Biochemical Pharmacology, Vol. 34, No. 16, pp. 2897–2903, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

THE EFFECT OF LEUCOVORIN ON THE SYNTHESIS OF METHOTREXATE POLY-γ-GLUTAMATES IN THE MCF-7 HUMAN BREAST CANCER CELL LINE

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(*Received* 26 *June* 1984; *accepted* 13 *December* 1984)

Abstract—The modulating effects of leucovorin on the synthesis of methotrexate (MTX) polyglutamates in the MCF-7 human breast cancer cell line have been investigated using a paired-ion high performance liquid chromatography (HPLC) system. Leucovorin decreased the intracellular level of MTX and profoundly affected polyglutamate synthesis irrespective of whether it was administered with or after MTX. Inhibition of MTX polyglutamate synthesis was also observed when concentrations of leucovorin too low to affect intracellular levels of MTX were employed. Leucovorin did not promote efflux of MTX from the MCF-7 cells and did not affect the distribution of the retained drug amongst the various polyglutamate forms.

Methotrexate (MTX) poly-γ-glutamates consist of one or more glutamyl residues linked to MTX through γ-carboxyl bonds and are analogous to the polyglutamyl derivatives of folic acid [1]. Methotrexate polyglutamates are synthesized by a wide variety of cell types including L1210 [2], rat hepatocytes [3], human fibroblasts [4] and human breast cancer [5]. The highly anionic nature of these metabolites suggests that they will not cross the cell membrane readily and hence longer chain derivatives will be preferentially retained. Although it has been reported that MTX polyglutamates could efflux from L1210 cells with the same facility as MTX [6]; it is generally accepted that MTX polyglutamates are retained by cells [5, 7]. More than 20 years ago it was reported that MTX could persist in murine tissue for up to 3 months after administration of the drug [8, 9]. In addition MTXG1 (MTX containing one additional γ-glutamyl residue) may persist in human liver for periods of up to 6 months after administration of the drug [10]. The chronic toxicity associated with MTX, leucoencephalopathy [11], hepatotoxicity [12] and pulmonary fibrosis [13], may be in part explicable by the long-term accumulation of these metabolites.

Methotrexate polyglutamates can bind to dihydrofolate reductase [14] and are at least as good inhibitors of this enzyme as the parent drug [15, 16]. The twin features of equal cytotoxicity and increased persistence in tissue makes the formation of these derivatives an important feature of the pharmacology of MTX.

Leucovorin (calcium N^5-formyl tetrahydrofolate) may rescue cells from the toxicity induced by MTX *in vitro* [17] and *in vivo* [18]. Following parenteral administration, leucovorin is converted to N^5-methyl tetrahydrofolate [19] which may compete with MTX for transport into cells [20]. Once inside the cell, the reduced folate may be used to restore thymidine and purine synthesis which had been inhibited by MTX. The ability of leucovorin to rescue cells is dependent on the concentration of MTX. Pinedo *et al.* [17] demonstrated that 10^{-7} M leucovorin could completely abolish the toxicity associated with 10^{-7} M MTX *in vitro*. Rescue from 10^{-5} M MTX required 10^{-3} M leucovorin and 10^{-4} M MTX resulted in toxicity which leucovorin was unable to reverse. Rosenblatt *et al.* [21] reported that leucovorin and N^5-methyl tetrahydrofolate could abolish the synthesis of MTX polyglutamates in human fibroblasts *in vitro*. However, these workers used an assay for MTX polyglutamates which could not resolve polyglutamates longer than MTXG1. Galivan and Nimec [22] showed that leucovorin prevented the synthesis of the higher mol.wt polyglutamate species following a short pulse with MTX in rat hepatoma cells.

In the present study we have used a high resolution high performance liquid chromatography (HPLC) method to characterize the effect of a wide range of concentrations of leucovorin (10^{-9}–10^{-5} M) on the synthesis of MTX polyglutamates in a human breast cancer cell line, MCF-7.

