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FURTHER STUDIES OF STEREOSPECIFICITY AT CARBON 6 FOR MEMBRANE TRANSPORT OF TETRAHYDROFOLATES

DIASTEREOISOMERS OF 5-METHYLTETRAHYDROFOLATES AS COMPETITIVE INHIBITORS OF TRANSPORT OF METHOTREXATE IN L1210 CELLS

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Abstract—The unnatural *d* diastereoisomer at carbon 6 of 5-methyltetrahydrofolate was only slightly less effective than the natural *l* diastereoisomer as a competitive inhibitor of the carrier-mediated membrane transport of [^{3}H]methotrexate into L1210 murine leukemia cells. The apparent K_i for a mixture containing equal amounts of both natural and unnatural diastereoisomers was not significantly different from that found for the unnatural form. These results show that the reduced folate carrier system in these cells has a strong affinity for the unnatural stereoisomer, a finding in contrast to that obtained with the corresponding diastereoisomer of 5-formyltetrahydrofolate.

Chemical reduction of folate introduces an asymmetric center at carbon 6 of the pteridine moiety and results in a racemic mixture of *l* and *d* diastereoisomers.§ Although it is generally accepted that there is stereochemical specificity of mammalian and bacterial folate enzymes for the *l* isomers of tetrahydrofolate and its one carbon derivatives as substrates [1–4], the stereochemical specificity of the reduced folate transport system in murine tumor cells is controversial. A study by White *et al.* [5] showed that the membrane carrier system for reduced folates in Ehrlich cells had a strong affinity for both the *d* and *l* diastereoisomers of 5-methyltetrahydrofolate. The affinity of the membrane carrier for the *l* isomer (K_m = 2.5 μM) was slightly greater than for the *d* isomer (K_m = 3.5 μM), and both isomers were strong competitive inhibitors of the carrier-mediated transport of methotrexate. In contrast, a later study with 5-formyltetrahydrofolate from this laboratory [6] reported a large difference in the stereochemical specificity of this transport mechanism in three different murine tumor cells.‖ The *d* isomer was 20-fold less effective than the *l* isomer as a competitive inhibitor of the carrier-mediated influx of methotrexate into L1210, S180 and Ehrlich cells. In addition, the *d* isomer was 100-fold less effective than the *l* isomer in preventing methotrexate inhibition of L1210 cell growth in culture.

These reported differences in the stereochemical specificity of the reduced folate transport mechanism in murine tumor cells for the *d* isomer of 5-methyltetrahydrofolate and 5-formyltetrahydrofolate [5, 6] may be of importance to understanding the properties of this membrane carrier system. In addition, if both isomers of 5-methyltetrahydrofolate have a similar affinity for membrane transport, the potential for the *d* isomer to inhibit the transport of either the *l* isomer or of folate analogs which share the same carrier, as well as the possible metabolic consequences, must be given serious consideration. However, these reported differences in the stereochemical specificity for the diastereoisomers of either reduced folate form may also be artifacts resulting from work done by different laboratories with different cell lines. To examine this possibility, a study was undertaken with the same L1210 cell line used in our previous study with 5-formyltetrahydrofolate [6] in order to compare the affinity of the reduced folate carrier system for the stereoisomers of 5-methyltetrahydrofolate. A few additional experiments with 5-formyltetrahydrofolates were also carried out in parallel to verify the results of our previous study [6] and to more conclusively validate the differences shown for the unnatural isomers of 5-methyltetrahydrofolate and 5-formyltetrahydrofolate.

MATERIALS AND METHODS

[^{3}H]Methotrexate was purchased from Moravek Biochemicals, City of Industry, CA. The purity of

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§The symbols *l* and *d* are used to denote the natural and unnatural configurations of tetrahydrofolate at carbon 6, respectively, and do not denote optical activity. "Racemic" is used to denote an equimolar mixture of the *l* and *d* forms.

