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SHORT COMMUNICATIONS

Analogs of tetrahydrofolate directed at folate-dependent purine biosynthetic enzymes. Characteristics of mediated entry and transport-related resistance in L1210 cells for 5,10-dideazatetrahydrofolate and two 10-alkyl derivatives

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Recent findings have suggested [1–5] that anabolism of methotrexate and other 4-amino-folate analogs to a polyglutamate in tumor cells may result in significant targeting of these agents to folate-dependent biosynthetic reactions necessary for thymidylate and purine biosynthesis. Although it is unclear to what extent these effects contribute to cytotoxicity of these analogs, these results have provided impetus [6–11] for the development of folate analogs that are not inhibitors of dihydrofolate reductase, but as close analogs of one-carbon donors may act directly on folate-dependent biosynthetic enzymes. A cytotoxic analog of tetrahydrofolate, 5-10-dideazatetrahydrofolate (DDTHF), was synthesized recently and found [8–11] to have significant antitumor activity in animal models naturally refractive to methotrexate. Additional studies with this analog suggests [8–11] that its cytotoxic activity is associated with effects on purine biosynthesis. Our own interest in these structures centers upon the issues of their membrane transport and transport-related acquired resistance in tumor cells which form the basis of this report.

Materials and methods

The syntheses of the new analogs of tetrahydrofolate employed here are described elsewhere [12, 13]. [3′,5′,9-^{3}H]Methotrexate (10–20 Ci/mmol) and [3′,5′,9-^{3}H]*dl*,L-5-formyltetrahydrofolate (3 Ci/mmol) were purchased from Moravek Biochemicals, City of Industry, CA, and purified >99% by HPLC [14]. Methotrexate was provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The various analogs under study were analyzed by HPLC [4] and found to be >97% pure. Cell culture procedures employed during studies of inhibition of growth of variant and parental L1210 cells and the derivation and cloning of the variants have been described [15–17].

Experiments measuring various parameters of membrane transport of folate compounds were performed [15–17] with cell suspensions in modified Krebs–Ringer solution with 7 mM glucose. Values for influx K_i for each analog were derived [15–17] from data measuring influx of [^{3}H]methotrexate in the presence or absence of various amounts of a competing analog. Analysis of these data was by the method of Hofstee [18].

Results and discussion

To provide a baseline for similar studies with DDTHF and its analogs, we compared the influx of [^{3}H]methotrexate and [^{3}H]5-formyltetrahydrofolate in parental and transport-defective, methotrexate-resistant L1210 cells. Data on the transport of these compounds, co-permeants [reviewed in Ref. 19] for the one-carbon, reduced folate transport system, are summarized in Table 1. As determined from values for K_m of initial influx, the affinity of this system for [^{3}H]5-formyltetrahydrofolate in parental cells was approximately 3-fold greater than for [^{3}H]methotrexate. The data also show that mediated entry of [^{3}H]methotrexate in L1210/R1 and L1210/R24 cells was altered both in respect to influx K_m and influx V_{max}. The former was increased 3- to 4-fold in the case of both variants, and the latter was decreased 5- to 7-fold (L1210/R1) and 15- to 18-fold (L1210/R24) relative to parental L1210 cells. In contrast, mediated entry of [^{3}H]5-formyltetrahydrofolate in L1210/R1 and L1210/R24 cells was altered only at the level of influx V_{max} (5-fold and 16-fold, respectively, relative to parental L1210 cells).

Since DDTHF is a close structural analog of the physiological permeant for this system (5-methyltetrahydrofolate), it was assumed that this compound and its analogs would compete to some extent with both naturally occurring folates and methotrexate for mediated entry by this system. Interestingly, as permeants for this classical transport systems, DDTHF and its 10-methyl and 10-ethyl analogs competed quite favorably (Table 2) with both methotrexate and 5-formyltetrahydrofolate. This was

Table 1. Influx of [^{3}H]methotrexate and [^{3}H]5-formyltetrahydrofolate by variant and parental L1210 cells

