5-Amino-4-Imidazolecarboxamide Riboside Potentiates Both Transport of Reduced Folates and Antifolates by the Human Reduced Folate Carrier and Their Subsequent Metabolism

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Abstract

Transport is required before reduced folates and anticancer antifolates [e.g., methotrexate (MTX)] exert their physiologic functions or cytotoxic effects. The folate/antifolate transporter with the widest tissue distribution and greatest activity is the reduced folate carrier (RFC). There is little evidence that RFC-mediated influx is posttranscriptionally regulated. We show that [^{3}H]MTX influx in CCRF-CEM human childhood T-leukemia cells is potentiated up to 6-fold by exogenous 5-amino-4-imidazolecarboxamide riboside (AICAr) in a AICAr and MTX concentration–dependent manner. Metabolism to more biologically active polyglutamate forms is also potentiated for MTX and other antifolates. That potentiation of influx by AICAr is mediated by effects on the RFC is supported by analyses ±AICAr showing (*a*) similarity and magnitude of kinetic constants for [^{3}H]MTX influx; (*b*) similarity of inhibitory potency of known RFC substrates; (*c*) lack of potentiation in a CCRF-CEM subline that does not express the RFC; and (*d*) similarity of time and temperature dependence. Potentiation occurs rapidly and does not require new protein synthesis. Effects of specific inhibitors of folate metabolism and the time and sequence of AICAr incubation with cells suggest that both dihydrofolate reductase inhibition and metabolism of AICAr are essential for potentiation. Acute folate deficiency or incubation of CCRF-CEM with AICAr-related metabolites (e.g., adenosine) does not initiate potentiation. AICAr increases growth inhibitory potency of MTX and aminopterin against CCRF-CEM cells when both AICAr and antifolate are present for the first 24 hours of a 120-hour growth period. AICAr is the first small molecule that regulates RFC activity. (Cancer Res 2006; 66(7): 3836-44)

Introduction

Reduced folates (e.g., tetrahydrofolate; Fig. 1) are a family of vitamin cofactors that participate in one-carbon transfers involved in synthesis of purines, thymidylate, serine, glycine, and methionine; in degradation of histidine; and in scavenging formaldehyde and formate (1). Because humans cannot synthesize folates *de novo*, they must be obtained from dietary sources and release from autotrophic enteric bacteria. Folates are absorbed in the gut and transported in the blood to tissues as 5-methyltetrahydrofolate. In tissues, two transporters (2) internalize folates: (*a*) the folate-binding protein family of endocytic, unidirectional, membrane receptor transporters, and (*b*) the reduced folate carrier (RFC; SLC19A1)-mediated, bidirectional facilitated diffusion system. Both transporters have been extensively characterized (2, 3).

Transporter expression is tissue dependent. The RFC is expressed in most, if not all, tissues (4) whereas expression of various folate-binding proteins is limited (5). Because of its wide distribution and high capacity, the RFC is likely the primary folate transporter (2). Under most conditions, RFC expression remains constant but expression levels in different tissues vary widely. RFC activity may increase in acute folate deficiency (6); the mechanism of this increase is unknown. RFC expression is transcriptionally regulated based on the activation of human RFC promoter constructs by ectopic expression of specific transcription factors (7, 8). Elucidation of mechanisms for regulating transport of natural folates is of fundamental importance to understanding nutritional effects of these vitamins.

Cellular uptake of "classic" anticancer folate antagonists or antifolates (i.e., containing *p*-aminobenzoylglutamate or similar structures) such as 4-amino-10-methylpteroylglutamic acid (methotrexate, MTX; Fig. 1), *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid (ZD1694), 5,10-dideazatetrahydrofolate (DDATHF), and pemetrexed is mediated by the RFC and/or folate-binding protein (9). Transport is an essential determinant of sensitivity to classic antifolates (10). Thus, discovery of mechanisms by which antifolate transport can be selectively increased in tumor cells should increase the clinical therapeutic benefit of classic antifolates. In addition, metabolism of classic antifolates to poly(γ-glutamate) forms (Fig. 1) by folylpolyglutamate synthetase can be limited by transport. Polyglutamyl antifolates are better retained and are often more potent inhibitors of their target enzyme than their respective monoglutamates (11); increased transport could enhance synthesis of these crucial metabolites. This may be especially critical in childhood acute lymphoblastic leukemia where clinical correlations show that the median difference in MTX polyglutamate accumulation between patients who respond to MTX-containing regimens and nonresponders is only about 3-fold (12, 13). Selectively increasing uptake of MTX in tumor even 3-fold should increase MTX polyglutamate synthesis and might increase long-term survivors.

Bochner and Ames (14) discovered that 5-amino-4-imidazolecarboxamide riboside (AICAr; Fig. 1) triphosphate regulates folate metabolism during folate stress in bacteria. Because MTX polyglutamate accumulation presents an ongoing folate stress, we tested exogenous AICAr, a nucleoside precursor of AICAr triphosphate, for its effects on MTX polyglutamate synthesis and MTX uptake in CCRF-CEM (15), a cell culture model for human childhood T-cell acute lymphoblastic leukemia. Exogenous AICAr

Note: W.H. Haile and C.-C. Yeh contributed equally to this work.

This work is dedicated to the memory of our mentor and friend Jesse C. Rabinowitz.

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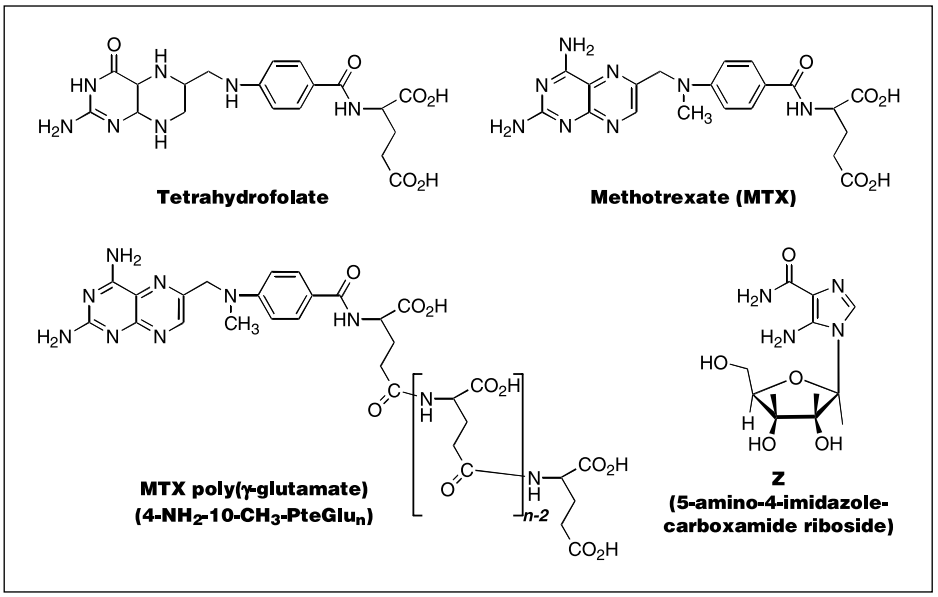

Figure 1. Structures of tetrahydrofolate (H_4Pte-Glu), MTX, MTX poly(γ-glutamate) metabolites, and AICAr.

potentiates metabolism of MTX to MTX polyglutamates and this occurs largely, if not exclusively, because AICAr potentiates influx of MTX by the RFC.

