

# The Proton-Coupled Folate Transporter: Impact on Pemetrexed Transport and on Antifolates Activities Compared with 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 article 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, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxyquinazolin-6-ylmethyl)-*N*-methyl-amino]-2-thenoyl)-L-glutamic acid (raltitrexed, Tomudex; ZD1694), or *N*<sup>α</sup>-(4-amino-4-deoxypteroyl)-*N*<sup>β</sup>-hemipthaloyl-L-ornithine (PT523) in

comparison with RFC irrespective of the folate growth source. PCFT, expressed at high levels in *Xenopus laevis* oocytes and in transporter-competent HepG2 cells, exhibited a high affinity for pemetrexed, with an influx  $K_m$  value of 0.2 to 0.8  $\mu\text{M}$  at pH 5.5. 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 as a result of impaired transport because of the redundancy of these genetically distinct routes.

Three folate transporters account for most, if not all, folate influx activities observed in mammalian cells. 1) The reduced folate carrier (RFC; SLC19A1) is an anion exchanger with a high affinity for reduced folates ( $K_m$  of  $\sim 1\text{--}5\ \mu\text{M}$ ) and a low affinity for folic acid ( $K_i$  of  $\sim 200\ \mu\text{M}$ ) 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). 2) 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- $\alpha$  is expressed in epithelial tissues, and folate

receptor- $\beta$  is expressed in cells of hematopoietic origin (Kamen and Smith, 2004; Salazar and Ratnam, 2007). Inactivation of folate receptor- $\alpha$  is embryonic lethal, whereas deletion of folate receptor- $\beta$  is not associated with an abnormal phenotype (Piedrahita et al., 1999). 3) 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, 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 epithelia in subjects with the autosomal recessive disorder hereditary folate malabsorption (Online Mendelian Inheritance in Man 229050) (Goldman et al., 2008) who have loss-of-function mutations in the *PCFT* gene (Qiu et al., 2006;

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**ABBREVIATIONS:** RFC, reduced folate carrier; PCFT, proton-coupled folate transporter; MTX, methotrexate; 5-formylTHF, leucovorin, 5-formyltetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate; CMV, cytomegalovirus; MES, 2-(*N*-morpholino)ethanesulfonic acid; ZD1694, raltitrexed (*N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxyquinazolin-6-ylmethyl)-*N*-methyl-amino]-2-thenoyl)-L-glutamic acid); PT523, *N*<sup>α</sup>-(4-amino-4-deoxypteroyl)-*N*<sup>β</sup>-hemipthaloyl-L-ornithine; GAT, glycine/adenosine/thymidine.

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* (Matherly and Goldman, 2003; Zhao and Goldman, 2003), and it 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 seems to be quite different. When 5-formylTHF (leucovorin) 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., 2004c; 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., 2004a; Qiu et al., 2006).

This article 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., 2004a), at levels comparable with 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 laevis* oocytes and HepG2 cells along with the impact of PCFT on the pharmacological activities of antifolates in HepG2 transfectants that express a high level of PCFT on a background of constitutive PCFT and RFC function.

## Materials and Methods

**Chemicals.** [ $^3\text{H}$ ],5',7,9- $^3\text{H}$ ]Folic acid, [ $^3\text{H}$ ],5',7- $^3\text{H}$ (N)]MTX, [ $^3\text{H}$ ],5',7,9- $^3\text{H}$ (N)](6S)-5-formylTHF, and [ $^3\text{H}$ ]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 [raltitrexed (Tomudex)] was obtained from AstraZeneca Pharmaceuticals LP (Wilmington, DE). 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 high-performance liquid chromatography 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 100  $\mu\text{g}/\text{ml}$  streptomycin or folate-free RPMI medium containing 25 or 40 nM (6R,S)5-formylTHF and the same supplements. 5-formylTHF was used 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, developed previously in this laboratory (Zhao et al., 2004a) that lack PCFT and RFC expression, were subcloned in 96-well plates at a density of one, two, four, or eight cells/well in RPMI 1640 medium in the absence of MTX. After 2 wk, single colonies were expanded for influx assays and cryopreservation. The R1-11 clone, which lacked low-pH folate transport activity and was stable for more than 3 months, was selected as the recipient for transfections.

R1-11 cells were then transfected with either RFC or PCFT cDNA 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 simian virus 40 promoter, whereas it is driven by the CMV promoter in the former vectors. These expression vectors were individually transfected into R1-11 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Stable transfectants were obtained and maintained in the presence of 100  $\mu\text{g}/\text{ml}$  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 20 ml of low-background glass scintillation vials (Research Products International, Mt. Prospect, IL) and grown for 3 days to reach early confluence. PCFT activity was measured as follows. Cells were washed twice with HBS (20 mM HEPES, 5 mM dextrose, 140 mM NaCl, 5 mM KCl, and 2 mM  $\text{MgCl}_2$ , pH 7.4) 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 0.5 ml of prewarmed (37°C) MBS (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM  $\text{MgCl}_2$  adjusted to pH 5.5, 6, or 7.0) or HBS, pH 7.0 to 7.4, containing 0.5  $\mu\text{M}$  [ $^3\text{H}$ ]MTX. Uptake was carried out over 2 min at 37°C and quenched by the addition of 5 ml of ice-cold HBS.

