Characterization of a Folate Transporter in HeLa Cells with a Low pH Optimum and High Affinity for Pemetrexed Distinct from the Reduced Folate Carrier

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ABSTRACT

Studies were undertaken to characterize a low pH transport activity in a reduced folate carrier (RFC)-null HeLa-derived cell line (R5). This transport activity has a 20-fold higher affinity for pemetrexed (PMX; \( K_t \approx 45 \text{ nmol/L} \)) than methotrexate (MTX; \( K_t \approx 1 \text{ \mu mol/L} \)) with comparable \( V_{\text{max}} \) values. The \( K_t \) values for folic acid, ZD9331, and ZD1694 were \( \approx 400-600 \text{ nmol/L} \), and the \( K_t \) values for PT523, PT632, and trimetrexate were \( >50 \text{ \mu mol/L} \). The transporter is stereospecific and has a 7-fold higher affinity for the 6\( S \) isomer than the 6\( R \) isomer of 5-formyltetrahydrofolate but a 4-fold higher affinity for the 6\( R \) isomer than the 6\( S \) isomer of dideazatetrahydrofolinic acid. Properties of RFC-independent transport were compared with transport mediated by RFC at low pH using HepG2 cells, with minimal constitutive low pH transport activity, transfected to high levels of RFC. MTX influx \( K_t \) was comparable at pH 7.4 and pH 5.5 (1.7 versus 3.8 \( \mu \text{mol/L} \)), but \( V_{\text{max}} \) was decreased 4.5-fold. There was no difference in the \( K_t \) for PMX (\( \approx 1.2 \text{ \mu mol/L} \)) or the \( K_t \) for folic acid (\( \approx 130 \text{ \mu mol/L} \)) or PT523 (\( \approx 0.2 \text{ \mu mol/L} \)) at pH 7.4 and pH 5.5. MTX influx in R5 and HepG2 transfecants at pH 5.5 was trans-stimulated in cells loaded with 5-formyltetrahydrofolate, inhibited by Cl\(^{-}\) (HepG2-B > R5), \( \text{Na}^+ \) independent, and uninhibited by energy depletion. Hence, RFC-independent low pH transport activity in HeLa R5 cells is consistent with a carrier-mediated process with high affinity for PMX. Potential alterations in protonation of RFC or the folate molecule as a function of pH do not result in changes in affinity constants for antifolates. Whereas both activities at low pH have similarities, they can be distinguished by folic acid and PT523, agents for which they have very different structural specificities.

INTRODUCTION

The major route for the membrane transport of folates and antifolates in mammalian cells is the reduced folate carrier (RFC) characterized largely in human and murine leukemia cell lines (1). RFC has a neutral pH optimum in these cells; however, there have been reports of process(es) with low pH optima that transport folates in solid tumors and cell lines of normal tissue origin (2–8). Recently, studies from this laboratory demonstrated the ubiquitous nature of low pH folate transport activity in a large number of human solid tumor cell lines from a variety of tissue origins (9). In the majority of these cell lines, transport at low pH was equal to or exceeded transport at physiologic pH. In only one cell line, the HepG2 hepatoma, was the low pH activity negligible. The low pH activity in HeLa cells is greater than transport at physiologic pH [(methotrexate (MTX) influx at pH 5.5 is 2\times greater than that at pH 7.4)]. In a derivative HeLa cell line, R5, obtained under MTX selective pressure, RFC was deleted from the genome, but transport at low pH was not reduced (9). Hence, in R5 cells, the low pH transporter is entirely independent of RFC. This low pH transport activity in R5 cells was shown to contribute to the inhibitory activity of MTX when cells were grown at a low pH (9).