‖ In the study (Ref. 6), the amount of *l*-5-formyltetrahydrofolate present in the *d*-5-formyltetrahydrofolate preparation was erroneously reported to be 0.05%. This value should have been reported as 0.5%.

the labeled analog was checked before use and, when necessary, was repurified on DEAE-cellulose paper. Racemic 5-methyltetrahydrofolate was purchased from the Sigma Chemical Co., St. Louis, MO. The *l*- and *d*-5-methyltetrahydrofolates were prepared from the corresponding tetrahydrofolates by reaction with formaldehyde followed by reduction with sodium borohydride [7]. *l*-Tetrahydrofolate was prepared enzymatically from folate using *Lactobacillus casei* dihydrofolate reductase [8]. This reaction is 100% stereospecific as judged by the utilization of the tetrahydrofolate so formed in the thymidylate synthase reaction. As expected, the *l*-5-methyltetrahydrofolate synthesized from *l*-tetrahydrofolate was twice as active as racemic 5-methyltetrahydrofolate in supporting the growth of *L. casei*. *d*-Tetrahydrofolate was prepared by depleting racemic tetrahydrofolate of the *l* stereoisomer by incubation with *L. casei* thymidylate synthase [9]. The *d*-5-methyltetrahydrofolate used in this study contained an amount of *L. casei* active material equivalent to a 5% contamination with *l*-5-methyltetrahydrofolate. The concentrations of the 5-methyltetrahydrofolates were determined by the absorbance at 289 nm using a molar absorption coefficient of $31.7 \times 10^3\ M^{-1}\ cm^{-1}$ [10]. Preparation and ultraviolet absorption spectra of the 5-formyltetrahydrofolates have already been described [6, 11].

L1210V murine lymphocytic leukemia cells were maintained by weekly passage in $B6D2F_1$ (C57Bl/6 × DBA/2)F_1 mice [12]. For experimental purposes, cells were flushed from the peritoneal cavity with a buffered-salts transport medium (107 mM NaCl, 20 mM Tris–HCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4). Heparin (liquaemin sodium, Organon, West Orange, NJ), 100 units/ml, was added to prevent clotting. The cell density was determined from an established correlation [13] between absorbance and cell number (A_{600} of 3.0 is equivalent to 2.4×10^7 cells). This correlation was rechecked regularly with a Neubauer improved counting chamber.

Methotrexate influx was measured by incubating $4–5 \times 10^7$ cells with labeled drug at 37°. The affinity of 5-methyltetrahydrofolate for the carrier system was measured by the ability to compete with [^{3}H]methotrexate for influx. To calculate K_m, K_i and V_{max} values, transport measurements were made at various external concentrations of [^{3}H]methotrexate. To ensure that only unidirectional influx was measured, the incubation time was adjusted for each concentration such that the intracellular accumulation did not exceed the dihydrofolate reductase drug-binding capacity. The final incubation volume in each case was 0.5 ml. Influx was terminated by placing the cell suspensions on ice and diluting 1:3 with cold (4°) 0.14 M sodium chloride–0.01 M sodium phosphate buffer, pH 7.4. One milliliter of the diluted cell suspension was removed and layered on 300 μl of SF1250 silicone fluid (density 1.045 g/ml; General Electric, Waterford, NY) in a 1.5 ml Eppendorf polypropylene centrifuge tube (Brinkmann Instruments, Inc., Westbury, NY). Both the silicone oil and the centrifuge tubes were at 0.4° prior to use. The cells were separated from the labeled transport medium by centrifugation through the silicone fluid in an Eppendorf model 5412 microcentrifuge (Brinkmann Instruments, Inc.) for 30 sec. After the aqueous and the silicone fluids were separated from the cell pellet, the cells were resuspended by vortex mixing in 0.5 ml of cold 0.14 M NaCl–0.02 M potassium phosphate buffer, pH 7.4. The resuspended cells were transferred quantitatively to 10 ml of hydrofluor scintillation mixture (National Diagnostics, Somerville, NJ), and the radioactivity was measured with a Packard Tri-carb No. 3385 liquid scintillation spectrometer. Incubation at 0° followed by identical dilution and processing was used to estimate cell surface absorption [14].