RFC transport activity was determined at pH 7.4. An anion-free buffer, MHS (20 mM HEPES and 235 mM sucrose, pH adjusted to 7.4 with MgO), was used to amplify RFC-mediated transport by eliminating competing inhibitory anions (Henderson and Zevly, 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  $\mu\text{M}$  [ $^3\text{H}$ ]MTX for 2 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 0.2 M NaOH at 65°C for 40 min. Radioactivity in 0.4 ml of lysate was measured on a liquid scintillation spectrometer and normalized to protein levels obtained with the bicinchoninic acid protein assay (Pierce Chemical, 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 (10 mM sodium acetate and 150 mM 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 5 nM [ $^3\text{H}$ ]folic acid in ice-cold HBS in the presence or absence of 500 nM nonlabeled folic acid. After three ice-cold HBS washes, membrane-bound [ $^3\text{H}$ ]folic acid was released with the addition of the acid buffer (0.5 ml) and radioactivity in the supernatant measured. The cells were dissolved in 0.5 ml of 0.2 NaOH, and 10  $\mu\text{l}$  of cell lysate was used for protein determination.

**Intracellular Accumulation of [ $^3\text{H}$ ]Folic Acid or [ $^3\text{H}$ ]5-formylTHF.** Cells were trypsinized and grown for a week in folate-free RPMI 1640 medium supplemented with 10% dialyzed FBS and GAT (0.2 mM glycine, 0.1 mM adenosine, and 0.01 mM thymidine) containing 2  $\mu\text{M}$  [ $^3\text{H}$ ]folic acid or 25 nM [ $^3\text{H}$ ](6S)-5-formylTHF. Cells were replated after 3 days with fresh medium and tritiated folate. After an additional 4 days, the medium was removed and the cells were washed three times with ice-cold HBS and processed as described under *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 to 2000 cells/well in a folate-free medium containing 10% dialyzed FBS and a spectrum of concentrations of either folic acid or (6*R*,*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  $\mu$ M folic acid or folate-

free RPMI 1640 medium supplemented with 25 or 40 nM (6*R*,*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 to 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.

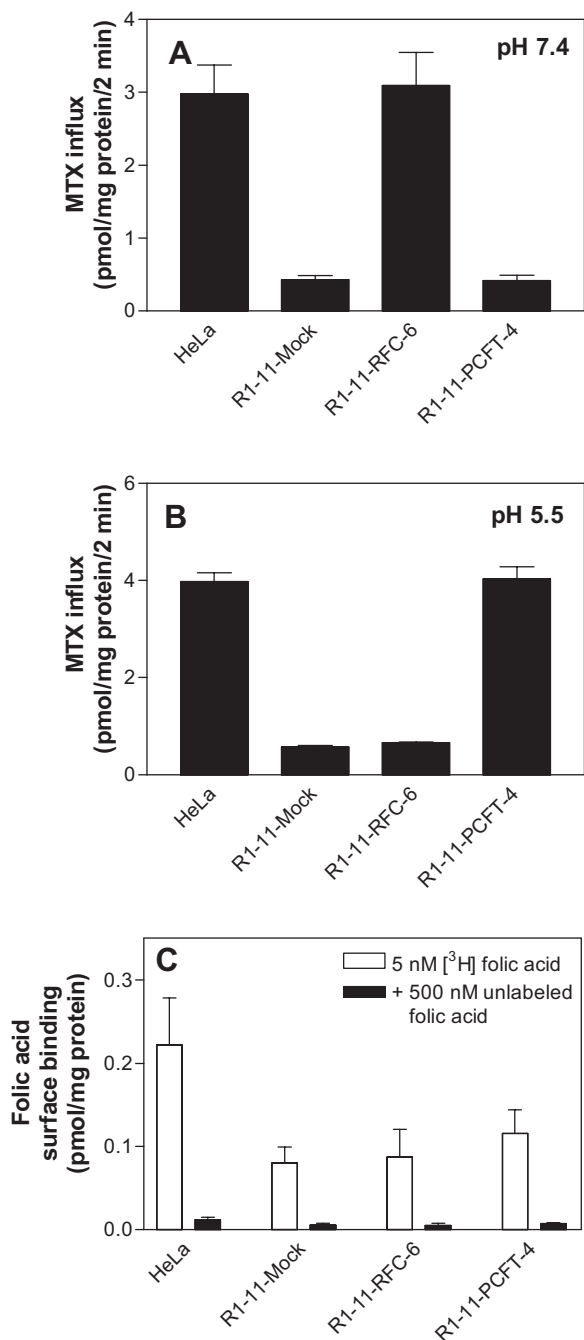
**Electrophysiological Analyses.** PCFT cRNA was transcribed in vitro, and *X. laevis* oocytes were prepared, injected with water or PCFT cRNA, and pemetrexed-induced currents were recorded at room temperature by a two-electrode voltage-clamp technique 3 to 7 days after injection as described previously (Qiu et al., 2006, 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 described previously (Cell Signaling Technology Inc., Danvers, MA) (Qiu et al., 2006).

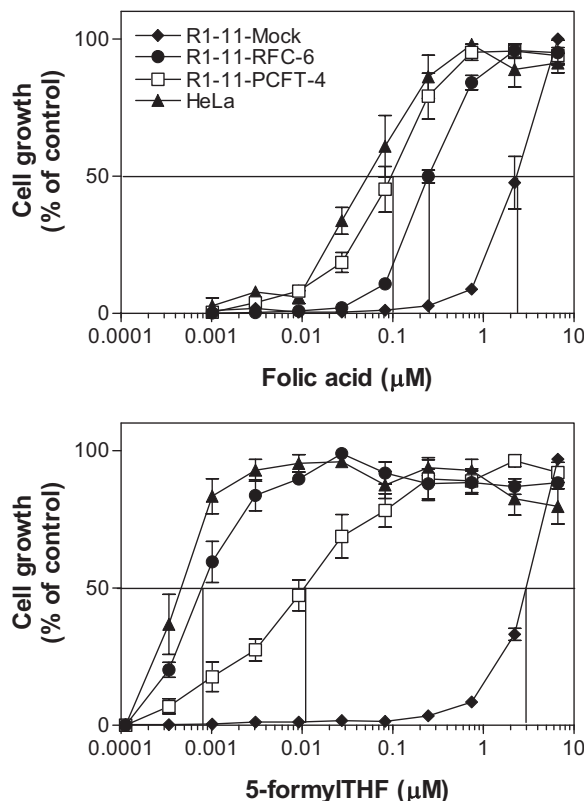
**Statistics.** Data were analyzed using repeated measures analysis of variance from Prism software (GraphPad Software Inc., San Diego, CA).

