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The Proton-Coupled Folate Transporter: Impact on Pemetrexed Transport and on Antifolates Activities Compared to the Reduced Folate Carrier

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Abstract

The reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT) are ubiquitously expressed in normal and malignant mammalian tissues and in human solid tumor cell lines. This paper addresses the extent to which PCFT contributes to transport of pemetrexed and to the activities of this and other antifolates relative to RFC at physiological pH. Either RFC or PCFT cDNA was stably transfected into a transporter-null HeLa cell variant to achieve activities similar to their endogenous function in wild-type HeLa cells. PCFT and RFC produced comparable increases in pemetrexed activity in growth medium with 5-formyltetrahydrofolate. However, PCFT had little or no effect on the activities of methotrexate, ZD1694 or PT523 in comparison to RFC irrespective of the folate growth source. PCFT, expressed at high levels in *Xenopus oocytes* and in transporter-competent HepG2 cells, exhibited a high affinity for pemetrexed with an influx K_m of 0.2 – 0.8 μM at pH 5.5 in these systems. PCFT increased the growth inhibitory activity of pemetrexed, but not that of the other antifolates in HepG2 cells grown with 5-formyltetrahydrofolate at physiological pH. These findings illustrate the unique role that PCFT plays in the transport and pharmacological activity of pemetrexed. Because of the ubiquitous expression of PCFT in human tumors, and the ability of PCFT to sustain pemetrexed activity even in the absence of RFC, tumor cells are unlikely to become resistant to pemetrexed due to impaired transport because of the redundancy of these genetically distinct routes.

Introduction

Three folate transporters account for most, if not all, folate influx activities observed in mammalian cells. (i) The reduced folate carrier (RFC – SLC19A1) is an anion exchanger with a high affinity for reduced folates ($K_m \sim 1\text{--}5 \mu M$) and a low affinity for folic acid ($K_i \sim 200 \mu M$) with optimum activity at physiological pH. RFC is ubiquitously expressed in normal mammalian tissues and tumors (Matherly and Goldman, 2003). Inactivation of RFC is embryonic lethal (Zhao et al., 2001). (ii) Folate receptors, that mediate an endocytic transport mechanism, have high affinity for folic acid ($\sim 1 \text{ nM}$) with somewhat lesser affinity ($\sim 5\text{--}10 \text{ nM}$) for reduced folates. Folate receptor- α is expressed in epithelial tissues, folate receptor- β is expressed in cells of hematopoietic origin (Kamen and Smith, 2004; Salazar and Ratnam, 2007). Inactivation of folate receptor- α is embryonic lethal while deletion of folate receptor- β is not associated with an abnormal phenotype (Piedrahita et al., 1999). (iii) This laboratory

recently identified a third transporter, the proton-coupled folate transporter (PCFT), a member of the superfamily of facilitative carriers (SLC46A1 #NM_080669) (Qiu et al., 2006) (Qiu et al., 2007;Zhao and Goldman, 2007). From a physiological perspective, PCFT is required for intestinal folate absorption and transport of folates across the choroid plexus-cerebrospinal fluid-barrier. This is based upon transport defects across these tissues in subjects with the autosomal recessive disorder hereditary folate malabsorption (OMIM 229050) who have loss-of-function mutations in the PCFT protein (Qiu et al., 2006;Zhao et al., 2007). Low-pH folate transport activity, now attributed to PCFT, has been described in many normal tissues and in a broad spectrum of human solid tumor cell lines (Zhao and Goldman, 2007). An additional function for PCFT has been proposed in folate receptor mediated transport as the route by which folates are exported from acidified endosomes into the cytoplasm during the endocytic cycle (Kamen et al., 1991;Prasad et al., 1994;Qiu et al., 2006;Wollack et al., 2008).

Impaired RFC function is an important mechanism of resistance to MTX and other antifolates *in vitro* (Zhao and Goldman, 2003) (Matherly and Goldman, 2003), and may be associated with clinical resistance to this agent in the treatment of acute lymphoblastic leukemia and osteogenic sarcoma (Matherly et al., 1995;Gorlick et al., 1997;Guo et al., 1999). The situation for pemetrexed appears to be quite different. When 5-formylTHF is the folate growth source, loss of RFC function does not result in resistance to this agent in human solid tumor cell lines. Indeed, modest collateral sensitivity has been observed under these conditions (Zhao et al., 2004b;Chattopadhyay et al., 2006). This has been attributed, in part, to an RFC-independent, pH-dependent pathway, which serves as an alternative transport route for this drug. It is likely that this alternative pathway is mediated by PCFT because of the similarities in their properties and expression patterns (Zhao et al., 2005;Qiu et al., 2006).

