Biochemical Pharmacology, Vol. 23, pp. 1827–1834. Pergamon Press, 1974. Printed in Great Britain.

REGENERATION OF TETRAHYDROHOMOFOLATE IN CELLS

POSSIBLE BASIS FOR ANTITUMOR ACTIVITY OF HOMOFOLATES

LAKSHMI C. MISHRA, AMAR S. PARMAR and J. A. R. MEAD

Microbiological Associates and National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

(*Received* 13 *September* 1973; *accepted* 16 *November* 1973)

Abstract—The purpose of this study was to determine if reduction of homofolates to tetrahydrohomofolate in mice and rat tumors *in vivo* could form the basis for the antitumor activity of reduced homofolates. A single dose (400 mg/kg) of dihydrohomofolate from the optimal therapeutic range was given to animals bearing advanced tumors, and their spleen or tumor tissues were analyzed for dihydro- and tetrahydrohomofolate content by DEAE cellulose column chromatography. Mice bearing leukemia L1210/FR-8, which is responsive to reduced homofolates, showed a measurable reduction of dihydrohomofolate in their spleen, whereas the mice bearing a less responsive tumor (L1210) or no tumor showed extensive metabolism and no detectable amount of dihydro- or tetrahydrohomofolate in their spleens. The drug was also extensively metabolized in rats bearing Walker carcinosarcoma 256 tumor, which might also explain the minimal response of the tumor to reduced homofolates. The reduction of the parent compound, homofolate, which is a minimally effective antitumor agent, was not detectable in mouse liver. These studies suggested an apparent correlation between the ability of the tumors to reduce homofolates to the tetrahydro level and their response to treatment with these drugs. Since the tetrahydro form of homofolate appears to be the active moiety, the levels of dihydrofolate reductase and catabolizing enzymes, and determinants of the sustained levels of the tetrahydro derivative in tumors might be the important factors in determining the responsiveness of tumors to homofolates.

DIHYDROHOMOFOLATE (H_2HF), an analog of dihydrofolate, has been shown to possess antitumor activity comparable to that of tetrahydrohomofolate (H_4HF)[1] against leukemia L1210 and methotrexate-resistant leukemia L1210/FR-8.[2] Since H_2HF is reduced to H_4HF, a pseudocofactor, in liver[3] and its antitumor activity is decreased by methotrexate,[2] an inhibitor of dihydrofolate reductase (DFR), it seemed likely that antitumor activity of H_2HF might be related to its conversion to H_4HF in tumors. Consequently, a tissue containing higher levels of DFR may regenerate larger amounts of the active moiety relative to other tissues and thus may be selectively inhibited. This study was initiated to examine this hypothesis in tumor lines sensitive and resistant to H_4HF having 20- to 30-fold differences in their DFR levels. A portion of this study has been presented elsewhere.[4]

MATERIALS AND METHODS

Homofolic acid (NSC-89249), obtained from Drug Research and Development, National Cancer Institute, was reduced to yield H_2HF, which was checked for purity according to the procedure described elsewhere.[2] For radioisotope studies, homofolate was tritiated by Amersham/Searle, Chicago, Ill., and reduced to form ^3H-H_2HF

according to the procedures previously reported.[2,3] The final product was 92 per cent pure and had a sp. act. of 6·53 mCi/m-mole. BDF_1 male mice, 10- to 12-weeks-old, weighing 20–25 g, and RAR female rats, weighing 60–100 g, maintained on Purina Chow and water *ad lib.*, were used in all the experiments. Leukemia L1210, and its methotrexate-resistant subline L1210/FR-8, were used in the present study. Mice were inoculated s.c. with 0·01 ml of tumor inoculum, which was prepared by suspending leukemic spleens (25% w/v) in saline. In all experiments, leukemic mice were used 1–2 days before their expected day of death. Walker carcinosarcoma 256 was transplanted i.m. into the right leg of rats, and the animals were used 9 days after transplant. The spleens of mice containing L1210/FR-8 tumors contained 20- to 30-fold higher levels of DFR than those of L1210 leukemia.[5] The median survival time of mice bearing L1210 or L1210/FR-8 tumor ranged from 9 to 10 days.