## 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 are much higher than their endogenous expression in HeLa cells



**Fig. 1.** RFC- and PCFT-mediated transport activities and folate receptor binding levels. A, [<sup>3</sup>H]MTX influx (0.5  $\mu$ M; 2 min) assessed at pH 7.4. An anion-free sucrose transport buffer was used to augment RFC activity. B, [<sup>3</sup>H]MTX influx (0.5  $\mu$ M; 2 min) assessed at pH 5.5. C, specific folic acid surface binding. Cells for these studies were grown in regular medium which contains 2  $\mu$ M folic acid. Data are the mean  $\pm$  S.E.M. from the three independent experiments.



**Fig. 2.** Growth requirements for folic acid (top) and for (6*R*,*S*)5-formylTHF (bottom). Cells were first depleted of intracellular folates by growth in folate-free medium supplemented with GAT for at least 1 week before transfer to medium containing different concentrations of folic acid or (6*R*,*S*)5-formylTHF. Growth was then measured after 6 days. The control represents the highest cell growth. Data are the mean  $\pm$  S.E.M. from three independent experiments for each panel.

(Zhao et al., 2004b; Qiu et al., 2006). To obviate this, a different vector, pZeoSV2(+), in which expression of protein was driven by a simian virus 40 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 Fig. 1A, 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. In contrast, MTX transport activity at pH 5.5, mediated by PCFT, was comparable in R1-11-PCFT-4 and HeLa cells (Fig. 1B). 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 with their endogenous activities in wild-type HeLa cells. Folate receptor-mediated transport did not contribute to MTX uptake under these conditions because cells for these experiments were grown in folic acid-containing medium that would block transport via this route.

Folate receptor levels in these cell lines were also assessed. As indicated in Fig. 1C, folic acid surface binding is highly specific because it was completely suppressed with 500 nM nonlabeled 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., 2004c).

TABLE 1

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

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

| Cell Line    | $2 \mu\text{M}$ [ $^3\text{H}$ ]Folic acid |                     | $25 \text{ nM}$ [ $^3\text{H}$ ]5-FormylTHF |                     |
|--------------|--|---------------------|---|---------------------|
|              | <i>pmol / mg protein</i>                   | Ratio to R1-11-Mock | <i>pmol / mg protein</i>                    | 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         | $136.5 \pm 11.9$                           | 14                  | $32.7 \pm 3.5$                              | 17                  |

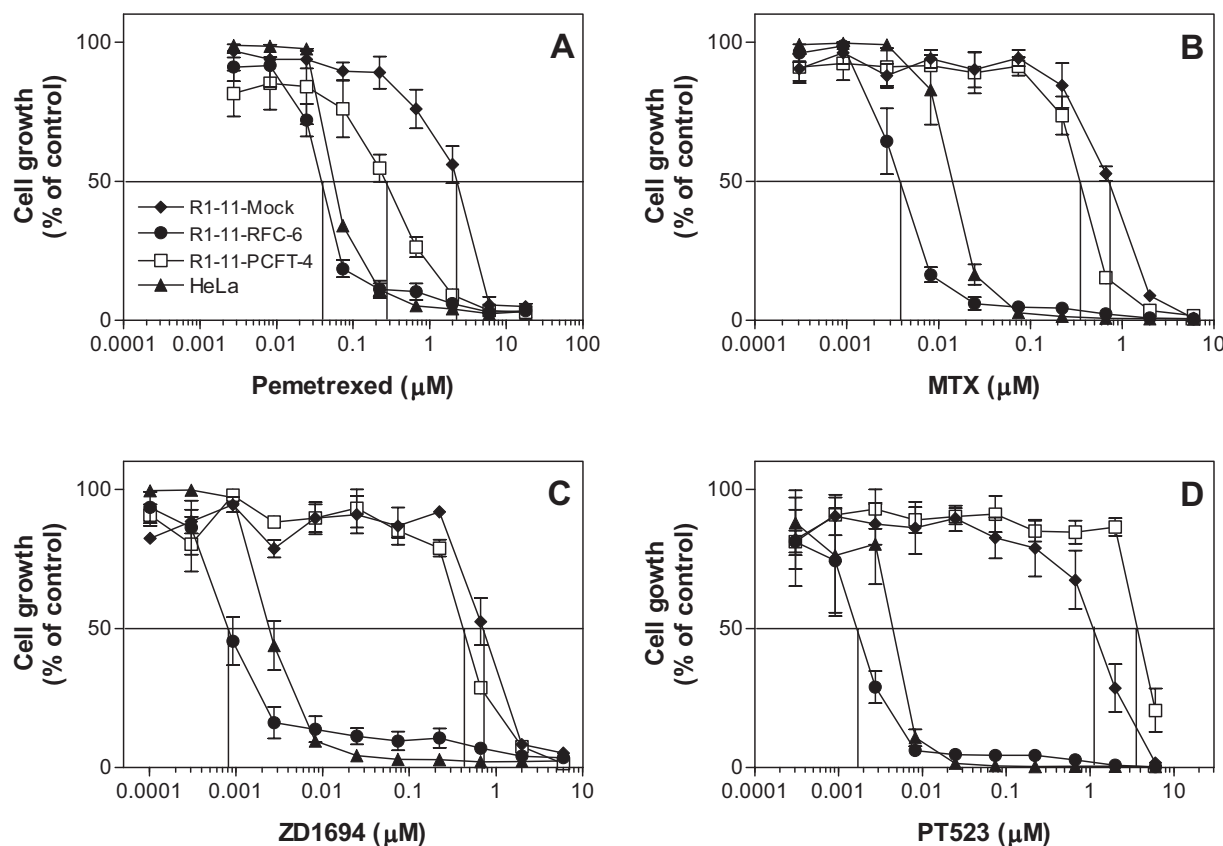


Fig. 3. Growth inhibition by antifolates when cells were grown in medium containing  $2 \mu\text{M}$  folic acid. Cells were grown with different drug concentrations for five days. The control represents maximal cell growth. Data are the mean  $\pm$  S.E.M. from three independent experiments for all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