	Influx properties					
	L1210		L1210/R1		L1210/R24	
Compound	V_{max} (nmol/min/g dry wt)	K_m (μM)	V_{max} (nmol/min/g dry wt)	K_m (μM)	V_{max} (nmol/min/g dry wt)	K_m (μM)
[^{3}H]Methotrexate	6.78 ± 0.8	3.92 ± 0.4	1.33 ± 0.3	12.3 ± 2	0.45 ± 0.07	11.8 ± 3
[^{3}H]5-Formylfolate-H_4*	6.93 ± 0.9	1.38 ± 0.2	1.41 ± 0.2	1.17 ± 0.2	0.43 ± 0.06	1.45 ± 0.3

Cells were washed twice in cold (0°) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4) and resuspended in transport buffer without serum. The procedures employed for measurements of initial influx at 37° at various concentrations of folate compound for determination of influx K_m or K_i and influx V_{max} and for processing of cells have been described in detail earlier [14–16]. Data shown (mean ± SEM) are the average of four experiments. Standard error of the mean did not exceed ±14%.

* Values are expressed as the concentration of the natural diastereomer in the mixture of diastereomers.

Table 2. Effect of various folate compounds on [^{3}H]methotrexate influx in variant and parental L1210 cells

Compound*	Influx properties		
	L1210 K_i (μM)	L1210/R1 K_i (μM)	L1210/R24 K_i (μM)
Methotrexate	3.85 ± 0.5	12.8 ± 0.2	13.2 ± 3
5,10-Dideaza-folate-H_4	2.48 ± 0.3	1.7 ± 0.2	2.05 ± 0.3
10-Methyl-5,10-dideaza-folate-H_4	2.21 ± 0.3	1.9 ± 0.3	1.90 ± 0.4
10-Ethyl-5,10-dideaza-folate-H_4	2.83 ± 0.4	2.3 ± 0.3	1.83 ± 0.4
Folic acid	407 ± 68	364 ± 52	
Folate -H_4†	23.1 ± 4	19.4 ± 3	
5-Formyl-folate-H_4†	1.24 ± 0.2	1.32 ± 0.2	1.41 ± 0.3

See the legend of Table 1 for experimental details. Data shown (mean ± SEM) are the average of four individual experiments. Standard error of the mean did not exceed ±15%.

* Chemical structures: 5,10-dideaza-folate-H_4, *N*-[4-[2-(2-amino-1,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoy]-L-glutamic acid; 10-methyl-5,10-dideaza-folate-H_4, *N*-[4-[1-[(2-amino-1,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl) methyl]ethyl]benzoyl]-L-glutamic acid; 10-ethyl-5,10-dideaza-folate-H_4, *N*-[4[1-[(2-amino-1,4,5,6,7,8 - hexahydro - 4 - oxopyrido[2,3 - *d*]pyrimidin - 6 - yl]methyl]propyl]benzoyl] - L-glutamic acid.

† Values are expressed as the concentration of natural diastereomer in the mixture of diastereomers.

determined indirectly from a series of experiments examining these structures as inhibitors of [^{3}H]methotrexate influx in both variant and parental cells. Like methotrexate, itself, these new analogs exhibited (data not shown) a characteristic hyperbolic downward inhibition curve to concentrations giving nearly complete inhibition of [^{3}H]methotrexate influx in L1210 cells. Under these conditions, a single value for influx K_i was derived (Table 2) for methotrexate (3.85 ± 0.5 μM) and 5-formyltetrahydrofolate (1.24 ± 0.2 μM) that closely approximated their corresponding values for influx K_m (Table 1). Values for influx K_i (Table 2) for DDTHF and its two analogs were all in the range of 2–3 μM. Of interest as well was our finding that the affinity of this system in L1210 cells for these new analogs was 1 log order of magnitude greater than for tetrahydrofolate, itself, and 2 log orders of magnitude greater than folic acid. Furthermore, like 5-formyltetrahydrofolate and in sharp contrast to methotrexate, the affinity of this system in variant cells (Table 2) for DDTHF and its 10-alkyl analogs was essentially unaltered from that seen in parental L1210 cells.