Materials and Methods

Materials. Methotrexate, (*S*)-2-(5-(((1,2-dihydro-3-methyl-1-oxobenzo(*F*)quinazolin-9-yl)methyl)-amino)-1-oxo-2-isoindolinyl)glutaric acid (BW-1843U89), ZD1694, and trimetrexate were gifts from Immunex (Seattle, WA), Glaxo Smith Kline (Research Triangle Park, NC), Astra Zeneca Pharmaceuticals (Macclesfield, United Kingdom), and Pfizer Warner-Lambert (Ann Arbor, MI), respectively. Aminopterin and AICAr were from Sigma Chemical Co. (St. Louis, MO). Solutions of drugs, nucleosides, and nucleobases were standardized using literature extinction coefficients. Fisher (Pittsburgh, PA) high-performance liquid chromatography grade acetonitrile was used. All common chemicals were reagent grade or higher. [^{3}H]MTX (15-33.5 Ci/mmol), [^{3}H]aminopterin (53 Ci/mmol), and [^{3}H]leucovorin (10-40 Ci/mmol) were from Moravek Biochemicals (Brea, CA) and were ≥96% pure when received as analyzed by high-performance liquid chromatography with internal standards. [^{3}H]DDATHF radiolabeled with L-[3,4-^{3}H]glutamate (0.5 Ci/mmol) during chemical synthesis was a gift of Drs. J. Tomsho and J.K. Coward (University of Michigan, Ann Arbor, MI; ref. 16). Radiolabeled compounds were repurified by high-performance liquid chromatography when analysis showed radiochemical purity <95% or analysis of intracellular drug pools (below) showed that <90% of intracellular radiolabel eluted as parent compound or its metabolites.

General methods. Experiments were repeated at least twice with similar results. Synthesis of MTX poly(γ-glutamate) metabolites (17) and protein [i.e., dihydrofolate reductase (DHFR)]-bound MTX (18) were quantitated as described. Reversed-phase analytic high-performance liquid chromatography was done as described (19).

Cell culture. Human T-lymphoblastic leukemia cell line CCRF-CEM (15) and its MTX-resistant RFC-deficient subline R2 (20) were cultured as described (17) and were negative for Mycoplasma contamination (Mycoplasma Plus PCR primers, Stratagene, La Jolla, CA). For 200- to 1,000-mL cultures, cells were expanded in T-75 flasks (60-mL medium) incubated on their sides at 37° and 5% CO_2. Logarithmically growing (3×10^5-5×10^5/mL) cells were inoculated into spinner flasks at $\geq 7 \times 10^4$ cells/mL. The spinner flask headspace was sparged for 1 to 2 minutes with 5% CO_2 through a sterile plugged pipette. Flasks were sealed and stirred (60-120 rpm) at 37°. CCRF-CEM growth inhibition in continuous and intermittent drug exposures was measured as described (17). EC_{50} values were determined from plots of percent control growth versus the logarithm of drug concentration.

Quantitation of folate and antifolate uptake. The method is essentially as described (19). Logarithmically growing cells were harvested at room temperature by centrifugation at 1,000 × *g* (5 minutes). After washing, cells were suspended at ≈2×10^7/mL. Transport medium (37°C) was usually RPMI 1640 containing 10% horse serum and 25 mmol/L HEPES-NaOH, pH 7.5 (pH measured at ambient temperature). This medium was used for consistency because the original observation of AICAr potentiation was made in this medium and the same medium is used for MTX metabolism studies (above). However, AICAr potentiation of uptake is also observed in both anion-free (MHS) and buffered balanced salts (ref. 21; see Results). Anion-free MHS contains 250 mmol/L sucrose and 20 mmol/L HEPES, titrated to pH 7.4 with MgO. HBSS (6) contains 107 mmol/L NaCl, 26.2 mmol/L $NaHCO_3$, 5.3 mmol/L KCl, 1.9 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 7 mmol/L glucose, and 20 mmol/L HEPES; the mixture was titrated to pH 7.4 with NaOH. Cells were preincubated with compounds of interest and uptake was initiated by addition of ^{3}H-permeant at the indicated concentration. Uptake was measured over 30 minutes (for rate and extent measurements) or over 5 minutes (initial velocity) and was linear for ≥5 minutes. Uptake was terminated by diluting duplicate 100 μL samples into 1-mL iced 0.9% NaCl. Cells were recovered by centrifugation in a horizontal rotor (10 seconds; 12,500 × *g*; Beckman Microfuge E), which pelleted >99.7% of cells. The supernatant was aspirated. The pellet was washed once with 1-mL iced 0.9% NaCl and recentrifuged. The washed pellet was solubilized in 1 mL of 0.3% Triton X-100 by incubation for 60 minutes at 37°C. The solubilized pellet was transferred to a scintillation vial, 10 mL of Liquiscint (National Diagnostics, Atlanta, GA) was added, and radioactivity was quantitated in a Beckman Model LS6500 liquid scintillation counter. At the longest incubation time used in an experiment, samples were harvested for high-performance liquid chromatography analysis of intracellular contents (above), except that cell pellets were extracted with 1 mL of boiling 0.1 mol/L Na-phosphate, pH 5.5 (17) after the second wash. The extract was clarified by centrifugation and the supernatant was filtered through an MPS-1 filter unit (Amicon, Beverly, MA) before analysis. Typically, >90% of intracellular radiolabel eluted with standards for MTX, MTX-γ-Glu, and MTX-γ-$(Glu)_2$.

MTX uptake inhibition. Uptake inhibition was assayed as described above in cells that were preincubated for 10 minutes with 1 μmol/L trimetrexate ± 0.5 mmol/L AICAr. Graded concentrations of competitor were mixed with [^{3}H]MTX and added at t_0. Duplicate samples were removed at 5 minutes and processed; uptake was linear over 5 minutes. IC_{50} values were determined graphically from plots of uptake relative to solvent-treated control versus competitor concentration.

MTX efflux. [^{3}H]MTX efflux from CCRF-CEM cells was measured as described (18). CCRF-CEM cells (2.2×10^7/mL; 3 mL) were incubated at 37° for 5 minutes ±0.5 mmol/L AICAr before [^{3}H]MTX addition to 2 μmol/L (3 μCi/mL). Samples (100 μL) were removed and processed (above) to monitor uptake; final uptake samples were taken at 32 minutes, just before efflux initiation. After 20-minute uptake, 2 × 1 mL were removed to separate tubes from ±AICAr uptake samples. These four samples were centrifuged at ambient temperature for 5 minutes at 1,000 × *g*. After aspiration of the supernatant, cell pellets were placed at 37°C and 2 mL of warmed transport medium ±AICAr were added to each pair. Warmed medium was added at 30-second intervals starting at 32 minutes and a zero-minute efflux sample was taken immediately after cells were evenly suspended. Additional samples of 200 μL were taken over 30 minutes and processed.