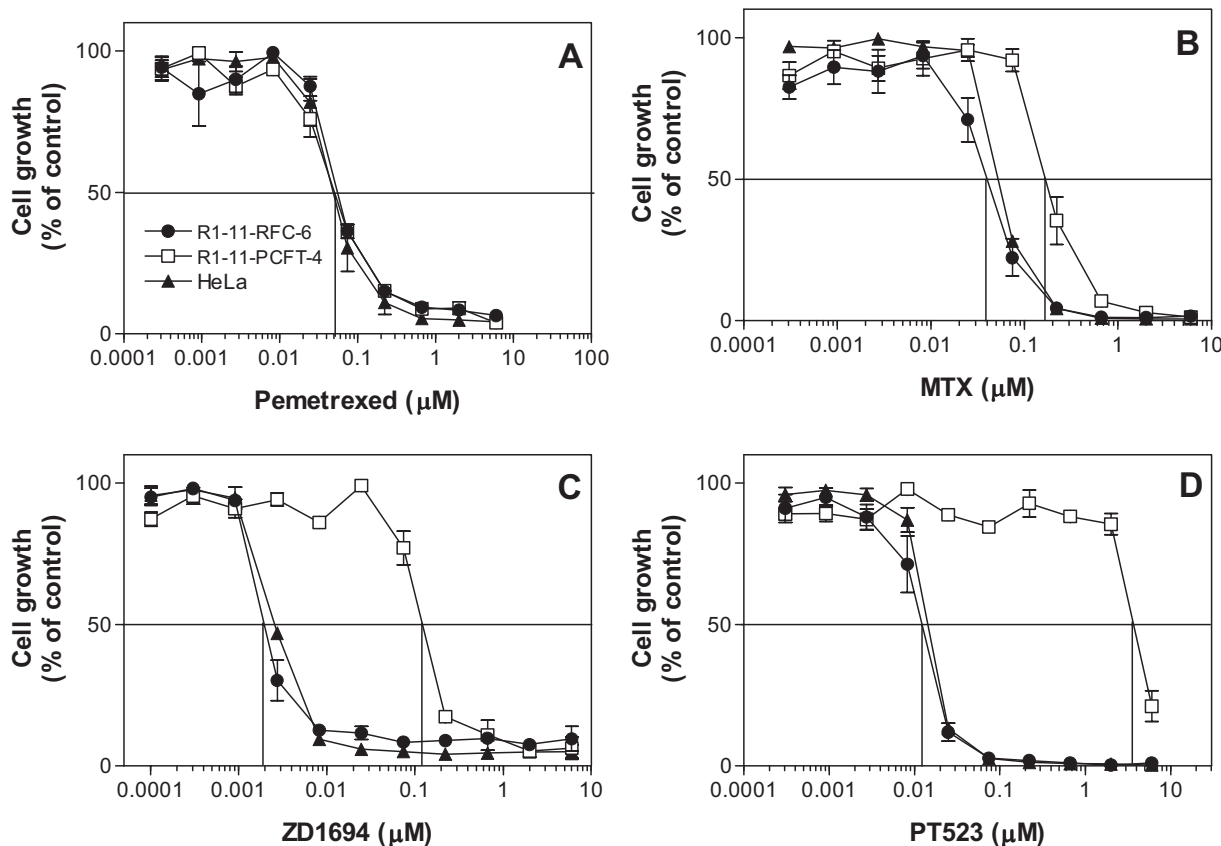
**Impact of Expression of RFC or PCFT on Cellular Folate Growth Requirement.** Cellular growth requirements for folic acid or 5-formylTHF were assessed in the transfectants along with HeLa cells. This was determined after cells had been depleted of intracellular folate pools by growth in folate-free medium supplemented with GAT and then exposed to medium containing different concentration of folates. Because 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 Fig. 2, top, the folic acid concentration required to support 50% 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  $EC_{50}$  value by a factor of  $\sim 10$ , 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  $\sim 20$ , 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  $EC_{50}$  value for folic acid compared with R1-11-PCFT-4 cells.

As indicated in Fig. 2, bottom, the  $EC_{50}$  value ( $\sim 3 \mu\text{M}$ ) for 5-formylTHF in R1-11-mock cells was similar to what was observed for folic acid (Fig. 2, top). 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  $0.01 \mu\text{M}$ , whereas expression of RFC decreased the  $EC_{50}$  value by a factor of

6000 to  $0.5 \text{ nM}$ . The  $EC_{50}$  value for 5-formylTHF in HeLa cells was slightly lower than that in R1-11-RFC-6 cells. The difference in the  $EC_{50}$  value for 5-formylTHF between R1-11-RFC-6 and R1-11-PCFT-4 cells was 20-fold compared with only a 2-fold difference in the folic acid  $EC_{50}$  values. Hence, 1) 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 ( $K_i$  or  $K_i$  of  $\sim 2$  and  $45 \mu\text{M}$  for RFC and PCFT, respectively; Zhao et al., 2004c). 2) 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). 3) Both RFC and PCFT are much more potent in decreasing the growth requirement for 5-formylTHF than for folic acid. This is probably due to a higher affinity of both RFC and PCFT for 5-formylTHF than for folic acid. For PCFT, the affinity for the natural isomer of 5-formylTHF is 4-fold higher than for folic acid at physiological pH (Zhao et al., 2004c).