The mechanism(s) underlying transport activities with low pH optima have not yet been clarified, although in intestinal cells there is some evidence to suggest that this may be related to RFC expression (10–14). The availability of the R5 cell line, in which transport at low pH is entirely RFC independent, provided an opportunity to characterize this process (one focus of this report). An additional issue addressed is the extent to which RFC-mediated transport may be altered at low pH due to changes in the protonation of the transporter or the folate/antifolate substrate molecule. These changes could obfuscate the properties of a RFC-independent low pH activity in cells in which both transport processes are expressed. To clarify this issue, HepG2 cells with low constitutive low pH activity (9) were transfected with RFC to achieve very high levels of carrier expression so that RFC-mediated transport activity at low pH was sufficient to allow its characterization. These properties were then compared with RFC-independent transport at low pH in HeLa R5 cells. Finally, these studies use and compare the transport properties of two antifolates: MTX, a classical inhibitor of dihydrofolate reductase; and pemetrexed (PMX), a novel new-generation antifolate, which, in its polyglutamyl forms, is a potent inhibitor of thymidylate synthase with lesser inhibitory potential at the level of glycaminide ribonucleotide formyltransferase (15).
MATERIALS AND METHODS

Chemicals. Radionabeled reagents included [3′,5′]-3H]MTX from Amersham (Piscataway, NJ) and [3H]PMX along with nonlabeled PMX, 6R- and 6S-dideazatetrahydrofolic acid (DDATHF), and LY309887 (6R-2′,5′-thienyl-5,10-dideazatetrahydrofolatic acid) provided by the Eli Lilly Co (Indianapolis, IN). All titrated reagents were purified before use and maintained at high purity by high-performance liquid chromatography (16). Nonlabeled folic acid was obtained from Sigma (St. Louis, MO). ZD1694 and ZD9331 were obtained from AstraZeneca (Wilmington, DE). Tridemtrate was provided by Dr. David Fry (Pfizer, New York, NY), aminopterin was provided by Dr. Barton Kamen (New Jersey Cancer Institute, New Brunswick, NJ), and PT523 and PT632 were provided by Dr. Andre Rosowsky (Dana-Farber Cancer Institute, Boston, MA). Nonlabeled MTX was provided by the National Cancer Institute (Bethesda, MD). The stereoisomers of 5-formyltetrahydrofolate (5-CHO-THF) were obtained from Schircks Laboratories (Jona, Switzerland). All other reagents were obtained in the highest purity available from commercial sources.

Cell Culture. Cells were maintained in RPMI 1640 containing 2.5 μmol/L folic acid, supplemented with 10% fetal calf serum (HyClone, Logan, UT), 2 mmol/L glutamine, 20 μmol/L mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. The R5 cell line, with a genomic RFC deletion, was maintained in the absence of MTX (9). A HepG2 cell line expressing high levels of RFC (HepG2-B) was obtained by transfection of human RFC cDNA (19). Experiments at pH 5.0 to pH 6.5 were performed in MBS (20 mmol/L 4-morpholinepropanesulfonic acid, 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L MgCl 2 , and 5 mmol/L glucose). NaCl-free sucrose buffer was used at pH 7.4 (20 mmol/L HBS) or pH 5.5 (20 mmol/L MBS) with 225 mmol/L sucrose, and pH was adjusted with Mg(OH) 2 . Na + -free MBS choline chloride buffer (20 mmol/L 4-morpholinepropanesulfonic acid, 140 mmol/L choline chloride, 5 mmol/L KCl, 2 mmol/L MgCl 2 , and 5 mmol/L glucose) was used at pH 5.5.

Before uptake determinations, cells were washed in ice-cold transport buffer, and then 1 mL of the buffer at 37°C was added, and cells were equilibrated at this temperature for 20 minutes. The buffer was then aspirated, and uptake was initiated by the addition of 0.5 mL of fresh buffer containing the radiolabeled antifolate and other reagents. For determination of transport kinetics, cells were incubated with 100 μmol/L racemic 5-CHO-THF in the appropriate buffer at 37°C for 25 minutes. The cells were then washed twice with ice-cold drug-free MBS buffer at pH 5.5, and then MBS at 37°C and pH 5.5 containing radioactive antifolate was added. Uptake was terminated by the addition of 5 mL of ice-cold HBS, and then cells were washed three times and lysed by the addition of 0.5 mL of 0.2N NaOH. Radioactivity was assessed on 0.4 mL of cell lysate to which chloroform was added. Protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Cell antifolate expression is measured as nanomoles per gram of protein.

