

Antileishmanial Activity of the Antiulcer Agent Omeprazole

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The benzimidazole compound omeprazole, used widely for the treatment of peptic ulcer disease, inhibits the growth of *Leishmania donovani*, the causative agent of visceral leishmaniasis. Promastigotes cultured at acidic pH and amastigotes within infected macrophages are reduced 90% or more with 150 μ M omeprazole. Anti-parasitic action of the drug is due to its inhibition of the P-type K^+, H^+ -ATPase on the surface membrane. This enzyme is important for pH homeostasis and the maintenance of proton motive force across the membrane in *Leishmania*. The drug is effective only at acidic pH, a condition that mimics the in vivo environment within the phagolysosomal vesicles where the amastigote form of the parasite resides. Omeprazole deserves consideration as an alternative to currently available chemotherapeutics, which have severe toxic side effects.

Visceral leishmaniasis (*Kala azar*), a disease characterized by enlargement of the spleen and liver, is caused by the parasitic protozoan *Leishmania donovani*. If left untreated, it can be fatal. Other species of *Leishmania* cause cutaneous and mucocutaneous forms of the disease which, though not life threatening, are chronic and often very disfiguring. According to the World Health Organization estimates, 12 million to 14 million people worldwide are affected.

Leishmania spp. are dimorphic organisms which exist as flagellated promastigotes in the sandfly gut and as aflagellated amastigotes in mammalian macrophages (9). Since they exist within the phagolysosomes of mammalian macrophages, amastigotes are relatively protected from the host immune responses and chemotherapeutics (1, 5, 6). The phagolysosomal environment is acidic (pH 4.0 to 5.0) in rat, human, and mouse macrophages (8, 15, 18). The acidity of the phagolysosomes is not affected by the presence of the parasite (4, 8). Amastigotes were shown to carry out activities such as respiration, nutrient uptake, and DNA and RNA synthesis optimally at pH 5.0 to 5.5 (15). Nutrient uptake occurs through a proton symport mechanism (10, 19), but it comes at a cost to the parasite in the accumulation of protons inside. This is countered by a P-type K^+, H^+ -ATPase situated on the surface membrane of the parasite, which pumps the protons out (2, 3, 12, 19). This enables the amastigote to maintain a stable and neutral internal pH within the proton-rich phagolysosomes.

In view of its role in maintaining a neutral intracellular pH, the K^+, H^+ -ATPase is an attractive target for chemotherapy. Current chemotherapy for leishmaniasis employs heavy metal compounds (antimony and arsenic) and the antibiotic amphotericin B, all of which induce toxic side effects for the host as well. An aminoglycoside antibiotic aminosidine (paromomycin) has recently shown some promise, although it has the drawback of poor penetration and induces painful inflammation in some cases (7).

Omeprazole (5-methoxy-2-[4-methoxy-3,5-dimethyl pyridinyl methyl sulfanyl]-1 H-benzimidazole) is the active ingredient of Prilosec, used to treat peptic ulcer disease. It is a specific

inhibitor of the human gastric K^+, H^+ -ATPase (11). At neutral pH, omeprazole permeates cell membranes and accumulates in acidic cellular compartments, such as lysosomes, where it undergoes protonation. The protonated form becomes an active sulfenamide compound and acts as a potent ATPase inhibitor (13). In the human stomach, activated omeprazole was shown to inhibit the gastric K^+, H^+ -ATPase and halt acid secretion by parietal cells (5).

Omeprazole and other benzimidazoles have antibacterial activity against *Helicobacter pylori* (14, 17), but the exact mechanism of action is unclear. Although *H. pylori* has a P-type ATPase that is affected by benzimidazoles, this inhibition apparently does not correlate to the antibacterial action of the drug.

