ACTIVITY OF AZITHROMYCIN AGAINST LEISHMANIA MAJOR IN VITRO AND IN VIVO

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Abstract. Azithromycin, an azalide antibiotic of the macrolide family, concentrates in the tissues and especially in macrophages. Because Leishmania parasites reside in these cells, we tested this antibiotic for a possible antileishmanial activity in vitro and in vivo. Azithromycin decreased the Leishmania major promastigote count in cell-free cultures at log phase approximately 50-fold. In macrophage cultures infected with L. major amastigotes, azithromycin caused a significant decrease in parasite levels with an ED_{50} of 12 μg/ml. The activity in vivo was evaluated after infection of the footpads of susceptible BALB/cByJ mice and resistant C57BL/6J mice with L. major. Treatment of BALB/cByJ mice with azithromycin, 100 to 200 mg/kg/d, resulted in a significant decrease in lesion size and in the number of parasites per lesion, whereas no effect was seen in the treated C57BL/6J mice. Azithromycin has activity against L. major in vitro and in vivo. Given the severity of the disease and the limitations of the available therapeutic agents, azithromycin may have a significant role in the treatment of this group of diseases.

INTRODUCTION

Parasites of the genus Leishmania are transmitted by sandflies that ingest the parasite in the amastigote stage resident within macrophages, then inoculate the promastigote stage into other hosts. New therapies are needed to supplement or replace currently available therapies. Proven therapies against human leishmaniasis include pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), amphotericin B, pentamide, and paromomycin. The drugs mentioned have the disadvantages of high cost, lack of oral formulation (e.g., amphotericin B can be used only intravenously), or serious side effects that require close monitoring of the patients. New therapeutic approaches using orally administered drugs are under investigation. Miltefosine in an oral formulation has shown clinical efficacy against visceral leishmaniasis in a Phase II clinical trial, although its activity against cutaneous forms has not been determined. Fluconazole has been shown to shorten the time to healing of cutaneous infections with Leishmania major.

Azithromycin, an azalide antibiotic from a family of macrolide substances, has the potential for effective antileishmanial activity. One of its key characteristics is that the drug concentrates in tissues, especially in macrophages, and may reach levels 100 to 200 times higher than those in serum. Other advantages include (1) the possibility of oral and injectable administration, (2) good oral bioavailability and long half-life, (3) relative safety for use in children and pregnant women (Food and Drug Administration category B), and (4) a benign toxicity profile.

Several different protozoan infections of humans have been shown in vitro and in vivo to be susceptible to azithromycin in varying degrees, including Acanthamoeba, Cryptosporidium parvum, Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax, and Toxoplasma gondii. Studies involving T. gondii showed direct effects of the drug on the parasite’s viability by inhibition of protein synthesis. In addition, it was shown that azithromycin concentrates in the lysosomes of T. gondii-infected macrophages. The site of azithromycin concentration in macrophages coincides with the intracellular location of Leishmania, suggesting a potential efficacy against these infections.

To test the susceptibility of Leishmania to different drugs, promastigotes were cultured in cell-free medium and amastigotes were cultured in vitro with either human monocyte- derived macrophages or mouse peritoneal macrophages in the presence of variable concentrations of antibiotics. Infection levels were measured by microscopic observation of the living promastigotes in cell-free culture or amastigotes in macrophages to determine the number of infected cells and the number of parasites per cell. In vivo studies showed that after infection with L. major, C57BL/6 mice develop CD4 Th1 cells, and the mice are resistant. The initial swelling of the infection site gradually subsides, and the number of parasites in the lesion decreases to 0. In contrast, after infection of BALB/c mice with L. major, Th2 cells dominate, and the mice develop an uncontrolled infection. Large lesions develop that ulcerate, become necrotic, and eventually cause the death of the mice.

The objective of the present study was to determine the effect of azithromycin in vitro on the growth and viability of L. major promastigotes in cell-free culture and amastigotes in murine macrophages. In vivo studies compared the effect of azithromycin on the infection in naturally susceptible BALB/cByJ mice and in naturally resistant C57/BL6J mice.