A study of the prevention of methotrexate toxicity in cell culture by diastereoisomers of 5-methyltetrahydrofolate, like that reported earlier with 5-formyltetrahydrofolate [6] from our laboratory, could not be carried out. Using a microbiological assay [15, 16], extensive degradation of 5-methyltetrahydrofolate was found to occur in the cell culture medium employed. Similar findings were also reported recently by other workers [16].

RESULTS AND DISCUSSION

A representative time course of the uptake of [^{3}H]methotrexate by L1210 cells is shown in Fig. 1. L1210 cells took up methotrexate at a linear rate for approximately 5 min before the rate of uptake decreased. The linear uptake is representative of unidirectional influx of drug until the tight-binding capacity of the intracellular dihydrofolate reductase for drug is exceeded (approximately 3.65 nmoles/g dry wt of cells). The gradual decrease in uptake reflects the slower accumulation of free intracellular drug as a result of increasing efflux. Both *l*- and *d*-5-methyltetrahydrofolate inhibited the uptake of [^{3}H]methotrexate. At the concentration employed (3 μM), the *l* isomer was slightly more effective as an inhibitor of [^{3}H]methotrexate influx than was the *d* isomer. The effect of several concentrations of *l*-

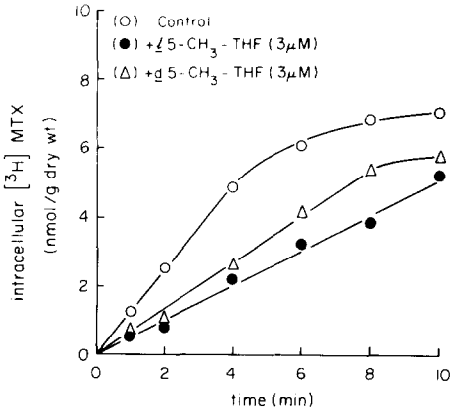

Fig. 1. Time course for [^{3}H]methotrexate accumulation by L1210 cells. L1210 cells were incubated at 37° with 0.8 μM [^{3}H]methotrexate in the presence and absence of 3 μM *l*- or *d*-methyltetrahydrofolate. These data are from a single experiment which was repeated several times with similar results (S.E. = < ±11%). Abbreviation: 5-CH_3-THF, 5-methyltetrahydrofolate.

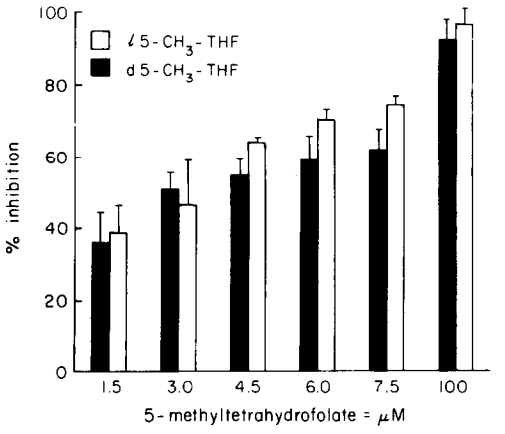

Fig. 2. Inhibition of the influx of [^{3}H]methotrexate into L1210 cells by increasing concentrations of *l*- or *d*-5-methyltetrahydrofolate. The initial influx of 0.8 μM [^{3}H]methotrexate into L1210 cells was measured at 37° for an interval of 4 min. The effect of increasing concentrations of *l*- or *d*-5-methyltetrahydrofolate on this initial influx was measured and expressed as percent inhibition of control influx. The height of each bar represents the mean of three separate experiments and the line above each bar represents the ± standard error. Abbreviation: 5-CH_3-THF, 5-methyltetrahydrofolate.

or *d*-5-methyltetrahydrofolate on the unidirectional influx of 0.8 μM [^{3}H]methotrexate is shown in Fig. 2. For these experiments, influx measurements were limited to 4 min to ensure unidirectional uptake. Again, both the *l* and *d* isomers of 5-methyltetrahydrofolate were effective inhibitors of methotrexate influx. As in the experiments described in Fig. 1, the *l* isomer was slightly more effective than the *d* isomer over the concentration range tested (1.5 to 7.5 μM). At a concentration of 100 μM, the inhibition of both isomers approached 100%.

