Antifolates Induce Inhibition of Amido Phosphoribosyltransferase in Leukemia Cells*  

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The pathway for de novo biosynthesis of purine nucleotides contains two one-carbon transfer reactions catalyzed by glycaminide ribotide (GAR) and 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylases in which N⁵⁰⁰-formyltetrahydrofolate is the one-carbon donor. We have found that the antifolates methotrexate (MTX) and piritrexim (PTX) completely block the de novo purine pathway in mouse L1210 leukemia cells growing in culture but with only minor accumulations of GAR and AICAR to less than 5% of the polyphosphate derivatives of N⁵⁰⁰-formylglycinamide ribotide (FGAR) which accumulate when the pathway is blocked completely by azaserine. This azaserine-induced accumulation of FGAR polyphosphates is completely abolished by MTX, indicating that inhibition of the pathway is at or before the GAR transformylase reaction 3; Lyons, S. D., and Christopherson, R. I. (1991) Biochem. Int. 24, 187–197. Three h after the addition of MTX (0.1 μM), cellular 5-phosphoribosyl-1- pyrophosphate has accumulated 3.4-fold while 6-methylmercaptopurine riboside (25 μM) induces a 6.3-fold accumulation. These data suggest that amido phosphoribosyltransferase catalyzing reaction 1 of the pathway is the primary site of inhibition. In support of this conclusion, we have found that dihydrofolate-Glu₅, which accumulates in MTX-treated cells, is a noncompetitive inhibitor of amido phosphoribosyltransferase with a dissociation constant of 3.41 ± 0.08 μM for interaction with the enzyme-glutamine complex in vitro. Folate-Glu₅, MTX-Glu₅, PTX, dihydrotiazine benzenesulfonyl fluoride, and AICAR also inhibit amido phosphoribosyltransferase.

Methotrexate (MTX) was synthesized as a structural analogue of folate (Seeger et al., 1949) and has been in clinical use as an anticaner drug for almost 40 years. MTX is a potent inhibitor of the enzyme dihydrofolate reductase (Wernheiser, 1961) with a Ki value for interaction with the free enzyme of 4 pM (Thillet et al., 1988). Dihydrofolate reductase is required for the biosyntheses of purine nucleotides, thymidine, serine, and methionine and the degradation of histidine. Dihydrofolate reductase also catalyzes the reduction of dihydrobiopterin to tetrahydrobiopterin, an electron carrier required for the conversion of phenylalanine to tyrosine. The toxicity of MTX against cancer cells may be relieved by leucovorin (N⁵-formyltetrahydrofolate) or hypoxanthine and/or thymidine. Thus, the anticaner activity of MTX may be attributed to inhibition of GAR or AICAR transformylases of de novo purine nucleotide biosynthesis in which N⁵⁰⁰-formyl- tetrahydrofolate is the one-carbon donor and/or inhibition of thymidylate synthase where N⁸,¹⁰-methylenetetrahydrofolate is the donor.

Inhibition of dihydrofolate reductase in human MCF-7 breast cancer cells exposed to MTX (1 μM) results in accumulation of polyglutamated dihydrofolate from undetectable levels to 20% of the total folate pool (3.9 pmol/mg protein; Allegra et al., 1986). Levels of N⁵⁰⁰-formyltetrahydrofolate were maintained at 84% of the control level after 21 h, indicating that MTX does not induce a cellular deficiency of one-carbon derivatives of tetrahydrofolate. Allegra et al. (1987) found that exposure of MCF-7 cells to MTX (10 μM) for 24 h induced a 3-fold accumulation of AICAR, and they concluded that the purine pathway is blocked at AICAR transformylase (reaction 9) by accumulated polyglutamated dihydrofolate. Pentaglutamyl derivatives of dihydrofolate and MTX are potent inhibitors of AICAR transformylase in vitro with K values of 2.7 and 5.9 μM, respectively (Allegra et al., 1985). Pentaglutamyl MTX also inhibits GAR transformylase in vitro with a Ki value of 2.5 μM (Chabner et al., 1985), and polyglutamated dihydrofolate may also inhibit this enzyme.

