The macrolide antibiotic megalomicin (MGM) has been shown to inhibit vesicular transport between the medial- and trans-Golgi, resulting in the undersialylation of cellular proteins (P. Bonay, S. Munro, M. Fresno, and B. Alarcón, J. Biol. Chem. 271:3719–3726, 1996). Due to the effects of MGM on the Golgi and on the replication of enveloped viruses, we decided to test whether it has any antiparasitic activity. The results showed that MGM has potent activity against the epimastigote stage of Trypanosoma cruzi, producing a 50% inhibitory concentration (IC50) of 0.2 μg/ml. Furthermore, MGM was also active against the intracellular replicative, amastigote form of T. cruzi, completely preventing its replication in infected murine LLC/MK2 macrophages at a dose of 5 μg/ml. Although less potent, MGM was also active against Trypanosoma brucei epimastigotes (IC50, 2 μg/ml) and Leishmania donovani and Leishmania major promastigotes (IC50, 3 and 8 μg/ml, respectively). MGM also blocked intracellular replication of the asexual stage of Plasmodium falciparum-infected erythrocytes at 1 μg/ml. Finally, MGM was active in an in vivo model, resulting in the complete protection of BALB/c mice from death caused by acute T. brucei infection and significantly reducing the parasitemia. These results suggest that MGM is a potential drug for the treatment of veterinary and human parasitic diseases.

There is wide variation in the availability and efficacy of drugs for the therapy and prophylaxis of parasitic diseases, in both humans and domestic animals (10). There are still major deficiencies in antiparasite chemotherapy for human African trypanosomiasis (15), Chagas’ disease (6, 20), and leishmaniasis (14), among others. Available drugs are inadequate because of low efficacy, high toxicity, or the requirement of long courses of parenteral administration. Thus, newer and better drugs are urgently needed. The flagellated protozoan Trypanosoma cruzi is the etiologic agent of American trypanosomiasis (Chagas’ disease), a major public health problem in many Latin American countries, with an estimated 15 million people infected. Infected people may experience an acute phase followed by a chronic phase that can be asymptomatic but frequently results in cardiomyopathy and irreversible dilation of the esophagus and colon. Both forms may have high mortality rates. Another member of the Trypanosomatidae family, Leishmania, infects over 12 million people worldwide. Leishmaniasis manifests as minor or severe cutaneous lesions or as a visceral form which, if untreated, has a fatality rate of nearly 100%. Its emergence as an opportunistic pathogen in AIDS patients (14) has further raised the public health awareness of leishmaniasis and the need to control this disease. In Africa, South America, and Asia, trypanosomiasis is a threat to both humans (African trypanosomiasis) and livestock; Trypanosoma brucei, Trypanosoma congolense and Trypanosoma vivax cause disease in cattle, sheep, and goats. Similarly, Trypanosoma evansi causes disease in camels, horses, and buffaloes. Resistance and cross-resistance to several currently used drugs has been reported for over 30 years (16).

Malaria remains one of the most important diseases of humans in terms of both mortality and morbidity, with Plasmodium falciparum being the most important infecting agent (24). Despite considerable therapeutic success with the antimalarial 4-aminooquinolines such as chloroquine, there are serious doubts about the future of this class of drugs as well as of other established antimalarial drugs, for example, pyrimethamine (23), due to the development and spread of parasite resistance in areas in which malaria is endemic.

We have characterized the antibiotic megalomicin (MGM) as an antiparasitic drug against several taxonomically distant parasites. MGM is a macrolide antibiotic complex produced by Micromonospora megalomica (19, 21, 22) that has been shown to have pleiotropic effects on vesicular transport in mammalian cells (7, 8), although it is nontoxic (22). In this study, we show that MGM strongly inhibits the proliferation and affects the viability of free T. cruzi epimastigotes and, more importantly, intracellular amastigotes. In addition, we show that MGM has a wide spectrum of activity and that it protects BALB/c mice against lethal infections with T. brucei. MGM may thus prove to be a promising new antiparasitic agent.