MATERIALS AND METHODS

[3′,5′,7-^{3}H]Methotrexate (TRK 224; sp. act. 11.9 Ci/mmole) was purchased from Amersham International (Amersham, U.K.). The purity of the label was not less than 98% as determined by paper chromatography using *n*-butanol:pyridine:water (1:1:1) as the eluent system. Methotrexate polyglutamate standards were purchased from Dr. C. M. Baugh (University of S. Alabama, Mobile, AL). The MCF-7 cells were a gift from Dr. C. R. Green (University of Liverpool, U.K.) and originated from Dr. M. Lippman (N.I.H. Bethesda, MD). Foetal calf serum was purchased from Randox Laboratories (Crumlin, U.K.) and was exhaustively dialysed against physiological saline before use. Tetrabutylammonium hydrogen sulphate and bovine liver

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dihydrofolate reductase (DHFR) was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Cell culture conditions. Approximately 5×10^5 cells were plated onto petri dishes in Eagles Minimal Essential Medium supplemented with Earles salts, 5% dialysed serum, 100 I.U. ml penicillin and 100 μg/ml streptomycin. The cells were grown in an air:CO_2 atmosphere (95:5) at 37° for 72 hr. The medium was replaced with fresh medium containing 10^{-7} M [^{3}H]MTX with and without leucovorin. Influx of MTX and MTX polyglutamate formation was measured at various times up to 48 hr. At each time point the medium containing the tritiated drug was removed and the monolayer washed twice with an aliquot (3 ml) of ice-cold phosphate-buffered saline, pH 7.4 and once with an aliquot of 0.15 M phosphate buffer, pH 7.4. Efflux of the drug from the cells and the polyglutamate distribution of the retained drug was measured by preloading the cells for 48 hr with medium containing 10^{-7} M [^{3}H]MTX. The monolayer was washed 3 times with sterile, ice-cold phosphate-buffered saline, pH 7.4. Drug-free medium (3 ml), with and without leucovorin, was added. Efflux of MTX from the cells was followed for periods of up to 24 hr.

Measurement of intracellular drug levels. The cell monolayer was dissolved in 1.0 M NaOH (1.0 ml). Radioactivity in an aliquot (500 μl) of the resulting solution was determined using a Rackbeta 1217 liquid-scintillation spectrometer. The remainder of the solution was assayed for protein content [23] using human serum albumin to prepare the standard solutions. Results were expressed as fmoles MTX/mg cell protein.

Estimation of poly-γ-glutamate formation. The cell monolayer was suspended in 0.15 M phosphate buffer, pH 7.4 (300 μl) and transferred to a tapered centrifuge tube which was placed in a boiling-water bath for 10 min to inactivate intracellular peptidases. The cell debris was pelleted by centrifugation at 10,000 *g* for 10 min and the supernatant was stored at −20° until required.

Methotrexate and MTX polyglutamates were separated using a paired-ion HPLC system (Waters Associates). The mobile phase was prepared by mixing the effluents of a model M-45 and a model 6000A pump under the direction of a model 720 system controller. Samples were injected onto a μ-Bondapak C_{18} reverse-phase column (0.39 × 30 cm) using a model U6K injector. Solvent A consisted of 10 mM potassium dihydrogen phosphate, pH 7.0 containing 10 mM tetrabutylammonium hydrogen sulphate and solvent B consisted of methanol. The column was irrigated at a flow rate of 1.5 ml min with 25–32% methanol over 10 min and 32–42% methanol over 20 min. The retention times of MTX and MTX polyglutamates were determined by measuring the absorbance of the effluent at 254 nm using a model 441 detector. Column effluent was collected into disposable scintillation-vial inserts using an LKB 2112 Redirac fraction collector and radioactivity determined as described earlier.

Estimation of dihydrofolate reductase levels. Approximately 5×10^6 cells were plated onto tissue-culture flasks (75 cm^2) and cultured for 3 days as described above. The cells were then exposed to medium containing 10^{-7} M [^{3}H]MTX for 48 hr and then harvested and suspended in 0.1 M potassium phosphate buffer, pH 6.0 (2.0 ml). Following cell disruption by three cycles of freezing and thawing, an aliquot (200 μl) was removed for determination of total radioactivity and cell protein as described above. The remainder was centrifuged at 10,000 *g* for 1 hr at 4° to pellet the cell debris. An aliquot (500 μl) of the supernatant was applied to a calibrated column of Bio-Gel P30 (0.9 × 80 cm). The column was irrigated with 0.1 M potassium phosphate buffer, pH 6.0 at a flow rate of 10 min/ml and radioactivity determined in effluent fractions (1.0 ml). Levels of DHFR were expressed as pmoles DHFR mg/cell protein.