Piritrexim (PTX) is also a potent inhibitor of dihydrofolate reductase (Duch et al., 1982; Kᵢ = 219 pM; Waltham and Nixon, 1989) but does not undergo polyglutamation and may not inhibit GAR or AICAR transformylases of the purine pathway. DTBSF is a folate analogue without a pteridine ring which inactivates dihydrofolate reductase (Kumar et al., 1981). We have used new chromatographic procedures, which enable quantification of intermediates of the de novo purine pathway (GAR, FGAR, AIR, SAICAR, and AICAR; Sant et al., 1989a), to compare the metabolic effects of MTX and PTX upon mouse L1210 leukemia cells growing in culture. The initial objective of this research was to determine which antifolates induced the maximum inhibition of amido phosphoribosyltransferase. Antifolates with 4 additional γ-glutamyl residues; PRA, 5-phosphoribosylamine; P-rib-PP, 5-phosphoribosyl-1-pyrophosphate; P-irin, piriteixin; SAICAR, N-succino-5-aminoimidazole-4-carboxamide ribotide; P-HPLC, high pressure liquid chromatography; Phe, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
of GAR or AICAR transformylase (reaction 3 or 9) was more severely inhibited by polyglutamated dihydrofolate accumulating in MTX-treated cells. However, we have shown recently that the primary site of inhibition induced by MTX in the pathway is at or before GAR transformylase (reaction 3; Lyons and Christopherson, 1991). Data presented in this paper indicate that MTX and PTX induce a primary blockade on the de novo purine pathway, leukemia cells were "pulse labeled" for 2 h with [14C]glycine before extraction of metabolites at appropriate times relative to drug addition. The time-dependent effects of MTX upon the azaserine-induced accumulation of dihydrofolate and dihydrofolate from Sigma and their purity was confirmed by anion exchange, gradient HPLC as described below for nucleotides using solvents purged with helium. MTX-Glu was supplied by Shireks Laboratories (Jona, Switzerland), PTX was a gift from Dr. N. J. Clendeninn of Burroughs Wellcome Co. (Research Triangle Park, NC), and folate-Glu, trimetrexate, and DTBSF were gifts from Dr. V. L. Narayanan of the National Cancer Institute. [14C]Glycine (9.62 mCi, 52.0 Ci/mol) was from the Australian Nuclear and Science Technology Organisation, Lucas Heights, Australia; sodium [14C]formate (100 mCi, 58.0 Ci/mol) was from Du Pont-New England Nuclear, and [14C]formate (1.90 mCi, 52.5 Ci/mol) was from Amersham Corp.

Synthesis of Dihydrofolate-Glu—Folate-Glu was reduced to dihydrofolate-Glu with sodium dithionite as described by Howard et al. (1974) and purified by HPLC using solvents purged with helium. The reduced product was applied to a Whatman Partisil 10 SAX column (25 x 0.94 cm) and eluted with a linear gradient from 50 to 1,000 mM ammonium formate adjusted to pH 3.0 with formic acid. Fractions containing dihydrofolate-Glu were pooled and desalted immediately by passage over a Brownlee RP-18 column (22 x 0.46 cm) eluted with 100 mM formic acid and then methanol and was lyophilized and crystallized from a minimal volume of methanol at -20°C. The product was then washed with a small volume of methanol, and the methanol-insoluble fraction was dissolved in D2O. 1H NMR spectra were recorded in D2O (pH 6.2) at 600 MHz on a Bruker AMX 600 spectrometer or at 600 MHz on a Bruker AMX 600 spectrometer. For folate-Glu: 1H NMR (400 MHz) 6 7.67 (d, J = 2.7 Hz, H2), 7.55 (d, J = 2.7 Hz, H2), 7.45 (d, J = 8.5 Hz, H2), 7.20 (d, J = 8.5 Hz, H2), 6.86 (d, J = 8.5 Hz, H2), 4.15 (m, H2), 3.06 (dd, J = 6.5 Hz, H3, Glu[1]), 2.25, 2.05, 1.98, 1.82, 1.80, 1.69, 1.62 (multiples of 3J, H3, H2, H1). For dihydrofolate-Glu: 1H NMR (600 MHz) 6 7.62 (d, J = 8.5 Hz, H2, Glu[1]), 8.00 (H2, methyl), 6.80 (d, J = 8.0 Hz, Glu[1]), 4.31 (m, H2), 4.04 (dd, J = 6.5 Hz, H2), 2.38, 2.28, 2.24, 2.18, 2.02, 1.86, 1.84 (s, polyglutamyl protons). Methanol was used as an internal standard at 6 = 3.20 ppm. The concentrations of diphotoacetate and dihydroacetate-Glu used for inhibition experiments were determined by absorption at 282 nm using an extinction coefficient of 28,000 1 mol-1 cm-1 (Bertino et al., 1985).

