Enhanced Receptor-Mediated Endocytosis and Cytotoxicity of a Folic Acid-Desacetylvinblastine Monohydrazide Conjugate in a Pemetrexed-Resistant Cell Line Lacking Folate-Specific Facilitative Carriers but with Increased Folate Receptor Expression

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ABSTRACT

The reduced folate carrier (RFC), proton-coupled folate transporter (PCFT), and folate receptors (FR) are folate-specific transporters. Antifolates currently in the clinic, such as pemetrexed, methotrexate, and pralatrexate, are transported into tumor cells primarily via RFC. Folic acid conjugated to cytotoxics, a new class of antineoplastics, are transported into cells via FR-mediated endocytosis. To better define the role of PCFT in antifolate resistance, a methotrexate-resistant cell line, M160-8, was selected from a HeLa subline in which the RFC gene was deleted and PCFT was highly overexpressed. These cells were cross-resistant to pemetrexed. PCFT function and the PCFT mRNA level in M160-8 cells were barely detectable, and FR-α function and mRNA level were increased as compared with the parent cells. While pemetrexed rapidly associated with FR and was internalized within endosomes in M160-8 cells, consistent with FR-mediated transport, subsequent pemetrexed and (6S)-5-formyltetrahydrofolate export into the cytosol was markedly impaired. In contrast, M160-8 cells were collaterally sensitive to EC0905, a folic acid–desacetylvinblastine monohydrazide conjugate also transported by FR-mediated endocytosis. However, in this case a sulfhydryl bond is cleaved to release the lipophilic cytotoxic moiety into the endosome, which passively diffuses out of the endosome into the cytosol. Hence, resistance to pemetrexed in M160-8 cells was due to entrapment of the drug within the endosome due to the absence of PCFT under conditions in which the FR cycling function was intact.

Introduction

The B9 folate vitamins use folate transporters for absorption across the intestine and other epithelia, and transport into systemic tissues to meet mammalian requirements for these one-carbon donors. The reduced folate carrier (RFC-SLC19A1), the proton-coupled folate transporter (PCFT-SLC46A1), and two membrane-anchored folate receptors (FR-α and FR-β) are the only known folate-specific transporters (Zhao et al., 2011). Both the reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT) are members of the major superfamily of facilitative transporters, but FRs transport folates by an endocytic process. The major differences between RFC and PCFT are 1) their energetics, the former an organic phosphate antiporter and the latter a proton symporter, 2) their pH optima, the former at pH ∼7.4, the latter ∼pH 5.5, and 3) their substrate specificities for folic acid and PT523 (N4-4-amino-4-deoxypteroyl)-N6-hemiphthaloyl-L-ornithine). The affinity of RFC is very low for folic acid and very high for PT523, the affinity of PCFT is high for the former and very low for the latter, at their pH optima. Although both RFC and PCFT are ubiquitously expressed in tissues, their physiologic roles are different. RFC mediates folate transport into systemic tissues whereas PCFT plays a key role in intestinal folate absorption and folate transport from blood across the choroid plexus into the cerebrospinal fluid (Zhao et al., 2011). RFC and PCFT are also ubiquitously expressed in human cancers (Zhao et al., 2004b; Kugel Desmoulin et al., 2011). FR-α is expressed in epithelia (proximal renal tubule, choroid plexus, retinal pigment epithelium) (Elnakat and Ratnam, 2004; Kamen and Smith, 2004) and is widely expressed in epithelial cancers (Weitman et al., 1992; Parker et al., 2005); FR-β is expressed in hematopoietic malignancies (Ross et al., 1999). Their selective expression in human cancers is the basis for the development of agents that are transported primarily by this route: 1) folate analogs with high affinity for FRs but very low affinity for RFC (Gibbs et al., 2005; Wang et al., 2011, 2013); 2) macromolecular conjugates (Zhao et al., 2010). Antifolate-resistant cancer cells may achieve resistance by downregulating RFC expression and/or activating PCFT expression. FRs are the target of the folic acid–desacetylvinblastine monohydrazide conjugate, EC0905. FR-α and FR-β have been studied in a number of cell lines, including FR-α+ FR-β+ M160-8 cells, FR-α− FR-β− M160-25 cells, and FR-α− FR-β+ M160-8 cells (Shah et al., 2012). In M160-8 cells, FR-α expression is not detectable, whereas FR-β expression is upregulated as compared with the parent cell line, M160-25.

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ABBREVIATIONS: 5-CHO-THF, 5-formyltetrahydrofolate; DAVLBH, desacetylvinblastine monohydrazide; FR, folate receptor (FOLR); GAT, 200 μM glycine, 100 μM adenosine, 10 μM thymidine; HBS, HEPES-buffered saline; PCFT, proton-coupled folate transporter; PCR, polymerase chain reaction; PT523, N4-4-amino-4-deoxypteroyl)-N6-hemiphthaloyl-L-ornithine; RFC, reduced folate carrier; (6S)-5-CHO-THF, the active isomer of 5-formyltetrahydrofolate.

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2) folic acid conjugated to cytotoxic agents that are endocytosed, released within, and diffuse out of endosomes to inhibit their intracellular targets (Xia and Low, 2010).

Impaired RFC-mediated transport is a common mechanism of resistance to methotrexate because of the reduced expression of or mutations in the RFC gene (Zhao and Goldman, 2003). A spectrum of RFC mutations have been identified with methotrexate-selective pressure in vitro and in human tumors in vivo (Zhao and Goldman, 2003). However, the loss of RFC function does not impact on the sensitivity of tumor cells to pemetrexed when 5-formyltetrahydrofolate (5-CHO-THF) is the folic source in the growth medium (Zhao et al., 2004c; Chattopadhyay et al., 2006; Desmoulin et al., 2012). This is due to transport mediated by PCFT, which has a high affinity for this agent, and the contraction of the intracellular folate pool due to decreased transport of 5-CHO-THF, for which PCFT has a lower affinity.