Binding of methotrexate polyglutamates to dehydrofolate reductase. An extract of MCF-7 cells containing MTX and MTX polyglutamates and 1 μM NADPH was incubated with bovine liver DHFR (3 μg) before being subjected to gel filtration on a Bio-Gel P30 column as described above with the exception that the irrigating buffer contained 0.1 mg/ml bovine serum albumin and 1.0 μM NADPH. The distribution of the radioactive MTX amongst its polyglutamyl forms was measured by HPLC as described above.

RESULTS

When cell extracts containing MTX polyglutamates were subjected to analysis using HPLC, peaks of radioactivity corresponding to authentic polyglutamate standards (MTX–MTXG5, which contains five additional γ-glutamyl residues) were detected. However, further peaks of radioactivity were detected which eluted from the column after MTXG5. These were provisionally designated MTXG6–MTXG8 on the basis of the relationship established by Cashmore *et al.* [24] between the logarithm of the adjusted retention time (t_r') and the

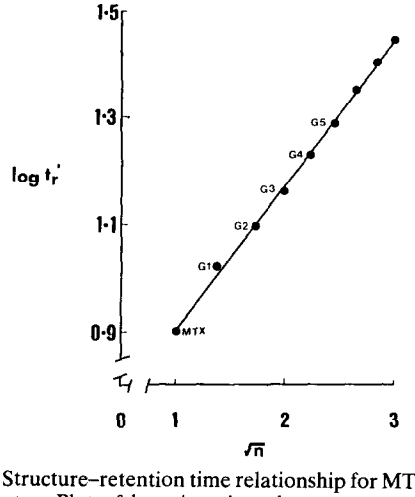

Fig. 1. Structure–retention time relationship for MTX polyglutamates. Plot of log t_r' against the square root of the total number of glutamate residues (N) for MTX for MTXG5 (N = 1–6, respectively). The function t_r' is given by the equation $t_r' = t_r - t_0$; where t_r and t_0 are the retention times of a polyglutamate species and the void volume of the column, respectively. Species eluting beyond MTXG5 are provisionally designated MTXG6–MTXG8.

Table 1. The percentage of radioactivity present in each of the polyglutamate forms was determined using HPLC

Species	Percentage of radioactivity
MTX	29.9
MTXG1	8.6
MTXG2	2.1
MTXG3	7.0
MTXG4	15.9
MTXG5	6.0
MTXG6	6.0
MTXG7	10.4
MTXG8	14.1
% of radioactivity eluting with DHFR	95.1
% of radioactivity eluting with MTX	4.9

The percentage of the radioactivity which could bind to DHFR was determined using gel filtration on a Bio-Gel P30 column (0.9 × 80 cm) and determining radioactivity in the column effluent. The values for polyglutamate composition are subject to an average error of about 7%.

square root of the total number of glutamate residues (Fig. 1). The correlation coefficient of regression for this relationship (0.999) is comparable with the value of 0.995 obtained by Jolivet *et al.* [25]. Table 1 shows that when cell extracts containing substantial amounts of the putative high-molecular-weight polyglutamates were incubated with DHFR and subjected to gel filtration on Bio-Gel P30 more than 95% of the radioactivity detected in the column effluent was associated with DHFR. The DHFR content of the MCF-7 cell line was determined to be 2.3 ± 0.2 pmoles/mg cell protein (mean ± S.D. of 3 determinations).

Table 2 shows that leucovorin at concentrations greater than that of MTX (10^{-7} M) caused a decrease in the 48 hr intracellular drug level, irrespective of whether the rescue agent was administered with MTX or delayed by 24 hr. In addition, leucovorin (10^{-6} M) decreased the intracellular drug level at all times during a 48-hr incubation with 10^{-7} M MTX (Fig. 2, $P < 0.01$). Leucovorin (10^{-9} and 10^{-8} M) exerted a marked effect on the synthesis of MTX polyglutamates in comparison with control values, when cells were exposed simultaneously to MTX and leucovorin (Fig. 3a). These concentrations of leucovorin were sufficient to cause a shift in the distribution of the intracellular drug toward the lower molecular weight species. When leucovorin (in at least equimolar concentrations) was presented to the cells, little polyglutamate synthesis was observed.

Cells treated only with MTX (10^{-7} M) synthesized substantial amounts of MTX polyglutamates (Fig. 4a). The intracellular levels of MTXGl and MTXG2 rose at first and then declined as the proportion of the higher molecular weight species increased. However, at all times the single most abundant species was the parent drug. In marked contrast, cells treated simultaneously with MTX (10^{-7} M) and leucovorin (10^{-6} M) showed virtually no polyglutamate synthesis (Fig. 4b). Approximately 90% of the intracellular drug was in the form of MTX at all times during the 48 hr incubation.

