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Inhibition of HKSV28 Cell Growth by 5,11-Methenyl-tetrahydrohomofolate

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SUMMARY

5,11-Methenyl-tetrahydrohomofolate (5,11-methenyl-H_4homofolate), a reduced folate analogue, inhibited hamster kidney HKSV28 cells grown *in vitro*. Hypoxanthine protected the cells from growth inhibition but thymidine had no effect, suggesting that the blockage was in the purine biosynthetic pathway. The measurement of formylglycinamide ribonucleotide levels in the presence of the drug showed that a decrease in these levels was a significant and primary effect of the compound, but the effect had an onset time of approximately 4 hr. In contrast, incubation of the cells with the hydrolysis products 11-formyl-H_4/H_2homofolate resulted in an immediate decrease in formylglycinamide synthesis. The latter formyl derivatives were shown to be potent competitive inhibitors of glycinamide ribonucleotide transformylase (EC 2.1.2.2) from chicken liver. However, the cell inhibition by 5,11-methenyl-H_4homofolate was not confined to glycinamide ribonucleotide transformylase, since the cells were not protected by the presence of aminoimidazole carboxamide. Although aminoimidazole carboxamide ribonucleotide transformylase (EC 2.1.2.3) from chicken liver was not inhibited by these derivatives, we propose that intracellular glutamate elongation of the 11-formyl-H_4/H_2homofolates might lead to inhibition of both transformylase enzymes.

INTRODUCTION

Homofolic acid is an analogue of folic acid, differing only by the insertion of a methylene unit in the chain linking C_6 to N_{10}. As the chemically reduced 6*RS*-H_4homofolate, it was found to be a potent inhibitor of *Escherichia coli* thymidylate synthetase (1) but was less active against the synthetase from L1210 cells (2). It did not inhibit the uptake of [^{3}H]deoxyuridine into the DNA of those cells (3). However, work by Divekar and Hakala (4) in sarcoma 180 cells showed that homofolate, when given a 24-hr preincubation period, retarded cell growth by decreasing levels of FGAR[1] in those cells. Since AICA protected against the inhibitiory effects of homofolate, the compound was postulated to act—after reduction to 6*S*-H_4homofolate—specifically at the level of the glycinamide ribonucleotide transformylase.

In contrast, investigations by Bertino and co-workers (5) on the retarding effect of 6*RS*-H_4homofolate on L1210 murine leukemia cell growth showed that the serine hydroxymethyltransferase from these cells is inhibited by the cofactor analogue. Since in whole cell studies, [3-^{14}C]serine incorporation into DNA and methionine was slowed in the presence of H_4homofolate, the ability of H_4homofolate to affect the growth of L1210 cells was traced, in part, to the inhibition of the serine hydroxymethyltransferase activity.

More recently, Caperelli *et al.* (6) reported the synthesis of 6*S*-5,11-methenyl-H_4homofolate (Fig. 1) from 6*S*-H_4homofolate[2] and triethylorthoformate. Although this compound was capable of inhibiting the 5,10-methenyl-H_4folate cyclohydrolase activity of the trifunctional protein from chicken liver, thus reducing the concentration of the N_{10}-formyl species required for *de novo* purine biosynthesis, it did not block the utilization of 5,10-methylene-H_4folate in the dehydrogenase/cyclohydrolase couple (7), yet it inhibited the growth of hamster kidney HKSV28 cells (SV40 virus-transformed cells).

In this paper we have examined the conversion of 5,11-methenyl-H_4homofolate to 11-formyl-H_4homofolate and its further oxidation products and measured their effect on both of the chicken liver transformylases of the *de novo* purine biosynthetic pathway. These studies, in con-

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[1] The abbreviations used are: FGAR, (α-*N*-formyl)-glycinamide-1-β-D-ribofuranose-5′-monophosphate; AICA, 5-aminoimidazole-4-carboxamide; AICA riboside, AICA-1-β-D-ribofuranose; AICAR, AICA-1-β-D-ribofuranose-5′-monophosphate; FAICAR, (*N*-formyl)-5-aminoimidazole-4-carboxamide-1-β-D-ribofuranose-5′-monophosphate; H_4folate, 6*S*,α*S*-5,6,7,8-tetrahydropteroylglutamate; GAR, glycinamide-1-β-D-ribofuranose-5′-monophosphate; trifunctional protein, 5,10-methenyl-5,10-methylene-10-formyl-H_4folate synthetase (combined).

[2] The absolute stereochemistry at C_6 of enzymatically reduced H_4homofolate is assumed to be *S* by analogy to H_4folate. However, this has not been rigorously proven by X-ray methods.

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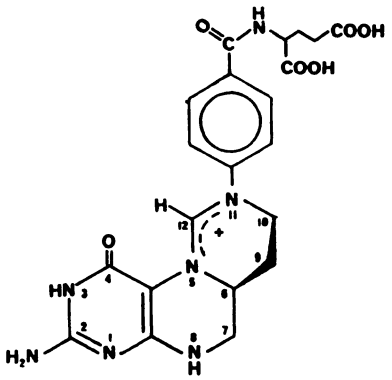

FIG. 1. *Structure of 6S-5,11-methenyl-H$_4$homofolate*

junction with whole cell experiments (HKSV28) in which nucleotide and FGAR pool levels were measured in the presence of 5,11-methenyl-H$_4$homofolate and its hydrolysis product(s), implicated these analogues as inhibiting primarily at the transformylase level.

MATERIALS AND METHODS

Homofolic acid was synthesized by the method of Kim *et al.* (8). Folic acid, NADP$^+$, NADPH, thymidine, hypoxanthine, AICA, AICA riboside, azaserine, methotrexate, and all nucleotide high-pressure liquid chromatography standards were purchased from Sigma Chemical Company (St. Louis, Mo.). Both 6*S*-H$_4$folate and 6*S*-H$_4$homofolate were synthesized enzymatically by the methods of Blakley (9) and Mathews and Huennekens (10). Dihydrofolate reductase (EC 1.5.1.3) isozyme 1, from TR 500 *Escherichia coli*, was a generous gift from Dr. C. R. Mathews, The Pennsylvania State University. GAR transformylase was donated by Dr. Gail Wasserman as a mixture containing GAR transformylase (0.73 unit/mg) and trifunctional protein (dehydrogenase 4.5 units/mg, synthetase 2.0 units/mg), and AICAR transformylase (0.9 units/mg) was a gift from Dr. W. Thomas Meuller, both of The Pennsylvania State University. GAR was prepared by C. Caperelli by the procedure of Chettur and Benkovic (11), and AICAR was obtained from Dr. W. T. Mueller. Elemental analyses were performed by M-H-W Laboratories (Phoenix, Ariz.). NMR spectra were obtained at 360.13 MHz on a Bruker WM-360, and, typically, 32k data points were accumulated in a sweep width of 4000 Hz. Solvent suppression was accomplished by setting the proton decoupler on the HOD peak during acquisition.