The P-type K^+, H^+ -ATPase on the surface membrane of *Leishmania* is a potential target for omeprazole. Accumulation of omeprazole within the acidic lysosomes of macrophages makes it attractive as an antileishmanial agent. Since *Leishmania* parasitophorous vacuoles fuse with lysosomes (1, 5), omeprazole-laden lysosomes could target the active drug directly to the parasite. The active sulfenamide binds to the K^+, H^+ -pump, crippling its activity. Impaired proton extrusion will lead to intracellular acidification of the amastigote, limiting its growth within the macrophage.

To our knowledge, this is the first report of omeprazole as a potential antiparasitic drug, especially against an intracellular parasite. Here we report the inhibitory effect of omeprazole on the growth of *L. donovani* in vitro and within primary mouse macrophages in culture. It is suggested that this antileishmanial activity is due to the inhibition of the P-type K^+, H^+ -ATPase on the surface membrane.

MATERIALS AND METHODS

Parasites. *Leishmania donovani* Sudan 1S promastigotes were maintained in medium 199 with 15% fetal bovine serum at pH 7.2 as described previously (12). Parasites were acid adapted by growing them in medium 199 supplemented with 15% fetal bovine serum and 20 mM MES (2-[N-morpholino]ethanesulfonic acid) at pH 5.1. They have been maintained in the acidic medium at 37°C with 5% CO₂ for several years. These cells are ovoid, mostly nonflagellated, and amastigote-like.

Macrophages. Peritoneal macrophages were obtained from CBA/caj mice without thioglycolate stimulation and cultivated in eight-well chamber slides at a density of 2.5×10^5 /ml in RPMI 1640 medium with 15% fetal bovine serum.

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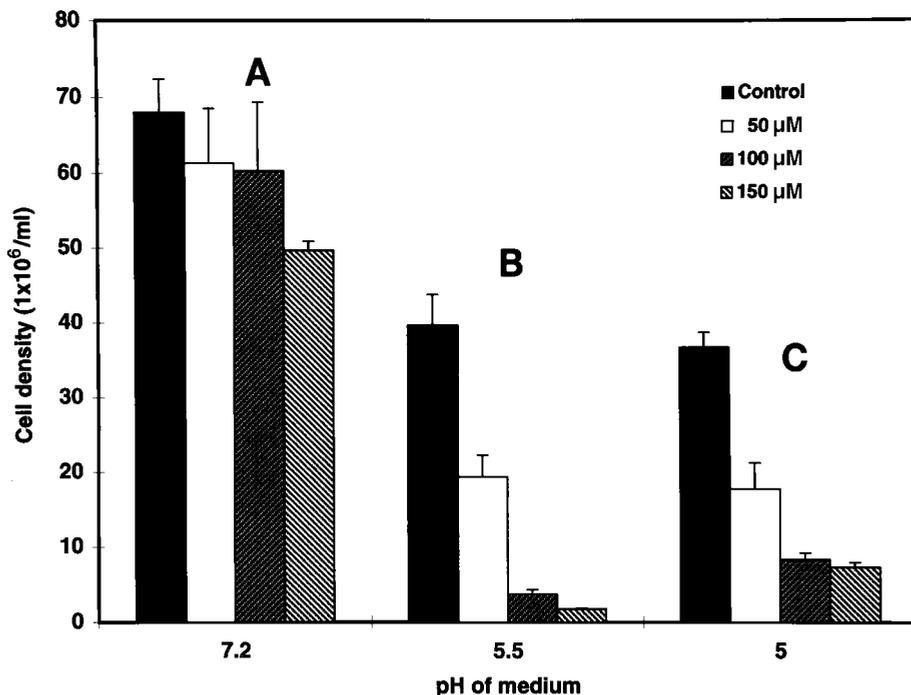


FIG. 1. Effect of pH on inhibition of growth by omeprazole. Parasites from pH-7.2 medium were washed and inoculated at a starting density of 5.7×10^6 cells/ml in fresh medium at pH 7.2 (A) and at pH 5.5 (B) with various doses of omeprazole (50, 100, and 150 μM) in 12-well tissue culture plates. Acid-adapted parasites (C) were incubated at a starting density of 7.5×10^6 cells/ml in the presence of 50, 100, and 150 μM omeprazole. All were incubated at 26°C with 5% CO_2 . Cells were counted in triplicate from each well, and the experiments were repeated four times. This figure represents results after 48 h of incubation.

