A Comparison of 4-Amino-4-deoxy-N^{10}-methylpteroic Acid and Methotrexate Transport by Mouse Leukemia Cells

DAVID KESSEL[1]

Laboratories of Pharmacology, Children's Cancer Research Foundation, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

(Received July 29, 1968)

SUMMARY

Studies of the uptake of methotrexate and an analogue, 4-amino-4-deoxy-N^{10}-methylpteroic acid, which lacks the carboxy-L-glutamate moiety, suggest that these compounds are taken up by different processes. The methotrexate-resistant P1534 murine leukemia accumulated methotrexate 13 times more slowly than the drug-sensitive L1210 cell line. In contrast, uptake of 4-amino-4-deoxy-N^{10}-methylpteroic acid proceeded at similar rates in both cell lines. Both compounds are potent inhibitors of the enzyme dihydrofolate reductase (5, 6, 7, 8-tetrahydrofolate: NADP-oxido reductase, EC 1.5.1.3. These findings suggest that drug resistance based on impaired uptake might be circumvented by the use of appropriate drug analogues.

INTRODUCTION

The antineoplastic drug methotrexate is tightly bound to its target enzyme, dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP-oxidoreductase, EC 1.5.1.3) (1), because of its 2,4-diaminopteridine structure (2–4); the *p*-aminobenzoyl and L-glutamate moieties also contribute to binding (2, 5–7). Uptake of methotrexate was found to be mediated by specific transport processes in animal tumor cells (8–10), human normal and leukemic leukocytes (11), rabbit choroid plexus (12), and rabbit kidney slices (13). Many methotrexate-resistant animal leukemia cell lines manifested impaired transport of the drug (10, 14–16).

Certain 2,4-diaminopyrimidines were found (12, 17) which also inhibit dihydrofolate reductase but apparently (18) enter cells by "passive diffusion." It was proposed by Baker *et al.* (17), and later confirmed by Mishra, Rosen, and Nichol (19), that folate antagonists structurally related to methotrexate but lacking the carboxy-L-glutamate moiety might serve as effective antitumor agents in cell lines unable to transport methotrexate. Baker *et al.* also suggested (17) that cleavage of the carboxy-L-glutamate from methotrexate would yield such a compound. A preparation of this derivative, 4-amino-4-deoxy-N^{10}-methylpteroic acid (Fig. 1), has been described (20). We have compared the characteristics of uptake of these two compounds in two murine leukemias: L1210, a cell line highly sensitive to methotrexate, and P1534, a cell line naturally resistant to methotrexate and showing (15) impaired uptake of the drug.

This work was supported by Contract PH-43-66-541 with the Cancer Chemotherapy National Service Center of the National Cancer Institute, National Institutes of Health.

[1] Present address, Department of Pharmacology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14620.

MATERIALS AND METHODS

Tumors. Sources of the L1210 and P1534 tumors (21) and methods of propagation and cell isolation (8) have been described.

Drugs. Methotrexate-3′,5′-^{3}H (2–9 C/mmole) was obtained from the Nuclear-Chicago Corporation and was purified by paper chromatography (22). Labeled 4-amino-4-deoxy-N^{10}-methylpteroic acid was

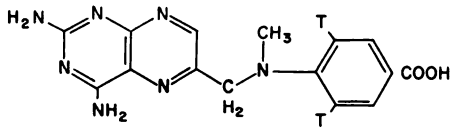

FIG. 1. *4-Amino-4-deoxy-N^{10}-methylpteroylglutamic acid*

prepared by microbial cleavage of the peptide bond of labeled methotrexate.[2] The product was purified by descending chromatography on Whatman No. 1 paper in 0.05 M phosphate buffer at pH 7.0. Authentic samples of methotrexate and 4-amino-4-deoxy-N^{10}-methylpteroate were provided by Dr. J. M. Ruegsegger, Lederle Laboratories. The latter compound was purified on a column of DEAE-cellulose; after washing with water to remove fluorescent impurities, the compound was eluted with a linear gradient (0.05–0.2 M) of Tris-HCl at pH 8.0. Tubes containing the major yellow peak, and free from blue fluorescence, were pooled. The chromatographic behavior of both labeled and unlabeled 4-amino-4-deoxy-N^{10}-methylpteroic acid, in four solvent systems, and the ultraviolet absorption spectra were similar to those described by Levy and Goldman (20).