This paper focuses on a direct comparison of the pharmacological effects of PCFT and RFC, introduced by transfection into a HeLa cell line devoid of both carriers (Zhao et al., 2005), at levels comparable to their endogenous activities. Because pemetrexed activity is modulated by alterations in cellular folates, the impact of these transporters on transport and accumulation of natural folates is also compared. In addition, the properties of PCFT-mediated pemetrexed transport were examined in xenopus oocytes and HepG2 cells along with the impact of PCFT on the pharmacological activities of antifolates in HepG2 transfectants that express high level of PCFT on a background of constitutive PCFT and RFC function.

Materials and methods

Chemicals

[3',5',7,9-³H]folic acid, [3',5',7-³H(N)]MTX, [3',5',7, 9-³H(N)](6S)-5-formylTHF and [³H] pemetrexed were purchased from Moravak Biochemicals (Brea, CA). Folic acid was purchased from Sigma Aldrich (St. Louis, MO) and MTX was obtained from the National Cancer Institute (Bethesda, MD). Both (6R,S) and (6S)5-formylTHF were obtained from Schircks Laboratories (Jona, Switzerland). ZD1694 was obtained from AstraZeneca. Pemetrexed was obtained from the pharmacy. PT523 was a gift from Andre Rosowsky (Dana-Farber Cancer Institute, Boston, MA). Tritiated compounds were purified, and/or the purity verified, before use by HPLC as described previously (Zhao et al., 2000).

Cell culture conditions and transfection

Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 Units/mL penicillin, and 100ug/mL streptomycin or folate-free RPMI-medium containing 25 nM or 40 nM (6R,S)5-formylTHF and the same supplements. 5-formylTHF was employed as the tetrahydrofolate cofactor substrate because of its stability relative to 5-methylTHF. Growth inhibition by pemetrexed was shown to be similar whether the medium contained 25 nM (6R,S)

5-formylTHF or was supplemented daily with 80 nM (6R,S)5-methylTHF (Chattopadhyay et al., 2007). The concentration of the active isomer of (6S)5-formylTHF in the growth medium was one-half the stated racemic level. HeLa R1 cells, previously developed in this laboratory (Zhao et al., 2005) that lack PCFT and RFC expression, were subcloned in 96 well plates at a density of 1, 2, 4, or 8 cells/well in RPMI medium in the absence of MTX. After two weeks, single colonies were expanded for influx assays and cryo-preservation. The R1-11 clone, which lacked low-pH folate transport activity and was stable for more than three months, was selected as the recipient for transfections.

R1-11 cells were then transfected with either RFC or PCFT to obtain the R1-11-RFC and R1-11-PCFT transfectants as follows. The cDNA of RFC (Wong et al., 1998) or PCFT (Qiu et al., 2006) was recloned from pcDNA3.1 to pZeoSV2 (+) (Invitrogen) to generate pZeoSV2 (+)-RFC and pZeoSV2(+)-PCFT. In the latter vectors, gene transcription is driven by the SV40 promoter whereas it is driven by the CMV promoter in the former vectors. These expression vectors were individually transfected into R1-11 cells using Lipofactamine 2000 (Invitrogen) according to the manufacturer's protocol. Stable transfectants were obtained and maintained in the presence of 100 µg/mL zeocin.

Transport assay

Transport studies followed a protocol designed for rapid uptake determinations with adherent cells (Sharif and Goldman, 2000). Cells ($\sim 4 \times 10^5$) were seeded in 20mL Low Background glass scintillation vials (Research Products International Corporation, Prospect IL) and grown for three days to reach early confluence. PCFT activity was measured as follows. Cells were washed twice with HBS (20mM HEPES, 5mM dextrose, 140mM NaCl, 5mM KCl, 2mM MgCl₂, pH7.4) and incubated in the same buffer at 37°C for 20 min. The incubation buffer was then aspirated and transport was initiated by the addition of 0.5mL of pre-warmed (37 °C) MBS (20mM MES, 140mM NaCl, 5mM KCl, 2mM adjusted to pH 5.5, 6 or 7.0) or HBS (pH 7.0 – 7.4) containing 0.5 µM [³H]MTX. Uptake was carried out over 2 min at 37°C and quenched by the addition of 5mL ice-cold HBS.

RFC transport activity was determined at pH 7.4. An anion-free buffer, MHS (20mM HEPES, 235mM sucrose, pH adjusted to 7.4 with MgO) was employed to amplify RFC-mediated transport by eliminating competing inhibitory anions (Henderson and Zevely, 1983). Cells were washed three times with, and incubated in, MHS buffer at 37°C for 20 min before they were exposed to prewarmed MHS containing 0.5 µM [³H]MTX for two min. Transport was terminated by adding 5 mL of ice-cold HBS.

Cells were washed three times with ice-cold HBS and dissolved in 0.5 mL of 0.2M NaOH at 65°C for 40 min. Radioactivity in 0.4mL of lysate was measured on a liquid scintillation spectrometer and normalized to protein levels obtained with the BCA Protein Assay (Pierce, Rockford, IL).

