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Inhibition of the Biosynthesis of 5'-Phosphoribosyl-*N*-formylglycinamide in Sarcoma 180 Cells by Homofolate

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SUMMARY

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Prior incubation of mouse Sarcoma 180 cells *in vitro* for 24 hr with 2-40 μM homofolate in either the presence or absence of 50 μM hypoxanthine inhibited the subsequent incorporation of [2-^{14}C]glycine into 5'-phosphoribosyl-*N*-formylglycinamide by 20-90%. Since hypoxanthine protects these cells against the growth-inhibitory effects of homofolate, this inhibition of 5'-phosphoribosyl-*N*-formylglycinamide synthesis was clearly a specific effect and not a consequence of growth arrest. Folinic acid (0.1-10 μM), when added after 24 hr of incubation with 10 μM homofolate, reversed only slightly the inhibition of 5'-phosphoribosyl-*N*-formylglycinamide synthesis. When present simultaneously with homofolate, folinic acid provided a competitive type of protection with respect to growth and 5'-phosphoribosyl-*N*-formylglycinamide synthesis. In view of the correlation between inhibition of growth and of 5'-phosphoribosyl-*N*-formylglycinamide synthesis, it appears that the latter is the site of action of homofolate in Sarcoma 180 cells. In the parent Sarcoma 180 cells and in a subline, which contains 300 times more dihydrofolate reductase than the parent cells, a 30-min prior incubation with homofolate was insufficient to inhibit 5'-phosphoribosyl-*N*-formylglycinamide synthesis significantly. The necessity for the prolonged initial incubations suggests that homofolate must undergo a time-dependent cellular conversion in order to inhibit 5'-phosphoribosyl-*N*-formylglycinamide synthesis.

INTRODUCTION

Because of the structural similarity of $N^{5,11}$-methylenetetrahydrohomofolate to $N^{5,10}$-methylenetetrahydrofolate, Friedkin *et al.* (1) suggested that it may act as a cofactor or antagonist of thymidylate synthetase. It was also anticipated that homofolate or dihydrohomofolate, which are reduced to tetrahydrohomofolate by dihydrofolate reductase [5,6,7,8-tetrahydrofolate:NAD(P) oxidoreductase, EC 1.5.1.3], may selectively kill antifolate-resistant tumors containing increased levels of this enzyme.

H_4homofolate, indeed, was found to be a potent inhibitor of TMP synthetase of *Escherichia coli* (2), but was 25 times less active against TMP synthetase of L1210 cells (3), and in a cellular system 100 μM H_4homofolate caused only marginal inhibition of deoxyuridine incorporation into DNA. Yet H_4homofolate increased the life span of mice bearing amethopterin-resistant L1210 leukemia (4), suggesting that the

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compound inhibited cellular multiplication by acting at a site other than TMP synthetase. Previous studies from this laboratory using mouse S-180 cells[1] in culture showed that hypoxanthine or AICA could prevent the growth inhibition caused by homofolate, implying thereby that the compound or its metabolite inhibited the synthesis of purines *de novo* at the introduction of C-8 (5). The purpose of the present study was to determine the possible effect of homofolate on this reaction in intact cells by measuring the formation of FGAR from [2-^{14}C]glycine. A preliminary report on this subject has been published (6).

MATERIALS AND METHODS

Compounds. [2-^{14}C]Glycine was obtained from Amersham/Searle. Horse serum and fetal calf serum were obtained from Grand Island Biological Company. Homofolate (NSC 79249) was provided by Drug Research and Development, National Cancer Institute. At pH 13 the material had the ultraviolet absorption spectrum reported for homofolate (2). On descending chromatography of 0.4 μmole on Whatman No. 3MM paper using 0.1 M ammonium bicarbonate as the solvent, more than 98% of the material was present as a single ultraviolet-absorbing spot at R_F 0.7 (7). No homopteroic acid was detected. This was confirmed by the ratio of absorbance (0.88) at 300 nm:280 nm (8). Homofolate was dissolved in 0.2 N NH_4OH and immediately diluted with an equal volume of double-strength Eagle's medium. The solution was stored in the refrigerator in the dark and prepared fresh every 10 days. (±)-L-Folinic acid was obtained from Lederle Laboratories, and the concentrations mentioned in the text below refer to the natural diastereoisomer. L-Azaserine (Parke, Davis and Company) was dissolved in 5 mM Tris-chloride, pH 8.0. It was stored frozen and prepared fresh every 10 days.

