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STEREOSPECIFICITY AT CARBON 6 OF FORMYLTETRAHYDROFOLATE AS A COMPETITIVE INHIBITOR OF TRANSPORT AND CYTOTOXICITY OF METHOTREXATE *IN VITRO*

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Abstract—The unnatural diastereoisomer of L-5-formyltetrahydrofolate was 20-fold less effective as a competitive inhibitor of [^{3}H]methotrexate influx than the natural diastereoisomer during carrier-mediated membrane transport in L1210, S180 and Ehrlich cells. Values derived for K_i were 1.84 to 2.29 μM for the natural derivative and 35.2 to 53.8 μM for the unnatural derivative. Values for K_i derived with a chemically synthesized mixture containing equal amounts of both natural and unnatural diastereoisomers were 2-fold greater than values obtained for the natural diastereoisomer. The unnatural diastereoisomer was 100-fold less effective and the chemically synthesized mixture was 2-fold less effective than the natural diastereoisomer in preventing inhibition by methotrexate of L1210 cell growth in culture. These results indicate that the unnatural diastereoisomer competes relatively ineffectively with the natural diastereoisomer or methotrexate for transport in these murine tumor cells.

Clinical chemotherapy protocols [1–4], which use high doses of methotrexate followed by leucovorin (5-formyltetrahydrofolate) "rescue" from host toxicity, employ chemically synthesized 5-formyltetrahydrofolate which is a mixture of equal amounts of both natural (*l*) and unnatural (*d*) diastereoisomers.* Although it is generally assumed [5] that only the natural diastereoisomers of tetrahydrofolate and methylated derivatives are biochemically active, this notion has not been established conclusively for membrane transport. In fact, data presented in a recent report [6] on the transport of diastereoisomers of 5-methyltetrahydrofolate suggest the opposite. If both of these isomers of 5-formyltetrahydrofolate are equally, or near equally, capable of membrane transport, then the potential for an effect of the unnatural derivative as an inhibitor of folate-dependent enzymes or as an inhibitor of transport of the natural derivative and folate analogs which share [7–9] the same carrier system is of importance. We have investigated the potential for biologic action of the unnatural diastereoisomer of 5-formyltetrahydrofolate by a comparison of the natural derivative and the mixture (*dl*), as well as the unnatural derivative, as competitive inhibitors of [^{3}H]methotrexate transport in L1210 leukemia, Sarcoma 180 and Ehrlich carcinoma tumor cells, and their ability to prevent inhibition by methotrexate of L1210 cell growth in culture. Our results indicate that the membrane transport system shared by 5-substituted reduced folates and 2,4-diamino analogs in these murine tumor cells exhibits stereospecificity at carbon 6 of this formylated reduced folate derivative.

EXPERIMENTAL

The murine tumor cells were obtained as intraperitoneal ascites suspensions from BD2F$_1$ mice and were processed for transport experiments in a manner described previously [9–11]. The suspending medium, conditions of incubation employed during measurements of unidirectional influx, and the time-course for total uptake of [^{3}H]methotrexate have also been described [10, 11]. Individual experimental details are provided in the legend for each figure or table. Methods for isolation and growth of L1210 cells in culture have been given earlier [12]. The medium employed was RPMI 1640 (Grand Island Biologicals, Grand Island, NY) supplemented with 10% fetal calf serum (Microbiological Associates, Bethesda, MD). The L1210 cell culture line employed here was derived from the same L1210 line (V) transplanted in BD2F$_1$ mice and used for transport experiments. [3′, 5′, 9-^{3}H]methotrexate (Amersham/Searle, Arlington Heights, IL) was purified by paper chromatography [13] to a final purity of >98 per cent. Samples of *dl*,L-5-formyltetrahydrofolate were obtained from Dr. Harry B. Wood, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and were determined by spectrophotometric [14] and microbiological [13] means to be >96 per cent pure. The individual diastereoisomers of L-5-formyltetrahydrofolate were prepared from the corresponding tetrahydrofolates [15]. Both diastereoisomers showed the characteristic ultraviolet absorption spectra of 5-formyltetrahydrofolate in neutral and acid solution. The natural and unnatural diastereoisomers of 5-

* The symbols *l* and *d* are used to denote, respectively, the natural and unnatural configurations of tetrahydropteroylglutamate at C-6 and do not denote optical activity or absolute configuration. In all cases, glutamyl residues have the L-configuration.

