Dihydrofolate Reductase Mutant with Exceptional Resistance to Methotrexate but Not to Trimetrexate

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Abstract: Two double (F31A/F34A, I60A/L67G) and one quadruple (F31A/F34A/I60A/L67G) mutant murine dihydrofolate reductases were constructed and evaluated for their ability to impart antifolate resistance. Both I60A/L67G and F31A/F34A/I60A/L67G were found to be unstable and devoid of catalytic activity. The K_i values for F31A/F34A, methotrexate (MTX), bis-MTX, and PT-523 were found to be 10100-, 4410-, and 617-fold higher than the wild-type enzyme, respectively, but only 13.5-fold higher for trimetrexate (TMTX). These findings suggest that F31A/F34A could be used for gene therapy to render normal cells resistant to MTX but sensitive to TMTX.

Introduction. Dihydrofolate reductase (DHFR) catalyzes the reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F). The enzyme is found in vertebrate and bacterial organisms, including mice, humans, and *E. coli*.[1] Since the amino acid sequence for DHFR shows 95% homology between humans and mice,[1] mouse DHFR (mDHFR) has been chosen for study. H_4F derivatives are necessary for generating purines, thymidylate, and some amino acids.[1] DHFR's crucial role in cell proliferation has made the enzyme an attractive target for anticancer drug design.[2] The inhibitor methotrexate (MTX) has been used extensively to treat such cancers as acute lymphoblastic leukemia and non-Hodgkin's lymphoma by competitively inhibiting DHFR.[3] Thus, MTX binds to DHFR's active site, preventing the generation of DNA precursors and subsequently inhibiting cancerous cell proliferation.[4]

The effectiveness of MTX as an anticancer agent is compromised by the drug's lack of specificity for tumor cells. Side effects of MTX treatment include toxicity for the gastrointestinal tract and for the bone marrow, since the cells at these sites are rapidly dividing.[5] Understanding the mode of inhibition of MTX on DHFR may lead to inhibitors with fewer side effects.

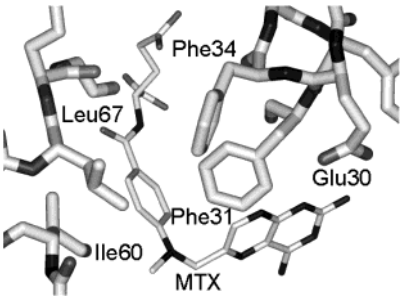

Figure 1. Model of murine DHFR active site. Although an X-ray structure of murine DHFR with MTX is not publicly available, on the basis of the nearly identical sequence similarity (95%) between the human and mouse enzymes, a model of the mDHFR active site was built. Placement of the active site residues was found to be identical to that for human DHFR and MTX (PDB file 1DLS).

Of particular interest are mutant DHFRs that are resistant to MTX. Such resistance may be of use in cancer therapy. Mutant DHFRs that are resistant to MTX yet retain sufficient catalytic activity may be used to render normal cells resistant to MTX.[6] The transfer of genes for such catalytically active MTX-resistant mutants has been shown to protect experimental animals from MTX toxicity.[6]

Previously, it had been shown that *E. coli* DHFR was remarkably able to retain significant catalytic activity but had reduced affinity for MTX when the four hydrophobic active site residues (L28, F31, I50, L54) responsible for folate and MTX binding were substituted by alanine or glycine as either double (L28A/F31A, I50A/L54G) or quadruple (L28A/F31A/I50A/L54G) mutants.[7] Consequently, although there is only 30% homology between the bacterial and murine DHFRs, we chose to construct and examine the effect of the corresponding mutations (F31A/F34A, I60A/L67G, and F31A/F34A/I60A/L67G) on murine DHFR catalysis and inhibitor binding (Figure 1).

All three mutant DHFRs were prepared, and their purification and kinetic evaluation were initiated. In contrast to the corresponding *E. coli* mutants, only the double mutant F31A/F34A was stable enough to be isolated and characterized.

Kinetic Characterization. The overall kinetic mechanism for mDHFR has been determined and shown to be characterized by the following: (1) release of H_4F is rate-limiting at neutral pH; (2) release of H_4F is from the mixed ternary $E{\cdot}NH{\cdot}H_4F$ complex; and (3) the high value of the internal equilibrium (K_{int} = 100−1000) strongly favors H_4F formation.[8] Consequently, two K_m values for NADPH and one K_m value for H_4F govern mouse DHFR steady-state kinetics.

Steady-state kinetic analysis of the wild-type mDHFR revealed only minor differences between the recombinant enzyme we have prepared and the protein previously reported.[8] Since the sequence of the wild-type enzymes and the buffer employed during the kinetic analysis are identical, observed discrepancies are likely attributable to differences in the enzyme preparations.