Folic acid surface binding

Cells were seeded in 12-well plates and grown for 3 days to near confluence. Cells were washed twice with ice-cold acid buffer (10mM sodium acetate, 150mM NaCl adjusted to pH 3.5 with acetic acid) followed by an ice-cold HBS wash to eliminate nonlabeled folic acid from the medium. Cells were then incubated for 15 min with 5nM [³H]folic acid in ice-cold HBS in the presence or absence of 500nM nonlabeled folic acid. After three ice-cold HBS washes, membrane-bound [³H]folic acid was released with the addition of the acid buffer (0.5mL) and radioactivity in the supernatant measured. The cells were dissolved in 0.5 mL of 0.2 NaOH and 10µL of cell lysate was used for protein determination.

Intracellular accumulation of [³H]folic acid or [³H]5-formylTHF

Cells were trypsinized and grown for a week in folate-free RPMI 1640 medium supplemented with 10% dialyzed FBS and GAT containing 2 μ M [³H]folic acid or 25 nM [³H](6S)-5-formylTHF. Cells were replated after 3 days with fresh medium and tritiated folate. After an additional 4 days the medium was removed, the cells washed three times with ice-cold HBS, and processed as described above for the transport assay.

Folate growth requirement

Cells were adapted in folate-free RPMI 1640 medium supplemented with 10% dialyzed FBS and GAT for 1 to 3 weeks to deplete endogenous folates. Cells were then transferred to 96-well plates at a density of 1000–2000 cells/well in a folate-free medium containing 10% dialyzed FBS and a spectrum of concentrations of either folic acid or (6R,S)5-formylTHF. Cells were allowed to grow for 5 days and cellular growth was measured by the sulforhodamine B protein assay (Skehan et al., 1990).

Cell growth inhibition by antifolates

Growth inhibition was assessed in RPMI 1640 medium containing 2 μ M folic acid or folate-free RPMI 1640 medium supplemented with 25 nM or 40 nM (6R,S)5-formylTHF, both with 10% dialyzed FBS. Cells were adapted to RPMI medium supplemented with 5-formylTHF for at least a week before assay. Cells were seeded in 96-well plates at a density of 1000–2000 cells/well in a medium containing a range of pemetrexed, MTX, ZD1694 or PT523 concentrations. Growth rates were quantified after 6 days by sulforhodamine B staining.

Electrophysiological analyses

PCFT cRNA was transcribed *in vitro*, and *Xenopus laevis* oocytes were prepared, injected with water or PCFT cRNA, and pemetrexed-induced currents recorded at room temperature by a two-electrode voltage clamp technique 3–7 days post injection as previously described (Qiu et al., 2006) (Qiu et al., 2007).

SDS-PAGE and Western blotting

The membrane fraction of HepG2 cells was isolated and the overexpressed PCFT protein was immunodetected with rabbit anti-PCFT peptide, targeted to the C-terminus, and a secondary goat anti-rabbit IgG-horseradish peroxidase conjugate as previously described (Cell Signaling Technology, Danvers, MA) (Qiu et al., 2006).

Statistics

Data was analyzed using the Repeated Measures ANOVA from GraphPad Prism software (San Diego, CA).

Results

Generation of stable transfectants that express wild-type levels of PCFT or RFC transport activities

When expression of PCFT or RFC is driven by a CMV promoter in the commercial pcDNA 3.1 expression vector, the level of expression and function of these transporters is much higher than their endogenous expression in HeLa cells (Qiu et al., 2006) (Zhao et al., 2004a). To obviate this, a different vector, pZeoSV2(+), in which expression of protein was driven by an SV40 promoter, was used. R1-11 cells were transfected with PCFT or RFC expression vectors or the empty vector. An RFC stable transfectant, R1-11-RFC-6, and PCFT stable transfectant, R1-11-PCFT-4, along with a mock transfectant, R1-11-Mock, were chosen for further studies.

Wild-type HeLa cells from which the R1-11 line was derived were also used throughout the following studies.

As indicated in Panel A of Fig. 1, MTX transport activity at pH 7.4 mediated by RFC was similar in HeLa and R1-11-RFC-6 cells. Transport at pH 7.4 was much lower in R1-11-mock and R1-11-PCFT-4 cells which do not express RFC. On the other hand, MTX transport activity at pH 5.5, mediated by PCFT, was comparable in R1-11-PCFT-4 and HeLa cells (Panel B). Little low-pH transport activity was detected R1-11-mock and R1-11-RFC-6 cells which do not express PCFT. Hence the levels of MTX transport activity mediated by PCFT or RFC are comparable to their endogenous activities in wild-type HeLa cells. Folate receptor-mediated transport did not contribute to MTX uptake under these conditions since cells for these experiments were grown in folic acid-containing medium which would block transport via this route.

