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The Intracellular Mechanism of Action of Metoprine (DDMP)*

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Abstract—*Cultured L1210 cells were exposed to equally toxic concentrations of methotrexate (MTX) and DDMP [2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine]. Protection of the cells against the toxic effects of both agents could be achieved with either folinic acid or with a purine/pyrimidine combination. The effects of either drug on the incorporation of radiolabelled precursors (thymidine or deoxyuridine) into acid precipitable material were similar. A MTX resistant subline with an elevated dihydrofolate reductase activity was also resistant to DDMP. DDMP was an effective inhibitor of purified dihydrofolate reductase (EC 1.5.1.4) (Ki = 71 pM) though at 100 μM it did not inhibit thymidylate synthetase (EC 2.1.1.45), serine hydroxymethyl transferase (EC 2.1.2.1), methionine synthetase (EC 2.1.1.13) or formyl tetrahydrofolate sythetase (EC 6.3.4.3). These studies suggest that, at the concentrations used, the intracellular locus of DDMP is the same as that of MTX. It is proposed that the observed biological differences between MTX and DDMP are due to the lipophilic nature of the latter giving rise to unique cell membrane transport and pharmacokinetic properties.*

INTRODUCTION

2,4-DIAMINO-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (metoprine, DDMP) is one of several diaminopyrimidines which inhibit the enzyme dihydrofolate reductase (EC 1.5.1.4, DHFR) [1]. It has been found to have antitumour activity in experimental systems [2, 3] and has undergone several clinical trials [4–8] in which antitumour activity has been observed. In an early study performed by Murphy *et al.* [4] DDMP was administered as a single agent without folinic acid. Major toxicities to the bone marrow, gastrointestinal tract and skin, which were reversible with folinic acid, were observed. Although its toxic side effects on the bone marrow and gastrointestinal tract may be prevented by the administration of folinic acid and the antitumour effect in animals has been similarly reversed in some systems [3, 16], the concurrent administration of folinic acid to patients treated with DDMP has sometimes been associated with an antitumour response [5–8]. This phenomenon has been attributed to differential transport of DDMP and folinic acid into normal and tumour tissues, on the basis of studies with cultured L5178Y cells resistant to methotrexate (MTX) by virtue of a transport defect [9–12]. There is however no definite data relating to the transport of DDMP into normal and tumour tissues in man. The major pharmacokinetic differences between DDMP and MTX are the longer plasma half-life of DDMP and its lipophilic character [13, 14]. This latter property makes DDMP a potentially useful clinical agent since it crosses the blood–brain barrier [13, 15]. Although the antifolate property of DDMP is well known, and its systemic toxicity may be reversed by the administration of reduced folates [5, 7, 14, 16], published data suggests that its inhibitory effect on the target enzyme DHFR is vastly inferior to that of MTX [9]. The possibility that DDMP has a second site of action which is responsible for its antitumour effects was therefore explored. The properties of DDMP in tissue culture were compared with those of MTX in the following ways:

(a) Comparison of folinic acid and purine/pyrimidine protection from DDMP and MTX.

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(b) Comparison of the effects of equally toxic doses of DDMP and MTX on nucleic acid synthesis as reflected by the incorporation of radiolabelled precursors.

(c) Comparison of the dose–response curves of MTX and DDMP for L1210 cells and a resistant subline (L1210/R71) with an increased DHFR content [17].

The inhibition kinetics of DDMP and MTX for purified L1210 DHFR were also investigated, and we explored the possibility that DDMP might inhibit several other folate metabolising enzymes, namely thymidylate synthetase (EC 2.1.1.45), serine hydroxymethyl transferase (EC 2.1.2.1), methionine synthetase (EC 2.1.1.13) or formyl tetrahydrofolate synthetase (EC 6.3.4.3).