6S-5,11-Methenyl-H$_4$homofolate. Typically, 67 μmoles of 6*S*-H$_4$homofolate were dissolved in 7.5 ml of 98% formic acid containing 165 μl of β-mercaptoethanol and heated at 80° under N_2 for 3 hr. The resulting deep yellow solution was lyophilized to dryness and the residue was dissolved in the minimal volume of 0.1 M HCl. Purification was accomplished by elution from phosphocellulose (Bio-Rad Cellex P, 1.5 × 25 cm, H$^+$ form) with 0.1 M HCl. Fractions were monitored at 320 nm (5), and the typical yield was 28–30 μmoles of the chloride salt. Both UV (λ_{max} = 320, 288 nm in 0.1 M HCl) and NMR spectral data confirmed this product to be the same material obtained by Caperelli *et al.* (6), using H$_4$homofolate and triethylorthoformate in acetic acid.

11-Formyl-homofolate. Homofolic acid (69 mg) was dissolved in 2 ml of 98% formic acid and left at 37° for 4 days. The crude product was precipitated with ether, collected by filtration, and dried to yield 67 mg of cream-colored solid. Thin-layer chromatography on cellulose plates using 3% NH_4Cl as developing solvent indicated one major fluorescent spot (R_F 0.78) and two minor absorbing spots at lower R_F. This crude material was chromatographed on a DEAE-cellulose column in the bicarbonate form (1.5 cm × 32 cm), employing a linear gradient from 0.1 to 0.3 M triethylammonium bicarbonate. Fractions (10 ml) were collected and the product was eluted in fractions 30–42. The product was separated from three leading impurities and trailing homofolic acid. The pooled fractions were lyophilized to dryness, and the compound was taken up in 10 ml of water and precipitated by adjusting the pH to 3.0. The precipitate was collected by centrifugation, washed twice with cold 0.001 M HCl, and dried *in vacuo* at room temperature. The yield was 26 mg of a cream-colored solid.

$C_{21}H_{21}N_7O_7 \cdot 2.5\ H_2O$
Calculated: C 47.73, H 4.92, N 18.56
Found: C 47.63, H 4.81, N 18.35

The UV spectrum in 50 mM Tris (pH 7.5) showed absorption maxima at 358 nm (ε = 5,000 M^{-1} cm^{-1}) and 262 nm (ε = 24,400 M^{-1} cm^{-1}). Chromatography on a Whatman Partisil 10 strong anion exchange column, eluting with a 20-min linear gradient from 0.01 to 0.1 M $NH_4H_2PO_4$ (pH 6.5), 2 ml/min flow rate, showed one major peak at 14.2 ml and a very minor contaminant at 8.0 ml.

11-Formyl-7,8-dihydrohomofolate. Sixty milligrams of homofolic acid were formylated as described above. Without further purification, the crude product was suspended in 5 ml of 10% ascorbic acid (pH 7.0) and brought into solution by the addition of 1 N NaOH, maintaining the same pH. Six hundred milligrams of $Na_2S_2O_4$ were added with stirring, and the reaction was allowed to proceed at room temperature for 45 min, during which time the color of the solution noticeably lightened. The solution was chilled on ice and adjusted to pH 3 with 2 N HCl. After further cooling, an off-white precipitate appeared and was collected by centrifugation, washed three times with cold 0.001 M HCl, and dried *in vacuo*. The residue was taken up in 5 ml of 0.05 M triethylammonium bicarbonate/0.1 M β-mercaptoethanol (pH 7.5), and loaded onto a DEAE-cellulose column (1.5 cm × 25 cm) equilibrated with the same buffer. Elution was performed with a linear gradient from 0.05 to 0.40 M triethylammonium bicarbonate/0.1 M β-mercaptoethanol (pH 7.5) (500 ml each). The product, collected in 10-ml fractions, appeared at 0.12 M HCO_3^- and was separated from four minor impurities. The pooled fractions (19–24) containing 11-formyl-7,8-dihydrohomofolate were lyophilized to dryness and the residue was dissolved in 4 ml of water. Acid precipitation yielded 7.4 mg of a pale yellow solid. The UV spectrum in 50 mM Tris buffer (pH 7.2) showed peaks at 230, 260, and 330 nm with estimated extinction coefficients of 24,500, 16,900, and 4,200 M^{-1} cm^{-1}, respectively, based on an assumed molecular weight of 520 (dihydrate).

11-Formyl-tetrahydrohomofolate. When 5,11-methenyl-H$_4$homofolate was added to 200 mM potassium carbonate/5 mM β-mercaptoethanol (pH 10.2 at 25°), hydrolysis proceeded rapidly with a half-time of 25 min (Fig. 2). The UV spectrum of the product contains a λ_{max} at 255 nm and a shoulder at approximately 300 nm, which is identical with the reported spectrum of 10-formyl-H$_4$folate (12). The absence of a peak at 285 nm suggests that 5-formyl-H$_4$homofolate, the presumed thermodynamic product of hydrolysis, is not formed initially. Acidification in 1.0 N HCl regenerated the original amidine spectrum (λ_{max} = 318 nm). To determine how rapidly hydrolysis would occur under cellular growth conditions, an experiment was performed in 40 mM sodium bicarbonate/1 mM potassium phosphate (pH 7.3 at 37°), and a half-life of 7.5 hr was calculated.

