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The relationship between folate transport activity at low pH and reduced folate carrier function in human Huh7 hepatoma cells

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Abstract

Transport of folates and antifolates in both hepatocytes and Huh7 human hepatoma cells is characterized by a low-pH optimum. Studies were undertaken to determine the extent to which this transport activity is mediated by the reduced folate carrier (RFC) in Huh7 human hepatoma cells. RFC expression was ablated by chemical mutagenesis and antifolate selective pressure with PT632 resulting in the PT632^R subline in which RFC mRNA could not be detected. Methotrexate (MTX) influx in these cells at pH 7.4 was reduced by 70%, leaving substantial residual RFC-independent influx while influx of MTX and folic acid at pH 5.5 was not significantly decreased. The influx K_t for folic acid and MTX at pH 5.5 in PT632^R cells was 0.36 and 1.5 μM, respectively. The affinity of the low pH transporter in PT632^R cells was highest for pemetrexed (K_i=140 nM), very low for PT632 (K_i=77 μM), and was stereospecific for the natural isomer (6S) of 5-formyltetrahydrofolate. In Huh7 cells transiently transfected with an RFC siRNA, RFC expression was reduced by 60% resulting in a 40% decrease in MTX influx at pH 7.4 but only a very small (5%) reduction in MTX or folic acid influx at pH 5.5. These data indicate that MTX transport in Huh7 cells at neutral pH is mediated largely by RFC while at pH 5.5 the predominant route of transport is independent of RFC.

Keywords: Folate transport; Folic acid; Methotrexate; Pemetrexed; Acidic pH

1. Introduction

Membrane transport of folates and antifolates into cells has been an area of considerable interest because of the important roles that natural folates play in biosynthetic processes, and that antifolates, primarily methotrexate (MTX), have played in cancer chemotherapy. The mechanisms of folate transport were recently reviewed [1]. Best characterized of these transport mechanisms has been the reduced folate carrier, (RFC, SLC19A1), an anion exchanger that has the properties of a classical facilitative carrier. Two folate receptors, FRα and FRβ, high-affinity membrane binding proteins, are endocytotic routes that transport folates unidirectionally into cells [1]. In addition, some members of the organic anion families of transporters (SLC21 and SLC22) transport folates and antifolates [2–4]. Beyond this, it has become clear that there is another folate transport activity (or activities) with a low pH optimum. This low-pH activity represents the most robust of the folate transport routes in small intestine [5], is present in a variety of other primary tissues or cells [6–9] and in the majority of human solid tumor cell lines, where transport at low pH is equal to, or greater than, transport at physiological pH [10].

While it has been suggested that transport at low-pH in small intestinal cells is mediated by RFC [11,12], studies from this laboratory have shown that when RFC function is eliminated due to mutations in the protein in rat small

Abbreviations: 5-CHO-THF, 5-formyltetrahydrofolate; DHFR, dihydrofolate reductase; MTX, methotrexate; RFC, reduced folate carrier; PT523, Nα-(-4-amino-4-deoxypteroyl)-Nδ-hemiphthaloyl-1-ornithine; PT632, 5,8-dideaza analog of PT523

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intestinal cells (IEC-6), there is no change in MTX influx at low pH [13]. Further, when RFC was deleted from the genome, transport of MTX in HeLa cells at low pH was not reduced [10]. This report extends studies on the relationship between RFC function and transport mediated at low pH to hepatic cells where the predominant folate transport activity in freshly isolated hepatocytes and hepatomas cells has a low pH optimum [14,15]. The model chosen was the Huh7 hepatoma cell line which exhibits a very high ratio of low-pH to neutral-pH MTX transport activity [10]. Two approaches were employed to determine the impact of suppression of RFC expression on MTX transport activity at low and physiological pH; chemical mutagenesis with antifolate selective pressure and gene silencing using RFC siRNA.

2. Materials and methods

2.1. Chemicals

[3′,5′7-^{3}H] MTX and [3′,5′,7′,9-^{3}H] folic acid were purchased from Moravek Biochemicals (Brea, CA). Non-labeled folic acid and 5-CH_3-THF were obtained from Sigma while 6S- and 6R-5-CHO-THF were purchased from Schircks Laboratories (Jona, Switzerland). PT 523 and PT 632 were gifts from Dr. Andre Rosowsky (Dana-Farber Cancer Institute, Boston). Pemetrexed was provided by Eli Lilly Co. (Indianapolis, IN) while trimetrexate was obtained from Warner-Lambert (Ann Arbor, MI). All other reagents were obtained in the highest purity available from commercial sources. Radiochemicals and nonlabeled folates and antifolates were purified by liquid chromatography [16].

