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Role of Lysine 411 in Substrate Carboxyl Group Binding to the Human Reduced Folate Carrier, as Determined by Site-Directed Mutagenesis and Affinity Inhibition[S]

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ABSTRACT

Reduced folate carrier (RFC) is the major membrane transporter for folates and antifolates in mammalian tissues. Recent studies used radioaffinity labeling with *N*-hydroxysuccinimide (NHS)-[^{3}H]methotrexate (MTX) to localize substrate binding to residues in transmembrane domain (TMD) 11 of human RFC. To identify the modified residue(s), seven nucleophilic residues in TMD11 were mutated to Val or Ala and mutant constructs expressed in RFC-null HeLa cells. Only K411A RFC was not inhibited by NHS-MTX. By radioaffinity labeling with NHS-[^{3}H]MTX, wild-type (wt) RFC was labeled; for K411A RFC, radiolabeling was abolished. When Lys411 was replaced with Ala, Arg, Gln, Glu, Leu, and Met, only K411E RFC showed substantially decreased transport. Nine classic diamino furo[2,3-*d*]pyrimidine antifolates with unsubstituted α- and γ-carboxylates (1), hydrogen- or methyl-substituted α-(2,3) or γ-(4,5) carboxylates, or substitutions of both α- and γ-carboxylates (6–9) were used to inhibit [^{3}H]MTX transport with RFC-null K562 cells expressing wt and K411A RFCs. For wt and K411A RFCs, inhibitory potencies were in the order 4 > 5 > 1 > 3 > 2; 6 to 9 were poor inhibitors. Inhibitions decreased in the presence of physiologic anions. When NHS esters of 1, 2, and 4 were used to covalently modify wt RFC, inhibitory potencies were in the order 2 > 1 > 4; inhibition was abolished for K411A RFC. These results establish that Lys411 participates in substrate binding via an ionic association with the substrate γ-carboxylate; however, this is not essential for transport. An unmodified α-carboxylate is required for high-affinity substrate binding to RFC, whereas the γ-carboxyl is not essential.

Folates are important cofactors for transferring one-carbon units in biosynthetic steps leading to thymidylate, purine nucleotides, and the amino acids serine and methionine (Stokstad, 1990). Because mammalian cells cannot synthesize folates de novo, these cofactors must be derived from exogenous dietary sources. Folates are hydrophilic anionic molecules with ionized α- and γ-carboxyl groups at physiologic pH that do not cross biological membranes by diffusion alone. Accordingly, mammalian cells have evolved sophisticated transport systems for facilitating folate uptake. Although folate uptake can occur by folate receptors, organic anion transporters, and a proton-coupled folate transporter (Matherly and Goldman, 2003; Zhao and Goldman, 2007), the best characterized folate transporter is the ubiquitously expressed reduced folate carrier (RFC) (SLC19A1) (Matherly et al., 2007). Indeed, given its widespread tissue expression, RFC is considered the major transport system for folates in mammalian cells and tissues.

Membrane transport by RFC is also important for antitumor activities of antifolates used for cancer chemotherapy

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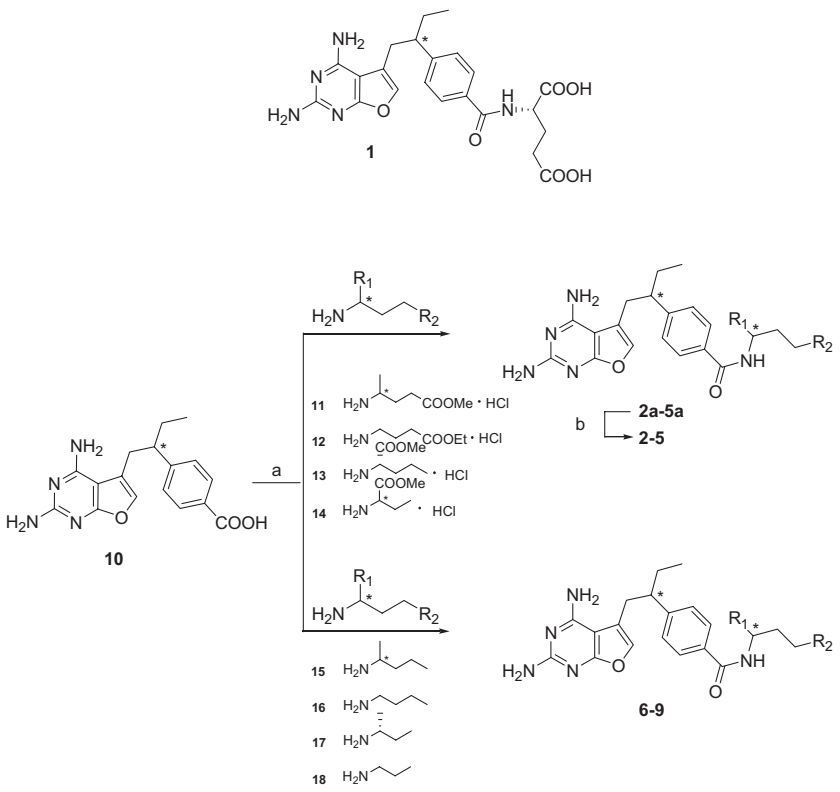
ABBREVIATIONS: RFC, reduced folate carrier; MTX, methotrexate; ZD9331, (2*S*)-2-{*O*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydro-quinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido}-4-(tetrazol-5-yl)-butyric acid; PT523, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine; TMD, transmembrane domain; hRFC, human reduced folate carrier; NHS, *N*-hydroxysuccinimide; ZD1694, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxyquinazolin-6-ylmethyl)-*N*-methyl-amino]-2-thenoyl)-L-glutamic acid; wt, wild type; HA, hemagglutinin; HSM, HEPES-sucrose-Mg^{2+}; DPBS, Dulbecco's phosphate-buffered saline; HBSS, Hanks' balanced salt solution; ICI-198583, (*S*)-2-[(1-{4-[(2-methyl-4-oxo-3,4-dihydro-quinazolin-6-ylmethyl)-prop-2-ynyl-amino]-phenyl}-methanoyl)-amino]-pentanedioic acid.

