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SHORT COMMUNICATIONS

On the biochemical mechanism of action of tetrahydrohomofolic acid

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TETRAHYDROHOMOFOLIC acid (H_4HF) has been reported to be a more effective antileukemic agent against a dichloromethotrexate-resistant subline of leukemia L1210 (L1210/FR-8), containing high levels of dihydrofolate (H_2F) reductase, than the parent line.[1] Previous studies[2] have shown that an optimum therapeutic dose[1] (400 mg/kg, i.p.) given daily for 4 days inhibited DNA biosynthesis in L1210/FR-8 tumor; however, a single dose was ineffective. Neither single nor daily dosage regimens had any effect on the parent leukemia L1210 line. In the present study, an attempt has been made to elucidate the inhibition in the biosynthetic pathway of DNA.

Since H_4HF has been shown to be a potent inhibitor of partially purified *Escherichia coli* thymidylate synthetase,[3] we attempted to ascertain if there was any correlation between deoxyuridine incorporation into DNA and the H_2F-reductase levels in the spleens of mice bearing advanced tumors. Using [^{14}C]6-orotic acid and [^{14}C]formate, we also studied the effect of H_4HF on the biosynthesis of DNA thymine.

BDF male mice, 10–12 weeks old, weighing 20–25 g, were used in this study. [^{14}C] 6-orotic acid (60·8 mc/m-mole) and [^{14}C] formate (35 mc/m-mole) were obtained from Nuclear Chicago, Des Plaines, Ill. [^{3}H]G-deoxyuridine (9400 mc/m-mole) was obtained from New England Nuclear Corp., Boston, Mass. Tetrahydrohomofolic acid was supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute. DNA thymine was estimated according to the procedure described by Danneberg *et al.*[4] Since the labeled deoxyuridine was found to be incorporated into DNA thymine and not into cytosine, its uptake into DNA was carried out according to the procedure used by Roberts and Wodinsky.[5] Leukemia L1210, or its dichloromethotrexate-resistant subline, L1210/FR-8, was inoculated subcutaneously as described elsewhere.[6] Since dihydrofolate reductase levels were found to be higher when animals lived for 12–13 days, a low tumor inoculum (0·1 ml of 2% (w/v) spleen), found to give a 12–13-day median survival time, was used in all the experiments. Dihydrofolate reductase levels in spleen were determined as described by Friedkin *et al.*[7]

Mice bearing 11-day-old tumors were injected with H_4HF, a total intraperitoneal dose of 1600 mg/kg divided into three equal doses, the second and third being given 8 and 22 hr after the first. Thirty min after the last dose, 250 μc/kg of the labeled deoxyuridine was injected and the animals

TABLE 1. EFFECT OF H_4HF ON H_2F-REDUCTASE AND DEOXYURIDINE UPTAKE INTO DNA OF SPLEENS OF MICE BEARING METHOTREXATE-SENSITIVE (L1210) AND METHOTREXATE-RESISTANT (L1210/FR-8) LEUKEMIAS*

Tissues	Spleen wt. (mg)	H_2F-reductase (mμmoles/hr/mg proteins)	(dis./min/mg DNA)
L1210			
Untreated	345 (98)	64 (8)	1660 (228)
Treated	332 (48)	67 (7)	620 (133)
(T × 100)/c	96	104	37
L1210/FR-8			
Untreated	569 (114)	2030 (544)	1325 (110)
Treated	539 (47)	2520 (510)	585 (87)
T × 100)/c	94	124	44

* Mice bearing 11-day-old tumors. A total dose of 1600 mg H_4HF/kg i.p. was divided into three equal doses, the second and third being given 8 and 22 hr after the first. Thirty min after the last injection, 250 μc deoxyuridine/kg (9400 mc/m-mole) was given i.p. and the animals were sacrificed after 1 hr. Each point represents the average (S.D.) of values obtained from eight animals.

were sacrificed after 1 hr. Spleens of individual mice were analyzed for both the deoxyuridine incorporation into DNA and for H_2F-reductase levels. The data summarized in Table 1 show that inhibition of deoxyuridine uptake into DNA in both the tumor lines, sensitive and resistant to H_4HF, was not markedly different (63 and 44 per cent). Also, no relationship between the reductase activity and the inhibition of the deoxyuridine uptake into DNA was seen in the individual mice. It appears likely that thymidylate synthetase is not the principal target site responsible for antitumor activity. This possibility was further supported by a series of parallel experiments in which [^{14}C]6-orotic acid was used to study thymine biosynthesis. The data are presented in Table 2. It can be seen from these data that inhibition of [^{14}C] 6-orotic acid into DNA thymine in the L1210/FR-8 line was >90 per cent, while no inhibition was observed in L1210 leukemia. In this experiment, a relationship between the inhibition of orotic acid uptake into DNA thymine by H_4HF and its antitumor effect on L1210/FR-8 and L1210 tumors was clearly demonstrated. The difference in results between the above two precursors suggests that inhibition of H_4HF might be on the pathway between orotic acid and deoxyuridine, and that thymidylate synthetase, shown to be the most sensitive enzyme to H_4HF *in vitro*,[3] may not be the primary target of the drug *in vivo*. Since the major route of [^{14}C]-formate uptake into DNA thymine is via the thymidylate synthetase reaction, the effect of H_4HF on the uptake of [^{14}C]formate into DNA thymine was also studied. As observed in the experiments with deoxyuridine, the inhibition of the uptake of [^{14}C]formate into DNA thymine was not as marked as that of orotic acid, and the inhibition seen in the L1210 tumor was comparable to that seen in the L1210/FR-8 tumor (Table 2). These results further support the view that thymidylate synthetase may not be the primary site of action of H_4HF responsible for antitumor activity.