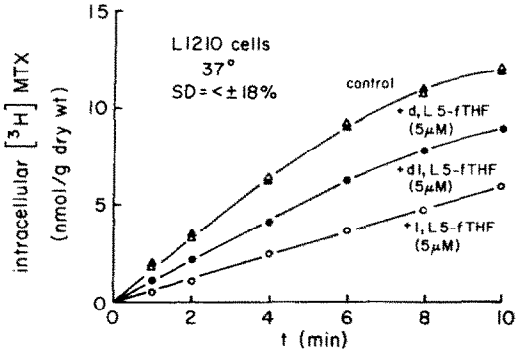

Fig. 1. Time-course for initial intracellular accumulation of [^{3}H]methotrexate at 37° by L1210 cells in the presence and absence of *l*-, *d*- or *dl*,L-5-formyltetrahydrofolate. The concentration of [^{3}H]methotrexate ([^{3}H]MTX) was 1 μM. The concentration of *l*,L-5-formyltetrahydrofolate folate (*l*,L-5-fTHF), *d*,L-5-formyltetrahydrofolate (*d*,L-5-fTHF) and *dl*,L-5-formyltetrahydrofolate (*dl*,L-5-fTHF) was 5 μM. Values for the controls are designated by the closed triangles. Average of four experiments done on separate days. See footnote in Table 1 for further experimental details.

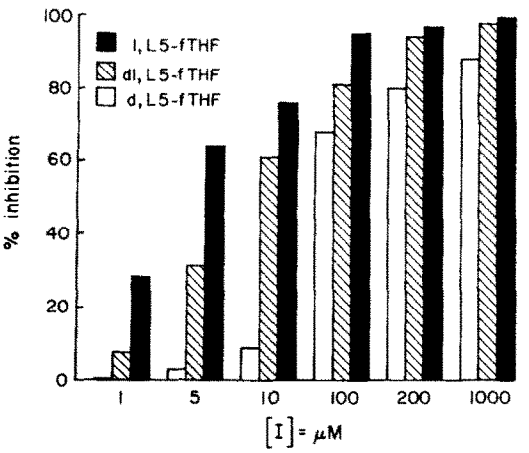

Fig. 2. Inhibition of initial influx of [^{3}H]methotrexate at 37° in L1210 cells by various concentrations of *l*-, *d*- or *dl*,L-5-formyltetrahydrofolate. The concentration of [^{3}H]methotrexate was 1 μM. The concentrations of *l*,L-5-formyltetrahydrofolate (*l*,L-5-fTHF), *d*,L-5-formyltetrahydrofolate (*d*,L-5-fTHF) and *dl*,L-5-formyltetrahydrofolate (*dl*,L-5-fTHF) are shown. Average of three experiments done on separate days (S.D. = ± 15 per cent). See footnote in Table 1 for further experimental details.

formyltetrahydrofolate were quantitively converted to 5,10-methenyltetrahydrofolate in 1.0 N HCl as judged by the formation of a peak at 348 nm [16]. The ratio of absorbance at 348 nm to that at 305 nm in 1.0 N HCl was 2.0 for both diastereoisomers. This is close to the reported value of 2.1 [16]. The natural diastereoisomer (*l*) of L-tetrahydrofolate was prepared from dihydrofolate [17] utilizing *Lactobacillus casei* dihydrofolate reductase. The unnatural diastereoisomer (*d*) of L-tetrahydrofolate was prepared from *dl*,tetrahydrofolate by incubation of the mixture with thymidylate synthetase [18]. This reaction converts *l*,L-tetrahydrofolate to dihydrofolate which was separated by DEAE-cellulose chromatography from the unreacted *d*,L-tetrahydrofolate. The sample of *d*,L-5-formyltetrahydrofolate used during these studies had no more than 0.05% *l*,L-5-formyltetrahydrofolate as a contaminant when determined by assay with *Pediococcus cerevisiae* [13]. The sample of the natural isomer showed full biological activity for this organism. The unnatural diastereoisomer of L-5-formyltetrahydrofolate (*d*) was analyzed by X-ray crystallography [19] and shown to have a configuration at carbon 6 opposite to that of the natural diastereoisomer (*l*). Details of the synthetic procedures will be published elsewhere.

