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Biochimica et Biophysica Acta 1567 (2002) 56–62

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Identification of lysine-411 in the human reduced folate carrier as an important determinant of substrate selectivity and carrier function by systematic site-directed mutagenesis

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Received 14 May 2002; received in revised form 26 August 2002; accepted 29 August 2002

Abstract

Site-directed mutagenesis was used to characterize the functional role of lysine-411, a conserved amino acid located in putative transmembrane domain (TMD) 11 of the human reduced folate carrier (hRFC). Lysine-411 was mutagenized to arginine, glutamate, and leucine, and the mutant constructs (K411R–, K411E–, and K411L–hRFC, respectively) were transfected into hRFC-deficient K562 cells. The mutant hRFC constructs were all expressed at high levels and restored 22–36% of the methotrexate (MTX) transport level in wild-type (K43-6) hRFC transfectants. Although 5-formyl tetrahydrofolate (5-CHO-H_4PteGlu) uptake levels for both the K411E– and K411L–hRFCs were also impaired (~ 33% and 28%, respectively), a complete restoration of the wild-type level was observed for K411R–hRFC. While loss of MTX transport activity for the K411R–hRFC transfectant was associated with an incomplete restoration of MTX sensitivity compared to K43-6 cells, these cells were similarly sensitive to Tomudex. The K411R–hRFC transfectants showed an approximately threefold decreased growth requirement for 5-CHO-H_4PteGlu compared to K43-6 cells. The 5-CHO-H_4PteGlu transport stimulation observed for the wild-type carrier in chloride-free buffer was also observed for K411R–hRFC, however, this response was decreased for the K411E– and K411L–hRFCs. The preservation of low levels of transport for the K411E– and K411L–hRFCs suggest that the amino acid at position 411 does not directly participate in the binding of anionic hRFC substrates. However, a functionally important role for a basic amino acid at position 411 was, nonetheless, implied by the increased MTX transport for wild-type hRFC over the K411 mutant hRFCs, and the highly selective uptake of 5-CHO-H_4PteGlu over MTX for K411R–hRFC.

Keywords: Reduced folate carrier; Methotrexate; Site-directed mutagenesis; Antifolate; Reduced folate

1. Introduction

Membrane transport of methotrexate (MTX) and related agents into tumor cells is key to chemotherapeutic effectiveness since the level of drug achieved within cells is an important determinant of the extent of binding to intracellular enzyme targets [1]. Membrane transport is also critical to the metabolism of antifolates to polyglutamyl forms required for drug retention and, for certain analogs (Lometrexol, Tomudex), high affinity binding to intracellular enzymes [1–3]. Thus, it is not surprising that impaired membrane transport is a common mode of antifolate drug resistance [1–3].

Decreased drug uptake generally involves alterations in the reduced folate carrier (RFC). For MTX, impaired RFC function has been reported in cultured murine and human tumor cells [4–12], and in murine tumor cells derived in vivo during MTX chemotherapy [13]. In addition, a wide range of RFC expression was described in lymphoblasts from patients with acute lymphoblastic leukemia (ALL) [14,15] and low levels of RFC were detected at relapse [14], suggesting an important role for RFC in clinical resistance. For osteosarcomas, decreased RFC expression was associated with a poor response to chemotherapy including MTX [16].

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0005-2736/02/$ - see front matter
PII: S0005-2736(02)00583-7

The characterization of the molecular alterations in RFC accompanying resistance has begun to shed light on particular amino acid residues or domains that are essential for transport substrate binding and/or membrane translocation. Thus, conserved amino acids located in the human RFC (hRFC) transmembrane domain (TMD) 1 (glycine-44 [10], glutamate-45 [12], serine-46 [11] and isoleucine-48 [17]), TMD 4 (serine-127 [10] and alanine-132 [18]), and TMD 8 (serine-313 [6]) have all been implicated as important to carrier function based on their capacities to confer resistance to antifolates. From the predicted membrane topology and homology comparisons between hRFC and the rodent carriers, potentially important charged amino acids in the TMDs have been identified. For instance, our recent studies suggested important structural roles for aspartate-88 (located in TMD 2) and arginine-133 (in TMD 4) in the maintenance of optimal hRFC function, via their participation in a charge-pair association [19]. By scanning leucine mutagenesis of the murine RFC (mRFC), important roles for arginine-131 (homologous to arginine-133 in hRFC) and arginine-366 (hRFC arginine-373) were likewise suggested [20].

Interestingly, replacement of lysine-404 (lysine-411 in hRFC) by leucine in mRFC resulted no appreciable change in MTX transport or kinetics [20]. However, K404L−mRFC exhibited a unique transport phenotype, including strikingly *decreased* binding affinities and transport for reduced folates [e.g., 5-formyl tetrahydrofolate (5-CHO-H_4PteGlu)] and a loss of the well documented inhibitory effect of chloride ion on RFC function [20]. In this report, we extend these results to hRFC and the homologous lysine-411. In contrast to the results with the murine carrier, we found that replacement of lysine-411 with leucine resulted in a significant and essentially comparable loss of transport activity with both MTX and 5-CHO-H_4PteGlu. While similar results were obtained upon substitution of glutamate for lysine-411 in hRFC, replacement with arginine restored a unique pattern of transport activity that was remarkably selective for 5-CHO-H_4PteGlu over MTX.