**Extraction and Assay of Amido Phosphoribosyltransferase**—Mouse L1210 leukemia cells (500 ml, 9 x 10^6 cells/ml) were harvested, washed twice, and the cell pellet was resuspended in an equal volume of sonication buffer (0.25 mM sucrose, 1.0 mM MgCl2, 20 mM K/Hepes, pH 7.0) at 4°C. The cells were lysed by sonication (50 watts, 50 s), and cellular debris was removed by centrifugation (10,000 x g, 10 min). The cell-free extract was concentrated 10-fold, and the sonication buffer was exchanged using a Diaflo ultrafiltration cell (Amicon Corporation, Danvers, MA). The extract was stored in small aliquots at -20°C. Assay mixtures for amido phosphoribosyltransferase contained in a total volume of 50 ml: 50 mM K/Hepes (pH 7.2), 1.0 mM dihydrothiol, 1.0 mM MgCl2, 500 mM Mg-F-Rib-PP, 1.0 mM L-glutamine (10 mM). Amido phosphoribosyltransferase activity was defined as the ability of the cell extract to form GTP (approximately 37 mg of protein), and appropriate concentrations of a potential inhibitor. Variations and further details of these procedures appear in figure legends. The reaction was initiated with L-[14C]glutamine, and four samples (7 ml) were transferred to polyethyleneimine-cellulose chromatograms (1.5 x 10 cm) at appropriate times up to 40 min. Chromatograms were developed immediately by ascending chromatography with 0.28% (v/v) formic acid at 4°C and autoradiographed overnight. Spots of L-[14C]glutamate formed were excised and quantified by scintillation counting. P-Rib-PP used in these assays was standardized by complete reaction of limiting concentrations with excess L-[14C]glutamate and quantified by absorption at 282 nm using an extinction coefficient of 28,000 1 mol-1 cm-1 (Bertino et al., 1985).
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The velocity equation derived from Scheme 1 is as follows.

\[
V = \frac{u_0 K_s K_i}{K_s + 2K_i} + \frac{\delta K_s}{\gamma K_s + \beta K_i} + \frac{\alpha K_i}{\gamma K_s + \alpha K_i K_s} + 2K_i + S^2
\]

When \( S \) is constant, Equation 1 may be written in the general form

\[ v = \frac{u_0}{1 + \delta I + \epsilon I^2} \]

where \( v \) is the uninhibited reaction velocity when \( I = 0 \), and \( \delta \) and \( \epsilon \) are constants. When the concentration of \( I \) is low and it is assumed that the binding of \( I \) does not affect the binding of \( S \) (\( \beta = 1 \)), then Equation 1 may be written in the form

\[ v = \frac{u_0 K_i}{I + K_i} \]

where \( K_i \) is an apparent inhibition constant for the noncompetitive, initial interaction of an inhibitor \( I \) with the enzyme-glutamine complex. Data obtained for inhibition of amido phosphoribosyltransferase giving linear Dixon plots were fitted to Equation 3.

RESULTS

Growth of mouse L1210 leukemia cells in the presence of \([^{14}C]glycine \) radiolabels GAR and subsequent intermediates of the \( de novo \) purine pathway as shown in Scheme 2. The incorporation of \([^{14}C]glycine \) into GAR, AICAR and subsequent purine nucleotides over a 2-h period, at various times relative to addition of an antifolate, gives a measure of the flux through the two transformylase reactions to ATP and GTP. The time-dependent effects of MTX (0.1 \( \mu \text{M} \)) upon the purine pathway in growing leukemia cells were determined by analysis of cell extracts by HPLC. Total levels of purine intermediates, derived from \( de novo \) and salvage synthesis, were determined by ultraviolet absorbance (Fig. 1, a–c) and intermediates derived from \( de novo \) synthesis over the previous 2 h, by incorporation of \([^{14}C]glycine \) (Fig. 1, d–f). After exposure of cells to MTX for 2 h, the HPLC elution profile at 260 nm shows significant accumulations of total SAICAR and AICAR, whereas ATP and GTP have decreased substantially (Fig. 1b). The peak corresponding to IMP appears to have increased, but this is because of an accumulation of \( N\)-(p-aminobenzoyl)-L-glutamate, which has the same retention time, whereas IMP decreases (Lyons and Christopherson, 1991). After 8 h, ATP and GTP have decreased further, and SAICAR, AICAR and IMP have virtually disappeared (Fig. 1c). Cellular levels of intermediates of nucleotide metabolism

![Figure 1](image-url)
with significant ultraviolet absorbance have been computed by integration of the peaks of Fig. 1, a-c, and are summarized in Table I. After exposure to MTX for 2 h, IMP synthesized de novo from [14C]glycine has decreased, ATP and GTP have almost disappeared, and SAICAR and AICAR have accumulated (Fig. 1e). A value of inhibition at AICAR trans-formylase (AICAR \rightarrow FAICAR, reaction 9, Scheme 2). After 8 h there is no [14C]glycine incorporated into purine intermediates (Fig. 1f), indicating that de novo synthesis (Scheme 2) over the previous 2 h was completely blocked.

