Methotrexate Resistance of Mouse Dihydrofolate Reductase: Effect of Substitution of Phenylalanine-31 by Serine or Tryptophan

David A. Evenson,[†] Joseph Adams,[‡] R. Scott McIvor,[†] and Carston R. Wagner*[,§]

Department of Laboratory Medicine and Pathology, Institute of Human Genetics, and Department of Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota 55455, and Department of Chemistry, San Diego State University, San Diego, California 92182

Received October 26, 1995

The antifolate methotrexate (MTX; 8-amino-10-methylpteryolglutamic acid) is a potent weapon in the chemotherapeutic arsenal against acute lymphoblastic leukemia and non-Hodgkin's lymphoma, as well as a variety of other tumors. Its primary mode of action is associated with the inhibition of dihydrofolate reductase (DHFR) and thus de novo thymidine and purine biosynthesis. Unfortunately, the therapeutic utility of MTX has been limited due to its toxicity toward normal proliferative tissues, such as the gastrointestinal tract and bone marrow. One approach that may overcome this drawback would be to render normal tissues MTX-resistant by introduction of a drug-resistant DHFR gene generated by site-directed mutagenesis.[1−5]

Recently, our laboratory has generated a series of MTX-resistant murine DHFRs by PCR-assisted saturation mutagenesis at phenylalanine-31.[6] Substitution at this position with serine resulted in a 5−6-fold increase in the IC_{50} of methotrexate, while a 4-fold increase in the activity of the mutant enzyme relative to wild-type was observed.[6] In contrast, substitution at position 31 with tryptophan resulted in a 20-fold increase in the IC_{50} of methotrexate and 7-fold decrease in DHFR activity.[6,7] In addition, mice transplated with bone marrow from transgenic mice expressing the Trp-31 mutant were effectively protected from the administration of low doses of MTX, which proved to be lethal for animals receiving normal marrow.[8] As a result, in an effort to delineate the parameters governing the impact of DHFR point site mutations on *in vitro* and *in vivo* drug resistance, we have chosen to contrast and compare the catalytic and ligand binding characteristics of the Ser-31 and Trp-31 mutant murine DHFRs relative to the wild-type enzyme.

DNA encoding the wild-type and mutant DHFRs was excised from the corresponding mammalian expression plasmids, pSV-DHFRwt, pSV-DHFRSer31, and pSV-DHFRTrp31, and subcloned into the cytoplasmic bacterial expression plasmid, pET22b(+). Transformed BL21 (DE 3) cells were grown in culture, and protein expression was induced with 1 mM IPTG, followed by an additional 3 h incubation. Typically, 2−3 g of cells was obtained. The wild-type and Trp-31 mutant DHFRs were purified by methotrexate affinity chromatography.[9] SDS−PAGE analysis of cell-free extracts demonstrated that greater than 10% of the cytoplasmic protein consisted of recombinant DHFR, whether wild-type or mutant enzyme.

Unfortunately, less than 1% of the Ser-31 enzyme was obtainable by the standard affinity purification protocol. Consequently, DNA encoding the mutant was subcloned into the novel expression plasmid, pPH70-d (Koning, K. R.; Bergstrom, C. P.; Hanna, P. E.; Wagner, C. R., manuscript in preparation), and expressed as a fusion protein to the L54F mutant *Escherichia coli* DHFR, which binds tightly to MTX.[10]. The recombinant fusion protein was subsequently purified by methotrexate affinity chromatography and the fusion protein eluted by raising the buffer pH from 6.0 to 9.0. Purified Ser-31 was obtained after thrombin digestion of the fusion protein followed by an additional passage over the affinity column at pH 6.0. Because Ser-31 does not bind to the affinity column, the bulk of the mutant DHFR was collected in the void volume, while the *E. coli* mutant remained bound to the MTX-resin. Trace amounts of bound folate or nucleotides was removed by anion-exchange chromatography and the purity of the protein demonstrated by SDS−PAGE and Immunoblot analysis.