Analytical procedure. H_2HF or homofolate, dissolved in 2% sodium bicarbonate containing 0·6% ascorbate, was given i.p. (800 mg/kg) to normal and tumor-bearing animals which were sacrificed after 1 hr. The spleens were pooled and analyzed by column chromatography on DEAE cellulose using 0·2 M ammonium acetate, pH 6·0, as an eluent according to a procedure described elsewhere.[3] Reduced homofolates in amounts as low as 25 μg gave identifiable peaks on chromatograms. Total reduced homofolates and the metabolites capable of liberating free diazotizable amine were measured in tissues by the previously standardized Bratton–Marshall reaction (BMR).[6]

For studies *in vitro*, leukemic and subcutaneous solid tumor tissues obtained from L1210 and L1210/FR-8 tumor-bearing mice were kept on ice and cut into pieces of 4–5 mm in size and suspended in four parts of Tyrode-ascorbate solution.[7] To suspend the spleen and tumor cells in solution, the tissue pieces were passed twice through a No. 21 hypodermic needle.[7] The cells were washed twice with four parts of ice-cold Tyrode-ascorbate solution before use. The leukemic spleen cells (1 g) were suspended in 4 ml Tyrode-ascorbate solution containing 5 mg H_2HF/ml, and the mixture was incubated for 30 min at 37°. The mixture was homogenized and clarified with alcohol. An aliquot (0·5 ml) of the clear supernatant was chromatographed on DEAE cellulose column. In parallel experiments, 1 ml of cell suspension (25% w/v in Tyrode-ascorbate) was mixed with 0·25 ml of 1 mg ^3H-H_2HF/ml of solution in Tyrode-ascorbate, and the mixture was incubated at 37° for various time intervals. The cell suspension was homogenized, clarified and centrifuged. The clear supernatant was mixed with 0·5 mg each of H_2HF and H_4HF and chromatographed. An aliquot (0·5 ml) of the fractions was mixed with 10 ml of scintillation fluid (Instagel) and the radioactivity of the mixture was determined in a Packard scintillation spectrometer. Fractions containing H_2HF and H_4HF were identified by u.v. absorption spectroscopy, and the total radioactivity present in H_2HF and H_4HF fractions was determined.

RESULTS

Figure 1 presents the elution pattern of spleen extracts which shows that both H_4HF and H_2HF were present in detectable amounts in L1210/FR-8 spleens but not in L1210 leukemic spleens. An unidentified material, probably a metabolite of H_2HF, which appeared in fractions 10–20 was relatively greater in spleens of L1210 leukemic mice than from those of L1210/FR-8 leukemic mice. The data summarized in Table

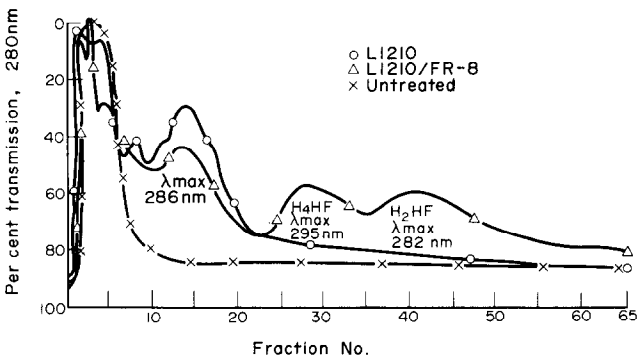

FIG. 1. Reduction of dihydrohomofolate (H_2HF) to tetrahydrohomofolate (H_4HF) in leukemic mouse spleens *in vivo*. Twelve to fourteen leukemic mice were given 800 mg H_2HF/kg, i.p., and sacrificed after 1 hr. Spleens (7·5 g) were analyzed by column chromatography.