From the data given in Tables 1 and 2, it is apparent that methotrexate-resistant L1210/R1 and L1210/R24 cells should exhibit cross-resistance to DDTHF and its 10-alkyl analogs. However, since these analogs would be expected to share the same alteration in influx V_{max}, but not the alteration in influx K_m (or K_i), seen with methotrexate in these variants, the cross-resistance to these new analogs should be less than for resistance to methotrexate, itself. Data on growth inhibition of these cells, given in Table 3, show that this was actually the case. The value for IC_{50} for growth inhibition by methotrexate was increased 20-fold (L1210/R1) and 75-fold (L1210/R24) relative to parental L1210 cells. However, the same values for DDTHF and its analogs were increased only 5-fold in L1210/R1 cells and 16- to 18-fold in L1210/R24 cells. With each variant, the increase in resistance to these new analogs (Table 3) correlated with the extent of reduction in influx V_{max} for these altered transport systems. This is in agreement with data (Table 2) showing no differences among variant and parental cells in influx K_i for these new analogs. The above data allow us to conclude that methotrexate and the three analogs of tetrahydrofolate utilize the same transport route in L1210 cells. Based upon unpublished findings, it had also been suggested in a prior report [9] that methotrexate and DDTHF share a transport route in CCRF-CEM cells.

Table 3. Inhibition of growth of variant and parental L1210 cells in culture

Compound	Growth inhibition		
	L1210 IC_{50} (nM)	L1210/R1 IC_{50} (nM)	L1210/R24 IC_{50} (nM)
Methotrexate	3.95 ± 0.5	79.1 ± 9.3	308 ± 48
5,10-Dideaza-folate-H_4	47.2 ± 3	240 ± 37	841 ± 101
10-Methyl,5,10-dideaza-folate-H_4	50.3 ± 8	275 ± 42	827 ± 92
10-Ethyl-5,10-dideaza-folate-H_4	34.6 ± 4	218 ± 24	686 ± 82

Cells were grown in the presence of drug for 72 hr following which a cell concentration was determined, and an IC_{50} (concentration at 50% inhibition) value was obtained by comparing cell number in the presence and absence of drug. See Materials and Methods for additional details. Data are the mean of three experiments. Standard error of the mean was less than ±15%.

It should be noted here that, because of the asymmetric centers at carbon 6 in the case of DDTHF and carbons 6 and 10 in the case of the two 10-alkyl analogs, these new analogs exist as mixtures of diastereomers. In light of earlier [19–21] results, it is quite probable that there is little stereospecificity for transport in the case of the unsubstituted reduced compounds. With DDTHF, results reported recently [10, 11] have shown that both of its diastereomers are equipotent in regard to inhibition of growth. Since these diastereomers appear to be similar in all other respects [10, 11], this result would not have been expected if there were major differences in the transport inward of the two diastereomers. In regard to the 10 position, we have found [22] no stereospecificity for membrane transport in the case of diastereomers of the 10-alkyl-10-deazaaminopterins. We would anticipate a similar result in the case of the 10-alkyl derivatives of DDTHF.

Variants of the L1210 cells and other murine and human tumor cells exist [15, 23–26] that exhibit, among alterations of this classical transport system, either reduced influx V_{max} or increased influx K_m alone. Therefore, the finding that cross-resistance in a transport-altered, methotrexate-resistant variant to DDTHF and its analogs may not be determined by a change in influx K_m alone has therapeutic significance not only in the case of acquired resistance and cross-resistance, but also in respect to the probable spectrum of clinical utility of these new agents. We have shown in earlier studies [27] that the variability in influx K_m (4-fold) for methotrexate found among a variety of therapeutically naive murine tumors is a determining factor in their relative responsiveness to this analog. We have also found [27] that this variability in influx K_m for methotrexate among these tumors is not seen for naturally occurring folates. Since this is the same situation for DDTHF and its analogs in L1210/R1 and L1210/R24 cells, it is reasonable to assume that the variability in value for influx K_m seen for methotrexate among drug-naive tumors may not occur with this new class of analogs.

