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In Vitro Activity of Sulfonamides and Sulfones against *Leishmania major* Promastigotes

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We examined the susceptibility of promastigotes of *Leishmania major* to sulfonamides and sulfones in vitro. In a completely defined medium only sulfamoxole, sulfaquinoxaline, and dapsone were inhibitory; the concentrations required for 50% inhibition of the rate of growth were 150, 600, and 600 μM, respectively. Eleven other sulfa drugs were ineffective at concentrations up to 2 mM. The growth inhibition was similar to that observed in procaryotes: the cells continued logarithmic growth for several cell doublings before inhibition was observed. Surprisingly, the addition of *p*-aminobenzoate or folate did not reverse the effects of the active sulfa drugs, the effects of sulfamoxole and methotrexate were additive rather than synergistic, and the addition of thymidine reversed methotrexate but not sulfa-drug inhibition. These results suggest that the mode of action of sulfa drugs on *L. major* is not by the classical route of inhibition of de novo folate synthesis. Promastigotes could be propagated for more than 40 passages in a completely defined medium in which the only added pterin was biopterin. The folate concentration in this medium was less than 10^{-10} to 10^{-11} M, as determined by a *Leishmania* bioassay. Although these data suggest that *L. major* may be capable of de novo synthesis of folate, the nonclassical mode of action of sulfa drugs, as well as other studies, favors the view that *L. major* is auxotrophic for folate.

Protozoan parasites of the genus *Leishmania* (order *Kinetoplastida*, family *Trypanosomatidae*) are the causative agents of human leishmaniasis, a widespread tropical disease transmitted by sand flies. Depending upon the species of parasite and possibly other factors, leishmaniasis manifests as mild cutaneous lesions, disfiguring mucocutaneous disease, or a fatal visceral form (19). Currently, organic antimonial drugs are the only chemotherapeutic agents widely used for the treatment of leishmaniasis (2, 19), and it is generally agreed that new agents are urgently needed.

Inhibitors of folate metabolism have been successfully used in the treatment of protozoal diseases, such as malaria and toxoplasmosis (1, 8, 17). These include sulfonamide and sulfone inhibitors of dihydropteroate synthetase, and inhibitors of dihydrofolate reductase (DHFR), such as pyrimethamine. There have been several reports of the apparent success of sulfonamides in the treatment of leishmaniasis in combination with DHFR inhibitors (5, 11, 15). However, experimental studies of *Leishmania* infections in laboratory animals or the amastigote stage propagated in cultured macrophages have generally failed to reveal any significant inhibition by sulfonamides, and the clinical efficacy of the sulfa drugs has been questioned (2, 6, 14, 16). In this report we describe the susceptibility of promastigotes (the insect stage) of *Leishmania major* to two sulfonamides and one sulfone in vitro.

External levels of folate and *p*-aminobenzoate (PABA) are known to affect the susceptibility of other species to sulfonamides (1), and consequently it was necessary to examine the nutritional requirements of *L. major* for PABA, pterins, and folates and their effects on the organism, which had not been previously reported. In a defined medium promastigotes of the related parasite *Leishmania mexicana* have recently been shown to require folates (18), as does the related monogenetic trypanosomatid *Crithidia fasciculata* (13). Interestingly, the folate requirement of *C. fasciculata* can be dramatically reduced by the addition of a pterin such as biopterin (13), suggesting that this organism may obtain pterins from folate rather than by de novo synthesis (9, 12, 13). In other species pterins are essential cofactors for enzymes such as phenylalanine hydroxylase, although their metabolic role(s) in *Leishmania* spp. has not been firmly established (9, 13).

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MATERIALS AND METHODS

Chemicals. Bovine serum albumin, folic acid, folinic acid, PABA, dapsone [bis-(4-aminophenyl)-sulfone], sulfamoxole [N^1-(4, 5-dimethyl-2-oxazolyl)sulfanilamide], sulfaquinoxaline [N^1-(2-quinoxalinyl)sulfanilamide], and all other sulfonamide drugs were obtained from Sigma Chemical Co. Biopterin was obtained from Calbiochem-Behring. Methotrexate (MTX) was obtained from Pfanstiehl Laboratories, Inc. Concentrated stocks of sulfa drugs were made and stored in dimethyl sulfoxide at −20°C. Aqueous solutions of MTX, folate, and PABA were adjusted to pH 7 and stored at −20°C.