2.2. Cell culture and selection of RFC-null sublines

Huh7 cells were obtained from the liver center of Albert Einstein College of Medicine and maintained in RPMI 1640 medium containing 2.0 μM folic acid, supplemented with 10% fetal calf serum (Hyclone), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml). For selection of RFC-null Huh7 sublines, Huh7 cells were treated with 2.4 mM ethylmethanesulfonate for 20 h to achieve about 90% cell kill. Cells were allowed to recover for 2 days after the mutagen was washed away and were then reseeded in 100 mm dishes in the presence of 5 or 15 nM PT 632. After 2 weeks, one surviving clone was detected in a plate containing 5 nM PT632 and another clone was visible in a plate containing 15 nM PT632. Both clones were expanded and maintained in medium containing the respective amount of PT632. Preliminary results indicated that both clones had similar resistance to antifolates, lack of RFC mRNA expression, and impaired influx of MTX and folic acid (see Results). The clone selected with 5 nM PT632 was chosen for detailed studies and named PT632^R. Cell cultures were monitored regularly with a mycoplasma detection kit (ATCC) and were shown to be free of this microorganism.

2.3. Cell growth inhibition

Cells were trypsinized, transferred to 96-well plates (1000 cells/well) and exposed continuously to a spectrum of antifolate concentrations for 6 days. Cell growth rate was quantified by sulforhodamine B staining [17].

2.4. Transport studies

Cells ($4-5\times10^5$) were seeded in 20-ml Low Background glass vials (Research International Corp., Prospect, IL) and grown for 3 days to reach confluency. For RFC-null cells, PT632 was present in the medium to maintain clonal stability but was not added to vials seeded for transport studies. Transport measurements were made in cells in monolayer cultures adherent to the glass vials as previously described [18]. Briefly, cells were washed twice in ice cold transport buffer following which 1 ml of the buffer at 37° was added and cells equilibrated at this temperature for 20 min. The buffer was then aspirated and uptake initiated by the addition of 0.5 ml of fresh buffer containing the radioactive substrate and other reagents. Uptake was terminated by the addition of 5 ml of ice cold HBS (see below) following which cells were washed three times with the same buffer and lysed in 0.5 ml of 0.2 N NaOH at 65 °C for 30 min. A 0.4-ml portion of cell lysate was assayed for radioactivity after addition of scintillation fluor (8 ml). Another 10 or 20 μl of lysate was processed for protein determination (BCA Protein Assay, Pierce, Rockford, IL). Cell antifolate is expressed as pmoles per milligram of protein.

The following buffers were used in these studies. HBS (HEPES-buffered saline) at pH 7.0, 7.4, and 8.0 adjusted with NaOH (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose). MBS (4-morpholinepropanesulfonic acid-buffered saline) at pH 5.0, 5.5, 6.0 and 6.5 adjusted with NaOH (20 mM 4-morpholinepropanesulfonic acid, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose). Na^+-free HBS at pH 7.4 or Na^+-free MBS at pH 5.5 were prepared by replacing 140 mM NaCl in HBS or MBS with equimolar choline chloride. Some HBS at pH 7.4 or MBS at pH 5.5 was made glucose-free. Sucrose buffer at pH 7.4 consisted of 20 mM HEPES, 225 mM sucrose, adjusted to pH 7.4 with $Mg(OH)_2$. Sucrose buffer at pH 5.5 consisted of 20 mM 4-morpholinepropanesulfonic acid, 225 mM sucrose, pH adjusted with $Mg(OH)_2$.

2.5. RNA isolation and Northern Blots

Total RNA was isolated from cells with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (30 μg) was resolved by

electrophoresis on 1.2% agarose gels containing formaldehyde. RNA was transferred to Nytran N-membranes (Schleicher and Schuell, Keene, NH) and fixed with a Stratalinker UV-cross linker (Stratagene, La Jolla, CA). The blot was first probed with human RFC cDNA then stripped and reprobed with β-actin cDNA as the loading control.