such as methotrexate (MTX), pemetrexed, and raltitrexed (Tomudex) (Jansen et al., 1999; Goldman and Zhao, 2002; Matherly et al., 2007). Losses of RFC function are common mechanisms of antifolate resistance in in vitro and in vivo models (Sirotnak et al., 1981; Schuetz et al., 1988; Gong et al., 1997; Jansen et al., 1998; Roy et al., 1998; Zhao et al., 1998a,b, 1999; Wong et al., 1999; Drori et al., 2000; Sadlish et al., 2000) and likely contribute to clinical resistance in patients with osteosarcoma (Guo et al., 1999) and B-precursor acute lymphoblastic leukemia who are treated with MTX (Ge et al., 2007). MTX has other clinical applications including treatment of autoimmune diseases and psoriasis (Giannini et al., 1992; Chládek et al., 1998).

The functional properties for RFC were first documented nearly 40 years ago in murine leukemia cells (Goldman et al., 1968). However, it is only since the cloning of RFCs from various species (Dixon et al., 1994; Williams et al., 1994; Moscow et al., 1995; Prasad et al., 1995; Williams and Flintoff, 1995; Wong et al., 1995) and the application of molecular biology approaches to engineer RFC for biochemical studies that a detailed picture of the molecular structure of this physiologically important carrier has emerged, including its membrane topology, its *N*-glycosylation, and identification of functionally and structurally important domains and amino acids (for review, see Matherly et al., 2007). In contrast, there is a dearth of information regarding the structural requirements of RFC substrates. Because RFC is an anion transporter, the role of the terminal glutamate in transport is of particular interest. Although modifications of the glutamate γ-carboxyl group in RFC substrates were tolerated (e.g., valine), including those for the antifolates ZD9331 and PT523 (Westerhof et al., 1995; Jansen, 1999), to date, no systematic study of the α- versus γ-carboxylates in binding and transport by RFC has been reported.

Cationic amino acids (Arg, Lys) localized within the RFC TMD-spanning segments can be envisaged to directly participate in binding of anionic (anti)folate substrates. Of particular interest are Arg373 in TMD10 and Lys411 in TMD11 [numbering refers to human RFC (hRFC) sequence (GenBank accession no. U19720)]. Both of these highly conserved amino acids were previously found to be important for RFC transport (Sharina et al., 2001; Sadlish et al., 2002; Witt and Matherly, 2002) and as likely candidates to participate in binding associations with ionized α- and γ-carboxyl groups in folate substrates. An unidentified nucleophilic amino acid in TMD11 in hRFC was implicated as the major site of covalent modification by the activated carboxyl group(s) in *N*-hydroxysuccinimide (NHS) [^{3}H]MTX (Witt et al., 2004; Hou et al., 2005), an established affinity inhibitor of RFC (Henderson and Zevely, 1984; Matherly et al., 1991).

In this report, we directly explore the role of Lys411 in TMD11 of hRFC in the binding and transport of anionic folate substrates and provide a structure-activity relationship of the substrate carboxylates for RFC transport. Our results establish that Lys411 participates in transport substrate binding to hRFC and is the primary site for covalent modification by NHS-MTX. Through the use of a novel series of furo[2,3-*d*]pyrimidine antifolates with substituted carboxyl groups on the terminal glutamate, we demonstrate that an ionizable α-carboxyl group, but less so a γ-carboxyl group, is a critical substrate feature for high-affinity binding to hRFC. To our knowledge, this is the first report to systematically charac-



Compound	R_1	R_2
1	COOH	COOH
2a	CH_3	COOMe
2	CH_3	COOH
3a	H	COOEt
3	H	COOH
4a	COOMe	CH_3
4	COOH	CH_3
5a	COOMe	H
5	COOH	H
6	CH_3	CH_3
7	H	CH_3
8	CH_3	H
9	H	H

Fig. 1. Synthesis of furo[2,3-*d*]pyrimidine antifolates 1 to 9. A scheme is shown for the structures and synthesis of antifolates 1 to 9. Reaction conditions are: a, *N*-methyl morpholine, 2-chloro-4,6-dimethoxy-1,3,5-triazine, dimethylformamide, room temperature, 6 h; and b, 1 N NaOH, MeOH, room temperature, 10 h and 1 N HCl. Compounds 13 and 17 have the same configuration as L-glutamate.

terize molecular features of substrate binding to RFC in light of specific structural motifs in the (anti)folate molecule and particular conserved amino acids lining the membrane translocation pathway.

Materials and Methods

Reagents. [3′,5′,7-^{3}H]MTX (46.8 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Unlabeled MTX was provided by the Drug Development Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD). Both labeled and unlabeled MTX were purified by high-performance liquid chromatography before use (Fry et al., 1982). The sources of the antifolate drugs were as follows: raltitrexed and ZD9331 were obtained from AstraZeneca Pharmaceuticals (Maccesfield, Cheshire, UK); pemetrexed was from Eli Lilly & Co. (Indianapolis, IN); and PT523 was a gift of Dr. Andre Rosowsky (Dana Farber Cancer Institute, Boston, MA). Synthetic oligonucleotides were obtained from Invitrogen (Carlsbad, CA). Tissue culture reagents and supplies were purchased from assorted vendors with the exception of fetal bovine and supplemented calf sera, which were purchased from Hyclone Technologies (Logan, UT). Molecular biology reagents were from Promega (Madison, WI) or Invitrogen.