The 360 MHz ^{1}H-NMR spectrum of the hydrolysis product in 200 mM potassium carbonate (D_2O) after a 2.5-hr incubation at 25° is consistent with an 11-substituted H$_4$homofolate derivative, since 11-formyl substitution results in $C_{3'5'}$2H appearing at 7.44 ppm. In derivatives of folic acid unsubstituted at N_{10}, this resonance is approximately 6.8 ppm (13; 14). Analysis of the aromatic proton region revealed that approximately 25% of the total pteridine was present as the H$_2$homofolate species. As expected, in the absence of mercaptoethanol, the H$_4$homofolate derivative was rapidly oxidized to the 7,8-H$_2$ form. Table 1 summarizes the pertinent NMR data determined for the series: 11-formyl-H$_4$, 7,8-H$_2$-, and homofolate.

General procedures for cell growth inhibition experiments. HKSV28 cells were maintained in Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum at 37° in an atmosphere of 10% CO_2. Dulbecco's modified Eagle's medium was obtained from Flow Laboratories (McLean, Va.), and fetal calf serum was purchased from Grand

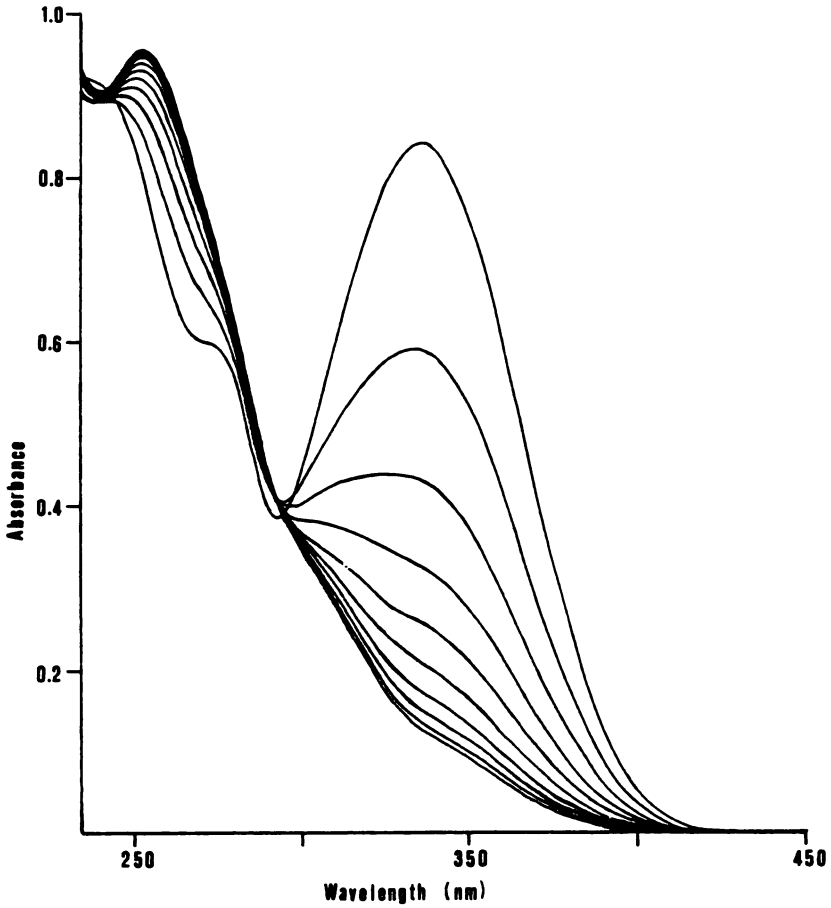

FIG. 2. *Spectral changes which occur upon hydrolysis of 5,11-methenyl-H_4homofolate in 200 mM sodium carbonate/5 mM mercaptoethanol (pH 10.2 at 25°)*

Scans were taken every 15 min.

Island Biological Company (Grand Island, N. Y.). For experiments in which glutamine-free medium was required, it was prepared from basic ingredients as described by the manufacturer. All required vitamins and L-amino acids were obtained from Sigma Chemical Company (St. Louis, MO.), and [2-^{14}C]-glycine (47.3 mCi/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). Cells were plated at an initial density of 2.5×10^4 cells/plate, unless otherwise noted. Effects of drugs on cellular growth were determined by counting duplicate plates after a designated period of time (generally approximately 95 hr). Drug was added after a 4-hr incubation period to allow the cells to attach to the plate. Cells were detached from the plate surface with a dilute solution of trypsin and counted by hemocytometer. Average values were generally reported as the mean of four determinations, with error bars equal to ± 1 SD.

TABLE 1

Chemical shift data for 11-formyl-H_4-, H_2-, and homofolate

Proton	Chemical shifts[a]		
	11-Formyl-homofolate	11-Formyl-H_2homofolate	11-Formyl-H_4homofolate
C_7H	8.422 (s) [b]	—	—
$C_{12}H$	8.336 (s)	8.432 (s)	8.445 (s)
$C_{2',6'}2H$	7.619 (d)	7.786 (d)	7.889 (d)
$C_{3',5'}2H$	7.030 (d)	7.350 (d)	7.443 (d)
$C_{10}2H$	4.419 (m)	4.254 (m)	NA[c]
C_nH	4.320 (dd)	4.331 (dd)	4.331 (dd)
C_72H	—	3.901 (s)	NA
C_6H	—	—	NA
C_92H	3.201 (m)	2.501 (m)	1.711 (m)
C_72H	2.325 (t)	2.325 (t)	2.321 (t)
$C_\beta2H$	2.20, 2.05 (m)	2.18, 2.04 (m)	2.17, 2.04 (m)

[a] Chemicals shifts are reported in parts per million relative to 3-(trimethylsilyl)-1-propanesulfonate.

[b] Letters in parentheses refer to multiplicity of the resonance: s, singlet; d, doublet; t, triplet; m, multiplet.

[c] NA, Not assigned.