2.6. Reduction of RFC expression by siRNA

Two complementary RNA oligonucleotides (GUGCUACCUUUGCUUCUACdTdT and GUAGAAGCAAAGGUAGCACdTdT) were synthesized and annealed by Ambion (Austin, Texas) and both were modified by the addition of two dTdT overhangs at the 3′ ends. The target region for RNA silencing was from 206 to 224 of human RFC cDNA (NM_194255). RNA molecules for negative control (Silence™ Negative control #1 siRNA) were purchased from the same company. Huh7 cells were seeded (0.35×10^6 cells/vial) in 20-ml Low Background glass vials (Research International Corp., Prospect, IL), which are equivalent to 12-well plates in cell growth area, and reached 40–50% confluence a day later. Transient transfection of siRNAs into Huh7 cells was performed using Oligofactamin™ reagent (Invitrogen, Carlsbad, CA) according to the recommended protocol with a concentration of siRNAs at 100 nM. Two days later, some vials were used for MTX influx determination and the other vials were pooled for extraction of total RNA as described above.

2.7. Quantitative PCR-analysis of RFC expression

RFC cDNA was synthesized from total RNA by Superscipt™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was carried out with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) by using SYBR Green 1 dye (Applied Biosystems) as the fluorescent probe. The housekeeping gene β-actin was used to control for variations in the amount of RNA and for determination of the efficiency of the reverse transcription reaction. The following primers, reported previously for RFC and β-actin [19], were employed: RFC sense primer, 5′-TCAAGACCATCATCACTTTCATTGT-3′; RFC antisense primer, 5′-AGGATCAGGAAGTACACGGAGTATAAC-3′; β-actin sense primer, 5′-CGTGCTGCTGACCGAGC-3′; β-actin antisense primer, 5′-GAAGGTCTCAAACATGATCTGGGT-3′. The following parameters were used for the PCR reaction: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 15 s at 55 °C and 1 min at 72 °C. Under these conditions, a single PCR product was observed for both the RFC and β-actin genes. All samples were amplified simultaneously in triplicate in one run. One cDNA sample was serially diluted one to one, six times, and the diluted samples served as standards for quantitative analysis of RFC and β-actin mRNA. The C_T value, or threshold cycle, at which the fluorescence signal rose above the baseline and began to increases exponentially, was used for calculations. As expected, the C_T values for the serially diluted samples were inversely proportional to the logarithm of the RFC or β-actin cDNA levels.

3. Results

3.1. pH dependence of folic acid and MTX influx in Huh7 cells

Influx characteristics for 0.5 μM tritiated folic acid and MTX were assessed in Huh7 cells over a pH range of from 5.0 to 8.0 (Fig. 1). Folic acid influx was negligible at physiological pH but increased as the pH was decreased; in particular, below a pH of 6.5. MTX influx was higher at pH 7.5–8.0 than folic acid influx, remained constant to pH 6.5 then increased in parallel with the increase in folic acid influx as the pH was decreased to 5.0. At pH 5.0 influx of MTX was ~5-fold greater than at pH 7.4. Hence, while folic acid and MTX influx were approximately the same at pH's below 6.5, MTX influx was far greater than folic acid influx at pH 7.4–8.0.

3.2. Effect of Na^+, azide and 5-CHO-THF on MTX influx at pH 7.4 in Huh7 cells

The higher rate of MTX transport relative to folic acid at pH 7.4 was attributed to RFC activity in Huh7 cells since this carrier has an affinity for MTX ~two orders greater than for folic acid [1]. To further explore the extent to which this flux at pH 7.4 is consistent with the properties of RFC-mediated transport a number of other parameters were assessed. As summarized in Table 1, MTX influx was largely preserved when extracellular sodium chloride was replaced with choline chloride—

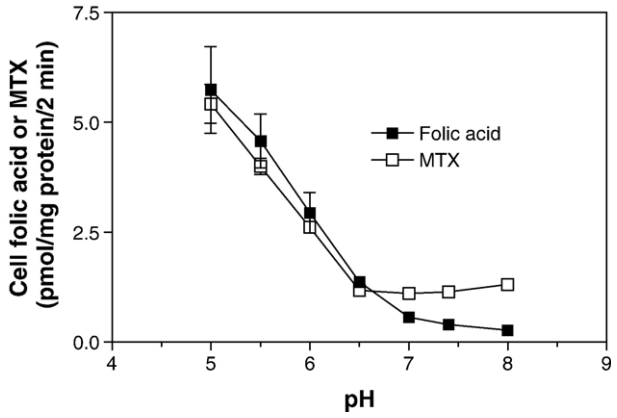

Fig. 1. MTX or folic acid influx over a pH range of 5.0 to 8.0 in Huh7 cells. The extracellular substrate concentration was 0.5 μM. [^{3}H]Folic acid and [^{3}H]MTX influx was determined at 2 min. The data are the mean ± S.E.M. of three independent experiments. Whenever the error bars are invisible, they are smaller than the symbols.

