Biochemical Pharmacology, Vol. 25, pp. 975–976. Pergamon Press, 1976. Printed in Great Britain.

SHORT COMMUNICATIONS

Relationship between intracellular dihydrofolate reductase and tightly bound intracellular methotrexate in human neoplastic cells

(*Received* 9 *December* 1974; *accepted* 20 *August* 1975)

The mode of anti-tumor action of methotrexate (MTX) is thought to be stoichiometric, high-affinity binding to the enzyme, dihydrofolate reductase (DHFR), with resultant depletion of folate coenzymes and eventual cell death [1]. Transport studies [2] suggest that non-effluxable intracellular MTX is specifically bound to DHFR and not to other intracellular proteins [3]. The present studies were undertaken to re-examine this question in human neoplastic cells using two independent techniques for assessing intracellular DHFR levels.

Determinations of high-affinity intracellular MTX binding were performed by efflux studies or enzymatic titration on peripheral neoplastic cells obtained either by leukophoresis or venipuncture from patients with Burkitt's lymphoma (BL; n = 4), acute lymphocytic leukemia (ALL; n = 4), and acute myelogenous leukemia (AML; n = 6). Patients had received no chemotherapy within 2 weeks of study and no MTX within 2 months of study.

Leukemic or lymphomatous cells were prepared for study by a 60-sec hypotonic lysis to free them from red cell contamination, two washes in 4° bicarbonate-buffered saline to remove plasma, and resuspension in 37° Eagle's minimal essential medium without serum or folates. Blast cell counts and cell viability (by trypan blue exclusion) were greater than 90 per cent in each case. Tritiated, purified MTX was then added to the suspension to achieve a final concentration of 1 μM and cellular uptake permitted for 80–120 min. Cells were then washed free of extracellular MTX and resuspended in a large volume of Eagle's medium to allow efflux. Cell pellets were isolated and processed as previously described [4] and intracellular MTX determined. Drug metabolism and membrane binding were negligible during the period of study [4]. The terminal efflux points were taken as the non-effluxable drug levels, as incubation in excess of 240 min led to decreased cell viability. As both AML and BL cells were closely approaching or had reached a steady-state at 240 min (120 min of uptake and 120 min of efflux) such an assessment seems justified. However, as the ALL cells had not reached a steady-state at this time, the terminal efflux point probably over-estimates the actual value of non-effluxable MTX (see Fig. 1).

Dihydrofolate reductase activity was determined by first suspending washed cells in two volumes of buffer (Tris–HCl; 0.05 M; pH 7.5 containing 0.1 M KCl), lysing cells by alternate freezing and thawing (three times), and then centrifuging the homogenate (27,000 *g* for 15 min at 4°). Preliminary studies revealed 14 per cent of the cells to be intact after the first freeze-thaw operation, 1 per cent after the second, and none after the third. DHFR activity (units/ml) rose from 0 to 0.045 after the first freeze-thaw, to 0.290 after the second freeze-thaw, and stabilized at 0.311 after the third, fourth, and fifth freeze-thaw procedures indicating complete recovery of the enzyme following the third freeze-thaw operation. Activity of DHFR (μmoles of dihydrofolate converted to the tetrahydrofolate per hr) in the supernatant fraction was assayed by determining the decrease in absorbance (340 nm) which accompanies the conversion of dihydrofolate and $NADPH_2$ to tetrahydrofolate and NADPH, respectively [5]. A standard assay system contained 100 μmole of Tris–HCl buffer (pH 7.5); 50 μmol KCl; 0.1 μmole $NADPH_2$ (Sigma Chemical Co.); 0.05 μmole dihydrofolate (prepared from folate by the method of Blakely [6]) containing 0.1 μmole of 2-mercaptoethanol, and enzyme extract in a final volume of 1 ml. When MTX was titrated against the enzyme a constant enzyme activity (0.2 μmoles/hr) was preincubated with MTX in buffer for 2 min at 37° before $NADPH_2$ and dihydrofolate were added to initiate the enzyme reaction. The residual enzyme activity was determined and plotted against MTX concentration to determine I_{50} values.

The conversion of I_{50} values to nmoles DHFR/g dry cell wt was performed using the following formula and assuming a 1:1 DHFR–MTX association:

$$\frac{\text{nmoles DHFR}}{\text{g dry cell wt}} = \frac{\dfrac{\text{pmoles MTX}}{\text{aliquot}} \times \text{DF}^* \times 2^\dagger}{(\text{no. of cells lysed}/6.62^\ddagger) \times 10^6 \text{ cells/mg dry cell wt}}$$

The results of the DHFR and non-effluxable MTX determinations are summarized in Table 1. The non-effluxable MTX levels exceed the DHFR level in 2 of 4 patients with BL, 3 of 4 patients with ALL, and 4 of 6 patients with AML. Further, the mean values of non-effluxable intracellular MTX exceed the DHFR levels for each cell type. However, in spite of this tendency, the mean values of the respective levels in each tumor type do not significantly differ as determined by paired *t*-test analysis.