2. Materials and methods

2.1. Reagents

[3′,5′,7-^{3}H] (20 Ci/mmol) and [3′,5′,7,9-^{3}H] (17 Ci/mmol) (6*S*) 5-CHO-H_4PteGlu were purchased from Moravek Biochemicals. Unlabeled MTX and (6*R*,*S*) 5-CHO-H_4PteGlu (leucovorin) were provided by the Drug Development Branch, National Cancer Institute, Bethesda, MD. Both labeled and unlabeled MTX were purified by HPLC prior to use [21]. Tomudex was provided by AstraZeneca Pharmaceuticals. Restriction and modifying enzymes were obtained from Promega (Madison, WI) or Roche (Indianapolis, IN). Synthetic oligonucleotides were obtained from Invitrogen. Tissue culture reagents and supplies were purchased from assorted vendors with the exception of dialyzed fetal bovine serum (Life Technologies) and iron-supplemented calf serum (Hyclone Laboratories, Inc.).

2.2. Cell culture

The transport-deficient K500E subline was selected from wild-type K562 cells, as previously described [9]. Cells were maintained in RPMI 1640 medium, 10% iron-supplemented calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere at 37 °C in the presence of 5% CO_2. Cells were routinely grown in media containing 0.5 μM MTX; however, prior to transport assays (see below), MTX was removed from the medium. The K43-6 subline was derived from K500E cells by transfection with full-length wild-type hRFC cDNA in pCDNA3 [9]. K43-6 cells were grown in the presence of 1 mg/ml G418.

For cytotoxicity determinations, cells were cultured in 24-well culture dishes at ~ 50,000 cells/ml in 2 ml of complete RPMI 1640 containing 10% dialyzed fetal bovine serum. Cells were counted with a hemacytometer after 96 h. The IC_{50} values were calculated as the concentrations of MTX necessary to inhibit growth by 50% compared to control cells grown under identical conditions except that the growth inhibitor was omitted. For assays of leucovorin growth requirements, cells were first cultured in complete folate-free RPMI 1640 media including dialyzed serum, plus 60 μM adenosine and 10 μM thymidine, for 2 weeks. The cells were then cultured in 24-well dishes in folate-free medium without nucleosides, supplemented with a range of nanomolar concentrations of leucovorin. Cell growth was measured as for the cytotoxicity assays and the concentrations of leucovorin capable of supporting 50% maximal growth were calculated.

2.3. Mutagenesis and stable transfections

Mutant hRFC constructs were prepared by PCR as previously described [19], using the complementary mutagenesis primers in Table 1. For all the K411 mutants, two separate amplicons were initially amplified, using the forward mutation primer and RFCout12 (5′gacaacccctttcccctgcactctgt3′; positions 1647 to 1619), and KS2 (5′cgcagcctcttcttcaaccgc3′; positions 622 to 642) with the reverse mutation primer. PCR conditions for the primary PCRs were 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 60 s. Secondary PCRs were performed with the KS2/RFCout12 primers and primary PCR products as templates, using the same conditions as for the primary PCRs. The secondary PCR products were digested with *Not*I and *Sfi*I, purified, and ligated into *Not*I- and *Sfi*I-digested hRFC cDNA in pCDNA3 (designated pC43) for transformation. All mutant hRFC constructs were confirmed by automated sequencing.

The mutant hRFC cDNAs were transfected into transport-impaired K500E cells with lipofectin [9]. Cells were

Table 1
PCR primers for preparing hRFC mutant constructs

Mutations	Forward mutation primer	Reverse mutation primer
K411R	5′ C TTTGCCACCATCGTCAGGAC CATCATCACT TTC 3′	5′ GAAAGTGATGATGGTCCTGACGATGGTGGCAAAG 3′
K411L	5′ C TTTGCCACCATCGTCCTGAC CATCATCACT TTC 3′	5′ GAAAGTGATGATGGTCAGGACGATGGTGGCAAAG 3′
K411E	5′ C TTTGCCACCATCGTCGAGAC CATCATCACT TTC 3′	5′ GAAAGTGATGATGGTCTCGACGATGGTGGCAAAG 3′

cloned in 0.35% Noble agar in complete medium and clones resistant to G418 (1 mg/ml) were isolated and expanded for further analysis [9].

2.4. Western blot analysis of mutant hRFC-transfectants

Plasma membranes were prepared by differential centrifugation [22]. Membrane proteins were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS [23], and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Pierce) [24]. The blots were developed with protein A-purified hRFC-specific antibody [10] and enhanced chemiluminescence (Roche), and exposed to X-ray film with multiple exposures. Densitometry was performed on a Kodak Digital Science Image Station 440CF.

