Biochemical Pharmacology, Vol. 33, No. 17, pp. 2711–2714, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

DETECTION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF METHOTREXATE AND ITS METABOLITES IN TUMOR TISSUE FROM OSTEOSARCOMA PATIENTS TREATED WITH HIGH-DOSE METHOTREXATE/LEUCOVORIN RESCUE

LAWRENCE L. SAMUELS, AARON FEINBERG, DONNA M. MOCCIO, FRANCIS M. SIROTNAK* and GERALD ROSEN

Memorial Sloan-Kettering Cancer Center, New York, NY 10021, U.S.A.

(*Received* 29 *August* 1983; *accepted* 10 *January* 1984)

Abstract—Methotrexate (MTX) polyglutamates were detected in osteogenic sarcoma tumor samples obtained from patients 24 or 48 h after receiving high-dose MTX/leucovorin rescue therapy. Tumor samples were assayed by high-performance liquid chromatography, and polyglutamyl metabolites, along with MTX, were quantitated using both direct u.v. absorption at 313 nm and an enzyme titration assay. Good agreement between these two methods was found although the more sensitive enzyme assay detected peaks in some samples not detected by u.v. absorbance. A wide variation in MTX:MTX polyglutamate levels (1:1 to 25:1) was found among the six clinical samples studied. Also, no correlation between the extent of polyglutamate formation and plasma levels (determined at the time of tumor sampling) was observed. High intracellular levels of a derivative which appears to be the 7-hydroxy metabolite of MTX were also detected in four of six samples. This material coeluted with authentic standard, showed spectral properties like standard 7-OH-MTX, and did not inhibit dihydrofolate reductase.

The intracellular metabolic conversion of methotrexate (MTX) to polyglutamates of varying chain length has been found to occur [1–16] in a variety of normal and neoplastic tissues. This conversion, which results from the addition of one or more glutamyl residues in a peptide linkage at the γ-carboxyl position, apparently has no effect [17] on the ability of this folate analog to inhibit the target enzyme, dihydrofolate reductase. However, the consequences of this conversion for other biochemical and pharmacologic parameters of antifolate action are less certain [4, 8, 10–12] and remain controversial. In view of this, the significance of this metabolism to antifolate-induced cytotoxicity and, more specifically, to therapy of human cancer with methotrexate remains undetermined.

Polyglutamation of methotrexate has also been shown to vary substantially in different normal proliferative and neoplastic animal tissues [4, 6, 11]. The detection of these metabolites has been reported as well in normal human tissues (liver and red blood cells) derived from patients treated with methotrexate [1, 14, 15]. Although polyglutamation of methotrexate has been demonstrated [5, 7, 12, 15] in human tumor cells exposed to this agent in culture, the extent of conversion of this folate analog in tumor tissue in patients under treatment has not been reported.

We now report on the chromatographic separation and detection of methotrexate polyglutamates in tumor tissue derived from osteogenic sarcoma patients treated with high-dose methotrexate/leucovorin rescue protocols. Polyglutamated derivatives were detected by direct u.v. absorption (313 nm) and by an enzyme inhibition assay [18] of eluted fractions. In addition, we also show that a large fraction of the total intracellular material absorbing at 313 nm present in this tissue at the time of analysis was not inhibitory to dihydrofolate reductase and exhibited elution properties and absorption spectra indistinguishable from that characteristic of the 7-hydroxy metabolite of methotrexate.

MATERIALS AND METHODS

Tumor sampling. Methotrexate was given by i.v. infusion to patients with osteogenic sarcoma at a dose of 8–12 g/m^2 over a period of 4 hr. Patients also received leucovorin (10 mg) every 6 hr beginning 24 hr after the start of methotrexate treatment. Urine was alkalinized with sodium bicarbonate, and output was monitored closely. After informed consent, tumor was obtained by needle biopsy (100–300 mg wet wt) or surgical resection 24 or 48 hr after methotrexate infusion. Additional details as to clinical management of these patients have been provided earlier [19].

Tumor tissue, after removal from the patient, was cooled to 0° and incubated in 50 vol. of cold (0°) 0.17 M NH_4Cl for 5 min to lyse red blood cells [20] and remove drug from interstitial space. This procedure was repeated if necessary. Following centrifugation at 0°, samples were homogenized for 30 sec in 0.01 M phosphate plus 0.14 M NaCl (pH

* Author to whom all correspondence should be addressed.