**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  $2 \mu\text{M}$  [ $^3\text{H}$ ]folic acid or  $25 \text{ nM}$  [ $^3\text{H}$ ](6*S*)5-formylTHF for 1 week. The concentrations of these folates were chosen because the former represents the folic acid concentration in growth medium, whereas 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



**Fig. 4.** Growth inhibition by antifolates when cells were grown in medium containing  $25 \text{ nM}$  (6*R,S*)5-formylTHF. Cells were first adapted in medium containing  $25 \text{ nM}$  5-formylTHF for 1 to 3 weeks, and then they were grown for an additional 5 days in the same medium at different drug concentrations. The control represents maximal cell growth. Data are the mean  $\pm$  S.E.M. from three independent experiments for all panels.

not grow in 25 nM 5-formylTHF alone (Fig. 2, bottom). As indicated in Table 1, folic acid and 5-formylTHF accumulation in R1-11-mock-1 cells was 1/14 and 1/17 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 it 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  $\mu$ M folic acid as shown in Fig. 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 IC<sub>50</sub> values (the concentration that inhibits cell growth by 50%) among R1-11-PCFT-4, R1-11-RFC-6, and R1-11-mock cells. The IC<sub>50</sub> for R1-11-PCFT-4 cells was decreased by a factor of 10 compared with 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 IC<sub>50</sub> value for R1-11-PCFT-4 cells was increased (although this did not reach statistical significance,  $P = 0.09$ ) by a factor of 3.5 compared with R1-11-mock-1 cells. In comparison, the IC<sub>50</sub> value for R1-11-RFC-6 cells was markedly decreased for pemetrexed, MTX, ZD1694, and PT523 (63-, 200-, 1000-, and 670-fold, respectively) compared with 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 Fig. 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 IC<sub>50</sub> value 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, IC<sub>50</sub> values 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 IC<sub>50</sub> values 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 2 that although 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 IC<sub>50</sub> value for PMX was not significantly different whether mediated by PCFT or RFC (average of 58 versus 69 nM, respectively), whereas, in all

cases, there was a marked (~25-fold) decrease in IC<sub>50</sub> value for ZD1694 in RFC- versus PCFT-transfected cells (average of 117 versus 4.7 nM, respectively).

**Pemetrexed Transport Properties Mediated by PCFT in HepG2 Cells and *X. laevis* Oocytes.** PCFT stable transfectants were generated in HepG2 cells that 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 compared with 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 Fig. 5A, pemetrexed initial uptake measured at pH 7.0 in each PCFT transfectant clone was 5- to 7-fold greater than uptake in mock transfectants. The level of PCFT expression was verified by Western blot (Fig. 5A, inset). Pemetrexed IC<sub>50</sub> values in the four transfectants were uniformly decreased by a factor of ~4 compared with the values in mock transfectants, indicating that this level of PCFT expression substantially increased sensitivity to this drug (Fig. 5B).

Pemetrexed transport characteristics were examined in one PCFT transfectant (clone 2) with one mock transfectant (clone 1) as a control. As indicated in Fig. 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 3, there was an ~2.6-fold increase in pemetrexed influx  $K_m$ , from pH 5.5 to 6.5 but a 6-fold increase from pH 6.5 to 7.4, with an ~2.8-fold increase over pH 7.0 to 7.4. Hence, there was an overall 16-fold increase in  $K_m$  value over the entire pH range, a level considerably less than the 38- and 43-fold increases observed for MTX and folic acid, respectively, over this pH range (Table 3). Likewise, there was only a modest decrease in  $V_{max}$  value (227–141 pmol/mg protein/2 min; ~38%) as the pH was increased from pH 5.5 to 7.4 compared with 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