The complete disappearance of SAICAR and AICAR synthesized de novo after an 8-h exposure to MTX (Fig. 1f) confirms that AICAR transformylase (reaction 9) is not the primary site of inhibition induced by MTX in the pathway (Lyons and Christopherson, 1991). GAR, the substrate for GAR transformylase (reaction 3), is not clearly separated from glycine by this HPLC procedure (Sant et al., 1989a) and was quantified from the same cell extract by thin layer chromatography (Table II). GAR initially accumulates from a level of 750 amol/cell (approximately 560 \( \mu \)mol) to 950 amol/cell (710 \( \mu \)mol) after 2 h while levels of the subsequent intermediates FGAR and AIR decrease, defining a second site of inhibition at GAR transformylase (GAR \rightarrow FGAR, reaction 3, Scheme 2). Eight h after the addition of MTX, GAR decreases to 400 amol/cell (300 \( \mu \)mol) while FGAR and AIR continue to decrease.

A second culture of leukemia cells was exposed to azaserine (25 \( \mu \)mol), pulse-labeled for 2 h with [14C]glycine, and extracts were prepared in parallel with the MTX-treated culture. Azaserine is a glutamine antagonist that acts as a potent inhibitor of FGAM synthetase (FGAR \rightarrow FGAM, reaction 4) resulting in the disappearance of purine nucleotides and accumulation of FGAR, FGAR-DP, and FGAR-TP to millimolar concentrations (Lyons et al., 1990). After exposure of cells to azaserine for 2 h, FGAR polyphosphates quantified from the incorporation of [14C]glycine (cf. Fig. 1e) had accumulated from a low level of FGAR (210 amol/cell, Table II) and undetectable levels of FGAR-DP and FGAR-TP to: FGAR, 11,100 amol/cell (8,380 \( \mu \)mol); FGAR-DP, 810 amol/cell (610 \( \mu \)mol); FGAR-TP, 3,370 amol/cell (2550 \( \mu \)mol). For the MTX-treated culture, the net accumulations of purine precursors after 2 h were: GAR, 200 amol/cell (150 \( \mu \)mol, Table II); SAICAR, 172 amol/cell (130 \( \mu \)mol, Table I), and AICAR, 360 amol/cell (270 \( \mu \)mol, Table I). MTX (0.1 \( \mu \)mol) or azaserine (25 \( \mu \)mol) rapidly and completely blocks the de novo purine pathway; and if the primary sites of inhibition in the pathway induced by MTX were the two transformylase reactions, then the total net accumulation of purine precursors should be equal to the accumulation of FGAR polyphosphates induced by azaserine after 2 h:

\[
\text{total purine precursors} = \text{GAR} + \text{SAICAR} + \text{AICAR} = 200 + 172 + 360 = 732 \text{ amol/cell}
\]

The net accumulation of purine precursors induced by MTX is only 4.9% of the accumulation of FGAR polyphosphates induced by azaserine. These observations could be explained if accumulated GAR and AICAR were degraded within leukemia cells, but they are stable when incubated with a whole lysate of mouse L1210 leukemia cells in complete RPMI 1640 medium (4.2 mg of protein/ml) or with an equivalent cell-free extract over 2 h at 37°C.2

### Table I

| Metabolite | Level of metabolite amol/cell |
|------------|-----------------------------|
| 0 h        | 2 h                         | 8 h                        |
| SAICAR     | 58                          | 230                        | 42                         |
| AICAR      | 180                         | 540                        | 130                        |
| IMP        | 1,000                       | 540                        | 130                        |
| sAMP\(^a\) | 110                         | 54                         | 35                         |
| AMP        | 360                         | 330                        | 120                        |
| GMP        | 88                          | 40                         | 2                          |
| ADP        | 800                         | 640                        | 380                        |
| GDP        | 210                         | 110                        | 46                         |
| ATP        | 1,500                       | 710                        | 600                        |
| GTP        | 310                         | 92                         | 69                         |

\(^a\) Approximate cellular concentrations (\( \mu \)mol) may be calculated by dividing by a cellular volume of 1.33 pl.

### Table II

| Metabolite | Level of metabolite amol/cell |
|------------|-----------------------------|
| 0 h        | 2 h                         | 8 h                        |
| P-Rib-PP\(^a\) | 83                      | 240                        | 55                         |
| GAR        | 750                         | 950                        | 400                        |
| FGAR       | 210                         | 190                        | 100                        |
| AIR        | 270                         | 130                        | 66                         |
| AICAR + IMP| 970                         | 740                        | 75                         |

\(^a\) Values taken from Fig. 2.