Cell culture. Promastigotes of the LT252 line of *L. major* (3) were used in all experiments. Cells were maintained at 26°C in a modified version of a completely defined medium (10). This medium is based on Dulbecco modified Eagle medium (high glucose; GIBCO Laboratories) and is supplemented with hemin, adenine, and biotin as described previously (10). Our modifications were adopted to eliminate folate and to exhaustively dialyze the stocks of bovine serum albumin supplement against Hanks balanced salt solution (GIBCO) before use. The basal medium, referred to as folate-depleted (FD) medium, was supplemented with 6 μM

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TABLE 1. Growth of *L. major* in FD media

Component added[a]			Ability to support continuous passage[b]
Biopterin	Folate	PABA	
−	−	−	−
−	−	+	−
−	+	−	+
+	+	+	+
+	+	−	+
+	−	+	+
+	−	−	+

[a] Added concentrations were as follows: biopterin, 6 μM; folate, 10 μM; PABA, 1 μM. The concentrations of folate and biopterin were those determined to give maximal growth.

[b] +, Cells were successfully propagated for at least 10 passages in which the cells were diluted 1:100; −, cells failed to grow. With biopterin alone (FD + B medium) continuous passage has been maintained for more than 40 passages.

biopterin (FD + B medium) in most experiments as indicated below. Promastigotes grew in FD + B medium with a doubling time of 17 to 24 h and were passaged at a 1:100 dilution when the cells entered the stationary phase. Numbers of cells were measured with a Coulter Counter (model ZBI; Coulter Electronics, Inc.). Different lots of albumin exhibited various abilities to support promastigote growth, and this was reflected in our experiments by small variations in the rate of promastigote growth. These variations did not affect the inhibition by sulfa drugs or MTX. The 50% effective concentration (EC_{50}) is defined as that concentration which reduces the rate of logarithmic growth by 50%. For sulfa drugs, the rate was measured after the initial lag in inhibition.

RESULTS

Growth of *L. major* in FD medium. To evaluate the requirement of *Leishmania* promastigotes for exogenous folates, we used a completely defined medium lacking added folate and PABA (FD medium). Promastigotes did not grow in FD medium or when 1 μM PABA was added, whereas continuous growth could be maintained after addition of 10 μM folate (Table 1). Surprisingly, continuous passage could be maintained if folate was replaced with 6 μM biopterin (FD + B medium), regardless of whether PABA was added (in contrast, *C. fasciculata* requires folate regardless of whether biopterin is added, although the minimal folate concentration is much less with biopterin [13]). The population growth rate of *L. major* was the same in all media in which continuous passage could be maintained, and growth in FD + B medium has been maintained for more than 40 passages. The folate concentration in FD + B medium is less than 10^{-10} to 10^{-11} M, as described below. In FD + B medium the EC_{50} for MTX was 2.5×10^{-9} M, in contrast to the previously reported EC_{50} of 500×10^{-9} M in medium 199-based medium (4).

Inhibition by sulfamoxole. The observation that *L. major* could be propagated indefinitely in FD + B medium suggested the possibility that promastigotes are capable of de novo synthesis of folate. Accordingly, we examined the susceptibility of promastigotes to inhibition by sulfonamides such as sulfamoxole (Fig. 1). After inoculation into medium containing the drug, logarithmic growth was maintained for several cell doublings, after which time the growth rate was reduced in a dose-dependent manner. Interestingly, this type of inhibition is similar to that seen in procaryotes after exposure to sulfonamides (1). The EC_{50} for sulfamoxole, defined as that concentration of the drug which inhibited the rate of growth by 50% after the initial lag in inhibition, was 150 μM.