Table 1
Properties of MTX transport in Huh7 cells at pH 7.4

Condition	MTX influx (pmol/mg/min)	Percentage of control
HBS (control)	0.78±0.04	100
HBS-choline chloride	0.72±0.03*	92
Sucrose buffer	2.58±0.32	331
Glucose-free HBS+azide	0.95±0.03#	122
HBS+100 μM 5-CHO-THF	0.23±0.13	29

[^{3}H]MTX influx is uptake as a function of time over 2 min at an extracellular concentration of 0.5 μM. Azide (10 mM) was added to the incubation buffer 15 min before, and was present in buffer containing [^{3}H]MTX. 5-CHO-THF was added with [^{3}H]MTX. Data are the mean±S.E.M. of three independent experiments. * and # indicate P values of 0.04 and 0.09, respectively, based upon a paired t test.

consistent with the known lack of sodium-dependence of RFC. However, consistent with the inhibitory effects of anions on RFC-mediated influx, MTX transport was increased by a factor of more than three in a sucrose buffer which lacked both sodium and chloride. RFC-mediated influx is either unchanged or increased in the presence of metabolic poisons [1]. Consistent with this, azide produced a 22% increase in MTX influx in these cells, although this change only reached a P value of 0.09. Finally, 100 μM 5-CHO-THF inhibited 0.5 μM [^{3}H]MTX influx by ~70%. Hence, these observations are consistent with an RFC-mediated process [1] and differ from what has been detected in freshly isolated hepatocytes [20] as considered further in Discussion.

3.3. Selection of RFC-null Huh7 cells

Studies were undertaken to assess the extent to which folic acid or MTX influx at acidic pH is mediated by RFC. This laboratory recently established a procedure to inactivate RFC while minimizing potential alterations in other folate transport pathways. The strategy employs antifolate selective pressure with PT632, an analog of PT523, dihydrofolate reductase inhibitors with higher affinity for RFC than MTX but with a much lower affinity for the transport activity at low pH [21,22]. Hence, loss of RFC activity is favored as a transport-mediated mechanism of resistance under these conditions. Mutagenesis of Huh7 cells with ethylmethanesulfonate followed by exposure to PT632 resulted in the isolation of the Huh7 subline, PT632^R, 400-fold resistant to PT632 and 20-fold resistant to MTX, as compared to the wild-type Huh7-cells (Fig. 2). PT632^R cells were, however, not cross-resistant to trimetrexate (TMQ), another dihydrofolate reductase inhibitor that enters cells by an RFC-independent process, likely passive diffusion. This resistance pattern was consistent with markedly impaired RFC function in PT632^R cells. As indicated in Fig. 3, RFC mRNA was expressed in wild-type Huh7 cells, but RFC mRNA was not detected in PT632^R cells even after overexposure of the film.