Subcellular fractionation. CCRF-CEM cells (2.6×10^7/mL; 5 mL) were preincubated with 2 μmol/L trimetrexate ± 0.5 mmol/L AICAr for 10 minutes and then [^{3}H]MTX was added (2 μmol/L; 0.2 μCi/mL). After 30 minutes, cells were diluted 9-fold in iced 0.9% NaCl, centrifuged at 1,000 × *g* for 5 minutes, and washed once with 40-mL iced saline. Subsequent steps were done at 4°C. Cells were homogenized as described (22), except that hypotonic buffer at 20-fold the packed cell volume was used, and the resulting homogenate was centrifuged for 6 minutes at 1,000 × *g* to yield a nuclear pellet and post-nuclear supernatant. Based on controls, >91% of cells are lysed during homogenization. The post-nuclear supernatant was centrifuged at 17,000 × *g* (15 minutes) to yield a combined mitochondria-lysosome pellet. The resulting supernatant was centrifuged at 100,000 × *g* (60 minutes) to yield the 100,000 × *g* supernatant (cytosol). The 100,000 × *g* pellet (microsomes) and combined mitochondria-lysosome pellet were each washed in isotonic buffer (22) and recentrifuged. Pellets were solubilized and quantitated as described above. Triton X-100 was added to the cytosol to 0.3% (v/v) and 1 mL was quantitated. Subcellular distribution of [^{3}H]MTX after 4 hours of incubation was determined similarly, except that no trimetrexate was present during the 10-minute preincubation and initial cell density was ≈5.5×10^5/mL.

Results

Effect of AICAr on MTX polyglutamate synthesis. For CCRF-CEM cells, the presence of 0.5 mmol/L AICAr increases both total drug (3-fold) and MTX polyglutamate ($n \geq 2$) accumulation (3.5-fold) after 4 hours of exposure to 2 μmol/L [^{3}H]MTX (Table 1). Of interest is that longer ($n \geq 3$), better-retained MTX polyglutamates are increased 6-fold by AICAr. If, after 4-hour uptake, cells are washed free of [^{3}H]MTX (and AICAr) and allowed to efflux for 2 hours, cells treated with AICAr retain higher drug levels (not shown), as expected for higher levels of longer MTX polyglutamates (23). Increased MTX polyglutamate accumulation is also observed at 1 and 10 μmol/L [^{3}H]MTX in the presence of 0.5 mmol/L AICAr (not shown). AICAr thus potentiates MTX polyglutamate accumulation in human leukemia cells. The more readily available aglycone of AICAr also potentiates [^{3}H]MTX uptake to a similar extent (below).

Effect of AICAr concentration on MTX influx. Because an effect of AICAr on influx could explain the observed potentiation of MTX accumulation, the effect of AICAr on [^{3}H]MTX uptake was assayed. AICAr was added 5 minutes before [^{3}H]MTX addition and influx was assayed over 0 to 30 minutes to quantitate initial influx velocity (v_i; 0-5 minutes) and extent (30 minutes; Fig. 2). AICAr does not affect v_i under these conditions. After 5 to 10 minutes of linear uptake, influx in the *absence* of AICAr plateaus, as is typical *in vitro* (e.g., ref. 24). In contrast, in the presence of AICAr, uptake continues after 10 minutes at a rate dependent on the AICAr concentration; there is little indication of a 30-minute plateau at high AICAr. AICAr at 0.5 to 1.0 mmol/L increases MTX accumulation at 30 minutes by ≥3-fold. High-performance liquid chromatography analysis shows that −AICAr at 30 minutes, 86% of intracellular radiolabel is MTX and 12% is MTX-γ-Glu, whereas +0.5 mmol/L AICAr, 83% is MTX and 7.5% is MTX-γ-Glu with <1% MTX-γ-$(Glu)_2$. Thus, neither degradation of [^{3}H]MTX to rapidly transported moieties (25) nor increased relative MTX polyglutamate synthesis accounts for the AICAr-induced potentiation of uptake. The similar magnitudes of increase (3-fold) in the level of MTX influx at 30 minutes and MTX polyglutamate accumulation at 4 hours (Table 1) +AICAr suggest that increased availability of substrate (MTX) for folylpolyglutamate synthetase is the primary mechanism of AICAr potentiation of MTX polyglutamate accumulation, although contributions by other factors are being evaluated. Preliminary data (not shown) indicate that 0.5 mmol/L AICAr also potentiates [^{3}H]MTX influx in K562 human chronic myelogenous leukemia cells with a similar lag and increase in 30-minute accumulation; thus, AICAr potentiation is not restricted to CCRF-CEM cells or to one cell lineage. AICAr also potentiates uptake of aminopterin, another antifolate DHFR inhibitor, to a similar extent and with a similar time course (not shown).

Effect of MTX concentration on uptake potentiation by AICAr. Influx was studied as a function of [^{3}H]MTX concentration ±0.5 mmol/L AICAr (Fig. 3). v_i values (0-5 minutes) are the same ±AICAr (note scale change), but AICAr potentiates 30-minute accumulation at 1 to 10 μmol/L MTX, with highest potentiation (6-fold) at 10 μmol/L. The ≈10-minute delay before onset of AICAr potentiation is seen at all MTX levels. Analysis of v_i data shows that −AICAr, $V_{max}/K_t = 0.44$, whereas +AICAr, $V_{max}/K_t = 0.47$, suggesting that kinetics at early times are unaffected by AICAr. These V_{max}/K_t values are consistent with those expected for the RFC (3, 6). Because

Table 1. Synthesis of MTX polyglutamates (4-NH_2-10-CH_3-$PteGlu_n$) by CCRF-CEM human leukemia cells in the presence and absence of 500 μmol/L AICAr

Conditions	Total drug (pmol/10^7 cells)	4-NH_2-10-CH_3-$PteGlu_n$ (pmol/10^7 cells)						
		n = 1 (MTX)	2	3	4	5	$n \geq 2$	$n \geq 3$
MTX	53.8	26.2	23.8	2.3	1.4	0.1	27.6	3.8
MTX + AICAr	165.7	70.1	73.0	16.6	5.4	0.6	95.6	22.6

NOTE: Logarithmically growing CCRF-CEM cells were exposed to 2 μmol/L [^{3}H]MTX for 4 hours and the intracellular radiolabel was analyzed by high-performance liquid chromatography for the presence of MTX polyglutamates (see Materials and Methods).

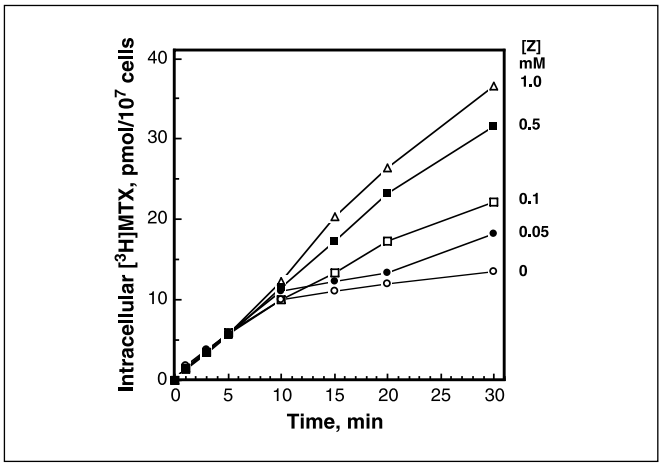

Figure 2. Concentration dependence of AICAr (Z) potentiation of [^{3}H]MTX uptake in CCRF-CEM cells. CCRF-CEM cells at 2×10^7 cells/mL were preincubated for 5 minutes with various concentrations of AICAr before addition of [^{3}H]MTX to a final concentration of 2 μmol/L (3 μCi/mL). Aliquots (100 μL) were removed at the indicated times and processed as described in Materials and Methods. AICAr concentrations are 0 (water; ○), 0.05 mmol/L (●), 0.1 mmol/L (□), 0.5 mmol/L (■), and 1.0 mmol/L (△).

rates >5 minutes are not v_i, the later portion of the +AICAr curve where potentiation is observed cannot be analyzed by Michaelis-Menten kinetics (see below).