Our study was designed to identify mutant forms of PCFT under methotrexate-selective pressure, augmented by chemical mutagenesis, to shed light on potential transport-related resistance mechanisms as well as provide insight into the structure/function of the carrier. This approach, used so productively to explore the structure-function of RFC in the past (Zhao and Goldman, 2003), has not yet been successful when directed to PCFT. We used a HeLa-derived stable transfectant that expresses high levels of PCFT in which the RFC gene was deleted. One of the cell lines that emerged was a methotrexate-resistant clone in which PCFT expression was lost but FR-α expression was increased. These cells were highly resistant to pemetrexed; however, the activity of a folic acid–desacetylvinblastine monohydrate (DAVLBH) conjugate was markedly augmented consistent with intact FR cycling.

### Materials and Methods

**Chemicals.** [3',5',7,9-H]Folic acid, [3',5',7-3H(N)]methotrexate, [3',5',7, 9-H(N)](6S)-5-CHO-THF, and generally-labeled [3H]pemetrexed were purchased from Moravek Biochemicals (Brea, CA). Unlabeled compounds used in this study were obtained from various sources: methotrexate (National Cancer Institute, Bethesda, MD), pemetrexed (LC Laboratories, Woburn, MA), raltitrexed (AstraZeneca, Cheshire, UK), PT253 (Dr. Andre Rosovsky, Dana-Farber Cancer Institute, Boston, MA), trimetrexate (Warner Lambert, Ann Arbor, MI), (6'R,S)- and (6S)-5-CHO-THF (Schires Laboratories, Jona, Switzerland), folic acid (Sigma-Aldrich, St. Louis, MO), and EC0905 and DAVLBH (Endocyte, West Lafayette, IN). Tritiated compounds were purified before use by liquid chromatography.

**Cells and Culture Conditions.** R1-11 cells are a HeLa subline in which the RFC gene is absent from the genome and PCFT mRNA is not expressed (Zhao et al., 2004b; Diop-Bove et al., 2009). Transfection of PCFT cDNA into R1-11 cells generated two different stable transfectants, R1-11PCFT-4 cells that express PCFT at a level similar to that of HeLa cells (Zhao et al., 2008), and R1-11-PCFT-h cells that express high levels of PCFT in which the RFC gene was deleted. One of the cell lines that emerged was a methotrexate-resistant clone in which PCFT expression was lost but FR-α expression was increased. These cells were highly resistant to pemetrexed; however, the activity of a folic acid–desacetylvinblastine monohydrate (DAVLBH) conjugate was markedly augmented consistent with intact FR cycling.

**Assessment of PCFT Function.** PCFT activity was determined by measuring the initial rate of uptake of [3H]methotrexate, [3H](6S)-5-CHO-THF in folate-free medium supplemented with GAT for 3 days. The cells were washed 3 times with ice-cold HEPES-buffered saline (HBS) and lysed in 0.5 ml 0.2 M NaOH at 65°C. Radioactivity in 0.4 ml of lysate was determined on a liquid scintillation spectrometer; the protein level in 0.02 ml of lysate was determined with the BCA protein assay (Pierce, Rockford, IL).

**Folate Acid Surface Binding.** Cells grown in 12-well plates were washed twice with ice-cold folate-free saline (20 mM HEPES, 5 mM dextrose, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, pH 7.4) and incubated in the same buffer at 37°C for 20 minutes. After aspiration of the incubation buffer, transport was initiated by the addition of 0.5 ml of prewarmed 20 mM 4-morpholinocethanesulfonic acid, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, pH 5.5, containing 0.5 μM tritiated compounds. After 1 or 2 minutes, influx was stopped by the addition of 5 ml of ice-cold HBS, the cells were washed 3 times with this solution, and then they were dissolved in 0.5 ml of 0.2 M NaOH at 65°C. Radioactivity in 0.4 ml of lysate was measured on a liquid scintillation spectrometer; the protein level in 0.02 ml of lysate was determined with the BCA protein assay (see above). Initial uptake rates are expressed in units of pmol/mg protein/min.

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washes, membrane-bound [3H]folate acid was released with the addition of 0.5 ml of cold acid buffer (pH 3.5), and the radioactivity in the acidic solution was determined. The cells were dissolved in 0.5 ml of 0.2 M NaOH, and 20 μl of lysate was used for protein determination with the BCA protein assay.

To assess the relative binding affinity of pemetrexed, (6S)-5-CHO-THF, and folate acid to the FR-α expressed in M160-8 cells, follic acid surface binding assays were performed in HBS (pH 7.4) or 20 nM 4-morpholineethanesulfonic acid, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, pH 5.5 (pH 6.5) with 20 nM [3H]folate acid in the presence or absence of unlabeled pemetrexed (25–400 nM), (6S)-5-CHO-THF (50–800 nM), or folic acid (6.25–100 nM). Surface binding in the absence of inhibitors is indicated as 100%.

**Assessment of Pemetrexed Accumulation over 2 Hours.** R1-11-PCFT-4, M160-8, R1-11-PCFT-4, and R1-11 cells grown in 12-well plates were washed twice with ice-cold acid buffer (pH 3.5) and then once with ice-cold HBS (pH 7.4). The cells were then preincubated for 20 minutes in 2 ml of prewarmed folate-free RPMI medium containing 10% dialyzed fetal bovine serum (∼pH 7.3) at 37°C. Cells were then exposed to 2 ml of folate-free RPMI medium containing 100 nM [3H]pemetrexed in the presence or absence of 1 μM folic acid for 2 hours in the 5% CO₂ incubator. The cells were washed three times with ice-cold acid buffer (pH 3.5) to remove surface bound drug, and then dissolved in 0.5 ml of 0.2M NaOH. The radioactivity of the internalized drug and the protein level were determined as described previously for the assessment for PCFT function.