Nucleotide and [^{14}C]FGAR analysis of cell extracts. Acid-soluble extracts were prepared by a modification of the procedure of Pogolotti and Santi (15). Cells were plated out at a density of 5×10^5 cells on 100-mm plates and were growth for 18–24 hr. At this point, the cell density was approximately 1–2 $\times 10^6$ cells/plate. The cells were harvested by aspirating the medium, rinsing with 5 ml of cold phosphate-buffered saline (0.90% NaCl, pH 7.2), and adding 0.5 ml of 0.5 M trichloroacetic acid. The plates were left at 4° for 30 min and then they were scraped with a rubber spatula to remove quantitatively the cellular debris and acid-soluble fraction. After washing each plate two times with 200 μl of 0.5 M trichloroacetic acid, the combined extracts were centrifuged and the aqueous portion was neutralized by extraction with an equal volume of 0.5 M tri-*n*-octylamine in Freon. For nucleotide analysis, the neutralized extracts were lyophilized and redissolved in a sufficient volume of water such that a 100-μl injection volume would represent approximately 10^6 cells. Analyses were performed on a Whatman Partisil 10 strong anion exchange column using a dual pump Altex 100 high-pressure liquid chromatography system. Elution was per-

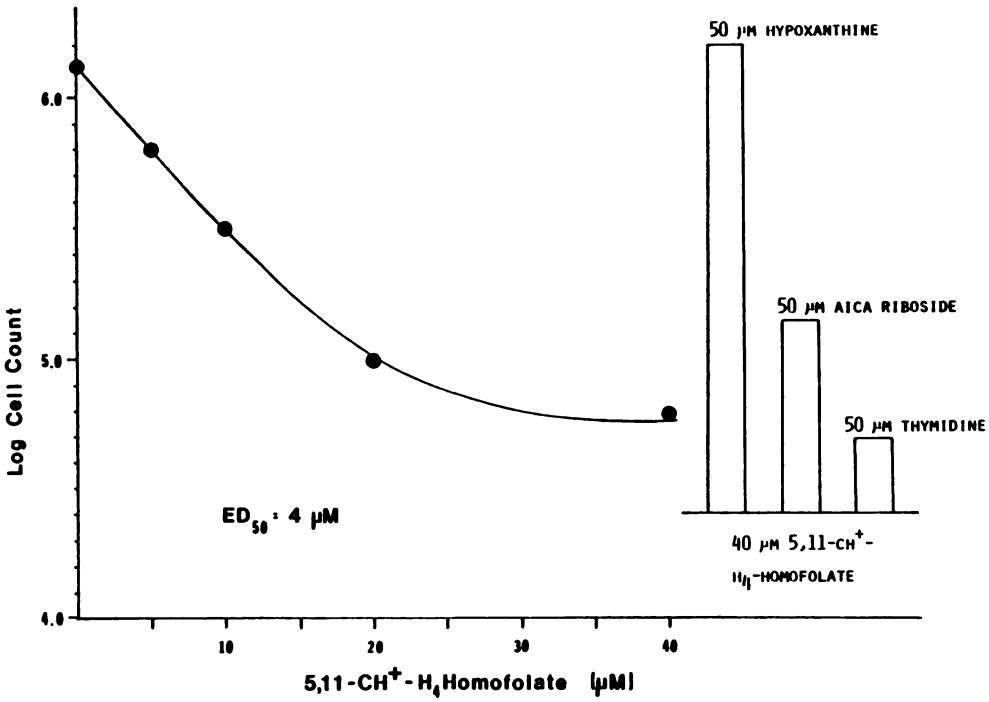

FIG. 3. *Inhibition of HKSV28 cell growth by 5,11-methenyl-H_4homofolate in the presence of hypoxanthine, AICA riboside, and thymidine* The *left* y-axis also applies to the *inset*.

formed using the method of Hartwick and Brown (16) as modified by Pogolotti and Santi (15).

To determine the effect of 5,11-methenyl or 11-formyl-H_4homofolate on FGAR synthesis, HKSV28 cells were plated out on eight 100-mm plates at a density of 5×10^5 cells/plate. Cells were grown for approximately 18 hr and then changed to medium free of L-glutamine and containing 12 μM azaserine. Drug plates contained 40 μM 5,11-methenyl-H_4homofolate or 40 μM 11-formyl-H_4homofolate which had been prepared by anaerobic hydrolysis of the amidine at pH 10.2, as described above. In the latter case, 50 μM ascorbate was added to the medium. Control plates contained no drug. After an initial 30-min incubation period, each plate was treated with 125 μl of 80 mM L-glutamine, 1.0 μCi of [2-^{14}C]glycine (47.3 mCi/mmole), and 40 mM potassium phosphate (pH 7.5). The specific activity after dilution with cold glycine in the medium was 0.69 mCi/mmole. Plates were quenched with 0.5 M trichloroacetic acid as described above after 2, 4, 6, and 8 hr, and the neutralized extract was loaded onto a QAE Sephadex A25 column (0.5 cm × 12.5 cm) equilibrated with 0.01 M NH_4HCO_3 (pH 3.8). Sixty drop fractions were collected and were counted after the addition of 15 ml of Liquescent scintillation cocktail (New England Nuclear Corporation). Typically, glycine eluted in the second and third fractions, and FGAR eluted in fractions 9–16. This procedure is a variation of the method described by Divekar and Hakala (4). The acid-insoluble material (protein, nucleic acids, and cell membranes) was dissolved in 1.0 ml of 0.2 N NaOH, and protein was determined by the biuret method described by Zamenhof (17). FGAR levels were reported as counts per minute per milligram of protein.

Enzymatic assays. The method of Smith *et al.* (12) was employed to assay GAR transformylase. Alternatively, the transformylase was also coupled to serine hydroxymethyltransferase to maintain H_4folate at a low steady-state concentration. This dramatically decreased the product inhibition of the transformylase[3] and allowed more accurate measurement of an initial velocity. The ratio of serine hydroxymethyltransferase to GAR transformylase was approximately 125:1, and the extinction change was increased to 22,500 M^{-1} cm^{-1} (due to the increased extinction of 5,10-methylene-H_4folate as compared with H_4folate). AICAR transformylase and dihydrofolate reductase were assayed by the methods of Mueller and Benkovic (18) and Hillcoat *et al.* (19), respectively.

[3] W. T. Mueller, The Pennsylvania State University, unpublished results.