2.5. Transport assay of mutant hRFC constructs

Initial rates of [^{3}H]-MTX (0.5 μM) or [^{3}H]-(6*S*)5-CHO-H_4PteGlu (1 μM) uptake were measured over 180 s as previously described [9,10,19,22]. Levels of intracellular radioactivity were expressed as pmol/mg protein, calculated from direct measurements of radioactivity and protein contents of the cell homogenates. Protein assays were by the method of Lowry et al. [25]. Kinetic constants (K_t, V_{max}) were calculated from Lineweaver–Burk plots for ^{3}H-MTX and ^{3}H-5-CHO-H_4PteGlu, and K_i values for Tomudex were determined from Dixon plots, with ^{3}H-MTX as the transport substrate. For the studies designed to assess the inhibitory effects of chloride on ^{3}H-5-CHO-H_4PteGlu uptake, transport assays were performed at 0.5 μM substrate in Hepes-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 5 mM glucose, pH 7.4) and an anion-free Hepes-sucrose buffer (20 mM Hepes, 235 mM sucrose, pH adjusted to 7.4 with MgO).

3. Results and discussion

Analysis of membrane topology models and sequence homologies between the rodent and human RFCs permits the identification of functionally important amino acids. Of particular interest are the conserved basic amino acids located in the predicted TMDs of hRFC, including argi-

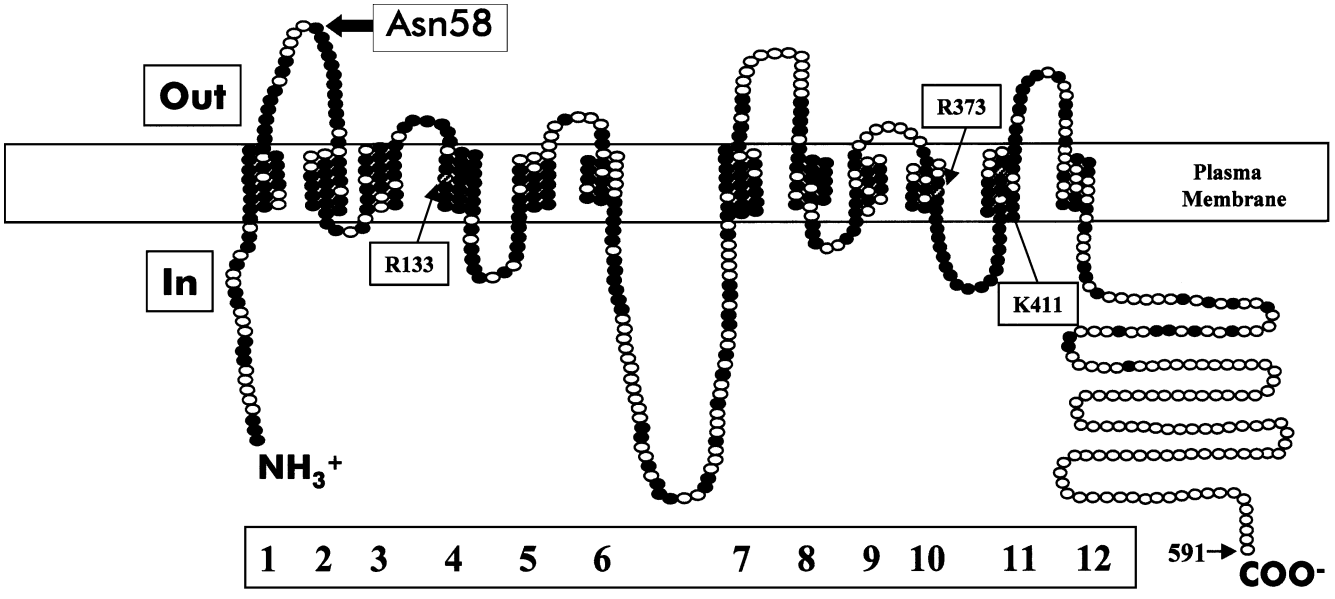

Fig. 1. Topology structure of the human reduced folate carrier. A topology model for hRFC (“TMPRED” [26]) is shown depicting 12 putative TMDs, internally oriented amino and carboxyl termini, and an externally oriented N-glycosylation site at asparagine 58. Conserved residues between the rodent and human RFCs are shown in black. The locations of the conserved arginines 133 and 373, and lysine-411 (as striped circles) are also noted. The numbers (1–12) in the lower bar designate the number of putative TMDs. The predicted membrane topology of TMDs 1–8 and the carboxyl terminus of hRFC has been experimentally verified [27,28].

nine-133 and -373, and lysine-411 (Fig. 1). Transport activity for mRFC with MTX and reduced folates was abolished upon replacement of the homologous arginine-131 and arginine-363 residues with leucines [20]. Likewise, for lysine-404 in mRFC, corresponding to lysine-411 in hRFC, leucine substitution resulted in a loss in binding affinity and transport of reduced folates. However, transport of MTX by the K404L–mRFC carrier was essentially unchanged from wild-type levels [20].