Kinetic Characterization

The complete kinetic mechanism for murine DHFR has been elucidated and shown to contain the following key features: (1) H_4F release limits steady-state turnover at neutral pH; (2) the preferred pathway for H_4F release is from the mixed ternary $E{\cdot}NH{\cdot}H_4F$ complex; (3) the overall reaction strongly favors H_4F formation due in part to the high value associated with the internal equilibrium ($K_{int} = 100$) for the reactive ternary complexes; (4) unlike the bacterial enzyme, the steady state kinetics are defined by two K_ms for NADPH; and (5) the observed apparent pK_a for the enzyme results from the intersection of the pH-independent rate of H_4F release and the pH-dependent rate of hydride transfer, while the intrinsic pK_a for the enzyme active site is coincident with the pK_a for hydride transfer.[11] Therefore, upon analysis by steady state and pre steady state kinetic experiments, the impact of mutant DHFRs on turnover can be attributed to the perturbation of one or more steps within the kinetic mechanism.[12]

Unexpectedly, significant differences were found to exist between the observed kinetic and thermodynamic parameters for wild-type mouse DHFR and previously reported results for this enzyme, despite identical buffer conditions.[11] The value of the k_{cat} was found to be nearly 7-fold lower, and only one K_m was observable for NADPH. Moreover, the apparent pK_a of the enzyme was 0.5 unit lower, while the intrinsic pK_a was 1.2 units higher than previously observed. Surprisingly, only a modest intrinsic isotope effect was detectable at both low and high pH. At neutral pH the rate of hydride transfer was slightly lower than that previously observed, but still high enough (i.e., 1400 s^{-1}) not to be considered rate-limiting given a turnover number of 4.1 s^{-1}.

Previously, the dissociation rate constant for H_4F from the $E{\cdot}NADPH{\cdot}H_4F$ complex (i.e., 40 s^{-1}) was found to be rate-limiting for the kinetic mechanism of mouse DHFR.[11] However, our observation of a dissociation rate constant of 38.0 ± 0.3 s^{-1} for H_4F release, given a

* To whom correspondence should be addressed.
[†] Department of Laboratory Medicine and Pathology, University of Minnesota.
[‡] Department of Medicinal Chemistry, University of Minnesota.
[§] Department of Chemistry, San Diego State University.

0022-2623/96/1839-1763$12.00/0

Table 1. Steady State and Pre Steady State Parameters for Murine Wild-Type and Mutant Dihydrofolate Reductases

	wild type[a]	wild type	F31S	F31W
k_{cat} (s^{-1})	28 ± 2	4.1 ± 0.5	12.4 ± 0.4	0.8 ± 0.1
$K_m(H_2F)$ (μM)	0.9 ± 0.3	0.4 ± 0.1	1.2 ± 0.2	0.5 ± 0.1
K_m(NH) (μM)	6.2 ± 1.0 49 ± 4	12.6 ± 2.8	37.0 ± 4.6	13.4 ± 2.0
k_{cat}/K_m (μM^{-1} s^{-1})	31	10.3 ± 1.3	10.3 ± 1.7	1.6 ± 0.3
pK_a (V)	9.46 ± 0.05	8.9 ± 0.1	7.5 ± 0.05	6.7 ± 0.2
p$K_{a_{intr}}$[b]	6.4 ± 0.05	7.6 ± 0.2	7.2 ± 0.2	7.4 ± 0.2
$D(v)$ pH 6.0[c]	1.0 ± 0.12 (pH 8.5)	0.6 ± 0.2	1.0 ± 0.1	1.2 ± 0.1
$D(v)$ pH 9.0[c]	3.3 ± 0.25 (pH 10)	1.6 ± 0.1	2.2 ± 0.6	1.2 ± 0.1
$k_{hydride}$ (pH 7.0)	2000	1400 ± 400	270 ± 10	900 ± 200

[a] Parameters were taken from ref 11. [b] Intrinsic pK_a values (p$K_{a_{intr}}$) were determined as described in ref 12. [c] $D(v) = k_{cat}^{H}/k_{cat}^{D}$.

k_{cat} of 4.1 s^{-1}, argues that the E·NADPH·H_4F complex is not a kinetically relevant species for the mouse DHFR. Therefore, the steady state and pre steady state kinetic results imply that a product release step other than H_4F from E·NADPH·H_4F or a kinetically significant conformational change that occurs prior to or after hydride transfer is probably rate-limiting and not hydride transfer.

Repeated sequencing of the wild-type and mutant DHFRs confirmed that the amino acid sequence of the enzyme was identical to that previously reported.[13] At least two isozymes of mouse DHFR have been reported and may account for the kinetic differences.[14] Ongoing rapid kinetic analysis of the wild-type and mutant enzymes should further clarify the rate-limiting step or steps in the kinetic mechanism of this mouse isozyme (Wagner, C. R.; Adams, J.; Evenson, D. A.; McIvor, R. S., manuscript in preparation).