Influx kinetics for [3H]MTX and [3H]PMX were determined with a time-course of uptake over 2 minutes, an interval over which initial uptake rates were constant. K i values for nonlabeled folates and antifolates were determined based on inhibition of 0.5 μmol/L [3H]MTX influx with concentrations of these agents adjusted to produce ~50% inhibition of MTX transport.

Transfections. The HepG2-B cell line was obtained by transfection of HepG2 cells with human RFC cDNA that included a hemagglutinin epitope at the COOH terminus (a gift from Dr. Larry Matherly; ref. 20). Transfection was carried out using LipofectAMINE Plus (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol after HepG2 cells were grown in 6-well plates to 50% to 75% confluence. After 2 days of growth, the cells were trypsinized, diluted 10-fold, and then incubated in RPMI 1640 containing G418 (600 μg/mL). After 2 weeks, surviving clones were picked up and replated, and RFC activity was assayed by determination of [3H]MTX influx. HepG2-B cells are the RFC-transfected clone. HepG2-D cells are the vector-transfected control clone.

RNA Isolation and Northern Blots. Total RNA was isolated from cells with TriZol reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA (25 μg) was resolved by electrophoresis on 1.2% agarose gels containing formaldehyde. RNA was transferred to Nytran N-membranes (Schleicher & Schuell, Keene, NH) and fixed with a Stratalinker UV-cross linker (Stratagene, La Jolla, CA). The blot was first probed with human RFC cDNA and then stripped and reprobed with β-actin cDNA as the loading control.

RESULTS

Influx Kinetics and Structural Specificity of the Low pH Transporter in Reduced Folate Carrier-Null HeLa R5 Cells. Uptake of MTX at pH 5.5 in RFC-null R5 cells is mediated by a RFC-independent process. Initial studies demonstrated that initial uptake rates for [3H]MTX and [3H]PMX were sustained for at least 2 minutes. This was the interval used for the determination of influx kinetics. As indicated in the top panel of Fig. 1, MTX influx was saturable with a K m of 1.03 ± 0.11 μmol/L and a V max of 1.37 ± 0.16 nmol/g protein/min. PMX had an ~20-fold higher affinity for this process, with an influx K m of 44.9 ± 5.0 nmol/L, but a comparable V max of 1.09 ± 0.14 nmol/g protein/min (Fig. 1, bottom panel). The K m for MTX

1 Unpublished observations.
Influx mediated by RFC in wild-type HeLa cells at pH 7.4 was ~2 μmol/L (data not shown).

Influx of 0.5 μmol/L [3H]MTX in R5 cells at pH 5.5 was markedly suppressed by 20 μmol/L folic acid but unaffected by 20 μmol/L PT523 (Fig. 2). At these concentration ratios, folic acid does not inhibit MTX influx mediated by RFC at pH 7.4, and PT523 markedly suppresses MTX influx mediated by RFC at pH 7.4 (see Fig. 5). Hence, these agents are good discriminators of RFC-mediated transport at neutral pH versus RFC-independent transport at low pH (see next section).

Table 1 indicates the inhibition constants for a variety of folates and antifolates in R5 cells determined at pH 5.5, based on their inhibition of [3H]MTX influx. The highest affinities were observed for PMX, the natural isomer (6S) of 5-CHO-THF, and the 6R isomer of DDATHF. Transport was stereospecific, with a Ki of 132 versus 873 nmol/L for the 6S versus 6R isomer of 5-CHO-THF, respectively, and a Ki of 113 versus 550 nmol/L for the 6R versus 6S isomer of DDATHF, respectively. The Ki for LY309887 was 285 nmol/L. This transporter had somewhat lower affinities for ZD9331, ZD1694, and folic acid (Ki values of 437, 531, and 622 nmol/L, respectively). Affinities for aminopterin and edatrexate were lower, with Ki values of ~1.3 and 1.7 μmol/L. The affinities for PT523, PT532, and TMR were very low (>50 μmol/L). The Ki for PMX, based on inhibition of [3H]MTX influx in these studies, was comparable with and only ~2-fold higher than the direct measurement of the [3H]PMX influx Ki described above.