**Comparison of Pemetrexed Bound to the Cell Surface and Pemetrexed Internalized as a Function of Incubation Time.** The R1-11-PCFT-4 and M160-8 cells’ growth media were aspirated, and the cells were exposed to prewarmed folate-free RPMI medium (∼pH 7.3) containing 100 nM [3H]pemetrexed for 0.5, 1, 2, 4, 6, and 16 hours in a 5% CO₂ incubator at 37°C. The cells were washed three times with ice-cold HBS (pH 7.4) before the surface-bound pemetrexed was released with a 5-minute exposure to 0.5 ml pH 3.5 ice-cold buffer. The cells were washed once with acid buffer (pH 3.5), and the tritium released with a 5-minute exposure to 0.5 ml pH 3.5 ice-cold buffer. The radioactivity of the remaining inside the cells was analyzed as indicated previously.

**Separation of Internalized Cytosolic and Membrane-Associated (Anti)folate Fractions.** PCFT-4 and M160-8 cells were seeded at a density of 0.3 × 10⁶ cells/well in 12-well plates. The next day, the medium was removed, and the cells grown in folate-free RPMI (pH 7.3) that contained 50 nM [3H]folate acid, 50 nM [3H]6S-5-CHO-THF, 50 nM [3H]pemetrexed, or 50 nM [3H]methotrexate. GAT was also included in the medium with methotrexate and pemetrexed to circumvent the cytotoxic effects of these drugs. Forty-eight hours later, the cells were washed once with ice-cold HBS (pH 7.4), once with ice-cold pH 3.5 buffer, and again with ice-cold HBS. Cells were treated with 0.5 ml of hypotonic buffer (0.5 M NaH₂PO₄, 1 mM EDTA, pH 7.0) for 10 minutes on ice and then detached from the plates by a cell lifter. The mixture was centrifuged for 5 minutes at 14,000g and 4°C. The supernatants containing the radioactivity in the cytosol were collected and counted, and the pellets containing the radioactivity in the membrane fraction were dissolved in 0.25 ml of 0.2 M NaOH before counting. The total protein was determined from cells seeded in additional wells at the same time and washed in the same way but not subjected to further treatment with hypotonic buffer.

**Measurements of PCFT and FR mRNA Levels by Real-Time Polymerase Chain Reaction and Sequencing of the Coding Region of FR-α.** Total RNA was isolated from PCFT-h, PCFT-4, M160-8, and R1-11 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s procedures. RNA (5 μg) was reverse-transcribed into cDNA using oligo(dT)₁₂₋₁₅ primers with Superscript II Reverse Transcriptase (Invitrogen) following the recommended procedure. Quantitative polymerase chain reaction (PCR) was performed with SYBR Green Master Mix (Applied Biosystems, Warrington, UK) and analyzed at the Albert Einstein Cancer Center Genomics Facility (Bronx, NY). The relative gene expression data were generated using the 2⁻ΔΔCt method. The primers used were 5’-CACTCTACCCACGCATCTGAC-3’ and 5’-GATCAGGCTTTCCCAGATCCT-3’ for PCFT (Gonen and Assaraf, 2010); 5’-CAAGGTCAAGGAACTACAGCCAG-3’ and 5’-CATGGCTGCAGGATACACTCCG-3’ for both FR-α and FR-β (Ashokkumar et al., 2007); 5’-AGGAGACTAGTGCCTGGCAGG-3’ and 5’-TGTGCTGCCTGGCCGCTGCATG-3’ for FR-α only (Jwala et al., 2012); 5’-CTGCTCCTTGTCGATGC-3’ and 5’-GCCAGCTCTGTTATCCA-3’ for FR-β only (Qi and Ratnam, 2006); and 5’-CCACCTTGTCAAGCACTGTA-3’ and 5’-CCCTGCTGCTCTAGCAAACTAT-3’ for G3PDH (Qi et al., 2006).

For sequencing the entire coding region of FR-α, a fragment covering the whole coding region was amplified using cDNA obtained from the M160-8 cells with Taq polymerase (Qiagen, Valencia, CA) and primers 5’-TCAAGGTCCTAGATTCTCCTGCGTCGCCATCAGG-3’ and 5’-TCTGATAGCAGGATGTCCTGATGCAAAGG-3’ which contain Hind III and Xba I restriction sites, respectively. After gel purification, the PCR product was either sequenced directly or digested with the restriction enzymes, cloned into pcDNA 3.1 (Invitrogen), then sequenced at the Albert Einstein Cancer Center Genomics Facility.

**Statistical Analysis.** Statistical comparisons were performed by one-tail paired t test using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). P ≤ 0.05 was considered statistically significant.

**Results**

**Rationale for the Selection Strategy.** A goal of this study was to obtain cell lines that harbor loss-of-function mutations in the PCFT gene that result in resistance to methotrexate. PCFT-h stable transfectants, generated by transfection of PCFT cDNA into R1-11 cells, were the starting point for chemical mutagenesis, followed by methotrexate-selective pressure. The following factors were considered in this strategy. 1) RFC is not expressed in the PCFT-h cells due to deletion of the gene so that influx in this cell line is mediated solely by PCFT (FR is minimally expressed in HeLa variants grown under these conditions). 2) Generation of a PCFT mutation that alters total PCFT-mediated uptake is more likely to occur in stable transfectants that usually have one copy of the gene than in tumor cells in which multiple copies of the gene may exist, as is the case in wild-type HeLa cells (endogenous PCFT expression is silenced in R1-11 cells) (Diop-Bove et al., 2009). 3) Mutations in the PCFT gene must be substrate-selective because a complete loss of PCFT function in the absence of RFC would result in reversion to R1-11 cells, which could not survive with 25 nM 5-CHO-THF as the sole folate source in the selection growth medium (Zhao et al., 2008).