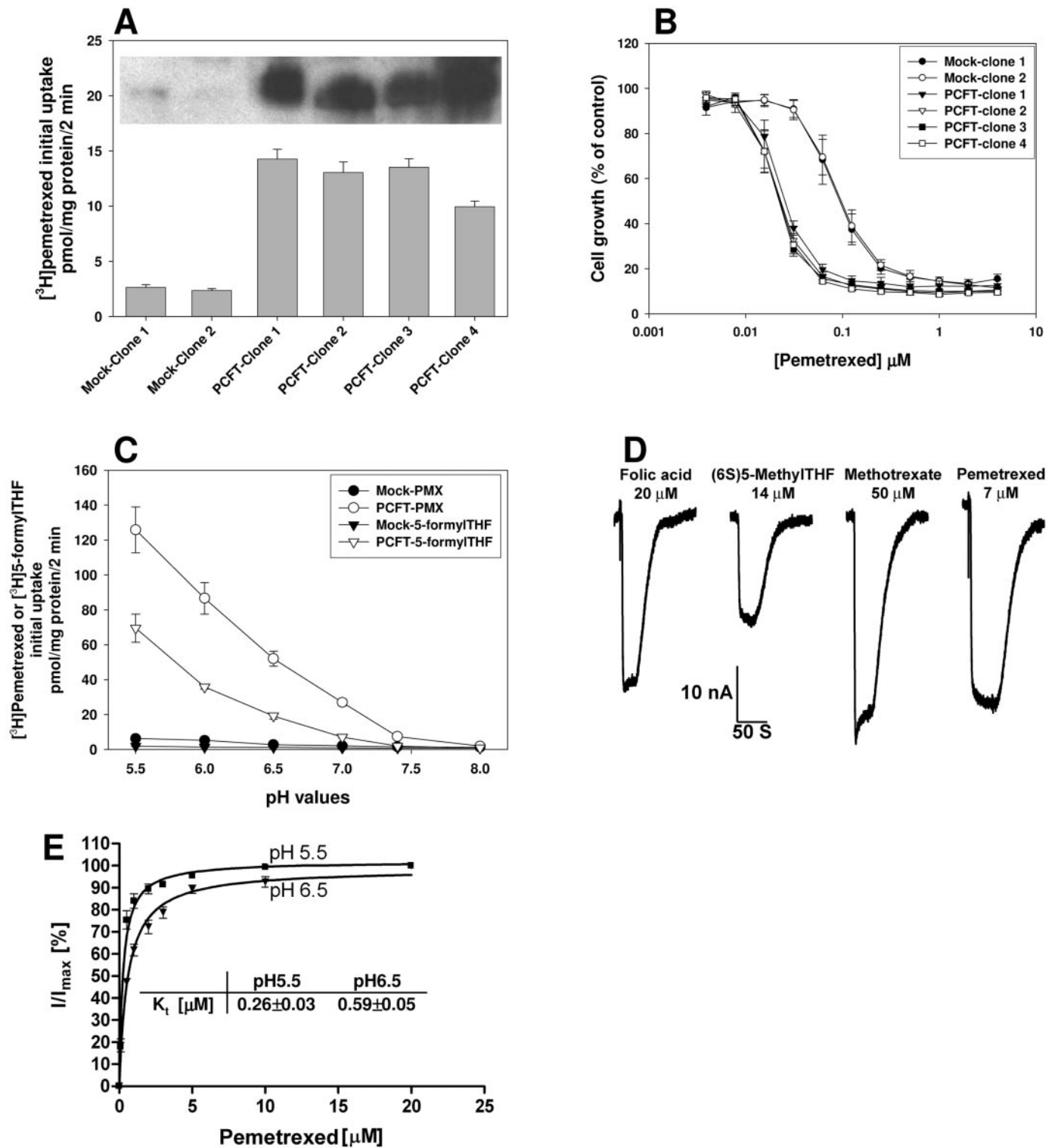
TABLE 2

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

Cells were maintained in folate-free medium containing 25 nM (6R,S)5-formylTHF. Data are the mean  $\pm$  S.E.M. from three separate experiments.

| Clone        | MTX Transport              |                 | IC <sub>50</sub> |               |
|--------------|----------------------------|-----------------|------------------|---------------|
|              | pH 5.5                     | pH 7.4          | Pemetrexed       | ZD1694        |
|              | pmol/mg protein/2 min      |                 | nM               |               |
| R1-11-mock   | 0.53 $\pm$ 0.02            | 0.76 $\pm$ 0.15 |                  |               |
| R1-11-PCFT-4 | 2.5 $\pm$ 0.3 <sup>a</sup> |                 | 68 $\pm$ 18      | 117 $\pm$ 9   |
| R1-11-PCFT-5 | 8.2 $\pm$ 1.1              |                 | 68 $\pm$ 33      | 125 $\pm$ 50  |
| R1-11-PCFT-8 | 2.8 $\pm$ 0.4              |                 | 38 $\pm$ 13      | 110 $\pm$ 10  |
| R1-11-RFC-5  |                            | 2.5 $\pm$ 0.1   | 68 $\pm$ 26      | 4.8 $\pm$ 2.6 |
| R1-11-RFC-6  |                            | 1.7 $\pm$ 0.2   | 94 $\pm$ 52      | 6.3 $\pm$ 3.4 |
| R1-11-RFC-7  |                            | 3.2 $\pm$ 0.16  | 49 $\pm$ 18      | 3.1 $\pm$ 1.6 |

<sup>a</sup> A folate-receptor-mediated contribution to MTX influx was excluded in some experiments with the demonstration that transport was not affected by coaddition of 50 nM folic acid.



**Fig. 5.** A, influx of  $0.5 \mu\text{M}$  [ $^3\text{H}$ ]pemetrexed was measured in multiple clones of mock- or PCFT-transfected HepG2 cells at pH of 7.0 for 2 min. Data are mean  $\pm$  S.E.M. from three independent experiments. Inset, representative Western blot of PCFT protein in these clones. B, pemetrexed growth inhibition in the mock- or PCFT-transfected HepG2 cells. Cell were grown with  $25 \text{ nM}$  (6*R,S*)5-formylTHF. Data are mean  $\pm$  S.E.M. from three independent experiments. C, initial uptake of  $0.5 \mu\text{M}$  [ $^3\text{H}$ ]pemetrexed or [ $^3\text{H}$ ](6*S*)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  $\pm$  S.E.M. from three independent experiments. D, comparison of current amplitude induced by saturating concentrations of  $20 \mu\text{M}$  folic acid,  $14 \mu\text{M}$  (6*S*)5-methylTHF,  $50 \mu\text{M}$  methotrexate, and  $7 \mu\text{M}$  pemetrexed, equal to approximately 20 to 25 times the  $K_m$  values for each substrate at pH 5.5 and room temperature in *X. laevis* oocytes expressing PCFT. E, electrophysiological analyses of pemetrexed uptake by PCFT as a function of substrate concentration in *X. laevis* oocytes at pH 5.5 and 6.5, where  $I$  is the current and  $I/I_{\text{max}}$  is the ratio of current measured to the maximal current. The  $K_m$  values are the mean  $\pm$  S.E.M. from three independent experiments.

concentrations of the various transport substrates in *X. laevis* oocytes injected with PCFT cDNA. It can be seen that a concentration of pemetrexed that was one sixth, one third, and one half 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 a small increase in influx  $K_m$  value of from 0.26 to 0.59  $\mu\text{M}$  over this pH range.