Synthesis of Furo[2,3-*d*]Pyrimidine Antifolates 1 to 9. Compound 10, 4-{1-[(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]propyl}-benzoic acid, was obtained as described previously (Gangjee et al., 2002). For compounds 2 to 5, 10 was coupled with the appropriate commercially available, modified, glutamate ester analogs with *N*-methylmorpholine and 2-chloro-4,6-dimethoxy-1,3,5-triazine in dimethylformamide at room temperature for 6 h to afford the desired esters in approximately 60% yield (see Fig. 1). Saponification with aqueous sodium hydroxide at room temperature followed by acidification to pH 4 in an ice bath afforded 2 to 5 in approximately 95% yield. For compounds 6 to 9, 10 was similarly coupled with the appropriate commercially available amines to give the desired products. A reaction scheme for the synthesis of analogs 1 to 9 is shown in Fig. 1. Detailed syntheses are provided in the Supplemental *Materials and Methods*.

Cell Culture. Transport-defective MTX-resistant HeLa cells, designated R5 (Zhao et al., 2004), were a generous gift of Dr. I. David Goldman (Bronx, New York). R5 cells were maintained in RPMI 1640 and supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere at 37°C in the presence of 5% CO_2. Transient transfections of wild-type (wt) and mutant hRFC constructs (see below) were performed with Lipofectamine Plus reagent (Invitrogen), as described previously (Hou et al., 2005, 2006). Cultures were split 24 h after transfection and assayed for transport and expression on Western blots after an additional 24 h.

The MTX transport-deficient K562 subline, designated K500E, was selected from wt K562 cells (American Type Culture Collection, Manassas, VA) and maintained in complete RPMI 1640 medium containing 10% supplemented calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 μM MTX in a humidified atmosphere at 37°C in the presence of 5% CO_2 (Wong et al., 1997). Mutant and wt hRFC constructs (see below) were transfected into K500E cells by electroporation (155 V, 1000-μF capacitance). After 24 h, cells were treated with G-418 (Geneticin; 1 mg/ml), and stable clones were selected by cloning in soft agar in the presence of 1 mg/ml G-418 (Wong et al., 1997). Transfected K500E cultures were maintained in complete RPMI 1640 medium with 1 mg/ml G-418.

Site-Directed Mutagenesis of hRFC. hRFC mutants were generated by site-directed mutagenesis using the QuickChange kit (Stratagene, La Jolla, CA) and a wt hRFC construct with a 5′-untranslated region from positions −1 to −49, the full-length hRFC open reading frame, and a hemagglutinin (HA) epitope at Gln587, cloned in pCDNA3 vector (Invitrogen) (Payton et al., 2007). Primers for site-directed mutagenesis were designed according to the instructions for the QuickChange kit and are summarized in Supplemental Table 1S. All mutations were confirmed by DNA sequencing at the Wayne State University DNA sequencing core.

Western Analysis of Mutant hRFC Transfectants. Plasma membranes were prepared by differential centrifugation (Matherly et al., 1991). For standard Western blotting, membrane proteins were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS (Laemmli, 1970) and electroblotted onto polyvinylidene difluoride membranes (Pierce, Rockford, IL) (Matsudaira, 1989). hRFC proteins were detected with HA-specific mouse antibody (Covance, Berkeley, CA) and secondary IRDye 800-conjugated antibody (Rockland Immunochemicals, Gilbertsville, PA). Detection and densitometry of the blots were performed with the Odyssey Imaging System (LI-COR, Lincoln, NE).

Membrane Transport Assays. Uptake of [^{3}H]MTX (0.5 μM) in transiently transfected R5 HeLa cells was measured over 2 min at 37°C in 60-mm dishes in an "anion-free" HEPES-sucrose-Mg^{2+} (HSM) buffer (20 mM HEPES, 235 mM sucrose, pH adjusted to 7.14 with MgO). Uptake of [^{3}H]MTX was quenched with ice-cold Dulbecco's phosphate-buffered saline (DPBS). Cells were washed with ice-cold DPBS (three times) and solubilized with 0.5 N NaOH. [^{3}H]MTX (1 μM) uptake into stably transfected K500E cells was measured over 180 s (wt and Lys411 mutant) in both HSM buffer and physiologic Hanks' balanced salt solution (HBSS) in a shaking water bath at 37°C, as described previously (Wong et al., 1997). For both cell line models, levels of intracellular radioactivity were expressed as picomoles per milligram of protein, calculated from direct measurements of radioactivity and protein contents of cell homogenates. Protein assays were based on the method of Lowry et al. (1951). For the stable transfected K500E cells, kinetic constants (K_t, V_{max}) were calculated from Lineweaver-Burk plots for [^{3}H]MTX, and K_i values for assorted antifolate substrates were determined from Dixon plots with [^{3}H]MTX (1 μM).