**Growth Inhibition of M160-8 Cells by Antifolates with Different Characteristics.** The cell line M160-8, obtained under selective pressure with 160 nM methotrexate, was randomly chosen for study because it had a resistance and sensitivity pattern to methotrexate and trimetrexate, respectively, representative of many of the lines identified in the initial screenings. As indicated in Fig. 1A, M160-8 cells (IC₅₀ ~2500 nM) were 83-fold resistant to methotrexate relative to R1-11-PCFT-h cells (IC₅₀ ~30 nM) and 5-fold resistant to both R1-11-PCFT-4 cells (IC₅₀ = 500 nM), which express a level of PCFT comparable to wild-type HeLa cells. In contrast, M160-8 cells (IC₅₀ = 4 nM) were 50-fold more sensitive to trimetrexate, a lipophilic dihydrofolate reductase inhibitor, than R1-11-PCFT-h cells (IC₅₀ = 200 nM) and 4-fold more sensitive to trimetrexate than R1-11-PCFT-4 cells (IC₅₀ = 16 nM) (Fig. 1B).

Because trimetrexate diffuses freely across the cell membrane and its activity is inversely proportional to the intracellular folate pool (Zhao et al., 2001), the high sensitivity of M160-8 cells to trimetrexate is consistent with a marked contraction of the intracellular folate pool as compared with R1-11-PCFT-h and
R1-11-PCFT-4 cells, as reported previously for other HeLa cell lines under these conditions (Zhao et al., 2004a) and confirmed experimentally (see below).

A similar but quantitatively smaller pattern was also observed for another dihydrofolate reductase inhibitor, PT523, which has an extremely low affinity for PCFT (Fig. 1C). The increased sensitivity of M160-8 cells to trimetrexate and PT523, as compared with R1-11-PCFT-h and R1-11-PCFT-4 cells, also indicated that the resistance to methotrexate cannot be attributed to alterations in dihydrofolate reductase, the target enzyme of these inhibitors. Rather, methotrexate resistance was due to a decrease in its transport moderated by a decrease in uptake of (6S)-5-CHO-THF, which resulted in a contraction of the intracellular folate pool.

The activity of pemetrexed and raltitrexed was also examined. Pemetrexed in its polyglutamate forms is primarily an inhibitor of thymidylate synthetase and, to a lesser extent, purine synthesis. Raltitrexed in its polyglutamate forms is an inhibitor of thymidylate synthase alone. Pemetrexed has a much higher affinity for PCFT than either methotrexate or

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**Fig. 1.** Growth inhibition by methotrexate (A), trimetrexate (B), PT523 (C), pemetrexed (D), and raltitrexed (E). Cells were seeded in 96-well plates before a spectrum of antifolate concentrations was added the next day and exposure was continued for 5 days. Growth inhibition is expressed as percentages of control growth in the absence of drugs. Data are the mean ± S.E.M. from three independent experiments for all panels.
statistically significant \((P = 0.049)\) that of R1-11-PCFT-4 cells (Fig. 2B). The 2-fold increase in (6S)-5-CHO-THF uptake in M160-8 cells relative to R-11 cells was not statistically significant \((P = 0.30)\). Pemetrexed uptake in R1-11-PCFT-h cells was 48-fold greater than that in PCFT-4 cells; uptake in M160-8 cells was 22% \((P = 0.047)\) that of R1-11-PCFT-4 cells, but this was 2.3-fold \((P = 0.015)\) greater than uptake in R1-11 cells. Hence, PCFT-mediated influx in M160-8 cells was negligible as compared with R1-11-PCFT-h and R1-11-PCFT-4 cells for all three substrates tested.

Consistent with the lack of PCFT function in M160-8 cells, the PCFT mRNA level in these cells was only 1.8% that of PCFT-4 cells and was essentially comparable to that of R11 cells (Table 1). As expected, the PCFT mRNA level in PCFT-h cells was 132-fold greater than that in PCFT-4 cells. FR uptake in M160-8 cells was 22% \((P = 0.047)\) that of R1-11-PCFT-h cells, and 3) the FR mRNA level in M160-8 cells was not significantly different from that in R-11 cells. Hence, PCFT-mediated influx in M160-8 cells was negligible as compared with R1-11-PCFT-h and R1-11-PCFT-4 cells for all three substrates tested.

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**Folate Receptor Expression.** The potential contribution of FR to the uptake observed in these cell lines was examined. Focusing first on surface binding (Fig. 3A), we found a low but comparable level of folic acid binding in R1-11-PCFT-h and R1-11-PCFT-4 cells. Surface binding in R1-11 cells was slightly but statistically significantly \((P = 0.035\) and 0.048, respectively), higher relative to both R1-11-PCFT-h and R1-11-PCFT-4 cells. Surface binding in M160-8 cells was increased 12-fold as compared with R1-11-PCFT-h cells from which M160-8 cells were selected. The surface binding was highly specific, as addition of 1 \(\mu M\) unlabeled folic acid completely blocked the binding of tritiated folic acid (5 nM) in all the cell lines. FR surface binding capacity in M160-8 cells at 3 pmol/mg protein or 0.75 pmol/million cells (based on 4.1 million cells/mg determined in this laboratory) is slightly lower than that in monkey kidney cells (~2 pmol/million cells) and much lower than that in human epidermoid carcinoma cells (~200 pmol/million cells) (Kamen and Smith, 2004).