RESULTS

The time-course for intracellular accumulation of methotrexate in murine tumor cells reported [9] from our laboratory shows an initial linear velocity with time, followed by a gradual decrease and exponential approach to steady state. Linear kinetics for initial uptake reflect the unidirectional influx of drug, since rapid binding of intracellular drug to dihydrofolate reductase will occur until the intracellular level approaches the drug binding equivalence of this enzyme. During the present study, measurements of accumulation of [^{3}H]methotrexate in murine tumor cells were restricted to initial uptake. A time-course for initial uptake of [^{3}H]methotrexate in L1210 cells in the presence or absence of 5 μM *l*-, *d*- or *dl*,L-5-formyltetrahydrofolate is shown in Fig. 1. The external concentration of radioactively labeled drug in this experiment was 1 μM. Accumulation was reduced in the presence of preparations of the (*l*) and (*dl*) forms of this reduced substituted folate, but to a 2-fold greater extent in the former case. Accumulation was essentially unaffected in the presence of the (*d*) isomer. Linear kinetics for accumulation of [^{3}H]methotrexate were observed in each case until the total intracellular level approached the drug binding equivalence (3.62 ± 0.44 nmoles/g dry wt) for dihydrofolate reductase [9]. The effects of various external concentrations of the (*l*), (*d*) and (*dl*) preparations of L-5-formyltetrahydrofolate on the unidirectional influx of [^{3}H]methotrexate (1 μM) by L1210 cells are shown in Fig. 2. Measurement of influx during these experiments was limited to a period of 2 min. Inhibition of [^{3}H]methotrexate influx was pronounced for both the (*l*) isomer and the mixture, within the concentration range of 1–50 μM. There was much less of an effect of the (*d*) isomer in this same concentration range. In the case of both the (*l*) isomer and the mixture, inhibition approached 100 per cent at the highest concentration of reduced folate employed (1 mM). However, the (*l*) isomer was a much more effective inhibitor of [^{3}H]methotrexate uptake overall. An analysis of the related data on initial velocity by the method of Dixon [20] is shown in Fig. 3. Values for K_i (competitive inhibition) derived in this way were 2.35 ± 0.43 μM for the (*l*) isomer, 47.8 ± 6.4 μM for the (*d*) isomer and 4.89 ± 0.95 μM for the mixture.

Additional kinetic data were derived for transport in L1210 cells by experiments measuring [21] the initial influx of [^{3}H]methotrexate at different external concentrations in the presence and absence of 5 μM of the (*l*) and (*dl*) preparations or 50 μM of the (*d*) isomer. Double-reciprocal plots [21] of these data are shown in Fig. 4. Inhibition of influx was again greatest with

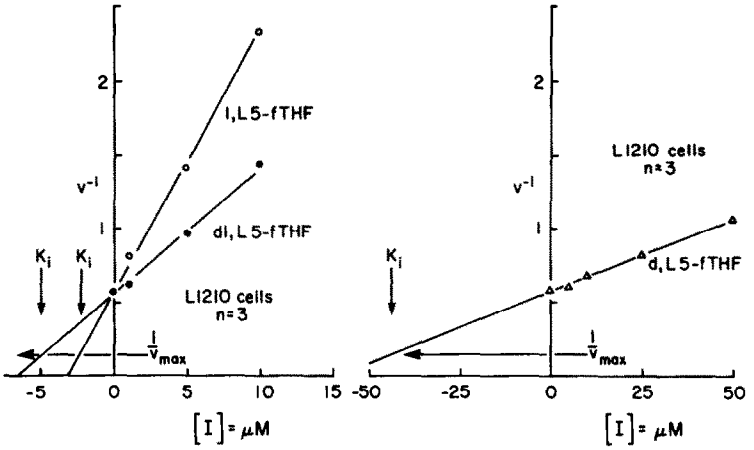

Fig. 3. Dixon plots of the inhibition of [^{3}H]methotrexate influx at 37° in L1210 cells by various concentrations of *l*-, *d*- or *dl*,L-5-formyltetrahydrofolate. The value for V_{max} employed in the plot was calculated as described by Dixon [20] from the control values for v and a value for K_m of 3.7 ± 0.6 μM (n = 3) determined from data similar to that shown in Fig. 4. The concentration of [^{3}H]methotrexate was 1 μM. The concentrations of *l*,L-5-formyltetrahydrofolate (*l*,L-5-fTHF), *d*,L-5-formyltetrahydrofolate (*d*,L-5-fTHF) and *dl*,L-5-formyltetrahydrofolate (*dl*,L-5-fTHF) are as shown. Initial velocity (v) = nmoles/g dry wt/min. Average of three experiments done on separate days (S.D. = < ± 15 per cent).

preparations of the (*l*) isomer. Values for K_i derived for these isomers and the mixture are shown in Table 1. These values compare closely to the same values for K_i derived from the Dixon plots shown in Fig. 3.