Replacement of phenylalanine-31 with serine resulted in a 3-fold increase in the pH independent k_{cat} and $K_m(H_2F)$. As a consequence, the value of k_{cat}/K_m was unchanged (Table 1). The observed steady state kinetic deuterium isotope effects (i.e., $D(v) = k_{cat}^{H}/k_{cat}^{D}$) on k_{cat} for the mutant exhibited a small increase at both low and high pH. Because the maximal isotope effect on k_{cat} (i.e., $D(v) \approx 3.0$) was not observed for either the Ser-31 or wild-type DHFR, the hydride transfer step can only be modestly rate limiting at low and high pH. Moreover, the intrinsic pK_a of the mutant is nearly identical to the apparent pK_a for catalysis. Taken together, the small isotope effect on k_{cat} at low and high pH, a nearly coincident intrinsic and apparent pK_a, and the reduction in the rate of hydride transfer by less than an order of magnitude implys that a step along the kinetic pathway other than hydride transfer is likely to be rate-limiting.

Determination of k_{cat} and K_m at neutral pH by varying the NADPH concentration (1−1000 μM) at a fixed H_2F concentration (100 μM) revealed a 3-fold increase in the K_m(NH) relative to wild-type and the same k_{cat} observed for the Ser-31 mutant under varying H_2F and fixed NADPH conditions. In contrast to previous observations for wild-type and mutant murine DHFR, varying the NADPH concentration did not reveal nonlinearity in the double-reciprocal plot resulting from two K_ms for the cofactor.[5,11] In addition, the 28-fold increase and 6.5-fold decrease in the thermodynamic dissociation constants of the Ser-31 mutant for NADPH and H_2F, respectively, argues that the residue substitution results in perturbation of the folate and NADPH binding sites.

Substitution of phenylalanine-31 with tryptophan resulted in a 5-fold decrease in the pH independent k_{cat} and little effect on $K_m(H_2F)$ (Table 1). Consequently, the 6.4-fold reduction in k_{cat}/K_m is largely the result of the reduced turnover number of the enzyme. The k_{cat} and K_m determined by varying the NADPH concentration (1−1000 μM) with a fixed H_2F concentration (100 μM) at neutral pH was shown to be nearly identical to the value obtained for wild-type. As shown for the wild-type and the Ser-31 mutant, nonlinearity for the double-reciprocal plots under variable cofactor conditions was not observed. Therefore, because the dissociation constant and K_m for NADPH and H_2F and Trp-31 remained essentially unchanged when compared to the wild-type enzyme, the active site surface is only minimally perturbed by substitution for Phe-31 with the larger indolyl side chain.

As observed for the Ser-31 mutant, the steady state kinetic deuterium isotope effects (i.e., k_{cat}^{H}/k_{cat}^{D}) on k_{cat} for the Trp-31 mutant at low and high pH remained close to unity. Similar to the wild-type enzyme and Ser-31 mutant, the intrinsic pK_a of the active site for Trp-31 is reasonably close to the apparent pK_a for catalysis, while the rate of hydride transfer is reduced by 1.4-fold. As a consequence, the pH rate profile for Trp-31, as observed for Ser-31, is likely to result from the pH dependence of an as yet to be determined conformational change or product release step and not hydride transfer.

Effects on Inhibitor Binding

X-ray crystallographic and mutagenic studies of murine DHFR have revealed that position 31 is part of an ensemble of amino acid side chains that comprise the hydrophobic surface of the active site responsible for folate and methotrexate binding.[4,5,15,16] Specifically, the phenylalanine side chain at this position provides an aromatic−aromatic interaction with the *p*-aminobenzoyl moiety of MTX. Interestingly, the active sites of the murine and human enzymes are nearly identical, as revealed by close inspection of the refined structures of the murine and human DHFRs containing MTX and the cofactor NADPH. In both cases, Phe-31 provides an important hydrophobic contact to the *p*-aminobenzoyl group of the bound antifolate.[15,17] Nevertheless, differences between the two mammalian enzymes, particularly with respect to the conformation of the bound inhibitor and Phe-31, are apparent.[17] In contrast to our observations with regard to the effect of substitutions at position 31 on drug resistance in transfected Chinese hamster ovary (CHO) cells, Blakely and co-workers have concluded from a comparison of mutants of the human enzyme that a reduction in the size of the amino acid side chain at position 31 was required in order to decrease the affinity of the enzyme for MTX.[2] To address the issue of side-chain bulk on MTX binding we determined the inhibition constants (K_i) and dissociation constants (K_d) for MTX and our mouse mutant DHFRs.