RESULTS

Determination of ED_{50} for 6S-5,11-methenyl-H_4homofolate and 11-formyl-homofolate, and the effects of hypoxanthine, AICA, and thymidine on growth inhibition. To determine the effectiveness of 5,11-methenyl-H_4homofolate in inhibiting HKSV28 cell growth, the ED_{50} value, at which cell growth is inhibited 50% relative to untreated cells, was measured. Figure 3 shows the results, from which an ED_{50} of 4 μM was obtained. Also presented are the results of simultaneously growing cells treated with 40 μM 5,11-methenyl-H_4homofolate and 50 μM hypoxanthine, 50 μM AICA riboside and 50 μM thymidine. Although hypoxanthine offers complete protection from the inhibitory effects of the drug, thymidine is apparently completely ineffective, and AICA riboside appears to offer partial protection. Similar experiments utilizing both AICA riboside and the free base AICA at 10, 20, 50, and 100 μM produced the same results, with protection never exceeding 10% of the control (data not shown). To determine whether AICA or its riboside was actually utilized by this cell line as precursors of purines in the *de novo* pathway, the cells were grown in the presence of 20 μM azaserine (20). AICA exhibited partial protection at 100 μM and complete protection at 300 μM (data not shown), but AICA riboside was not utilized effectively. The experiment was then repeated using 40 μM 5,11-methenyl-H_4homofolate and 300 μM AICA, but no protection was observed (Fig. 4).

11-Formyl-homofolate, however, gave different results.

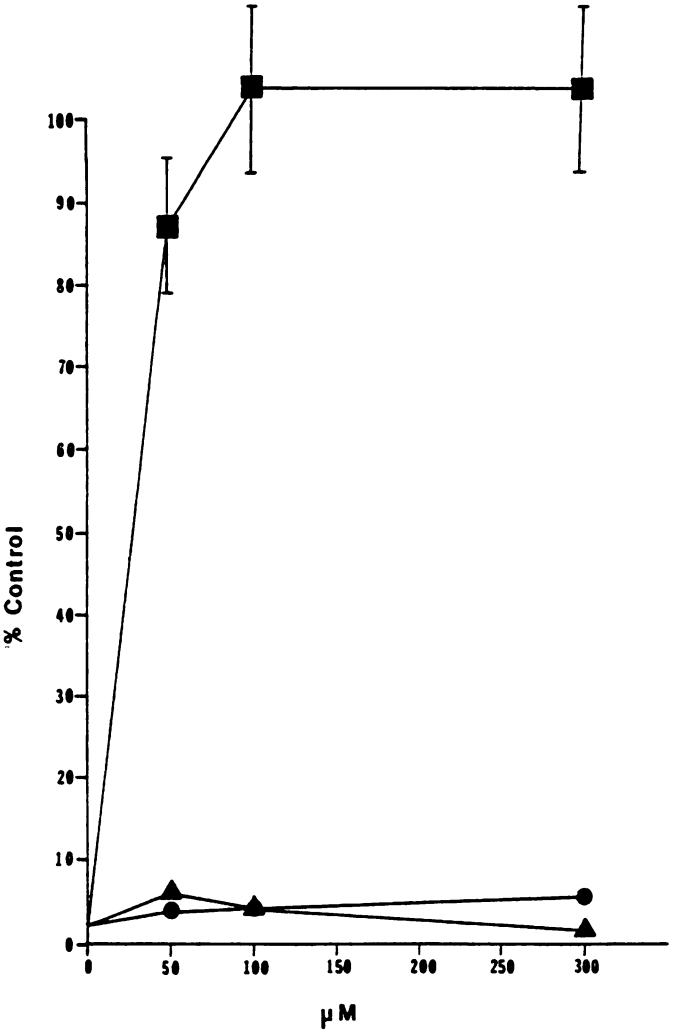

FIG. 4. *Effects of hypoxanthine (■), AICA (●), and AICA riboside (▲) on 5,11-methenyl-H₄homofolate-induced growth inhibition of HKSV28 cells*

The ED_{50} for this compound, when determined under conditions identical with those for 5,11-methenyl-H_4homofolate, was approximately 14 μM. Furthermore, although hypoxanthine and thymidine exhibited the same protection characteristics as observed with 5,11-methenyl-H_4homofolate-induced growth inhibition, in contrast, AICA was shown to offer complete protection at 300 μM (data not shown).

Effects of 5,11-methenyl-H_4homofolate, 11-formyl-homofolate, and methotrexate on ribonucleotide pool levels in HKSV28 cells. Cell extracts were prepared as described under Materials and Methods. Cells were treated with 40 μM 5,11-methenyl-H_4homofolate or 50 μM 11-formyl-homofolate and incubated for 5 hr. When methotrexate was used, its concentration was 10 μM and the incubation period was 1 hr. Samples were injected as 100-μl aliquots representing approximately 10^6 cells. Nucleotides were quantitated by peak height comparison to standards, and the results appear in Table 2. All drugs showed a decrease in purine nucleotides and an increase in pyrimidine nucleotides, specifically UTP and UDP. In cells treated with 11-formyl-homofolate, changes in ATP and CTP levels were significantly different from those observed in 5,11-methenyl-H_4homofolate-treated cells. The results for methotrexate were consistent with those reported by Pogolotti and Santi (15) with L1210 leukemia cells. The agreement between the control levels of nucleotides for all three experiments and the generally high ratio of ATP to ADP observed (≥10) suggest that these data are reliable, and that significant triphosphate hydrolysis had not occurred during sample preparation.

Effects of 11-formyl-H_4homofolate on GAR and AICAR transformylase. To determine whether inhibition of one or both of the transformylases of purine biosynthesis might be responsible for the growth inhibition observed with 5,11-methenyl-H_4homofolate-treated cells, both enzymes, purified from chicken liver, were assayed in the presence of 6*S*-11-formyl-H_4homofolate using 6*S*-10-formyl-H_4folate as substrate. Sufficient transformylase activity from the cells themselves was not readily available for this type of screen. No inhibition was observed with AICAR transformylase and the following initial concentrations of substrates and inhibitor: AICAR 47 μM, 10-formyl-H_4folate 42 μM, and 11-formyl-H_4homofolate 58 μM. 11-Formyl-H_4homofolate was also shown not to serve as a cofactor at the same concentration. In contrast, 11-formyl-H_4homofolate was a potent competitive inhibitor of GAR transformylase, with a $K_i \cong 1$ μM.