Nonadherent cells were washed off after a 24-h incubation at 37°C and 5% CO_2 . Promastigotes were loaded in each well at a ratio of 10 promastigotes to 1 macrophage. After overnight infection, the macrophages were washed to remove all external promastigotes. Omeprazole was added at different dosages. The slides were washed after 48 h, dried, and stained with Wright-Giemsa stain (Diff-Quik; Baxter Scientific Products).

Intracellular pH determination. Intracellular pH (pHi) was determined by measuring the fluorescence of the pHi indicator BCECF (2',7'-bis [carboxyethyl-5 (and -6)-carboxy] fluorescein) as previously described (12, 16). In brief, promastigotes were harvested at stationary phase and loaded with BCECF in fresh medium 199 at pH 7.2. They were treated with nigericin (10 $\mu\text{g}/\text{ml}$) for acid loading, and treatment was stopped by the addition of bovine serum albumin (5 mg/ml). The cells were washed twice with choline buffer containing 115 mM choline chloride, 10 mM glucose, 5 mM MgCl_2 , 10 mM MES, 10 mM MOPS (3-[*N*-morpholino]propanesulfonic acid) (pH 7.1, adjusted with Trizma base) to remove excess dye, nigericin, and BSA. Cells treated with omeprazole were incubated for 10 min before they were scanned in the choline buffer (pH 5.7) with a fluorescence spectrophotometer (Perkin-Elmer pmf-66) at excitation and emission wavelengths of 503 and 535 nm, respectively. Once fluorescence intensity was stabilized, 5 mM KCl was added and the change in fluorescence was recorded. Procedures for parasites cultured at pH 5.1 were the same except that after BCECF loading at pH 7.2, they were washed with choline buffer at pH 5.7.

ATPase assay. Activity of purified ATPase was determined by measuring the release of inorganic phosphate (P_i) from [γ - ^{32}P]ATP as described previously (3). Purified enzyme (0.5 μg) was incubated for 20 min at room temperature in 100 μl of buffer containing 1 mM MES, 1 mM MOPS, and 50 mM sucrose at pH 7.4. The reaction was started by adding 100 μl of reaction buffer containing 40 mM MOPS, 40 mM sucrose, 4 mM MgCl_2 , 2 mM [γ - ^{32}P]ATP, and 20 mM KCl at pH 7.4. In experiments to determine the effect of omeprazole, the enzyme was incubated with various amounts of omeprazole for 20 min at room temperature at pH 5.5 before adding the reaction mixture with [γ - ^{32}P]ATP. After a 10-min incubation at 37°C, the reaction was stopped by the addition of 5% (final) trichloroacetic acid, and 50 μl of the mixture was removed for analysis of P_i (3).

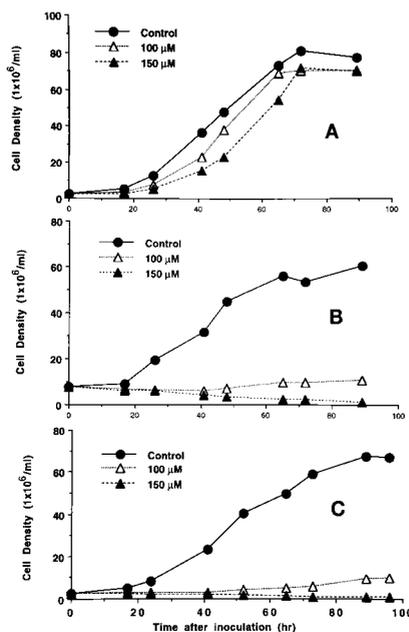


FIG. 2. Effect of omeprazole on the growth of *L. donovani* at various pHs. Growth was followed by inoculating the cells into media with or without omeprazole in wells at starting densities of $2.5 \times 10^6/\text{ml}$ at pH 7.0 (A), $7.5 \times 10^6/\text{ml}$ at pH 5.5 (B), and $2.5 \times 10^6/\text{ml}$ at pH 5.0 (C). Cells from each well were counted at regular intervals. (A) Promastigotes grown at pH 7.0 and incubated in pH 7.0 medium with or without drug. (B) Promastigotes grown at pH 7.0 and incubated in pH 5.5 medium with or without drug. (C) Acid-adapted parasites incubated in pH 5.0 medium with or without drug. Each point represents the average for three experiments.