3.1. Transport properties of mutant K411–hRFCs

The novelty of the K404L mRFC phenotype led us to further explore the possible role of this highly conserved cationic amino acid in the maintenance of hRFC structure and transport function. Accordingly, we systematically mutagenized lysine-411 in hRFC to arginine, glutamate, and leucine, and transfected the mutant hRFC constructs (designated K411R–, K411E–, and K411L–hRFC, respectively) into MTX resistant K562 (i.e., K500E) cells, characterized by a 7.7-fold increased dihydrofolate reductase content and nearly undetectable levels of hRFC transcripts and protein [9]. G418-resistant clones were isolated, expanded, and plasma membrane proteins prepared, for analyses of hRFC expression on Western blots with hRFC-specific antibody [10]. A Western blot for representative clonal isolates expressing the K411–hRFC mutant proteins is shown in Fig. 2A, for comparison with K43-6 cells, expressing high levels of wild-type hRFC (~ 20-fold over the levels in K562 parent cells; 9). Only slight differences in levels of carrier expression between wild-type and mutant hRFC transfectants were observed, suggesting that amino

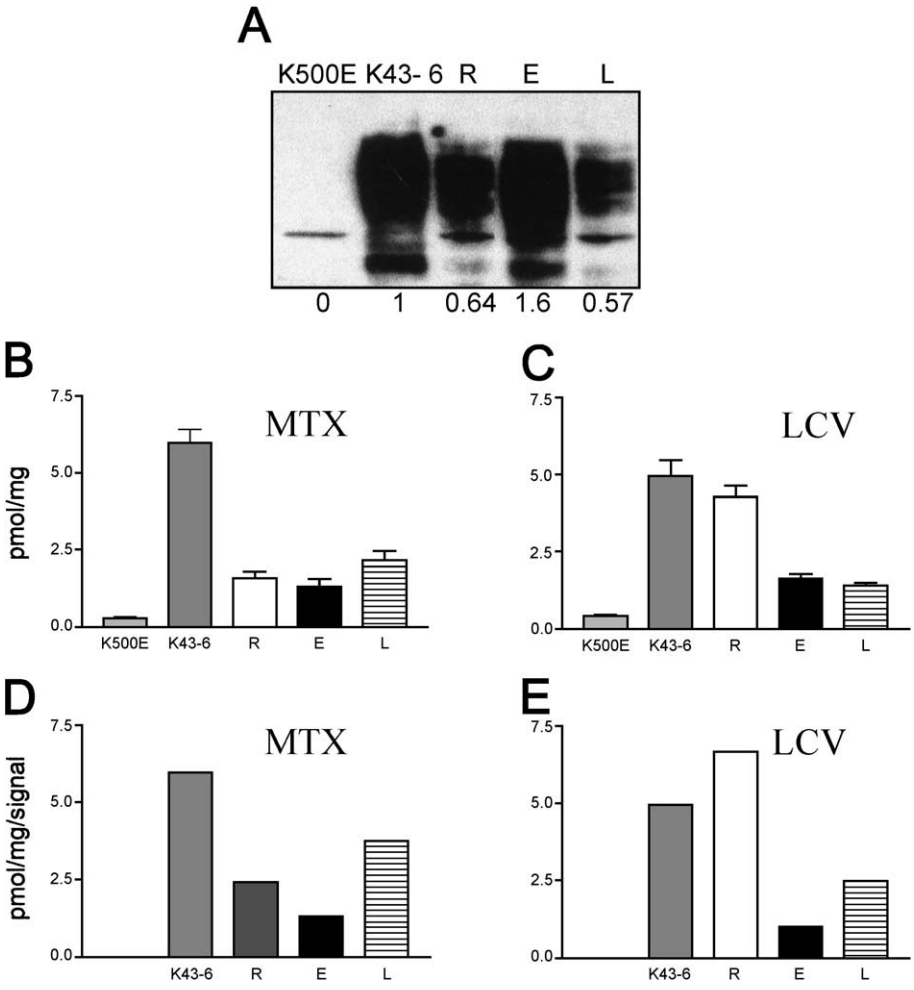

Fig. 2. Immunoblot analysis and transport activity of wild type and mutant hRFCs expressed in K500E cells. *Panel A*: Data are shown for a western blot of plasma membrane proteins from hRFC-null K500E cells and transfectants expressing wild type hRFC (K43-6) and mutant hRFCs including K411R–, K411E–, and K411L–hRFC (the latter are labeled R, E, and L, respectively). Proteins (20 μg) were fractionated on a 7.5% gel in the presence of SDS and electroblotted onto a PVDF membrane. Detection was with anti-hRFC antibody and an enhanced chemiluminescence kit (Roche). Molecular weight standards (in kDa) are shown. The relative levels of hRFC protein by densitometry are noted. *Panels B–E*: Data are shown for the relative uptakes of ^{3}H-MTX and ^{3}H-5-CHO-H_4PteGlu (abbreviated LCV) over 180 s at 37 °C. In panels B and C, are shown the absolute uptake rates in pmol/mg cell protein (mean values from 3–4 experiments ± S.E.). In panels D and E, are shown the normalized transport data, using the relative hRFC expression levels from the western blot.

acid substitutions at position 411 have no major adverse effects on hRFC expression or membrane targeting.