Inhibition by other sulfonamides and sulfones. We next examined the inhibition of promastigote growth by 13 additional drugs, including the sulfonamides sulfadimethoxine, sulfanilamide, sulfaguanidine, sulfadiazine, sulfabenzamide, sulfamonomethoxine, sulfamethoxazole, sulfanitran, sulfathiazole, sulfamethizole, sulfisoxazole, and sulfaquinoxaline and the sulfone dapsone. Only sulfaquinoxaline (EC_{50}, 600 μM) and dapsone (EC_{50}, 600 μM) inhibited promastigote growth; the other drugs were inactive at 2 mM, the highest concentration tested. The effect of sulfaquinoxaline and dapsone on promastigote growth was similar to that shown in Fig. 1 for sulfamoxole, with a lag of 2 to 3 cell doublings before inhibition (data not shown).

Sulfonamide inhibition is not reversed by PABA or folate. We examined the effect of increasing PABA or folate concentrations on sulfa-drug inhibition of promastigote growth in FD + B medium. In other species, PABA can reverse the inhibition of dihydropteroate synthetase by sulfa drugs, whereas folate bypasses the block directly (1). Surprisingly, high concentrations of folate (12.5 μM) or PABA (1,000 μM) could not reverse the inhibition by sulfamoxole, dapsone, or sulfaquinoxaline when drug concentrations which only partially inhibited growth (75% of control growth) were used (data not shown). The lack of reversal was observed even if the promastigotes were propagated for several passages in the presence of folate or PABA before drug treatment. The ineffectiveness of folate was not due to a failure in uptake, since folate could sustain promastigote growth (Table 1) and we have shown that promastigotes possess a high-affinity carrier capable of mediating efficient uptake of exogenous folate (5a). Similarly, radiolabeled PABA is taken up by promastigotes in a temperature-dependent manner (T. E. Ellenberger and S. M. Beverley, unpublished data).

Sulfonamides and antifolates do not exhibit synergy. The combination of a sulfonamide inhibitor of dihydropteroate synthetase with a DHFR inhibitor yields synergistic inhibition of the growth of bacteria and malaria parasites if both drugs are effective when given alone (1, 8, 17). We examined the effect of sulfamoxole and the DHFR inhibitor MTX (4), singly or in combination, on promastigote growth in FD + B

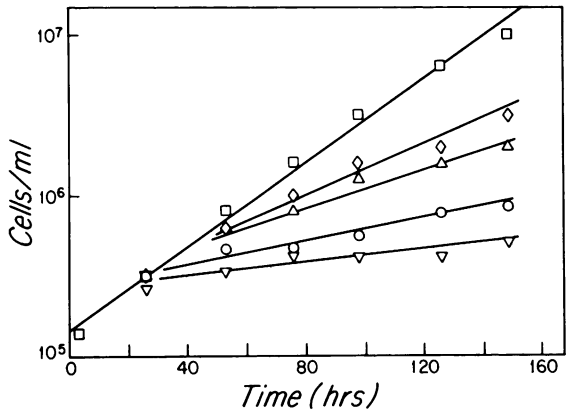

FIG. 1. Growth of *L. major* promastigotes in the presence of sulfamoxole. Promastigotes were inoculated into FD + B medium containing various concentrations of sulfamoxole, and growth was monitored at the indicated times with a Coulter Counter. Symbols: □, no drug; ◇, 75 μM; △, 112 μM; ○, 150 μM; ▽, 225 μM.

TABLE 2. Absence of synergistic inhibition of promastigote growth by sulfamoxole and MTX

Concn (μM) of drug added		% Inhibition	
Sulfamoxole	MTX	Observed[a]	Expected if additive
56	0	1.4	
75	0	6.3	
0	0.25	2.8	
0	0.5	7.1	
0	1.0	12	
56	0.25	4.0	4.2
56	0.5	7.3	8.5
56	1.0	13	14
75	0.25	14	9.1
75	0.5	17	13

[a] The percent inhibition was measured as the reduction in growth rate in FD + B medium (after the initial lag) relative to controls. Data from a single representative experiment are shown; duplicate experiments gave similar results.

