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ENHANCEMENT OF METHOTREXATE CYTOTOXICITY TOWARDS THE MDA.MB.436 HUMAN BREAST CANCER CELL LINE BY DIPYRIDAMOLE

THE ROLE OF METHOTREXATE POLYGLUTAMATES

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Abstract—MTX and dipyridamole are synergistic in their toxicity towards the MDA.MB.436 human breast cancer cell line. Dipyridamole increases net MTX uptake into the cells and increases the intracellular levels of MTXG7 to G10, the highest molecular weight polyglutamyl derivatives of MTX detected. During a recovery period, after completion of exposure to MTX with and without dipyridamole, levels of MTXG7 to G10 remained elevated in dipyridamole treated cells by comparison with controls. Dipyridamole, which has no intrinsic effect on cell growth, transforms a cytostatic response of MDA.MB.436 cells towards MTX into a cytotoxic response. The effect of dipyridamole is not mediated through an increase in prostacyclin biosynthesis.

The poly-γ-glutamyl derivatives of methotrexate (MTX), first detected in human lymphocytes by Baugh *et al.* [1], have now been observed in a wide variety of cell types [2–4] including human breast cancer [5–12]. The polyglutamates bind to dihydrofolate reductase (DHFR) with an equal affinity to MTX but show decreasing values of k_{off} (the dissociation rate constant) with increasing chain length [13]. Furthermore, the polyglutamates are retained by cells, their half life of retention increasing with increasing γ-glutamyl chain length [10]. Hence the synthesis of high molecular weight polyglutamates *in vitro* and *in vivo* may explain the persistence of MTX and the toxicity of the drug observed previously [14].

Dipyridamole (Persantin) is an anti-platelet drug and is indicated for use in cases of thromboembolism and angina. It acts by increasing prostacyclin biosynthesis and inhibition of cAMP phosphodiesterase [15]. It is also known to inhibit the active transport systems of nucleosides [16]. Furthermore, the action of avicin, an inhibitor of *de novo* nucleotide biosynthesis is potentiated by dipyridamole *in vitro* [17].

Two recent reports by Cabral *et al.* [18] and Nelson and Drake [19] have shown that dipyridamole may potentiate the cytotoxicity of MTX *in vitro* and *in vivo*. Cabral and co-workers reported that dipyridamole inhibited cytidine and thymidine uptake by Sarcoma-180 cells. They also observed that 10^{-5} M-dipyridamole enhanced MTX accumulation and diminished MTX efflux in these cells [18]. Nelson and Drake reported that dipyridamole potentiated MTX toxicity against CHO cells *in vitro* but failed to enhance significantly the toxicity of MTX against Ridgeway osteogenic sarcoma and L1210 cells which had been implanted into AKR and $BD2F_1$ mice, respectively [19].

The present study was initiated to investigate the interactions between MTX and dipyridamole in a human breast cancer cell line (MDA-MB-436). The influence of dipyridamole on the formation and retention of MTX polyglutamates and on the drug-induced growth delays was studied.

MATERIALS AND METHODS

3′,5′,7-[^{3}H] Methotrexate (TRK 224; specific activity 11.9 Ci/mmole) was purchased from Amersham International PLC (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. MTX polyglutamate standards were purchased from Dr. C. M. Baugh (University of South Alabama, Mobile, AL, U.S.A.). The MDA-MB-436 cell line, the characteristics of which have previously been described [20], were obtained from Flow Laboratories Ltd. (Irvine, Scotland). Foetal calf serum was supplied by Randox Laboratories (Crumlin, U.K.) and was dialysed extensively against physiological saline before use. Dipyridamole, indomethacin and arachidonic acid were obtained from the Sigma Chemical Corporation.

Cell culture conditions. Cells (5.10^3 cm^{-1}) were plated onto petri dishes (20 cm^2 surface area, Sterilin Ltd.) in Eagles Minimal Essential Medium supplemented with Earles salts, 5% dialysed serum, penicillin (100 i.u./ml) and streptomycin (100 μg/ml). The cells were grown for 72 hr in an air:CO_2 (95:5) atmosphere at 37° and were then exposed to

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fresh medium containing 10^{-7} M-[^{3}H]-MTX with or without dipyridamole, indomethacin or arachidonic acid, as appropriate.

