± 1.2       | 3.44 ± 0.03  | 17.8 ± 2.8 |
| L221A/clone (2)                             | 1.46 ± 0.88          | 8.98 ± 0.34      | 7.41 ± 0.25  | 11.6 ± 3.1 |
| L221I/clone (1)                             | 1.98 ± 0.40          | 8.51 ± 0.18      | 7.53 ± 0.25  | 38.1 ± 4.1 |
| L221S/clone (2)                             | 1.80 ± 0.06          | 7.38 ± 0.28      | 7.63 ± 0.26  | 3.49 ± 0.55 |
| F225W/clone (2)                             | 1.76 ± 0.01          | 1.0              | 6.11 ± 10.3 | 73.8 ± 2.6 |
| F225L/clone (1)                             | 1.33 ± 0.29          | 1.64 ± 0.56      | 7.54 ± 0.44  | 16.5 ± 2.8 |
| F225Y/clone (1)                             | 1.53 ± 0.17          | 2.38 ± 0.45      | 7.32 ± 0.17  | 3.01 ± 0.06 |

a The ratio is the IC₅₀ value of mutant to wild-type.

The kinetic parameters of the F225W mutant were not very different from those of the wild-type human protein. The Kₘ values for dUMP and the kₗₑₐₜ were slightly decreased and increased, respectively, while the Kₘ for CH₂H₄folate was increased 8-fold. By comparison with the I108A variant, inhibition of F225W mutant by raltitrexed or Thymitaq differed slightly from the inhibition of wild-type TS. The Kᵢ value of the F225W TS variant for BW1843U89, as expected, was 68-fold greater than the Kᵢ for wild-type enzyme. This was reflected in the cell toxicity studies, where cells transfected with F225W were 17-fold more resistant to this compound than the wild-type TS transfected cells.

CFU-GM and HPP-CFC Colony Assays—Transduction of mouse marrow cells with the I108A mutant cDNA resulted in more raltitrexed- or Thymitaq-resistant CFU-GM colonies as compared with mouse marrow cells either untransduced or transduced with wild-type human TS. The Kᵢ values for raltitrexed and Thymitaq were slightly decreased in CFU-GM and HPP-CFC colonies, which represent growth of early progenitors, were also detected in marrow transduced with the I108A mutant, but not in untransduced cells or cells transduced with wild-type human TS, when marrow was incubated with 10⁻⁸ M raltitrexed or 10⁻⁹ M Thymitaq.

**Discussion**

Ile-108 Mutants and the Role of Ile-108—Ile-108, as a highly conserved amino acid residue, shows flexibility of its side chain when antiglobulins occupy the folate-binding site. When TS inhibitors such as Thymitaq were designed by molecular modeling, it was predicted that the side chain of Ile-108 would move toward the distal phenyl ring of Thymitaq to make the desired nonpolar interaction, resulting in the calculated minimum-energy conformation. This movement and modeled interaction were subsequently observed by crystal structure studies, which also demonstrated that Thymitaq binds to the active site in a similar orientation as the cofactor (24, 26, 27). The shift of the Ile-108 side chain upon Thymitaq binding suggested that the spatial relationship of Ile-108 to bound Thymitaq may be little

or the electrostatic environment, resulting in altered binding affinities with substrate and inhibitors, thus leading to drug resistance.

To investigate the effect of amino acid substitution at Ile-108, Leu-221, and Phe-225 on drug resistance, isoleucine 108 was replaced with alanine, phenylalanine, glycine, glutamate, and asparagine; leucine 221 was replaced with phenylalanine, arginine, alanine, isoleucine, and serine; and phenylalanine 225 was replaced with serine, leucine, tyrosine, and tryptophan. A mammalian expression plasmid (pcDNA3hTS) carrying human TS cDNA with minor modification of the N-terminal nucleotide codon to enhance enzyme expression, was used as a template for creating mutant TS vectors (30).

**Mutational Activity of TS Mutants Assayed with a Mammalian Expression System**—The mutant enzymes were expressed in mouse TS-negative cells (F3thy21) by stable transfection of human TS cDNA variants. The host cells are unable to survive without exogenous thymidine. Eight TS mutants (I108A, I108F, L221A, L221I, L221S, F225W, F225L, and F225Y) exhibited behavior indistinguishable from the wild-type enzyme in the complementation experiments. In contrast, cells were unable to survive transfection with six other human TS variants (I108G, I108E, I108N, L221F, L221R, and F225S) (data not shown). For each transfected, surviving clones were isolated and then expanded to cell lines by growth in normal media.