Kinetic data on the inhibition of [^{3}H]methotrexate influx by isomers of 5-methyltetrahydrofolate are shown in Fig. 3. Lineweaver–Burk analysis [17] indicated (Fig. 3A) that both *l* and *d* isomers of 5-methyltetrahydrofolate were strong competitive inhibitors of labeled methotrexate influx. The apparent K_m for methotrexate influx derived in a series of six experiments was 4.4 ± 0.5 μM. The K_i values calculated from the Lineweaver–Burk double-reciprocal plots and also by separate analysis by the method of Dixon [18] (Fig. 3B) are summarized in Table 1. The apparent K_i for *l*-5-methyltetrahydrofolate was lower ($P < 0.05$) than the apparent K_m for methotrexate influx (4.4 ± 0.5 μM) or the apparent K_i for *d*-5-methyltetrahydrofolate ($P < 0.05$), indicating a slightly greater affinity for the membrane carrier system.

These results show that the reduced folate transport system in L1210 cells had a high affinity for both the *l* and *d* stereoisomers of 5-methyltetrahydrofolate which was similar to the results reported by White *et al.* [5] with Ehrlich ascites tumor cells. In both studies, the affinity of the carrier system for the *l* isomer was slightly better than for the *d* isomer. These results contrast with the difference in stereochemical specificity of the reduced folate transport system in these same L1210 cells and in Ehrlich and S180 cells reported [6] for the *l* and *d* stereoisomers of 5-formyltetrahydrofolate. In this earlier study [6], the apparent K_i (2.1 ± 0.5 μM) of *l*-5-formyltetrahydrofolate as a competitive inhibitor of carrier-mediated [^{3}H]methotrexate influx was similar to the apparent K_i (3.2 ± 0.9 μM) reported herein for *l*-5-methyltetrahydrofolate. However, the apparent k_i (39.2 ± 6.4 μM) for the *d* isomer of 5-formyltetrahydrofolate as a competitive inhibitor of carrier-mediated methotrexate influx was approximately 20-fold greater than for the *l* isomer. Thus, the reduced folate carrier system has greatly different affinity for the *d* isomers of the two reduced folate