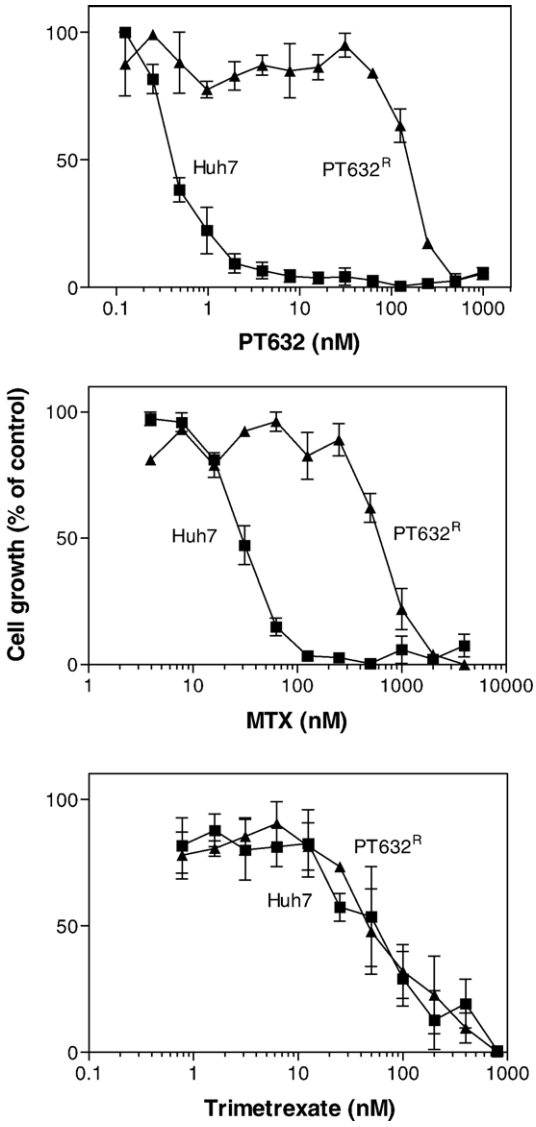

Fig. 2. Antifolate growth inhibition in wild-type and PT632^R cells. Cells were grown for 6 days with the various antifolates following which cell mass was assessed. Controls were cells not exposed to drug. Data are the mean±S.E.M. of three independent experiments.

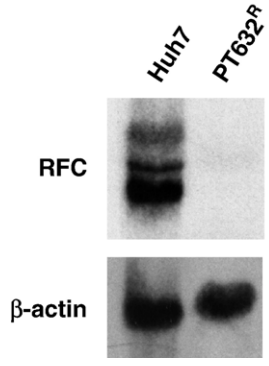

Fig. 3. Northern blot analysis of RFC mRNA in PT632^R and wild-type Huh7 cells. Total RNA was probed successively with the full-length human RFC (upper panel) and β-actin cDNA (bottom panel). The X-ray films shown are one of two separate analyses.

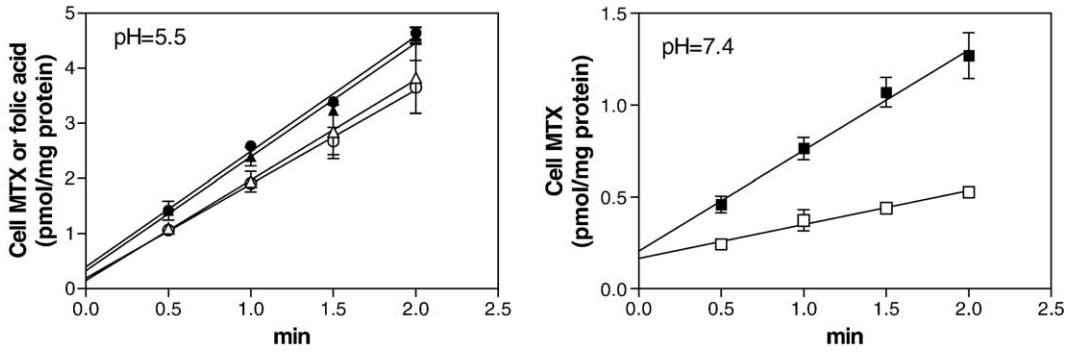

Fig. 4. Influx of MTX or folic acid in PT632^R and Huh7 cells at pH 5.5 (left panel) and 7.4 (right panel). The extracellular substrate concentration was 0.5 μM under all conditions. PT632^R (open symbols) and Huh7 (filled symbols) were exposed to [^{3}H]MTX (triangles), [^{3}H] folic acid (circles) at pH 5.5 or [^{3}H]MTX (squares) at pH 7.4, at time zero. Data are the average ± S.E.M. of three independent experiments. Whenever the error bars are invisible, they are smaller than the symbols.

3.4. MTX and folic acid influx in RFC-null PT632^R cells

MTX influx in PT632^R cells at pH 7.4 was markedly decreased as compared to wild-type Huh7 cells (Fig. 4). However, despite the complete loss of RFC expression in PT632^R cells, about a third of wild-type MTX influx activity was retained. In contrast, MTX and folic acid influx at pH 5.5 in PT632^R cells was not significantly decreased (12%, $P=0.58$; 18%, $P=0.28$, respectively) as compared to Huh7 cells based upon three separate experiments and a paired t-test analysis. Hence, loss of RFC expression had no, or only a very small, effect on influx at acidic pH; the major portion of this transport activity was RFC-independent.