MATERIALS AND METHODS

L1210, L1210/R71 and L5178Y murine leukaemia cells were maintained in logarithmic growth in RPMI 1640 with 30 mM Hepes buffer (Flow Laboratories), supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Under these conditions the doubling times of the L1210 lines were 15 hr and for the L5178Y line 17 hr.

MTX and folinic acid (Lederle Laboratories, Gosport, Hants, England), DDMP (Burroughs Wellcome, Beckenham, Kent, England), and nucleosides and bases (Sigma Chemical Co., London, England) were serially diluted in unsupplemented medium which was sterilised by millipore filtration and added to cultures in 1:100 dilution to obtain the desired final concentrations. DDMP was initially dissolved in two molar equivalents of lactic acid, and nucleic acid bases in NaOH: the pH was adjusted and dilutions made in unsupplemented medium prior to addition to the cultures. In protection experiments, agents were added at the same time as the drug. All experiments included control cultures containing drug alone or protection agents alone. At the appropriate time, after addition of the drug and/or protection agents, cells were counted on a Coulter Model F and results expressed as cell density or as percentage of control culture containing no additives. The ID_{50} value of the dose–response curves corresponds to that concentration of drug which doubles the generation time of the population of cells, and is calculated according to the formula in Fig. 1 where *Co* is the initial cell density and *Ct* is the cell density in untreated control cultures at the time the cells are counted. The ID_{50} is that concentration of drug corresponding to the *P* value obtained by using the formula indicated in Fig. 1. A full discussion of this concept is provided by Jackson *et al.* [18].

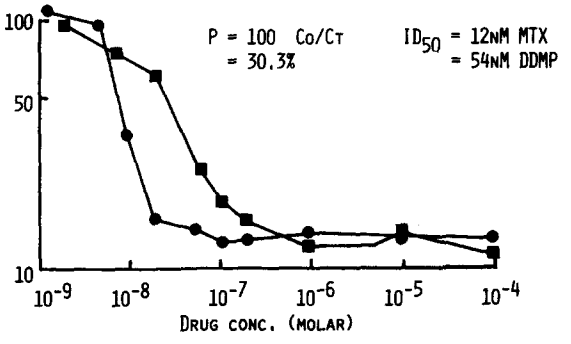

Fig. 1. Dose–response curves of L1210/S to methotrexate (●) and DDMP (■). The formula P = 100 Co/Ct *is used to calculate the concentration of drug which halves the growth rate* (ID_{50}). *See text for details.*

Incorporation of radiolabelled precursors into acid precipitable material was measured by incubation of cells in medium at approximately 2×10^5/ml with the appropriate compound at a concentration of 1 μCi/ml, 56 nM for 30 min. Triplicate 1 ml aliquots of the cell suspension were then removed, placed on ice and mixed with 1 ml of ice cold saline. This mixture was then filtered on a Whatmans GF/C glass fibre disc attached to a vacuum pump. The residue on the disc was washed sequentially with 5 ml of ice cold saline, 10% trichloracetic acid and distilled water. The discs were then dried and counted in PCS scintillant (Hopkin and Williams). In all cell culture experiments, independent triplicate determinations of each point were made.

The inhibition constants (*Ki*) for MTX and DDMP were determined using L1210 DHFR purified by affinity chromatography as described by Jackson *et al.* [19]. In each case the values for the *Ki* were calculated as described by Jackson *et al.* [19] except that the data were processed using the non-linear least squares regression program BM DX 85 [20]. On each occasion the spectrophotometric assay was observed for a sufficiently long period to ensure that the steady state rate was achieved, in order to eliminate effects due to the relative rates of binding of substrate and of inhibitor. Significant depletion of substrates did not occur during this observation period. The methods used for the other enzymes were as follows: thymidylate synthetase, Roberts

[21]; serine hydroxymethyl transferase, Scrimgeour and Huennekens [22]; methionine synthetase, Weissbach *et al.* [23]; and formyl tetrahydrofolate synthetase, Rabinowitz and Pricer [24].