The K_i values for MTX and murine wild-type and mutant DHFRs were determined by Dixon plot analysis and are given in Table 2. For Ser-31, the K_i for MTX was modestly increased by 4.8-fold relative to the K_i for wild-type, which was similar to the increase observed for the IC_{50} value. In contrast, a 1100-fold increase in the K_i was previously reported for this mutant, and an

Table 2. Thermodynamic Dissociation Constants, K_d (nM) and Inhibitor Constants for Various Ligands to Mutant and Wild-Type DHFRs

constant	human[a]	WT[b]	WT	hF31S[a]	F31S[c]	F31S	F31W
K_d(NH)	40	1.85 ± 0.16	0.05 ± 0.02	25		1.4 ± 0.2	0.6 ± 0.07
K_d(DHF)	50	0.81 ± 0.07	1.3 ± 0.2	5		0.2 ± 0.09	1.4 ± 0.5
K_d(NP)		3.71 ± 0.22					
K_d(MTX)	0.0048		10.0 ± 0.7	0.443		220 ± 6	10.0 ± 0.3
K_i(MTX)	0.0034[d]	0.004[c]	0.027 ± 0.005	0.289[d]	4.4	0.130 ± 0.007	3.5 ± 0.1
IC_{50}(MTX)			4.5[e]			20[e]	100[e]
K_d/K_i	1.29		370	1.53		1690	2.85
$K_i(k_{cat}/K_m) \times 10^3$	0.31	0.12	0.28	3.82		1.34	5.60

[a] Taken from ref 19. [b] Taken from ref 11. [c] Taken from ref 4. [d] Taken from ref 2. [e] Taken from ref 6.

85-fold increase in the K_i for MTX was reported for the human Ser-31.[2,4] As was observed for the IC_{50} values, the Trp-31 mutant had a larger effect on inhibitor binding resulting in a 130-fold increase in the K_i for MTX. In contrast to previous mutagenic analyses of the role of position 31 for MTX binding to human DHFR, these results argue that for mouse DHFR an increase in the surface area of the side chain at position 31 is partially responsible for a decrease in the affinity of the binary E·NADPH complex for MTX, which is consistent with the substantial increase in the K_i previously observed for the mouse DHFR Arg-31 mutant.[4]

Surprisingly, while there is a substantial difference between the K_is for wild-type and the Trp-31 mutant, little difference exists between the respective dissociation constants of these enzymes for MTX. In contrast, a 22-fold increase in the K_d for MTX was observed for the Ser-31 mutant, which is consistent with the effect imparted by the same mutation in the human enzyme and is consistent with the failure of MTX affinity chromatography to purify this mutant (Table 2). Consequently, destabilization of MTX binding to E is enhanced by a smaller amino acid side chain at position 31.

Because the affinity of the enzymes for MTX by the ternary and binary complexes is a reflection of the K_i and K_d values, respectively, the role of NADPH on MTX binding can be assessed (Table 2). With the exception of human DHFR, enhancement of MTX binding to DHFR by NADPH has been previously demonstrated for mammalian and bacterial enzymes.[18] For the mouse enzyme, inhibitor binding to the mouse E·NADPH complex was enhanced 370-fold by NADPH. As a result, the active site surface of mouse DHFR is able to increase the effect of the cofactor stabilization of MTX binding by 287-fold over the effect observed for human DHFR, despite their high degree of sequence and structural similarity. This result may be partially due to the ability of mouse DHFR to stablize NADPH binding nearly 800-fold better than human DHFR.[19]

Comparison of the ratio, K_d/K_i, for the human and mouse Ser-31 mutants revealed that whereas the wild-type and mutant human DHFRs exhibited no synergistic effect of NADPH on MTX binding, the mouse Ser-31 mutant was able to further enhance this effect nearly 5-fold above that observed for wild-type and over 1100-fold above that observed for human Ser-31. The Trp-31 mutant, however, reduced by approximately 130-fold the enhancement of inhibitor binding by the cofactor. An increase in the synergistic effect could not be correlated with further stabilization of cofactor binding, since the dissociation constant for NADPH was increased by 28-fold and 12-fold for Ser-31 and Trp-31, respectively. Therefore, accommodation of the larger indolyl side chain at position 31 by the active site resulted in destabilization of the ternary inhibitor complex and not the binary complex, while accommodation of the smaller methylene hydroxyl side chain of Ser-31 by the DHFR active site resulted in further stabilization of the ternary inhibitor complex relative to the binary complex.