Following exposure of the cells to the test media for 48 hr the cell monolayer was washed twice with ice-cold isotonic phosphate buffered saline pH 7.4 (3.0 ml) and once with ice-cold 0.15 M sodium phosphate, pH 7.4 (3.0 ml).

The measurement of intracellular drug levels, dihydrofolate reductase levels and extraction of MTX polyglutamates from the cell monolayers was accomplished as described previously [11]. Cells for growth delay experiments were plated onto 24-well multiwell dishes (Flow Laboratories, FB-16-24-TC) at a density of 5.10^3 cells/cm^2 in medium as described above. Following a 72 hr incubation, cell number was determined electronically using a Coulter Counter (Model ZBl, Coulter Electronics, England). The cells were then exposed to fresh medium containing 10^{-7} M-MTX and/or 10^{-5} M-dipyridamole, as appropriate. After a 48 hr exposure to the test media, the cell number was determined and the medium replaced with drug free medium. Thereafter, the cells were re-fed and cell number determined every three days for 18 days.

High pressure liquid chromatography. MTX and MTX polyglutamates were separated using a paired-ion HPLC system (Waters Associates). The mobile phase was prepared by mixing the effluents of two model 580 pumps under the direction of a model 680 Automated Gradient Controller as described earlier [11]. Column effluent was collected as 27 drop fractions into disposable scintillation vial inserts using an LKB 2112 Redirac fraction collector.

Scintillation fluid (4 ml of 0.2% PPO in toluene:Triton X-100 2:1 (v/v)) was added and radioactivity determined using a Rackbeta 1217 liquid scintillation spectrometer. Recovery of radioactivity from the HPLC column was 92.0 + 8.9% (mean ± SEM).

RESULTS

The MDA-MB-436 cell line can synthesize poly-γ-glutamyl derivatives of MTX with up to 10 additional γ-glutamyl residues (MTXG10) as described earlier [12].

Dipyridamole (10^{-8}–10^{-6} M) failed to affect the intracellular level of MTX following co-exposure of the cells to dipyridamole and [^{3}H]-MTX for 48 hr (6.75 ± 0.39 pmoles/mg cell protein). However, 10^{-5} M dipyridamole increased that intracellular level of MTX to 9.01 ± 0.44 pmoles/mg cell protein, equivalent to an increase in the free intracellular drug level of about 40%. (The DHFR level is 0.91 ± 0.10 pmoles/mg cell protein [12].) This concentration of dipyridamole produced a 56% increase in the amount of MTXG7 to MTXG10 present in the cells at the end of the incubation period (Fig. 1). A smaller increase (34%) in the amount of MTXG1 to MTXG3 was also observed. At lower concentrations of dipyridamole (10^{-8}–10^{-6} M) there were no significant alterations in the intracellular amounts of MTX to MTXG6. However, there were slight increases in the levels of the highest molecular weight polyglutamates. At 10^{-4} M, dipyridamole caused cell death during the 48 hr exposure to the drug combination (data not shown).

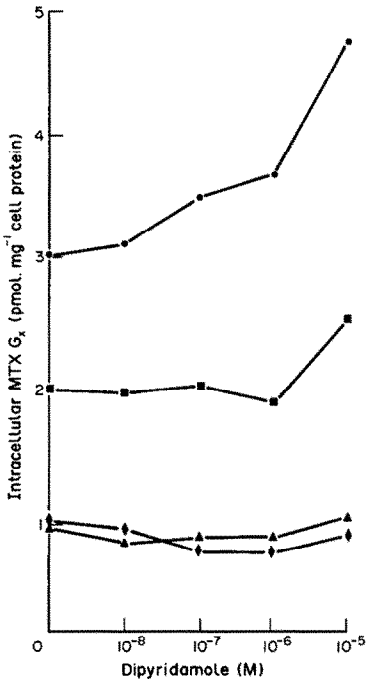

Fig. 1. The effect of dipyridamole on intracellular polyglutamate formation. MDA.MB.436 cells were cultured for 72 hr before being exposed to 10^{-7} M-[^{3}H]-MTX for 48 hr in the presence and absence of dipyridamole (10^{-8}–10^{-5} M). The values represent a mean from two experiments and are subject to an average error of less than 7%. Key: MTX (◆); MTXG1 to G3 (■); MTXG4 to G6 (▲); MTXG7 to G10 (●).