Transfectants expressing the mutant and wild-type hRFCs were assayed for cellular uptake with ^{3}H-MTX and ^{3}H-5-CHO-H_4PteGlu (Fig. 2B–E). All K411 substitutions restored only low levels of ^{3}H-MTX transport, ranging from ~ 22% of wild type for the K411E–hRFC transfectant to 36% for the K411L–hRFC transfectant. These values were slightly changed (to 14% and 63%, respectively) when transport was normalized for modest differences in hRFC expression (Fig. 2D). These effects of the K411 substitutions on MTX uptake were reflected in both the MTX K_t (~ 2–3-fold increase) and V_{max} values (50–60% decrease) (Table 2).

Somewhat different results were obtained with ^{3}H-(6*S*)5-CHO-H_4PteGlu as the transport substrate. Thus, significantly impaired ^{3}H-5-CHO-H_4PteGlu uptake was observed for both the K411E– and K411L–hRFCs (~ 33% and 28%, respectively), and a *complete* restoration of wild-type ^{3}H-5-CHO-H_4PteGlu uptake was observed for K411R–hRFC (Fig. 2C). When adjusted for the slight differences in hRFC protein, these uptake values changed to 21% for K411E–hRFC and 50% for K411L–hRFC, and to 135% of wild-type levels for K411R–hRFC (panel E). Elevated ^{3}H-5-CHO-H_4PteGlu uptake for K411R–hRFC was associated with threefold decreased K_t and V_{max} values, compared to K43-6 cells (Table 2). For the K411E– and K411L–hRFCs, K_t values were essentially unchanged from the wild-type value accompanying a three- to sixfold decreased V_{max} values.

These results suggest a somewhat modified spectrum of transport properties for K411L–hRFC compared to the homologous K404L–mRFC. Whereas the K404L–mRFC selectively retained high level MTX transport over 5-CHO-H_4PteGlu, for the corresponding hRFC mutant, a significant and similar loss of transport occurred with both substrates. Although the charge properties at position 411 appear to have only a slight effect on carrier function for MTX, for 5-CHO-H_4PteGlu, transport was clearly fostered by the presence of a positively charged residue at this position. The dramatic stimulation of 5-CHO-H_4PteGlu transport over MTX by K411R–hRFC in large part reflects ~ 3-fold increased binding affinity for the reduced folate and ~ 3-fold decreased binding affinity for the antifolate substrate compared to the wild-type carrier.

3.2. K411R–hRFC confers a selective pattern of antifolate resistance and decreased growth requirements for leucovorin

The loss of MTX transport activity in cells expressing K411R–hRFC was associated with an incomplete restoration of MTX sensitivity compared to the K43-6 cells (IC_{50}'s of 90 and 38 nM, respectively, versus 880 nM for K500E cells; Fig. 3A). Interestingly, the transfectant expressing K411R–hRFC showed an increased affinity for the antifolate Tomudex ($K_i = 0.940 \pm 21$ μM) over K43-6 cells ($K_i = 7.03 \pm 2.14$ μM). This was reflected in the complete restoration of high level Tomudex sensitivity for the K411R–hRFC transfectant in outgrowth assays (IC_{50}'s of 4.2 versus 6.1 nM for K43-6 cells and 31 nM for K500E cells) (Fig. 3B). The decreased K_t of K411R–hRFC for 5-CHO-H_4PteGlu (Table 2) was accompanied by a decreased growth requirement for leucovorin [(6*R*,S)5-CHO-H_4PteGlu] for the K411R–hRFC transfectant (~ 3-fold lower than that for K43-6 cells and 19-fold lower than for K500E cells; Fig. 3C).

3.3. Chloride dependence for the mutant K411–hRFC transporter

A characteristic feature of RFC involves an inhibition of transport by chloride ions and marked stimulation in anion-free buffers [20,29,30]. For K404L–mRFC, only 10% of the maximum level of stimulation in wild-type cells was found when transport assays were performed in Hepes–sucrose anion-free buffer [20]. For wild-type hRFC in K43-6 cells, uptake of ^{3}H-5-CHO-H_4PteGlu was, likewise, stimulated (3.1-fold) in anion-free Hepes–sucrose buffer over Hepes-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 5 mM glucose, pH 7.4). For

Table 2
Kinetic constants for MTX and CHO-H_4PteGlu transport by hRFC wild-type and mutant K411–hRFCs

Transfectant	Substrate	K_t (μM)	V_{max} (pmol/mg/3 min)	V_{max}/K_t	Normalized V_{max}	Normalized V_{max}/K_t
K43-6	MTX	1.80 ± 0.50	33.59 ± 6.01	18.66	33.59	18.60
K411R–hRFC	MTX	5.02 ± 0.79	21.77 ± 2.29	4.34	34.02	6.78
K411E–hRFC	MTX	5.80 ± 0.86	14.39 ± 2.9	2.48	8.99	1.55
K11L–hRFC	MTX	3.42 ± 1.03	16.12 ± 4.10	4.71	28.28	8.26
K43-6	5-CHO-H_4PteGlu	2.28 ± 0.51	29.51 ± 4.32	12.94	29.51	12.94
K411R–hRFC	5-CHO-H_4PteGlu	0.75 ± 0.16	11.56 ± 1.66	15.41	18.06	24.07
K411E–hRFC	5-CHO-H_4PteGlu	1.84 ± 0.48	7.29 ± 1.05	3.96	4.56	2.47
K411L–hRFC	5-CHO-H_4PteGlu	1.31 ± 0.08	4.98 ± 1.31	3.80	8.74	6.66