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Memorial Sloan-Kettering Cancer Center
New York, NY 10021, U.S.A.
FRANCIS M. SIROTNAK*
GLENYS M. OTTER

Southern Research Institute
Birmingham, AL 35255, U.S.A.
J. ROBERT PIPER

SRI International
Menlo Park, CA 94025, U.S.A.
JOSEPH I. DEGRAW

REFERENCES

1. R. L. Kisliuk, Y. Gaumont and C. M. Baugh, *J. biol. Chem.* **249**, 4100 (1974).
2. C. J. Allegra, B. A. Chabner, J. C. Drake, R. Lutz, D. Rodbard and J. Jolivet, *J. biol. Chem.* **260**, 9720 (1985).
3. J. E. Baggot, *Fedn Proc.* **42**, 667 (1983).
4. C. J. Allegra, J. C. Drake, J. Jolivet and B. A. Chabner, *Proc. Am. Ass. Cancer Res.* **25**, 76 (1984).
5. T. Ueda, G. E. Duchman, M. G. Nair, J. I. DeGraw, F. M. Sirotnak and Y-C. Cheng, *Molec. Pharmac.* **30**, 149 (1986).
6. T. R. Jones, A. H. Calvert, A. L. Jackman, S. J. Brown, M. Jones and K. R. Harrap, *Eur. J. Cancer* **17**, 11 (1981).
7. R. C. Jackson, A. L. Jackman and A. H. Calvert, *Biochem. Pharmac.* **32**, 3783 (1983).
8. R. G. Moran, E. C. Taylor and G. P. Beardsley, *Proc. Am. Ass. Cancer Res.* **26**, 231 (1985).
9. G. P. Beardsley, E. C. Taylor, G. Grindey and R. G. Moran, In *Chemistry and Biology of Pteridines* (Eds. G. A. Cooper and V. M. Whitehead) p. 953. Walter de Gruyter, Berlin (1986).
10. G. P. Beardsley, E. C. Taylor, G. Shih, G. A. Poore, G. B. Grindey and R. G. Moran, *Proc. Am. Ass. Cancer Res.* **27**, 259 (1986).
11. R. G. Moran, E. C. Taylor, G. Shih and G. P. Beardsley, *Proc. Am. Ass. Cancer Res.* **28**, 274 (1986).
12. J. R. Piper, G. S. McCaleb, R. L. Kisliuk, Y. Gaumont, J. A. Montgomery and F. M. Sirotnak, *J. med. Chem.*, in press.
13. J. I. DeGraw, W. T. Colwell, P. H. Christie, R. L. Kisliuk, Y. Gaumont and F. M. Sirotnak, *J. med. Chem.*, in press.
14. A. R. Cashmore, R. M. Dreyer, C. Horvath, J. O. Knipe, J. K. Coward and J. R. Bertino, *Meth. Enzym.* **66**, 459 (1980).
15. F. M. Sirotnak, L. J. Goutas, L. E. Kelleher and J. A. Montgomery, *Cancer Res.* **42**, 924 (1982).
16. F. M. Sirotnak, L. J. Goutas and L. S. Mines, *Cancer Res.* **45** 4732 (1985).
17. L. L. Samuels, D. M. Moccio and F. M. Sirotnak, *Cancer Res.* **45**, 1488 (1985).
18. B. H. J. Hofstee, *J. biol. Chem.* **184**, 1296 (1959).
19. F. M. Sirotnak, P. L. Chello, D. M. Moccio, R. L. Kisliuk, G. Combepine, Y. Gaumont and J. A. Montgomery, *Biochem. Pharmac.* **28**, 2993 (1979).
20. J. C. White, B. D. Bailey and I. D. Goldman, *J. biol. Chem.* **253**, 342 (1978).
21. P. L. Chello, F. M. Sirotnak, E. Wong, R. L. Kisliuk, Y. Gaumont and G. Combepine, *Biochem. Pharmac.* **31**, 1527 (1982).
22. J. I. DeGraw, P. H. Christie, H. Tagawa, R. L. Kisliuk, Y. Gaumont, F. A. Schmid and F. M. Sirotnak, *J. med. Chem.* **29**, 1056 (1986).
23. W. E. Flintoff and C. R. Nagaines, *Archs Biochem. Biophys.* **223**, 433 (1983).
24. R. C. Jackson, D. Niethammer and F. M. Heunnekens, *Cancer Biochem. Biophys.* **1**, 151 (1975).
25. J. T. McCormick, S. S. Susten and J. H. Freishem, *Archs Biochem. Biophys.* **213**, 311 (1981).
26. D. Neithammer and R. C. Jackson, *Eur. J. Cancer* **11**, 845 (1975).
27. F. M. Sirotnak and R. C. Donsbach, *Cancer Res.* **36**, 1151 (1986).

* Correspondence: F. M. Sirotnak, Ph.D., Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.