The inhibition of unidirectional influx of [^{3}H]methotrexate by the (*l*) and (*d*) isomers of 5-formyltetrahydrofolate and the (*dl*) preparation was also studied in S180 and Ehrlich cells using identical conditions (see legend of Fig. 3). Values for K_i derived for each isomer and the mixture with each tumor cell type are summarized in Table 1. Values for K_i derived in S180 cells were comparable to those derived in L1210 cells, but values for K_i in Ehrlich cells were somewhat lower. However, in all three cases, the value derived for the mixture was almost exactly 2-fold greater than the corresponding value for the (*l*) isomer. As with L1210 cells, the (*d*) isomer was relatively ineffective as an inhibitor of [^{3}H]methotrexate influx in both Sarcoma 180 and Ehrlich cells.

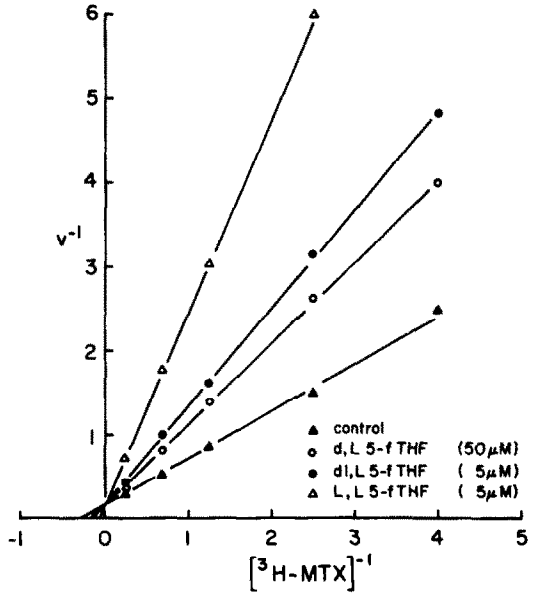

Fig. 4. Lineweaver–Burk plots of the inhibition of [^{3}H]methotrexate influx at 37° in L1210 cells by *l*-, *d*- or *dl*,L-5-formyltetrahydrofolate. The concentrations of *l*,L-5-formyltetrahydrofolate (*l*,L-5-fTHF) and *dl*,L-5-formyltetrahydrofolate (*dl*,L-5-fTHF) were 5 μM. The concentration of *d*,L-5-formyltetrahydrofolate (*d*,L-5-fTHF) was 50 μM. Initial velocity (v) = nmoles/g dry wt/min. Average of three experiments done on separate days (S.D. < ± 26 per cent). See footnote in Table 1 for further experimental details.

In related experiments, we measured the prevention of methotrexate inhibition of growth of L1210 cells in culture by (*l*), (*d*) and (*dl*) preparations of L-5-formyltetrahydrofolate. These results are summarized in Table 2. In all three experiments, the (*l*) isomer was far more effective than the (*d*) isomer in preventing the inhibition of growth by methotrexate. The amount of the (*d*) isomer required for 50 per cent reduction of inhibition was about 100-fold greater than that amount of the (*l*) isomer. The average value for the mixture of both isomers was 2-fold greater than the (*l*) isomer.

DISCUSSION

The results presented here are interpreted to mean that the membrane transport system for 5-substituted reduced folates (and 2,4-diamino folate analogs) in these murine tumor cells exhibits stereospecificity for carbon 6 of this formylated derivative. The 2-fold differences in K_i derived for preparations of the natural diastereoisomer (*l*) and the mixture (*dl*) by two methods of kinetic analysis are precisely what would be expected if it is presumed that the unnatural diastereoisomer (present in an amount equal to that natural diastereoisomer in the mixture) was not able to effectively utilize this carrier system for transport. The studies carried out with a preparation of the unnatural diastereoisomer (*d*), which show a 20-fold larger value for K_i, validate this interpretation. Moreover, this

Table 1. Summary of the kinetic analysis (Lineweaver–Burk) of competitive inhibition of [^{3}H]methotrexate influx in murine tumor cells by diastereoisomers of L-5-formyltetrahydrofolate *

Tumor cells	K_i for competitive inhibition		
	l-L-Formyltetrahydrofolate (μM)	*dl*-L-Formyltetrahydrofolate (μM)	*d*-L-Formyltetrahydrofolate (μM)
L1210	2.13 ± 0.46 (5)	4.52 ± 1.24 (5)	39.2 ± 6.4 (3)
S180	2.29 ± 0.22 (5)	4.63 ± 0.51 (5)	53.8 ± 4.6 (3)
Ehrlich	1.84 ± 0.6 (5)	3.70 ± 0.59 (5)	35.2 ± 7.8 (3)