Quantitative PCR with a pair of primers that amplified both FR-\(\alpha\) and FR-\(\beta\) cDNA showed an 8-fold increase in the FR mRNA level in M160-8 cells as compared with R1-11-PCFT-4 and R1-11-PCFT-h cells. Using primers specific for FR-\(\alpha\) cDNA or primers specific for FR-\(\beta\) cDNA, it was determined that 1) the FR-\(\beta\) mRNA level in R1-11-PCFT-h cells was 2% that of the FR-\(\alpha\) mRNA level, 2) the FR-\(\beta\) mRNA level in M160-8 cells was not significantly different from that in R1-11-PCFT-h cells, and 3) the FR-\(\alpha\) mRNA level in M60-8 cells was increased 6.8-fold as compared with R1-11-PCFT-h cells. Therefore, only FR-\(\alpha\) was overexpressed in M160-8 cells. A 12-fold surface overexpression of FR-\(\alpha\) and a 6.8-fold increase in FR-\(\alpha\) mRNA level in M60-8 cells as compared with R1-11-PCFT-h cells is consistent with overexpression of FR-\(\alpha\) at the transcriptional or, more likely, posttranscriptional level, as previously reviewed by Elnakat and Ratnam (2004).
To exclude the possibility of FR-α mutations, because the M160-8 cells were obtained after the PCFT-h cells had been treated with the mutagen, the entire FR-α coding region was PCR-amplified from cDNA obtained from these cells. Automated sequencing of the total PCR product did not reveal any mutation in the coding region. This result was further confirmed by sequencing 11 independent FR-α cDNA clones constructed in the vector. Seven of 11 clones had no mutation at all, but 4 of 11 each had one unrelated mutation that was generated by Taq polymerase. Thus, FR-α in M160-8 cells was wild type.

Studies then assessed the extent to which FR was active in terms of delivering pemetrexed into the cells. After cells were incubated with 100 nM [3H]pemetrexed in folate-free RPMI medium (pH 7.4) for 2 hours, surface-bound pemetrexed was stripped off and washed away with acidic buffer (pH 3.5) so that what remained was pemetrexed that had been internalized. As indicated in Fig. 3B, pemetrexed uptake into R1-11-PCFT-h cells was ~10-fold greater than that in R1-11-PCFT-4 cells, as expected due to the high level of PCFT expression. The pemetrexed uptake into R1-11 cells that do not express PCFT was negligible. Pemetrexed uptake into M160-8 cells was virtually the same as that into R1-11-PCFT-4 cells.

However, although addition of 1 μM unlabeled folic acid did not suppress uptake into the R1-11-PCFT-h or R1-11-PCFT-4
cells (P = 0.095 and 0.13, respectively), it markedly suppressed (75%) pemetrexed uptake into M160-8 cells (P = 0.028). Hence, pemetrexed uptake into R1-11-PCFT-h and R1-11-PCFT-4 cells was mediated solely by PCFT while uptake in M160-8 cells was predominantly FR-α-mediated over a 2-hour interval. (6S)-5-CHO-THF Accumulation and Growth Requirement. M160-8 cells were selected under conditions in which 5-CHO-THF was the sole folate source in the growth medium. Accumulation of (6S)-5-CHO-THF was compared in M160-8 cells, R1-11-PCFT-h, R1-11-PCFT-4, and R1-11 cells. As indicated in Fig. 3C, (6S)-5-CHO-THF accumulation in R1-11-PCFT-h cells was 40% higher (P = 0.0048) than that in R1-11-PCFT-4 cells. This is expected due to the higher expression of PCFT in the former cells and is consistent with the weaker growth inhibition produced by trimetrexate and PT523 (Fig. 1). (6S)-5-CHO-THF accumulation in M160-8 cells was only 30% that in R1-11-PCFT-4 cells but was 6.6-fold higher than that in R1-11 cells. Because the cells were not stripped with acidic buffer and thus the FR on the cell surface of M160-8 cells was occupied by tritiated (6S)-5-CHO-THF, the value determined in M160-8 cells was higher than the level actually accumulated inside the cells.

The growth requirement for (6S)-5-CHO-THF in these cells is indicated in Fig. 3D. A near normal growth rate was reached at the lowest concentration of (6S)-5-CHO-THF for R1-11-PCFT-h cells, but 10 nM (6S)-5-CHO-THF was required for near maximum growth of R1-11-PCFT-4 cells. For both M160-8 and R1-11 cells, the growth rate was decreased even at the second highest concentration (667 nM). However, statistically significantly higher growth rates (P < 0.05) were observed throughout the (6S)-5-CHO-THF concentration range in M160-8 as compared with R1-11 cells. Hence, functional FR in M160-8 cells renders a growth advantage as compared with R1-11 cells to account for the ability 5-CHO-THF to sustain the viability of these cells; however, it is far from the efficiency observed in R1-11-PCFT-4 cells that express constitutive levels of PCFT. An Analysis of the Time-Dependence of Pemetrexed Surface Binding and Cellular Uptake. Pemetrexed uptake in R1-11-PCFT-4 and M160-8 cells was further monitored over an extended interval to 16 hours in folate-free RPMI medium (pH 7.4). R1-11-PCFT-4 cells instead of R1-11-PCFT-h cells were chosen for this study because pemetrexed influx in the latter cells was too rapid to make a meaningful comparison with M160-8 cells. In these experiments, both surface-bound pemetrexed, stripped off by the acidic buffer, and pemetrexed remaining inside cells after the acidic wash were assessed. As indicated in Fig. 4A, pemetrexed uptake into R1-11-PCFT-4 cells increased rapidly over the interval of observation, consistent with continued uptake and subsequent formation and retention of polyglutamate derivatives within these cells (Zhao et al., 2004c). Pemetrexed bound at the cell surface was negligible. Pemetrexed uptake into M160-8 cells increased very slowly and achieved a much lower level (∼10 pmol/mg protein) than in R1-11-PCFT-4 cells. As indicated in the expanded scale of Fig. 4B, pemetrexed associated with the cell surface peaked early then decreased slowly while the drug delivered into the cells was initially lower than the bound fraction exceeding that level beyond 4 hours. Hence, FR-α-mediated pemetrexed uptake in M160-8 cells was much slower than the PCFT-mediated process in R1-11-PCFT-4 cells under these conditions.