1 indicate that, although significant uptake of the drug and drug-derived material, determined by BMR assay, occurred in spleens of mice bearing advanced tumors, H_2HF and H_4HF were demonstrable only in the spleens of mice bearing L1210/FR-8 tumors. The total amount of both the reduced homofolates isolated from the spleens ranged from 20 to 59 per cent of the total BMR positive material, and the ratio of H_4HF to H_2HF ranged from 1:1·7 to 1:0·58. In a parallel experiment, however, reduction of H_2HF in liver from mice bearing L1210 and L1210/FR-8 tumors was found to be complete which was similar to that reported for liver in normal mice.[3] When spleen from non-leukemic mice, obtained after H_2HF administration, was analyzed by column chromatography and BMR assay, significant uptake of BMR-positive material (0·6 mg/kg) but no detectable amounts of H_2HF and H_4HF were observed. It was intriguing to note that, even though the specific activity of the enzyme as determined by assay *in vitro* in liver and spleen of normal and L1210

TABLE 1. REDUCTION OF DIHYDROHOMOFOLATE (H_2HF) IN SPLEENS FROM MICE BEARING L1210 AND L1210/FR-8 TUMORS

	L1210			L1210/FR-8			
Expt. No.*	BMR-positive material† (μg/g)	H_2HF (μg/g)	H_4HF‡ (μg/g)	BMR-positive material (μg/g)	H_2HF (μg/g)	H_4HF (μg/g)	$\frac{H_4HF \times 100}{H_4HF + H_2HF}$ (%)
1	125	—	—	204	23	35	60
2	127	—	—	223	17	29	63
3	160	—	—	187	28	20	42
4	137	—	—	243	28	37	57
5	159	—	—	162	60	36	37
6	126	—	—	197	41	34	45
Average	139			202	32	31	49

* Twelve to fifteen mice bearing 8- to 9-day-old tumors were given 800 mg/kg of H_2HF, i.p., and sacrificed after 1 hr. Spleens were pooled, homogenized and analyzed for their H_2HF/H_4HF content and BMR-positive material as described in the text.

† Bratton–Marshall reagent.

‡ H_4HF, tetrahydrohomofolate.

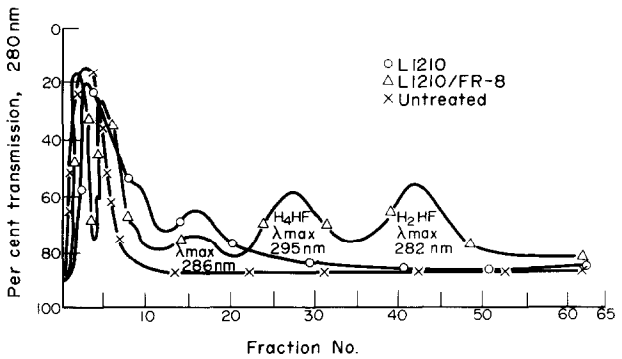

FIG. 2. Reduction of dihydrohomofolate (H_2HF) to tetrahydrohomofolate (H_4HF) in leukemic mouse spleens *in vitro*. Spleen cells from advanced leukemic mice (1 g) were suspended in 4 ml of 5 mg H_2HF/ml of solution in Tyrode-ascorbate solution and incubated for 30 min. The mixture was homogenized, and an aliquot (0·5 ml) of the homogenate was clarified and chromatographed.

tumor-bearing mice was comparable,[5] the difference in the ability of these two tissues to generate H_4HF was considerable.

Experiments were also conducted *in vitro* to determine if the H_2HF is actually reduced to H_4HF in L1210/FR-8 spleens or if the reduction product is transported to the spleens from other organs. The chromatographic data presented in Fig. 2 paralleled the observations *in vivo* indicating that H_2HF is reduced in L1210/FR-8 spleen cells and that the difference between the reduction of H_2HF by L1210 and L1210/FR-8 leukemic spleens observed *in vivo* is real. In a control experiment, in which incubation was omitted, H_2HF was found to be the major drug moiety in both the tumor lines. Results of the experiments carried out using tritiated H_2HF are summarized in Table 2. It can be seen that 30 min after incubation of both spleen and