MATERIALS AND METHODS

Drugs. MGM (see Fig. 2) was obtained from cultures of M. megalomica (ATCC 27598) by a procedure already described (21). Briefly, 50 ml of medium 172 was inoculated with spores of the actinomycete and incubated at 27°C with high aeration for 5 days and was then used as an inoculum for a 400-ml culture. After 3 days, this culture was used to inoculate 4 liters of medium 172 under the same conditions. Five days later, the pH of the cultures was raised to 9.5 with sodium hydroxide and the culture was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was evaporated to 1/100 of the initial volume and was then extracted twice with 0.14 N hydrochloric acid. The acid extract was again raised to pH 9.5 and extracted twice with equal volumes of ethyl acetate. The ethyl acetate extracts were pooled and evaporated completely, dissolved in acetonitrile, and precipitated by the rapid addition of 1 liter of distilled water and raised to pH 9.5 with sodium hydroxide. The precipitate was collected by filtration through Whatman paper. As previously described (21), this procedure results in a mixture of megalomicins especially rich in the C complex. The purity of the preparation was assessed by high-performance liquid chromatography and thin-layer chromatography, and its activity was estimated in a growth inhibition assay.
assay on Sarcoïdia lutea. The purity of the preparation was estimated to be 90%. Erythromycins A, B, and C were a kind gift of J. Corbalán (The Lilly Company, Windlesham, Surry, United Kingdom).

In vitro culture and parasites. We used the following parasite strains: bloodstream stage and epimastigotes of T. cruzi (3 and 4) and promastigotes of Leishmania major M-HOM-Su-73-Saskh and Leishmania donovani M-HOM-In-80-DD8 (World Health Organization reference strains). All were grown in LIT medium (11) (NaCl, 0.4%; KCl, 0.04%; PO₄HNa₂, 0.8%; glucose, 0.2%; liver infusion, 0.3%; tryptose, 0.5% [adjusted to pH 7.2]) at 28°C. Metacytic promastigotes were obtained by in vitro metacyclogenesis in triatomine artificial urine medium plus 0.035% sodium carbonate, as previously described (9).

To monitor the growth of amastigotes, the murine macrophage cell line LLC/MK2 was grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and infected with metacytic trypomastigotes (obtained as described above) at an infection index of 10 (parasites/host) (5).

P. falciparum clone 3D7 was cultured in infected B (Rh+) erythrocytes obtained from healthy Spanish donors not exposed to malaria, maintained at 5% hematocrit in petri dishes with standard RPMI 1640 medium supplemented with 20 mM l-glutamine, 25 mM HEPES, 25 mM sodium bicarbonate (Gibco Life Technologies Ltd., Paisley, Scotland), 25 mg of gentamicin (Sigma Chemical Co., St. Louis, Mo.) per liter, and 10% human B (Rh+) serum (heat inactivated), and grown in a low-oxygen-concentration atmosphere (5% CO₂–5% O₂–90% N₂) at 37°C as described by Trager and Jensen (18).

Growth inhibition assays for Trypanosomatidae species. Trypanosoma spp. and Leishmania spp. (10⁶ parasites/ml) were seeded in cultures of LIT medium in the presence or absence of different concentrations of MGM (from a 100-fold stock in ethanol). Control cultures were treated with the same dose of the solvent ethanol (not higher than 1%, vol/vol). Aliquots of the cultures were taken at the indicated times, and the total number of parasites was counted in a Neubauer hemocytometer. To estimate viability, parasite suspensions were washed with 1% glucose plus 2% bovine serum albumin in phosphate-buffered saline (PBS) and resuspended in the same solution plus 12 mM fluorescein diacetate (FDA) and 5 mg of propidium iodide (PI) per ml and incubated at room temperature for 15 min. Viability was determined by counting the number of living parasites (green fluorescence) and dead parasites (red fluorescence) with a Zeiss Axioskop microscope.