MATERIALS AND METHODS

Antibiotics. Azithromycin (Zithromax; Pfizer, New York, NY) was obtained in lyophilized form as 500 mg/vial for intravenous administration. Amphotericin B (Fungizone; GIBCO, Rockville, MD) was obtained as sterile powder. The drugs were reconstituted with sterile water and diluted to the appropriate concentration either in sterile phosphate-buffered saline (PBS) for injection (0.5 ml/mouse) or in medium (Roswell Park Memorial Institute with supplements) for in vitro treatments.

Parasites. L. major MHOM/IL/80/Friedlin amastigotes were isolated from SCID mice and cultured at 26°C to log phase in Grace’ medium (GIBCO, Rockville, MD) supplemented with 20% fetal bovine serum (FBS), 2 mM of glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The parasites were centrifuged at 1,500g for 15 minutes, and the pellet was suspended in 10% dimethyl sulfoxide in PBS. Aliquots were kept frozen in liquid nitrogen until use. The cryopreserved promastigotes were thawed at 37°C and
washed with warm RPMI (low glucose, 2 mg/ml) (Sigma, St. Louis, MO) supplemented with 10% FBS, 25 mM of N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES), 50 μM of β-mercaptoethanol, 2 mM of glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The pellet was suspended in Grace’s medium and incubated to stationary phase (26°C for 5–6 days). The metacyclic promastigotes were obtained by negative selection with peanut agglutinin (Arachis hypogaea; Sigma, St. Louis, MO).26 The parasites were washed 4 times with PBS by centrifugation 1,500g for 15 minutes, then the pellet was suspended at a density of 1 × 10^6 promastigotes/100 μl in RPMI.

**Treatment of promastigotes in cell-free culture.** Promastigotes of *L. major* were grown in axenic cell-free culture in Grace’s medium to mid-log phase (14 × 10^6/ml) at 27°C (about 2.5 days). The suspension was diluted to 1 × 10^6 parasites/ml in Grace’s medium containing 10–1,000 μg/ml of azithromycin or 1–10 μg/ml of amphotericin B. After exposure to the drug for 3–6 days, aliquots were taken and inoculated into 96-well culture plates to determine the number of live parasites, by the limiting dilution method. The aliquots were inoculated into the wells at 10-fold dilutions (20 μl of sample and 180 μl of medium per well). Each sample was plated in triplicate, and the plates were kept at 27°C for 7 days. The highest dilution with live parasites was recorded, and the results are expressed as log_{10} dilution.27

**Animals.** BALB/cByJ or C57BL/6J female mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 6–8 weeks of age. The maintenance and care complied with the National Institutes of Health guidelines for humane use of laboratory animals.

**Treatment of amastigotes within macrophages.** Monocyte-derived macrophages were harvested from the marrow of femora of naïve C57BL/6J mice and cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing high glucose (4.5 mg/ml) and supplemented with 20% FBS, 30% L-929 cell supernatant as a source of granulocyte-macrophage colony-stimulating factor,28 1 mM of pyruvate, 50 μM of β-mercaptoethanol, 2 mM of glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were harvested after 6 days at 37°C/5% CO_2 by washing the plates with ice-cold PBS (endotoxin-free; GIBCO, Rockville, MD). After sedimentation at 170g for 10 minutes, the pellet was suspended in DMEM to a density of 1 × 10^6 cells/250 μL. Sterile round glass coverslips (12 mm diameter) were placed in each well of 24-well culture plates.27 A suspension of macrophages (1 × 10^6 in 250 μl of DMEM) was added and incubated at 37°C/5% CO_2 overnight. The medium was aspirated, and the wells were washed with 2 ml of warm DMEM to remove nonadherent cells. A suspension of 1 × 10^6 metacyclic promastigotes in 100 μl of RPMI was added on top of the coverslip in each well (for a macrophage/parasite ratio of 1:1). The plates were incubated for 3 hours at 37°C/5% CO_2, then the medium was aspirated, and the wells were washed with 2 ml of warm PBS to remove free parasites. Fresh RPMI (2.5 ml) with or without antibiotics (azithromycin or amphotericin B) at the appropriate concentration was added in duplicate wells. The plates were incubated at 37°C/5% CO_2 for 5 days with daily change of medium. The medium was aspirated; the wells were washed with PBS; and the coverslips were removed, dried in the air, and glued to microscope slides. After staining with Wright-Giemsa (Biochemical Sciences, Swedesboro, NJ), the cells on the glass disks were counted along the equator from end to end. The total number of macrophages (200–500), the number of infected macrophages, and the total number of parasites were recorded. The results are presented as a multiplication index (MI) calculated as the ratio of the mean number of parasites per 100 macrophages in experimental cultures versus control cultures [MI = (mean number of parasites per 100 treated macrophages / mean number of parasites per control 100 macrophages) × 100].