Folate receptor levels in these cell lines were also assessed. As indicated in Panel C of Fig. 1, folic acid surface binding is highly specific as it was completely suppressed with 500 nM non-labeled folic acid. There was no difference in folate receptor binding capacity among the transfectants ($P > 0.05$) but these levels were significantly lower than that in HeLa cells ($P < 0.05$) which was similar to the level reported previously (Zhao et al., 2004b).

Impact of expression of RFC or PCFT on cellular folate growth requirement

Cellular growth requirements for folic acid or 5-formylTHF were assessed in the transfectants along with HeLa cells. This was determined after cells had been depleted of intracellular folate pools by growth in folate-free medium supplemented with GAT and then exposed to medium containing different concentration of folates. Since cells lacked any intracellular folate pools at the time they were exposed to folates, the data obtained under these conditions may exceed actual folate growth requirement when cellular folate pools are at steady-state.

As indicated in the upper panel of Fig. 2, the folic acid concentration required to support 50% of cell growth (EC_{50} values) in R1-11-Mock cells was $\sim 2 \mu\text{M}$, the concentration in commercial RPMI 1640 medium. Expression of RFC decreased the EC_{50} by a factor of ~ 10 despite the low affinity of RFC for this folate (influx $K_i \sim 200 \mu\text{M}$). Expression of PCFT reduced the EC_{50} for this folate by a factor of ~ 20 , consistent with its higher affinity for this folate, (influx $K_m \sim 56 \mu\text{M}$ at pH 7.5 (Qiu et al., 2006)). HeLa cells that express both RFC and PCFT had a further small reduction in EC_{50} for folic acid as compared to R1-11-PCFT-4 cells.

As indicated in the lower panel of Figure 2, the EC_{50} ($\sim 3 \mu\text{M}$) for 5-formylTHF in R1-11-Mock cells was similar to what was observed for folic acid (upper panel). However, expression of either PCFT or RFC resulted in a much greater reduction of EC_{50} than observed for folic acid. Expression of PCFT decreased the EC_{50} by a factor of 300 to $0.01 \mu\text{M}$ while expression of RFC decreased the EC_{50} by a factor of 6000 to 0.5 nM . The EC_{50} for 5-formylTHF in HeLa cells was slightly lower than that in R1-11-RFC-6 cells. The difference in the EC_{50} for 5-formylTHF between R1-11-RFC-6 and R1-11-PCFT-4 cells was 20-fold as compared to only a 2-fold difference in the folic acid EC_{50} 's. Hence: (i) RFC is much more potent than PCFT in decreasing the growth requirement for 5-formylTHF, consistent with its much higher affinity for this folate at physiological pH (K_t or K_i of $\sim 2 \mu\text{M}$ and $45 \mu\text{M}$ for RFC and PCFT, respectively (Zhao et al., 2004b)). (ii) PCFT is somewhat more potent than RFC in decreasing the growth requirement for folic acid, consistent with its higher affinity for this folate at physiological pH (K_i of $200 \mu\text{M}$ and K_m $56 \mu\text{M}$ for RFC and PCFT, respectively (Qiu et al., 2006)). (iii) Both RFC and PCFT are much more potent in decreasing the growth requirement for 5-formylTHF than for folic acid. This is likely due to a higher affinity of both RFC and PCFT for 5-formylTHF than for folic acid. In the case of PCFT, the affinity for the natural

isomer of 5-formylTHF is four-fold higher than for folic acid at physiological pH (Zhao et al., 2004b).

Impact of expression of RFC and PCFT on intracellular folate pools

Intracellular folate pools were determined by growing cells in folate-free medium supplemented with 2 μ M [3 H]folic acid or 25 nM [3 H](6S)5-formylTHF for one week. The concentrations of these folates were chosen because the former represents the folic acid concentration in growth medium while the latter falls within the low-normal range of serum reduced folate levels. GAT was added to the assay medium to supplement R1-11-mock cells that do not grow in 25 nM 5-formylTHF alone (bottom panel of Figure 2). As indicated in Table I, folic acid and 5-formylTHF accumulation in R1-11-mock-1 cells was 1/14th and 1/17th that in HeLa cells, respectively, indicating that RFC and PCFT can account for the accumulation of these folates under these conditions. Expression of RFC in R1-11-Mock increased folic acid accumulation by a factor of 3 and 5-formylTHF accumulation by a factor of 12. In contrast, expression of PCFT augmented folic acid accumulation by a factor 12 and 5-formylTHF by a factor 11. Hence, PCFT is the predominant transporter leading to folic acid accumulation but is still efficient in achieving 5-formylTHF accumulation at physiological pH.