Growth inhibition assay for P. falciparum. To determine drug susceptibility, asynchronous cultures of P. falciparum (approximately 99% asexual erythrocytic stages and 1% unreplicating gametocytes) (18) were grown essentially as described previously (12). Briefly, complete medium with 5% hematocrit and 0.3% starting parasitemia was added to a final volume of 2 ml in 24-well culture plates (Falcon; Becton Dickinson, Paramus, N.J.). MGM was added to the blood medium mixture to total concentrations of 1 and 3 μg/ml per well from a stock solution at 10 mg/ml in ethanol. Control cultures contained less than 1% ethanol per well, which did not affect the parasite growth compared to a culture without ethanol (not shown). To avoid growth saturation and keep parasitemia at an optimum growth level for 10 days, half of the blood medium mixture was replaced daily with fresh medium containing the corresponding amount of MGM. Thin blood films were made daily from duplicate cultures and stained with Giemsa, and parasites in each preparation were counted three times from about 2,000 erythrocytes at the time points indicated in Fig. 6. The parasite count in each of the MGM-treated wells was expressed as a percentage of the parasitemia of the control cultures against the culture time.

In vivo infections. Twenty 8-week-old male BALB/c mice were inoculated intraperitoneally with 10⁶ P. brucei trypomastigotes in 100 μl of PBS (obtained by pooling the blood of several mice at the peak of parasitemia). MGM was administered intraperitoneally in two doses of 1 mg per mouse (50 mg/kg of body weight), emulsified in 400 μl of a 1:1 PBS-incomplete Freund’s adjuvant suspension to half of the parasite-inoculated mice at 24 and 72 h after infection. As a control group, the other 10 infected mice were administered only emulsified incomplete Freund’s adjuvant. Each day, blood samples were taken from the retro-orbital sinus of half of the mice in each group and examined in a Neubauer hemocytometer to count the number of parasites.

RESULTS

Effects of MGM on the replication of T. cruzi epimastigotes. To test the possible antiparasitic effects of MGM, T. cruzi epimastigote cultures were initiated (by diluting an exponential growth culture) in the presence of two concentrations of the drug. The effect of MGM on the parasites was assessed by counting the total number of epimastigotes at each time point (Fig. 1A) as well as by estimating the viability of the parasites by FDA-PI, as described above (Fig. 1B). As shown in Fig. 1, MGM at a concentration of 50 μg/ml completely inhibited the replication of T. cruzi and caused a drastic drop in the viability of the epimastigotes initially seeded. The number of viable parasites after 10 days in culture with 50 μg of MGM per ml was reduced by 99.9%. In the presence of 5 μg of MGM per ml, there was an initial increase in the number of parasites to twice that of the number originally seeded, without any further increase. After 10 days in the continuous presence of the drug, the parasite viability was reduced to 0.7%.

The structure-function specificity of the MGM action was determined by assessing the activity of erythromycins, which
are structurally related to MGM (Fig. 2). As shown in Fig. 3, MGM caused a drastic inhibition of *T. cruzi* replication, with a calculated 50% inhibitory concentration (IC_{50}) of 0.2 µg/ml. However, erythromycins did not significantly affect *T. cruzi* replication, suggesting that the antiparasitic effect of MGM is specific to its unique structure. Unlike MGM, erythromycins do not have the D-rhodosamine sugar moiety, which may be required for the antiparasitic activity.

**Inhibition of the intracellular replication of *T. cruzi* by MGM.** Intracellular replication of *T. cruzi* inside some host cells, mainly macrophages and muscle cells, is an absolute requirement for its dissemination in the host and for the propagation of the disease. Thus, an anti-Chagasic drug must be able, in order to be an effective therapeutic agent, to penetrate the host cells and inhibit the intracellular replication of the parasite. Therefore, the capability of MGM to inhibit replication of the intracellular, amastigote form of *T. cruzi* was evaluated. Toward this end, murine LLC/MK2 macrophages were infected with *T. cruzi* trypomastigotes, and the number of living cells was evaluated as an inverse index of intracellular replication of the parasites (5). As shown in Fig. 4A, the number of living macrophages in the infected cultures dropped to 23%, compared with the uninfected control, after 4 days in culture due to the intracellular replication of *T. cruzi* amastigotes. In contrast, when MGM was added at 5 and 50 µg/ml, the percentages of living macrophages in the infected culture remained at 75 and 86, respectively. To exclude the possibility that MGM could be acting on the parasites before they actually entered the macrophages, a parallel experiment was performed in which MGM was added 1 day after the beginning of the infection, when most of the parasites had been internalized and the excess free parasites had been removed (Fig. 4B). Under these conditions, the viabilities of the infected macrophages at day 4 postinfection of the host cells were maintained at 86 and 90% at 5 and 50 µg of MGM per ml, respectively.