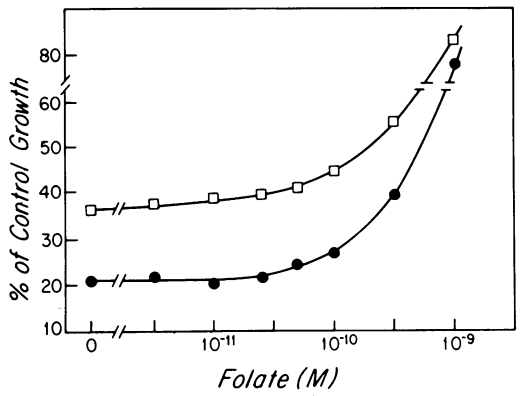

FIG. 2. Reversal of MTX inhibition by exogenous folate. Promastigotes were inoculated into FD + B medium containing 2.5×10^{-9} M MTX, supplemented with various concentrations of folate as indicated, and numbers of cells were measured after 66 (□) or 96 (●) h. Growth was measured as a percent relative to controls propagated in the absence of MTX.

medium. The inhibitory effect of these drugs was additive and not synergistic (Table 2).

Conditions which reverse MTX toxicity do not reverse sulfa-drug action. Because sulfa drugs and DHFR inhibitors both act by reducing the availability of reduced folates necessary for intermediary metabolism in many species, agents which rescue promastigotes from DHFR inhibitors will also rescue cells from sulfa-drug inhibition. We first examined the ability of a reduced folate, folinic acid, to reverse MTX inhibition of *L. major*. MTX inhibition of promastigote growth in FD + B medium was reversed by the addition of folinic acid (data not shown). Surprisingly, folate could also reverse MTX toxicity in a dose-dependent manner, with detectable effects starting at 10^{-11} M and saturation occurring at about 10^{-9} M (Fig. 2; this experiment may be interpreted as a bioassay for folate or folate equivalents, suggesting that the folate level in the FD + B medium was 10^{-10} to 10^{-11} M). This observation suggested that folinic acid may not be suitable for use in these experiments, which were designed solely to eliminate the dependence of the cell upon reduced folates.

Thymidine supplementation at concentrations of 10 to 1,000 μM in FD + B medium could completely reverse the inhibition of promastigote growth by MTX (Table 3; FD + B medium contains adenine since *L. major* is auxotrophic for purines). An interesting contrast is provided by mammalian cells, which are inhibited by high thymidine concentrations. Despite the complete reversal of MTX inhibition, thymidine supplementation had no effect on sulfamoxole inhibition of leishmanial growth.

DISCUSSION

Our results indicate that the sulfonamides sulfamoxole and sulfaquinoxaline and the sulfone dapsone inhibit the growth of promastigotes of *L. major* in vitro. In contrast to the mode of action of sulfonamides in other susceptible species such as bacteria or malaria parasites (1, 8), the mode of action of the sulfa derivatives in *L. major* appears not to be at the level of de novo synthesis of folate. This conclusion stems from our findings that (i) neither PABA nor folates could relieve sulfa-drug inhibition, even partially; (ii) there was no synergy between sulfa drugs and MTX; and (iii) agents which reversed MTX inhibition did not relieve sulfa-drug inhibition. Consistent with this hypothesis is the fact that dihydropteroate synthetase, the classical target of sulfa drugs in bacteria and protozoa, has not been demonstrated in *L. major* or any other trypanosomatid parasite thus far. It therefore appears likely that sulfa drugs attack another, thus far uncharacterized, target in *L. major*. Interestingly, sulfonamide inhibitors of protein kinase C and calmodulin of other species have been reported (7). We have isolated sulfamoxole-resistant mutants (unpublished data) which may prove useful in eventually identifying the presumptive target of sulfa-drug action.