A Comparison of the Impact of RFC or PCFT on Antifolate Activities

Antifolate activities were first assessed in cells growing in medium containing 2 μ M folic acid as shown in Figure 3. As expected, R1-11-Mock cells were highly resistant to pemetrexed, MTX, ZD1694 and PT523. The effects of PCFT expression are best seen by a comparison of antifolate IC₅₀'s (the concentration that inhibits cell growth by 50%) among R1-11-PCFT-4, R1-11-RFC-6, and R1-11-mock cells. The IC₅₀ for R1-11-PCFT-4 cells was decreased by a factor of 10 as compared to R1-11-Mock for pemetrexed, but only by a factor of ~2 for MTX and ZD1694 (P=0.05 and 0.17, respectively). The PT523 IC₅₀ for R1-11-PCFT-4 cells was *increased*, (although this did not reach statistical significance, P=0.09), by a factor of 3.5 as compared to R1-11-mock-1 cells. In comparison, the IC₅₀ for R1-11-RFC-6 cells was markedly decreased for pemetrexed, MTX, ZD1694 and PT523 (63-, 200-, 1000- and 670-fold, respectively) as compared to R1-11-Mock cells. Hence, when cells were grown with folic acid as the sole folate source, expression of RFC markedly increased activity of all antifolates that require carrier-mediated transport into cells, whereas expression of PCFT only produced a substantial enhancement of pemetrexed activity.

Antifolate activities in cells adapted and grown in folate-free medium supplemented with 25 nM 5-formylTHF are illustrated in Figure 4. As indicated above, R1-11-mock cells could not grow in this medium. Hence comparisons were only possible among HeLa, R1-11-PCFT-4 and R1-11-RFC-6 cells. It can be seen that the IC₅₀ was the same for pemetrexed in the three cell lines, indicating that under these conditions RFC and PCFT are equally effective in restoring pemetrexed activity. However, IC₅₀'s for MTX, ZD1694 and PT523 were 4-, 75- and 20-fold higher, respectively, in R1-11-PCFT-4 cells than in R1-11-RFC-6 cells. In all cases, the IC₅₀'s for R1-11-RFC-6 and HeLa cells were comparable. Hence, when cells are grown with 5-formylTHF as the growth source, RFC alone can fully restore activity of all the antifolates tests. However, pemetrexed was the only antifolate for which PCFT fully restored activity.

To exclude the possibility that these observations were specific to the clones studied, the pattern of growth inhibition of pemetrexed or ZD1694 was also assessed in an additional two stable PCFT transfected clones and an additional two stable RFC transfected clones with 5-formylTHF as the folate growth source. It can be seen from Table II that while there was some variability in the extent to which MTX transport activities were increased, the same pattern of change was observed in all the clones. The IC₅₀ for PMX was not significantly different

whether mediated by PCFT or RFC (average of 58 nM vs 69 nM, respectively) while, in all cases, there was a marked (~25 fold) decrease in IC_{50} for ZD1694 in RFC vs PCFT transfected cells (average of 117 nM vs 4.7 nM, respectively).

Pemetrexed transport properties mediated by PCFT in HepG2 cells and xenopus oocytes

PCFT stable transfectants were generated in HepG2 cells which express endogenous RFC and PCFT as reported previously (Qiu et al., 2006). The expression of PCFT in this case was driven by the CMV promoter in the pCDNA 3.1 vector and resulted in a 30-fold increase in PCFT activity as compared to the mock transfectant (Qiu et al., 2006). Two independent mock transfectants and four PCFT transfectants were selected for initial studies to exclude clonal variability. As indicated in Figure 5A, pemetrexed initial uptake measured at pH 7.0 in each PCFT transfectant clone was 5–7 fold greater than uptake in Mock transfectants. The level of PCFT expression was verified by Western blot (inset). Pemetrexed IC_{50} 's in the four transfectants were uniformly decreased by a factor of ~4 as compared the values in Mock transfectants indicating that this level of PCFT expression substantially increased sensitivity to this drug (Figure 5B).

Pemetrexed transport characteristics were examined in one PCFT transfectant (clone 2) with one mock transfectant (clone 1) as a control. As indicated in Figure 5C, influx of both pemetrexed and 5-formylTHF was maximal at pH 5.5 and decreased as the pH of the transport buffer increased. Transport of both substrates was negligible in the Mock transfectant. However, unlike 5-formylTHF transport, there was significant residual pemetrexed transport in the PCFT transfectant at pH 7.0.

Pemetrexed influx kinetics was determined in the HepG2 PCFT transfectant as a function of pH. As indicated in Table II, there was a ~2.6-fold increase in pemetrexed influx K_m , from pH 5.5–6.5 but a 6-fold increase from pH 6.5 – 7.4 with a ~2.8 fold increase over pH 7.0 – 7.4. Hence, there was an overall 16-fold increase in K_m over the entire pH range, a level considerably less than the 38- and 43- increases observed for MTX and folic acid, respectively, over this pH range (Table III). Likewise there was only a modest decrease in V_{max} (227 to 141 pmol/mg protein/2min, ~38%) as the pH was increased from pH 5.5 to 7.4 as compared to the 2.6 fold fall in V_{max} reported for folic acid over this pH range (Qiu et al., 2006).