**Influx Kinetics and Structural Specificity in HepG2 Cells Transfected with Reduced Folate Carrier.** HepG2 cells, with very low constitutive low pH activity and only modest RFC activity at physiologic pH (9), were transfected with human RFC to achieve high-level carrier expression. The Northern analysis on one transfectant, the HepG2-B clone, is indicated in the top panel of Fig. 3 and demonstrates the high level of RFC message. RFC mRNA in wild-type HepG2 cells was very low, and detection required a long interval of exposure to the X-ray film. Consistent with the high level of carrier expression was the marked increase in initial uptake rates for MTX in the HepG2-B transfectant (Fig. 3, middle panel), which exceeded that of the vector-transfected control cells by a factor of ~65 at pH 7.4 (7.82 ± 0.91 vs 0.12 ± 0.05 nmol/g cell MTX).
protein/min) and by a factor of ~8 at pH 5.5 (1.51 ± 0.05 versus 0.18 ± 0.02 nmol/g protein/min). The bottom panel of Fig. 3 is the pH profile for MTX influx in wild-type and HepG2-B cells over a pH range of 5.0 to 8.0. It can be seen that although the pH optimum is ~7.4 to 8.0 in HepG2-B cells, there is still a prominent RFC-mediated transport component at pH 5.5 in the transfected cells that allowed for measurement of RFC-mediated transport characteristics at this pH.

Influx kinetics for MTX in HepG2-B cells mediated by RFC at pH 7.4 is indicated in the top panel of Fig. 4. The $K_i$ was 1.70 ± 0.21 μmol/L, a level comparable with RFC-mediated transport 2 μmol/L at pH 7.4 in HeLa cells (data not shown). The influx $K_i$ was only slightly increased to 3.77 ± 0.12 μmol/L when the pH was decreased to 5.5 (Fig. 4, bottom panel). Hence, the affinity of RFC for MTX was essentially constant over this pH range. On the other hand, the $V_{max}$ decreased by a factor of 4.5 (59.1 ± 7.2 to 12.8 ± 1.6 nmol/g protein/min) with this fall in pH. The influx $K_i$ for folic acid at pH 7.4 and pH 5.5 was 142 and 125 μmol/L, respectively, and the influx $K_i$ for PT523 at pH 7.4 and pH 5.5 was 0.24 and 0.19 μmol/L, respectively (the average of two experiments). Consistent with these inhibition constants, as indicated in the top panel of Fig. 5, influx of 0.5 μmol/L [³H]MTX was essentially unaffected by 20 μmol/L folic acid at either pH 7.4 (7.16 ± 0.06 versus 7.08 ± 0.27 nmol/g protein/min) or pH 5.5 (0.86 ± 0.15 versus 0.80 ± 0.13 nmol/g protein/min) but was markedly suppressed (Fig. 5, bottom panel) by equimolar PT523 at pH 5.5 (2.15 ± 0.67 versus 0.18 ± 0.09 nmol/g protein/min) and pH 7.4 (8.85 ± 0.91 versus 0.46 ± 0.13 nmol/g protein/min). There was a small ordinate intercept at pH 5.5 in this series of experiments that was depressed by folic acid without a change in initial uptake rate (Fig. 5, top panel). This likely reflects a very low level of MTX bound to the cell surface. The $K_{in}$ for PMX was 1.10 and 1.28 μmol/L, respectively, at pH 7.4 and pH 5.5 (the average of two experiments).