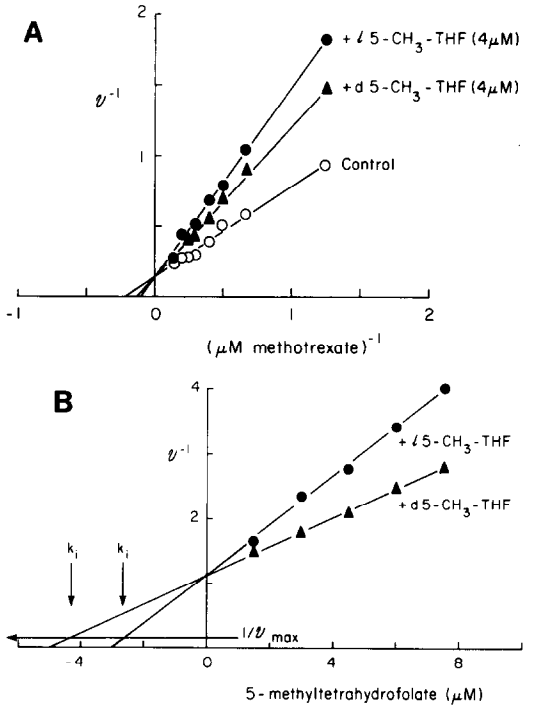

Fig. 3. Kinetic analysis of the competitive inhibition of influx [^{3}H]methotrexate by *l*- or *d*-5-methyltetrahydrofolate. (A) Lineweaver–Burk plots of the inhibition of [^{3}H]methotrexate influx at 37° by 4 μM *l*-5-methyltetrahydrofolate and by 4 μM *d*-5-methyltetrahydrofolate. The initial influx at various external concentrations of [^{3}H]methotrexate was measured under the conditions discussed in Materials and Methods. Each point is the mean calculated from three experiments performed on different days. The standard error was < ± 12%. The initial velocity (v) represents nmoles methotrexate per g dry wt of cells per min. (B) Dixon plots of the inhibition of [^{3}H]methotrexate influx at 37° by several concentrations of *l*- and *d*-5-methyltetrahydrofolate. These data were obtained in experiments separate from those in (A). L1210 cells were incubated at 37° with 0.8 μM [^{3}H]methotrexate for 4 min in the presence of 5-methyltetrahydrofolate. Each point is the mean calculated from three experiments performed on different days. The standard error was < ±12%. The initial velocity (v) represents the nmoles of methotrexate taken up per g dry wt of cells per min. The value for V_{max} was calculated from Lineweaver–Burk kinetic analyses performed at the same time as each of the Dixon kinetic analyses. Abbreviation: 5-CH_3-THF, 5-methyltetrahydrofolate.

Table 1. Kinetic constants for the competitive inhibition of [^{3}H]methotrexate influx into L1210 tumor cells by diastereoisomers of 5-methyltetrahydrofolate*

Method of kinetic analysis	K_i for competitive inhibition† (μM)		
	5-Methyltetrahydrofolate		
	l	Racemic	*d*
Lineweaver–Burk‡	3.1 ± 0.9	4.1 ± 0.6	4.6 ± 0.8
Dixon§	2.7 ± 0.6	3.8 ± 0.9	4.3 ± 0.7

* Kinetic constants were determined according to the procedures discussed in Materials and Methods and shown in Fig. 3.

† Each value is the mean ± S.D. calculated from three separate experiments.

‡ $K_i = I/((K_p/K_m)-1)$ where K_p is the apparent K_m in the presence of the competing reduced folate.

§ See Fig. 3B for the methods used to determine K_i.

forms which differ only with regard to the substituent on the 5-nitrogen atom.

The difference observed in stereochemical specificity for the *d* isomers of 5-formyl- and 5-methyltetrahydrofolate has certain therapeutic implications. Both the *l* and *d* isomers of 5-formyltetrahydrofolate are present in equal amounts in the chemically synthesized racemic mixture used for rescue during high-dose methotrexate therapy. Since the *d* isomer has a lower affinity for the reduced folate membrane carrier system, its presence would not initially interfere with reversal of methotrexate cytotoxicity by the *l* isomer. Rothenberg *et al.* [19] have reported that the *d* isomer of 5-formyltetrahydrofolate is retained in human plasma because of slow excretion in the urine. Repeated administration of large amounts of racemic 5-formyltetrahydrofolate might result in accumulation of enough of the *d* isomer to interfere with rescue. If a rescue protocol were designed which utilized racemic 5-methyltetrahydrofolate, the presence of the *d* isomer might interfere with the membrane transport of both the folate analog and the natural isomer, thereby blocking rescue.

The *d* isomer of 5-methyltetrahydrofolate may also interfere with intracellular folate metabolism, since it is known that [20, 21] *d*-5,10-methylenetetrahydrofolate is capable of binding to thymidylate synthase from *L. casei* and acting as a competitive inhibitor of the *l* isomer. Further, *d*-tetrahydrofolate is a substrate for rat liver folylpolyglutamate synthetase [22] and the polygammaglutamyl derivatives of *d*-tetrahydrofolate are potent inhibitors of *L. casei* thymidylate synthase [10]. This lack of stereochemical specificity for transport of this folate encourages caution in utilizing a racemic mixture of the preparation to define growth requirements for cells in culture or in interpreting published results for which a racemic preparation was utilized for such ends. A lack of stereochemical specificity for either isomer of 5-methyltetrahydrofolate has also been reported for the reduced folate transport system in *L. casei* [23] and in a mutant of *Pediacoccus cerevisiae* [24].

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