Since MTX and leucovorin are rarely co-administered in the clinic, the effect of delayed administration of leucovorin was investigated (Fig. 3b). At leucovorin concentrations less than that of MTX, little effect on polyglutamate synthesis was observed, although there was a slight increase in the contribution of MTX to the total intracellular drug level. Equimolar MTX and leucovorin resulted in an

Table 2. Effect of leucovorin (10^{-9}–10^{-5} M) on the intracellular level of MTX achieved after a 48-hr incubation with 10^{-7} M MTX

Leucovorin concentration (M)	Intracellular drug concentration (fmoles MTX/mg cell protein) Simultaneous MTX + leucovorin	Delayed administration of leucovorin
0	5789 ± 115	5789 ± 115
10^{-9}	5849 ± 78 ($P > 0.1$)	5701 ± 241 ($P > 0.1$)
10^{-8}	5689 ± 143 ($P > 0.1$)	5843 ± 187 ($P > 0.1$)
10^{-7}	5837 ± 68 ($P > 0.1$)	5634 ± 74 ($P > 0.1$)
10^{-6}	4978 ± 35 ($P < 0.001$)	5112 ± 95 ($P < 0.002$)
10^{-5}	4632 ± 115 ($P < 0.001$)	5125 ± 74 ($P < 0.002$)

Leucovorin was administered following 24 hr of a 48-hr exposure of MCF-7 cells to MTX (10^{-7} M). Values shown mean ± S.D. P values were assigned using Student's *t*-test

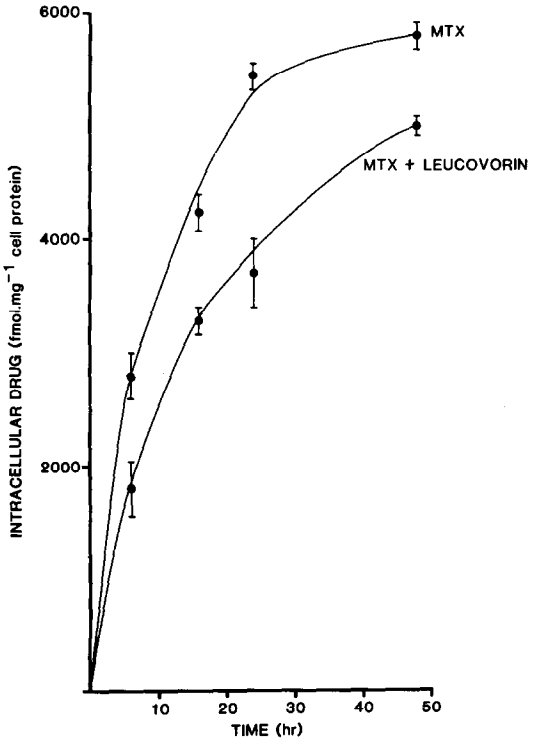

Fig. 2. Influx of total drug following exposure of MCF-7 cells to 10^{-7} M [^{3}H]MTX in the presence or absence of leucovorin (10^{-6} M). Mean ± S.D. of 3 determinations.

increase in the proportion of the lower molecular weight species ($G < 4$) at the expense of the higher molecular weight species. Marked effects on polyglutamate synthesis were observed at high concentrations of leucovorin (10^{-6} and 10^{-5} M) with the progressive abolition of the higher molecular weight species.

When MCF-7 cells were exposed to 10^{-7} M MTX for 48 hr before being transferred to drug-free medium with or without leucovorin (10^{-6} M), no difference in overall drug efflux was observed (Fig. 5). Similarly no pronounced difference in the polyglutamate distribution of the retained drug was observed during the efflux period (Fig. 6).