Effect of preincubation time with AICAr. Above, AICAr was preincubated with cells for 5 minutes before [^{3}H]MTX addition; 5 minutes was chosen because most nucleosides are rapidly transported (26). Because onset of potentiation is delayed (Fig. 2), the effect of preincubation time with 0.5 mmol/L AICAr on onset time and extent of potentiation was tested. Whether preincubation of cells with AICAr was for 5 to 20 minutes or even if AICAr was added together with [^{3}H]MTX, the time course was identical to Fig. 2 (i.e., potentiation does not occur until ≈10 minutes after [^{3}H]MTX addition). Even if AICAr is added 10 minutes *after* [^{3}H]MTX, there is a ≥5-minute delay before potentiation of [^{3}H]MTX uptake occurs and full potentiation occurs only after 5 to 10 minutes more (not shown).

Effect of AICAr on MTX efflux. Increased MTX accumulation over 30 minutes could result from decreased efflux. [^{3}H]MTX efflux from CCRF-CEM cells was assayed after uptake ±0.5 mmol/L AICAr. Efflux in each sample was measured ±0.5 mmol/L AICAr (cross-over design). No significant difference was noted in efflux rates ($t_{1/2}$ ranged from 14.6 to 18.7 minutes; not shown) suggesting that AICAr affects influx only. The efflux times are slower than those reported for CCRF-CEM (4.5-5.5 minutes; ref. 6), but a buffered balanced salts medium, which enhances efflux (21), was used previously.

Effect of AICAr on protein-bound intracellular MTX. Protein (DHFR)-bound MTX (18) levels are essentially identical ±0.5 mmol/L AICAr over 30 minutes of uptake; at 30 minutes, protein-bound levels are 6.4 ± 0.1 and 5.4 ± 0.4 pmol/10^7 cells −AICAr and +AICAr (n = 2), respectively. Thus, the increased influx rate +AICAr is not a result of induction of a high-affinity binding protein that sequesters MTX.

Subcellular location of [^{3}H]MTX accumulated during AICAr potentiation. Cellular folate transporters are in the plasma membrane and, once translocated, MTX binds to DHFR. After DHFR is saturated, unbound MTX accumulates in the cytosol and is polyglutamylated (11). Mitochondria and lysosomes have folate/antifolate transporters that are not fully characterized. Mitochondria (27) do not transport MTX or MTX polyglutamates (28) whereas lysosomes transport MTX polyglutamates, but MTX only poorly (29). If AICAr activates organellar MTX transport and continued uptake +AICAr is a function of sequestration, MTX should be located in a subcellular organelle, most likely mitochondria or lysosomes. The subcellular distribution of [^{3}H]MTX after 30 minutes ± 0.05 mmol/L AICAr was determined (not shown) after separation of CCRF-CEM cytosol from a nuclear pellet, a mitochondrial/lysosomal fraction (22) and a microsomal fraction. There is no qualitative difference between the distribution ±AICAr and the total recovered radioactivity is primarily cytosolic (−AICAr, 88.9%; +AICAr, 94.8%; ±AICAr, >99.3% if the nuclear pellet, which contains unlysed cells, is excluded from analysis). High-performance liquid chromatography analysis of the cytosol shows it is >96% MTX and MTX-γ-Glu (not shown). A similar subcellular distribution analysis was done after 4-hour incubation with [^{3}H]MTX ± AICAr and the radioactivity was again cytosolic in both cases (>93% cytosolic; >96% if the nuclear pellet is excluded from analysis). Potentiation in uptake observed +AICAr is not caused by sequestration of MTX or its metabolites in a subcellular compartment.

DHFR inhibition and AICAr potentiation. Studies were undertaken to determine whether MTX itself, inhibition of DHFR, or other inhibition of folate metabolism is sufficient to initiate AICAr potentiation. Trimetrexate is a potent, lipophilic DHFR inhibitor that rapidly enters cells and does not use the RFC (9). The v_i for [^{3}H]MTX uptake is the same for CCRF-CEM cells preincubated for 10 minutes (the lag in potentiation) ±2 μmol/L trimetrexate (Fig. 4); the extent of [^{3}H]MTX accumulation at 30 minutes +trimetrexate is 90% to 100% that −trimetrexate (range obtained in two independent experiments). It may be speculated that trimetrexate does not affect [^{3}H]MTX uptake over 30 minutes because trimetrexate neither uses nor inhibits the RFC and because the >10-fold lower affinity for DHFR of trimetrexate compared with MTX (30) may lead to rapid displacement of trimetrexate from DHFR by MTX. If AICAr + trimetrexate is present in the preincubation, there is no delay in potentiation of [^{3}H]MTX uptake and v_i is immediately increased (Fig. 4). Thus, MTX per se or its metabolites are not required to initiate AICAr potentiation; however, inhibition of folate metabolism is required and DHFR inhibition is sufficient. AICAr + trimetrexate preincubation has the same effect on [^{3}H]aminopterin transport (not shown). The delay in onset of inhibition with MTX + AICAr (Fig. 2) probably occurs because it takes ≈5 minutes for 2 μmol/L MTX to saturate CCRF-CEM cell DHFR (18) and essentially 100% saturation is required to inhibit folate metabolism (31).

These findings are reinforced by studies with a reduced folate, leucovorin. Preincubation of CCRF-CEM cells with AICAr alone does *not* potentiate uptake of 1 μmol/L (6*S*)-[^{3}H]leucovorin (Fig. 5), consistent with a requirement for inhibition of folate metabolism in AICAr potentiation. Preincubation of cells with trimetrexate alone causes a small increase in v_i (1.4-fold) and 30-minute accumulation (1.6-fold) of [^{3}H]leucovorin (Fig. 5). If cells are preincubated with AICAr + trimetrexate, however, potentiation of [^{3}H]leucovorin uptake is observed without a lag, and the v_i (3.6-fold) and extent (3.8-fold) of [^{3}H]leucovorin uptake are substantially increased (Fig. 5). These data are also consistent with DHFR inhibition being sufficient to initiate AICAr potentiation, showing that reduced folates are subject to AICAr potentiation and that MTX itself need not be involved.