Figure 5D illustrates currents obtained with saturating concentrations of the various transport substrates in *Xenopus* oocytes injected with PCFT cDNA. It can be seen that a concentration of pemetrexed that was 1/6th, 1/3rd, and 1/2th that of MTX, folic acid, and 5-methylTHF, respectively, produced a comparable or greater current, consistent with the high affinity of this agent for PCFT. Figure 5E illustrates pemetrexed-induced currents as a function of concentration at pH 5.5 and 6.5. It can be seen that there was only small increase in influx K_m of from 0.26 to 0.59 μ M over this pH range.

Finally, the impact of PCFT expression in HepG2 cells on the activities of pemetrexed and other antifolates with 5-formylTHF (40 nM) as the folate growth source was measured. There was no difference in IC_{50} 's for ZD1694 (4.6 ± 0.5 vs 5.5 ± 1.2 nM; $P=0.33$), MTX (19 ± 2 vs 16 ± 2 nM; $P=0.42$) and PT523 (3.6 ± 0.7 vs 4.5 ± 0.4 nM; $P=0.26$) in Mock- versus PCFT-transfected cells, respectively, based upon the average of four independent experiments.. However, the PCFT transfectant was more than three-time more sensitive ($P=0.014$) to pemetrexed than the mock-transfectant (17 ± 4 vs 62 ± 6 nM, respectively.). This is similar to what was observed in the HeLa transfectants (Figure 4).

Discussion

It has been recognized since shortly after antifolates were introduced in the clinics that the membrane transport of these agents is an important determinant of their pharmacological activity and that impaired transport is an important mechanism of resistance. These observations were based both in studies of tumor cell growth *in vitro* and tumor cells derived from patients treated with MTX (Matherly and Goldman, 2003). The focus of this work has been directed virtually exclusively on RFC which has been shown to undergo profound changes in expression and amino acid residue integrity under antifolate selective pressure (Zhao and Goldman, 2003; Matherly and Goldman, 2003). With the introduction of pemetrexed, the role of membrane transport became more complicated when studies demonstrated that the activity of this agent could be fully preserved in the complete absence of RFC due, in part, to the presence of another genetically distinct transport mechanism (Zhao and Goldman, 2003). That transporter was recently identified as PCFT, a carrier with very high affinity for folate that has optimal activity at low pH (Qiu et al., 2006). In this paper, pemetrexed transport properties were assessed as a function of pH, along with the impact of PCFT relative to RFC under physiological pH conditions in which PCFT function is far from its optimum.

Using [³H]pemetrexed, these studies confirmed the high affinity of this agent for PCFT suggested from earlier studies. Hence, the influx K_m for pemetrexed at pH 5.5 of 0.8 μ M was one fourth that for MTX, 3.4 μ M, in PCFT-transfected HepG2 cells while the influx K_m was 0.26 μ M and 2 μ M, respectively, for these substrates in *Xenopus* oocytes injected with PCFT cDNA. The major difference between PCFT-mediated pemetrexed transport and transport of the other antifolates studied is the small changes in pemetrexed influx K_m and V_{max} as the pH was increased. Hence, at pH 7.4 the pemetrexed influx K_m was 13 μ M as compared to a MTX K_m of 130 μ M, values consistent with influx K_i 's estimated for the low-pH folate transport activity in an RFC-null HeLa cell line (Zhao et al., 2004b). The similarity between the substrate specificity for PCFT and the low-pH folate transport activities studied in human cell lines provides additional evidence that the low-pH transport activity, ubiquitously present in tissues and cells, is encoded by PCFT.

This retention of PCFT-mediated pemetrexed transport at physiological pH translated into enhanced pharmacologic activity. Hence, using a cell line with a complete lack of constitutive carrier expression, PCFT and RFC were shown to produce equally potent pemetrexed growth inhibition when cells were grown with 25 nM 5-formylTHF as the folate growth source. This was not the case for ZD1694, PT523, and MTX; in this case, RFC restored the levels of activity to that of wild-type HeLa cells, PCFT did not. These results are consistent with what was observed when RFC was inactivated in HeLa or HCT-15 cells; preservation of pemetrexed activity but high-level resistance to MTX, ZD1694, PT523 or PT632 (an analog of PT523) (Zhao et al., 2004b; Chattopadhyay et al., 2006).