DISCUSSION

The data presented in Fig. 1 and Table 1 have permitted the tentative identification of peaks of radioactivity which elute from the HPLC column after MTXG5 as MTX polyglutamates, designated MTXG6–MTXG8. The retention times of the radioactive species are consistent with their being polyglutamates (Fig. 1) according to the relationship established by Cashmore *et al.* [24]. Jolivet *et al.*[25] have used this relationship to confirm the identity of polyglutamate species detected in extracts from the MCF-7 cell line. The correlation coefficient of regression obtained in this study (0.999, Fig. 1) is comparable with that previously reported [25]. Furthermore, when cell extracts containing substantial amounts of the species (>30% of total radioactivity)

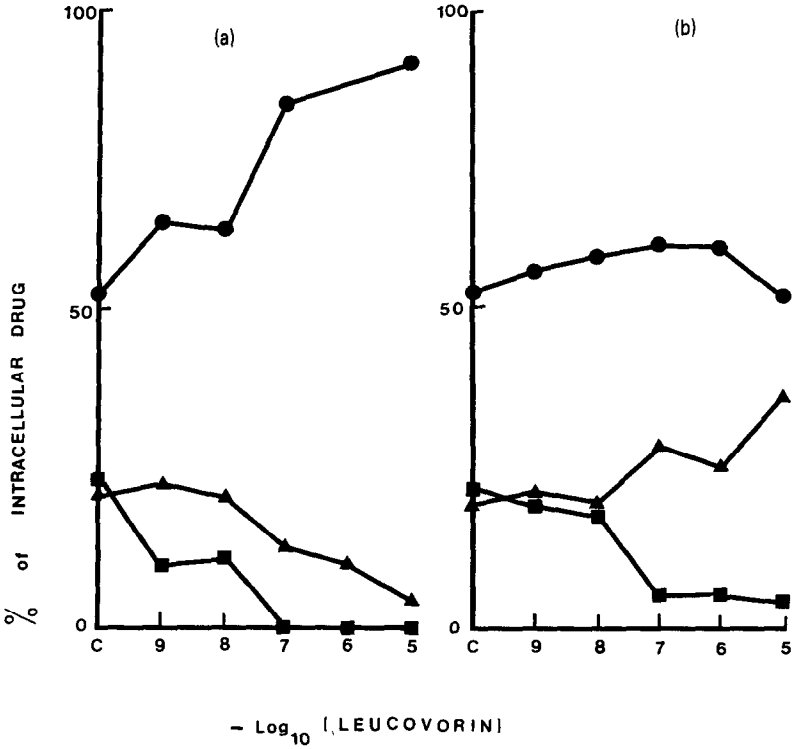

Fig. 3. The effect of leucovorin (10^{-9}–10^{-5} M) on the distribution of MTX amongst its polyglutamate forms. Cells were exposed to 10^{-7} M [^{3}H]MTX for 48 hr. The cells were exposed to leucovorin either at the start of the incubation period with MTX (a, left panel) or 24 hr after commencement of the incubation with MTX (b, right panel). Key: ●, MTX; ▲, MTXG1–G3; ■, MTXG4+.

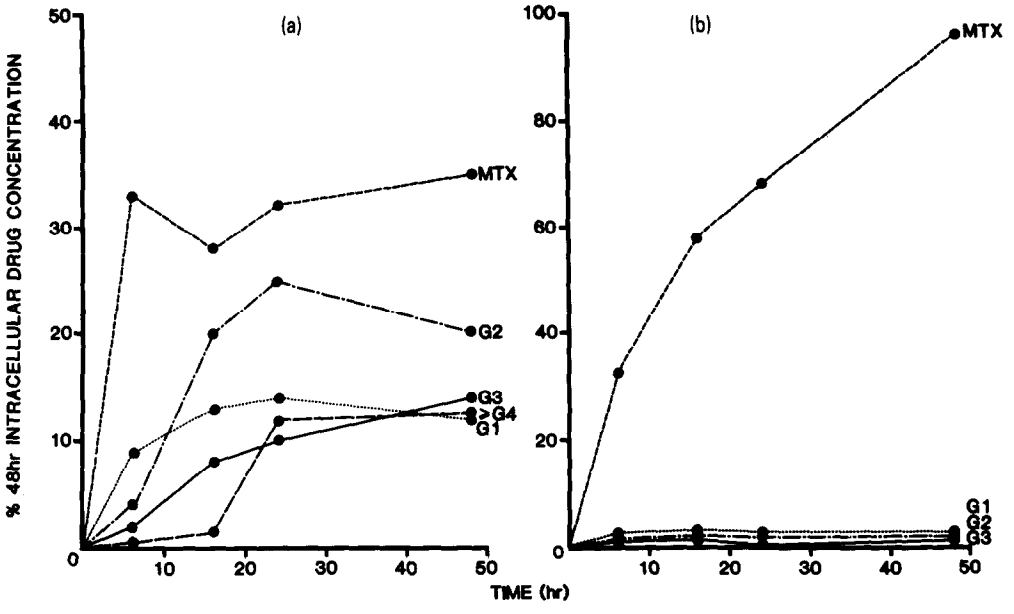

Fig. 4. Accumulation of intracellular MTX and polyglutamates following exposure of MCF-7 cells to 10^{-7} M [^{3}H]MTX in the absence (a, left panel) and presence (b, right panel) of 10^{-6} M leucovorin.