The most critical data on the effects of MGM are provided by the direct counting of intracellular replicative forms inside infected macrophages. MGM seems to affect the intracellular replication of the amastigotes, since a drastic reduction in the number of intracellular replicative forms in MGM-treated macrophages compared with nontreated macrophages was detected by optical microscopy. As shown in Table 1, nontreated macrophages at day 4 postinfection contain from 12 to 24 intracellular amastigotes compared with the 2 to 7 and 1 to 3 found in cells maintained in the presence of 5 or 50 µg of MGM per ml, respectively, during and postinfection. A dramatic reduction in the number of intracellular amastigotes was
noted even when MGM was added to the cultures at 1 day postinfection, further suggesting that MGM inhibits intracellular replication of *T. cruzi*.

**Antiparasitic spectrum of MGM.** In addition to the effect on the replication of *T. cruzi*, the in vitro activity of MGM against other parasitic protozoa was studied. As estimated from the data of Fig. 5, the IC50 of MGM for *T. brucei* epimastigotes was 2 μg/ml; for *L. donovani* and *L. major*, the IC50 were 3 and 8 μg/ml, respectively. Therefore, it appears that MGM is active against other pathogenic *Trypanosomatidae* parasites related to *T. cruzi*, the inhibitory activity being higher against the closely related *T. brucei* and lower against the more distant species of the genus *Leishmania*.

The antiparasitic activity of MGM on non-*Trypanosomatidae* protozoa was determined by testing on the growth of asexual intraerythrocytic stages of *P. falciparum*. This parasite completes a replicative cycle in infected erythrocytes every 48 h, and cultures do not have to be highly infected for optimal growth (<10% parasitemia) because of the toxicity of the metabolites secreted by the parasite into the culture medium. MGM was added when cultures were at 0.3% infected erythrocytes. Culture samples were taken daily, and the levels of parasitemia in control cultures were given a value of 100%; the MGM-treated cultures were compared to the controls at each time point. Thus, as shown in Fig. 6, the growth of intraerythrocytic stages of *P. falciparum* was inhibited from the first day of incubation in the presence of 1 and 3 μg of MGM per ml.

### TABLE 1. Effect of MGM on intracellular replication of *T. cruzi* amastigotes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intracellular amastigotes/cell &lt;sup&gt;a&lt;/sup&gt; (no. of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>None</td>
<td>1–4 (80)</td>
</tr>
<tr>
<td>MGM (5 μg/ml)</td>
<td>1–3 (95)</td>
</tr>
<tr>
<td>MGM (50 μg/ml)</td>
<td>1–3 (75)</td>
</tr>
<tr>
<td>MGM (50 μg/ml) at day 1 postinfection</td>
<td>1–5 (50)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are ranges. All experiments were done in triplicate.

<sup>b</sup> By Student’s *t* test, all data for day 4 with MGM are significantly different from untreated controls (*P* ≤ 0.001).
Antiparasitic activity. This is reflected in the low IC50 shown not only against several members of the *Trypanosomatidae* family (*T. cruzi*, *T. brucei*, and *Leishmania* spp.) but also against the more distantly related *P. falciparum*, which is an obligate intracellular parasite of human erythrocytes. Despite its effects on vesicular transport in mammalian cells (7, 8), MGM was selectively toxic for these parasites. Thus, MGM inhibits the growth of *T. cruzi* epimastigotes at concentrations 200- to 500-fold lower than those which are toxic for mammalian cells (2, 22). Indeed, MGM inhibited the intracellular replication of *T. cruzi* (Fig. 4) and *P. falciparum* (Fig. 6) without inherent toxicity for uninfected host cells, as measured by its proliferation rate (not shown). In addition, it proved to be very active in an experimental in vivo mouse model of *T. brucei* infection, suggesting that MGM has a potential pharmacological application. It is worth noting that two single intraperitoneal doses of MGM were sufficient to completely prevent *T. brucei* lethality. It is likely that MGM, by slowing *T. brucei* replication and reducing its viability, can allow the immune system to overcome infection. In untreated control BALB/c mice, *T. brucei* replication takes place at a very high rate, thus preventing an effective immune response.