TABLE 2. INTRACELLULAR REDUCTION OF ^{3}H-DIHYDROHOMOFOLATE (^{3}H-H_2HF) IN SPLEEN CELL SUSPENSION *in vitro*

Tissue*	Time (min)	Amount of radioactivity (cpm × 10^{-6}/g) L1210/FR-8 H_4HF†	L1210/FR-8 H_2HF	L1210 H_4HF	L1210 H_2HF
Spleen	0	0	2·88	0	2·91
			100%		100%
	15	0·38	2·48	0·13	2·51
		13%	87%	4%	96%
	30	1·26	1·66	0·40	2·51
		43%	57%	13%	87%
Tumor	0	0	4·07	0	4·15
			100%		100%
	30	1·51	2·56	0·80	3·25
		37%	63%	19%	81%

* One ml of cell suspension (25% w/v) was mixed with 0·25 ml of 1 mg ^{3}H-H_2HF/ml of solution in Tyrode-ascorbate (sp. act. 6·53 mCi/m-mole), and the mixture was incubated at 37° in a water bath for various time intervals. The mixture was homogenized, clarified and chromatographed with 0·5 mg each of H_2HF and H_4HF. Radioactivity present in the H_2HF and H_4HF fractions was determined.

† H_4HF, tetrahydrohomofolate.

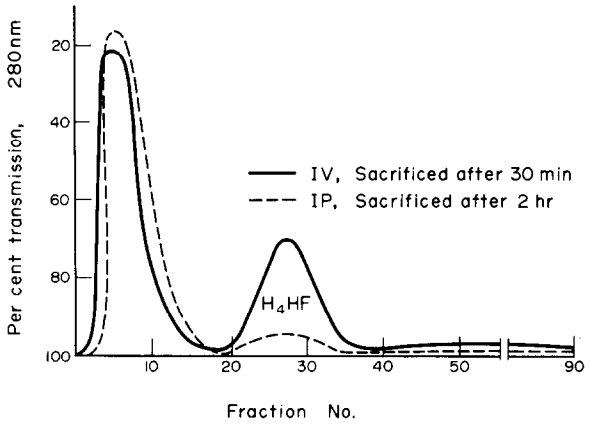

FIG. 3. Reduction of dihydrohomofolate (H_2HF) in rat liver *in vivo*. Two rats were given 400 mg H_2HF/kg, i.v., and sacrificed at specified time intervals. Livers (5·5 g) were analyzed by column chromatography.

tumor cell suspensions, the amounts of radioactivity in the H_4HF fraction were significantly greater in the L1210/FR-8 tumor than the L1210 tumor. Incubation of cells for 60 min resulted in considerable decomposition of reduced homofolates.

Since H_4HF has shown minimal antitumor activity against Walker carcinosarcoma 256 tumor,[2] the reduction of H_2HF was also studied in rats bearing this tumor. The levels of H_4HF in liver, for periods up to 2 hr after administration of 400 mg H_2HF/kg, i.p., were found to be less than 5 μg/g, not adequate for the isolation and characterization by the analytical procedure used (Fig. 3). To achieve greater uptake of H_2HF in rat liver, the same dose of the drug was given i.v. and the rats were sacrificed after 30 min. H_4HF was clearly demonstrable on the chromatogram and the uptake was estimated to be 40 μg/g of tissue. To study the reduction of H_2HF in tumor, the drug was therefore given i.v., and the rats were sacrificed after 30 min. The chromatogram of the tumor tissue homogenates presented in Fig. 4 shows large amounts of H_2HF-like material with smaller amounts of H_4HF-like material, suggesting that the reduction of H_2HF may be poor in the Walker tumor. Although the H_4HF-like material appeared before the H_2HF, both the materials appeared nearly

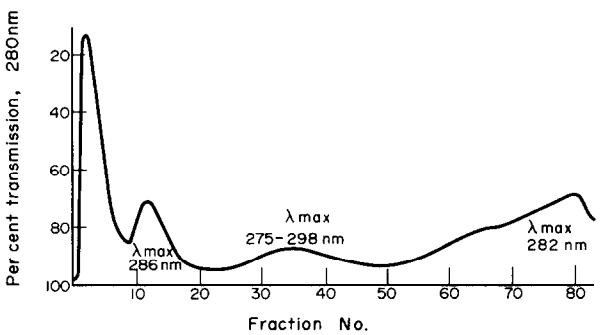

FIG. 4. Chromatogram of the Walker carcinosarcoma 256 tumor obtained from dihydrohomofolate (H_2HF)-treated rats. Two rats bearing 8-day-old Walker tumors (i.m.) were given 400 mg H_2HF/kg, i.v., and sacrificed after 30 min. Tumor (20 g) was excised and analyzed by column chromatography.