tentatively identified as MTXG6–MTXG8 were subjected to gel filtration using Bio-Gel P30 together with DHFR, over 95% of the radioactivity detected in the column effluent eluted at a position consistent with the radioactivity being bound to DHFR. Therefore it may reasonably be concluded that the peaks of radioactivity emerging from the HPLC column beyond MTXG5 are MTXG6–MTXG8.

The DHFR content of this cell line (2.3 ± 0.2 pmoles/mg cell protein) is in reasonable agreement with that (4 pmoles/mg cell protein) of Jolivet *et al.* [25]. The intracellular level of MTX exceeded that of DHFR following approximately a 10 hr incubation with 10^{-7} M MTX (Fig. 2). The intracellular level of MTX was still slightly in excess of that of DHFR following a 24 hr efflux period (Fig. 5).

Leucovorin, at concentrations equal to or greater than that of MTX, was able to affect the intracellular drug level following a 48 hr incubation with 10^{-7} M MTX (Table 2). However, leucovorin concentrations which were too low to affect the intracellular drug level could affect the polyglutamate distribution of the intracellular drug. When the cells were exposed to 10^{-9} and 10^{-8} M leucovorin simultaneously with MTX, the proportion of the intracellular drug present as MTX was increased (Fig. 3a). This is in contrast with the data of Rosenblatt *et al.* [21] who suggested that the reduced folates acted by competing with MTX for transport into the cell. In addition Fig. 3a suggests that leucovorin may compete with folylpolyglutamate synthetase, although it may also compete with MTX for transport into the cell at higher concentrations (Table 2). At leucovorin concentrations equal to or greater than that of MTX, which could affect the intracellular drug level (Table 2), profound effects on the synthesis of MTX polyglutamates were observed (Fig. 3a). No polyglutamates with chain lengths $G > 4$ were synthesized throughout the 48-hr incubation period (Figs 3a and 4).

When administration of the rescue agent was delayed by 24 hr, less marked effects on polyglutamate synthesis were observed. This is consistent with the observations of Galivan and Nimec [22] who showed that addition of folinic acid to rat hepatoma cells *in vitro* after MTX treatment resulted in a partial rescue that was concentration and time dependent. However, Fig. 3b shows that low concentrations of leucovorin (10^{-9} and 10^{-8} M) could

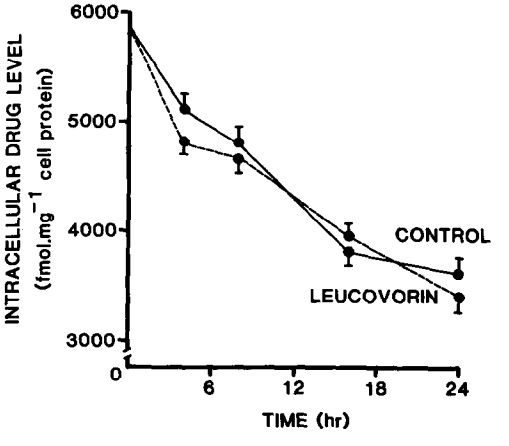

Fig. 5. Total drug efflux from MCF-7 cells following a 48-hr exposure to 10^{-7} M [^{3}H]MTX in the absence and presence of 10^{-6} M leucovorin. Mean ± S.D. of 3 determinations.