3.5. Characteristics of MTX influx at pH 5.5 in PT632^R cells

Influx kinetics for both MTX and folic acid in PT632^R cells were determined at pH 5.5. Influx of both substrates was saturable (Fig. 5). Best fit of the data to the Michaelis–Menten equation revealed a folic acid influx K_t of 0.36 ± 0.05 μM and V_{max} of 3.0 ± 0.1 pmol/mg protein/min, and a MTX influx K_t of 1.5 ± 0.3 μM and V_{max} of 6.0 ± 0.5 pmol/mg protein/min.

The affinity of the low pH transport activity for other folate/antifolates was assessed based upon inhibition of [^{3}H]MTX influx in PT632^R cells by these substrates. The concentration for each substrate (except PT523 and PT632) was chosen such that ~50% of MTX influx activity was inhibited. For PT523 and PT632 a higher concentration (20 μM) did not reach 50% of inhibition. Based upon the measured MTX influx K_t, and the assumption that inhibition was competitive, the influx K_i for each substrate was computed and is listed to the right of the horizontal bar in Fig. 6. Influx K_is for folic acid and MTX computed in this way were comparable to K_ts that were measured directly. Pemetrexed had the highest affinity, while PT532 and PT632 had the lowest affinities for this transport activity among the substrates tested. This transport activity was also stereospecific; the K_i for the physiological 6-(S)-5-CHO-THF isomer was 1/5th that of the nonphysiological isomer. The physiological blood folate, 5-CH_3-THF, also had high affinity for the low pH transporter.

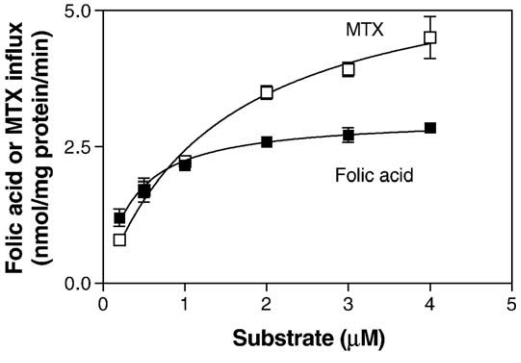

Fig. 5. Influx kinetics for [^{3}H]MTX and [^{3}H]folic acid in PT632^R cells at pH 5.5. Influx was determined based upon a time-course of uptake over 2 min at each substrate concentration indicated. The lines are the best fit of data to the Michaelis–Menten equation. Data are the average ± S.E.M. of three independent experiments. Whenever the error bars are invisible, they are smaller than the symbols.

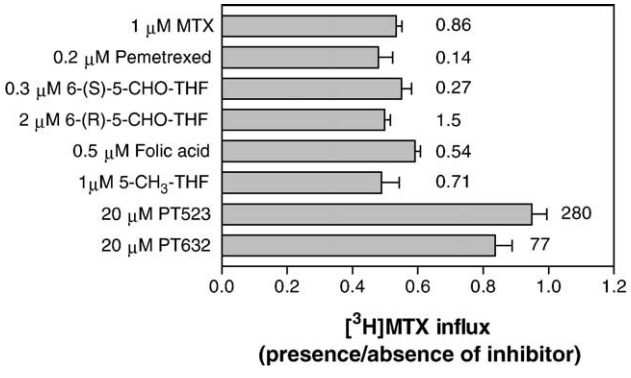

Fig. 6. Inhibition constants for folate and antifolate influx based upon suppression of MTX influx at pH 5.5. Concentrations of inhibitors were adjusted to achieve ~50% inhibition of 0.5 μM MTX uptake at 2 min. [^{3}H]MTX and inhibitors were co-added to cell suspensions. Influx K_is were calculated using the Michaelis–Menten equation assuming that inhibition was competitive. Calculated K_is in μM are indicated to the right of each bar. Data are the average ± S.E.M. of three independent experiments.