The Pattern of Folate/Antifolate Uptake Associated with the Cytosolic and Membrane Fractions. One obvious difference between PCFT- and FR-mediated pemetrexed uptake is that PCFT delivers pemetrexed directly to the cytosol while during FR-mediated endocytosis the drug is internalized bound to FR within the endosome, after which, with acidification, it is released then exported across the endosomal membrane into the cytosol. Although pemetrexed located in the endosome is “intracellular,” it is not accessible to its target enzyme in the cytosol and is thus pharmacologically inert.

We examined the distribution of pemetrexed associated with M160-8 and R1-11-PCFT-4 cells after a 2-day exposure by separating the cytosol from the membrane fraction. Again, R1-11-PCFT-4 cells rather than R1-11-PCFT-h cells were chosen for this study due to the similarity of their influx activity to that of M160-8 cells. This was also assessed for methotrexate, folic acid, and (6S)-5-CHO-THF. As indicated in Fig. 5, accumulation of pemetrexed in either the cytosol or membrane fraction was greater than that of 5-CHO-THF and was much greater than those of methotrexate and folic acid in R1-11-PCFT-4 cells; the level in the cytosol was much higher than in the membrane fraction regardless of the substrate. In contrast, the accumulation of pemetrexed and (6S)-5-CHO-THF in the cytosol of M160-8 cells was much lower than that in R1-11-PCFT-4 cells, and the distribution of the various

![Fig. 4.](image-url) Comparison of cell surface-bound and internalized pemetrexed after exposure to tritiated pemetrexed for various intervals (A). Confluent M160-8 and PCFT-4 cells were exposed to 100 nM [3H]pemetrexed in folate-free RPMI medium (pH 7.3, 37°C) in a 5% CO2 incubator for 0.5, 1, 2, 4, 6, and 18 hours before the cells were washed 3 times with HBS (pH 7.4) to remove unbound pemetrexed. Both the cell surface–bound pemetrexed, which was released by 0.5 ml of acid buffer (pH 3.5) at 0°C, and pemetrexed uptake into cells were assessed. (B) Amplification of the y-axis to facilitate comparison between cell surface–bound pemetrexed among M160-8, R1-11-PCFT-4 cells and pemetrexed uptake in M160-8 cells. Data are the mean ± S.E.M. from three independent experiments.
substrates between the cytosol and membrane fractions was also very different. Hence, pemetrexed was equally divided in the cytosol and membrane fractions. The level of 5-CHO-THF in the cytosol was slightly lower than that in the membrane fraction, but the difference did not reach statistical significance ($P = 0.25$). However, the folic acid level in the membrane fraction was 4.5-fold greater ($P = 0.035$) than in the cytosol of M160-8 cells, indicating that only a small amount of folic acid escaped the endosomes over 2 days. In contrast, for methotrexate, the membrane fraction was only 10% that of the cytosol. The levels of pemetrexed, folic acid, and (6S)-5-CHO-THF in the membrane fraction of M160-8 cells were similar. Hence, even after 2 days, FR-α-mediated uptake of pemetrexed in M160-8 cells was only 15% the level achieved in PCFT-4 cells and half the level associated with the cells within the membrane fraction.

It should be pointed out that the membrane fraction prepared in our study contained a variety of cell organelles, including endosomes. Hence, folic acid/antifolates found in the membrane fractions of R1-11-PCFT-4 cells reflect these compounds bound to, or transported into, these organelles.

**Relative FR-Binding Affinities among Pemetrexed, Folic Acid, and (6S)-5-CHO-THF.** To further assess the basis for the impaired FR-mediated transport in M160-8 cells, the relative affinity of these substrates for the receptor was evaluated by their ability to compete with [3H]folic acid for binding to the surface of M160-8 cells. As indicated in Fig. 5B, the concentration of folic acid, pemetrexed, and (6S)-5-CHO-THF required to inhibit [3H]folic acid (20 nM) surface binding by 50% in M160-8 cells was 18, 70, and 140 nM, respectively. Hence, the affinity of pemetrexed for FR-α in M160-8 cells was 25% that of folic acid but twice that of (6S)-5-CHO-THF. The relative affinity of pemetrexed or (6S)-CHO-THF and folic acid was also assessed at pH 6.5, reflective of the average pH found in FR-containing endosomes (Yang et al., 2007). As indicated in Fig. 5C, the affinity of pemetrexed relative to folic acid was preserved, but the affinity of (6S)-CHO-THF was slightly reduced relative to folic acid at this pH as compared with neutral pH.

**Growth Inhibition by EC0905.** EC0905 is a hybrid molecule in which folic acid and DAVLBH are connected through a hydrophobic linker containing a cleavable disulfide bond (Dhawan et al., 2013) (Fig. 6). This molecule is similar to EC145 (vintafolide), which has advanced to phase III and phase II clinical trials for the treatment of ovarian and nonsmall-cell lung cancers, respectively (Vlahov et al., 2006; Reddy et al., 2007; Pribble and Edelman, 2012; Naumann et al., 2013). This chemical modification does not alter the interaction between folic acid and FR-α so that the complex is endocytosed; with a sufficient increase in the endosomal reducing potential, the cleavable bond is broken, and DAVLBH diffuses out of the endosome to disrupt cellular microtubules and achieve its cytotoxic effect (Yang et al., 2006).