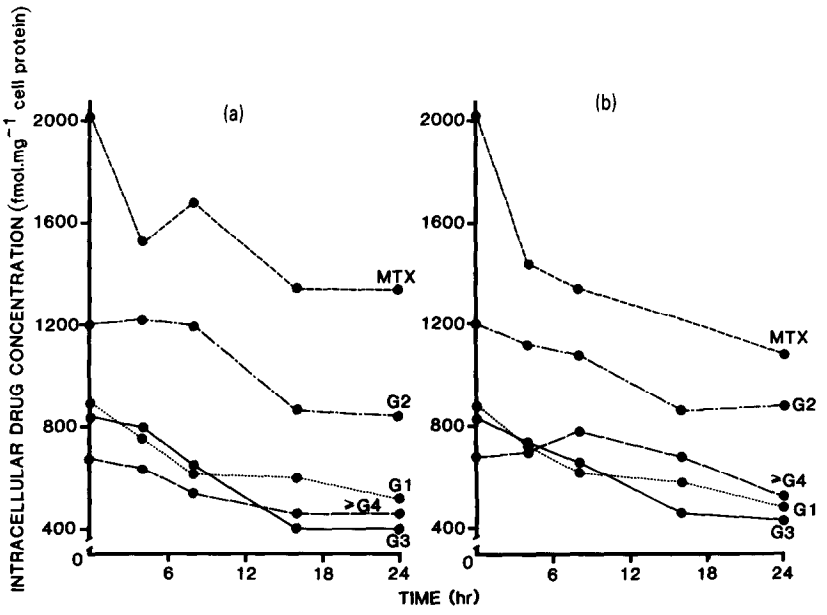

Fig. 6. Efflux of MTX and polyglutamates from MCF-7 cells following a 48-hr exposure to 10^{-7} M [^{3}H]-MTX in the absence (a, left panel) or presence (b, right panel) of leucovorin (10^{-6} M).

increase the contribution of MTX to total intracellular drug slightly, even when administration of the rescue agent was delayed by 24 hr. Higher concentrations of leucovorin (10^{-6} and 10^{-5} M), which could affect the intracellular drug level (Table 2), reduced polyglutamate synthesis more markedly (Fig. 3b) with a progressive elimination of the synthesis of the higher molecular weight polyglutamates ($G > 4$). The difference between the polyglutamate distribution of the intracellular drug following a 48-hr exposure to 10^{-7} M MTX shown in Figs 3a and 4a is caused by variations in initial cell density. A separate publication deals with this phenomenon [26].

Although leucovorin may compete with MTX for transport into this cell line (Table 2), it did not promote drug efflux from the cells (Fig. 5) nor did it markedly affect the polyglutamate distribution of the retained drug during efflux (Fig. 6). This contrasts with the data of Nimec and Galivan [22] who reported that leucovorin promoted the loss of MTX polyglutamates from the H-35 rat hepatoma cell line. During the first 4 hr of efflux only about 30% of the intracellular MTX was lost from the cells (Fig. 6). This contrasts with previous results from this laboratory [5] and results obtained by other workers [27], which showed that most of the intracellular MTX is lost after a few hours of efflux from cells preloaded with MTX polyglutamates, providing the intracellular drug level is in excess of that of DHFR. The data presented in Fig. 6 suggests that, given the absence of a rapid efflux of the parent drug, most of the MTX is DHFR-bound. Under such conditions a low rate of MTX efflux is to be expected, since the rate-limiting step in the efflux of MTX is the dissociation of the drug from DHFR. This, however, may not be the only explanation. Balinska *et al.* [28] exposed H-35 rat hepatoma cells to 10^{-5} M MTX for 16 hr when the intracellular drug level (48 μM) was in excess of that of DHFR (0.8 μM [14]). During a subsequent 6-hr efflux period, less than 40% of the parent drug was lost from the cells. At the end of the efflux period, the intracellular drug level was still in excess of the DHFR level. In this case the MTX cannot all be DHFR-bound since the intracellular level of the parent drug at the end of the efflux period was 5-fold that of DHFR.

During the 24-hr immediately preceding the efflux period, little change in the distribution of the intracellular drug amongst its polyglutamyl forms occurred (Fig. 4a). An electrochemical and chemical equilibrium had been established. Hence no further synthesis occurred during the efflux period (Fig. 6). This is in contrast with the data of Jolivet and Chabner [27] who demonstrated continuing synthesis of MTXG4 during the first 6 hr of efflux at the expense of MTX and the lower molecular weight polyglutamates. However, these authors used culture conditions (2×10^{-6} M MTX for 24 hr with MCF-7 cells) which did not permit an equilibrium to be achieved. During the 12 hr preceding the commencement of the efflux period, major changes in the intracellular polyglutamate pattern were observed [27].