**Trans-Stimulation.** One important characteristic of carrier-mediated processes is trans-stimulation: augmentation of
the unidirectional flux of a substrate into cells by the opposite flux of the same or another substrate out of cells via the same carrier (21, 22). This has been demonstrated at physiologic pH for RFC-mediated transport (23). This was also the case for MTX influx in RFC-null R5 cells loaded with 5-CHO-THF at pH 5.5 (from 0.87 ± 0.15 to 1.19 ± 0.17 nmol/g protein/min, a 45% increase; Fig. 6, top panel). Comparable trans-stimulation (45%) was also demonstrated in the HepG2-B transfectant loaded with 5-CHO-THF not only at pH 7.4 (data not shown) but also at pH 5.5 (a 43% increase from 0.71 ± 0.10 to 1.03 ± 0.25 nmol/g protein/min; Fig. 6, bottom panel). This is strong evidence that the RFC-independent, low pH transport activity in R5 cells is a carrier-mediated process and that trans-stimulation mediated by RFC remains intact at low pH.

Anion Sensitivity. RFC is an anion exchanger and is inhibited at physiologic pH by a variety of structurally unrelated inorganic and organic anions (24–26). Likewise, when chloride is removed from the transport buffer, influx mediated by RFC is enhanced (24, 25, 27). As indicated in the top panel of Fig. 7, influx mediated by the low pH transporter in R5 cells was anion sensitive. When sodium chloride was removed, influx was stimulated by a factor of 2.7. Influx of MTX mediated by RFC in HepG2-B cells at pH 5.5 was much more sensitive to the presence of chloride in the buffer (Fig. 7, bottom panel). When sodium chloride was removed, influx of MTX increased by a factor of 7.5 (from 1.09 ± 0.11 to 8.18 ± 1.20 nmol/g protein/min). In neither case was influx altered at all by the substitution of sodium with choline, consistent with the lack of sodium dependence by either process, confirming that the influx stimulation in the absence of sodium chloride is related solely to the absence of chloride.

Energy Dependence. Influx mediated by RFC at physiologic pH is energy independent; the process is unchanged or increased under conditions of energy deprivation (28). As indicated in the top panel of Fig. 8, there was no change in influx in R5 cells in the absence of glucose with or without the addition of 10 mmol/L azide at pH 5.5. In HepG2-B cells at pH 5.5 (Fig. 8, bottom panel) and at pH 7.4 (data not shown), influx was stimulated by a factor of 1.50 ± 0.30 in the presence of azide in glucose-free buffer, but it was unchanged in the absence of glucose.
DISCUSSION

RFC is highly expressed in normal human tissues and tumors, and this transporter in human and murine leukemia cells has a neutral pH optimum (1). However, recent studies from this laboratory indicate that in human solid tumors, in general, there is very prominent transport activity at low pH that is usually equal to or often exceeds transport mediated by RFC at physiologic pH (9). The mechanism(s) underlying this low pH transport activity has not been clarified, although there is some evidence suggesting that in cells of intestinal origin this transport activity is related, at least in part and in some way, to RFC (11–14). This study addresses the mechanism of folate transport at low pH using a HeLa cell line with prominent low pH activity in which RFC was deleted from the genome and therefore cannot contribute at all, qualitatively or quantitatively, to the transport characteristics observed. The current studies indicate that the low pH transporter in R5 cells has the properties of a carrier-mediated process similar to what has been observed for members of the superfamily of facilitative carriers (29). This transporter shares many of the characteristics of RFC, but there are some important distinguishing features beyond the difference in pH optima.