As indicated in Fig. 7A, M160-8 cells (IC50, ~0.25 nM) were 240-times more sensitive to continuous exposure to EC0905 than either R1-11-PCFT-h or R1-11-PCFT-4 cells (IC50, ~60 nM). Inclusion of 20 μM folic acid, which blocks binding to the FR, completely abolished the increased sensitivity of M160-8 cells to EC0905 whereas there was no change in the sensitivity of R1-11-PCFT-h or R1-11-PCFT-4 cells to this agent (Fig. 7B). M160-8, R1-11-PCFT-h, and R1-11-PCFT-4 cells were equally sensitive to DAVLBH in the absence (Fig. 7C) or presence (Fig. 7D) of 20 μM folic acid. The results with wild-type HeLa cells, from which R1-11-PCFT-4 and R1-11-PCFT-h were derived, were the same as for R1-11-PCFT-4 and R1-11-PCFT-h cells (data not shown).

It is noteworthy that the EC0905 IC50 (~0.25 nM) in M160-8 cells was 8.5% that of the DAVLBH IC50 (~3 nM), consistent with the effectiveness of FR-α-mediated delivery of DAVLBH into the cells as compared with the passive diffusion of the drug alone. Inhibition of the growth of R1-11-PCFT-4 or R1-11-PCFT-h cells by EC0905 (IC50, ~70 nM) is consistent with a small non–FR-α-mediated component of uptake of this drug or by its partial cleavage to DAVLBH in the medium followed by passive diffusion of DAVLBH into these cells.

**Discussion**

This study was designed to select PCFT mutants with impaired transport of methotrexate but sufficient uptake of 5-CHO-THF, the folic source in the medium, to sustain survival of the cells. However, what occurred under methotrexate-selective pressure was the loss of PCFT function in the M160-8 cell line due to a complete loss of PCFT mRNA with a level of residual activity indistinguishable from that of R1-11 cells that lack both RFC and PCFT, and from which the PCFT-h cells and PCFT-4 cells were derived. Because R1-11 cells cannot survive with 25 nM 5-CHO-THF, the folic source during the selection procedure, the M160-8 cells apparently kept their folate requirement by up-regulating FR-α expression and activity.

The high cross-resistance to pemetrexed in M160-8 cells as compared with R1-11-PCFT-4 cells was unexpected. 1) The primary target of pemetrexed, thymidylate synthase, was not altered in M160-8 cells, as the selection was conducted against methotrexate, a dihydrofolate reductase inhibitor, and the M160-8 and R1-11-PCFT-4 cells had comparable sensitivity to raltitrexed, a pure thymidylate synthetase inhibitor. 2) Pemetrexed activity increases as the intracellular folate pools decreases (Zhao et al., 2001, 2004c; Chattopadhyay et al., 2006). In this case, pemetrexed activity decreased under conditions in which the folate pool decreased. Hence, the marked increase in the growth inhibitory activity of trimetrexate in M160-8 cells was indicative of a marked contraction of the intracellular folate pools, as confirmed by the low level of [3H]5-CHO-THF that accumulated in these cells. 3) FR-α overexpressed in M160-8 cells was clearly functional to the extent that EC0905 was endocytosed and released into the cytosol. However, that function did not extend to pemetrexed or to (6S)-5-CHO-THF.

For transport processes mediated by the facilitative carriers PCFT or RFC, folate or antifolate substrates are delivered directly to the cytosol. For transport mediated by FR-α, an endocytic process, folate substrates are delivered first into the endosomes, from which they must exit to reach the cytosol. Hence, effective FR-α-mediated pemetrexed transport would require successful execution each of following steps.

1. Binding of pemetrexed to the receptor. This appeared to be intact because the level of folic acid and pemetrexed bound to the surface of M160-8 cells was similar (Figs. 3A and 4B).

2. Formation of the endosomes so that pemetrexed bound to FR is enclosed in the vesicles. This step was highly efficient in M160-8 cells because within 2 hours there was as much pemetrexed accumulated inside cells as bound to the surface.
3. Dissociation of pemetrexed from FR-α upon the acidification of the endosome. Because pemetrexed has a much lower affinity than folic acid for FR-α at both neutral and acidic pH, pemetrexed dissociation from FR-α in the endosomes should have occurred. This is even more relevant to (6S)-5-CHO-THF, which has an even lower affinity for FR-α than pemetrexed.

4. Transport of unbound pemetrexed out of the endosome. Further evidence that steps 1 and 2 were intact is the marked enhancement of the pharmacologic activity of EC0905 in the M160-8 cells. The folic acid domain of the EC0905 molecule need not dissociate from FR; rather, if the endosomal reducing potential is sufficient to cleave the sulfhydryl bond, the lipophilic DAVLBH is released and then passively diffuses out of the endosome (Yang et al., 2006). Because both pemetrexed and (6S)-5CHO-THF appeared to be trapped in the endosomes in M160-8 cells, step 4 was likely defective for both substrates, consistent with the lack of PCFT expression in M160-8 cells.

FR-α has a high affinity for folic acid with a $K_d$ of $<1$ nM. It has a lower but still high affinity for 5-methyltetrahydrofolate, the physiologic blood folate, and (6S)-5-CHO-THF, the sole folate source in the medium throughout the current study (Wang et al., 1992; Brigle et al., 1994). During endocytosis, folic acid is not considered to dissociate from FR in the endosome at the level of pH achieved within these vesicles (Kamen and Smith, 2004), consistent with the observation that little $[^{3}H]$folic acid escaped the vesicles (Fig. 5). A pH of 3.5 was required to release most FR-bound folic acid in MA104 cells (Kamen and Smith, 2004). An earlier study reported pemetrexed to have an affinity for murine FR-α 50% higher than folic acid based upon a competitive inhibition assay of surface bound folic acid at pH 7.4 in murine leukemia cells (Westerhof et al., 1995). However, the same assay conducted in M160-8 cells at both pH 7.4 and 6.5 indicated that the affinity of pemetrexed for FR-α is only 20% that of folic acid. The basis for this discrepancy is not clear. It could be due to differences between the human and murine receptors; the possibility that this was due to mutations in FR-α in M160-8 cells was excluded.