Effects of 11-Formyl-7,8-H_2homofolate on GAR and AICAR transformylase and dihydrofolate reductase. For AICAR transformylase, concentrations of 11-formyl-H_2homofolate as high as 80 μM showed no inhibition versus a substrate concentration of 18 μM 10-formyl-H_4folate. However, 11-formyl-7,8-H_2homofolate was shown to be a significant competitive inhibitor of GAR transformylase, with a $K_i \cong 4$ μM.

To determine whether 11-formyl-7,8-H_2homofolate could be intracellularly reduced to the tetrahydro form, it was assayed as a substrate with dihydrofolate reductase isozyme 1 from *Escherichia coli* TR 500. The results showed that it is a substrate for the bacterial enzyme, with an apparent K_m equal to approximately 80 μM. When compared with the natural cofactor 7,8-H_2folate, which has a K_m equal to 8.9 μM (21), the relative V_{max}

TABLE 2

Changes in ribonucleotide levels in HKSV28 cells treated with 5,11-methenyl-H_4homofolate, 11-formyl-homofolate, and methotrexate

Nucleotide	% Change		
	5,11-Methenyl-H_4homofolate	11-Formyl-homofolate	Methotrexate
UDP-Glc	−3	+14	+10
UDP-Gal	−3	+31	+34
UDP	+14	+12	+44
CDP	+30	+18	0
ADP	−33	−3	−18
GDP	−19	−9	+81
UTP	+29	+36	+34
CTP	0	+30	+4
ATP	−52	−7	−39
GTP	−59	−30	−66

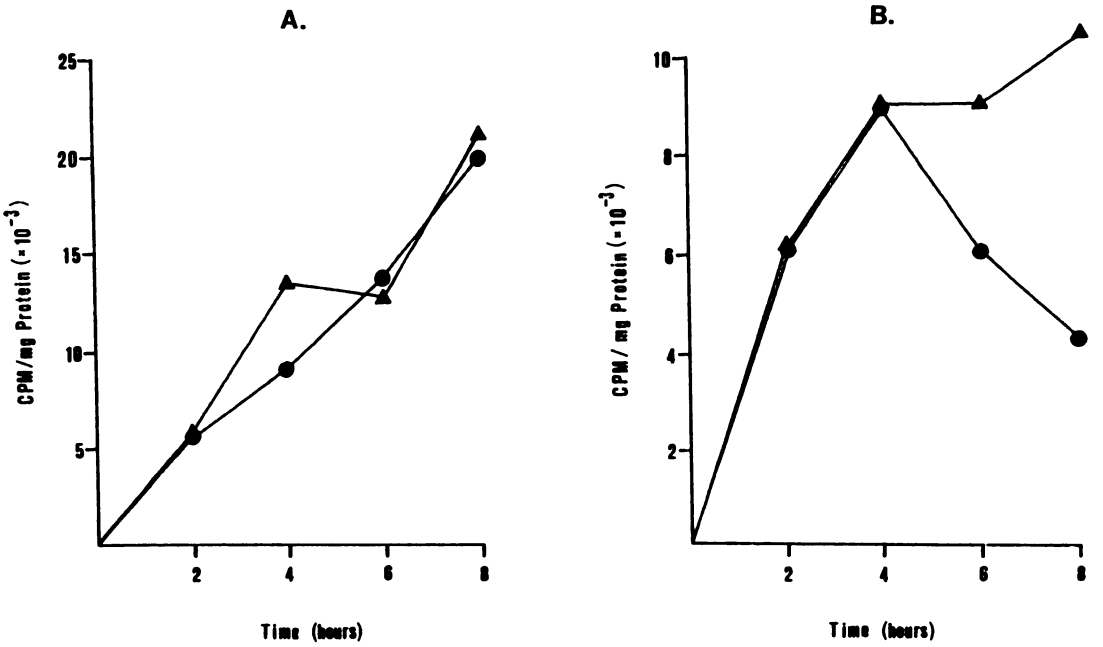

FIG. 5. *Incorporation of [^{14}C]glycine into acid-insoluble material (A) and FGAR (B) in cells treated with 5,11-methenyl-H$_4$homofolate* ●, Drug-treated cells; ▲, control cells.

was 2.7%. Although the source of this enzyme is not eukaryotic, dihydrofolate reductases in general have a very wide substrate specificity (22).

Effects of 11-formyl-homofolate on GAR and AICAR transformylase. To determine whether the fully oxidized species, 11-formyl-homofolate, was capable of inhibiting either transformylase, it was assayed against both enzymes as described under Materials and Methods. For both GAR and AICAR transformylase, no inhibition was observed.

Measurement of FGAR levels in cells treated with 5,11-Methenyl-H$_4$homofolate. As an intermediate in purine biosynthesis, FGAR is normally present intracellularly at very low levels, but in the presence of azaserine, it is elevated for at least 10–12 hr (20, 23). The effects of drugs on FGAR levels can be measured by growing cells in medium contain [^{14}C]glycine and azaserine, and then quantitating the FGAR as described under Materials and Methods.

Figure 5 shows that 5,11-methenyl-H$_4$homofolate has no effect on FGAR levels until after 4 hr, and that after 8 hr the levels are approximately 50% decreased.[4] However, as is shown in Fig. 6, prehydrolysis of the amidine to the formamide resulted in immediate inhibition of FGAR synthesis ranging from 47% at 2 hr to 76% at 8 hr. Also included in these figures is the time-dependent incorporation of [^{14}C]glycine into acid-insoluble material, which, because of the azaserine block, should reflect only protein synthesis. This is essentially linear and does not appear to be significantly affected in the case of either drug.

[4] The observation that levels of FGAR actually decreased instead of simply increasing at a slower rate than the control suggests that the azaserine block was not total and that a steady-state concentration was obtainable.