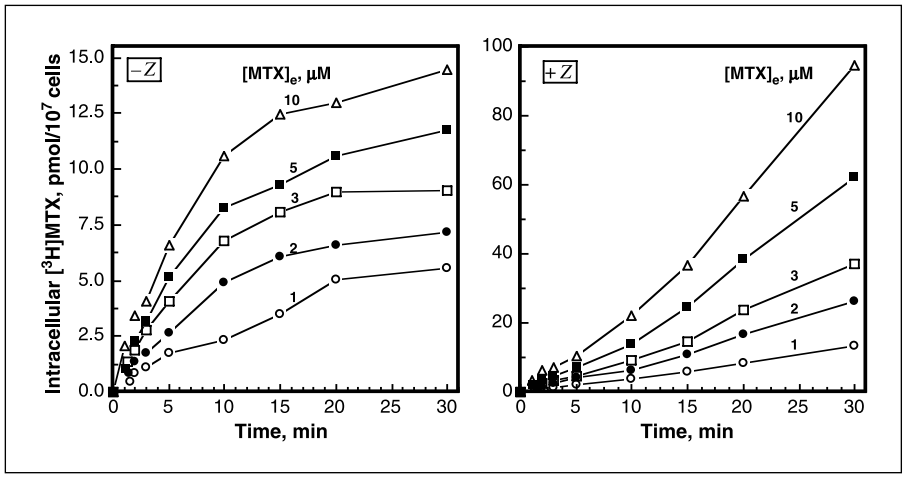

Figure 3. Dependence of [^{3}H]MTX uptake in CCRF-CEM cells on MTX concentration in the presence and absence of AICAr. Cells were preincubated with 500 μmol/L AICAr (*Z*) for 5 minutes and then [^{3}H]MTX was added at the indicated concentration (3 μCi/mL). Aliquots (100 μL) were removed at the indicated times and processed as described in Materials and Methods.

Effect of thymidylate synthase or glycinamide ribonucleotide formyltransferase inhibition on AICAr potentiation. The above data show that DHFR inhibition is sufficient to induce influx potentiation by AICAr, but not whether it is necessary. MTX indirectly inhibits two key folate-dependent pathways (9), thymidylate and purine syntheses; thus effects on AICAr potentiation of inhibitors of enzymes in these pathways, thymidylate synthase and glycinamide ribonucleotide formyltransferase, were evaluated. AG337, a specific, lipophilic inhibitor of thymidylate synthase (32), at 2 or 20 μmol/L does not potentiate uptake of [^{3}H]leucovorin ±AICAr (Fig. 5). However, AG337 ablates potentiation of leucovorin uptake induced by trimetrexate + AICAr, as well as the minor increase in uptake seen +trimetrexate alone (Fig. 5). Because no specific lipophilic purine synthesis inhibitor is available, an approach using [^{3}H]DDATHF, a specific antifolate inhibitor of glycinamide ribonucleotide formyltransferase that uses the RFC (9), was employed. If purine synthesis inhibition initiates AICAr potentiation, DDATHF uptake should be potentiated +AICAr after a lag period (cf., Fig. 2). However, when [^{3}H]DDATHF (1 μmol/L) is added after 10-minute preincubation of CCRF-CEM cells ±0.5 mmol/L AICAr, no potentiation by AICAr is observed over 30 minutes (not shown). After 30-minute uptake +AICAr, 16.6 ± 1 pmol DDATHF/10^7 cells accumulates (n = 2), of which 42% is $\geq Glu_3$ and 19% is $Glu_{5\text{-}6}$; DDATHF polyglutamates are required to inhibit glycinamide ribonucleotide formyltransferase. DDATHF uptake is susceptible to AICAr potentiation, however, because preincubation with trimetrexate + AICAr increases the initial rate of DDATHF uptake 2.5-fold and the extent at 30 minutes by 3.2-fold; trimetrexate alone causes a small (15%) increase in extent at 30 minutes. Because neither thymidylate synthase nor glycinamide ribonucleotide formyltransferase inhibition initiates AICAr potentiation, but DHFR inhibition does, inhibition of DHFR is sufficient and necessary.

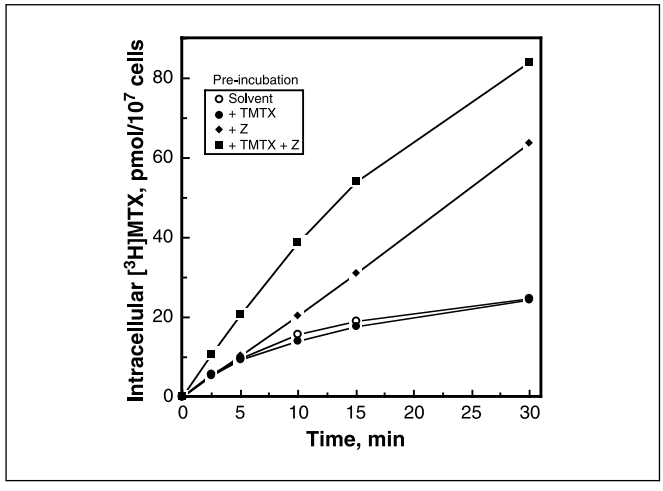

Figure 4. Effect of the lipophilic DHFR inhibitor trimetrexate (*TMTX*) on potentiation of [^{3}H]MTX uptake by 0.5 mmol/L AICAr. CCRF-CEM cells were preincubated for 10 minutes with solvent alone (○), 1 μmol/L trimetrexate (●), 0.5 mmol/L AICAr (♦), or trimetrexate + AICAr (■). At 10 minutes, [^{3}H]MTX (2 μmol/L and 0.75 μCi/mL final) was added and uptake was quantitated as described in Materials and Methods.

Maintenance of potentiation. When potentiation is initiated in CCRF-CEM cells by 10-minute preincubation with trimetrexate + 0.5 mmol/L 5-amino-4-imidazolecarboxamide (AICAr-base), the ν_i of [^{3}H]MTX uptake increases 2-fold and the 30-minute extent increases 3.4-fold, when both remain in the medium. If trimetrexate and AICAr-base are removed after preincubation, potentiation is sustained. [^{3}H]MTX uptake at 30 minutes loses potentiation with $t_{1/2}$ = 16 to 21 minutes (n = 2). The potentiated ν_i is unchanged for 20 to 30 minutes and then declines slowly ($t_{1/2}$ = 58-69 minutes; n = 2).

Effect of AICAr on MTX influx kinetic constants. MTX influx is mediated by the RFC or membrane-bound folate-binding protein (2). The micromolar MTX concentration dependence during potentiation and the time dependence (Figs. 2 and 3) are consistent only with RFC participation (33). Because preincubation with AICAr + trimetrexate eliminates the lag before potentiation occurs (Fig. 4), it is possible to determine kinetic constants for transport during potentiation. For MTX in the absence of trimetrexate or AICAr, K_t = 3.0 ± 0.8 μmol/L with V_{max} = 5.8 ± 0.9 pmol/min/10^7 cells (n = 2 for all). Trimetrexate increases the K_t (5.3 ± 0.6 μmol/L) and V_{max} (7.2 ± 0.6 pmol/min/10^7 cells) for MTX slightly. AICAr addition does not further affect K_t (5.1 ± 0.1 μmol/L) but increases V_{max} (19.8 ± 2 pmol/min/10^7 cells) by ≈3-fold. These K_t values are consistent with RFC mediating AICAr potentiated influx. Because AICAr affects only V_{max}, it indicates that more transporter is active or individual transporters are translocating at an increased rate. Assuming a value of 0.55 pL/CCRF-CEM cell (34), influx at 30 minutes −AICAr reaches equilibration only at 1 μmol/L; influx +0.5 mmol/L AICAr is concentrative ($[MTX]_i$ / $[MTX]_e \geq 1.9$) at 1 to 10 μmol/L MTX.