20 fractions later than that observed in the chromatograms of mouse spleen and rat liver. The large amount of Walker tumor tissue (20 g) used was the only difference between these experiments which may have influenced the elution of the reduced homofolates. The unidentified material which appeared in fractions 10–20 in the chromatogram of L1210 and L1210/FR-8 spleens (Figs. 1 and 2) also appeared in the chromatogram of the Walker tumor (Fig. 4).

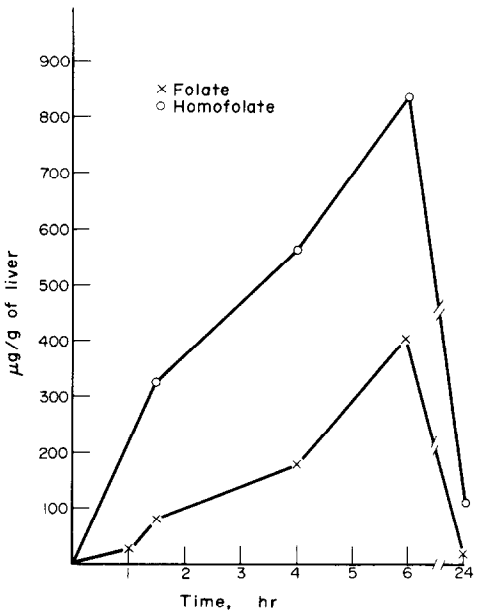

FIG. 5. Uptake of folate and homofolate in mouse liver. The mice were given the compounds (400 mg/kg, i.p.) and sacrificed at various time intervals. The livers from two mice were pooled and analyzed for folate and homofolate contents.

In contrast to H_2HF and H_4HF, homofolate has been reported to have no definitive antitumor effect against the mouse leukemias.[1] It appeared likely that the lack of antitumor activity of homofolates may be related to lack of or inadequate reduction of the drug to form H_4HF *in vivo*. The liver from normal mice sacrificed at various time intervals after i.p. administration of homofolate (400 mg/kg) was first analyzed, since H_2HF is reduced by liver from tumor- or non-tumor-bearing mice regardless of the sensitivity of the tumor to H_2HF therapy. Homofolate was characterized by u.v. absorption spectrum and estimated from the absorbance of the eluate at 275 nm. At all time intervals, homofolate was found to be the major drug moiety present in the liver and tetrahydrohomofolate was undetectable; similar results were obtained when folate was administered. Since reduction of homofolate was not detectable in liver, no attempt was made to study its reduction in tumor and spleen tissues of tumor-bearing mice. Levels of homofolate and folate in liver at various time intervals are shown in Fig. 5. It is interesting to note that the peak uptake of homofolate in liver was twice that of folate, and the disappearance of homofolate was also somewhat slower than the folate.

DISCUSSION

To exploit the increased levels of DFR in tumor for overcoming the resistance to methotrexate therapy, Misra *et al.*[8] suggested that an analog of folate, which is a substrate of DFR and, in its reduced form, an inhibitor of tetrahydrofolate utilization, might prove useful. H_2HF appears to be such an analog.

This study has shown an apparent correlation between the ability of tumor-infiltrated spleen cells and tumor cells to generate H_4HF from H_2HF *in vivo*, and their responsiveness to therapy with these compounds. The study has revealed that, even though liver and spleen from L1210 tumor-bearing and normal mice contained comparable levels of enzyme activity as determined by assay *in vitro*,[5] marked generation of H_4HF was seen only in liver; none was detectable in spleen. These results strongly suggest that the generation of H_4HF in cells may not be primarily the function of DFR levels but the net result of DFR and catabolic activities of the cells. It is, therefore, likely that the ability of a tumor tissue to generate H_4HF *in vivo* would be more predictive of usefulness of these compounds than the specific activity of the enzyme in that tumor.