Cells. The parent S-180 cell line of Foley and Drolet (9) was maintained in monolayer culture in Eagle's medium (10) containing 1 μM folic acid and supplemented with 5% horse serum. The amethopterin-resistant subline (AT/3000), containing 300-fold more dihydrofolate reductase than S-180, was maintained in a medium containing 50 μM amethopterin (11). In order to free the cells of amethopterin, prior to use they were grown for 2 weeks in the absence of amethopterin in a medium supplemented with hypoxanthine, thymidine, and glycine (12). The cultures of both cell lines were tested and found to be free of PPLO contamination (13).

FGAR biosynthesis. FGAR biosynthesis was measured by the method of Henderson (14) with minor modifications (15) as described earlier. Monolayer cultures of S-180 cells were prepared in T15 flasks by inoculation 1 or 2 days previously with 4–5 $\times$ 10^6 cells in 2 ml of Eagle's medium (10) containing 1 μM folate supplemented with 5% horse serum (16). The cells were refed 1 day before use with the same medium. For FGAR synthesis the cells, equal to about 1 mg of protein, were first incubated for 30 min at 36° with 2 ml of medium devoid of glutamine, folic acid, and serum but containing 12 μM L-azaserine and homofolate. Then 50 μl of 80 mM glutamine and 50 μl of 16 mM [2-^{14}C]glycine (0.63 μCi/μmole) were added, and the incubation was continued for 2 hr. At the end of the incubation the medium was poured off, and the cell layer was rinsed twice with ice-cold, serum-free medium and extracted with 2 ml of 5% TCA as described (16). After removal of TCA with ether extraction, a 1-ml aliquot was chromatographed on a Dowex 1-formate column, and the FGAR fraction was counted for ^{14}C in a Packard Tri-Carb liquid scintillation spectrometer with an external standard. The remaining cell layer was dissolved in 0.2 N NaOH and analyzed for ^{14}C and protein (16). The results are expressed as disintegrations per minute per milligram of cellular protein. When 24-hr preliminary incubations were

[1] The abbreviations used are: S-180, Sarcoma 180; AICA, 5-amino-4-imidazolecarboxamide; AICAR, 5-amino-1-β-D-ribosyl-4-aminoimidazolecarboxamide 5'-phosphate; GAR, glycinamide ribonucleotide (5'-phosphoribosyl-*N*-glycinamide); FGAR, formylglycinamide ribonucleotide (5'-phosphoribosyl-*N*-formylglycinamide); L-azaserine, *O*-diazoacetyl-L-serine; PPLO, pleuropneumonia-like organisms; TCA, trichloracetic acid.

used, the cells, equal to about 0.5 mg of protein, were incubated in Eagle's medium containing either 1 μM folic acid or varied concentrations of folinic acid, 5% horse or fetal calf serum, and varied concentrations of homofolate with or without 50 μM hypoxanthine. After the initial incubation the medium was poured off, and the cells, equal to about 1 mg of protein, were incubated for 10 min with 2 ml of medium devoid of glutamine, folic acid, and serum but containing 12 μM azaserine. Glutamine and [2-^{14}C]glycine were then applied as above, and incubation was continued for 2 hr at 36°.

RESULTS

Incorporation of [2-^{14}C]glycine into S-180 cells. The incorporation of [2-^{14}C]glycine into acid-soluble and insoluble fractions and into FGAR was linear for at least 2 hr (Fig. 1). The formation of FGAR reached a plateau at about 4 hr, while the uptake of glycine into acid-soluble and insoluble fractions (radioactivity in the latter fraction represents incorporation into protein, since nucleic acid synthesis is blocked by azaserine) continued up to 6 hr, although at a diminished rate. At the end of 6 hr of incubation about 15% of extracellular glycine had been taken up by the cells. The incorporation of glycine into S-180 cells was unaffected by the presence or absence of 1 μM folate in the incubation medium, indicating that the cells contained ample pools of folate cofactors. In subsequent experiments, 2-hr periods were chosen for measuring FGAR biosynthesis. The rate of FGAR synthesis varied considerably from experiment to experiment and was 26,000 ± 5,800 dpm/mg of protein in 2 hr (22 experiments). If intracellular glycine pools are not taken into account, this represents a synthesis of 9.5 nmoles of FGAR per milligram of protein per hour.