RESULTS

Comparison of folinic acid and purine/pyrimidine protection

The dose–response curves of MTX and DDMP against L1210 cells are shown in Fig. 1. The ID_{50} of MTX was 12 nM, while that of DDMP was 54 nM. In subsequent protection experiments DDMP and MTX were used in the ratio of their ID_{50}'s so that protection from equitoxic levels was being assessed. Figure 2 shows the protective effect

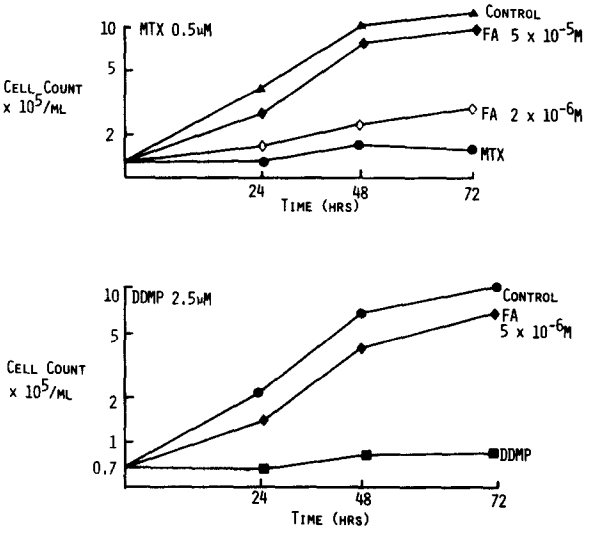

Fig. 2. Protection by folinic acid (FA) of the cytotoxic effects for MTX and DDMP on L1210/S over 72 hr.

of folinic acid on L1210 cells treated with toxic doses of MTX and DDMP. Folinic acid protected from DDMP more readily than from MTX, the concentrations required to achieve a growth rate comparable with that of the control being 5 μM for cells treated with 2.5 μM DDMP and 50 μM for cells treated with 0.5 μM MTX. This effect was explored further and is illustrated in Fig. 3. As the folinic acid concentration is increased beyond a critical level, there is a proportionately greater protective effect from DDMP toxicity. Protection from MTX is not conferred until a greater critical concentration ratio is reached, beyond which point protection increased with increasing folinic acid concentration. The vertical lines indicate the protective effect of equimolar concentrations of folinic acid.

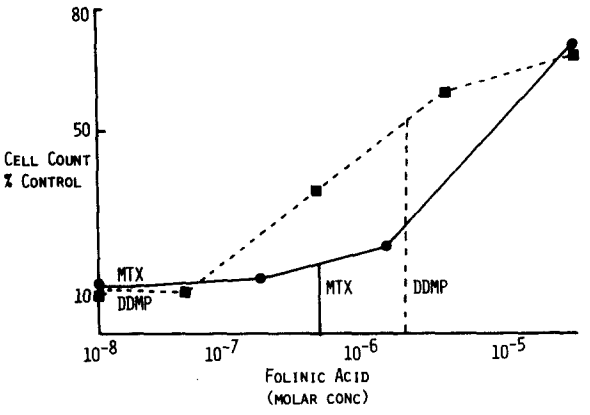

Fig. 3. Relationship of the molar concentration of folinic acid required to protect L1210/S cells from MTX and DDMP in tissue culture. The vertical lines extending down to the abscissa indicate equimolar concentrations of drug and folinic acid.