**Treatment of mice infected with *Leishmania major*.** Metacyclic promastigotes were obtained as described previously. Suspensions of 1 × 10^6 to 2 × 10^6 parasites per 50 μl of sterile PBS (endotoxin-free) were injected with a 30G needle into the right rear footpad of the mice. On the same day, a solution of azithromycin, 2–4 mg/0.5 ml PBS, was injected subcutaneously along the back of the animal, representing a dose of 100–200 mg/kg. The drug was administered daily, 5 times a week, for the duration of the experiment. Once a week, the thickness of the infected footpad and the contralateral footpad was measured with a dial micrometer (Starett Co, Athol, MA), and the difference between the 2 footpads was recorded.

At the end of the experiment (4–8 weeks), the number of parasites in each lesion was determined as described previously.27 Briefly, each lesion was excised aseptically, homogenized in 2 ml of sterile PBS (supplemented with penicillin/streptomycin) to disrupt the tissue cells and obtain free parasites. After low-speed centrifugation (170g for 5 minutes) to remove debris, the supernatant was collected and centrifuged at 1,500g for 15 minutes. The pellet was suspended in 2 ml of Grace’s medium and cultured using the limiting dilution method described previously.

**Data analysis.** *In vitro* and *in vivo* experiments were carried out 2–4 times. The data were analyzed for statistical significance with the Mann-Whitney U test or Student’s t-test when appropriate; *P* < 0.05 was considered significant.

**RESULTS**

Effect of azithromycin on *Leishmania major in vitro*. In cell-free cultures of promastigotes, azithromycin significantly reduced the parasite counts after 6 days from 8.0 ± 0.1 log_{10} in the control to 6.3 ± 0.5 log_{10} in the cultures with 100 μg/ml of azithromycin and 6.8 ± 0.2 log_{10} in cultures with 1,000 μg/ml of azithromycin. No effect was seen with 10 μg/ml of azithromycin. Under the same conditions, amphotericin B, 1 μg/ml, killed most of the parasites (0.1 ± 0.1 log_{10}).

Bone marrow macrophages infected with amastigotes were treated with azithromycin at different concentrations. After 5 days in culture, the number of infected macrophages decreased with increasing concentration of azithromycin in a dose-dependent manner. At 0 concentration of the drug (control), approximately 50% of the cells were infected; from the regression line (r² = 0.76), an ED_{50} of 12 μg/ml was calculated (Figure 1). The number of parasites per cell varied from 1 to > 25 in the same culture. To compare different cultures in the different experiments, the results are presented as the multiplication index. It seems that the decrease in the number of parasites per cell is also dose-dependent. The value for 50% decrease compared with the control was 3–15 μg/ml, in agreement with the level of 12 μg/ml found for the infection.
rate as described previously. Amphotericin B was highly effective at controlling the amastigotes, as it was in killing the promastigotes (Figure 2).

**Effect of azithromycin on *Leishmania major* in vivo.** Footpad swelling developed in control infected BALB/cByJ and C57/BL6J mice within 2 weeks postinfection. Treatment of infected BALB/cByJ mice with azithromycin resulted in reduced swelling, whereas treatment of infected C57/BL6J mice had no effect (Figure 3). These results were observed with infecting doses of $1 \times 10^5$ to $2 \times 10^6$ per mouse and treatment with 100 or 200 mg/kg of azithromycin. The number of parasites per lesion in the azithromycin-treated BALB/cByJ mice ($5.28 \pm 0.08 \log_{10}$) was significantly lower than in controls ($6.58 \pm 0.30 \log_{10}$) at 4 weeks postinfection ($P < 0.01$). The number of parasites per lesion at 4 weeks in treated infected C57BL/6 mice did not differ from controls. Values were not obtained at 7 weeks because the lesions in the control animals became necrotic, and reliable parasite counts could not be obtained.