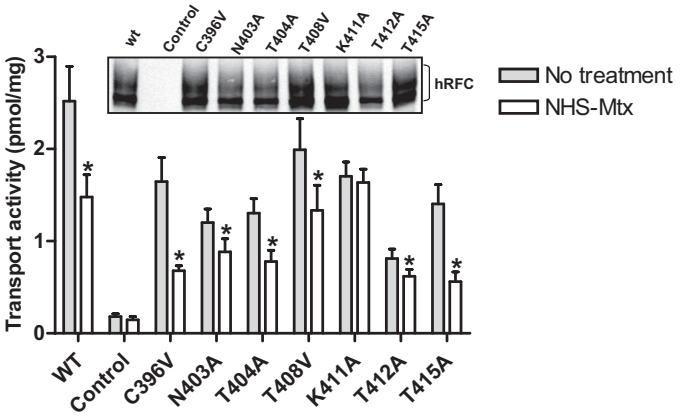


Fig. 2. NHS-MTX inhibition of nucleophilic amino acids in TMD11 of hRFC. Seven nucleophilic amino acids in TMD11 (Cys396, Asn403, Thr404, Thr408, Lys411, Thr412, Thr415) were mutated to non-nucleophilic amino acids (e.g., Ala or Val), and the wt and mutant hRFC constructs were transiently transfected into R5 HeLa cells for comparison with the vector control (labeled "Control"). hRFC proteins were assayed on Western blots with 2.5 μg of membrane proteins and with HA-specific mouse antibody and secondary IRDye 800-conjugated antibody (inset). Detection used the Odyssey Imaging System. In the main panel, the R5 transfectants were treated with NHS-MTX (5 μM) and then assayed for transport of [^{3}H]MTX (0.5 μM) over 2 min. In results from five separate experiments, statistically significant inhibitions ($p < 0.05$ by Wilcoxon test; asterisks) resulting from NHS-MTX treatments compared with untreated samples were measured for wt and TMD nucleophilic substitutions, which ranged from 24% (for Thr412) to 60% (for Thr415). Only the K411A hRFC mutant was completely inert to the NHS-MTX treatment. In the figure, single-letter abbreviations for the amino acid replacements at Lys411 are used. WT, wild type.

Affinity Labeling of hRFC with NHS-MTX Ester. The preparation of unlabeled and radiolabeled NHS-MTX was performed exactly as described previously (Matherly et al., 1991; Witt et al., 2004). For treatments of R5 transfectants, NHS-MTX in 20 μl of dry DMSO was added to 60-mm dishes of R5 cells in 2 ml of HSM buffer at room temperature for 5 min. For nonradioactive NHS-MTX, the final concentration was 5 μM. After treatment with NHS-MTX, cells were washed three times with DPBS at 0°C and assayed for [^{3}H]MTX (0.5 μM) transport (see above).

For radioaffinity labeling experiments, NHS-[^{3}H]MTX was prepared at a radiospecific activity of 46.8 Ci/mmol; the final concentration of NHS-[^{3}H]MTX with the cells was 700 nM. After radioaffinity labeling, plasma membranes were prepared by differential centrifugation. Membrane pellets were solubilized in 1% SDS and fractionated on 1.5-mm 7.5% polyacrylamide gels with SDS, the gels were sliced into 2-mm segments, and the pieces were suspended into 1 ml of Soluene-350 (PerkinElmer Life and Analytical Sciences, Waltham, MA) overnight at room temperature. Five milliliters of Ready-value scintillation cocktail (Beckman Coulter, Fullerton, CA) were added, and radioactivity was detected on a model 6500 Beckman liquid scintillation counter.

NHS esters of furo[2,3-*d*]pyrimidine antifolate analogs were prepared from the parent compounds exactly as for MTX. For treatments of the stable transfected K500E cells with the NHS-furo[2,3-*d*]pyrimidine antifolates, cells were treated in 2 ml of HSM buffer, as described above for the R5 cells, albeit in suspension in a shaking water bath at room temperature. Cells were washed with ice-cold DPBS and assayed for MTX (1 μM) transport in HBSS.

Results

Identification of Lys411 as the Primary Site of Covalent Labeling by NHS-MTX. NHS-MTX is an established affinity inhibitor of hRFC (Henderson and Zevely, 1984; Matherly et al., 1991). NHS esterification activates the carboxyl groups of MTX and related compounds for electrophilic reaction with biological nucleophiles. NHS-[^{3}H]MTX and -aminopterin, have been extensively used for covalently labeling the carrier in cultured cells (Henderson and Zevely, 1984; Schuetz et al., 1988; Matherly et al., 1991; Yang et al., 1992). Previous studies from our laboratory used NHS-[^{3}H]MTX with hRFC TMD1–6 and TMD7–12 half-molecules to localize covalent binding of [^{3}H]MTX (via the substrate carboxyl groups) to TMDs 11 and 12 (Witt et al., 2004; Hou et al., 2005). Because TMD11 (but not TMD12) is directly involved in substrate binding of hRFC substrates (Hou et al., 2005), we reasoned that one or more of the seven nucleophilic amino acids in TMD11 must be a direct target for covalent modification by the NHS-MTX activated ester and, by extension, inhibition of transport activity.

Accordingly, we mutated each of the seven nucleophilic amino acids in TMD11 (Cys396, Asn403, Thr404, Thr408, Lys411, Thr412, Thr415) to non-nucleophilic amino acids (Ala or Val). Mutant and wt hRFC proteins were expressed in hRFC-null R5 HeLa cells, and all were found to be capable of transporting MTX (0.5 μM) within an approximately 3-fold range of activities (Fig. 2). When the R5 transfectants were treated with NHS-MTX (5 μM) then assayed for [^{3}H]MTX transport, for six of seven hRFC mutants and wt hRFC, transport was inhibited. Statistically significant inhibitions resulting from NHS-MTX were measured that ranged from 24% (for Thr412) to 60% (for Thr415). Only the K411A hRFC mutant was completely and reproducibly inert to NHS-MTX treatment.