DISCUSSION

The object of this project was to determine the mechanism by which a novel antifolate, 5,11-methenyl-H$_4$homofolate, inhibits the growth of a line of transformed mammalian cells. The methods employed were (a) synthesis of the possible hydrolysis and oxidation products of the drug and evaluation of them as possible inhibitors versus purified folate-requiring enzymes, and (b) construction of a hypothesis from the above results which could be tested by performing experiments with cultured cells. Although the data do not unambiguously confirm a mechanism of action, they are consistent with the scheme presented later. From the initial dose-response experiments with 5,11-methenyl-H$_4$homofolate, it was shown that this compound was an effective inhibitor of cell growth, with an ED$_{50}$ of 4 μM. Furthermore, the inhibition was maintained over a 4-day period, indicating some degree of stability of the active species. Hypoxanthine was capable of protecting the cells from growth inhibition, but thymidine had no effect. This suggested that the blockage was in the purine biosynthetic pathway, and that pyrimidine synthesis (i.e., thymidylate synthetase and/or dihydrofolate reductase) was not inhibited. It was also shown that neither AICA nor AICA riboside (which can be converted to AICAR by the action of adenine phosphoribosyltransferase and adenosine kinase, respectively) could alleviate the growth inhibition. The fact that AICAR did protect the cells against the effects of azaserine proved that this cell line was capable of utilizing it by the salvage pathway, and suggested, therefore, that the block at least included AICAR transformylase. Since Caperelli *et al.* (6) had shown that 5,11-methenyl-H$_4$homofolate only weakly inhibited the 5,10-methenyl-H$_4$folate cyclohydrolase activity of the trifunctional protein and that GAR transfor-

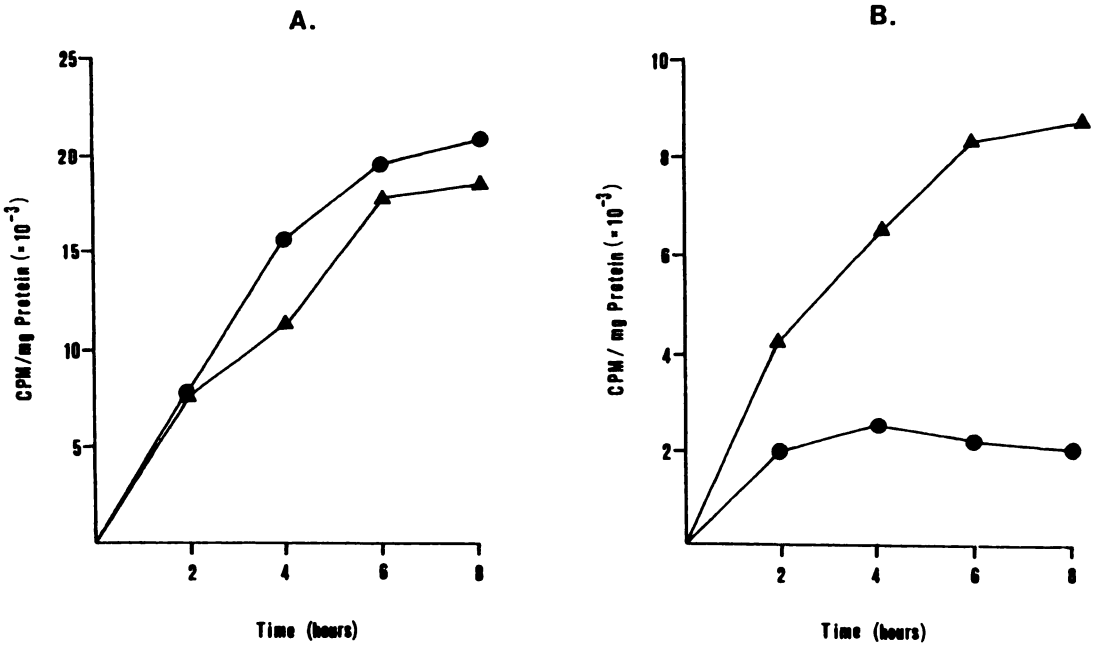

FIG. 6. *Incorporation of [^{14}C]glycine into acid-insoluble material (A) and FGAR (B) in cells treated with prehydrolyzed 5,11-methenyl-H_4homofolate*

●, Drug-treated cells; ▲, control cells.

mylase was not inhibited, it appeared likely that a derivative of 5,11-methenyl-H_4homofolate might be the active species involved in cell growth inhibition. In fact, it seemed likely that hydrolysis of the amidine to the formamide might generate a species capable of inhibiting the two transformylases of *de novo* purine biosynthesis, since both require 10-formyl-H_4folate (12).

Initially, one would predict hydrolysis to 11-formyl-H_4homofolate, which could then undergo a two-electron oxidation to form 11-formyl-7,8-H_2homofolate followed by a second oxidation to the fully oxidized pteridine, 11-formyl-homofolate. Since the instability of 11-formyl-H_4homofolate toward air oxidation precluded its facile use in the cellular studies, we decided to synthesize first 11-formyl-homofolate and investigate its ability to inhibit cell growth. Significantly, 11-formyl-homofolate exhibited an ED_{50} of 14 μM (compared with 4 μM for 5,11-methenyl-H_4homofolate under identical conditions) and its inhibitory effects were alleviated by 300 μM AICA. Furthermore, an analysis of the ribonucleotide pool changes induced by both drugs showed significant differences. Both compounds decreased levels of purines and increased levels of pyrimidines. The decrease in ATP levels was quite large for 5,11-methenyl-H_4homofolate-treated cells (−52%), whereas 11-formyl-homofolate exhibited very little effect (−7%). For CTP, the opposite was true (0 versus + 30%). The general increase in pyrimidine levels is probably due to a slowdown in their utilization in RNA synthesis because of reduced purine levels (24), or it may arise from elevated rates of pyrimidine synthesis due to an excess of pyrophosphorylribose phosphate. The conclusions from these data are that 5,11-methenyl-H_4homofolate does not act simply through hydrolysis and oxidation to 11-formyl-homofolate.

The measurement of FGAR levels in the presence of 5,11-methenyl-H_4homofolate showed that a decrease in FGAR levels was a significant and primary effect of the drug. Since [^{14}C]glycine incorporation into protein and nucleic acids was not decreased significantly, general cytotoxicity of the drug was not likely to be the cause of a decrease in FGAR synthesis. When the cells were treated with 5,11-methenyl-H_4homofolate, FGAR did not decrease for the first 4 hr, but after 8 hr levels were reduced to 50% of control. However, if the amidine was prehydrolyzed, then the rate of FGAR synthesis was decreased immediately, and a lower steady-state level was established. This would suggest that a time-dependent hydrolysis was necessary to generate a species capable of inhibiting the synthesis of FGAR. This time scale is consistent with a half-time of 7.5 hr for hydrolysis of 5,11-methenyl-H_4homofolate under cellular growth conditions and suggests that 11-formyl-H_4- and 7,8-H_2homofolate are the active species.