7.4) and then immediately boiled for 15–20 min. After centrifugation, the supernatant fraction was removed and held at −70° until analysis by reverse-phase HPLC. As a control for endogenous foly-polyglutamate hydrolase activity, we processed under identical conditions 500 mg of mouse small intestine, a tissue with high hydrolase levels, and found no hydrolysis of authentic MTX + G_3 standard. Similar results were found using murine tumors, which have less hydrolase activity (L. L. Samuels and F. M. Sirotnak, unpublished observation).

HPLC assay. A Waters Associates (Milford, MA) high-performance liquid chromatographic system equipped with a systems controller, data module integrator, WISP autosampler, two pumps, and variable wavelength u.v. detector was used for separation of methotrexate and its metabolites. Samples were injected onto a Waters μBondpak C-18 reverse phase column. The solvent system employed, modified from Cashmore *et al.* [21], consisted of a 20-min linear gradient from 5–15% acetonitrile in 0.1 M sodium acetate buffer, pH 5.1. The flow rate was maintained at 1 ml/min. Column eluate was monitored at 313 nm for maximum u.v. absorption by methotrexate and its derivatives with minimum background interference. Peak areas were integrated by a Waters data module and quantitated by the external standard method, which has a sensitivity of 10–20 pmoles.

Fractions of 1 ml were collected and assayed by dihydrofolate reductase titration inhibition, a method which can detect 0.5 pmole drug [18]. Plasma levels of methotrexate were derived by a dihydrofolate reductase titration inhibition assay [18].

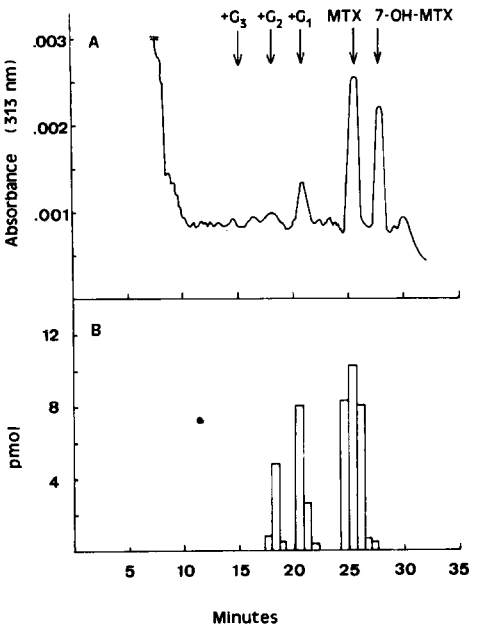

Fig. 1. Elution profile for methotrexate and its metabolites in osteosarcoma tissue. Tissue extract was derived from a patient (Sh.A.) 24 hr after treatment with methotrexate. Additional clinical and experimental details are provided in the text. Elution profiles were obtained by direct u.v. detection (313 nm) (A) and dihydrofolate reductase inhibition titration (B). Abbreviations: MTX, methotrexate; MTX + G_1, methotrexate + glutamate; MTX + G_2, methotrexate plus diglutamate; and MTX + G_3, methotrexate plus triglutamate.

RESULTS AND DISCUSSION

The elution profile shown in Fig. 1A was obtained by direct u.v. detection at 313 nm of material eluting during chromatography of an extract of tumor tissue obtained from patient Sh.A. 24 hr after methotrexate therapy. Fractions of eluate were collected and also assayed for drug content by enzyme titration. This elution profile is shown in Fig. 1B. Of the total amount of drug detected by either method (Table 1), approximately 62% of that which inhibited dihydrofolate reductase coeluted with authentic methotrexate. The remainder eluted earlier as additional peaks which were coincident with authentic standards for methotrexate derivates with one (25%) or two (13%) additional glutamyl residues. Otherwise, good agreement was obtained between values derived by each method. However, in addition to methotrexate and the two polyglutamated derivatives, a substantial quantity of u.v. absorbing material, which did not inhibit dihydrofolate reductase, coeluted after methotrexate with authentic 7-OH-methotrexate. This fraction also showed an absorption spectra which was characteristic of this metabolite. An elution profile was also obtained with extract from another sample of tumor tissue removed from patient X.V. 24 hr after methotrexate therapy. In this sample (Table 1) peak fractions coeluting with methotrexate and derivatives bearing two and three additional glutamyl residues were the predominant forms detected by u.v. absorption. All of these were further authenticated by enzyme titration. A small amount of methotrexate + G_1 (one additional glutamyl residue) was also