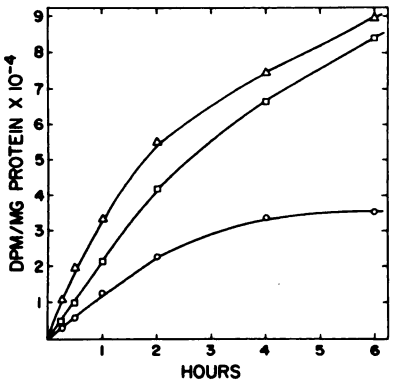

FIG. 1. *Incorporation of [2-^{14}C]glycine into various fractions of S-180 cells*

Monolayers of S-180 cells were incubated with 12 μM azaserine for 10 min in folate-free Eagle's medium. After addition of 2 mM glutamine and 400 μM glycine (0.63 μCi/μmole), the incubations were continued for 15 min–6 hr. Radioactivity, as disintegrations per minute per milligram of protein, was determined in acid-soluble (△) and insoluble fractions (□) and in FGAR (○) as described in MATERIALS AND METHODS.

Inhibition of FGAR biosynthesis by homofolate in folic acid medium. The formation of labeled FGAR from [2-^{14}C]glycine was not significantly inhibited in S-180 cells which had been incubated for only 30 min with 5–200 μM homofolate and 12 μM azaserine (Fig. 2A). At 0.5 and 1 mM homofolate, which are 100–200 times the 50% growth-inhibitory concentration (Fig. 2B), both FGAR biosynthesis and the incorporation of glycine into protein (not shown) were inhibited by about 40% and 85%, respectively. The inhibition of protein synthesis suggested general cytotoxicity, and microscopic examination of these cultures revealed detachment of cells from the glass.

At physiological pH homofolate possesses a net negative charge, like amethopterin, which is taken up very slowly by S-180 cells (17), suggesting that homofolate also would penetrate at a slow rate. Moreover, since no intracellular reduction of H_2homofolate had been observed in 45 min in L1210 cells (18), it was felt that the 30-min initial incubation used above may have been too short to allow homofolate to exert an effect. To allow for optimal uptake and possible intracellular reduction of homofolate, S-180 cells were first incubated with 5–200 μM homofolate for 24 hr. The effect of these prior incubations on subsequent FGAR biosynthesis was measured by incubating the cells in fresh medium containing azaserine and [2-^{14}C]glycine but no homofolate or folate (Fig. 2A). Under these conditions only 5 μM homofolate (50% growth-inhibitory concentration) was re-

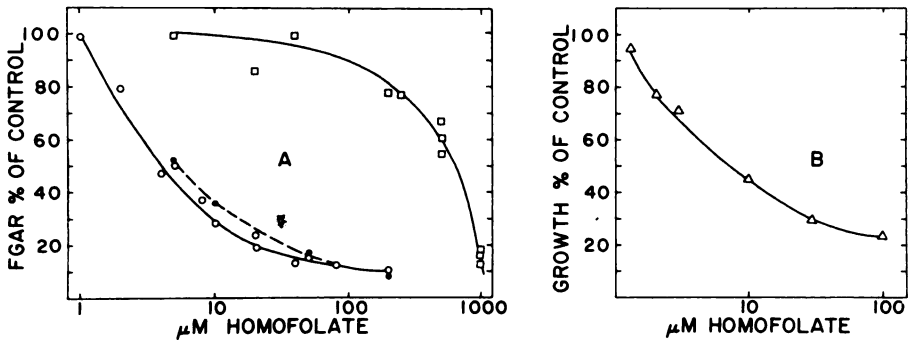

FIG. 2. *Effect of homofolate on FGAR biosynthesis and growth of S-180 cells*

A. FGAR synthesis. Before FGAR synthesis with 400 μM [2-^{14}C]glycine (0.63 μCi/μmole) was measured, the cells were incubated for 30 min with homofolate (□), for 24 hr with homofolate (●), or for 24 hr with homofolate and 50 μM hypoxanthine (○). For details, see MATERIALS AND METHODS. B. Growth inhibition (data from ref. 19). S-180 cells were grown in Eagle's medium with varied concentrations of homofolate, as indicated, for 6 days.

quired to inhibit FGAR formation by 50%, while maximal inhibition (90%) occurred at 40 μM. At 20 μM homofolate and below, the total cellular protein after initial incubation was comparable to the controls, whereas at higher concentrations a 15–30% reduction was observed. Under these conditions ^{14}C incorporation into protein was also inhibited 13–65%. Thus, in spite of a good correlation observed between the inhibition of growth and FGAR synthesis, these experiments could not ascertain whether inhibition of FGAR synthesis is a primary or secondary (due to growth arrest) effect of homofolate.

