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# Effects of methotrexate and other antifolates on the growth and dihydrofolate reductase activity of leishmania promastigotes

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The drugs currently in use against the leishmaniases are relatively toxic and not always effective [1], so there is a pressing need for a new therapy for this group of diseases. The enzyme dihydrofolate reductase is the target for various drugs including ones with activity against bacterial infections and malaria [2]. Dihydrofolate reductase inhibitors, both established drugs and novel compounds, have been tested against *Leishmania* species in culture as amastigotes or promastigotes [3–8] and in experimental infections of mice [9, 10], but in most cases with little success. Interestingly, however, cycloguanil pamoate has been used successfully in the treatment of cutaneous and mucocutaneous disease [11]. Therefore it is of interest to study further the effects of this group of inhibitors, particularly since the finding that leishmanial dihydrofolate reductase activity is mediated by the same protein as thymidylate synthetase [12], whereas in man two distinct enzymes occur. In this paper we report that the efficacy of methotrexate against leishmanias differs with the species used and that three novel 2,4-diaminopyrimidines are good leishmanicides.

## *Materials and methods*

Leishmania strains and conditions of culture in HOMEM medium supplemented with 10% heat-inactivated foetal calf serum (HIFCS) were as described previously.* *Leishmania m. mexicana* (MNYC/BZ/62/M379) promastigotes were also grown in Medium 199 (M199) supplemented to 20% with HIFCS. Average generation times were calculated as before.* Promastigote densities were estimated by counting samples of cultures using an improved Neubauer haemocytometer. In all growth inhibition experiments, cultures were established with an initial density of $1–5 \times 10^5$ cells $ml^{-1}$.

Stock solutions of novel 2,4-diaminopyrimidines, designated M&B compounds, were prepared in dimethyl sulphoxide and subsequently diluted twofold in distilled water. Methotrexate was dissolved in 0.1 M sodium phosphate, pH 7.0, and pyrimethamine was dissolved in 1% (v/v) lactic acid. These solutions were sterilized by filtration (0.22 μm pore size filter) and added to cultures in volumes of up to 0.1 ml (0.5 ml for methotrexate) per 5 ml medium. It was confirmed that the solvents used in the solutions did not affect the normal growth of the promastigotes.

Pyrimethamine and M&B compounds were tested in doubling dilutions for their effect upon *L. m. mexicana* promastigotes and the lowest concentration that killed all cells by 60–80 hr (minimum lethal concentration) was determined.

Resistance to methotrexate and M&B 35769 was developed by growing *L. m. mexicana* in cultures containing inhibitory but non-lethal concentrations of the drugs. The drug concentration was increased periodically until lines growing routinely in 500 μg methotrexate $ml^{-1}$ and 20 μg M&B 35769 $ml^{-1}$ were developed.

Supernatants from homogenates of *L. m. mexicana* promastigotes were prepared and dihydrofolate reductase activity in these extracts was assayed as described previously.* For inhibition studies, the enzyme was preincubated with inhibitor for 6 min at 30° prior to adding NADPH and dihydrofolate to start the reaction. Concentrations of inhibitors that reduced the activity by 50% ($I_{50}$s) were determined from plots of % inhibition versus $\log_{10}$ inhibitor concentration.

The M&B (May & Baker) compounds used were: M&B 33563, 2,4-diamino-5-(3-[2-cyclohexylphenoxy]propyl-1-oxy)-6-methylpyrimidine monohydrochloride; M&B 35769, 2,4-diamino-5(3-[4-4′-chlorophenylphenoxy]-propyl - 1 - oxy) - 6 - methylpyrimidine monohydrochloride; M&B 39434, 2,4-diamino-5(3-[2-cyclohexyl-4-methoxycarbonyl - phenoxy] - propyl - 1 - oxy) - 6 - ethylpyrimidine. Other dihydrofolate reductase inhibitors were gifts of the following companies: methotrexate, Lederle Laboratories, Gosport, U.K.; pyrimethamine, The Wellcome Research Laboratories, Beckenham, Kent, U.K. M199 medium was purchased from Gibco Ltd, Paisley. All other materials were as described previously.*

## *Results*

The differential effects of methotrexate upon *L. donovani*, *L. major* and *L. m. mexicana* promastigotes in HOMEM plus HIFCS cultures are illustrated in Fig. 1. *Leishmania donovani* and *L. m. mexicana* grew in very high (500 μg $ml^{-1}$, 1.1 mM) concentrations of the drug. By contrast, *L. major* could not sustain growth in concentrations of methotrexate greater than 5 μg $ml^{-1}$. The sensitivity of *L. m. mexicana* to methotrexate was also assessed in Medium 199 plus 20% HIFCS; promastigotes increased fourfold in density even in the presence of 500 μg methotrexate $ml^{-1}$. Despite the insensitivity of *L. m. mexicana* promastigotes to methotrexate, the dihydrofolate reductase from this organism was inhibited 50% by the drug at a concentration of only $2 \times 10^{-9}$ M (Table 1).