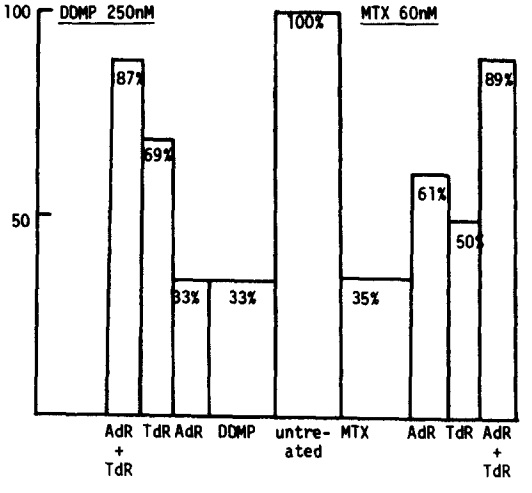

Fig. 4. Protection of L5178Y cells from MTX and DDMP by simultaneous addition of a purine and/or a pyrimidine.

$$\left.\begin{array}{l} AdR = \text{deoxyadenosine} \\ TdR = \text{thymidine} \end{array}\right\} 40\,\mu\text{M}$$

The ordinate shows the cell count at 48 hr expressed as the percentage of an untreated control.

Figure 4 compares the relative ability of salvageable purine (deoxyadenosine 40 μM) and pyrimidine (thymidine 40 μM) to protect L5178Y cells from equitoxic concentrations of MTX and DDMP. Both purine and pyrimidine were necessary for protection from either drug and the combination protected DDMP and MTX treated cells to the same extent. Studies in L1210 cells also demonstrated a similar effect of purine and pyrimidine protection on equitoxic concentrations of MTX and DDMP, although complete reversal of the toxic effects of either drug was not achieved (Fig. 5).

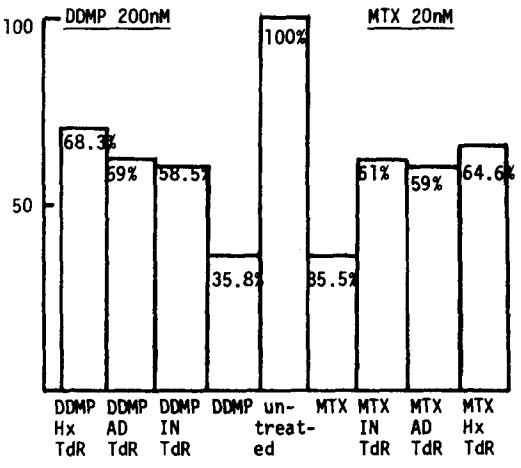

Fig. 5. Protection of L1210/S cells from MTX and DDMP by simultaneous addition of a purine and pyrimidine.

TdR = thymidine
AD = adenine
Hx = hypoxanthine
IN = inosine
} 40 μM

The ordinate is as in Fig. 4.

The effects of DDMP and MTX on the incorporation of tritiated thymidine (^{3}H-TDR) and deoxyuridine (^{3}H-UdR) into acid precipitable material

These results are illustrated in Fig. 6 where the concentrations of MTX and DDMP were chosen to be cytostatic (i.e., so that the cell counts remained virtually unchanged during the 24-hr observation period). The sensitivity of the L1210 cells at this time was slightly different from their sensitivity in the previous experiments. The incorporation of ^{3}H-UdR was slower to fall with MTX than with DDMP. This delay was repeatedly seen when minimally toxic concentrations of MTX were used, and correlated with the time taken for the intracellular MTX level to reach a plateau (data not shown). After 10-hr the ^{3}H-UdR incorporation was suppressed equally with either drug. The ^{3}H-TdR incorporation rose above control levels following treatment with either drug and fell slowly after 10-hr. This rise has been observed previously and is probably artefactual owing to a reduction in the size of the TTP pool. We do not consider the differences between the rises observed in the MTX and DDMP treated cells to be significant.