Because loss of RFC activity by NHS-MTX treatment is the result of a covalent modification of the carrier (Henderson and Zevely, 1984; Schuetz et al., 1988; Matherly et al., 1991; Witt et al., 2004), the lack of a transport effect on the K411A mutant suggested that Lys411 is a likely target for electrophilic attack by the activated NHS-MTX ester. To directly test this possibility, we transiently transfected R5 cells with wt and K411A hRFC constructs, then treated the cells with NHS-[^{3}H]MTX (700 nM) so as to radiolabel the carrier. Plasma membranes were prepared and detergent-solubilized, and the solubilized radiolabeled proteins were fractionated on a 7.5% polyacrylamide gel for direct counting. Similar to previous reports (Matherly et al., 1991; Witt et al., 2004), for wt

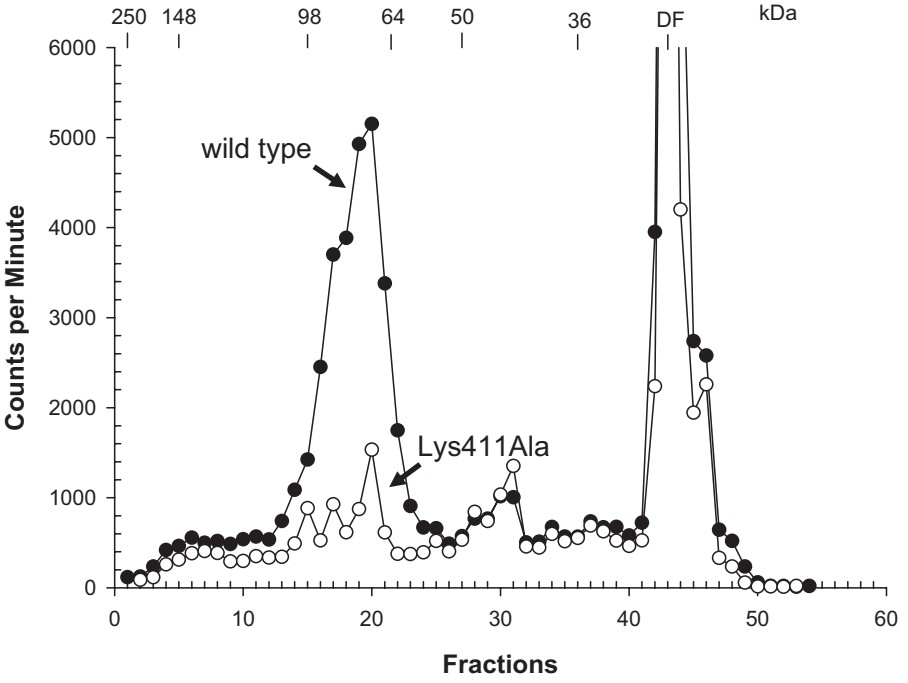


Fig. 3. Radioaffinity labeling of wt hRFC and K411A hRFC with NHS-[^{3}H]MTX. R5 cells were transfected with wt and K411A hRFC constructs. Cells were treated with NHS-[^{3}H]MTX (700 nM), as described under *Materials and Methods*. Membranes were prepared, and proteins (150 μg) were fractionated on 7.5% polyacrylamide gels in the presence of SDS. The gels were sliced into 2-mm segments (labeled "Fractions" on the abscissa), and the radioactivity was extracted and directly counted. The positions of molecular mass standards (in kilodaltons) are shown. hRFC migrates on SDS gels as a broadly banding species centered at 80 to 85 kDa as previously reported (Matherly et al., 1991; Witt et al., 2004). DF, dye front.

hRFC expressed in R5 cells, incorporation of radioactivity from NHS-[^{3}H]MTX involved a broadly migrating hRFC protein centered at ~80 to 85 kDa (Fig. 3). Substitution of Lys411 by Ala dramatically and nearly completely abolished incorporation of radioactivity into hRFC, clearly establishing Lys411 as the primary target for covalent modification by NHS-MTX and implicating Lys411 as involved in the binding of (anti)folate substrates via ionic associations with the α- and/or γ-carboxyl groups of the terminal glutamate.

Effects of Conservative and Nonconservative Replacements of Lys411 in TMD11 of hRFC. The results in Fig. 3 clearly identify Lys411 as the primary modification site for NHS-[^{3}H]MTX and, by inference, as important for substrate carboxyl group binding to hRFC. It is interesting that both conservative and nonconservative substitutions at Lys411, including Ala, Glu, Leu, Met, Gln, and Arg, were well tolerated when mutant hRFC constructs were transiently expressed in hRFC-null R5 HeLa cells, along with wt hRFC, and assayed for MTX transport (Fig. 4). Indeed, when transport results were normalized to hRFC protein on Western blots (lower panel), only K411E showed a substantial decrease in drug uptake (~50% of wt). Similar results were reported elsewhere for hRFCs with a smaller number of Lys411 mutants (Witt and Matherly, 2002; Hou et al., 2005).

Further Characterization of the Role of Lys411 in Substrate Binding with Diamino Furo[2,3-*d*]Pyrimidine Antifolates with Substituted Carboxyl Groups. Gangjee et al. (2002) previously reported the synthesis and biological activities of a dihydrofolate reductase inhibitor and RFC substrate, *N*- [4-[1-ethyl-2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]-benzoyl]L-glutamic acid (designated 1; Fig. 1), as a growth inhibitor of CCRF-CEM leukemia cells in culture. To further characterize the relative importance of the α- and γ-carboxyl groups of (anti)folate substrates for binding with Lys411 of hRFC, we synthesized a series of furo[2,3-*d*]pyrimidine analogs with methyl- or hydrogen-substituted α- (2, 3) or γ- (4, 5) carboxyl groups and with substitutions of both α- and γ-carboxyl groups (6–9) (Fig. 1). We initially used these analogs as reversible inhibitors (at 10 μM) of radioactive MTX (1 μM) uptake with hRFC-null K562 (K500E) cells stably transfected with wt and K411A hRFC. Kinetic constants (V_{max} and K_t) for MTX with wt and K411A hRFC are summarized in Table 1, and a Western blot of wt and K411A hRFC proteins in the transfected cells is shown in Supplemental Fig. 1S.