The low pH transport carrier in R5 cells has an affinity for MTX of ~1 μmol/L at pH 5.5, a $K_v$ that is only slightly less than that reported for RFC at physiologic pH in these and other human cell lines (1). However, whereas RFC has a 2-fold higher affinity for PMX (30, 31), the low pH transporter has an affinity for PMX (~45 nmol/L) that is ~20-fold greater than that for MTX at this pH. This transporter has affinities for the natural (6S) isomer of 5-CHO-THF and the 6R isomer of the glycine-ribonucleotide formyltransferase inhibitor DDATHF comparable with that of PMX. Affinities for ZD1694 and ZD9331 were somewhat lower ($K_v$ ~500 nmol/L) and slightly less than their affinities for RFC. On the other hand, the affinity of folic acid for the low pH route at pH 5.5 in R5 cells ($K_v$ ~620 nmol/L) was 2 orders of magnitude greater than the affinity of folic acid for RFC. The transporter is stereospecific, with a 6-fold higher affinity for the natural isomer of 5-CHO-THF as compared with the 6R form and a 5-fold higher affinity for the 6R isomer as compared with the 6S isomer of DDATHF. The low pH transporter has a very low affinity for PT523, PT632, and trimetrexate agents that either lack or have a modified glutamate moiety, although the affinity for ZD9331 is orders of magnitude lower.

![Fig. 7](image_url) The impact of inorganic ions on influx of MTX in R5 and HepG2-B cells at pH 5.5. In Na+-free buffer, choline chloride was substituted for NaCl. In NaCl-free buffer, sucrose was substituted for both ions. Top panel, R5 cells; bottom panel, HepG2-B cells. The extracellular [3H]MTX concentration was 0.5 μmol/L. The data are the average of three independent experiments ± SE.

![Fig. 8](image_url) The impact of energy metabolism on influx of MTX in R5 or HepG2-B cells at pH 5.5. For depletion of glucose, cells were incubated in the absence of this substrate for 20 minutes. Azide was used at a concentration of 10 mmol/L; cells were pretreated with this agent 15 minutes before transport determinations. Top panel, R5 cells; bottom panel, HepG2-B cells. The extracellular [3H] MTX concentration was 0.5 μmol/L. The data are the average of three independent experiments ± SE.
magnitude greater. The high affinity of PT523 for RFC at low and high pH and its low affinity for the low pH transporter, along with the high affinity of folic acid for the low pH transporter and its low affinity for RFC, make these agents good discriminators of these processes. Hence, in cells in which a high level of RFC activity is present along with the low pH transport activity, the absence of inhibition by PT523 but potent inhibition by folic acid at low pH would be consistent with a RFC-independent transport process as described in these studies.

The observation that influx of MTX is trans-stimulated at low pH in R5 cells that have been loaded with 5-CHO-THF is the most definitive evidence that this RFC-independent transporter in R5 cells is a carrier-mediated process. These studies also demonstrate using the RFC-transfected HepG2-B model that the trans-stimulation phenomenon for RFC is present at both physiologic and low pH. The data indicate that the low pH transport system in R5 cells is anion sensitive, albeit to a lesser extent than that observed for RFC. This suggests that in addition to a proton symport that appears to be a basis for the uphill transport of folates into cells and membrane vesicles of intestinal origin at low pH, an anion-exchange component may also play a role in transport mediated by the low pH transporter (10, 32). For instance, the hydroxyl gradient that occurs across the cell membrane under acidic extracellular conditions would also favor uphill transport into cells. For RFC, uphill transport appears to be related to an exchange with intracellular organic phosphates that are asymmetrically present at high concentrations within cells. This type of exchange was recently confirmed by the demonstration that both thiamine pyrophosphate and monophosphate are good substrates for RFC, and when cells are loaded with MTX, efflux of thiamine anions is inhibited, resulting in their enhanced intracellular accumulation (33, 34). Also, as observed for RFC (28), the unidirectional flux of folates into cells via the low pH transporter was not impaired by energy depletion. The many similar features between the low pH transporter in R5 cells and transport mediated by RFC suggest that these carriers may share structural as well as functional similarities and raise the possibility that the low pH route may represent another member of the SLC19 family of membrane carriers. However, beyond RFC (SLC19A1), the other two members of this family (SLC19A2 and SLC19A3) are thiamine carriers that do not transport folates (35, 36), and a genome-wide search does not suggest that there are other members of this transport family.