Despite the rather drastic nature of the active site amino substitutions, the F31A/F34A mutant was able

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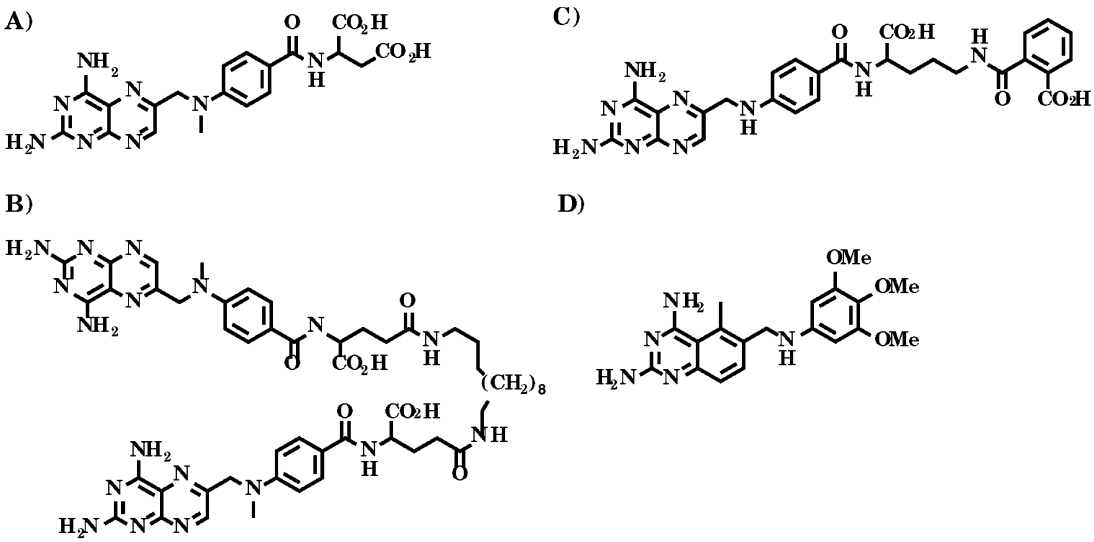

Figure 2. Chemical structures of DHFR inhibitors: (A) MTX; (B) bis-MTX: (C) PT-523; (D) TMTX.

Table 1. Steady-State Kinetic Parameters for Murine Wild-Type and F31A/F34A DHFRs

	wild-type[a]	wild-type	F31A/F34A
$k_{cat}(H_2F)$, s^{-1}	28 ± 2	50.2 ± 3.98	4.03 ± 0.08
K_m (H_2F), μM	0.9 ± 0.3	1.91 ± 0.26	7.55 ± 0.56
k_{cat}/K_m (H_2F), μM^{-1} s^{-1}	31	26.3	0.53
k_{cat1}(NH), s^{-1}	17 ± 2	31.1 ± 2.0	2.45 ± 0.13
k_{cat2}(NH), s^{-1}	36 ± 2	60.5 ± 1.6	3.07 ± 0.90
K_{m1}(NH), μM	6.2 ± 1	1.2 ± 0.2	1.67 ± 0.19
K_{m2}(NH), μM	49 ± 4	16.9 ± 1.7	7.87 ± 1.44

[a] Parameters were taken from ref 8.

to retain significant catalytic efficiency for both substrates, with a $k_{cat}(H_2F)$ that was 12.5-fold lower and a $K_m(H_2F)$ that was 4-fold higher than observed for the wild-type enzyme (Table 1). While not significantly perturbing K_{m1}(NADPH) and K_{m2}(NADPH), the wild-type values for k_{cat1}(NADPH) and k_{cat2}(NADPH) are 12.7- and 19.7-fold greater, respectively, than the values found for the mutant enzyme. Consequently, despite significant perturbation of the active site, the mutant enzyme exhibited significant catalytic efficiency for both substrates.

Effects on Inhibitor Binding. As mentioned previously, MTX's mode of therapy centers on its ability to competitively inhibit DHFR. To determine the effect of the active site mutations F31A and F34A on inhibitor binding and to test for selectivity between different inhibitors, the K_i values were determined for four structurally similar DHFR inhibitors: MTX, bis-methotrexate (bis-MTX),[9] PT-523,[10] and trimetrexate (TMTX) (Figure 2). The data were collected and analyzed using Dixon plot analysis and are summarized in Table 2.

Compared to wild-type DHFR, the K_i values for the F31A/F34A mutant and MTX and bis-MTX were increased by similar factors of 10 100 and 4410, respectively. In contrast, the K_i values for PT-523 and TMTX were 617- and 13.5-fold higher for the mutant than the wild-type enzyme, respectively. These results indicate that the selectivity of PT-523 and TMTX relative to MTX has increased by 16.3- and 747-fold, respectively.