Other transport properties of 0.5 μM μM MTX at pH 5.5 were assessed in PT632^R cells. Replacement of sodium chloride with choline chloride had no effect on MTX influx (3.1±0.1 pmol/mg/min vs. 3.2±0.3 pmol/mg/min; $n=3$), respectively. MTX influx was increased by 85% in a sucrose buffer lacking both sodium and chloride (5.8±0.1 pmol/mg/min, $n=3$) as compared the standard MBS buffer (3.1±0.1 pmol/mg/min, $n=3$). These properties of MTX transport at low pH are largely consistent with what has been observed in HeLa R5 cells that lack RFC function [22]. Incubation of PT632^R cells with azide in glucose-free buffer decreased MTX influx by 60% (1.2±0.1 pmol/mg/min, $n=3$) as compared to the standard MBS buffer containing glucose in the absence of azide. Similar suppression of MTX influx was also observed in the wild-type Huh7 cells at pH 5.5 after exposure to azide in glucose-free buffer. This inhibitory effect by azide was different from what was observed in HeLa R5 cells in which influx was unchanged [22] but similar to what was shown in rat IEC-6 intestinal epithelial cells [23], human NCM460 colonic epithelia cells, [24] and human MIA PaCa-2 pancreatic epithelial cells [25].

3.6. Effect of suppression of RFC expression by siRNA on MTX influx

An siRNA approach was used to suppress RFC-mediated transport in order to circumvent changes that may occur during the selection process used to derive the PT632^R cells. Three independent transient transfections resulted in reductions of RFC mRNA expression by 61, 64, and 60% as compared to the vector-control transfected cells, based upon the quantitative PCR assay. The same batches of transfectants were also used to determine MTX influx activity. As indicated in Fig. 7, MTX influx at pH 7.4 was reduced by 39% in siRNA transfectants as compared to the vector-control transfectants. At pH 5.5, changes in influx of tritiated folic acid and MTX in the siRNA transfectants (~5%) were very small although the former reached a level of significance of $P=0.023$.

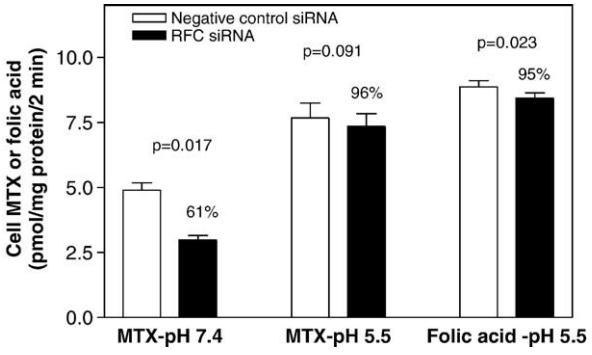

Fig. 7. MTX or folic acid influx at pH 7.4 and 5.5 in wild-type Huh7 cells and cells transiently transfected with an RFC siRNA construct. Wild-type cells were transfected with either RFC siRNA or a scrambled RNA unrelated to any mammalian sequence (negative control siRNA). Influx was determined with 0.5 μM [^{3}H]MTX or [^{3}H]folic acid within 2 min. Data are the average of influx determinations ±S.E.M. from three independent transfections. P values were derived from the paired Student's t-test.

4. Discussion

While studies on transport of folates and antifolates have focused on RFC and the folate receptors, there has been increasing interest in transport of these substrates into cells mediated by other mechanisms. Among the most intriguing are low-pH folate transport activities that have been observed in a variety of human tissues and cell lines. This is characteristic of folate absorption in the intestine and transport into cells and membrane vesicles of intestinal origin [1,5]. Recently, it has been shown to be the dominant folate transport activity in the majority of human solid tumor cell lines [10]. It had been assumed that this transport activity is mediated by RFC; however differences between the pH optimum for RFC-mediated transport and substrate specificities in these tissues versus cell lines of hematopoietic origin were unexplained [11,12].

There is recent evidence that in at least some cells, this low pH activity can be entirely RFC-independent: (i) Transport at low pH was not diminished (indeed it was slightly increased) in a HeLa subline (R5 cells) in which RFC had been deleted from the genome [10]. (ii) In intestinal epithelial cells (IEC-6) in which RFC was mutated and function was lost, there was no change in folate transport at low pH [13]. The current study provides additional evidence that the low pH folate transport activity in hepatoma Huh7 cells is almost exclusively independent of RFC since it was negligibly affected when RFC expression was abolished in cells selected for antifolate resistance. The RFC-independence of the low-pH activity in Huh7 cells was further verified by reduction of RFC expression with siRNA. A 60% decrease in mRNA was associated with a 40% decrease in MTX influx at pH 7.4 but only a negligible decrease in activity at pH 5.5. This is in contrast to the larger reduction of RFC expression by siRNA recently reported in pancreatic MIA PaCa-2 pancreatic epithelial cells [25], that was associated with a 50% decrease in folic acid uptake at acidic pH.