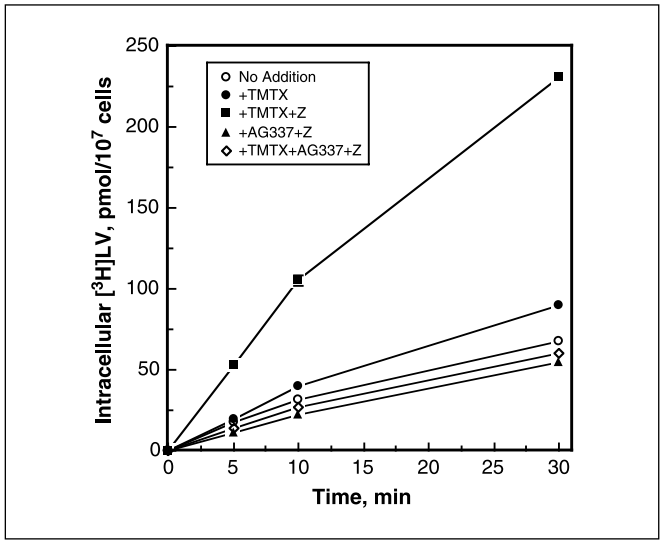

Figure 5. Effect of the lipophilic DHFR inhibitor trimetrexate and lipophilic thymidylate synthase inhibitor AG337 on potentiation of [^{3}H]leucovorin uptake by 0.5 mmol/L AICAr. CCRF-CEM cells were preincubated for 10 minutes with solvent alone (○), 2 μmol/L trimetrexate (●), trimetrexate + 0.5 mmol/L AICAr (■), 20 μmol/L AG337 + AICAr (▲), or trimetrexate + AG337 + AICAr (◇). At 10 minutes, [^{3}H]leucovorin (1 μmol/L and 0.25 μCi/mL) was added and uptake was quantitated as described in Materials and Methods. Note that for the sake of clarity, three conditions are not shown: AICAr alone is essentially equivalent to solvent control (○); AG337 alone is essentially equivalent to AG337 + AICAr (▲); and trimetrexate + AG337 is essentially equivalent to trimetrexate + AG337 + AICAr (◇).

Effect of RFC competitors on AICAr potentiation of [^{3}H]MTX uptake. If RFC mediates increased [^{3}H]MTX influx + AICAr, uptake should be inhibited by alternate substrates of the RFC (3, 6). Inhibition of ν_i of uptake was measured after preincubation of CCRF-CEM cells either +trimetrexate (inhibition at the RFC) or +trimetrexate + AICAr (inhibition during AICAr potentiation). Efficient RFC substrates (aminopterin, (6*R,S*)-leucovorin, ZD1694, BW1843U89, and DDATHF; refs. 2, 3) have similar potencies as competitors of [^{3}H]MTX influx ±AICAr (Table 2) and their potencies are similar to their published K_t values for the RFC (2, 3, 6, 9). Folic acid, a poor RFC substrate (2, 3), is a poor competitor of [^{3}H]MTX influx ±AICAr. Folic acid competition, unlike the folate analogues, plateaus at 60% to 70% ±AICAr, emphasizing its poor affinity (not shown). Inhibitory potencies of aminopterin, (6*R,S*)-leucovorin, and folic acid in the absence of trimetrexate and AICAr (not shown) were comparable to those +trimetrexate alone (Table 2). These data are consistent with RFC mediating AICAr potentiated influx although induction by AICAr of a novel influx mechanism for MTX and folates having a substrate specificity similar to the RFC cannot be excluded.

Effect of AICAr on [^{3}H]MTX transport in RFC-defective cells. An MTX-resistant CCRF-CEM subline (R2; ref. 20) does not transport MTX because active RFC is not expressed (35); R2 retains trimetrexate sensitivity (36). After preincubation with trimetrexate ± 500 μmol/L AICAr (e.g., Fig. 4), there was essentially no [^{3}H]MTX uptake by R2 (not shown). Because CCRF-CEM and R2 should be isogenic except for the RFC alteration, lack of AICAr potentiation in R2 is consistent with the RFC, and not an undescribed transporter, mediating AICAr potentiated influx. Neither parental CCRF-CEM nor R2 expresses folate-binding protein (37), further supporting the suggestion that folate-binding protein is not involved.

Temperature dependence of AICAr potentiation of MTX uptake. The Q_{10} (37°C versus 27°C) for ν_i of uptake −AICAr (+trimetrexate) is 4.6 ± 0.3 (n = 2) and for +0.5 mmol/L AICAr (+trimetrexate) the Q_{10} is 5.9 ± 0.1 (n = 2); influx ±AICAr is thus facilitated and values ±AICAr are similar. Q_{10} values for 30-minute uptake are also similar (−AICAr, 3.45 ± 0.15; +AICAr, 3.65 ± 0.15). The data are consistent with RFC mediating AICAr potentiated influx.

Medium effects on AICAr potentiation of [^{3}H]MTX influx. Both ν_i and extent of [^{3}H]MTX influx by the RFC are affected by the transport medium because extracellular anions inhibit (21). If AICAr reverses anion inhibition in standard transport medium, then AICAr potentiation should not occur in anion-deficient MHS. If AICAr potentiation requires a component of standard medium, but is unrelated to anion inhibition, potentiation should not occur in the balanced salt solution HBSS. [^{3}H]MTX influx (ν_i and 30-minute extent; −trimetrexate) in standard medium and HBSS is essentially identical (not shown). Influx (−trimetrexate) in anion-free MHS has an ≈1.4-fold greater ν_i and a 2.7-fold higher 30-minute extent, as reported (21). AICAr (0.5 mmol/L AICAr; −trimetrexate) potentiates influx in all three media, albeit with different characteristics (not shown). Standard medium shows a 5- to 10-minute lag (Fig. 2) before potentiation occurs. HBSS and MHS show no or less lag, and ν_i values are 1.4- to 1.9-fold those observed −AICAr. Potentiated accumulation at 30 minutes is similar in standard medium and MHS (≈2-fold), but is higher (≈4-fold) in HBSS. Potentiation in all buffers seems to rule out exchange (2) of MTX for an anion generated after AICAr transport (i.e., AICAr monophosphate) as a mechanism. MHS and HBSS contain no folate; because AICAr potentiation occurs, the data also rule out potentiation by acute sensing of extracellular folate deficiency that AICAr mimics. Consistent with this idea is the finding (not shown) that AICAr potentiation occurs to the same extent if uptake by folate-replete cells is measured in standard medium lacking folic acid.

Table 2. Inhibition of initial velocity of [^{3}H]MTX influx by antifolates and folic acid

Competitor/inhibitor	IC_{50} (μmol/L)	
	+Trimetrexate	+Trimetrexate + AICAr
MTX	11.1 ± 0.2	10.7 ± 0.8
Aminopterin	3.7 ± 0.2	2.6 ± 0.3
(6*R,S*)-leucovorin	2.6 ± 0.1	2.7 ± 0
PteGlu	18.6 ± 0.6	18.5 ± 1.1
Aminopterin	4.0 ± 0.6	4.4 ± 0.4
ZD1694	2.4 ± 0.5	3.4 ± 0.5
BW1843U89	1.0 ± 0.1	1.3 ± 0.4
DDATHF	1.0 ± 0.2	1.2 ± 0.4

NOTE: Inhibition studies were carried out as described in Materials and Methods after a 10-minute preincubation with 1 μmol/L trimetrexate alone (inhibition of the RFC) or 1 μmol/L trimetrexate + 0.5 mmol/L AICAr (inhibition of AICAr potentiated influx). All studies were carried out in standard transport medium. Inhibitory potency was measured in three series of experiments and aminopterin was included in each series as an internal control. Values are average ± range of two determinations.