Kinetic constants for MTX and 5-CHO-H_4PteGlu were determined from Lineweaver Burk plots for the transfectants expressing the wild-type lysine-411 (K43-6) and the mutant K411R–, K411E– and K411L–hRFC constructs, as described in the text. The normalized V_{max} and V_{max}/K_t values are also shown, normalized for the hRFC expression level on Western blots (i.e., Fig. 2A). The data shown are mean values from three to five independent experiments with standard errors of the mean.

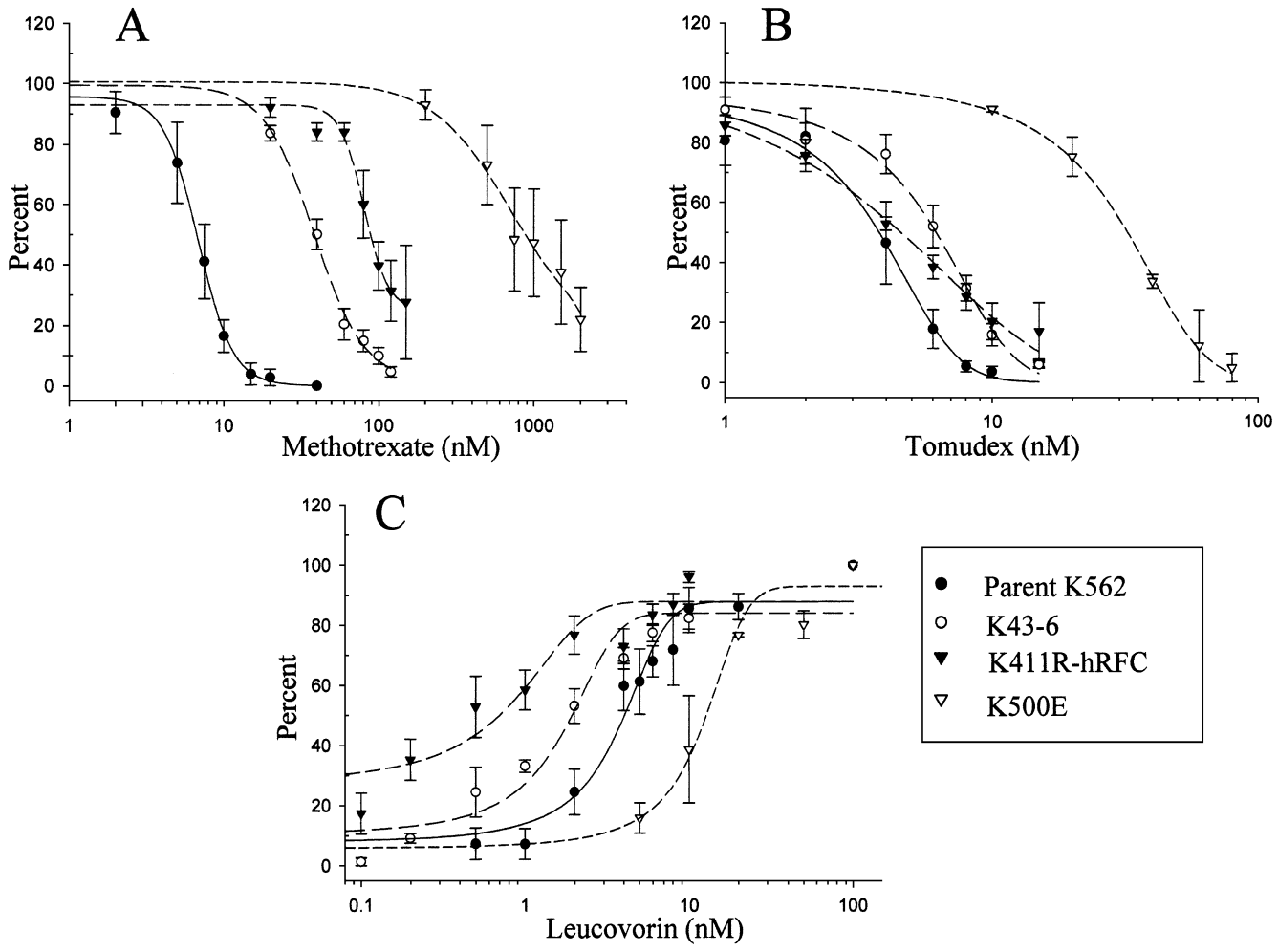

Fig. 3. Cytotoxicity of MTX and Tomudex; growth requirements for Leucovorin [(6*R*,*S*)5-CHO-H_4PteGlu). In panels A and B are shown the MTX and Tomudex cytotoxicity curves for parental K562, K43-6, and K500E cells, for comparison with the K411R–hRFC transfectants. Results are presented as the percent cell outgrowth at each drug concentration relative to cells cultured in the absence of drug. IC_{50} values for MTX were: parental K562, 6.8 nM; K43-6 cells, 38 nM; K411R–hRFC transfectant, 90 nM; K500E cells, 880 nM. For Tomudex, the IC_{50} values were: parental K562, 3.6 nM; K411R–hRFC transfectant, 4.2 nM; K43-6 cells, 6.1 nM; K500E cells, 31 nM. In panel C are shown the growth curves for the cell lines cultured in folate-free medium with dialyzed fetal bovine serum, supplemented with low nanomolar concentrations of leucovorin. Results are presented as the percent growth at various low concentrations of leucovorin relative to the level in the presence of 100 nM leucovorin. The 50% growth response values for leucovorin are as follows: parental K562, 4.2 nM; K43-6 cells, 1.9 nM; K411R–hRFC transfectant, 0.68 nM; K500E cells 13 nM.

the mutant hRFCs, the extent of stimulation was variable, and ranged from 3.2-fold for K411R–hRFC, to 2.2-fold for K411E–hRFC and 1.9-fold for K411L–hRFC. While these results suggest that the maintenance of a positive charge at position 411 is not absolutely required for chloride inhibition of transport, nonetheless, a positively charge amino acid at the position is essential to achieving a maximum inhibitory response by excess chloride ion.

4. Conclusion

The earlier finding of a significantly preferred substrate activity for MTX over reduced folates for the K404L–mRFC was not observed with the homologous K411L–hRFC construct. This implies that the structural or functional role of this residue differs between the mouse and human transporters. For hRFC, lysine-411 does not likely directly participate in the binding of anionic substrates, since low level transport was independent of charge at this position for K411L–, K411E–, and K411R–hRFCs with MTX, and for K411L– and K411E–hRFCs with 5-CHO-H_4PteGlu. However, an indirect involvement of a basic amino acid at position 411 in substrate binding was, nonetheless, implied by our finding that substitution of arginine for lysine-411 in K411R–hRFC substantially increased 5-CHO-H_4PteGlu binding while similarly decreasing the binding of MTX. Thus, MTX and 5-CHO-H_4PteGlu must bind somewhat differently to hRFC. The continued characterization of essential structural and functional features of hRFC should facilitate the design of new, more potent antifolate chemotherapeutic agents, based on their capacities

for membrane transport. If hRFC mutants with significantly increased specificities for reduced folate over antifolate substrates can be identified, these may be useful tools for gene therapy applications in combination with antifolates.

Acknowledgements

This study was supported by grant CA53535 from the National Cancer Institute, National Institutes of Health.