To express the combined effects of the active site mutations F31A and F34A on catalytic efficiency and inhibitor binding, the $K_i(k_{cat}/K_m)$ values were calculated (Table 2).[11] The F31A/F34A mutant conferred resistance

Table 2. Inhibitor Constants for Various Ligands of Wild-Type and F31A/F34A Murine DHFRs

	wild-type	F31A/F34A
K_i(MTX), nM	0.033 ± 0.011	333 ± 32
K_i(bis-MTX), nM	0.048 ± 0.002[a]	212 ± 25
K_i(PT-523), nM	0.042 ± 0.002	25.9 ± 0.94
K_i(TMTX), nM	0.060 ± 0.005	0.810 ± 0.062
$K_I(k_{cat}/K_m) \times 10^3$ (MTX)	0.87	177
$K_I(k_{cat}/K_m) \times 10^3$ (bis-MTX)	1.26	112
$K_I(k_{cat}/K_m) \times 10^3$ (PT-523)	1.1	13.7
$K_I(k_{cat}/K_m) \times 10^3$ (TMTX)	1.58	0.43
K_{imut}/K_{iwt} (MTX)		10,100
K_{imut}/K_{iwt} (Bis-MTX)		4410
K_{imut}/K_{iwt} (PT-523)		617
K_{imut}/K_{iwt} (TMTX)		13.5

[a] Parameters were taken from ref 9.

to MTX, bis-MTX, and PT-523 that was 203-, 89.0-, and 12.5-fold greater, respectively, than the wild-type enzyme. In contrast, the mutant enzyme was 3.7-fold more sensitive to TMTX than the wild-type. Thus, even with decreased catalytic efficiency, the F31A/F34A mutant enzyme is able to impart highly selective resistance to MTX.

A rationale for the behavior of the F31A/F34A mutant toward the antifolates examined in this study will require structural analysis and additional kinetic studies. Nevertheless, it is clear from these results that compounds most resembling MTX are likely to be the most susceptible to reduced binding affinity. The addition of a hydrophobic hemiphthaloyl-L-ornithine group to the terminus of MTX, as observed for PT-523, partially restores (16.3-fold) the loss of these key side chain interactions with the 4-aminophenyl moiety. Replacement of the 2-(4-methylaminobenzoylamino)-glutamyl group with trimethoxyaniline further enhances binding to the mutant by 746-fold. It is probable that the ability of the two ortho methoxy moieties of TMTX to access hydrophobic binding interactions, not available to MTX, bis-MTX, or PT-523, is responsible for reducing the impact of the F31A and F34A mutations on TMTX binding.

In contrast, the K_m for H_2F and the mutant DHFR was found to be only 4-fold greater than the value determined for wild-type DHFR, consistent with the lack of an observable difference between the dissociation

constants (K_d) for H_2F and the two enzymes (data not shown). In addition, the K_m for H_2F and K_i for MTX are closer in magnitude by nearly 3000-fold than the values found for the wild-type enzyme. Given that the optimum binding orientation for the pteridine ring in MTX when bound to wtDHFR is 180° from that observed for H_2F, it is probable that the orientation of MTX in the active site of the mutant is similar to that of H_2F. This conclusion would imply that the side chains of F31 and F34 have a role in determining the optimal binding conformation of MTX and other MTX-based ligands such as bis-MTX and PT-523. Consequently, the ability of these residues to stabilize active site binding of the non-H_2F conformation of the inhibitor may be responsible for the 60000-fold enhancement of MTX affinity for the mDHFR over that observed for H_2F. Ongoing structural and kinetic studies should address the validity of these conclusions.

Summary. In the search for a mutant DHFR that is capable of catalysis yet resistant to DHFR inhibitors, catalytic and inhibition constant values have shown that the F31A/F34A mutant is catalytically active and also largely resistant to MTX, bis-MTX, and PT-523 but not to TMTX. In addition, the side chains of F31 and F34 may be partially responsible for maintaining not only hydrophobic aromatic−aromatic interactions but also the highest affinity conformation of the pteridine ring of MTX for the active site. Both the double mutant I60A/L67A and the quadruple mutant F31A/F34A/I60A/L67A were found to be unstable and devoid of catalytic activity, implying that these residues are important for maintaining the structural and catalytic integrity of murine and human DHFR. While mutants highly resistant to MTX and TMTX have been identified, to our knowledge, this is the first example of a mutant highly resistant to MTX but not to TMTX.[6,12] The ability of the F31A/F34A mutant to confer cellular resistance to MTX while maintaining sensitivity to TMTX is under investigation and will be reported in due course.

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Supporting Information Available: Experimental methods, protein purification elution profiles, SDS−PAGE results, purification table summary, kinetics, and Lineweaver−Burk and Dixon plots. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added after ASAP Posting. This manuscript was released ASAP on 6/7/2003 with an error in the linker region of Figure 2B. The correct version was posted on 6/26/2003.

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