Specificity of potentiation for AICAr. AICAr-base, the aglycone of AICAr, potentiates [^{3}H]MTX influx after a 10-minute preincubation with CCRF-CEM cells (not shown) to a similar extent; the ribose of AICAr is thus not essential for potentiation. However, adenosine, deoxyadenosine, guanosine, deoxyguanosine, adenosine + guanosine, inosine, deoxyinosine, adenine, guanine, hypoxanthine, and thymidine (each 0.5 mmol/L) and L-methionine (5 mmol/L) have no potentiating effect. Potentiation of [^{3}H]MTX uptake is not a general property of nucleosides or nucleobases, but is limited to AICAr and closely related compounds. Preincubation with excess glutamine (5 mmol/L above the normal 2 mmol/L), which increases MTX polyglutamate accumulation over hours (38) and is also required for purine synthesis, has no effect on [^{3}H]MTX influx over 30 minutes (not shown). The presence of 5 mmol/L Na-formate (a one-carbon unit salvageable by 10-formyltetrahydrofolate synthetase to form 10-formyltetrahydrofolate, the cosubstrate for AICAr monophosphate formylation) or 5 mmol/L L-methionine during preincubation with AICAr-base did not affect potentiation (not shown).

Requirement for protein synthesis. CCRF-CEM protein synthesis is completely inhibited by 10 μg/mL cycloheximide within 5 minutes (39). When CCRF-CEM cells are preincubated with 10 μg/mL cycloheximide for 10 minutes, and 0.5 mmol/L AICAr is added for the last 5 minutes, [^{3}H]MTX influx potentiation occurs as in Fig. 2 (not shown). This rules out a requirement for new protein synthesis both in the RFC and in metabolism of AICAr, because of the incubation sequence, and suggests that the RFC is activated directly or indirectly.

Effect of AICAr on growth inhibitory potency. Because AICAr increases MTX uptake and MTX poly-glutamate accumulation, AICAr should increase MTX growth inhibitory potency against CCRF-CEM. AICAr (50 μmol/L) does not increase MTX potency during continuous (120 hours) drug exposure (Table 3). However, following "pulse" exposure (MTX and 100 μmol/L AICAr are present only 0 to 24 hours of 120 hours), potency increases 2-fold relative to MTX alone. The concentration-response curve of MTX alone in 0 to 24 hours of pulse exposure has a shallower slope than that for continuous exposure, but addition of 100 μmol/L AICAr restores the steeper slope (not shown). Thus, the EC_{80} is 3- to 4-fold lower +AICAr. AICAr potentiation of MTX uptake and metabolism is thus translated into increased cytotoxicity. EC_{50} potency of aminopterin is also increased ≈2-fold +AICAr in 0 to 24 hours of exposure (Table 3). AICAr, which could potentially serve as a purine source, did not reverse growth inhibitory effects of MTX or aminopterin. Potency of MTX against RFC-defective R2 in continuous exposure (EC_{50}, 2,200 nmol/L) was unaffected by 50 μmol/L AICAr (EC_{50}, 2,200 nmol/L). If AICAr activated an RFC-independent pathway, increased influx via this pathway should increase MTX potency against R2. This result supports the RFC, and not a novel pathway with similar properties that is activated by AICAr, as responsible for the increased uptake that occurs +AICAr. After our studies on AICAr were initiated, Ha and Baggott (40) reported that 100 μmol/L AICAr modestly increased growth inhibitory potency of MTX during continuous exposure; MTX transport or metabolism was not examined and growth inhibition was suggested to occur because of transmethylation pathway inhibition.

Table 3. Effect of AICAr on growth inhibitory potency of MTX and aminopterin in continuous and "pulse" exposure

Drug	Drug exposure time (h/total h)	[AICAr] (μmol/L)	EC_{50} (nmol/L)	*n*
MTX	0-120/120	0	14.5 ± 0.5	2
		50	13.5 ± 0.5	2
	0-24/120	0	70 ± 16	5
		100	30 ± 2	3
Aminopterin	0-24/120	0	6.1 ± 1.3	2
		100	3.7 ± 0.3	2

NOTE: A range of concentrations of MTX ± the indicated concentration of AICAr was incubated with CCRF-CEM cells for 120 hours of a growth period of 120 hours or for only 0 to 24 hours (pulse) of the same growth period. Inhibitory potency was quantitated (EC_{50}) as the concentration effective at inhibiting growth by 50% relative to an untreated culture as described in Materials and Methods. AICAr at 50 μmol/L is nontoxic to CCRF-CEM cells during 120-hour exposure (EC_{50}, 130 ± 20 μmol/L; n = 2). AICAr at 100 μmol/L is nontoxic to CCRF-CEM cells during 0 to 24/120 hours exposure (EC_{50}, 410 ± 50 μmol/L; n = 2). Values presented are average ± SD for n > 2 and average ± range for n = 2.

Discussion

During a program to identify MTX poly(γ-glutamate) synthesis modulators in CCRF-CEM cells, we found that AICAr enhances accumulation of these cytotoxic metabolites up to 3-fold during a 4-hour incubation (Table 1). AICAr causes a rapid increase in MTX influx (Fig. 2) that is also ≈3-fold, suggesting that increased metabolite accumulation at 4 hours results from increased substrate availability for polyglutamylation. We evaluated AICAr because MTX polyglutamate accumulation presents a folate stress and Bochner and Ames (14) showed that AICAr triphosphate (or related metabolites) regulates folate metabolism during folate stress.

Folates and antifolates containing an intrinsic glutamate or glutamate-like moiety use two transporters, the RFC and folate-binding protein, although the RFC is considered the more prominent (2, 3). Transport by the RFC and folate-binding protein can be differentiated by their unique characteristics (2, 3). That influx potentiation by AICAr is mediated by the RFC is supported by analysis of transport ±AICAr. First, K_t values for [^{3}H]MTX uptake are similar ±AICAr and are in the low micromolar range as is typical for the RFC, not in the nanomolar range typical for the folate-binding protein. The values are similar to those reported for the CCRF-CEM RFC (6, 24). AICAr potentiation affects only the V_{max} of transport. Second, efficient alternate substrates for the RFC (aminopterin, leucovorin, ZD1694, BW1843U89, and DDATHF) compete with [^{3}H]MTX uptake with similar relative potency ±AICAr (low micromolar range; Table 2). Folic acid is a poor competitor of uptake ±AICAr. The RFC prefers leucovorin ≥ aminopterin > MTX ≫ PteGlu whereas the folate-binding protein prefers PteGlu ≫ leucovorin > MTX = aminopterin (41). For each competitor, there is no evidence of biphasic competition ±AICAr, consistent with a single uptake mechanism. Third, no AICAr potentiation occurs in CCRF-CEM subline R2 in which RFC protein is not expressed. The data also rule out induction by AICAr of a novel transporter that shares properties with the RFC because such a transporter should still be present in R2. Fourth, uptake +AICAr responds to medium composition in a manner comparable to that of the RFC. Fifth, the time dependence of transport corresponds to that of the RFC (2, 3) and not the folate-binding protein, which transports slowly

(33). Finally, the temperature dependence (Q_{10}) of uptake ±AICAr is similar and the magnitude suggests a carrier-mediated process, such as the RFC. Neither sequestration of antifolate in a subcellular compartment nor increased protein binding contributes to the increased influx rate.