**Cytotoxicity Studies**—From the transfecteds of human TS wild-type or variants, cell lines derived from clones with levels of TS protein similar to wild-type as measured by Western blotting (data not shown), were chosen for cytotoxicity studies with three antifolates (raltitrexed, Thymitaq, and BW1843U89) and FdUrd. The selected transfecteds and the IC₅₀ values of drugs tested are presented in Table I. As indicated, statistically significant differences in the IC₅₀ values were as follows: 1) the I108A mutant transfected cells were resistant to raltitrexed and Thymitaq with respective IC₅₀ values 54- and 80-fold greater than wild-type transfecteds. Less resistance was observed with BW1843U89 and FdUrd (6- and 16-fold, respectively); 2) F225W transfecteds demonstrated 17- and 8-fold resistance to BW1843U89 and FdUrd, respectively, but were not resistant to raltitrexed and Thymitaq.

**Kinetic Properties of Mutant Enzymes**—To correlate cytotoxicity results with enzyme properties, the human TS I108A and F225W mutant proteins were evaluated to interpret the effects of the mutations on the catalytic and inhibitor-binding properties of the enzyme. After expression and purification (see “Experimental Procedures”), the mutant enzymes were estimated to be 80–95% homogenous, as determined by densitometric scanning (data not shown).

Kinetic studies with I108A demonstrated that this mutation dramatically changes the catalytic properties of human thymidylate synthase (Table II). The I108A variant had a greatly increased Kᵢₘ for CH₂H₄folate and a slightly elevated Kᵢₘ for dUMP, and a catalytic efficiency (kₑₐₜ/Kᵢ) of TS I108A mutant that was reduced about 8-fold. These data indicate that Ile-108 is involved in the binding of CH₂H₄folate, and only two hydrophobic amino acid substitutions, alanine and phenylalanine yielded sufficient TS activity to permit growth of TS-negative cells in normal medium. Moreover, increased Ki values of this mutation for the TS-directed inhibitors such as raltitrexed and Thymitaq correlated with the cytotoxicity studies.

In contrast, the kinetic parameters of the F225W mutant were not very different from those of the wild-type human protein. The Kᵢₘ values for dUMP and the kₑₐₜ were slightly decreased and increased, respectively, while the Kᵢₘ for CH₂H₄folate was increased 8-fold. By comparison with the I108A variant, inhibition of F225W mutant by raltitrexed or Thymitaq differed slightly from the inhibition of wild-type TS. The Kᵢ value of the F225W TS variant for BW1843U89, as expected, was 68-fold greater than the Kᵢ for wild-type enzyme. This was reflected in the cell toxicity studies, where cells transfected with F225W were 17-fold more resistant to this compound than the wild-type TS transfected cells.

**Discussion**

Ile-108 Mutants and the Role of Ile-108—Ile-108, as a highly conserved amino acid residue, shows flexibility of its side chain when antiglobulins occupy the folate-binding site. When TS inhibitors such as Thymitaq were designed by molecular modeling, it was predicted that the side chain of Ile-108 would move toward the distal phenyl ring of Thymitaq to make the desired nonpolar interaction, resulting in the calculated minimum-energy conformation. This movement and modeled interaction were subsequently observed by crystal structure studies, which also demonstrated that Thymitaq binds to the active site in a similar orientation as the cofactor (24, 26, 27). The shift of the Ile-108 side chain upon Thymitaq binding suggested that the spatial relationship of Ile-108 to bound Thymitaq may be little
Drug-resistant Mutants of Human Thymidylate Synthase

**TABLE II**

Kinetic parameters and drug binding affinities for wild-type and mutant human thymidylate synthases

| Parameter | $K_m$ (dUMP) | $K_m$ (CH$_2$H$_4$folate)$^a$ | $k_{cat}$ | $K_i$ |
|-----------|--------------|-------------------------------|----------|--------|
|           | $\mu M$     | $\mu M$                       | s$^{-1}$ | nM     |
| Wild-type | 3.9          | 13                            | 1.7      | 13     |
| I108A     | 12           | 390                           | 0.22     | 340    |
| F225W     | 2.4          | 108                           | 2.8      | 87     |

|           | Raltitrexed | Thymitaq | BW1843U89 | FdUMP |
|-----------|-------------|----------|-----------|-------|
|           |             |          |           |       |
| Wild-type | 0.34        | 2.6      |           |       |

$a$ The values shown are corrected, as the racemic mixture of this cofactor was used.