Cells exposed to 10^{-7} M [^{3}H]-MTX with and without 10^{-5} M-dipyridamole for 48 hr were transferred to drug-free medium for a 24 hr efflux period. The dipyridamole treated cells retained more MTX than the control cells throughout the efflux period ($P < 0.05$, Student's t-test; data not shown). The polyglutamate pattern of the retained drug is shown in Fig. 2. MTX to MTXG6 decreased to similar levels in the treated and control cells by the end of the efflux period. However, MTXG7 to MTXG10 levels in dipyridamole treated cells were still 40% in excess of those in the control cells at the end of the efflux period. Lower concentrations of dipyridamole (10^{-8}–10^{-6} M) failed to affect ($P > 0.1$, Student's t-test) either the amount of drug retained after the 24 hr efflux period (3.84 ± 0.10 pmoles/mg cell protein) or the polyglutamate distribution of the retained drug (Fig. 3).

The effect on cell number of a 48 hr exposure of MDA-MB-436 cells to MTX (10^{-7} M) and dipyridamole (10^{-5} M) separately and together is shown in Fig. 4. Dipyridamole-treated cells were unaffected by the drug and grew at the same rate as control cells. MTX, under the conditions employed, was cytostatic, rather than cytotoxic, with no change in cell number being observed for five days after the start of the treatment. In marked contrast, however,

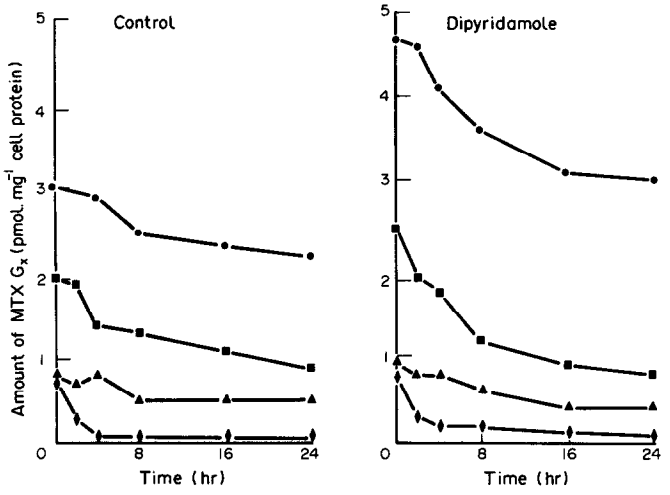

Fig. 2. MTX polyglutamate content during efflux of MTX from MDA.MB.436 cells preloaded with MTX in the presence and absence of dipyridamole. MDA.MB.436 cells were cultured for 72 hr before being exposed to 10^{-7} M-[^{3}H]-MTX alone or with 10^{-5} M dipyridamole for 48 hr. The cells were transferred to a drug-free medium and intracellular polyglutamate formation assessed at various times during a 24 hr recovery period. Values represent a mean from 2 experiments and are subject to an average error of less than 7%. Key: MTX (◆); MTXG1 to G3 (■); MTXG4 to G6 (▲); MTXG7 to G10 (●).