Table 1. Summary of data on HPLC analysis of methotrexate and methotrexate polyglutamates in two samples of osteosarcoma tissue, comparing two methods of detection*

Sample	Method of detection	Total (nmoles/g wet wt)	Intracellular drug (MTX + MTX + G_n) MTX (%)	+ G_1 (%)	+ G_2 (%)	+ G_3 (%)
Sh.A.	Ultraviolet		76.1	14.6	9.2	
	DHFR	1.08	62.1	25.1	12.8	
X.V.	Ultraviolet		44.6		13.5	41.8
	DHFR	0.52	52.8		19.1	28.0

* Experimental details are provided in the text. DHFR = dihydrofolate reductase.

detected by u.v. absorption but this fraction was not collected for an enzyme inhibition determination. This sample also showed a large peak of u.v. absorbing material which coeluted with 7-OH-methotrexate, exhibited spectral properties similar to a known standard of this metabolite, and did not inhibit dihydrofolate reductase. Following treatment of an aliquot of this sample with carboxypeptidase [22], peak fractions coeluting with methotrexate + G_2 and methotrexate + G_3 were no longer detectable, and there was a proportional increase in the peak corresponding to methotrexate.

Additional data from HPLC analysis of extracts from tumor derived from a series of other patients are summarized in Table 2 along with data derived from the two patients discussed above. For the additional samples, methotrexate and its polyglutamates were quantitated by dihydrofolate reductase inhibition assays on each fraction. Also, in almost every case, a peak of u.v. absorbance was detected which was coincident with the peak fractions of inhibitory activity. 7-OH-Methotrexate content in each sample was determined by examining the u.v. fractions coeluting with this metabolite, determining the u.v. spectra, and showing that this material had no inhibitory activity for dihydrofolate reductase. Polyglutamates of methotrexate were detected in all of the additional samples even when analyzed 48 hr after methotrexate therapy. However, among this group of samples both the total amount of active drug (methotrexate + methotrexate polyglutamates) and the fraction existing in each sample as a polyglutamate varied widely from patient to patient (methotrexate:methotrexate polyglutamate ratios varied from 1:1 to 25:1). Total intracellular levels of methotrexate and the percent conversion to polyglutamates did not correlate with plasma levels measured at the same sampling interval. Of interest, as well, was the amount of drug which appeared to exist as the 7-OH metabolite. In four of the samples this was found at a high level (varying from 40 to 60% of the total intracellular drug). However, in two of the samples examined this metabolite was not detected.

The large variation in the total level of intracellular drug among the samples examined is of interest. The high intracellular level of what appears to be the 7-OH metabolite in some patients, but not in others, is also of interest. During the period of 24–48 hr after methotrexate therapy, levels of the 7-OH metabolite in plasma vary considerably [23, 24] and are often higher than the levels of methotrexate itself [23, 24]. We have also noted [25] that this metabolite competes almost as effectively as methotrexate for entry into tumor cells by the high-affinity reduced folate transport system. Assuming a similar competitive interaction in osteogenic sarcoma cells, higher relative levels of 7-OH-methotrexate in plasma would tend to restrict entry of methotrexate and (or) reduce intracellular levels of drug already accumulated by a counter-transport effect [26]. The variable levels of this metabolite in the intracellar compartment could reflect one or more of these factors. Moreover, the endogeneous generation of this metabolite from intracellular methotrexate in this tissue cannot be excluded, although this metabolism appears to be associated [27] with enzymatic activities present in the liver. In view of the above considerations, the presence of methotrexate polyglutamates in this tumor tissue and the large variability observed between samples probably do not solely reflect the level of folypolyglutamyl synthetase activity in these tissues. Consequently, our ability to derive conclusions as to the significance, if any, of polyglutamation to responsiveness in this category of patient and treatment protocol must await further studies.

Acknowledgements—Supported in part by Grants CA 08748 and CA 22764 from the National Cancer Institute, N.I.H. Dr. Samuels was supported by Training Grant CA 09207 from the National Cancer Institute, N.I.H.