2 M. E. Sant, S. D. Lyons, and R. I. Christopherson, unpublished experiments.

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**Table III**

| Metabolite | Level of metabolite amol/cell |
|------------|-----------------------------|
| 0 h        | 2 h                         | 8 h                        |
| P-Rib-PP\(^a\) | 83                      | 240                        | 55                         |
| GAR        | 750                         | 950                        | 400                        |
| FGAR       | 210                         | 190                        | 100                        |
| AIR        | 270                         | 130                        | 66                         |
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induced a blockade at GAR transformylase. Purine intermediates, perhaps preexisting distal to GAR in the pathway, are converted through to AICAR where there is also a blockade at AICAR transformylase (Scheme 2). A primary blockade at reaction 1 by MTX would result in accumulation of P-Rib-PP; distal sites of inhibition at reactions 3 and 9 would result in small accumulations of GAR and AICAR with depletion of the end products of the pathway as observed (Fig. 1; Tables I and II).

Treatment of leukemia cells with MTX (0.1 μM) induces an accumulation of P-Rib-PP from an initial cellular concentration of 83 amol/cell (62 μM) to 240 amol/cell (180 μM) after 2 h, 280 amol/cell (210 μM) at 3 h, and 55 amol/cell (41 μM) at 8 h (Fig. 2). Such an accumulation of P-Rib-PP has been reported (Bökerkírki etc., 1986) and is consistent with potent inhibition of reaction 1 (P-Rib-PP → PRA, Scheme 2). PTX (0.1 μM) also induced the disappearance of purine intermediates synthesized de novo and a similar accumulation of P-Rib-PP to that for MTX (Fig. 2). The total accumulation after 2 h of purine precursors (GAR + SAICAR + AICAR) was only 2.3% of FGAR polyphosphates (FGAR + FGAR-DP + FGAR-TP). Allegra et al. (1987) have attributed total inhibition of de novo purine biosynthesis by MTX to inhibition of AICAR transformylase by accumulated polyglutamated dihydrofolate. However, if the primary site of blockade of the purine pathway was reaction 9, an accumulation of AICAR of 84-fold would be induced by 0.1 μM MTX after 2 h rather than the 3-fold observed here (Table I) and by Allegra et al. (1987). Mouse leukemia cells grown in the presence of [14C]formate (50 μM, 58.0 Ci/mol) or [14C]bicarbonate (100 μM, 57.0 Ci/mol) and exposed to MTX (25 μM) for 8 h also showed the disappearance of the intermediates of Scheme 2 (Lyons and Christopherson, 1991).

MMPR, as the 5'-monophosphate derivative, is a potent inhibitor of amido phosphoribosyltransferase which catalyzes reaction 1 of the de novo purine pathway (Hill and Bennett, 1969; Nelson and Parks, 1972). MMPR (25 μM) induced metabolic effects similar to MTX (0.1 μM) and PTX (0.1 μM) with accumulation of P-Rib-PP (Fig. 2) and the disappearance of purine intermediates synthesized de novo. Lyons and Christopherson (1991) showed that MTX (25 μM) abolished the accumulation of FGAR polyphosphates induced by azaserine (25 μM) in mouse L1210 leukemia cells grown in the presence of [14C]formate. Fig. 3 shows data from similar experiments in which [14C]formate was added to cultures with azaserine (25 μM) and 10 μM concentrations of MTX, PTX, or MMPR. [14C]Formate is incorporated into purine nucleotides during 2 h of radiolabeling (Fig. 3a), and azaserine completely blocks this incorporation and induces accumulation of FGAR, FGAR-DP, and FGAR-TP (Fig. 3b). The presence of MTX (10 μM, Fig. 3c), PTX (10 μM, Fig. 3d), or MMPR (10 μM, Fig. 3e) blocks the accumulation of FGAR polyphosphates confining the primary site of inhibition to the first three reactions (P-Rib-PP → PRA → GAR → FGAR).

FIG. 2. Effect of MTX, PTX, and MMPR on P-Rib-PP levels in mouse L1210 leukemia cells. Cultures (100 ml) were grown as for Fig. 1, MTX (0.1 μM), PTX (0.1 μM), or MMPR (25 μM) was added, and samples (10 ml) were taken at the indicated times. P-Rib-PP concentrations were calculated assuming a cellular volume of 1.38 pL: C, control; + MTX; + PTX; + MMPR.