Recently, Nahas and Friedkin[9] have reported lack of reduction of H_2HF to H_4HF in either L1210 or L1210/MTX ascites cells containing moderately high levels of DFR. Their negative observation is difficult to explain, particularly in the light of the fact that a cell lysate effectively reduced H_2HF to H_4HF. It is possible that the isolated L1210/FR-8 ascitic leukemic cells and the conditions *in vitro* might be qualitatively different in behavior than the leukemic organs and solid tumor cells used *in vivo* in the present study. On the basis of radioactivity and microbial assay methods, Nahas and Friedkin[10] reported that H_4HF is not metabolized in mice to any significant extent and up to 75 per cent of the dose is excreted in urine essentially unmetabolized. Although the reduced homofolates are very stable in liver,[11] demonstrable degradation of H_2HF and H_4HF was found in spleens of leukemic and non-leukemic mice. The role of catabolic activity of cells as a determinant of antitumor activity of reduced homofolates is also evident from the fact that 5-methyl-H_4HF, which was more stable than H_4HF *in vivo*, was more effective than H_4HF against L1210 leukemia.[12]

Other factors, like folate deficiency which could result from large doses of homofolates, may also influence the antitumor activity. Folate and its analogs have been shown to displace folates in rats and man.[13,14] On the basis of greater uptake of homofolates in liver relative to the natural vitamin, it is likely that the high doses of homofolates may cause mild folate deficiency in cells, resulting in some growth inhibitory effect. Mice bearing L1210 tumor kept on folate-deficient diets were found to be more responsive to H_4HF therapy than the mice kept on a regular diet.[2]

Acknowledgement—This study was supported in part by Contract NO1-CM-33728 with the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare, Bethesda, Md. 20014.

REFERENCES

1. J. A. R. Mead, A. Goldin, R. L. Kisliuk, M. Friedkin, L. Plante, E. J. Crawford and G. Kwok, *Cancer Res.* **26,** 2374 (1966).
2. L. C. Mishra and J. A. R. Mead, *Chemotherapy* **17,** 283 (1972).
3. L. C. Mishra, A. S. Parmar and J. A. R. Mead, *Biochem. Pharmac.* **20,** 2871 (1971).
4. L. C. Mishra, A. S. Parmar and J. A. R. Mead, *Proc. Fifth Int. Cong. on Pharmacology*, Abstr. 952, San Francisco (1972).

5. M. FRIEDKIN, E. CRAWFORD, S. R. HUMPHREYS and A. GOLDIN, *Cancer Res.* **22,** 600 (1962).
6. L. C. MISHRA, A. S. PARMAR and J. A. R. MEAD, *Cancer Res.* **30,** 642 (1970).
7. D. LIVINGSTON, E. J. CRAWFORD and M. FRIEDKIN, *Biochemistry, N.Y.* **7,** 2814 (1968).
8. D. K. MISRA, S. R. HUMPHREYS, M. FRIEDKIN, A. GOLDIN and E. J. CRAWFORD, *Nature, Lond.* **189,** 39 (1961).
9. A. NAHAS and M. FRIEDKIN, *Molec. Pharmac.* **8,** 353 (1972).
10. A. NAHAS and M. FRIEDKIN, *Cancer Res.* **29,** 1937 (1969).
11. L. C. MISHRA, A. S. PARMAR and J. A. R. MEAD, *Chem.-Biol. Interact.* **4,** 97 (1971).
12. L. C. MISHRA, A. S. PARMAR, J. A. R. MEAD, R. KNOTT, A. TAUNTON-RIGBY and O. M. FRIEDMAN, *Proc. Am. Ass. Cancer Res.* **13,** 76 (1972).
13. G. E. NEAL and D. C. WILLIAMS, *Biochem. Pharmac.* **14,** 903 (1965).
14. D. G. JOHNS and I. H. PLENDERLEITH, *Biochem. Pharmac.* **12,** 1071 (1963).