**TABLE III**

Resistance to raltitrexed (ZD1694) and Thymitaq (AG337) in mouse marrow CFU-GM colonies and HPP-CFC colonies of the transfection with plasmids containing wild-type TS and I108A mutant

| Bone marrow cell expressed with$^a$ | No drug | CFU-GM Assay | HPP-CFC assay |
|------------------------------------|---------|--------------|---------------|
|                                    |         | Raltitrexed  | Thymitaq      | Raltitrexed  | Thymitaq   |
| No DNA                             | 82$^b$  | $2 \times 10^{-8}$ M | $1 \times 10^{-8}$ M | $2 \times 10^{-7}$ M | $1 \times 10^{-7}$ M |
| wt TS                              | 108     | 0            | 14 (17%)$^c$  | 0            | 10 (12%)   |
| I108A TS                           | 144     | 14 (10%)     | 64 (44%)      | 12 (8%)      | 60 (42%)   |

|$^a$ DOTAP-mediated gene transfer of wild-type or mutant TS into mouse marrow cells is described under "Experimental Procedures."

|$^b$ Number of colonies formed in thymidine phosphorylase-treated serum.

|$^c$ The percentage of resistant colonies was calculated by dividing the drug-resistant colonies number with the colonies number in the absence of drug.

different with other antifolates due to the changed molecular structure of inhibitor. We postulated that some mutations at position 108 would greatly affect the binding of cofactor and inhibitors (raltitrexed, Thymitaq, and BW1843U89) that occupy the cofactor-binding site. The first two mutations we decided to make at this position were Phe and Ala substitutions. Because alanine has a fixed side chain, which is smaller than isoleucine, it may not reach the phenyl ring of Thymitaq. Loss or weakening of these interactions in the variants must also reduce the binding energy, leading to dramatic changes of affinity of Thymitaq to TS. In the case of phenylalanine, another non-polar amino acid with less flexibility, we expected that if the stacking of phenyl-phenyl rings between inhibitor and altered human TS is impaired, TS will adopt a new conformation by reorientation of amino acid residues, thus causing a large change in enzyme behavior.

The results of cytotoxicity assays confirmed that when Ile-108 was replaced by residues with a smaller side chain (Ala), the binding of raltitrexed and Thymitaq was weakened, leading to respective IC$_{50}$ values 66- and 80-fold greater than the IC$_{50}$ value of wild-type human TS. On the other hand, in the presence of BW1843U89, the IC$_{50}$ value of I108A was relatively less (6-fold), which suggests that the large molecular structure of BW1843U89 may retain contact with the side chain of alanine. Moreover, since Ile-108 is not one of the residues in the nucleotide binding pocket, the I108A mutant did not affect FdUMP binding as much as it did raltitrexed and Thymitaq binding. In contrast, little effects on catalysis and inhibitor binding were observed for the I108F mutant (data not shown). This variant behaved the same as wild-type TS, suggesting that this side chain hydrophobic substitution did not change the spatial interaction between enzyme, cofactor, or inhibitors. In order to further study the role of Ile-108 on cofactor and antifolate binding, we replaced this residue by Asn (a polar amino acid), Glu (an acidic amino acid), and Gly (a non-polar amino acid without a side chain). None of these three mutant enzymes allowed growth of mouse TS-deficient cells in the absence of thymidine, indicating that these changes greatly decreased the catalytic efficiency of human TS.

The drastic differences in folate binding and catalytic activity among these five variants indicate that position 108 is quite sensitive to mutagenesis and plays a critical role in folate binding. I108A mutation confers significant Thymitaq and raltitrexed resistance. This is due to its distinctive effects on binding affinity among the three antifolates and different properties on catalysis as compared with the other four mutants (Phe, Gly, Asn, and Glu). We hypothesize that the changes in the side chain due to this mutation may cause the loss of hydrophobic interactions between I108A and the phenyl ring of raltitrexed and Thymitaq, resulting in reduced binding affinity of these molecules.

Phe-225 Mutants and the Role of Phe-225—Phe-225 was also targeted for site-directed mutagenesis. Based on the crystal structure of TS, this position contributes the major hydrophobic force on binding the PABA moiety of CH$_2$H$_4$folate, and undergoes a dramatic side chain shift to accommodate different ligands upon ternary complex formation. For example, the orientation of the phenyl group of Phe-225 toward CB3717 and H$_2$folate is changed on binding to these ligands (10, 14). The aromatic ring of Phe-225 stacks against the propargyl group of CB3717, which could be important for the stability of CB3717 binding to TS. However, this specific interaction and Phe-225 side chain is rotated away when H$_2$folate replaces CB3717, resulting in weaker and more expansive van der Waals contacts to allow H$_2$folate to dissociate from TS (14). These observations indicate that when TS forms a complex with either CH$_2$H$_4$folate, H$_2$folate, CB3717, raltitrexed, or Thymitaq, which all have a pterin or quinazoline ring, the mobile phenyl group of Phe-225 has enough space to accommodate the above ligands by different hydrophobic interactions and it is not necessary to form aromatic-aromatic non-polar interaction by face-to-face interactions between Phe-225 and the folate molecule. BW1843U89 binds differently, as a consequence of an expanded benzoquinazoline ring, and results in reorientation of the Phe-225 phenyl ring (a $90^\circ$ turn, relative to the orientations found in the CB3717 and H$_2$folate complexes) to form an idealized aromatic $\pi$ stacking with the isoindolyl ring of BW1843U89 (18).