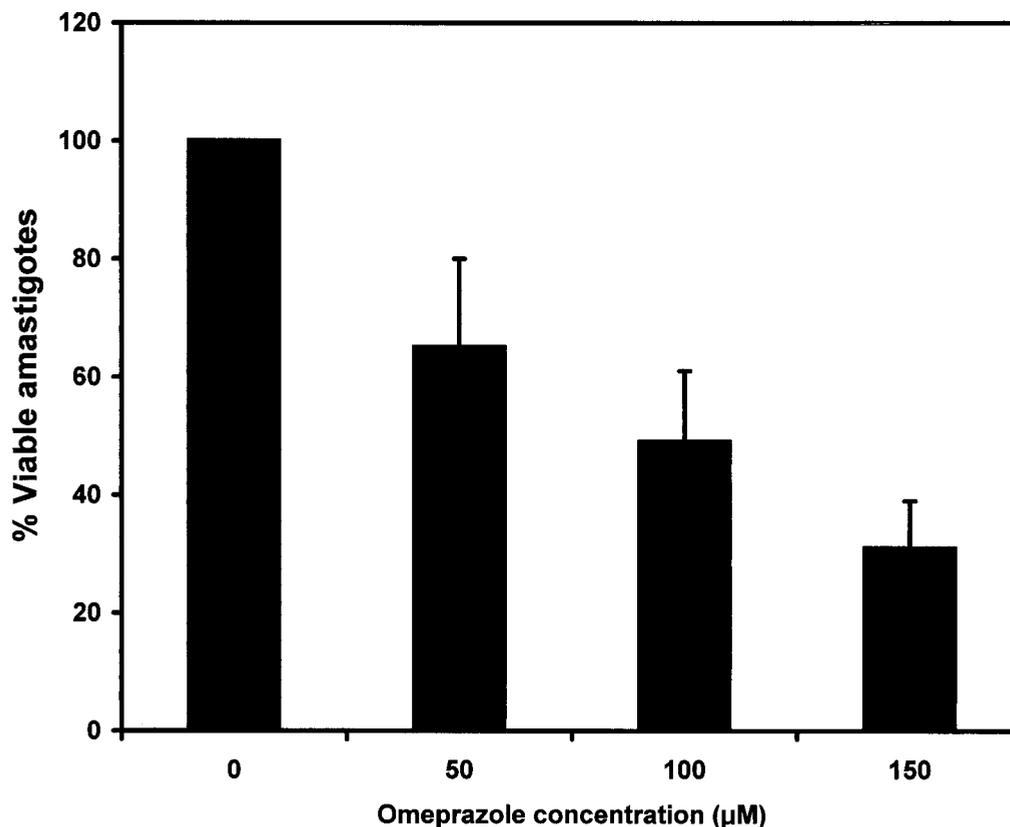


FIG. 3. Dose-related response of intracellular amastigotes to omeprazole.