Recently, the expression $K_i(k_{cat}/K_m)$ has been used to describe the combination of the effects of a mutation on inhibitor binding (K_i) and catalytic efficiency (k_{cat}/K_m)[2] (Table 2). Within this context, the Ser-31 and Trp-31 mutants are 4.8 and 20 times better at conferring resistance than the wild-type enzyme, respectively. If the effect of the substitutions on the K_i and k_{cat}/K_m values are compared, it is apparent that a significant reduction in the affinity of the E·NH complex for MTX is associated with a reduction in enzyme catalytic efficency for the Trp-31 mutant but not the Ser-31 mutant. A similar trend is observed when the IC_{50} and k_{cat}/K_m values are compared, thus indicating that the E·NH complex was the predominant physiological species in MTX-resistant CHO transfectants.[6] In contrast, although substitution of position 31 with serine in human DHFR enhances by 12-fold the enzyme's ability to confer resistance, the value of k_{cat}/K_m was reduced 5.8-fold.[2]

Summary

Steady state and preliminary pre steady state studies of the mouse DHFR indicate that the wild-type enzyme used for our mutagenic studies follows a significantly different *in vitro* kinetic pathway than previously reported.[5,11] In particular, turnover does not appear to be governed by H_4F release from the E·NADPH complex. The discrepancies in catalysis and binding behavior of the mouse DHFRs maybe due to the isomeric nature of the DHFRs studied.

The enhanced ability of the two mutations at position 31 to confer resistance to MTX, as expected, decreased the affinity of the enzyme for the inhibitor. A correlation between the increased size of the side chain at position 31 and decreased inhibitor affinity was observed. This findings is consistent with previous mutagenesis studies of mouse DHFR but is at odds with conclusions drawn from an analysis of the role of the position in inhibitor binding to human DHFR.[2,4]

It is generally agreed that a highly efficient enzyme is desired for most cellular metabolic functions; however, because substitution of position 31 with tryptophan impairs catalytic efficiency, it appears that there exists a high physiological tolerance for significantly impaired DHFR. Indeed, mice who have recieved transplants of bone marrow expressing the Trp-31 mutant or the

severely impaired Arg-22 mutant are capable of surviving lethal doses of MTX.[10] Nevertheless, the consequences *in vivo* of a reduction in the observed *in vitro* catalytic effectiveness of DHFR remain to be determined. Additional mutagenic studies attempting to select catalytically silent mutations that reduce inhibitor binding may further enhance the therapeutic potential of drug-resistant DHFR genes for improved folate antagonist mediated antitumor activity.

Acknowledgment. We are grateful to Ms. Jenny Fisher and Ms. Laurie McLeod for their excellent technical assistance. This work was supported by grant CA60803 (R.S.M.) from the National Institutes of Health.