Classical inhibitors of dihydropteroate synthetase generally share the sulfanilamide nucleus, to which different side groups are attached (1). The failure of 11 sulfonamides to inhibit the growth of *L. major* suggests the possibility that it is the specific side group attached to the sulfanilamide nucleus which is the active moiety responsible for inhibition. Two observations suggest that this is not the case: (i) the activity of dapsone [bis-(4-aminophenyl)-sulfone], which is a symmetric molecule bearing only the aminophenyl sulfone moieties; and (ii) the activity of sulfamoxole and sulfaquinoxaline, which contain very different side groups. It is possible that the side group modulates the activity of the sulfanilate nucleus by mechanisms such as uptake, etc.

Several laboratories have examined the activity of sulfa drugs on *Leishmania* amastigotes grown in cultured macrophages or in animals (2, 6, 14, 16). Generally, these investigations have failed to demonstrate significant inhibition, and our experiments showed that many of the sulfa drugs used in

TABLE 3. Thymidine reversal of inhibition by MTX but not sulfamoxole

Thymidine concn (μM)	Doubling time (h) with[a]:		
	No drug	MTX (2.5 nM)	Sulfamoxole (150 μM)
0	17	34	24
1	17	20	24
10	17	17	23
100	17	17	22
1,000	17	17	23

[a] Population doubling times were measured in FD + B medium. Data from a single representative experiment are shown; duplicate experiments gave similar results.

these studies were also inactive against *L. major* promastigotes. Preliminary tests of dapsone, sulfamoxole, and sulfaquinoxaline against *L. major* amastigotes grown in cultured human macrophages suggest that these compounds are inactive at concentrations up to 1 mM (J. Berman and S. M. Beverley, unpublished data). There are several possible reasons why sulfa drugs which inhibit promastigotes fail to inhibit amastigotes in vivo. Amastigotes may lack the presumptive sulfa-drug target found in promastigotes, or perhaps sulfa drugs are not sufficiently accumulated within the phagolysosome. This latter problem could be approached since the pharmacokinetic properties of sulfa drugs can be dramatically affected by the side group attached to the sulfanilamide nucleus (1). However, at present the potency of the most active sulfa drug, sulfamoxole, is insufficient to warrant its use in chemotherapy.

The observation that folate can rescue *L. major* from MTX toxicity may provide an explanation for the differential sensitivity of promastigotes to MTX in medium 199 and FD + B medium (500×10^{-9} versus 2.5×10^{-9} M) since medium 199 contains about 1,000-fold more folate (25×10^{-9} versus 0.1×10^{-9} to 0.01×10^{-9} M). Folate reversal of MTX toxicity may be mediated at the level of uptake, since we have shown that high-affinity uptake of folate (oxidized and reduced) and MTX is mediated by a single carrier with similar affinities for these ligands (5a). In any event, the reversal by folate clearly cannot be due to the supply of reduced folate equivalents. It is possible that folinate may similarly counteract MTX inhibition in addition to providing a potential source of reduced folate.

We showed that *Leishmania* promastigotes could be propagated indefinitely in a medium whose folate concentration was less than 10^{-10} to 10^{-11} M, as estimated by a *Leishmania*-based bioassay (Fig. 2). Similar results have been reported for the related protist *C. fasciculata* (9), and at face value these data suggest that trypanosomatids may be capable of de novo synthesis of folate. However, we have been unable to demonstrate incorporation of radiolabeled PABA into folates (T. E. Ellenberger and S. M. Beverley, unpublished data), a finding consistent with our results concerning the nonclassical mode of sulfonamide action in *L. major*. Consequently, if *L. major* is capable of de novo synthesis of folate, it may be by a pathway other than the classical route via PABA and dihydropteroate synthetase. Alternatively, *L. major* may be auxotrophic for folate. The demonstration of high-affinity uptake of folates by promastigotes (5a) suggests the possibility that even in FD + B medium sufficient folate remains for the minimal metabolic requirement (see also reference 13). This hypothesis further requires that in the absence of biopterin the hypothetical residual folate is insufficient to provide a source of both folate and pterin (analogous to the folate-sparing effect of biopterin in *C. fasciculata* [12, 13]), since growth was not maintained in FD + B medium in the absence of both pterins (Table 1). It is evident that the question of whether *L. major* is capable of de novo synthesis of folate remains unresolved, although we favor the view that *L. major* is auxotrophic for folate.

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