* Scott *et al.*, *Molec. biochem. Parasitol.* **23**, 139 (1987).

Table 1. Dihydrofolate reductase activity in *L. m. mexicana* promastigotes and its inhibition by methotrexate

| Promastigote type | Dihydrofolate reductase activity* | Concentration of methotrexate required to inhibit enzyme activity 50% ($I_{50}$, μM) |
|---|---|---|
| Parent (M379) | 7.4 ± 3.2 (16) | $2 \times 10^{-3}$ |
| Methotrexate-resistant† | 6.7 ± 1.6 (4) | $2 \times 10^{-3}$ |

* Activity in 10,000 *g* supernatants and expressed in nmoles $min^{-1}$ (mg protein)$^{-1}$ (mean ± SD from the number of determinations given in parentheses).
† Grown in the presence of 500 μg methotrexate $ml^{-1}$.

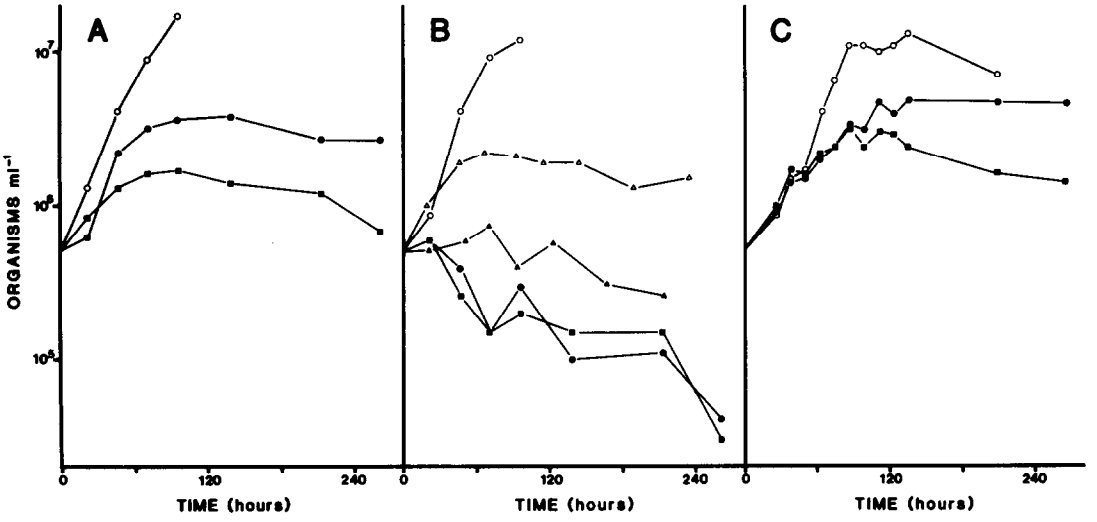


Fig. 1. Effect of methotrexate upon *Leishmania* species promastigotes growing in HOMEM medium with HIFCS. The growth of typical cultures is shown for each drug concentration. Species: A, *L. donovani*; B, *L. major*; C, *L. m. mexicana*. Methotrexate concentrations ($\mu g\ ml^{-1}$): ○, 0; △, 5; ▲, 10; ●, 100; ■, 500.

The promastigote lines of *L. m. mexicana* which were selected for their greater resistance to methotrexate grew to a density of $10^7$ cells $ml^{-1}$ in HOMEM plus HIFCS containing 500 $\mu g$ drug $ml^{-1}$. The average doubling times for a line growing in 500 $\mu g$ methotrexate $ml^{-1}$ (19 ± 2 hr, average ± SD from 6 subpassages) and a line growing in drug-free medium (21 ± 2 hr, average ± SD from six subpassages) were very similar. Methotrexate-resistant *L. m. mexicana* displayed neither increased dihydrofolate reductase activity nor decreased sensitivity of the enzyme to inhibition by methotrexate (Table 1).

Several 2,4-diaminopyrimidine dihydrofolate reductase inhibitors, including the established antimalarial pyrimethamine, were tested for their effects upon the growth of *L. m. mexicana* promastigotes. It was found that, in contrast to methotrexate, some of these were lethal to *L. m. mexicana* at low concentrations, despite being less potent than methotrexate as inhibitors of the parasite's dihydrofolate reductase (Table 2). M&B 35769 was similarly active against promastigotes of *L. donovani* and *L. major*. The M&B 35769-resistant line of *L. m. mexicana* could grow in 120 $\mu M$ (50 $\mu g$/ml) M&B 35769—10 times the normal minimum lethal concentration. The line was studied in an attempt to elucidate the mechanism of resistance. Comparison with the parent line, however, revealed that there had been no change in the specific activity of the enzyme, its sensitivity to M&B 35769, or the apparent $K_m$s for dihydrofolate and NADPH (3.7 $\mu M$ and 5.7 $\mu M$, respectively).

* Scott *et al.*, *Molec. biochem. Parasitol.* **23**, 139 (1987).