These findings relate to the marked difference in affinities of the antifolates for PCFT and RFC at physiological pH and the high degree of preference of PCFT for pemetrexed (Westerhof et al., 1995; Zhao et al., 2004b). The lesser decrease in the pemetrexed IC_{50} produced by PCFT versus RFC in cells grown with folic acid is attributed to the greater increase in cell folates produced by the former and the inhibitory effect this has on the formation of pemetrexed polyglutamate derivatives (Andreassi and Moran, 2002; Zhao et al., 2004b). Based upon these findings: (i) It is highly unlikely that tumors will become resistant to pemetrexed due to a loss of transport since this would require two separate events affecting two genetically unrelated carriers that have comparable ability to transport this agent into cells. (ii) Tumors that become resistant to MTX *in vivo* due to loss of RFC activity should remain transport-competent for pemetrexed, although activity would be determined on the basis of different parameters since these agents have quite different mechanisms of action. Likewise, sensitivities to the other

antifolates studied will be influenced by cellular properties such as activating enzymes, in some cases, and specific intracellular targets.

High-level PCFT expression also increased pemetrexed activity, but not the activities of other antifolates, even in HepG2 that constitutively express these transporters. However, the level of enhancement of pemetrexed activity was less than observed in cells that lack endogenous carriers. It remains unclear, however, as to the extent to which PCFT might influence the transport of a broader spectrum of antifolates under the acid pH conditions of solid tumors (Wike-Hooley et al., 1984; Helmlinger et al., 1997) where the affinity of these drugs for this transporter would be higher than at physiological pH.

It was recognized early-on, from work in other laboratories, that there must be a folic acid transporter independent of RFC (Yang et al., 1983; Henderson and Strauss, 1990). It now appears that PCFT is that elusive folic acid transporter that functions most efficiently at low pH but delivers sufficient levels of this folate to sustain folate pools even at physiological pH. Indeed, PCFT is much more efficient than RFC under these conditions (Table 1). Hence, the marked reduction of the low-pH transport activity and PCFT transcript in HeLa R1 cells (that lack RFC and PCFT) is accompanied by a reduction in pemetrexed transport and folic acid accumulation at neutral pH (Zhao et al., 2005). PCFT transfection in this study restored not only the low-pH folate transport activity but almost fully restored folic acid accumulation at neutral pH. The impact of RFC on folic acid accumulation was much smaller in comparison.

In all HeLa cell transfectants used in this study folate receptor was expressed at comparable levels so that it does not appear that this uptake route played a role as a determinant of folate pool differences, sensitivities to antifolates, or folate growth requirements. This does not preclude their importance under other conditions since folate receptor expression may have been too low to be of consequence, or the receptors that were expressed were not functional, a phenomenon that has been reported previously in some cell lines (Kamen and Smith, 2004). It is possible, however, that the low level of folate receptor expression in R1-11-Mock cells was sufficient to provide the very low levels of folate necessary to permit cell replication in the complete absence of PCFT and RFC.

Abbreviations

5-formylTHF, Leucovorin, 5-formyltetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate; GAT, (0.2 mM glycine, 0.1 mM adenosine, 0.01 mM thymidine); MTX, methotrexate; PCFT, proton-coupled folate transporter; PT523, *N*^α-(4-amino-4-deoxypteroyl)-*N*^δ-hemiphthaloyl-L-ornithine; RFC, reduced folate carrier; ZD1694, raltitrexed, Tomudex, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxyquinazolin-6-ylmethyl)-*N*-methyl-amino]-2-thenoyl)-L-glutamic acid.

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Figure 1.

RFC and PCFT-mediated transport activities and folate receptor binding levels. Panel (A) [³H]MTX influx (0.5 μ M, 2min) assessed at pH 7.4. An anion-free sucrose transport buffer was used to augment RFC activity. (B) [³H]MTX influx (0.5 μ M, 2min) assessed at pH 5.5. (C) Specific folic acid surface binding. Cells for these studies were grown in regular medium which contains 2 μ M folic acid. Data are the mean $\pm$ SEM from the three independent experiments.

Figure 2.

Growth requirements for folic acid (upper panel) and for (6R,S)5-formylTHF (lower panel). Cells were first depleted of intracellular folates by growth in folate-free medium supplemented with GAT for at least one week before transfer to medium containing different concentrations of folic acid or (6R,S)5-formylTHF. Growth was then measured after 6 days. The control represents the highest cell growth. Data are the mean $\pm$ SEM from three independent experiments for each panel.

Figure 3. Growth inhibition by antifolates when cells were grown in medium containing 2 μ M folic acid. Cells were grown with different drug concentrations for five days. The control represents maximal cell growth. Data are the mean $\pm$ SEM from three independent experiments for all panels.

Figure 4.

Growth inhibition by antifolates when cells were grown in medium containing 25 nM (6R,S) 5-formylTHF. Cells were first adapted in medium containing 25 nM 5-formylTHF for 1–3 weeks then grown for an additional five days in the same medium at different drug concentrations. The control represents maximal cell growth. Data are the mean $\pm$ SEM from three independent experiments for all panels.