To avoid the secondary effects of growth arrest on FGAR synthesis, similar experiments were carried out in the presence of hypoxanthine, which is known to protect S-180 cells against homofolate (5). However, concentrations of hypoxanthine greater than 30 μM have been shown to cause feedback inhibition of FGAR formation in Ehrlich ascites cells (14). In preliminary experiments it was established that 50 μM hypoxanthine in the initial incubation medium protected the cells against cytotoxic effects of 5–200 μM homofolate, as revealed microscopically and by lack of effect on the total cellular protein. By itself 24-hr prior incubation medium had no significant effect on subsequent FGAR synthesis. In cells first incubated with 50 μM hypoxanthine there were 33,200 dpm/mg of protein in the FGAR fraction as compared with 34,400 dpm/mg in the hypoxanthine-free controls (average of duplicates ± 5%). As shown in Fig. 2A, the pattern of inhibition of FGAR synthesis by homofolate was identical with that observed in the absence of hypoxanthine. However, in contrast to the experiments without hypoxanthine, glycine incorporation into protein was not inhibited. Thus the inhibition of FGAR synthesis appears to be a primary and specific effect of homofolate. In a preliminary abstract (6) we reported that under these conditions 40–200 μM homofolate caused only 27–46% inhibition of FGAR biosynthesis. The reason for this discrepancy may have been contamination of the cell cultures with PPLO which occurred at that time and may have caused breakdown of homofolate.

To find out whether higher intracellular dihydrofolate reductase activity could hasten the presumed reduction of homofolate, 30-min initial incubation experiments were carried out with an amethopterin-resistant subline of S-180 cells (AT/3000) which contains 300 times more dihydrofolate reductase than the parent S-180 cells (11). Preliminary incubation with 20–200 μM homofolate caused no greater inhibition of FGAR biosynthesis than was observed for S-180 cells (data not shown). Thus, as observed previously with respect to growth inhibition (5, 19), dihydrofolate reductase was not the limiting factor in the action of homofolate.

Inhibition of FGAR biosynthesis by homofolate in folinic acid medium. The

growth inhibition by homofolate could be competitively prevented by folic or folinic acid in the medium (5). To determine whether this was also true for FGAR biosynthesis, S-180 cells were incubated (24 hr) with 1–200 μM homofolate in Eagle's medium containing either 0.01 or 0.1 μM folinic acid. In the controls FGAR biosynthesis was found to be identical whether the cells were incubated with 0.01 or 0.1 μM folinic acid or with 1 μM folic acid. It may be noted that the patterns of inhibition of FGAR synthesis by homofolate in 0.01 μM folinic acid (Fig. 3A) and 1 μM folic acid (Fig. 2A) media, both of which support optimal growth of these cells (20), were similar. In 0.1 μM folinic acid medium 30–50 times more homofolate was required to inhibit FGAR synthesis than in 0.01 μM folinic acid medium (Fig. 3A).

Reversal of homofolate inhibition of FGAR biosynthesis by folinic acid. Previous studies showed that H_4homofolate toxicity in monkeys (8) could be completely reversed by subsequent addition of folinic acid. It was of interest to determine whether the inhibition of FGAR biosynthesis caused by homofolate could also be similarly reversed. S-180 cells were therefore incubated with 10 μM homofolate and 50 μM hypoxanthine for 24 hr, after which various concentrations of folinic acid were applied together with azaserine before measuring FGAR formation. As shown in Fig. 3B, there was only a slight even though reproducible reversal at 0.1–10 μM folinic acid. Concentrations of folinic acid higher than 10 μM (1000 times that required for optimal growth under normal conditions) were not tested.

DISCUSSION

Folate cofactors participate in three synthetic reactions involving nucleic acid precursors:

$$\text{GAR} + N^{5,10}\text{-methenyl-H}_4\text{folate} \rightarrow \text{FGAR} + \text{H}_4\text{folate} \quad (1)$$

$$\text{AICAR} + N^{10}\text{-formyl-H}_4\text{folate} \rightarrow \text{5-formyl-AICAR} + \text{H}_4\text{folate} \quad (2)$$

$$\text{dUMP} + N^{5,10}\text{-methylene-H}_4\text{folate} \rightarrow \text{dTMP} + \text{H}_2\text{folate} \quad (3)$$

Homofolate is a structural analogue of folate and is known to be reduced by dihydrofolate reductases of mammalian cells to H_4homofolate (19, 21). It would be logical, therefore, to assume that the actual inhibitor would be H_4homofolate or a derivative which presumably interferes with one of these reactions. Earlier studies from this laboratory (5) showed that AICA or hypoxanthine alone reversed the growth-inhibitory action of homofolate on S-180 cells but thymidine had no effect. This indicated that reactions 2 and 3 are not significantly inhibited by the drug in these cells.