The structural information indicates that the interactions of Phe-225 with the folate cofactor and various antifolates...
appear to be quite different. We therefore replaced Phe-225 by tryptophan, tyrosine, leucine, or serine. The results of transfection of mouse TS-negative cells demonstrated that this highly conserved residue could be substituted by Trp, Leu, and Tyr but not by Ser. Also, the TS-negative mouse cells containing F225S and F225L mutant enzymes did not show drastic changes in inhibition by three antifolates (ralitrexed, Thymitaq, and BW1843U98) or FdUrd as compared with wild-type TS. In contrast, although F225W conferred resistance to BW1843U98 and FdUrd, with increased IC50 values of 17- and 8-fold, respectively, resistance was not encountered with ralitrexed or Thymitaq. Thus, the replacement of Phe-225 by the above amino acids had very different effects on enzyme activity and binding affinity of these inhibitors. The F225 mutant exhibited severely diminished TS catalytic activity, presumably because the hydroxyl side chain of serine decreased the hydrophobic interaction between TS and the PABA moiety of folate. Substitutions with tyrosine, an aromatic amino acid also containing a hydroxyl moiety, retained TS function. The aromatic group of Tyr may contribute hydrophobic contacts to folate compounds, which account for the loss of binding energy for BW1843U98 to TS F225W mutant.

As shown in earlier equilibrium dialysis studies, folate and dUMP derivatives enhance each others binding (40, 41). This effect is even more dramatically shown in the effect that BW1843U98 has on the binding of dUMP and even dGMP (GMP) where the stacking energy between the benzoquinazoline ring of BW1843U98 and TS, and also indicated nucleotides greatly enhances their binding (42). Any mutation that impairs the binding of folate analogues would naturally impair the binding of dUMP or FdUMP and would thus explain the resistance of the indicated cell lines containing I108A and F225W to both BW1843U98 and FdUMP.

Mutations at Position 221—In the recently determined crystal structure of the human TS, Leu-221, an almost invariant residue in all reported TS sequences, was shown to be one of the residues involved in hydrophobic contacts with the folate cofactor or inhibitors (4). We introduced five mutations (phenylalanine, alanine, isoleucine, serine, or arginine) at position 221 of the human TS. Mouse TS-negative cells were rescued by transfection of these cDNA’s with Ala, Ile, and Ser variants but not Phe and Arg variants. The loss of TS function of the positively charged Arg variant may be explained by the loss of hydrophobic interactions. Substitution with phenylalanine, a hydrophobic amino acid, because of its bulky side chain (Phe, 142 Å2 and Leu, 107 Å2) (43), may explain the lack of TS complementation by the L221F variant. However, when the volume of the non-polar side chain was unchanged (by Ile substitution) or smaller (by Ala substitution) and hydroxyl group added (by Ser substitution), these mutations resulted in catalytically active TS. The L221A, L221I, and L221S variants of human TS also did not show significant differences in inhibitor binding as measured by inhibition of cell growth. Leu-221, unlike Phe-225 and Ile-108, may therefore marginally reorient when cofactor or inhibitors having different molecular structures bind to TS.

In summary, we have generated novel human TS mutants that confer antifolate resistance, and provide evidence that ralitrexed, Thymitaq, and BW1843U98 bind differently to TS. Further x-ray crystallographic studies of these mutants (I108A and F225W) may provide more detailed information to answer the questions of how the mutated residues participate in inhibitor binding or interfere with inhibitor binding through steric hindrance (for F225W) and the loss of hydrophobic interaction (for I108A). Information on the difference between wild-type and mutant human TS structures may also be of value in the design of new TS inhibitors with desirable properties. Data presented using DOTAP transduction of mouse marrow cells with the I108A mutant cDNA, encourage the further development of this mutant incorporated into retroviral constructs, to protect hematopoietic progenitor cells from toxic effects of the TS inhibitor ralitrexed (44), now widely used to treat colorectal cancer.

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