Incubations. Tumor cells were resuspended in medium buffered with 75 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (23) at pH 7.2 and containing, at levels found in Ehrlich ascitic fluid (24), 71 mM NaCl, 20 mM KCl, 1.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 1 mM Na_2HPO_4. Incubations were carried out in 10×30 mm siliconized glass tubes which contained 200-μl aliquots of 5–8% cell suspensions. After warming to incubation temperatures, labeled compounds (final level, 0.2 μM) were added (volume, <10 μl). Incubations were terminated by rapidly chilling tubes to 0°; the cells were collected by centrifugation for 30 sec at $500 \times g$, resuspended in 300 μl of fresh medium at 0°, and again collected by centrifugation. The cell pellets were finally suspended in 250 μl of 0.9% NaCl, and a 200-μl aliquot was removed and mixed with 10 ml of a liquid phosphor. Radioactivity was measured by liquid scintillation counting. For further details of this procedure, see refs. 8 and 22. Drug distribution ratios were calculated on the basis of measurements of the intracellular cell space permeable to tritiated water, but not to labeled sulfate (25).

[2] A similar procedure has been reported (20); details of the method used here will be published elsewhere. Occasional "spontaneous" decomposition at 4° of solutions of labeled methotrexate to yield 4-amino-4-deoxy-N^{10}-methylpteroic acid has been traced to airborne microbial contaminants. This can be monitored by paper chromatography; in 0.05 M sodium phosphate buffer at pH 7.0, 4-amino-4-deoxy-N^{10}-methylpteroate migrates at only about 50–60% of the rate of methotrexate. The labeled pteridines were stored, after rapid freezing, at −20°, and no stability problems were encountered.

RESULTS

The uptake of methotrexate-^{3}H by L1210 and P1534 cells at 27° and 37° is shown in Figs. 2 and 3. The initial rate of uptake, in either cell line, was decreased about 15-fold by a 10° drop in incubation temperature.

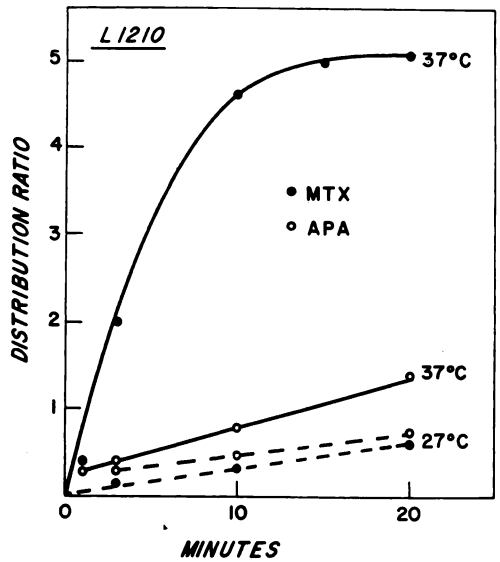

FIG. 2. *Time course of methotrexate (MTX) and 4-amino-4-deoxy-N^{10}-methylpteroate (APA) uptake by L1210 cells*

The conditions of incubation are described under MATERIALS AND METHODS. The drug levels were 0.2 μM. The distribution ratio is the ratio of the drug concentration in cell water to the drug concentration in the incubation medium.

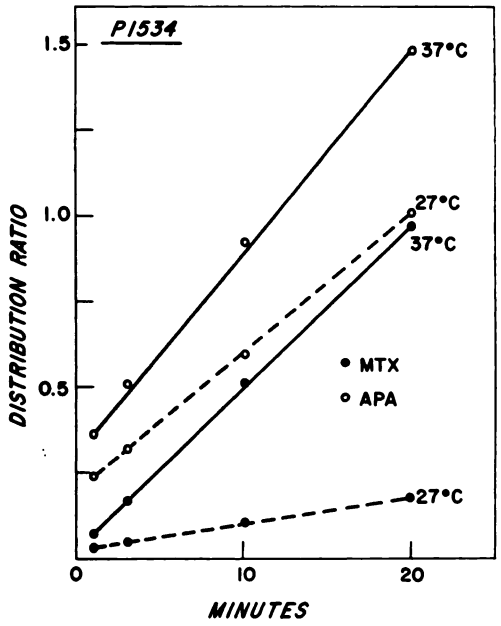

FIG. 3. *Time course of methotrexate (MTX) and 4-amino-4-deoxy-N^{10}-methylpteroate (APA) uptake by P1534 cells*

The incubation conditions are described under MATERIALS AND METHODS. The drug levels were 0.2 μM.