Indirect support for this hypothesis stems from the finding that both 11-formyl-H_4homofolate and 11-formyl-7,8-H_2homofolate are competitive inhibitors of GAR transformylase. Furthermore, the apparent K_i values of 1 and 4 μM, respectively, are of the same order of magnitude as the observed ED_{50} of 4 μM for 5,11-methenyl-H_4homofolate. However, the inability of AICA to protect against their effects would suggest that AICAR transformylase might also be affected, since the experiments with azaserine had clearly demonstrated the ability of this cell line to utilize AICA in purine biosynthesis.

One possible explanation for this might be that the inhibition characteristics observed with chicken liver transformylases do not apply to the enzymes present in mammalian cells. Another point to consider is the question of glutamine chain length, which has been shown to

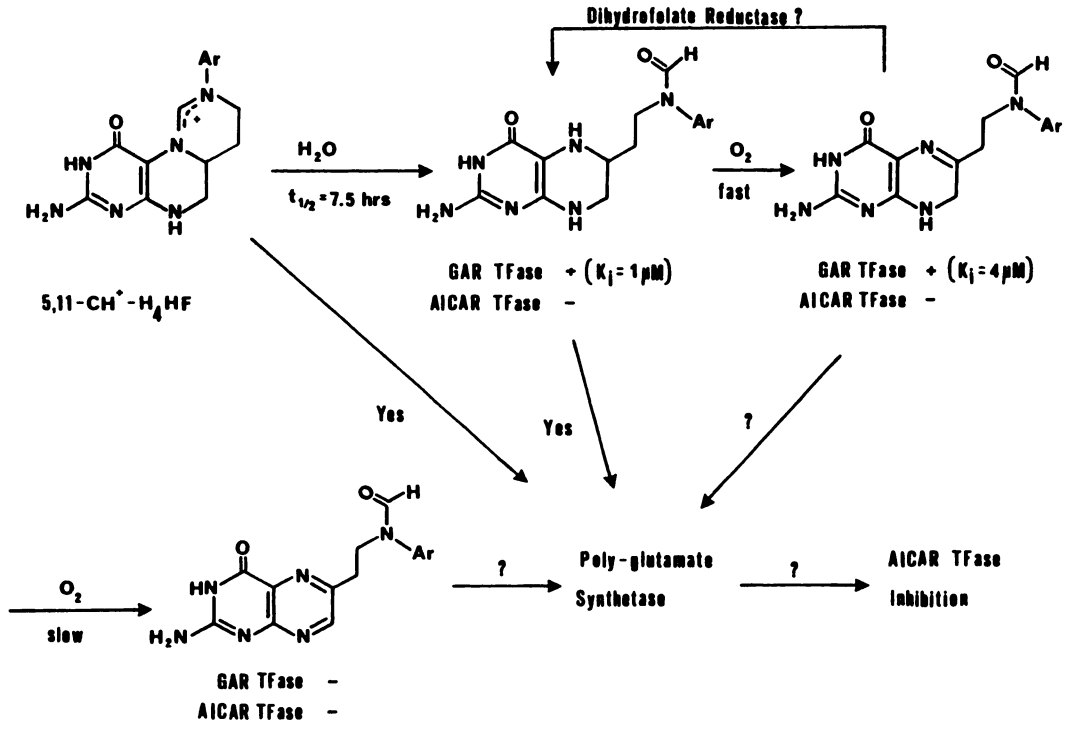

FIG. 7. *Proposed mechanism of action of 5,11-methenyl-H_4homofolate on the growth inhibition of HKSV28 cells* See text for explanation. *Ar*, $pC_6H_4CONHCH(CO_2H)CH_2CH_2CO_2H$.

play a significant role in the intracellular utilization of folate cofactors (25, 26). In general, for any enzyme utilizing the generalized folate derivative pteroyl $(Glu)_n$, an increase in n results in a decrease in the apparent K_m, or an increase in the tightness of binding. Recently, Baggott (27) showed that the methotrexate inhibition of AICAR transformylase became competitive and stronger as n was increased from 1 to 5, and that the unreduced 10-formyl-pteroyl $(Glu)_5$ but not $(Glu)_1$ inhibited the enzyme against 10-formyl-H_4pteroyl $(Glu)_5$ as the assay substrate. Thus it is possible that conjugated derivatives of 11-formyl-H_4/H_2homofolate might exhibit inhibition of AICAR transformylase not observed with the monoglutamate species.

From the data presented above, a likely mode of action of 5,11-methenyl-H_4homofolate can be formulated and is detailed in Fig. 7. Initial hydrolysis of the amidine is required, at least for inhibition of FGAR levels, and this proceeds under cellular growth conditions with a $t_{1/2}$ of 7.5 hr. 11-Formyl-H_4homofolate is capable of inhibiting GAR transformylase, but its conversion to the more stable 11-formyl-7,8-H_2homofolate is quite rapid. It is not clear whether this conversion is intra- or extracellular. Once in the cell, however, the 7,8-dihydro derivative could conceivably be reduced enzymatically back to the 5,6,7,8-tetrahydro form by dihydrofolate reductase. Conjugation by folylpolyl-γ-glutamyl synthetase[5] to an extended glutamate metabolite might generate species capable of inhibiting both transformylases of the purine biosynthetic pathway (at least at the tetra and dihydro levels) or only GAR transformylase at the fully oxidized level. In any case, these compounds appear to be specific for the transformylases in *de novo* purine biosynthetic pathway and, as such, should be useful biological probes.

[5] In collaboration with Dr. John McGuire of the Yale University School of Medicine, Department of Pharmacology (personal communication), we showed that 11-formyl-H_4homofolate is a substrate for rat liver folylpolyl-γ-glutamyl synthetase, and was probably extended to the penta-glutamate form.

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