In conclusion, the higher molecular weight polyglutamates which efflux from human breast cancer cells less readily than the lower molecular weight species [5] and which are at least as active as MTX, may contribute to both the acute and chronic toxic reactions to MTX. Leucovorin can prevent the accumulation of the higher molecular weight polyglutamates either when adminstered with, or more relevantly, after MTX. Furthermore, the effect of leucovorin on polyglutamate synthesis may be

observed even at concentrations of the rescue agent which are too low to affect the intracellular drug level. This study illustrates an additional mechanism whereby leucovorin may rescue cells from the toxic reactions associated with MTX.

Acknowledgements—The authors wish to thank Action Cancer (Northern Ireland) and the Medical Research Council (U.K.) for their generous financial support.

REFERENCES

1. J. J. McGuire and J. R. Bertino, *Molec. cell. Biochem.* **38**, 19 (1981).
2. V. M. Whitehead, *Cancer Res.* **37**, 408 (1977).
3. D. A. Gerwitz, J. C. White, J. K. Randolph and I. D. Goldman, *Cancer Res.* **39**, 2914 (1979).
4. D. S. Rosenblatt, V. M. Whitehead, N. Vera, A. Pottier, M. Dupont and M.-J. Vuchich, *Molec. Pharmac.* **14**, 1143 (1978).
5. D. G. Kennedy, R. Clarke, H. W. Van den Berg and R. F. Murphy, *Biochem. Pharmac.* **32**, 41 (1983).
6. R. G. Poser, F. M. Sirotnak and P. L. Chello, *Biochem. Pharmac.* **29**, 2701 (1980).
7. M. Balinska, Z. Nimec and J. Galivan, *Archs Biochem. Biophys.* **216**, 466 (1982).
8. J. R. Fountain, D. J. Hutchinson, G. B. Waring and J. H. Burchenal, *Proc. Soc. exp. Biol. Med.* **83**, 369 (1953).
9. S. Charache, P. T. Condit and S. R. Humphreys, *Cancer* **13**, 236 (1960).
10. S. A. Jacobs, C. J. Derr and D. G. Johns, *Biochem. Pharmac.* **26**, 2310 (1977).
11. H. E. M. Kay, P. J. Knapton, J. P. O'Sullivan, D. G. Wells, R. F. Harris, E. M. Innes, J. Stuart, F. C. M. Schwartz and E. N. Thompson, *Archs Dis. Child.* **47**, 344 (1972).
12. G. H. Millward-Sadler and T. J. Ryan, *Br. J. Derm.* **90**, 661 (1974).
13. R. L. Kaplan and D. H. Waite, *Archs Derm. Syph.* **114**, 1800 (1978).
14. J. Galivan, *Molec. Pharmac.* **17**, 105 (1980).
15. S. A. Jacobs, R. H. Adamson, B. A. Chabner, C. J. Derr and D. G. Johns, *Biochem. biophys. Res. Commun.* **63**, 692 (1975).
16. M. Chauvet, M. Bordeaux, C. Briand, M. Dell'Amico, R. Gilli and M. Diarra, *Biochem. Pharmac.* **32**, 1059 (1983).
17. H. M. Pinedo, D. S. Zaharko, J. M. Bull and B. A. Chabner, *Cancer Res.* **36**, 4418 (1976).
18. J. F. Bender, W. R. Grove and C. L. Fortner, *Am. J. Hosp. Pharmac.* **34**, 961 (1977).
19. P. F. Nixon and J. R. Bertino, *New Engl. J. Med.* **286**, 175 (1972).
20. F. M. Sirotnak and R. C. Donsbach, *Cancer Res.* **36**, 1151 (1976).
21. D. S. Rosenblatt, V. M. Whitehead, M.-J. Vuchich, A. Pottier, N. Vera-Matiaszuk and D. Beaulieu, *Molec. Pharmac.* **19**, 87 (1981).
22. J. Galivan and Z. Nimec, *Cancer Res.* **43**, 551 (1983).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
24. A. R. Cashmore, R. N. Dreyer, C. Horvath, J. O. Knipe, J. K. Coward and J. R. Bertino, *Meth. Enzym.* **66**, 459 (1980).
25. J. Jolivet, R. L. Schilsky, B. D. Bailey, J. C. Drake and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
26. D. G. Kennedy, H. W. Van den Berg, R. Clarke and R. F. Murphy, *Biochem. Pharmac.*, in press.
27. J. Jolivet and B. A. Chabner, *J. clin. Invest.* **72**, 773 (1983).
28. M. Balinska, J. Galivan and J. K. Coward, *Cancer Res.* **41**, 2751 (1981).