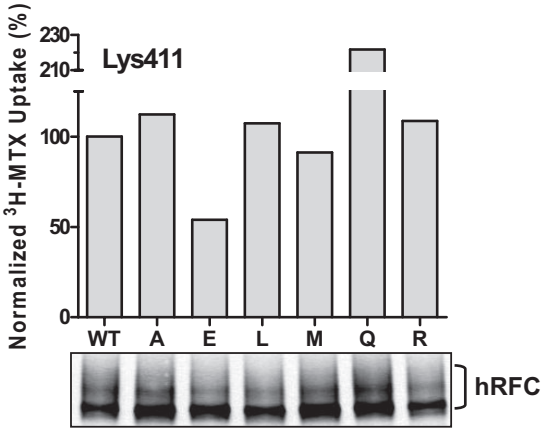


Fig. 4. Site-directed mutagenesis of Lys411 and expression in R5 HeLa cells. Transport activity and Western blot data are shown for position 411 substitutions, as described in the text. Lower panel, results for a Western blot of proteins (2.5 μg) solubilized from membrane preparations from R5 HeLa cells transiently transfected with wt and Lys411 mutant hRFC constructs. hRFC proteins were detected with HA-specific mouse antibody and secondary IRDye 800-conjugated antibody. Detection and densitometry of the blots were performed with the Odyssey Imaging System. Upper panel, uptake data for [^{3}H]MTX (0.5 μM) over 2 min at 37°C, normalized to hRFC protein levels from the Westerns. Results presented are representative of three experiments. Single-letter abbreviations for the amino acid replacements at Lys411 are used. WT, wild type.

In physiologic HBSS buffer, the γ-substituted 4 and 5 analogs showed potent inhibitions of MTX transport for both wt and K411A hRFC (Fig. 5, A and B), whereas the parental drug, 1, and α-substituted 2 and 3 analogs were weaker inhibitors. hRFC transport activity was stimulated (up to 5-fold) in the absence of competing anions (i.e., in anion-free HSM buffer) (data not shown), presumably reflecting decreased competition for anionic substrate binding by anions (e.g., Cl^-) in HSM buffer. Consistent with this, the inhibitory effects on [^{3}H]MTX uptake by all of the anionic antifolates with one or two carboxyl groups (compounds 1–5) were enhanced in HSM buffer (Fig. 5, C and D). Although the increase was greatest for 1, compounds 4 and 5 remained the most potent inhibitors. In contrast, analogs with neither α- nor γ-carboxyl groups (6–9) were exceedingly poor inhibitors of MTX uptake in both HBSS and HSM buffers. K_i values for the antifolates 4 and 5 with wt and K411A hRFCs (in physiologic buffer) are summarized in Table 1. Results are consistent with higher affinity binding for antifolates 4 and 5 than for pemetrexed and raltitrexed and similar binding affinities to those for the classic hRFC high-affinity substrates PT523 and ZD9331.

These results indicate that although both substrate α- and γ-carboxyl groups participate in substrate binding to hRFC, it is the binding of the α-carboxyl group that predominates and is indeed essential for high-affinity binding. From the results with the 1 versus 4/5 antifolates in HBSS, it appears that the γ-carboxyl can negatively affect α-carboxyl group binding to the carrier. Finally, the presence of a cationic amino acid at position 411 is clearly not necessary for reversible binding of the furo[2,3-*d*]pyrimidine antifolates 1 to 5 to hRFC.

Affinity Inhibition of wt and K411A hRFC by NHS Esters of Diamino Furo[2,3-*d*]Pyrimidine Antifolates with Substituted Carboxyl Groups. To further explore the associations between the α- and γ-carboxyl groups of (anti)folate substrates and Lys411, compound 1 and its α- and γ-methyl-substituted congeners, 2, 4, and 6, were treated

TABLE 1

K_i values for furo[2,3-*d*]pyrimidine antifolates 4 and 5 and classical antifolates with wt and K411A hRFCs

Kinetic constants were determined from Lineweaver-Burk and Dixon plots for the wt and K411A hRFC transfectants using [^{3}H]MTX. The data shown are the mean values from three experiments ± S.E.M.

Parameter	Antifolate	Wild-Type hRFC	K411A hRFC
		μM	
K_t	MTX	1.23 ± 0.23	1.58 ± 0.17
V_{max}	MTX	4.76 ± 0.87[a]	2.76 ± 0.32[a]
K_i	4	1.5 ± 0.2	3.2 ± 0.2
K_i	5	2.5 ± 0.2	4.4 ± 0.6
K_i	Pemetrexed	9.9 ± 0.7	15.1 ± 1.5
K_i	Tomudex	5.3 ± 0.4	11.2 ± 0.2
K_i	ZD9331	2.9 ± 0.2	5.4 ± 0.5
K_i	PT523	2.4 ± 0.2	5.0 ± 0.6

[a] Picomoles per minute per milligram of protein.

with NHS, using methods identical to those for preparing NHS-MTX. The activated NHS-antifolate esters (5 μM) were added to the K500E transfectants expressing wt and K411A hRFC (in HSM buffer) to determine effects on MTX transport activity resulting from covalent modification of the carrier, analogous to the experiment in Fig. 2 with NHS-MTX and transfected R5 cells. In striking contrast to the results for reversible inhibition of transport by the unmodified furo[2,3-*d*]pyrimidine antifolates (Fig. 5), for the NHS-activated analogs, the order of inhibition with wt hRFC was 2 > 1 > 4 with potencies ranging from 70% inhibition down to 30% inhibition (Fig. 6). Not surprisingly, there was no inhibition by analog 6, which has no carboxyl groups. For K411A hRFC, affinity inhibition of MTX transport by 1 to 6 was largely abolished.