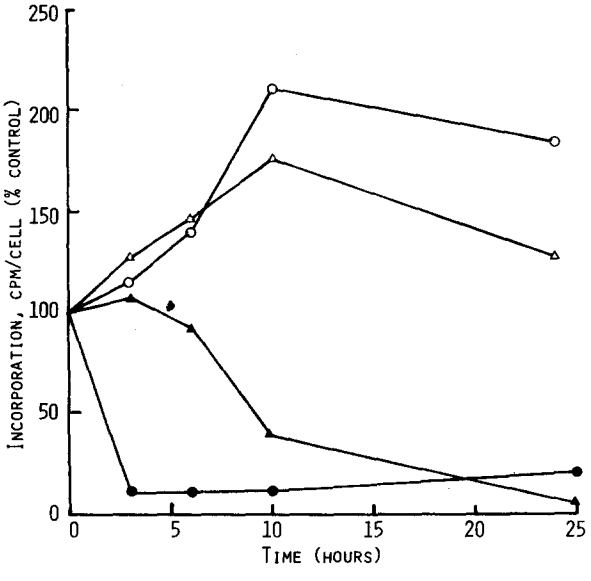

Fig. 6. Effects of DDMP (500 nm) and MTX (10 nM) on the incorporation of ^{3}H-TDR and ^{3}H-UdR into acid precipitable material of L1210 cells in culture. DDMP, TdR (○); DDMP, UdR (●); MTX, TdR (△); MTX, UdR (▲).

Comparison of the dose–response curves of L1210 and the resistant subline L1210 R71 possessing raised DHFR activity

The dose–response curves are shown in Fig. 7. The ID_{50} of the resistant line was increased 316 times for MTX and 116 times for DDMP, suggesting substantial cross-resistance between the two drugs. The fact that the cross-resistance was not complete may reflect the kinetics of the binding of MTX and DDMP rather than presence of a second locus of action of DDMP. This is discussed further below.

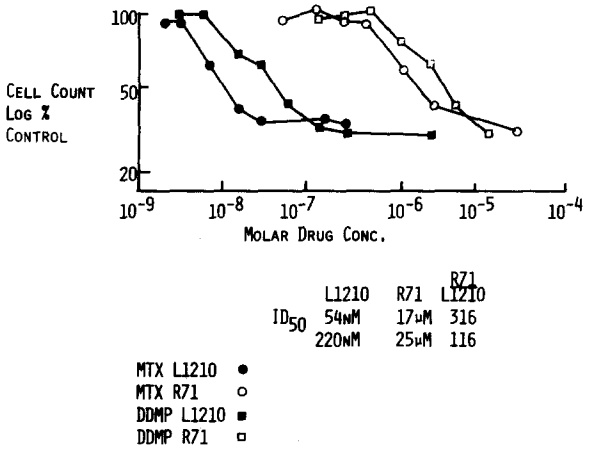

Fig. 7. Dose–response curves of L1210 and L1210 R71 to MTX and DDMP. Cell counts were made at 48 hr.

The inhibition of MTX and DDMP with DHFR

Figure 8 shows the DDMP inhibition curve for R71 DHFR. The individual points represent measured values of enzyme activity at various concentrations of the drug. The line represents a computer generated "best fit".