RESULTS

Effect of omeprazole on parasites in vitro. Parasites cultured at neutral pH as well as those that were acid adapted long term were used to investigate the effect of omeprazole on growth in vitro. Growth was followed at various pHs and at different concentrations of omeprazole. Omeprazole was not active at pH 7.2, and although it may permeate the outer membrane, its effect on the parasite was negligible even at the high dose of 150 µM (Fig. 1A). In contrast, at pH 5.5 (Fig. 1B) it was in its pharmacologically active form. At 50 µM, growth is reduced by 50% while at 150 µM growth is inhibited 90 to 95% in 48 h. Acid-adapted cells also suffered similarly high levels of growth inhibition, with omeprazole concentrations of 50, 100, and 150 µM when tested at pH 5 (Fig. 1C). During a 96-h incubation, parasites grown at neutral pH showed no growth inhibition in the presence of 100 to 150 µM omeprazole at pH 7.2 (Fig. 2A) but the same cells suffered severe growth inhibition at pH 5.5 (Fig. 2B). Parasites cultured long term in pH-5.1 medium and then treated with omeprazole in pH-5.0 medium were also severely inhibited (Fig. 2C). It is clear that omeprazole in its active protonated form limits parasite growth severely in vitro.

Effect of omeprazole on parasites within cultured macrophages. To test the antileishmanial property of omeprazole further, macrophages infected with *L. donovani* were treated with various concentrations of omeprazole, ranging from 0 to 150 µM. As shown in Fig. 3, intracellular parasites (amastigotes) suffer significant inhibition in a dose-dependent man-

ner. Figure 4 shows the results of a typical experiment: untreated control cultures (Fig. 4A) show 1 to 30 parasites per macrophage, but infected cultures treated with 150 µM omeprazole (Fig. 4B) show very few or no intracellular parasites. In another set of experiments, peritoneal macrophages were infected in vivo by injecting 2×10^9 parasites into the peritoneal cavity of CBA/caj mice. Two days postinfection, macrophages were harvested by flushing the peritoneal cavity with physiological saline followed by centrifugation at $2,000 \times g$. They were washed twice and incubated in eight-well chamber slides in RPMI 1640 medium with 15% fetal bovine serum. Unattached cells were washed off, and the slides were incubated in the same medium containing 150 µM omeprazole. The results after 48 h of incubation were essentially similar to those shown in Fig. 4B. Omeprazole thus shows a marked effect on amastigote survival within the macrophages.

Effect of omeprazole on the K^+, H^+ -ATPase. We have previously demonstrated the presence of a K^+, H^+ -ATPase on the surface membrane of *L. donovani* (12). This pump plays a key role in the maintenance of the pH_i in *L. donovani*. Intracellular pH changes can be monitored with various fluorescent dyes (13), among which BCECF is the most widely used. It was thought that inhibition of the K^+, H^+ -ATPase by omeprazole could block H^+ extrusion, resulting in proton accumulation and intracellular acidification, which would terminate or limit the growth of the parasite. To explore this possibility, the effect of omeprazole on intracellular pH changes was examined.