Thus, for NHS antifolate activation and covalent modification of the carrier, the γ-carboxyl is clearly preferred, in contrast to the results for reversible binding in which the furo[2,3-*d*]pyrimidine antifolates bearing ionizable α-carboxyl groups were more potent inhibitors than those with γ-carboxyl groups alone or with both α- and γ-carboxyl groups. Because affinity labeling was nearly completely abolished for K411A for both NHS-MTX (see above) and the NHS-esters of the furo[2,3-*d*]pyrimidine antifolates, the γ-carboxyl groups of transport substrates must associate with Lys411 in TMD11 of hRFC.

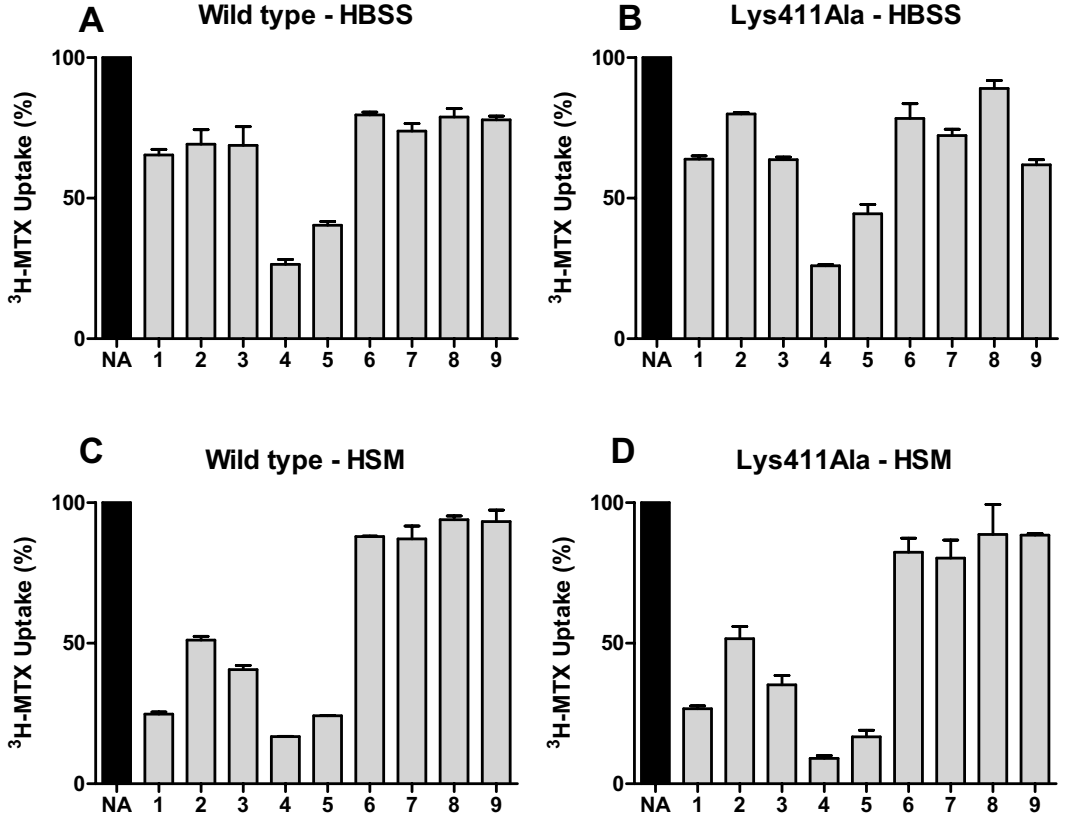


Fig. 5. Inhibition of MTX transport in K500E transfectants expressing wt and K411A hRFCs with diamino furo[2,3-*d*]-pyrimidine antifolates 1 to 9. hRFC-null K500E cells were stably transfected with wt hRFC and K411A hRFC, as described under *Materials and Methods*. Clones were isolated, expanded, and assayed for hRFC levels on Western blots (Supplemental Fig. 1S), and kinetic constants were determined for MTX (Table 1). In the experiment shown, wt and K411A were assayed for [^{3}H]MTX uptake (1 μM) in the presence of the α- and γ-carboxyl-substituted diamino furo[2,3-*d*]pyrimidine antifolates 1 to 9 (10 μM), as described in the text. The structures for compounds 1 to 9 are shown in Fig. 1. The experiments shown in A and B used HBSS for transport, whereas the experiments shown in C and D used anion-free HSM buffer. Results are shown as mean values ± S.E.M. for three experiments. NA, no additions.