REFERENCES

1. C. M. Baugh, C. L. Krumdieck and M. G. Nair, *Biochem. biophys. Res. Commun.* **52**, 27 (1973).
2. J. Galivan, *Cancer Res.* **39**, 735 (1979).
3. D. A. Gewirtz, J. C. White, J. K. Randolph and I. D. Goldman, *Cancer Res.* **39**, 2914 (1979).
4. R. G. Poser, F. M. Sirotnak and P. L. Chello, *Cancer Res.* **41**, 4441 (1981).
5. R. L. Schilsky, B. D. Bailey and B. A. Chabner, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2919 (1980).
6. V. M. Whitehead, *Cancer Res.* **37**, 408 (1977).
7. D. S. Rosenblatt, V. M. Whitehead, M. M. Dupont, M-J. Vuchich and N. Vera, *Molec. Pharmac.* **14**, 210 (1978).
8. D. W. Fry, J. C. Yalowich and I. D. Goldman, *J. biol. Chem.* **257**, 1890 (1982).

Table 2. Summary of HPLC analyses for methotrexate and its metabolites of various samples of osteosarcoma tissue*

Sample	Interval post R_x (hr)	Plasma MTX (μM)	Total (nmoles/g wet wt)	Interacellular drug MTX (%)	MTX + G_n (%)	7-OH-MTX (%)
Sh.A.	24	1.2	2.19	36.5	12.8	50.7
X.V.	24	4.3	0.94	29.4	31.5	39.1
L.S.	24	1.3	8.25	44.4	8.2	47.4
R.C.	24	2.2	3.12	95.9	4.1	<1
S.A.	48		0.77	78.5	21.5	<1
J.R.	48	0.6	6.07	37.4	3.3	59.3

* Experimental details are provided in the text.

Abbreviations: MTX, methotrexate; MTX + G_n, methotrexate polyglutamates; and 7-OH-MTX, 7-OH-methotrexate.

9. J. Galivan, *Molec. Pharmac.* **17**, 105 (1980).
10. R. G. Poser, F. M. Sirotnak and P. L. Chello, *Biochem. Pharmac.* **29**, 2701 (1980).
11. M. Balinska, J. Galivan and J. K. Coward, *Cancer Res.* **41**, 2751 (1981).
12. J. Jolivet, R. L. Schilsky, B. D. Bailey, J. C. Drake and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
13. S. A. Jacobs, C. J. Derr and D. G. Johns, *Biochem. Pharmac.* **26**, 2310 (1977).
14. G. R. Krakower, P. A. Nylen and B. A. Kamen, *Analyt. Biochem.* **122**, 412 (1982).
15. A. Witte, V. M. Whitehead, D. S. Rosenblatt and M. S. Vuchich, *Devl Pharmac. Ther.* **1**, 40 (1980).
16. V. M. Whitehead, M. M. Perrault and S. Stelener, *Cancer Res.* **35**, 2985 (1975).
17. S. A. Jacobs, R. H. Adamson, B. A. Chabner, C. I. Derr and D. G. Johns, *Biochem. biophys. Res. Commun.* **63**, 692 (1975).
18. W. L. Werkheiser, *J. biol. Chem.* **236**, 888 (1961).
19. G. Rosen, B. Caparros, A. G. Huvos, C. Kosloff, A. Nirenberg, A. Cacavio, R. C. Marcove, J. M. Lane, B. Mehta and C. Urban, *Cancer, N.Y.* **49**, 1221 (1982).
20. K. Shortman, N. Williams and P. Adams, *J. Immun. Meth.* **1**, 273 (1972).
21. A. R. Cashmore, R. M. Dreyer, C. Horvath, J. O. Knipe, J. K. Coward and J. R. Bertino, *Meth. Enzym.* **66**, 459 (1980).
22. 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).
23. H. Breithaupt and E. Küenzlen, *Cancer Treat. Rep.* **66**, 1733 (1982).
24. C. Canfell and W. Sadée, *Cancer Treat. Rep.* **64**, 165 (1980).
25. D. M. Moccio, F. M. Sirotnak, L. L. Samuels, T. Ahmed, A. Yagoda, J. I. DeGraw and J. R. Piper, *Cancer Res.* **44**, 352 (1984).
26. I. D. Goldman, N. S. Lichtenstein and V. T. Oliverio, *J. biol. Chem.* **243**, 5007 (1968).
27. D. J. Johns, A. T. Ionatti, A. C. Sartorelli and J. R. Bertino, *Biochem. Pharmac.* **15**, 555 (1965).