Figure 5.

(A) Influx of $[^3\text{H}]$ pemetrexed (0.5 μM) was measured in multiple clones of Mock- or PCFT-transfected HepG2 cells at pH of 7.0 for 2 min. Data are mean $\pm$ SEM from three independent experiments. Inset: A representative Western blot of PCFT protein in these clones. (B) Pemetrexed growth inhibition in the Mock- or PCFT-transfected HepG2 cells. Cells were grown with 25 nM (6S) 5-formylTHF. Data are mean $\pm$ SEM from three independent experiments. (C) Initial uptake of 0.5 μM $[^3\text{H}]$ pemetrexed or $[^3\text{H}]$ (6S)5-formylTHF in Mock- and PCFT-HepG2 (PCFT-clone2 and Mock-clone-1) cell lines as a function of pH over 2 min. The data are mean $\pm$ SEM from three independent experiments. (D) A comparison of current amplitude induced by saturating concentrations of folic acid (20 μM), (6S)5-methylTHF (14 μM),

methotrexate (50 μM) and pemetrexed (7 μM), equal to about 20–25 time K_m values for each substrate at pH 5.5 and room temperature in *Xenopus* oocytes expressing PCFT. (E) Electrophysiological analyses of pemetrexed uptake by PCFT as a function of substrate concentration in *Xenopus* oocytes at pH 5.5 and 6.5, where I is the current and I/I_{max} is the ratio of current measured to the maximal current. The K_m values are the mean $\pm$ SEM from three independent experiments.

Table I

Accumulation of folates in cells grown with tritiated folic acid and 5- formylTHF

Cell line	2 μ M [3 H]folic acid		25 nM [3 H]5-formylTHF	
	pmol/mg protein	Ratio to R1-11-Mock	pmol/mg protein	Ratio to R1-11-Mock
R1-11-Mock	9.5 $\pm$ 1.4	1	1.9 $\pm$ 0.3	1
R1-11-RFC-6	27.4 $\pm$ 2.7	3	23.2 $\pm$ 4.8	12
R1-11-PCFT-4	112.8 $\pm$ 10.0	12	21.0 $\pm$ 0.6	11
HeLa	136.5 $\pm$ 11.9	13	32.7 $\pm$ 3.5	17

Cells were grown in folate-free medium containing GAT for a week in the presence of 2 μ M [3 H]folic acid or 25 nM [3 H]6(S)5-formylTHF. Medium including the tritiated folates was replaced after three days. Data are the mean $\pm$ SEM from the three independent experiments for each panel.

Table II

MTX transport activity mediated by PCFT or RFC and growth inhibition by pemetrexed and ZD1694 in independent clones of PCFT or RFC transfectants

Clone	MTX transport at pH 5.5 ^a	MTX transport at pH 7.4 ^a	Pemetrexed IC ₅₀ (nM)	ZD1694 IC ₅₀ (nM)
R1-11-mock	0.53±0.02	0.76±0.15		
R1-11-PCFT-4	2.5±0.3 ^b		68±18	117±9
R1-11-PCFT-5	8.2±1.1		68±33	125±50
R1-11-PCFT-8	2.8±0.4		38±13	110±10
R1-11-RFC-5		2.5±0.1	68±26	4.8±2.6
R1-11-RFC-6		1.7±0.2	94±52	6.3±3.4
R1-11-RFC-7		3.2±0.16	49±18	3.1±1.6

^a MTX transport activity is expressed in pmol/mg protein/2 min.

^b A folatereceptor- mediated contribution to MTX influx was excluded in some experiments with the demonstration that transport was not affected by co-addition of 50 nM folic acid. Cells were maintained in folate-free medium containing 25 nM (6R,S)-5- formylTHF. Data are the mean±SEM from three separate experiments.

Table III

Pemetrexed influx kinetics as a function of pH

	Pemetrexed influx		*MTX influx	*Folic acid influx
	K_m (μ M)	V_{max} (pmol/mg protein/2 min)	K_m (μ M)	K_m (μ M)
pH5.5	0.8 ± 0.1	227 ± 6	3.4	1.3
pH 6.0			3.4	1.5
pH6.5	2.1 ± 0.2	174 ± 6	7.3	2.7
pH7.0	4.6 ± 0.9	175 ± 10	16.3	6.0
pH7.1	5.9 ± 0.9	165 ± 8		
pH7.2	12.5 ± 0.8	161 ± 4		
pH7.4	12.7 ± 1.2	141 ± 5	131	56.2

Pemetrexed K_m and V_{max} values are based upon analysis of initial uptake rates in PCFT-transfected HepG2 cells. Data are the mean $\pm$ SEM from three independent experiments.

* The K_m 's for MTX and folic acid were reported previously (Qiu et al., 2006) and are listed in the table for the purpose of comparison.