FIG. 3. Effects of MTX, PTX, and MMPR on the azaserine-induced accumulation of FGAR polyphosphates. [14C]Formate (50 μM, 58.0 Ci/mol) was added to cultures of mouse L1210 leukemia cells (51 ml) with a no drug; b, 25 μM azaserine; c, 25 μM azaserine + 10 μM MTX; d, 25 μM azaserine + 10 μM PTX; e, 25 μM azaserine + 10 μM MMPR. Procedures for extraction after 2 h and analysis were as for Fig. 1. Peaks A, B, and C are derived from the corresponding phosphorylated forms of FGAR (Lyons et al., 1990).
and presence of

This time-dependent inhibition was reversible. However, enzyme has two distinct allosteric, inhibitory sites for AMP and GMP or IMP. The spatial relationship of the inhibitory site for dihydrofolate and dihydrofolate-Glu5 to the catalytic site of the enzyme was investigated with full inhibition patterns with P-Rib-PP as the varied substrate (Fig. 5). Lines at higher inhibitor and lower P-Rib-PP concentrations show upward curvature, consistent with positive cooperativity with respect to P-Rib-PP (Hill and Bennett, 1969; Wood and Seegmiller, 1973). The inhibition patterns intersect to the left of the 1/ν axis, indicating that dihydrofolate and dihydrofolate-Glu5 bind at a site distinct from the catalytic site.

Dixon plots (1/ν versus the concentration of dihydrofolate or dihydrofolate-Glu5) at constant L-glutamine (1 mM) and P-Rib-PP (500 μM) concentrations also showed upward curvature at higher concentrations, consistent with positive cooperativity for the binding of inhibitor. High concentrations of dihydrofolate (1 mM) completely inhibited enzyme activity, indicating that enzyme-dihydrofolate complexes are catalytically inactive. The data of Fig. 5 were fitted to Equation 1 which describes the model of Scheme 1 with the simplifying assumptions described under "Analysis of Kinetic Data" in "Experimental Procedures." The standard errors were large for some of the parameter values obtained, and the theoretical lines of Fig. 5 should be considered as simulations rather than fits by nonlinear regression. However, the experimental data obtained are consistent with the model of Scheme 1. For dihydrofolate (Fig. 5a), values for the interaction factors α and γ are less than 1.0, indicating that the substrate P-Rib-PP and inhibitor are both bound with positive homotropic cooperativity as shown by upward curvature in Lineweaver-Burk plots (Fig. 5) and Dixon plots, respectively. The value for β of 5.4 indicates negative heterotropic cooperativity for binding of substrate or inhibitor to an enzyme complex with the other ligand (Scheme 1). The Kν value for binding the first molecule of P-Rib-PP of 109 μM is decreased to 16.8 μM (αKν) for the second interaction. The Kν for dihydrofolate decreases from 312 μM (Kν) to 286 μM (γKν). For dihydrofolate-Glu5, values for the interaction factors α and γ are again less than 1.0, whereas β is greater than 1.0. The Kν value for P-Rib-PP decreases from 138 to 44.6 μM (αKν) for the second interaction. The Kν for dihydrofolate-Glu5 decreases from 16.1 to 9.2 μM (γKν).

With the assumption that amido phosphoribosyltransferase contains an allosteric site for folate analogues as well as for

![FIG. 4. Time-dependent effects of MTX on the azaserine-induced accumulation of FGAR polyphosphates.](image-url)

![FIG. 5. Inhibition patterns for dihydrofolate and dihydrofolate-Glu5 with respect to P-Rib-PP as the varied substrate for amido phosphoribosyltransferase. Assays contained 37 μg of protein, were initiated with 1.0 mM L-[14C]glutamine, and samples were taken at 10, 20, 30, and 40 min as described under "Experimental Procedures." Data were fitted to Equation 1 which describes the model of Scheme 1. a. For dihydrofolate the parameter values used to generate the lines were: V = 1.06 μM/min, α = 0.154, β = 3.42, γ = 0.918, Kα = 109 μM, Kβ = 312 μM, b. For dihydrofolate-Glu5: V = 0.869 μM/min, α = 0.324, β = 1.64, γ = 0.573, Kα = 138 μM, Kβ = 16.1 μM.)
6-hydroxy and 6-amino purine nucleotides, Dixon plots were obtained for GMP, folate, folate-Glu₅, N⁵-formyltetrahydrofolate, dihydrofolate, dihydrofolate-Glu₅, MTX-Glu₅, PTX, DTBSF, and AICAR, and the data were fitted to Equation 3. Most of these Dixon plots showed upward curvature at higher inhibitor concentrations consistent with positive cooperativity for the binding of inhibitor as described by Equation 2. However, data from the initial linear portions of these plots were fitted to Equation 3, which describes simple noncompetitive inhibition, and the values obtained are listed in Table III. Dihydrofolate-Glu₅ and PTX are the most potent (noncompetitive) inhibitors of amido phosphoribosyltransferase with apparent Kᵢ values of 3.4 and 6.0 μM for dissociation from the free enzyme compared with dihydrofolate with a Kᵢ of 310 μM. The Kᵢ value of 3.4 μM obtained by fitting data to Equation 3 by nonlinear regression may be considered more reliable than the value used in Fig. 5b to simulate theoretical lines from Equation 1.