It is also important to note that MGM was active when injected 1 day after infection, further supporting an interest in its potential curative value in infected humans or animals. With regard to this, MGM toxicity in mice has been determined as giving a 50% lethal dose of 270 mg/kg when administered intraperitoneally and of 5,000 mg/kg when administered orally (22). Thus, the toxicity of MGM is similar to the widely used, and structurally related, erythromycin antibiotics (intraperitoneal 50% lethal dose in mice, 490 mg/kg) (19). Erythromycins differ from MGM basically in that this antibiotic contains a D-rhodosamine substitution in the erythronolide ring. The fact that erythromycins did not have antiparasitic activity indicates that the D-rhodosamine moiety may be a structural determinant necessary for antiparasitic activity.

**DISCUSSION**

Our results show that MGM exhibits potent wide-spectrum antiparasitic activity. This is reflected in the low IC50 shown not only against several members of the *Trypanosomatidae* family (*T. cruzi*, *T. brucei*, and *Leishmania* spp.) but also against the more distantly related *P. falciparum*, which is an obligate intracellular parasite of human erythrocytes. Despite its effects on vesicular transport in mammalian cells (7, 8), MGM was selectively toxic for these parasites. Thus, MGM inhibits the growth of *T. cruzi* epimastigotes at concentrations 200- to 500-fold lower than those which are toxic for mammalian cells (2, 22). Indeed, MGM inhibited the intracellular replication of *T. cruzi* (Fig. 4) and *P. falciparum* (Fig. 6) without inherent toxicity for uninfected host cells, as measured by its proliferation rate (not shown). In addition, it proved to be very active in an experimental in vivo mouse model of *T. brucei* infection, suggesting that MGM has a potential pharmacological application. It is worth noting that two single intraperitoneal doses of MGM were sufficient to completely prevent *T. brucei* lethality. It is likely that MGM, by slowing *T. brucei* replication and reducing its viability, can allow the immune system to overcome infection. In untreated control BALB/c mice, *T. brucei* replication takes place at a very high rate, thus preventing an effective immune response.

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**FIG. 7. Therapeutic effects of MGM on *T. brucei* infected mice.** The plot represents parasitemia (dotted line) and survival (continuous line) of two groups of BALB/c mice infected with trypomastigotes (10⁵ per mouse), one group treated with 1 mg of MGM in incomplete Freund’s adjuvant (closed circles) at days 1 and 3 postinfection (representing a dose of 50 mg/kg) and a second group, as a control, injected with only incomplete Freund’s adjuvant (open circles). Values are means ± standard deviations of 10 animals per group. Differences in parasitemia at days 7, 9, 11, and 13 are significant (P < 0.05).
asites tested and mammalian cells, since the compound can inhibit the replication of *Trypanosoma cruzi* amastigotes inside macrophages and *P. falciparum*-infected erythrocytes (Fig. 4 and 5).

Although the antibacterial activity of MGM was described 26 years ago, this antibiotic has not been used clinically probably because, in general, its in vitro and in vivo activities are weaker than those of erythromycins (22) and MGM gives cross-resistance with erythromycins. Nevertheless, our previous work has demonstrated that MGM has wide-spectrum antiviral activity that is not related to the inhibition of protein synthesis (1, 2). We first found it had interesting activity against herpesvirus and other enveloped viruses. However, it is the recent characterization of the activity of MGM against human immunodeficiency virus type 1 that opens new possibilities for the treatment of a major human disease (17). The antiparasitic activity of MGM described in this work adds to the list of interesting effects of MGM on different microbial systems. However, MGM was active against *T. cruzi* at concentrations well below those that have an effect on mammalian Golgi and lysosomes. Thus, whereas the antiviral effect of MGM seems to depend on cellular targets, MGM could exert its antiparasitic effect on specific parasitic targets. Finally, the antiparasitic activity of MGM on the in vivo model makes the possible use of MGM against human trypanosomiasis feasible.

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