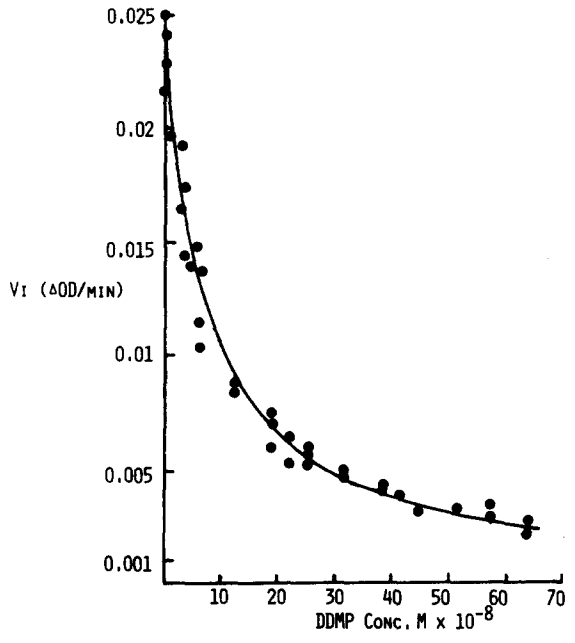

Fig. 8. Inhibition of DHFR (R71) by DDMP. Et = *enyme concentration;* S = *substrate concentration;* Vo = *uninhibited reaction velocity;* Km = *substrate affinity;* K*iapp* = 78.7 nM; Et = 0.103 nM; Vo = 0.023 (ΔO.D./min); Ki = (K*iapp*)/(1 + S/Km) = 71 pM.

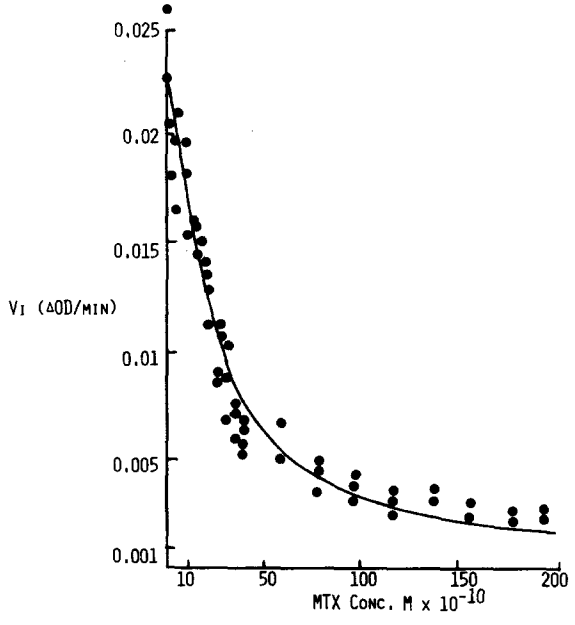

Fig. 9. Inhibition of DHFR (R71) by MTX. K*iapp* = 1.41 nM; Et = 1.55 nM; Vo = 0.023 (ΔO.D./min); Ki = 1.4 pM.

Using appropriate calculations, the *Ki* of DDMP was calculated at 71 pM. The MTX inhibition curve for R71 is depicted in Fig. 9 and the *Ki* was calculated as 1.43 pM. The enzyme inhibition curve of DDMP for L1210 DHFR is not shown, but a *Ki* value of 192 pM was computed. The *Ki* of MTX for DHFR from L1210 has been previously reported as 5.3 pM when measured under similar conditions [19].

If one considers the ratios of *Ki* L1210/S: L1210/R71, a value of 3.7 is obtained for MTX and 2.7 for DDMP. It is unlikely that such a minor difference in the *Ki* ratio for sensitive and resistant lines accounts for the differential shifts in MTX and DDMP dose–response curves.

The inhibitory properties of DDMP on other folate metabolizing enzymes

DDMP at a final concentration of 0.1 mM was added to the assay systems. Yoshida cells were used as the source for tetrahydrofolate formylase and serine hydroxymethyl transferase, L1210 cells for thymidylate synthetase and the cytosol of mouse liver was used as the source of methionine synthetase. No inhibition of any of these enzymes was seen.

DISCUSSION

These results are compatible with DHFR being the only cytotoxic locus of action of DDMP in the cultured cells studies, and at the DDMP concentrations used.

The protection experiments demonstrate that the cytotoxic effect of DDMP can be substantially reversed by the simultaneous addition of folinic acid. Although folinic acid is more effective, on a comparative molar basis in preventing cytotoxicity from DDMP than from MTX, this can theoretically be explained as due to a transport phenomenon since MTX and folinic acid share a common carrier [17]. Although it is reported that DDMP and folinic acid may share a common transport system, the degree of competition between these two agents was less than that between MTX and folinic acid [11]. Furthermore, the lipophilic nature of DDMP makes it likely that entry into the cell can occur by another independent route. This is supported by evidence that DDMP is effective in tumours resistant to MTX by virtue of a transport defect which also affects transport of reduced folates [25].