DISCUSSION

Data presented in this paper have necessitated a reevaluation of the mechanism by which antifolates block the de novo biosynthesis of purine nucleotides. Four different types of experiments indicate that antifolates induce a primary blockade of the de novo pathway at reaction 1 (Scheme 2). 1) MTX and PTX abolish the azaserine-induced accumulation of FGAR polyphosphates in growing leukemia cells (Fig. 3). 2) Purine precursors such as SAICAR and AICAR synthesized de novo from [1⁴C]glycine disappear after an 8-h exposure to MTX (Fig. 1), and the net accumulation after 2 h of such precursors is less than 5% of the FGAR polyphosphates which accumulate with azaserine. 3) Dihydrofolate-Glu₅, MTX-Glu₅, PTX, and DTBSF inhibit amido phosphoribosyltransferase in vitro. Dihydrofolate polyglutamates accumulate to 20% of the total folate pool in MTX-treated cells (Allegra et al., 1986). The total cellular concentration of reduced folates in mouse L1210 leukemia cells is about 10 μM (Seither et al., 1989), suggesting that dihydrofolate polyglutamates would accumulate to approximately 2 μM in MTX-treated cells. The fractional inhibition of amido phosphoribosyltransferase can be calculated by substitution of appropriate values into Equation 1, the velocity equation which describes the dependence of amido phosphoribosyltransferase activity upon the concentration of P-Rib-PP and dihydrofolate-Glu₅, both of which are bound with positive cooperativity. At an initial cellular P-Rib-PP concentration of 56 μM (average value from Fig. 2), an increase in cellular dihydrofolate-Glu₅ from undetectable levels to 2 μM after the MTX addition would decrease enzymic activity to 51% (Table IV). However, after 2 h, P-Rib-PP has increased to 180 μM, and Equation 1 then predicts 117% of the initial activity (Table IV). If the concentration of dihydrofolate polyglutamates after 2 h were approximately equal to the total cellular pool of reduced folates of 10 μM (Seither et al., 1989), then enzymic activity would have decreased to 16%. After 8 h, P-Rib-PP has decreased to 41 μM, and Equation 1 predicts 36% of the initial activity.

| Inhibitor          | Kᵢ    |
|--------------------|-------|
| GMP                | 1,580 ± 196 |
| Folate-Glu₅        | 316 ± 89   |
| Dihydrofolate      | 312 ± 61   |
| Dihydrofolate-Glu₅| 3.41 ± 0.08|
| N⁵-formyltetrahydrofolate | 216 ± 61 |
| MTX-Glu₅           | 550 ± 66   |
| PTX                | 5.97 ± 1.09|
| DTBSF              | 143 ± 29   |
| AICAR              | 1,910 ± 550|

MTX does induce a 3-fold accumulation of AICAR (Table I) as reported by Allegra et al. (1987), but this AICAR is probably derived only from the pools of intermediates between the two transformylase reactions (FGAR → FGAM → AIR → CAIR → SAICAR → AICAR) which existed before the MTX addition. MTX and PTX induce small accumulations of GAR, SAICAR, and AICAR derived from [¹⁴C]glycine which are inconsistent with complete blockade of the purine pathway. These experiments with growing cells indicate that antifolates induce primary inhibition of the pathway at reaction 1 or 2 (P-Rib-PP → PRA → GAR). MMPR, like MTX and PTX, abolishes the azaserine-induced accumulation of FGAR polyphosphates (Fig. 3) and induces a similar accumulation of P-Rib-PP (Fig. 2), suggesting that reaction 1 is the primary site of inhibition. PRA is very unstable (t₁/₂ = 38 s at 37 °C and pH 7.5; Schendel et al., 1988), and amido phosphoribosyltransferase is therefore more easily assayed than GAR synthetase. Amido phosphoribosyltransferase was known to be the regulatory enzyme of the pathway, and we have now demonstrated potent inhibition of this enzyme by dihydrofolate-Glu₅ in vitro. Dihydrofolate polyglutamates accumulate to 20% of the total folate pool in MTX-treated cells (Allegra et al., 1986). The total cellular concentration of reduced folates in mouse L1210 leukemia cells is about 10 μM (Seither et al., 1989), suggesting that dihydrofolate polyglutamates would accumulate to approximately 2 μM in MTX-treated cells. The fractional inhibition of amido phosphoribosyltransferase can be calculated by substitution of appropriate values into Equation 1, the velocity equation which describes the dependence of amido phosphoribosyltransferase activity upon the concentration of P-Rib-PP and dihydrofolate-Glu₅, both of which are bound with positive cooperativity. At an initial cellular P-Rib-PP concentration of 56 μM (average value from Fig. 2), an increase in cellular dihydrofolate-Glu₅ from undetectable levels to 2 μM after the MTX addition would decrease enzymic activity to 51% (Table IV). However, after 2 h, P-Rib-PP has increased to 180 μM, and Equation 1 then predicts 117% of the initial activity (Table IV). If the concentration of dihydrofolate polyglutamates after 2 h were approximately equal to the total cellular pool of reduced folates of 10 μM (Seither et al., 1989), then enzymic activity would have decreased to 16%. After 8 h, P-Rib-PP has decreased to 41 μM, and Equation 1 predicts 36% of the initial activity.