It is of interest that the high affinity of the low pH transporter in R5 cells for PMX (~45 nmol/L) is only somewhat less than what was observed for PMX transport in human mesothelioma cell lines (~30 nmol/L; ref. 17). Subsequent studies have clarified that this transport activity in mesothelioma was related to the presence of Mycoplasma adherent to these cells and likely represents transport into this organism, although the possibility that Mycoplasma might have induced the expression of a novel transporter in these human cells was not excluded (18). Whereas Mycoplasma contamination of HeLa cells and HepG2 cells can produce this high-affinity PMX transport, this is of much lower magnitude than that observed in mesothelioma cells. However, the low pH activity reported here is unrelated to this process. First, cells are regularly assessed for Mycoplasma with a highly sensitive polymerase chain reaction-based assay and are free of this organism. Second, there are features of the structural specificities that are quite different. For instance, the $K_r$ values for MTX, ZD1694, and ZD9331 observed in Mycoplasma-contaminated mesothelioma cells of ~100 μmol/L are more than 3 orders of magnitude higher than those observed at low pH in R5 cells (17).

In a previous study (9), residual MTX transport activity at physiologic pH was noted in R5 cells that contributed to the preservation of MTX activity despite the complete loss of RFC. This RFC-independent transport activity at pH 7.4 also had a relatively higher affinity for PMX ($K_r$~12 μmol/L) than for MTX, ZD1694, and folic acid ($K_r$~90–100 μmol/L) and a very low affinity ($K_r$~250 μmol/L) for PT523 (37). R5 cells were, in fact, collaterally sensitive to PMX when grown in medium in which 5-CHO-THF was the folate growth source. This was attributed to the partial preservation of transport along with the marked contraction of folate cofactor pools under these conditions (9, 37, 38). The mechanism underlying this transport activity at neutral pH was not clarified, but it is possible that this may represent residual activity of the low pH route within the physiologic range with pH-dependent alterations in binding constants for folates and antifolates. This is currently under further study.

These studies with HepG2 cells represent the first analysis of RFC-mediated transport properties at low and neutral pH in the same cell line in general and in a human solid tumor cell line in particular. Due to the prevalence and prominence of low pH folate transport activity in human solid tumors, these studies were possible because of the unique lack of this activity in HepG2 cells. The data indicate clearly that the absolute and relative affinities of RFC for its substrates are minimally affected by pH with very similar properties over a 2-order difference in proton concentration. The reduction in pH did, however, produce a substantial decrease in the maximum transport velocity that may be due to an alteration in mobility of the carrier alone or complexed to folate substrates. Hence, the unique characteristics of transport at low pH, when the low pH carrier and RFC activities are present in the same cell, cannot be attributed to pH-dependent alterations in the protonation of RFC or the folate molecule. This is consistent with the $pK_a$ values of the glutamate carboxyl and amino groups of the folate molecules that would not result in an appreciable change in dissociation and charge over the range of pH values evaluated in this study (39). These observations also appear to exclude alterations in protonation of RFC or folate substrates as a basis for transport activity at low pH in intestine or cell lines of intestinal origin, which has been attributed to RFC (11, 13).

PMX is a new-generation antifolate that, in its polyglutamyl forms, is a potent ($K_r$, 1.3 μmol/L) inhibitor of thymidylate synthase with lesser inhibitory activity ($K_r$, 65 nmol/L) at the level of glycaminide ribonucleotide formyltransferase (15). The drug has activity in mesothelioma and was recently approved by the Food and Drug Administration for the treatment of this disease in the United States; it is also active in the treatment of patients with a variety of other solid tumors (40, 41). If the low pH transport activity prevalent in other human solid tumor cell lines has a similar high affinity for PMX as determined in HeLa cells, this could have important pharmacological consequences in view of the low pH present in the
interstitium of solid tumors (42–44). This advantage for PMX would be most important long after infusion of the drug (2–3 days), when blood levels fall within the range of the affinity constant of the low pH transporter, and transport via this mechanism would tend to sustain uptake and continued formation of active polyglutamyl derivatives to maintain suppression of PMX target enzymes.

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