In both of the cell lines studied, purines and TdR prevented cytotoxicity equally well from equitoxic concentrations of MTX and DDMP. If DDMP acted on *de novo* nucleic acid synthesis by a mechanism other than folate depletion it would be necessary to postulate a simultaneous effect both on the purine and pyrimidine pathways. Greco and Hakala [26] showed that the toxicity of

DDMP to various cultured mammalian cell lines was not totally reversed by hypoxanthine, thymidine and glycine when higher levels of DDMP were used (7–50 μM). Such high levels were not used in this study, so the existence of a second, lower affinity site cannot be excluded. However the cross-resistance of the R71 subline does suggest that any effects of DDMP on a second locus could only be at a high concentration (>10 μM). Such concentrations are probably only achieved transiently in patients treated with DDMP [8, 27].

The effects of DDMP and MTX on incorporation of radiolabelled precursors into the acid precipitable fraction of cells in culture were similar. Others have demonstrated that DDMP and MTX produce similar changes in incorporation of nucleic acid precursors but no attempt was made to use concentrations of drug of similar cytotoxicity [28]. Further support for the idea that DDMP and MTX act in the same biochemical manner is provided by evidence that both produce similar changes in deoxyribonucleotide triphosphate pools [28]. It would be of interest to quantitate these changes in the presence of equitoxic drug concentrations.

Studies of cross-resistance with the R71 subline showed a slightly greater shift in the dose–response curve for MTX than DDMP. However, an identical shift of dose–response curves would only occur if the drugs had an equal affinity for the enzyme. Since DDMP has a lesser affinity for DHFR a substantial amount of free intracellular drug will be present at a time when the enzyme is inhibited by 95%, an event necessary for the cytotoxic effect [29], while at the corresponding MTX concentration only a slight excess of MTX over DHFR will be required. Thus as the DHFR content of resistant cells rises a parallel rise in the amount of intracellular MTX will be required to maintain cytotoxicity. The proportional rise in the intracellular DDMP will be lower as a considerable amount of free drug is present in the sensitive line. Preliminary experiments with ^{3}H-MTX and ^{14}C-DDMP revealed no difference in the initial uptake velocity of the two cell lines for these isotopes which might have accounted for the differences in the shifts of the dose–response curves.

A significant observation from this work is the demonstrated high affinity of DDMP for DHFR. Although this affinity is still almost 100-fold less than that of MTX for the same enzyme it nevertheless represents an example of a very tight binding inhibitor. The DHFR concentration in the assays used in these studies was approximately 1 nM, which is considerably lower than the *Ki* for DDMP. This underlines the necessity for the use of "Zone B kinetics" as described by Straus and Goldstein [30], which takes account of the proportion of inhibitor bound to the enzyme. The difference in *Ki* of DDMP for DHFR from sensitive and resistant cells was about 3-fold and approximated the shift of the *Ki* for MTX, suggesting that DDMP and MTX may interact with the enzyme in a similar manner. No inhibition of various other folate metabolising enzymes was seen. DDMP does however inhibit the enzyme histamine-*N*-methyl transferase [31] and in this respect is different from MTX. However, it is likely that the effect of DDMP on histamine metabolism, although responsible for some of its systemic toxicity, does not contribute to the antitumour action. In the absence of evidence for a second site of action of DDMP the clinical observation of 'selective folinic acid protection' of the normal tissues [7] may be attributed either to differences in the cell membrane transport characteristics of DDMP and folinic acid as originally suggested by Hill *et al.* [9], or to pharmacokinetic differences in the distribution of these two agents into the various tissues as suggested by Nichol [32].

The high affinity of DDMP for DHFR coupled with its lipophylic characteristics may suggest an important future role for this drug in selected clinical cases of MTX resistance.

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