* Cells, removed as ascites suspensions from the peritoneal cavity of $BD2F_1$ mice, were washed once in cold (0°) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4) and resuspended in a solution of buffered salts [9] with 2 mM glucose but no serum. Initial influx measurements were made with various external concentrations of [^{3}H]methotrexate and the incubation time was adjusted at each concentration so that the intracellular accumulation did not exceed the dihydrofolate reductase drug-binding capacity. A double reciprocal plot of the data (v/[drug]) was constructed to obtain values for maximum velocity (V_{max}) and the apparent Michaelis constant (K_m). Values for K_i were derived from similar data measuring the influx of [^{3}H]methotrexate in the presence of reduced folate and using the following calculation, ($K_i = [I]/[K_p - 1]$ where K_p is the apparent K_m in the presence of the competing folate. After influx measurements, incubation was terminated by a 10-fold dilution of cells in cold (0°) buffered isotonic saline solution, washing three times with the same cold solution. No loss of drug occurs at this temperature [9–11]. Also, since drug accumulation is nonexchangeable because of binding to dihydrofolate reductase, no loss would be expected to occur. [^{3}H]Methotrexate uptake was determined by scintillation counting of radioactivity and the intracellular accumulation was determined after correction for cell surface absorption [9–11]. The numbers in parentheses indicate the number of experiments.

markedly reduced ability of the (*d*) isomer to be transported would appear to account, to a great extent, for the reduced ability of the mixture at equimolar concentrations and the greatly reduced ability of the (*d*) isomer to prevent the inhibition of methotrexate on growth of L1210 cells. The greater difference in the concentration-dependence of effects of (*l*) and (*d*) isomers on growth inhibition by methotrexate when compared to the relative concentration-dependence for effects on transport is of interest. This finding, however, was not entirely unexpected, since the net effect of folate derivatives on growth inhibition is also a reflection of intracellular biochemical events occurring after transport. Our results are consistent with the assumption that the known pharmacologic effects associated with preparations of *dl*-L-5-formyltetrahydrofolate administered to patients are related primarily to the action of the natural diastereoisomer. However, the (*d*) isomer is not completely inert.

Table 2. Reversal by L-5-formyltetrahydrofolate of the inhibition of growth of L1210 cells in culture by methotrexate *

Exp.	Concentration for 50 per cent reversal of inhibition		
	l-L-5-Formyltetrahydrofolate (nM)	*dl*-L-5-Formyltetrahydrofolate (nM)	*d*-L-5-Formyltetrahydrofolate (nM)
1	7.9	22.0	1244
2	15.8	22.9	1378
3	13.2	27.8	1336
Ave.	12.3 ± 3.9	24.2 ± 3.1	1319 ± 97

* Logarithmic phase L1210 cells (10^4 cells/ml) in RPMI 1640 medium (formulated with 2.2 μM folic acid), 10 per cent with respect to fetal bovine serum, were dispensed into 16 × 125 mm culture tubes (final volume 5.0 ml) containing methotrexate (final concentration 0.1 μM) and various concentrations of each reduced folate derivative. Cells grown with 0.1 μM methotrexate undergo 1 to 1.5 divisions within a 72-hr period; non-treated cells divide six to seven times within a similar interval. Cell growth in control tubes with and without methotrexate (no reduced folate derivatives) was monitored every 24 hr to verify that the growth pattern was normal. After 72 hr, the cell density in all experimental groups was determined with a model ZBI Coulter Counter. Cell counts were averaged and the means were plotted on full logarithmic paper against the concentration of reduced folate to determine the amount necessary to produce a 50 per cent reversal of inhibition. Cell counts from triplicate culture tubes within the same experimental group agreed within 10 per cent.

Specificity of carrier-mediated membrane transport for diastereoisomers of methylated reduced folate coenzymes has been reported [22] for bacteria. These workers described a transport system in *P. cerevisiae* which was specific for the natural diastereoisomer of 5-formyltetrahydrofolate. However, a mutant capable of transporting folate appears [23] to accumulate both diastereoisomers of 5-methyltetrahydrofolate. Other workers have also reported [24] the transport of both diastereoisomers of 5-methyltetrahydrofolate by *L. casei*. This was based upon a kinetic analysis of uptake of radiolabeled preparations of the natural isomer and a racemic mixture (*dl*) and the fact that more than half of the latter was removed from the extracellular compartment. Differences between the results reported here on stereospecificity at carbon 6 and those observed earlier [6] during studies of transport of natural and unnatural diastereoisomers of L-5-methyltetrahydrofolate in Ehrlich cells are unexplained. Whether or not the differences in the results of these two studies are in some way related to the use of a different substituted reduced

folate derivative in each case will require further investigation.

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