Although the increase in influx by the RFC contributes (via mass-action) to the increased accumulation of MTX polyglutamates observed at 4 hours +AICAr, other contributions (e.g., increased folylpolyglutamate synthetase activity and/or decreased γ glutamyl hydrolase activity) cannot yet be ruled out. These and other potential factors contributing to AICAr potentiation are now being studied. AICAr potentiates MTX influx (Fig. 2); however, it does not increase the relative proportion of drug metabolized to MTX polyglutamates at 30 minutes, suggesting that AICAr does not acutely activate folylpolyglutamate synthetase.

DHFR inhibition is necessary and sufficient for AICAr to induce potentiation of influx by the RFC. MTX and aminopterin initiate AICAr potentiation, but because ≥5 minutes is required to completely inhibit DHFR under these conditions (18), there is a lag before potentiation occurs (Fig. 2). The lipophilic DHFR inhibitor trimetrexate also initiates AICAr potentiation. DHFR inhibitors act primarily by indirectly inhibiting *de novo* thymidylate synthesis (at thymidylate synthase) and purine synthesis (at glycinamide ribonucleotide formyltransferase and AICAr formyltransferase) through depletion of their requisite reduced folate cofactors; however, direct inhibition of these pathways with specific inhibitors (AG337 and DDATHF, respectively) does not initiate potentiation by AICAr (Fig. 5). Of interest is the finding that simultaneous direct inhibition of thymidylate synthase and DHFR abrogates AICAr potentiation (Fig. 5), suggesting that binding of antifolate to DHFR is insufficient to initiate potentiation, and one or more biochemical consequences of DHFR inhibition are required (e.g., depletion of reduced folates or accumulation of dihydrofolate forms). Antagonism of DHFR inhibition by thymidylate synthase inhibition is well described (42). Of note is that even when DHFR inhibition is established before AICAr addition, there is still an ≈5-minute lag before potentiation occurs, consistent with uptake and metabolism of AICAr being essential. Transport and metabolism of AICAr are under study.

Anion-exchange is an intrinsic property of the RFC in which an anion (e.g., phosphate or AMP; ref. 24) on one side of the plasma membrane exchanges for a folate on the other side (2, 3). Thus, exogenous AICAr could be transported and anabolized to AICAr monophosphate, which could exchange for MTX. Two lines of evidence suggest that anion-exchange is not involved in AICAr potentiation of uptake by the RFC. AICAr alone does not potentiate influx of [^{3}H]leucovorin (Fig. 5) whereas trimetrexate + AICAr does potentiate its uptake, arguing against anion-exchange because AICAr should be transported and anabolized to AICAr monophosphate whether or not trimetrexate is present. Likewise, the lack of potentiation by various purines argues against an anion-exchange mechanism. The latter finding also suggests that metabolism of AICAr (as AICAr monophosphate) to purines downstream in *de novo* purine synthesis (e.g., IMP, AMP, or ATP) is not responsible for potentiation because this should also occur with other purines.

Although RFC is transcriptionally regulated (2, 3), posttranscriptional regulation of RFC activity has been described rarely. Protein tyrosine phosphorylation has been correlated with decreased MTX transport in L1210 (43), and both dibutyryl-cyclic AMP and protein tyrosine kinase inhibitor treatment decreased folic acid uptake by a normal colon cell line (44). It is unclear if these reports are relevant to AICAr potentiation. Because protein synthesis inhibition does not abrogate AICAr potentiation, synthesis of new RFC cannot account for the increased V_{max} and the effect of AICAr must be posttranscriptional. AICAr is unique in this regard. This finding also shows that cells are constitutively competent to carry out whatever metabolism of AICAr is required. Nonexclusive mechanisms for the increased RFC V_{max} are (*a*) the presence of cryptic RFC that is recruited to the plasma membrane by AICAr treatment; (*b*) allosteric activation of RFC by AICAr or a AICAr metabolite; and (*c*) involvement of AICAr or a AICAr metabolite in eliciting a posttranslational modification that activates RFC. Whereas these possibilities are all attractive, AICAr is well known as an exogenous activator of AMP-activated protein kinase (45). AMP-activated protein kinase maintains cellular ATP pools in conditions of stress by decreasing activity of ATP-using pathways and increasing activity of ATP-generating pathways. Among the proteins phosphorylated by AMP-activated protein kinase are three transport proteins: GLUT-1 and GLUT-4, the activities of which are enhanced, and the cystic fibrosis transmembrane conductance regulator, the activity of which is inhibited (45). AMP-activated protein kinase itself must be phosphorylated by a kinase before it can be activated by AMP, allowing it to phosphorylate target proteins. Recently, the AMP-activated protein kinase was identified as tumor suppressor LKB1 (46), which functions as a master regulator in metabolism (47). Studies are ongoing to determine whether AMP-activated protein kinase mediates increased RFC activity induced by AICAr and whether LKB1 is required. If LKB1 is essential to AICAr potentiation, it will provide the first link between a tumor suppressor and folate metabolism and expands the scope of involvement of intermediary metabolism in tumor progression. In this regard, functional folate deficiency correlates with increased occurrence of several tumors, including childhood acute lymphoblastic leukemia (48).

AICAr monophosphate is an intermediate in *de novo* purine synthesis and its conversion to formyl-AICAr monophosphate is folate dependent. Increased AICAr monophosphate (or other AICAr metabolite) could be a physiologic signal for incipient folate deficiency. Exogenous AICAr, after metabolism, may signal in the same manner. Acute extracellular folate deficiency, by transfer of cells to media containing no folate, did not significantly affect transport ±AICAr, suggesting that the extracellular folate level is not sensed. The effect of induced folate deficiency is now being studied. AICAr potentiation may be pharmacologically significant because AICAr increases growth inhibitory potency of MTX and aminopterin in a 24-hour exposure (Table 3) and typical clinical regimens for childhood acute lymphoblastic leukemia use interspersed 24-hour infusion of high-dose MTX (49). Our data may underestimate the potential of this regimen because a suboptimal AICAr concentration (100 μmol/L; Fig. 2) was included in the assays because CCRF-CEM is slightly sensitive to AICAr toxicity on extended exposure. In addition, because potentiation by AICAr is MTX concentration dependent (Fig. 3), at low MTX concentrations such as those used for 24-hour exposures (0.01-0.2 μmol/L), potentiation should be less pronounced. However, because in the high-dose MTX regimens used for childhood acute lymphoblastic leukemia, serum MTX levels are >10 μmol/L for >24 hours (50) and AICAr has been evaluated as a treatment for cardiac ischemia and

plasma concentrations up to 270 μmol/L are achievable without toxicity (51), greater potentiation by AICAr may occur in patients. Clinical data suggest that a selective increase of 2- to 3-fold in intracellular MTX may be sufficient to increase long-term survivors of childhood acute lymphoblastic leukemia (12, 13). Our results are encouraging with regard to the hypothesis that combination with AICAr could enhance the therapeutic effect of MTX against childhood acute lymphoblastic leukemia.

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