### Table IV

**Predicted changes in amido phosphoribosyltransferase activity in leukemia cells treated with MTX**

Fractional changes in amido phosphoribosyltransferase activity caused by accumulation of dihydrofolate-Glu₅ (I) and P-Rib-PP (S) were calculated by substitution of appropriate values into Equation 1 (α = 0.324, β = 1.64, γ = 0.573, Kᵢ = 138 μM, Kᵢ = 3.4 μM) with appropriate concentrations for S and I. The fractional change of enzymic activity (v/v₀) predicted in growing cells was calculated using S = 56 μM and I = 0 to calculate v/v₀. v was calculated by substituting concentrations of P-Rib-PP at the indicated times relative to addition of MTX (0.1 μM, Fig. 2) and the indicated dihydrofolate-Glu₅ concentrations into Equation 1.

| Time [P-Rib-PP] | [Dihydrofolate-Glu₅]* | v/v₀ |
|-----------------|-----------------------|------|
| h μM            | μM                    |      |
| 0               | 56                    | 1.0  |
| 2               | 56                    | 0.51 |
| 10              | 56                    | 0.064|
| 8               | 180                   | 2.117|
| 10              | 180                   | 0.16 |
| 8               | 41                    | 0.36 |
| 10              | 41                    | 0.044|

* Seither et al. (1989); Allegra et al. (1986).
activity with 2 μM dihydrofolate-Glu₄ and 4.4% with 10 μM dihydrofolate-Glu₆.

Thus, inhibition of amido phosphoribosyltransferase predicted from enzyme kinetic studies in vitro does not quantitatively account for the total blockade at reaction 1 observed in growing cells. Inhibition by MTX-Glu₆ (Kᵢ = 550 μM) and AICAR (Kᵢ = 1910 μM; Table III) would not make significant contributions to the blockade, given their likely cellular concentrations (Table I). The more potent inhibition of amido phosphoribosyltransferase by PTX (Kᵢ = 6.0 μM; Table III) suggests that accumulated PTX may significantly inhibit reaction 1 at later times, but again the inhibition would not be complete as observed in culture. A similar discrepancy would exist for the established feedback inhibition of amido phosphoribosyltransferase by purine nucleoside monophosphates such as GMP (Kᵢ = 1580 μM; Table III). Amido phosphoribosyltransferase may be part of a multienzyme complex in intact cells (Rowe et al., 1978) which may change the kinetic properties of the enzyme, and the local concentrations of these regulatory metabolites may be higher because of their compartmentation in vivo. The data of Table III and Fig. 5 establish that there are strong and specific interactions of dihydrofolate-Glu₆, PTX and some other folate analogues with amido phosphoribosyltransferase.

Inhibition experiments with amido phosphoribosyltransferase in vitro provide evidence for an inhibitory, allosteric site that binds dihydrofolate-Glu, and certain folate analogues. This interaction must have a physiological role in the regulation of de novo purine nucleotide biosynthesis in normal cells. Inhibition of amido phosphoribosyltransferase by dihydrofolate polyglutamates could reduce the flux through the pathway at reaction 1 when there is insufficient Nᵦ-formyltetrahydrofolate to convert GAR → FGAR and AICAR → FAICAR. The total concentration of the cellular pool of reduced folates is about 10 μM (Seither et al., 1989), and a decrease in tetrahydrofolate derivatives would result in an equivalent increase in dihydrofolate derivatives. The rate of reduction of dihydrofolate to tetrahydrofolate may be low because of low dihydrofolate reductase activity during particular phases of the cell cycle or levels of NADPH required for the reduction may be low in cells starved for a carbon source or oxygen. Inhibition of reaction 1 under these conditions would prevent accumulation of GAR and unnecessary consumption of P-Rib-PP, l-glutamine, glycine, and ATP.

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