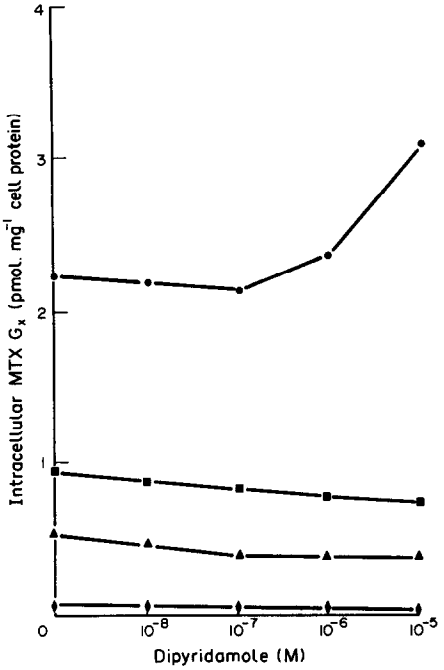

Fig. 3. The effect of dipyridamole on MTX polyglutamate retention by MDA.MB.436 cells following a 24 hr recovery period. MDA.MB.436 cells were cultured for 72 hr before being exposed to 10^{-7} M-[^{3}H]-MTX and dipyridamole (0 and 10^{-8}–10^{-5} M) for 48 hr. The cells were transferred to drug free medium and intracellular polyglutamates assessed at the end of a 24 hr recovery period. Values represent a mean from two experiments and are subject to an average error of less than 7%. Key: MTX (◆); MTXG1 to G3 (■); MTXG4 to G6 (▲); MTXG7 to G10 (●).

the number of dipyridamole plus MTX-treated cells fell by 67% during the same period.

Neither arachidonic acid (10^{-10}–10^{-7} M), a precursor of prostacyclin, nor indomethacin (10^{-9}–10^{-5} M), an inhibitor of fatty acid cyclooxygenase, affected either the intracellular level of MTX or the polyglutamate distribution of the intracellular drug in the absence and presence of 10^{-5} M dipyridamole (data not shown).

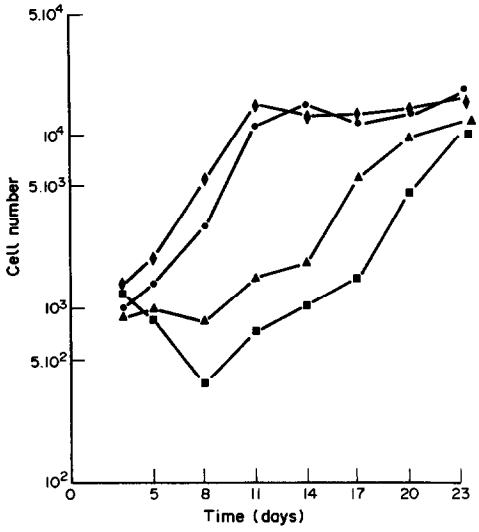

Fig. 4. The effect of MTX plus dipyridamole in combination on cell growth. MDA.MB.436 cells were cultured for 72 hr before being exposed to drug free medium (◆), 10^{-7} M MTX (▲), 10^{-5} M dipyridamole (●) and 10^{-7} M MTX plus 10^{-5} M dipyridamole (■) for 48 hr. Cell number was determined and the cells transferred to drug free medium. Cell number was determined and the cells re-fed every 3 days for 18 days. Values represent a mean from quadruplicate wells and are subject to an average error of less than 10%.

DISCUSSION

The synthesis of poly-γ-glutamyl derivatives of MTX, with up to 10 additional γ-glutamyl residues [11, 12] by the MDA.MB.436 human breast cancer cell line is highly dependent on the rate of cell proliferation [12]. The metabolites can bind to DHFR [11] and are highly retained by the cells since retention half life increases with the γ-glutamyl chain length [10].

The present study illustrates that dipyridamole can alter the intracellular kinetics of MTX and its poly-γ-glutamyl derivatives. The intracellular level of MTX was increased by 33% when cells were co-cultured with dipyridamole (10^{-5} M) and MTX (10^{-7} M) for 48 hr. This increase was almost entirely accounted for by a 56% increase in the intracellular levels of MTXG7 to G10 (Fig. 1).

Throughout a 24 hr recovery period the increase in MTXG7 to G10 was maintained in dipyridamole treated cells (Fig. 2). Hence total intracellular drug levels in dipyridamole-treated cells exceeded those in control cells at all times during the recovery period (data not shown). Lower concentrations of dipyridamole (10^{-8}–10^{-6} M) failed to exert a significant effect on drug influx and efflux or on polyglutamate synthesis (Fig. 1) or retention (Fig. 2). This is in agreement with Cabral *et al.* [18], who observed that the enhancement of MTX accumulation in Sarcoma 180 cells was optimal at a dipyridamole concentration of 10^{-5} M.