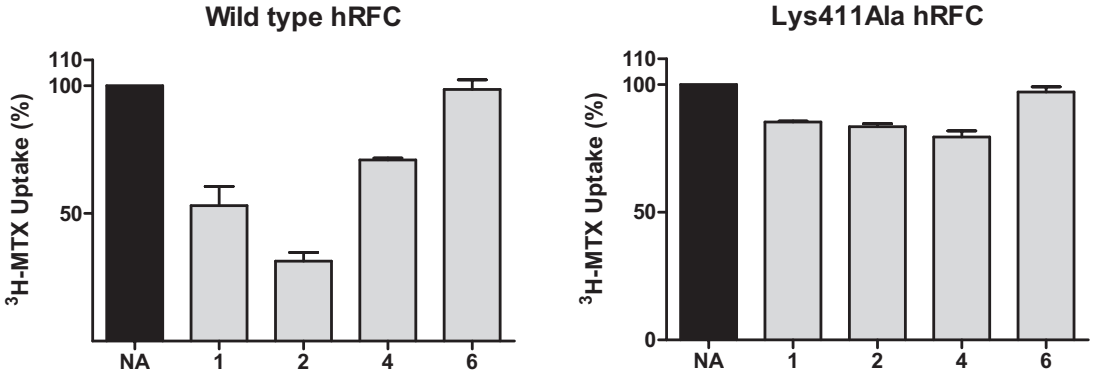


Fig. 6. Inhibition of MTX transport in stable K500E-transfected cells expressing wt and K411A hRFC with activated NHS esters of antifolates 1, 2, and 4. Analogs 1, 2, and 4 with α- and/or γ-carboxyl groups (structures are shown in Fig. 1) and 6 with both α- and/or γ-carboxyl groups substituted with methyls were treated with NHS, using methods identical to those for preparing NHS-MTX. The activated NHS-antifolate esters (5 μM final concentrations) were added to the K500E transfectants expressing wt and K411A hRFC for 5 min; the cells were washed and assayed for the effects on [^{3}H]MTX (1 μM) uptake over 180 s. Data are expressed as relative [^{3}H]MTX uptakes (in picomoles per milligram of protein; mean values ± S.E.M.; n = 3) after treatment with the NHS esters of the furo[2,3-*d*]pyrimidine antifolates, and are compared with the level in untreated cells. NA, no additions.

Discussion

Folates are composed of distinct structural motifs including pteridine, *p*-aminobenzoate, and glutamate. The glutamate moiety is of particular importance in that its α- and γ-carboxyl groups are ionized at physiologic pH, thus limiting diffusion of folates and classic antifolates across biological membranes. Because RFC is a transporter of organic anions, in this study, we focused on the mechanistic role of the substrate carboxyl groups in transport by hRFC. Analysis of membrane topology models and sequence homologies for RFCs from assorted species identified the highly conserved cationic residues, Arg373 in TMD10 and Lys411 in TMD11, as possibly functionally important (Matherly et al., 2007). By site-directed mutagenesis, both these residues were previously implicated as important to RFC transport (Sharina et al., 2001; Sadlish et al., 2002; Witt and Matherly, 2002) and as likely candidates to participate in binding associations with ionized α- and γ-carboxyl groups in folate substrates.

This article further focuses on Lys411 and provides important new insights into the relationship between antifolate α- and γ-carboxyl groups and this residue in TMD11, identified as an important substrate binding domain and component of the transmembrane translocation pathway in hRFC for anionic folate and antifolate substrates (Hou et al., 2005). As previously implied (Hou et al., 2006; Matherly et al., 2007), the present results establish that Lys411 lies in the proximity of the aqueous substrate binding pocket in hRFC, where it is subject to electrophilic attack by NHS-activated MTX ester and can participate in an interaction with (anti)folate substrate, primarily through an ionic association with the γ-carboxyl group. Remarkably, this interaction is apparently not essential for transport function because the γ-carboxyl group is not only expendable, but indeed its replacement by an uncharged hydrogen or a methyl group in a series of furo[2,3-*d*]pyrimidine antifolates actually enhances high-affinity reversible binding of substrate to the carrier, as long as an ionizable α-carboxyl group is intact. Furthermore, Lys411 can be replaced by any of a number of amino acids of varying bulk and charge with relatively nominal effects on overall transport activity. From the apparently critical role of a cationic amino acid at position 373 (Sharina et al., 2001; Sadlish et al., 2002; Hou et al., 2006), we suggest that substrate binding involves an ionic association between the α-carboxyl group of (anti)folate substrates and Arg373 in TMD10 of hRFC. Because substrate binding is partially preserved for antifolate analogs 2 and 3 with blocked α- and ionizable γ-carboxylates, we propose that in the absence of the α-carboxylate, the γ-carboxylate can adopt a folded conformation so as to mimic the α-carboxylate.

Our studies are the first to systematically examine the structure-activity relationships for the α- and γ-carboxyl groups of hRFC substrates. They are consistent with previous findings that replacement of glutamate by valine in ICI-198583 was well tolerated (Westerhof et al., 1995) and that ZD9331 and PT523, both of which have substitutions for the γ-carboxylate, are excellent substrates for hRFC (Jansen, 1999). However, comparisons with ZD9331 and PT523 are inexact in that the anionic character of the γ-carboxylate is at least partly preserved for these drugs because the benzoic acid in PT523 has the equivalent of a γ-carboxylate, and the tetrazole in ZD9331 is an isosteric anionic replacement for the γ-carboxylate.

Future studies will continue to focus on identification of functionally important amino acids in hRFC and key substrate-specific determinants of binding and translocation as important steps to understanding the mechanism of folate transport. Indeed, molecular insights from RFC structure-function studies should foster the design of new antifolate inhibitors that rely on RFC for cellular entry, or with substantially enhanced transport by other folate transporters over RFC, and the development of strategies for biochemically modulating the carrier that could be therapeutically exploited in the context of nutritional interventions or antifolate chemotherapy.

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