The results suggest that the high-affinity intracellular sites to which MTX binds may be exclusively DHFR in that DHFR and non-effluxable MTX levels do not significantly differ in the three cell types studied. Although a suggestion of high-affinity binding in excess of DHFR sites is seen in individual patient data, an overall analysis does not support this view. Because marked variability is observed among individual patients, additional patient data will be necessary in order to resolve this point. Further, it is important to recognize that the interaction between MTX and DHFR in the intact cell may not be adequately reflected by the cell-free assay used to assess

*The dilution factor (DF) used in the assay. The term (pmoles MTX)/aliquot × DF represents the I_{50} value.

† The factor (2) converts the I_{50} to the I_{100}.

‡ The conversion factor is used to convert cell number to dry wt. It has been calculated for L1210 murine leukemia [2] and is not, as yet, available for these human cell types.

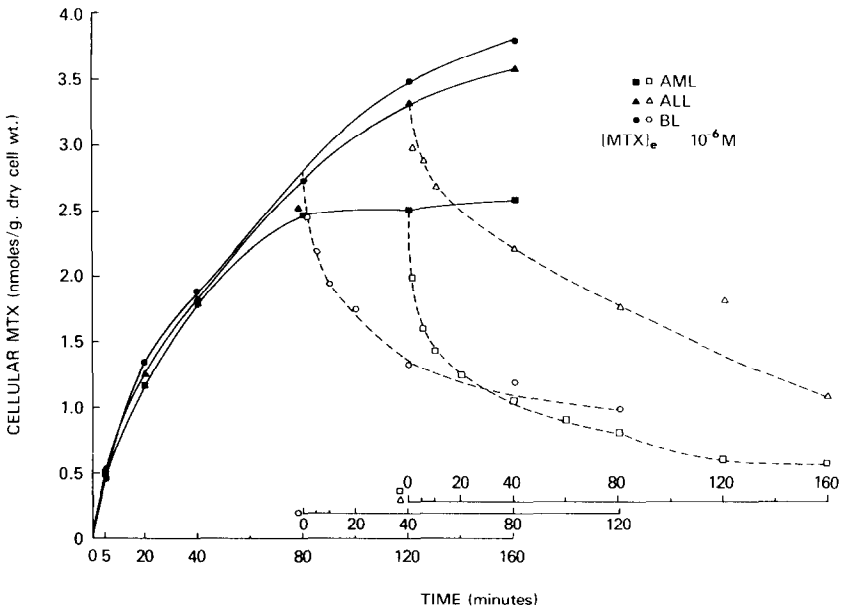

Fig. 1. Uptake and efflux of MTX in human neoplastic cells. Specific procedural details are contained in the text. Each point represents the mean value of duplicate determinations at each time for the patients listed in Table 1. Uptake curves are represented by a solid line and solid symbol and efflux curves by an interrupted line and open symbol. The time course for uptake is on the abscissa with that for efflux represented by separate numbered lines above the abscissa. The symbol at the beginning of each of these lines designates the cell type to which it corresponds.

Table 1. Levels of intracellular DHFR or MTX observed in neoplastic cells.

Cell type		DHFR (nmoles/g dry cell wt)	Non-effluxable MTX (nmoles/g dry cell wt)
Burkitt's lymphoma	I	1.07	2.24
	II	0.65	1.21
	III	1.46	0.42
	IV	0.85	0.51
		1.01 ± 0.17	1.09 ± 0.42
Acute lymphocytic leukemia	I	0.19	1.51
	II	0.21	0.70
	III	1.72	2.16
	IV	0.55	0.24
		0.67 ± 0.36	1.15 ± 0.43
Acute myelocytic leukemia	I	0.09	0.30
	II	0.71	1.00
	III	0.41	0.75
	IV	0.11	0.60
	V	0.58	0.45
	VI	—	0.25
		0.33 ± 0.13	0.56 ± 0.13

* Specific procedural and technical details are described in the text. The mean ± S.E. appears at the bottom of each column of figures. The mean value of the DHFR and non-effluxable MTX levels do not differ significantly in the cell types studied.

DHFR levels. This observation is especially relevant in light of recent investigations [7] which suggest that intracellular MTX in excess of the tightly bound fraction is necessary for a maximal cytotoxic effect. Furthermore, the non-effluxable MTX may consist, to a significant degree, of polyglutamate metabolites of the drug. Formation of these derivatives has recently been demonstrated in L1210 leukemia cells [8]. Although these derivatives are as potent as the parent compound as inhibitors of DHFR [8], they may possess different transport characteristics and may not freely efflux from cells.

Laboratory of Chemical Pharmacology and the Medicine Branch National Cancer Institute, Bethesda, Md. 20014, U.S.A.

RICHARD A. BENDER
DAVID R. MAKULU

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