A possible role for PCFT in FR-mediated folate transport was implied by elucidation of the genetic basis of two related autosomal recessive disorders, hereditary folate malabsorption (Online Mendelian Inheritance in Man or OMIN-229050) and cerebral folate transport deficiency (OMIN-61308). Folate levels in the cerebrospinal fluid are very low in these disorders even when the blood folate level is normal, as occurs in subjects with cerebral folate deficiency and when subjects with hereditary folate malabsorption are treated with folates, consistent with a defect in the transport of folates across the choroid plexus. Hereditary folate malabsorption is caused by loss-of-function mutations in the PCFT gene (Diop-Bove et al., 2011), whereas cerebral folate transport deficiency is due to loss-of-function mutations in the FR-α gene (Cario et al., 2009; Dorn et al., 2009; Grapp et al., 2012). One explanation for a role for

Fig. 5. Comparison of folates or antifolates accumulated in the cytosolic and membrane fractions, and inhibitory effects of pemetrexed, (6S)-5-CHO-THF and folic acid on binding of $[^{3}H]$folic acid to FR at the cell surface at pH 7.4 and 6.5. (A) Cells were exposed to 50 nM tritiated pemetrexed, 5-CHO-THF, folic acid, or methotrexate for 2 days in folate-free RPMI-medium in a 5% CO₂ incubator. GAT was added to cell cultures during exposure to pemetrexed and methotrexate to prevent cytotoxicity mediated by these agents. The data for PCFT-4 cells are indicated on the left side of the graph, and the values for M160-8 cells are shown on the right side of the graph. (B) $[^{3}H]$Folic acid bound to the surface of M160-8 cells was assessed with 20 nM $[^{3}H]$folic acid in the presence or absence of unlabeled pemetrexed, (6S)-5-CHO-THF, or folic acid. Data are the mean ± S.E.M. from three independent experiments in both panels. (C) Comparison of 20 nM $[^{3}H]$folic acid bound to the surface of M160-8 cells at pH 7.4 and pH 6.5 in the presence or absence of various concentrations of unlabeled folic acid, pemetrexed, or (6S)-5-CHO-THF; *P < 0.05 for the comparison in A and C.
these two distinct transporters, encoded by different genes, in this process is that PCFT and FR-α functions are coupled and that PCFT is required for the export of folates from endosomes within the choroid plexus. Evidence that PCFT plays a role in endosomal export was observed in HeLa cells in which FR-α was expressed in the presence or absence of PCFT (Zhao et al., 2009). Lack of PCFT markedly impaired but did not completely eliminate transport of 5-methyler tetrahydrofolate or (6S)-5-CHO-THF out of endosomes, consistent with PCFT-dependent and independent processes. The presence of an alternative folate endosomal export mechanism seems likely, based upon the observation that an FR-α–targeted antifolate is active even in the absence of PCFT (Wang et al., 2011, 2013).

The level of FR-α expressed on M160-8 cells is between 3 and 4 pmol/mg protein, based on the binding of folic acid or pemetrexed to the cell surface (Figs. 3A and 4). This is equivalent to 0.73–0.98 pmol/million cells, using the conversion factor of 4.1 million cells per mg protein determined for M160-8 cells in this laboratory. Although the level of FR-α in M160-8 cells is much lower as compared with that in human epidermoid carcinoma cells (200 pmol/million cells (Kamen and Smith, 2004), FR-α–mediated transport in M160-8 cells
was sufficient to produce marked sensitivity to EC0905. It is possible that a high rate of FR-α-mediated endocytosis, the role of PCFT, and the transport of pemetrexed (PMX) and EC0905 in M160-8 cells. In wild-type cells, pemetrexed is exported from endosomes via PCFT. It can be also enter cells by this mechanism even at neutral pH. In M160-8 cells, pemetrexed is endocytosed by the receptor, but, upon dissociation, it is trapped within the endosome in the absence of PCFT. This is the case also for (6S)-5-CHO-THF (not shown). In contrast, there is a high level of FR-mediated transport of EC0905. In this case, the disulfide bond linking folinic acid and DAVLBH is reduced within the endosome, releasing the lipophilic DAVLBH, which exits the endosome by passive diffusion, a PCFT-independent process.

Fig. 8. A diagram illustrating FR-mediated endocytosis, the role of PCFT, and the transport of pemetrexed (PMX) and EC0905 in M160-8 cells. In wild-type cells, pemetrexed is exported from endosomes via PCFT. It can be also enter cells by this mechanism even at neutral pH. In M160-8 cells, pemetrexed is endocytosed by the receptor, but, upon dissociation, it is trapped within the endosome in the absence of PCFT. This is the case also for (6S)-5-CHO-THF (not shown). In contrast, there is a high level of FR-mediated transport of EC0905. In this case, the disulfide bond linking folinic acid and DAVLBH is reduced within the endosome, releasing the lipophilic DAVLBH, which exits the endosome by passive diffusion, a PCFT-independent process.

In summary, a unique cell line, M160-8, was obtained in which both facilitative folate transporters, RFC and PCFT, were absent, but functional FR-α was expressed. These cells were resistant to pemetrexed due to retention of the drug in the endosomal compartment, as illustrated in Fig. 8. There was comparable impairment of FR-α-mediated transport of 5-CHO-THF that was consistent with a high growth requirement. Because both pemetrexed and (6S)-5-CHO-THF have a much lower affinity for FR-α than does folic acid and should dissociate from FR-α in the endosomes, the retention of these substrates in this compartment is attributed to the lack of PCFT-mediated export from endosomes into the cytosol. Despite the failure of this aspect of FR-α-mediated transport and the modest level of FR-α expression in these cells, there was robust FR-α-mediated endocytosis of EC0905 as reflected in the high level of growth inhibition by this agent, a process independent of PCFT-mediated endosomal export.

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