**DISCUSSION**

These experiments show that azithromycin has *in vitro* and *in vivo* activity against the promastigotes and amastigotes of *L. major*. There was a statistically significant reduction in the survival of promastigotes in cell-free culture and of amastigote cultures in bone marrow–derived macrophages. The effect of azithromycin against amastigotes was dose dependent, with an ED$_{50}$ of 12 $\mu$g/ml. Because much larger amounts of drug were needed to control the promastigotes in cell-free culture, the more pronounced effect in macrophage culture may be due to the ability of the macrophage to concentrate the drug actively inside the cells. Treatment of susceptible BALB/cByJ mice reduced the lesion size and parasite numbers in the lesions; however, no effect was seen in the normally resistant C57/BL6J mice.

The mechanism by which azithromycin controls *L. major* infections is unknown. There is evidence that azithromycin can have a direct effect on the survival of *T. gondii* by the inhibition of protein synthesis. Evidence in the present report also suggests that azithromycin may have a direct effect on the promastigotes. Alternatively, azithromycin has been shown to enhance the ability of macrophages to eliminate many different pathogens. Macrophage activation might be the killing mechanism induced with azithromycin against amastigotes in macrophages. It is also possible that azithromycin has a direct killing effect and an immunomodulatory effect.

To our knowledge, the use of monocyte-derived bone marrow macrophages for drug susceptibility tests against *Leishmania* has not been reported previously. The advantage of using these cells rather than peritoneal macrophages is the higher yield of cells per mouse. Previous studies showed variable results in determining the susceptibility of *Leishmania* to different drugs depending on the source of the macrophages. Specifically, it was shown that *Leishmania* are susceptible to pentavalent antimony *in vivo* and *in vitro* in mouse peritoneal macrophages and in human monocyte-derived macrophages but not in tumor macrophages. Pentamidine was shown to have significantly better activity when used in cultures of *Leishmania* with human macrophages than in cultures using mouse peritoneal macrophages. Monocyte-derived bone marrow macrophages are an alternative source for *in vitro* testing of drugs for efficacy against *Leishmania*.

Amphotericin B was used as a positive control in these studies because it was reported previously to control *Leishmania tropica* and *Leishmania donovani* infections in human monocyte-derived macrophage cultures. The concentration used in the present study followed previous reports and was based on the approximate plasma levels in humans treated with the drug. When comparing the efficacy of azithromycin with amphotericin B, the latter was more effective at eliminating the parasites. The elimination of parasites achieved
FIGURE 3. Effect of treatment of BALB/cByJ and C57/BL6J mice with 100 mg/kg of azithromycin on the development of footpad swelling caused by infection with 2 × 10^6 L. major amastigotes. The values represent the mean ± SD difference between the thickness of the infected footpad and the contralateral normal footpad. *P < 0.05.

with azithromycin was incomplete, a characteristic similar to that observed in vitro with antimony gluconate and pentamidine tested in similar systems. These 2 drugs are effective and clinically indicated for therapy of leishmaniasis.

The in vivo results showed that azithromycin was functional only in the susceptible BALB/cByJ mice and not in the resistant C57BL/6J mice. These results were seen in experiments in which different doses of azithromycin and different infection doses were tested. Similar results have been reported for the treatment of BALB/c and C57BL/6J mice infected with L. tropica with amphotericin B. The drug was effective in BALB/c mice and had no effect in C57BL/6J mice. It is not clear why there is this dichotomy in responses. One hypothesis to explain these findings is that azithromycin augments a weak immune response but is ineffective at boosting a functioning protective immune response to higher levels. Azithromycin has been shown to have immunomodulatory activity by preventing the production of proinflammatory mediators and cytokines. In addition, effects on macrophage functions include stimulation of phagocytosis, chemotaxis, and cytocidal activity. On this basis, it has been suggested that the ability of azithromycin to accumulate in the tissues and especially in phagocytes can act synergistically with the host immune system. It is possible that azithromycin in mice infected with L. major enhances the immune response, but this effect is patent only when the inherent immune response is suboptimal. The diversity of human responses to infection with Leishmania is one of the integral characteristics of this disease; it is possible that the portion of the population that requires drug intervention because of an inadequate immune response may benefit from treatment with azithromycin.

In conclusion, it has been shown that azithromycin has activity against L. major in vitro and in vivo. Given the severity of the disease and the limitations of the available therapeutic agents, azithromycin may have a significant role in the treatment of this group of diseases.

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