Finally, the impact of PCFT expression in HepG2 cells on the activities of pemetrexed and other antifolates with 40 nM 5-formylTHF as the folate growth source was measured. There was no differences in  $\text{IC}_{50}$  values for ZD1694 ( $4.6 \pm 0.5$  versus  $5.5 \pm 1.2$  nM;  $P = 0.33$ ), MTX ( $19 \pm 2$  versus  $16 \pm 2$  nM;  $P = 0.42$ ), and PT523 ( $3.6 \pm 0.7$  versus  $4.5 \pm 0.4$  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 3 times more sensitive ( $P = 0.014$ ) to pemetrexed than the mock transfectant ( $17 \pm 4$  versus  $62 \pm 6$  nM, respectively). This is similar to what was observed in the HeLa transfectants (Fig. 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 (Matherly and Goldman, 2003; Zhao 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 article, 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.

These studies with [ $^3\text{H}$ ]pemetrexed confirm the high affinity of this agent for PCFT suggested from earlier studies. Hence, the influx  $K_m$  value for pemetrexed at pH 5.5 of 0.8  $\mu\text{M}$  was one fourth that for MTX, 3.4  $\mu\text{M}$ , in PCFT-transfected HepG2 cells, whereas the influx  $K_m$  value was 0.26 and 2  $\mu\text{M}$ , respectively, for these substrates in *X. laevis* 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  $V_{\text{max}}$  values as the pH was increased. Hence, at pH 7.4 the pemetrexed influx  $K_m$  value was 13  $\mu\text{M}$  compared with influx  $K_i$  values estimated for the low-pH folate transport activity in an RFC-null HeLa cell line (Zhao et al., 2004c). 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 pharmacological 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., 2004c; 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., 2004c). The lesser decrease in the pemetrexed  $\text{IC}_{50}$  value 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., 2004c). These findings suggest the following: 1) It is highly unlikely that tumors will become resistant to pemetrexed because of a loss of transport because this would require two separate events affecting two genetically unrelated carriers that have comparable ability to transport this agent into cells. 2) 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 because 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

TABLE 3

Pemetrexed influx kinetics as a function of pH

Pemetrexed  $K_m$  and  $V_{\text{max}}$  values are based upon analysis of initial uptake rates in PCFT-transfected HepG2 cells. Data are the mean  $\pm$  S.E.M. from three independent experiments. The  $K_m$  values for MTX and folic acid were reported previously (Qiu et al., 2006) and are listed in the table for the purpose of comparison.

| pH  | Pemetrexed INFLUX |                       | Influx, $K_m$ |            |
|-----|-------------------|-----------------------|---------------|------------|
|     | $K_m$             | $V_{\text{max}}$      | MTX           | Folic Acid |
|     | $\mu\text{M}$     | pmol/mg protein/2 min | $\mu\text{M}$ |            |
| 5.5 | $0.8 \pm 0.1$     | $227 \pm 6$           | 3.4           | 1.3        |
| 6.0 |                   |                       | 3.4           | 1.5        |
| 6.5 | $2.1 \pm 0.2$     | $174 \pm 6$           | 7.3           | 2.7        |
| 7.0 | $4.6 \pm 0.9$     | $175 \pm 10$          | 16.3          | 6.0        |
| 7.1 | $5.9 \pm 0.9$     | $165 \pm 8$           |               |            |
| 7.2 | $12.5 \pm 0.8$    | $161 \pm 4$           |               |            |
| 7.4 | $12.7 \pm 1.2$    | $141 \pm 5$           | 131           | 56.2       |

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 seems 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) (Qiu et al., 2006) is accompanied by a reduction in pemetrexed transport and folic acid accumulation at neutral pH (Zhao et al., 2004a). PCFT transfection in this study restored not only the low-pH folate transport activity but also 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 seem 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 because folate receptor expression in the current study 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.