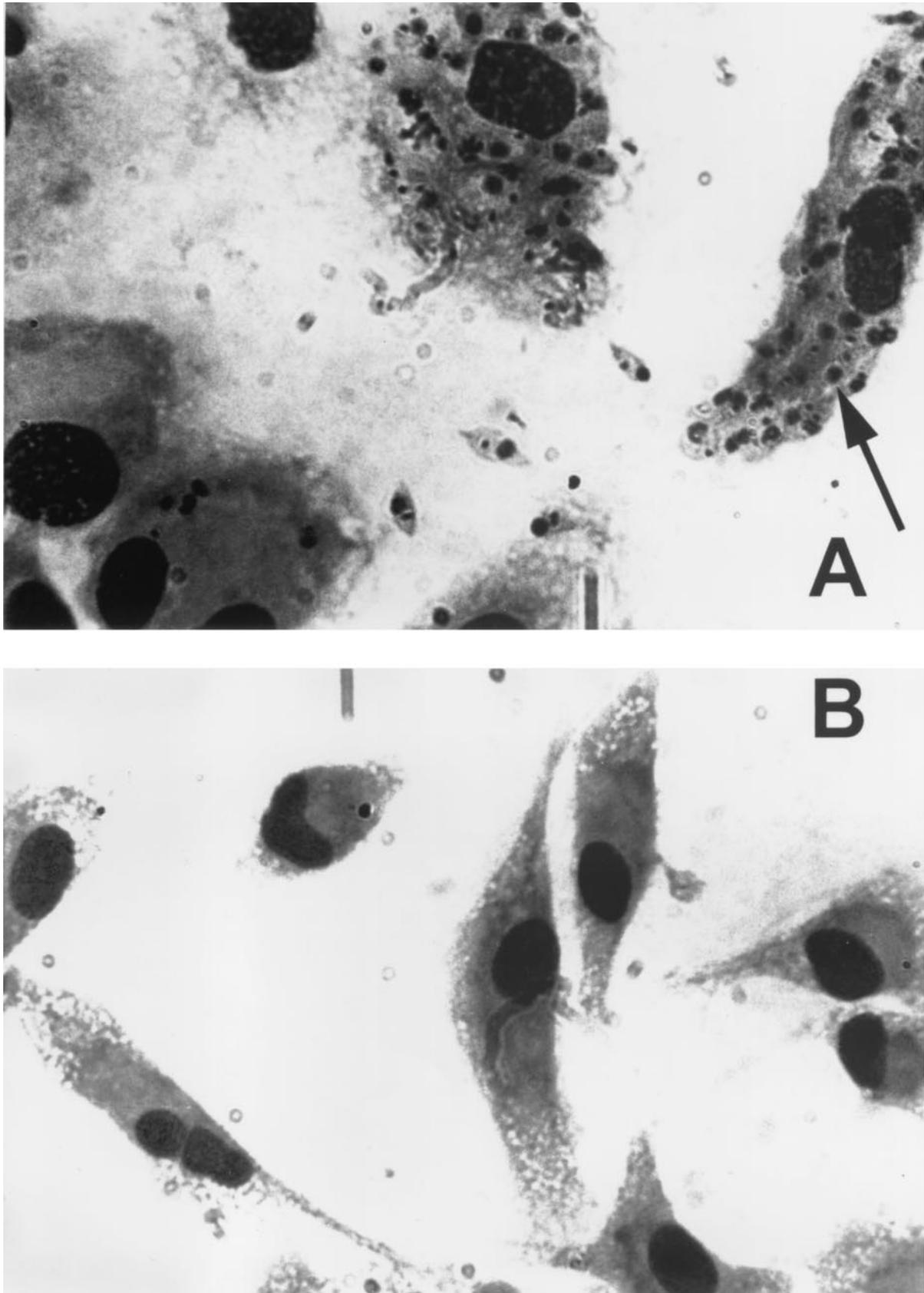


FIG. 4. Effect of omeprazole on *L. donovani* in infected macrophages in vitro. (A) *Leishmania*-infected macrophages (control). The arrow points to amastigotes within the acidic phagolysosomes of the macrophage. (B) *Leishmania*-infected macrophages treated with 150 μ M omeprazole for 48 h.

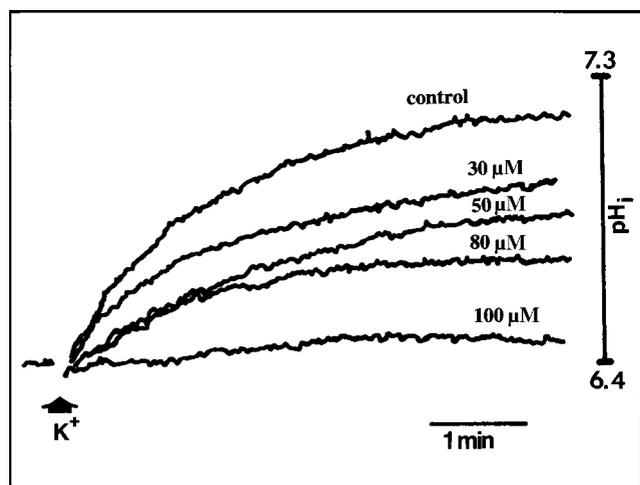


FIG. 5. Effect of omeprazole on H^+ extrusion and pH_i in *L. donovani*. Tracings show fluorescence in promastigotes treated with various amounts of omeprazole (30, 50, 80, and 100 μM).