References

[1] I.D. Goldman, L.H. Matherly, The cellular pharmacology of methotrexate, Pharmacol. Ther. 28 (1985) 77–100.
[2] G. Jansen, Receptor- and carrier-mediated transport systems for folates and antifolates. Exploitation for folate chemotherapy and immunotherapy, in: A.L. Jackman (Ed.), Anticancer Development Guide: Antifolate Drugs in Cancer Therapy, Humana Press, Totowa, NJ, 1999, pp. 293–321.
[3] L.H. Matherly, Molecular and cellular biology of the human reduced folate carrier, in: K. Moldave (Ed.), Progress in Nucleic Acid Research and Molecular Biology, Harcourt/Academic Press, San Diego, 2001, pp. 131–161.
[4] M. Gong, J. Yess, T. Connolly, S.P. Ivy, T. Ohnuma, K.H. Cowan, J.A. Moscow, Molecular mechanism of antifolate transport deficiency in a methotrexate resistant MOLT-3 human leukemia cell line, Blood 89 (1997) 2494–2499.
[5] G. Jansen, R. Mauritz, S. Drori, H. Sprecher, I. Kathmann, M. Bunni, D.G. Priest, P. Noordhuis, J.H. Schornagel, H.M. Pinedo, G.J. Peters, Y.G. Assaraf, A structurally altered human reduced folate carrier with increased folic acid transport mediates a novel mechanism of antifolate resistance, J. Biol. Chem. 273 (1998) 30189–30198.
[6] K. Roy, B. Tolner, J.H. Chiao, F.M. Sirotnak, A single amino acid difference within the folate transporter encoded by the murine RFC-1 gene selectively alters its interaction with folate analogues. Implications for intrinsic antifolate resistance and directional orientation of the transporter within the plasma membrane of tumor cells, J. Biol. Chem. 273 (1998) 2526–2531.
[7] H. Sadlish, R.C. Murray, F.M.R. Williams, W.F. Flintoff, Mutations in the reduced folate carrier affect protein localization and stability, Biochem. J. 346 (2000) 509–516.
[8] J.D. Schuetz, L.H. Matherly, E.H. Westin, I.D. Goldman, Evidence for a functional defect in the translocation of the methotrexate transport carrier in a methotrexate resistant murine L1210 leukemia cell line, J. Biol. Chem. 263 (1988) 9840–9847.
[9] S.C. Wong, R. McQuade, S.A. Proefke, A. Bhushan, L.H. Matherly, Human K562 transfectants expressing high levels of reduced folate carrier but exhibiting low transport activity, Biochem. Pharmacol. 53 (1997) 199–206.
[10] S.C. Wong, L. Zhang, T.L. Witt, S.A. Proefke, A. Bhushan, L.H. Matherly, Impaired methotrexate transport in methotrexate resistant CCRF-CEM cells involves early translation termination and increased turnover of a mutant reduced folate carrier, J. Biol. Chem. 274 (1999) 10388–10394.
[11] R. Zhao, Y.G. Assaraf, I.D. Goldman, A reduced folate carrier mutation produces substrate-dependent alterations in carrier mobility in murine leukemia cells and methotrexate resistance with conservation of growth in 5-formyltetrahydrofolate, J. Biol. Chem. 273 (1998) 7873–7879.
[12] R. Zhao, Y.G. Assaraf, I.D. Goldman, A mutated reduced folate carrier (RFC1) with increased affinity for folic acid, decreased affinity for methotrexate, and an obligatory anion requirement for transport function, J. Biol. Chem. 273 (1998) 19065–19071.
[13] F.M. Sirotnak, D.M. Moccio, L.E. Kelleher, L.J. Goutas, Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate resistant L1210 clonal cell lines derived in vivo, Cancer Res. 41 (1981) 4442–4452.