## References

- Andreassi JL 2nd and Moran RG (2002) Mouse folylpoly-gamma-glutamate synthetase isoforms respond differently to feedback inhibition by folylpolyglutamate cofactors. *Biochemistry* **41**:226–235.
- Chattopadhyay S, Tamari R, Min SH, Zhao R, Tsai E, and Goldman ID (2007) Commentary: a case for minimizing folate supplementation in clinical regimens with pemetrexed based on the marked sensitivity of the drug to folate availability. *Oncologist* **12**:808–815.
- Chattopadhyay S, Zhao R, Krupenko SA, Krupenko N, and Goldman ID (2006) The inverse relationship between reduced folate carrier function and pemetrexed activity in a human colon cancer cell line. *Mol Cancer Ther* **5**:438–449.
- Goldman ID, Min SH, Sandoval C, and Kronn D (2008) Hereditary folate malabsorption. *Gene Reviews*. Available at <http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=folate-mal>.
- Gorlick R, Goker E, Trippett T, Steinherz P, Elisseyyeff Y, Mazumdar M, Flintoff WF, and Bertino JR (1997) Defective transport is a common mechanism of acquired methotrexate resistance in acute lymphocytic leukemia and is associated with decreased reduced folate carrier expression. *Blood* **89**:1013–1018.
- Guo W, Healey JH, Meyers PA, Ladanyi M, Huvos AG, Bertino JR, and Gorlick R (1999) Mechanisms of methotrexate resistance in osteosarcoma. *Clin Cancer Res* **5**:621–627.
- Helmlinger G, Yuan F, Dellian M, and Jain RK (1997) Interstitial pH and PO<sub>2</sub> gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nat Med* **3**:177–182.
- Henderson GB and Strauss BP (1990) Characteristics of a novel transport system for folate compounds in wild-type and methotrexate-resistant L1210 cells. *Cancer Res* **50**:1709–1714.
- Henderson GB and Zevely EM (1983) Use of non-physiological buffer systems in the analysis of methotrexate transport in L1210 cells. *Biochem Int* **6**:507–515.
- Kamen BA and Smith AK (2004) A review of folate receptor alpha cycling and 5-methyltetrahydrofolate accumulation with an emphasis on cell models in vitro. *Adv Drug Deliv Rev* **56**:1085–1097.
- Kamen BA, Smith AK, and Anderson RGW (1991) The folate receptor works in tandem with a probenecid-sensitive carrier in MA104 cells in vitro. *J Clin Invest* **87**:1442–1449.
- Matherly LH and Goldman DI (2003) Membrane transport of folates. *Vitam Horm* **66**:403–456.
- Matherly LH, Taub JW, Ravindranath Y, Proefke SA, Wong SC, Gimotty P, Buck S, Wright JE, and Rosowsky A (1995) Elevated dihydrofolate reductase and impaired methotrexate transport as elements in methotrexate resistance in childhood acute lymphoblastic leukemia. *Blood* **85**:500–509.
- Piedrahita JA, Oetama B, Bennett GD, van Waes J, Kamen BA, Richardson J, Lacey SW, Anderson RG, and Finnell RH (1999) Mice lacking the folic acid-binding protein Folbp1 are defective in early embryonic development. *Nat Genet* **23**:228–232.
- Prasad PD, Mahesh VB, Leibach FH, and Ganapathy V (1994) Functional coupling between a bafilomycin A<sub>1</sub>-sensitive proton pump and a probenecid-sensitive folate transporter in human placental choriocarcinoma cells. *Biochim Biophys Acta* **1222**:309–314.
- Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, Sandoval C, Zhao R, Akabas MH, and Goldman ID (2006) Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell* **127**:917–928.
- Qiu A, Min SH, Jansen M, Malhotra U, Tsai E, Cabelof DC, Matherly LH, Zhao R, Akabas MH, and Goldman ID (2007) Rodent intestinal folate transporters (SLC46A1): secondary structure, functional properties, and response to dietary folate restriction. *Am J Physiol Cell Physiol* **293**:C1669–C1678.
- Salazar MD and Ratnam M (2007) The folate receptor: what does it promise in tissue-targeted therapeutics? *Cancer Metastasis Rev* **26**:141–152.
- Sharif KA and Goldman ID (2000) Rapid determination of membrane transport parameters in adherent cells. *BioTechniques* **28**:926–928, 930, 932.
- Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, and Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82**:1107–1112.
- Westerhof GR, Schornagel JH, Kathmann I, Jackman AL, Rosowsky A, Forsch RA, Hynes JB, Boyle FT, Peters GJ, Pinedo HM, et al. (1995) Carrier- and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecular-structure and biological activity. *Mol Pharmacol* **48**:459–471.
- Wike-Hooley JL, Haveman J, and Reinhold HS (1984) The relevance of tumour pH to the treatment of malignant disease. *Radiother Oncol* **2**:343–366.
- Wollack JB, Makori B, Ahlawat S, Koneru R, Picinich SC, Smith A, Goldman ID, Qiu A, Cole PD, Glod J, et al. (2008) Characterization of folate uptake by choroid plexus epithelial cells in a rat primary culture model. *J Neurochem* **104**:1494–1503.
- Wong SC, Zhang L, Proefke SA, and Matherly LH (1998) Effects of the loss of capacity for N-glycosylation on the transport activity and cellular localization of the human reduced folate carrier. *Biochim Biophys Acta* **1375**:6–12.
- Yang CH, Dembo M, and Sirotnak FM (1983) Relationships between carrier-mediated transport of folate compounds by L1210 leukemia cells: evidence for multiplicity of entry routes with different kinetic properties expressed in plasma membrane vesicles. *J Membr Biol* **75**:11–20.
- Zhao R, Babani S, Gao F, Liu L, and Goldman ID (2000) The mechanism of transport of the multitargeted antifolate, MTA-LY231514, and its cross resistance pattern in cell with impaired transport of methotrexate. *Clin Cancer Res* **6**:3687–3695.
- Zhao R, Chattopadhyay S, Hanscom M, and Goldman ID (2004a) Antifolate resistance in a HeLa cell line associated with impaired transport independent of the reduced folate carrier. *Clin Cancer Res* **10**:8735–8742.
- Zhao R, Gao F, Hanscom M, and Goldman ID (2004b) A prominent low-pH methotrexate transport activity in human solid tumor cells: contribution to the preservation of methotrexate pharmacological activity in HeLa Cells lacking the reduced folate carrier. *Clin Cancer Res* **10**:718–727.
- Zhao R and Goldman ID (2003) Resistance to antifolates. *Oncogene* **22**:7431–7457.
- Zhao R and Goldman ID (2007) The molecular identity and characterization of a proton-coupled folate transporter-PCFT; biological ramifications and impact on the activity of pemetrexed. *Cancer Metastasis Rev* **26**:129–139.
- Zhao R, Hanscom M, Chattopadhyay S, and Goldman ID (2004c) Selective preservation of pemetrexed pharmacological activity in HeLa cells lacking the reduced folate carrier; association with the presence of a secondary transport pathway. *Cancer Res* **64**:3313–3319.
- Zhao R, Min SH, Qiu A, Sakaris A, Goldberg GL, Sandoval C, Malatack JJ, Rosenblatt DS, and Goldman ID (2007) The spectrum of mutations in the PCFT gene, coding for an intestinal folate transporter, that are the basis for hereditary folate malabsorption. *Blood* **110**:1147–1152.
- Zhao R, Russell RG, Wang Y, Liu L, Gao F, Kneitz B, Edelmann W, and Goldman ID (2001) Rescue of embryonic lethality in reduced folate carrier-deficient mice by maternal folic acid supplementation reveals early neonatal failure of hematopoietic organs. *J Biol Chem* **276**:10224–10228.

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