That the interaction between MTX and dipyridamole is synergistic, is shown by Fig. 4. Dipyridamole alone failed to affect cell growth, but, in combination with MTX, transformed the cytostatic effect of MTX into a cytotoxic effect. A marked 67% reduction in cell number was observed in the five days after the start of exposure to the drug combination, as opposed to a maintenance of cell number during the same period following exposure of the cells to MTX alone.

The present study confirms the observations of Cabral *et al.* [18] and of Nelson and Drake [19] that dipyridamole can increase the cytotoxicity of MTX and that this correlates with an increase in intracellular drug levels [18]. However our results suggest an additional mechanism by which this effect is mediated. Not only are the intracellular drug levels increased, but the synthesis of the highest molecular weight polyglutamates (G7 to G10) is also increased. This may explain the decreased rate of loss of drug from cells observed previously [18]. However, the conditions used in that study (influx and efflux periods of less than 1 hr [18]) do not favour extensive polyglutamate formation. Accordingly, their results may have an alternative explanation. The mechanism by which dipyridamole increases intracellular drug levels and modulates polyglutamate biosynthesis in unclear. However, it is unlikely to be mediated by an increased rate of biosynthesis of prostacyclin, since arachidonic acid and indomethacin did not affect either the uptake of MTX or the biosynthesis of MTX polyglutamates (data not shown). Further studies are required to determine the precise mechanism of dipyridamole-induced changes in MTX transport and metabolism.

REFERENCES

1. C. M. Baugh, C. L. Krumdieck and M. G. Nair, *Biochem. biophys. Res. Commun.* **52**, 27 (1973).
2. V. M. Whitehead, M. M. Perrault and S. Stelcner, *Cancer Res.* **35**, 2985 (1975).
3. V. M. Whitehead, *Cancer Res.* **37**, 408 (1977).
4. D. S. Rosenblatt, V. M. Whitehead, M. M. Dupont, M. J. Vuchich and N. Vera, *Molec. Pharmac.* **14**, 210 (1978).
5. R. L. Schilsky, B. D. Bailey and B. A. Chabner, *Proc. natn Acad. Sci. U.S.A.* **77**, 2919 (1980).
6. J. Jolivet and R. L. Schilsky, *Biochem. Pharmac.* **30**, 1387 (1981).
7. J. Jolivet, R. L. Schilsky, B. D. Bailey, J. C. Drake and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
8. J. Jolivet and B. A. Chabner, *J. clin. Invest.* **72**, 773 (1983).
9. K. H. Cowan and J. Jolivet, *J. biol. Chem.* **259**, 10793 (1984).
10. D. G. Kennedy, R. Clarke, H. W. van den Berg and R. F. Murphy, *Biochem. Pharmac.* **32**, 41 (1983).
11. D. G. Kennedy, H. W. van den Berg, R. Clarke and R. F. Murphy, *Biochem. Pharmac.* **34**, 2897 (1985).
12. D. G. Kennedy, H. W. van den Berg, R. Clarke and R. F. Murphy, *Biochem. Pharmac.* **34**, 3087 (1985).
13. N. J. Clendeninn, K. H. Cowan, B. T. Kaufman, M. V. Nadkarni and B. A. Chabner, *Proc. Am. Ass. Cancer Res.* **240**, 276 (1983).
14. J. Jolivet, K. H. Cowan, G. A. Curt, N. J. Clendeninn and B. A. Chabner, *New Engl. J. Med.* **309**, 1094 (1983).
15. L. A. Harker and R. A. Kadatz, *Thromb. Res. Suppl.* **IV**, 39, (1983).
16. G. Weber, *Cancer Res.* **43**, 3466 (1983).
17. P. H. Fischer, R. Pamukcu, G. Bittner and J. K. V. Willson, *Cancer Res.* **44**, 3355 (1984).
18. S. Cabral, S. Leis, L. Bover, M. Nembrot and J. Mordoh, *Proc. natn Acad. Sci. U.S.A.* **81**, 3200 (1984).
19. J. A. Nelson and S. Drake, *Cancer Res.* **44**, 2493 (1984).
20. R. Clarke, N. W. van den Berg, D. G. Kennedy and R. F. Murphy, *Eur. J. Cancer clin. Oncol.* **19**, 19 (1983).