Parasites cultivated both at pH 7.2 and pH 5.1 were used in this experiment. They were initially loaded with protons as described in Materials and Methods so that the pH_i declined to approximately pH 6.4. Protons are not released unless K^+ is added to the suspension (12). Upon addition of 5 mM KCl, the acid-loaded parasites pumped out H^+ and fully recovered within 5 min (Fig. 5). However, cells incubated with omeprazole for 10 min at pH 5.7 following acid loading suffered inhibition of proton extrusion in a concentration-dependent manner (Fig. 5). It is worth noting that once the cells are acid loaded, omeprazole inhibits proton extrusion even when the cells are suspended at neutral pH. The drug is protonated to its active form as the intracellular pH drops below neutral.

Since omeprazole is a specific inhibitor of the gastric K^+, H^+ -ATPase both in vitro and in vivo (11, 13), its effect on the purified plasma membrane K^+, H^+ -ATPase in *L. donovani* was tested. The enzyme is indeed sensitive to omeprazole at acid pH. At 80 μM omeprazole, activity is inhibited about 50%. Activity (nmol of P_i /min/mg of protein) in the absence and presence of the drug was $3,710 \pm 62$ and $1,900 \pm 47$, respectively ($n = 4$). Blockage of the enzyme by omeprazole confirms not only that the ATPase is indeed a K^+, H^+ -ATPase but also that this enzyme is responsible for the K^+ -induced proton extrusion in *L. donovani*. When omeprazole blocks the K^+, H^+ -ATPase on the plasma membrane, excess protons cannot be extruded, leading to intracellular acidification. Growth of the parasite is seriously impeded or terminated as the intracellular pH drops.

DISCUSSION

This report demonstrates that omeprazole is effective in killing free-living parasites (promastigotes) at acidic pH as well as intracellular parasites (amastigotes) within infected macrophages. Since omeprazole is a lysosomotropic drug, it is attractive as a potential chemotherapeutic agent for the treatment of intracellular parasites, such as *Leishmania*. It is effective only at an acidic pH and does not affect growth at a neutral pH. This

is presumably due to the conversion of the compound from a benzimidazole into the more toxic sulfenamide form at the acidic pH. In the gastric K^+, H^+ -ATPase system, more omeprazole binds to the enzyme at pH 5.5 than at neutral pH (11, 13). The activated compound binds to sulfhydryl groups of the ATPase on the luminal side of the membrane and stops the extrusion of protons, thus preventing stomach acidification. *Leishmania* has a functionally similar K^+, H^+ -ATPase, and presumably omeprazole inhibits the enzyme in a similar fashion. Our results show that parasites pretreated with omeprazole are unable to extrude protons and maintain a neutral intracellular pH. This and the inhibition of the isolated enzyme strongly suggest that the target of the drug is in fact the K^+, H^+ -ATPase on the plasma membrane of the parasite.

The finding that omeprazole inhibits *Leishmania* amastigotes within macrophages is promising. The parasite thrives sequestered within phagolysosomes, where most other pathogens are destroyed. Omeprazole accumulates in the acidic milieu of phagolysosomes and is activated to inhibit the growth of the parasite.

Omeprazole is widely used in the treatment of peptic ulcer disease. It inhibits the K^+, H^+ -ATPase in gastric mucosa (11), and its pharmacological properties and therapeutic potential for humans are firmly established. It does not have the severe toxic side effects associated with currently available antileishmanial drugs. It could be a good candidate in the topical treatment of cutaneous leishmaniasis. The development of a proper delivery system (e.g., in liposomes) to target the drug directly to the spleen and the liver should make it useful in the treatment of visceral leishmaniasis as well. The potential value of the drug for treating human leishmaniasis, however, needs further evaluation in animal models. Other benzimidazole derivatives, such as lansoprazole, deserve evaluation for potential antileishmanial properties. The antiparasitic activity of omeprazole adds to the versatility of omeprazole as a chemotherapeutic agent. This is reminiscent of the analgesic aspirin, which is also useful in treating heart disease.

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