Some component of RFC-mediated transport has been shown to be present at low pH. This was based upon the increased transport activity at low pH observed when RFC was transfected into HepG2 cells which have very low levels of constitutive MTX transport activity at neutral or low pH [22]. However, RFC-mediated transport was shown to have the same properties at low and neutral pH, while in the current study RFC-independent transport at low pH had different properties in terms of low affinity for PT632, high affinity for folic acid and suppression by energy inhibition. It is possible that despite its very low affinity for the low pH transport route in Huh7 cells, the selective pressure with PT632, a very potent dihydrofolate reductase inhibitor, led to a concurrent small decrease in this transport activity. This was seen after

MTX selective pressure in R5 HeLa cell that lack genomic RFC where the low pH activity was virtually abolished [26].

The low-pH transport activity in the Huh7 PT632^R line shares many of the characteristics of transport at pH 5.5 in HeLa cells which has the properties of a facilitative carrier mechanism. The spectrum of affinities for folates and antifolates is similar. The low pH transporter(s) in both systems have an affinity for the natural 6(S) isomer of 5-CHO-THF six times greater than for the unnatural 6(R) isomer [22]. As observed for all low-pH folate transporters, folic acid has an affinity (<1 μM) that is ~two orders of magnitude greater than its affinity for RFC at pH 7.4 [22].

RFC-independent low-pH folate transport is of considerable importance. From a physiological perspective this RFC-independent process plays a critical role in folate intestinal absorption, particularly in view of the acidic pH present at the surface microclimate of intestinal cells [27,28]. This transport activity also has important ramifications with respect to cancer chemotherapeutics since there is a low pH in the core of human solid tumors so that delivery of antifolates to these regions via this transporter could be an important determinant of activity [10,29–31]. This may be of particular importance to the efficacy of the new-generation antifolate, pemetrexed, that is active in the treatment of a variety of human solid tumors and was recently approved for the treatment of mesothelioma and non-small cell lung cancer [32,33]. In HeLa cells, the K_t for PMX influx at pH 5.5 is 45 nM—representing the highest affinity for any folate or antifolate studied [22]. In the Huh7 cells, the PMX influx K_i was 140 nM—the lowest K_i among all the folates and antifolates tested, consistent with the high affinity of the low-pH transporter for this agent.

It is of interest that there was substantial residual transport of MTX at pH 7.4 in RFC-null PT632^R cells, consistent with what was observed in HeLa R5 cells in which genomic RFC was lost [10]. In the latter case, the properties of this residual transport activity were compatible with a carrier-mediated process in which the affinities of all the folate and antifolate substrates were reduced while maintaining relative activities similar to what was observed with transport at pH 5.5 [22,34]. A subsequent study demonstrated that when cells were subjected to further MTX selective pressure, transport at both low and physiological pH were coordinately decreased and subsequently increased to near-usual levels when cells were grown in the absence of the drug. These observations suggested that these transport activities reflect the same underlying process with pH-dependent differences in affinities for various folate and antifolate substrates [26]. This is in contrast to the unchanged structural specificity for RFC-mediated transport at neutral and low pH, as described above [22].

The low-pH folate transport activity in Huh7 hepatoma cells as well as HeLa cells [10] was greater than the activity at pH 7.4. This is similar to what was has been observed in freshly isolated hepatocytes [14,15]. However, while MTX transport characteristics in Huh7 cells at pH 7.4 were typical of an RFC-mediated process, this is not the case for freshly isolated hepatocytes [20]. Hence: (i) MTX-influx at pH 7.4 in Huh7 cells was not sodium-dependent, influx in hepatocytes is highly sodium-dependent [14,15,35,36]. (ii) MTX influx in Huh7 cells is inhibited by 5-CHO-THF and 5-CH_3-THF. MTX influx in the rat hepatoma H35 rat hepatoma cell line is also inhibited by 5-CH_3-THF [37]. But this is not the case in freshly isolated hepatocytes [36]. (iii) MTX influx in Huh7 cells at pH 7.4 was slightly enhanced by metabolic poisons whereas MTX influx in hepatocytes is suppressed by metabolic inhibitors [35,36].

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