References

(1) Schweitzer, B. I.; Dicker, A. P.; Bertino, J. R. Dihydrofolate Reductase as a Therapeutic Target. *FASEB J*. **1990**, *4*, 2441−2452.
(2) Chunduru, S. K.; Cody, V.; Luft, J. R.; Pangborn, W.; Appleman, J. R.; Blakley, R. L. Methotrexate-Resistant Variants of Human Dihydrofolate Reductase-Effects of Phe31 Substitutions. *J. Biol. Chem*. **1994**, *269*, 9547−9555.
(3) Nakano, T.; Spencer, H. T.; Appleman, J. R.; Blakley, R. L. Critical Role of Phenylalanine-34 of Human Dihydrofolate Reductase in Substrate and Inhibitor Binding and in Catalysis. *Biochemistry* **1994**, *33*, 9945−9952.
(4) Thillet, J.; Absil, J.; Stone, S. R.; Pictet, R. Site-Directed Mutagenesis of Mouse Dihydrofolate Reductase. Mutants with Increased Resistance to Methotrexate and Trimethoprim. *J. Biol. Chem*. **1988**, *263*, 12500−12508.
(5) Wagner, C. R.; Thillet, J.; Benkovic, S. J. Complementary Perturbation of the Kinetic Mechanism and Catalytic Effectiveness of DHFR by Sidechain Interchange. *Biochemistry* **1992**, *31*, 7834−7840.
(6) Morris, J. A.; McIvor, R. S. Saturation Mutagenesis at Dihydrofolate Reductase Codons 22 and 31—A Variety of Amino Acid Substitutions Conferring Methotrexate Resistance. *Biochem. Pharmacol*. **1994**, *47*, 1207−1220.
(7) McIvor, R. S.; Simonsen, C. C. Isolation and Characterization of a Variant Dihydrofolate Reductase cDNA From Methotrexate-Resistant Murine L5178Y cells. *Nucleic Acids Res*. **1990**, *18*, 7025−7032.
(8) May, C.; Gunther, R.; McIvor, R. S. Protection of Mice From Lethal Doses of Methotrexate by Transplantation With Transgenic Marrow Expressing Drug-Resistant Dihydrofolate Reductase Activity. *Blood* **1995**, *86*, 2439−2448.
(9) Kaufman, B. T. Methotrexate-Agarose in the Purification of Dihydrofolate Reductase. *Methods Enzymol*. **1974**, *34*, 272−281.
(10) Huang, Z.; Wagner, C. R.; Benkovic, S. J. Nonadditivity of mutational effects at the folate binding site of *Escherichia coli* dihydrofolate reductase. *Biochemistry* **1994**, *33*, 11576−11585.
(11) Thillet, J.; Adams, J. A.; Benkovic, S. J. The Kinetic Mechanism of Wild-Type and Mutant Mouse Dihydrofolate Reductases. *Biochemistry* **1990**, *29*, 5195−5202.
(12) All steady state and pre steady state kinetic measurements (Table 1) were obtained at 25 °C in buffer containing 50 mM 2-morpholinoethanesulfonic acid (Mes), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine, and 800 mM sodium chloride (MTEN buffer, pH 5−10) as previously described.[11] Rapid kinetic experiments were carried out with an Applied Photophysics Stopped-Flow apparatus as previously described.[11] Methotrexate binding data was determined in MTEN (pH 7.0) (Table 2). The intrinsic pK_a of the enzymes was determined by DAM inhibition studies as previously described.[20] Thermodynamic dissociation constants (K_d) for folate, NADPH, and methotrexate and the DHFRs were measured by fluorescence titration[11] (Table 2). All K_i values listed in Table 2 for methotrexate were determined by Dixon plot analysis.[18]
(13) Chang, A. C. Y.; Nunberg, J. H.; Kaufman, R. J.; Erlich, H. A.; Schimke, R. T.; Cohen, S. N. Phenotypic Expression in *E. coli* of a DNA Sequence Coding for Mouse Dihydrofolate Reductase *Nature* **1978**, *275*, 617−624.
(14) Blakley, R. L. In *Folates and Pterins*; Blakley, R. L., Benkovic, S. J., Eds.; Wiley: New York, 1986; pp 191−273.
(15) Stammers, D. K.; Champness, J. N.; Beddell, C. R.; Dann, J. G.; Eliopoulos, E.; Geddes, A. J.; Ogg, D.; North, A. C. T. The Structure of Mouse L1210 Dihydrofolate Reductase-Drug Complexes and the Construction of a Model of Human Enzyme. *FEBS Lett*. **1987**, *218*, 178−184.
(16) Prendergast, N. J.; Appleman, J. R.; Delcamp, T. J.; Blakley, R. L.; Freisheim, J. H. Effects of Conversion of Phenylalanine-31 to Leucine on the Function of Human Dihydrofolate Reductase. *Biochemistry* **1989**, *28*, 4645−4650.
(17) Oefner, C.; D'Arcy, A.; Winkler, F. K. Crystal Structure of Human Dihydrofolate Reductase Complexed With Folate. *Eur. J. Biochem*. **1988**, *174*, 377−385.
(18) Stone, S. R.; Morrison, J. R. Mechanism of Inhibition of Dihydrofolate Reductases From Bacterial and Vertebrate Sources by Various Classes of Folate Analogs. *Biochim. Biophys. Acta* **1986**, *869*, 275−285.
(19) Schweitzer, B. I.; Srimatkandada, S.; Gritsman, H.; Sheridan, R.; Venkataraghavan, R.; Bertino, J. R. Probing the Role of Two Hydrophobic Active Site Residues in the Human Dihydrofolate Reductase by Site-Directed Mutagenesis. *J. Biol. Chem*. **1989**, *264*, 20786−20795.
(20) Stone, S. R.; Morrison, J. F. Kinetic mechanism of the reaction catalyzed by dihydrofolate reductase from *Escherichia coli*. *Biochemistry* **1982**, *21*, 3757−3765.

JM950793D