In contrast, the rate of uptake of 4-amino-4-deoxy-N^{10}-methylpteroate by either cell line was decreased only 2-fold by lowering the incubation temperature from 37° to 27°.

The uptake of methotrexate was 13-fold slower in P1534 than in L1210 cells, but the rate of uptake of 4-amino-4-deoxy-N^{10}-methylpteroate was approximately the same in both cell lines. At 37°, uptake of the latter compound was somewhat more rapid than that of methotrexate in P1534 cells, but the rate of methotrexate uptake was considerably greater in the L1210 cells.

Inhibition studies (Table 1) showed that an excess of unlabeled methotrexate profoundly inhibited uptake of labeled methotrexate by either cell line; such data had previously been interpreted to indicate a saturable transport process (8). Unlabeled 4-amino-4-deoxy-N^{10}-methylpteroate inhibited only slightly the uptake of 4-amino-4-deoxy-N^{10}-methylpteroate-^{3}H or methotrexate-^{3}H; unlabeled methotrexate was similarly ineffective as an inhibitor of 4-amino-4-deoxy-N^{10}-methylpteroate-^{3}H uptake.

TABLE 1
Inhibition of pteridine uptake

L1210 or P1534 cells were incubated for 10 min at 37° with either labeled methotrexate (MTX) or 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA) at 0.2 μM. Unlabeled compounds at 0.2 or 1.0 mM were added at the beginning of the incubations where specified. Results are reported in terms of the cell to medium distribution ratio of labeled compounds. Results of a typical experiment are shown; five other such experiments yielded data which did not vary from reported values by more than ±10%.

^{3}H-Labeled compound	Other additions	Distribution ratio in L1210 cells	Distribution ratio in P1534 cells
MTX	None	4.95	0.50
	0.2 mM MTX	0.75	0.30
	1 mM MTX	0.22	0.10
	0.2 mM APA	4.25	0.43
	1 mM APA	3.70	0.38
APA	None	0.70	0.90
	0.2 mM MTX	0.55	0.75
	1 mM MTX	0.45	0.65
	0.2 mM APA	0.54	0.73
	1 mM APA	0.33	0.60

Other experiments indicated that labeled methotrexate or 4-amino-4-deoxy-N^{10}-methylpteroate, when taken up under conditions specified in the legend to Figs. 2 and 3, could not readily be washed from the cells by resuspension in fresh medium at 0°. The half-time of drug loss at 37° was estimated as 3–5 hr.

DISCUSSION

Studies on 4-amino-4-deoxy-N^{10}-methylpteroic acid, a methotrexate analogue lacking the carboxy-L-glutamate moiety of the parent compound, have revealed several differences in transport of these two folate antagonists and suggest that different processes are involved. The slow and highly temperature-sensitive ($Q_{10} = 15$) uptake of methotrexate can be taken as an indication of the high energy of activation needed to bring this lipophobic molecule [charge, −2 at pH 7.0 (26)] across the cell

membrane, even though a specific mediated transport process is involved (8, 10). In contrast, uptake of 4-amino-4-deoxy-N^{10}-methylpteroic acid was less temperature-sensitive ($Q_{10} = 2$); deletion of the carboxy-L-glutamate moiety to yield a singly negatively charged molecule at pH 7.0[3] greatly decreased this energy barrier.

Since neither methotrexate nor 4-amino-4-deoxy-N^{10}-methylpteroic acid could be removed from cells by extensive washing, it was concluded that these compounds are tightly bound to cell components, presumably to the enzyme dihydrofolate reductase. Therefore, initial uptake measurements are taken to represent unidirectional flux.[4]

Methotrexate uptake occurred approximately 13 times more rapidly in L1210 than in P1534 cells at 37°. This difference presumably accounts for the relative insensitivity of P1534 cells to methotrexate (15). The rate of 4-amino-4-deoxy-N^{10}-methylpteroate uptake at 37° was similar in both cell lines. In L1210, the initial rate of methotrexate uptake exceeded that of 4-amino-4-deoxy-N^{10}-methylpteroic acid 11-fold at 37°. In contrast, uptake of the latter compound was 40% faster than methotrexate in P1534 cells. An apparently instantaneous mode of 4-amino-4-deoxy-N^{10}-methylpteroate uptake was suggested by extrapolation of the plots of uptake with respect to zero time. This also might represent rapid surface adsorption of the compound.