[14] R. Gorlick, E. Goker, T. Trippett, P. Steinherz, Y. Elisseyeff, M. Mazumdar, W.F. Flintoff, J.R. Bertino, Defective transport is a common mechanism of acquired methotrexate resistance in acute lymphoblastic leukemia and is associated with decreased reduced folate carrier expression, Blood 89 (1997) 1013–1018.
[15] L. Zhang, J.W. Taub, M. Williamson, S.C. Wong, B. Hukku, J. Pullen, Y. Ravindranath, L.H. Matherly, Reduced folate carrier gene expression in childhood acute lymphoblastic leukemia: relationship to immunophenotype and ploidy, Clin. Cancer Res. 4 (1998) 2169–2177.
[16] W. Guo, J.H. Healey, P.A. Meyers, M. Ladanyi, H.G. Huvos, J.R. Bertino, R. Gorlick, Mechanisms of methotrexate resistance in osteosarcoma, Clin. Cancer Res. 5 (1999) 621–627.
[17] A. Tse, K. Brigle, S.M. Taylor, R.G. Moran, Mutations in the reduced folate carrier gene which confer dominant resistance to 5,10-dideazatetrahydrofolate, J. Biol. Chem. 273 (1992) 25953–25960.
[18] K.E. Brigle, M.J. Spinella, E.E. Sierra, I.D. Goldman, Characterization of mutation in the reduced in the reduced folate carrier in a transport defective L1210 murine leukemia cell line, J. Biol. Chem. 270 (1995) 22974–22979.
[19] X.Y. Liu, L.H. Matherly, Functional interactions between arginine 133 and aspartate 88 in the human reduced folate carrier. Evidence for a charge-pair association, Biochem. J. 358 (2001) 511–516.
[20] I.G. Sharina, R. Zhao, Y. Wang, S. Babani, I.D. Goldman, Mutational analysis of the functional role of conserved arginine and lysine residues in transmembrane domains of the murine reduced folate carrier, Mol. Pharmacol. 59 (2001) 1022–1028.
[21] D.W. Fry, J.C. Yalowich, I.D. Goldman, Rapid formation of polygamma-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high pressure liquid chromatography in the Ehrlich ascites tumor cell in vitro, J. Biol. Chem. 257 (1982) 1890–1896.
[22] L.H. Matherly, C.A. Czajkowski, S.M. Angeles, Identification of a highly glycosylated methotrexate membrane carrier in K562 human erythroleukemia cells up-regulated for tetrahydrofolate cofactor and methotrexate transport, Cancer Res. 51 (1991) 3420–3426.
[23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[24] P. Matsudaira, Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes, J. Biol. Chem. 262 (1987) 10035–10038.
[25] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[26] K. Hoffman, W. Stoffel, TMBASE—a database of membrane spanning protein segments, Biol. Chem. Hoppe-Seyler 374 (1993) 166.
[27] P.L. Ferguson, W.F. Flintoff, Topological and functional analysis of the human reduced folate carrier by hemagglutinin epitope insertion, J. Biol. Chem. 274 (1999) 16269–16278.
[28] X.Y. Liu, L.H. Matherly, Analysis of membrane topology of the human reduced folate carrier protein by hemagglutinin epitope insertion and scanning glycosylation insertion mutagenesis, Biochim. Biophys. Acta 1564 (2002) 333–342.
[29] I.D. Goldman, The characteristics of the membrane transport of amethopterin and the naturally occurring folates, Ann. N.Y. Acad. Sci. 186 (1971) 400–422.
[30] C.H. Yang, F.M. Sirotnak, M. Dembo, Interaction between anions and the reduced folate/methotrexate transport system in L1210 cell plasma membrane vesicles: directional symmetry and anion specificity for differential mobility of loaded and unloaded carrier, J. Membr. Biol. 79 (1984) 285–292.