The results of the present study showed that homofolate inhibited incorporation of

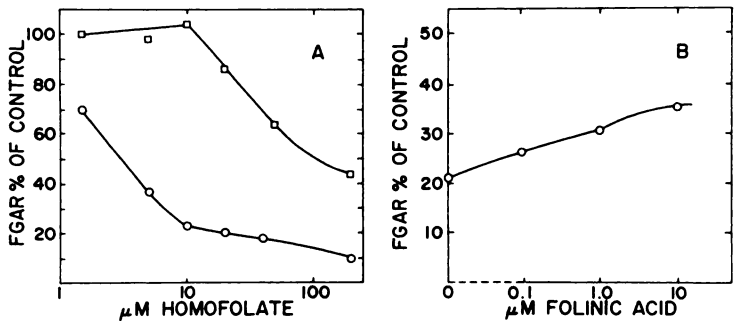

FIG. 3. *Effects of folonic acid and homofolate on FGAR synthesis in S-180 cells*

A. Folinic acid was present together with homofolate and 50 μM hypoxanthine during 24 hr of initial incubation. O, 0.01 μM folinic acid; □, 0.1 μM folinic acid. B. Folinic acid was applied after 24 hr of initial incubation with 10 μM homofolate and 50 μM hypoxanthine. In both A and B the incorporation of 400 μM [2-^{14}C]glycine (0.63 μCi/μmole) into FGAR was measured after 2 hr of incubation in the presence of 2 mM glutamine but in the absence of homofolate. Each point represents an average of duplicates, which varied by no more than 10%. For details, see MATERIALS AND METHODS.

[2-^{14}C]glycine into FGAR in S-180 cells, even when the cytotoxicity of the analogue was prevented by supplying preformed hypoxanthine. Moreover, in Eagle's medium containing 1 μM folic acid, every concentration of homofolate tested caused almost identical inhibition of FGAR synthesis and of growth (Fig. 2), 50% inhibition being obtained at 5 μM. Similarly, in Eagle's medium containing varied concentrations of folinic acid, the concentrations of homofolate required to produce 50% inhibition of growth (5) and of FGAR synthesis were almost identical (Fig. 3A). Thus, in view of the direct correlation between inhibition of growth and of FGAR synthesis under a variety of experimental conditions, it appears that the blockade of FGAR formation is indeed the mechanism of growth-inhibitory action of homofolate in S-180 cells.

It is conceivable that homofolate or its reduced derivative inhibits the intracellular formation of FGAR by inhibiting reaction 1 either directly, by competing with the cofactor ($N^{5,10}$-methenyl-H_4folate) for GAR transformylase (5'-phosphoribosyl-*N*-formylglycinamide:tetrahydrofolate 5,10-formyltransferase, EC 2.1.2.2), or indirectly, by inhibiting the formation of the cofactor. However, the latter possibility seems unlikely in view of the fact that the drug does not inhibit N^{10}-formyl-H_4folate-dependent formylation of phosphoryibosyl-AICA (see ref. 5 for discussion). Attempts to demonstrate net accumulation of GAR in cells in which FGAR formation was inhibited by homofolate were unsuccessful (6), presumably because the equilibrium of the GAR synthetase (5'-phosphoribosylamine:glycine ligase, EC 6.3.1.3) reaction is not favorable for GAR formation (22).

In short-term incubations (30 min) FGAR biosynthesis was not inhibited by homofolate in sensitive or amethopterin-resistant S-180 cells, possibly because of slow uptake and/or reduction. Nahas and Friedkin (18) were unable to detect in 45 min any reduction of 1 mM [^{3}H]H_2homofolate in sensitive or amethopterin-resistant L1210 cells containing 13-fold elevated dihydrofolate reductase activity. Yet, with respect to H_2folate, these authors observed a 6-fold higher uptake and 100% conversion to H_4folate in resistant cells as compared with 80% in the parent L1210 cells. Contrary to these observations, Mishra *et al.* (23) noted significant conversion of H_2homofolate to H_4homofolate in 30 min in both sensitive and amethopterin-resistant L1210 cells *in vitro*. However, no reduction of homofolate was observed in mouse liver even after 24 hr, although this tissue reduced H_2homofolate completely in 1 hr. In a cell-free enzyme system of S-180 cells a slow reduction of homofolate was observed (19), and it would be of interest to know whether such reduction occurs in intact cells.

It appears that the intracellular breakdown of the inhibitory homofolate derivative formed during 24 hr of preliminary incubation is a slow process, since the inhibition of FGAR synthesis persisted for 2 hr after removal of the extracellular drug (Fig. 2A). Moreover, the inhibition of FGAR biosynthesis was insignificantly affected by subsequent addition of high concentrations of folinic acid, indicating that the homofolate metabolite is a potent and perhaps an irreversible inhibitor. In view of this it would be of interest to isolate and identify the homofolate metabolite formed within these cells.

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