The ability of a cell line with impaired methotrexate uptake to accumulate 4-amino-4-deoxy-N^{10}-methylpteroic acid at a rate greater than that found for methotrexate confirms the prediction of Baker *et al.* (17) that deletion of the carboxy-L-glutamate moiety should yield a compound not affected by impairment of the methotrexate transport system. 4-Amino-4-deoxy-N^{10}-methylpteroic acid might therefore be a useful compound for the treatment of certain methotrexate-resistant leukemias. It should be noted that even the replacement of the glutamate moiety of methotrexate with aspartate appreciably weakens the drug–dihydrofolate reductase binding (7). This might have consequences for a chemotherapeutic process which is presumably based on stoichiometric inhibition of this enzyme (1).

[3] Confirmed by paper electrophoresis at pH 7.0.

[4] Both L1210 and P1534 cells contain approximately equal levels of the enzyme dihydrofolate reductase (27). 4-Amino-4-deoxy-N^{10}-methylpteroic acid inhibits the enzyme from either source ($K_i = 5 \times 10^{-8}$ M). Other details of this determination are given in ref. 27.

ACKNOWLEDGMENTS

Miss Marjorie Myers and Mrs. Judy Henrikson assisted with these studies.

REFERENCES

1. W. C. Werkheiser, *J. Biol. Chem.* **236**, 888 (1961).
2. S. F. Zakrzewski, *J. Biol. Chem.* **238**, 1485 (1963).
3. S. F. Zakrzewski, *J. Biol. Chem.* **238**, 4002 (1963).
4. B. R. Baker, *Cancer Chemother. Rep.* **4**, 1 (1959).
5. J. R. Bertino, J. P. Perkins and D. G. Johns, *Biochemistry* **4**, 839 (1965).
6. S. F. Zakrzewski, *J. Biol. Chem.* **235**, 1780 (1960).
7. J. A. R. Mead, N. H. Greenberg, A. W. Schrecker, D. R. Seeger and A. S. Tomcufcik, *Biochem. Pharmacol.* **14**, 105 (1965).
8. D. Kessel and T. C. Hall, *Cancer Res.* **27**, 1539 (1967).
9. A. Y. Divekar, N. R. Vaidya and B. M. Braganca, *Biochim. Biophys. Acta* **135**, 927 (1967).
10. F. M. Sirotnak, S. Korita and D. J. Hitchison, *Cancer Res.* **28**, 75 (1968).
11. D. Kessel, T. C. Hall and D. Roberts, *Cancer Res.* **28**, 564 (1968).
12. R. Rubin, E. Owens and D. P. Rall, *Cancer Res.* **28**, 689 (1968).
13. R. Rubin, E. S. Henderson, E. S. Owens and D. P. Rall, *Cancer Res.* **27**, 553 (1967).
14. G. A. Fischer, *Biochem. Pharmacol.* **11**, 1233 (1962).
15. D. Kessel, T. C. Hall, D. Roberts and I. Wodinsky, *Science* **150**, 752 (1965).
16. B. M. Braganca, A. Y. Divekar and N. R. Vaidya, *Biochim. Biophys. Acta* **135**, 937 (1967).
17. B. R. Baker, D. V. Santi, P. I. Almaula and W. C. Werkheiser, *J. Med. Chem.* **7**, 24 (1964).

18. R. C. Wood, R. Ferone and G. H. Hitchings, *Biochem. Pharmacol.* **6**, 113 (1961).
19. L. C. Mishra, F. Rosen and C. A. Nichol, *Proc. Amer. Ass. Cancer Res.* **9**, 49 (1968).
20. C. C. Levy and P. Goldman, *J. Biol. Chem.* **242**, 2933 (1967).
21. D. Roberts, I. Wodinsky and T. C. Hall, *Cancer Res.* **25**, 1899 (1965).
22. D. Kessel and T. C. Hall, *Biochem. Pharmacol.* **16**, 2395 (1967).
23. N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa and R. M. M. Singh, *Biochemistry* **5**, 467 (1966).
24. K. H. Ibsen and R. W. McKee, *Cancer Res.* **27**, 1648 (1967).
25. D. Kessel, *Biochim. Biophys. Acta.* **163**, 179 (1968).
26. M. T. Hakala, *Biochim. Biophys. Acta* **102**, 210 (1965).
27. D. Roberts, I. Wodinsky and T. C. Hall, *Cancer Res.* **25**, 1899 (1965).