*Discussion*

The sensitivity of *L. m. mexicana* dihydrofolate reductase to inhibition by methotrexate, in terms of the $I_{50}$, was very similar to that reported for *L. major* [13]. (In the original publication the parasite was named *L. tropica*, it is now referred to as *L. major*—see ref. 14). In contrast, promastigotes of *L. m. mexicana* and *L. donovani* in culture were remarkably insensitive to methotrexate, growing in over 1 mM of the drug. Coderre *et al.* [13] found this concentration to be lethal for *L. major*, which we have confirmed in this study. As these differences cannot be explained by varied levels of enzyme activity,* it is possible that the kinetics of drug uptake differ. Methotrexate, unlike other inhibitors used in this study (Table 2), is not lipid-soluble and requires transport into cells. In bacterial and mammalian systems, methotrexate is moved into the cells as effectively as folates [15]. It appears that in the case of *L. m. mexicana* and, presumably, *L. donovani* promastigotes, methotrexate is not so well transported. Alternatively, the differing effects may be due to varying activities of drug-metabolizing enzymes. The related trypanosomatid *Crithidia fasciculata* possesses an enzyme which cleaves methotrexate to inactive metabolites [16].

The effectiveness of several 2,4-diaminopyrimidines in killing *L. m. mexicana* promastigotes (Table 2) demonstrates that antifolates do have potential as agents against this parasite. M&B 35769 has good antimalarial activity [17] and is undergoing clinical trials. Its activity against leishmania promastigotes *in vitro* makes it, in this respect, one of the most potent antileishmanial 2,4-diaminopyrimidines yet reported. We are at present pursuing these

Table 2. Effect of 2,4-diaminopyrimidines on *L. m. mexicana* promastigotes

| Inhibitor | Minimum lethal concentration ($\mu M$) | Concentration required to inhibit dihydrofolate reductase activity 50% ($\mu M$) |
|---|---|---|
| Pyrimethamine | 400 | 9 |
| M&B 33563 | 13 | 0.3 |
| M&B 35769 | 12 | 2 |
| M&B 39434 | 24 | 0.2 |

findings by assessing its activity against leishmania amastigotes growing *in vitro* in macrophages and against leishmanias in mice. Preliminary results indicate that it shows good effect in the former system but little or none in the latter (unpublished).

It has also been shown that dihydrofolate reductase/thymidylate synthetase is overproduced in *L. major* promastigotes as a resistance mechanism to both the dihydrofolate reductase inhibitor methotrexate and a quinazoline thymidylate synthetase inhibitor [13, 18]. The development of resistance to methotrexate in *L. m. mexicana* promastigotes, however, was not accompanied by the substantial rise in dihydrofolate reductase activity. As the *L. m. mexicana* strain was relatively insensitive to methotrexate before further selection, biochemical changes responsible for the acquisition of resistance might be expected to be small. It may be that the development of further methotrexate resistance in *L. m. mexicana* promastigotes, inexplicable in terms of increased activity or decreased drug sensitivity of the enzyme, could be due to a further reduction in its transport into the parasite, as has recently been reported for methotrexate-resistance in *Crithidia fasciculata* [19], or a further increase in drug metabolism. M&B 35769 is unlikely to require transport across the membrane of *L. m. mexicana*, being lipophilic, but resistance to it may be effected by increased drug metabolism.

In summary, *L. m. mexicana* and *L. donovani* promastigotes were found to be naturally insensitive to methotrexate in culture, growing in the presence of 1 mM of the drug. In contrast, growth of *L. major* promastigotes was inhibited almost totally by 0.02 mM methotrexate. The drug was, however, a potent inhibitor ($I_{50}$, 2 nM) of *L. m. mexicana* dihydrofolate reductase in cell-free extracts. Further resistance to 1 mM methotrexate was developed in *L. m. mexicana* promastigotes until growth occurred at normal rates. This resistance was not associated with changes in the properties or levels of dihydrofolate reductase activity. Four 2,4-diaminopyrimidines proved more effective than methotrexate against the growth of *L. m. mexicana* promastigotes, although they were less potent inhibitors of dihydrofolate reductase activity. Resistance to one 2,4-diaminopyrimidine was developed in culture until the organism could grow in 10 times the normal lethal concentration. This resistance was also not associated with changes in the properties or levels of the *L. m. mexicana* dihydrofolate reductase.

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# Concomitant changes of ethanol partitioning and disordering capacities in rat synaptic membranes

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During the last decade, a great number of investigators have found that membrane disordering and reorganizing could be an essential step in the progression of events leading to intoxication by ethanol and could be involved in the development of tolerance and dependence [1, 2]. Membrane fluidity as membrane ordering index has been extensively studied mainly on brain membranes of experimental animals [3–6]. Membrane molecular order is a result of the complex interactions of multiple membrane entities and can be modulated by compositional and/or structural alterations in these components.

It has been accepted after Meyer and Overton's work [7] that the membrane concentration is more responsible for the observed results than the initial concentration in the