TABLE 2. EFFECT OF TETRAHYDROHOMOFOLIC ACID ON INCORPORATION OF PRECURSORS INTO DNA THYMINE IN LEUKEMIC SPLEENS

Experiments	L1210 (counts/min/μmole of thymine)	(%)	L1210/FR-8 (counts/min/μmole of thymine)	(%)
(1) [^{14}C] 6- Orotic acid				
Control	480	100	910	100
H_4HF*	580	>100	80	9
(2) [^{14}C] Formate				
Control	9360	100	8130	100
H_4HF	4160	44	3180	39

* H_4HF (533 mg/kg i.p.) was injected into mice bearing 11-day-old tumors at 0-, 8- and 22-hr intervals. Thirty min after the last injection, 250 μc/kg of labeled percursors was injected and the animals were sacrificed after 1 hr. Spleens of three mice were analyzed for DNA thymine.

Livingston *et al.*[8] have reported that suspensions of leukemic spleen incubated with H_4HF did not show any inhibition of deoxyuridine uptake into DNA, suggesting that thymidylate synthetase might not be the site of action of H_4HF. The present study supports their speculation, except that we observed moderate inhibition of deoxyuridine uptake into DNA in both L1210 and L1210/FR-8 tumors, which might be due to the system *in vivo* and the high dose of H_4HF (equal to LD_{100} given as a single injection) used in the present study.

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Metabolism of drugs by isolated hepatocytes

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THE STUDY of the mechanisms involved in the control of the drug-metabolizing enzymes of the hepatocyte would be greatly facilitated by the development of a system *in vitro* which could be maintained for long periods and at the same time permit manipulation of the cellular environment. Juchau *et al.*[1] reported both the metabolism of 3,4-benzo(α)-pyrene in the isolated perfused liver and the induction of increased metabolism by polycyclic hydrocarbons. Unfortunately, this method is so cumbersome that it is difficult to maintain simultaneously multiple incubations which receive different treatments. Further, although Juchau *et al.*[1] were able to achieve induction with 3,4-benzo(α)-pyrene, this preparation is not viable for a sufficient length of time to study the induction by other agents such as phenobarbital.

More recently Henderson and DeWaide[2] have demonstrated the ability of citrate-disassociated cells to metabolize a number of substrates. Several workers have shown, however, that this method of cell isolation yields cells which are inferior in metabolic activity and morphological integrity[3,4] when compared with those prepared by the enzymatic method of Howard and Pesch.[5] The latter procedure, which utilizes perfusion and subsequent incubation with a mixture of collagenase and hyaluronidase, gives a lower cell yield than the citrate method; but the cells incorporate both acetate and amino acids at a faster rate, are metabolically active for a longer period, and require no additions of cofactors for lipid synthesis.[4] In view of these observations, we have examined the ability of freshly prepared hepatocytes separated by this method to metabolize drugs.

All animals used in these experiments were 200–300 g, fed, male, Sprague–Dawley rats obtained from Charles River, Inc. In experiments where animals were induced with 3-methylcholanthrene, they received the drug (40 mg/kg in corn oil, i.p.) at 72 and 48 hr before sacrifice. Animals induced with phenobarbital received the drug in their drinking water (1 g/l.) for 4 days.

Hepatocyte suspensions were prepared by the method of Howard and Pesch.[5] The animals were anesthetized with ether and the livers were perfused *in situ* with 20 ml of cold Ca^{2+}-free Hanks' solution which contained collagenase (120 units/ml) and hyaluronidase (1 mg/ml). The liver was removed, minced with scissors, and incubated in a water-bath shaker with 30 ml of the above enzyme solution for 45 min at 37° under O_2–CO_2 (95:5). The dispersed cells were centrifuged at 50 *g* for 5 min at 5°, resuspended in standard Hanks' solution and recentrifuged. The cells were then suspended in standard Hanks' solution (7·65 ml). The incubation mixture consisted of 7·15 ml of the cell suspension, 0·64 ml