Antiamoebic and Toxicity Studies of a Carbamic Acid Derivative and Its Therapeutic Effect in a Hamster Model of Hepatic Amoebiasis

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Amoebiasis is an important public health problem in developing countries. *Entamoeba histolytica*, the causative agent of amoebiasis, may develop resistance to nitroimidazoles, a group of drugs considered to be the most effective against this parasitic disease. Therefore, research on new drugs for the treatment of this common infection still constitutes an important therapeutic demand. In the present study we determined the effects of a carbamate derivative, ethyl 4-chlorophenylcarbamate (C4), on trophozoites of *E. histolytica* strain HM-1: IMSS. C4 was subject to various toxicity tests, including the determination of mutagenicity for bacterial DNA and changes in the enzymatic activities of eukaryotic cells. Genotoxicity studies were performed by the mutagenicity Ames test (plate incorporation and preincubation methods) with *Salmonella enterica* serovar Typhimurium, with or without metabolic activation produced by the S9 fraction of rat liver. C4 toxicity studies were performed by measuring enzymatic activity in eukaryotic cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide–formazan test with Fischer 344 rat hepatocytes. C4 did not induce either frame-shift mutations in *S. enterica* serovar Typhimurium TA97 or TA98 or base pair substitutions in strains TA100 and TA102. The compound was not toxic for cultured rat hepatic cells. Trophozoites treated with 100 µg of C4 per ml were inhibited 97.88% at 48 h of culture; moreover, damage to the amoebae was also confirmed by electron microscopy. The antiamoebic activity of C4 was evaluated by using an in vivo model of amoebic liver abscess in hamsters. Doses of 75 and 100 mg/100 g of body weight reduced the extent of the amoebic liver abscess by 84 and 94%, respectively. These results justify further studies to clearly validate whether C4 is a new suitable antiamoebic drug.

Entamoeba histolytica is the etiologic agent of amoebiasis, a disease exclusive to humans (25). It is preferentially distributed in tropical and subtropical areas of Africa, Asia, and Latin America, especially in developing countries with poor sanitary conditions (18). In some countries, such as Mexico, it is among the top 10 causes of death; worldwide, only malaria and schistosomiasis cause greater numbers of deaths (35). Epidemiological data on the prevalence of this infection indicate that about 500 million people worldwide are infected with the invasive parasite *E. histolytica* and the noninvasive parasite *Entamoeba dispar* (9, 26, 34). Of these, 90% are asymptomatic carriers, whereas the other 10% of infected individuals show clinical symptoms such as colitis, dysentery, and amoebic liver abscess (ALA) (36).

Antiamoebic drugs have been classified as luminal if their site of action is in the large intestine and extraluminal if their site of action is in other organs, mainly the liver (11). Although most antiamoebic drugs have been shown to be relatively efficient for the treatment of clinical cases, the long-term use of medications produces undesirable side effects in patients. The chemical products used to eradicate *E. histolytica*, such as nitroimidazoles, have been shown to have several important problems; for example, they might be mutagenic and toxic for the host when they are used at high doses, amoeba strains are able to develop resistance to these drugs, and insufficient information is available about the drug levels at the tissue sites of infection in vivo (4, 5, 7, 12, 13, 27, 28). On the basis of these considerations, it is important to search for new alternative antiamoebic drugs from either biological or chemical sources that can eliminate the parasite without generating complications or secondary reactions in the host.

In this work we report on a new carbamate derivative drug, ethyl 4-chlorophenylcarbamate (C4), as an antiamoebic agent. The drug showed good activity against axenic *E. histolytica* cultures and significantly reduced the development of ALAs in hamsters. C4 was neither mutagenic for bacterial cells nor toxic to rat hepatocytes.
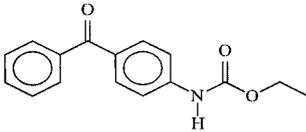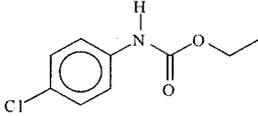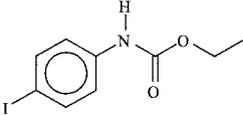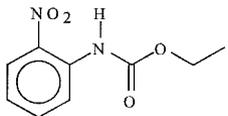
MATERIALS AND METHODS

Chemicals. Carbamic acid derivatives (Table 1) were synthesized by the method described by Angeles et al. (2). Briefly, aryl- and alkyl-amines with sodium hydride and diethylcarbonate were used in dry benzene as the solvent; they were then purified by column chromatography and recrystallized. Structures were elucidated by common spectroscopy techniques (infrared, nuclear magnetic resonance, and mass spectroscopies). 2-Aminoanthracene (2AA), cyclophosphamide (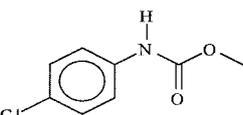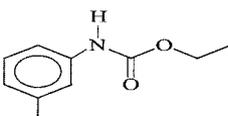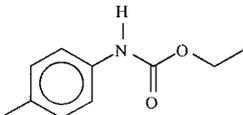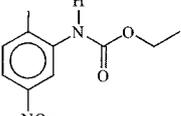CP), metronidazole [1-(2-hydroxy-ethyl)-2-methyl-5-nitroimidazole], mitomycin C (MC), *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG), and picrolonic acid (PA) were obtained from Sigma-Aldrich Co. (St. Louis, Mo.). The antiamoebic activity of C4 is the subject of a patent (24).

Amoeba strain and growth conditions. Trophozoites of *E. histolytica* strain HM-1:IMSS (from a human in Mexico, obtained from the Instituto Mexicano del

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TABLE 1. Carbamic acid derivatives examined in this work

Full chemical name	Structure	% Inhibition of *E. histolytica* growth[a]
Ethyl 4-benzoylphenylcarbamate		100
C4		97.88
Ethyl 4-iodophenylcarbamate		81.66
Ethyl 2-nitrophenylcarbamate		76.87
Ethyl 4-nitrophenylcarbamate		74.76
Ethyl 4-hydroxyphenylcarbamate		60.65
Methyl 4-chlorophenylcarbamate		59.67
Ethyl 3-nitrophenylcarbamate		56.57
Ethyl 4-methylphenylcarbamate		55.86
Ethyl 2-methyl-5-nitrophenylcarbamate		35.42
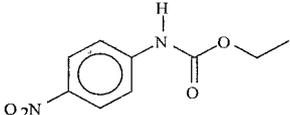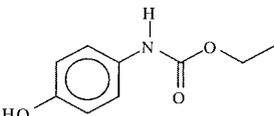
[a] The percentage was obtained at 48 h of exposure to a concentration of 100 μg of each carbamate per ml. Inhibition of 0% corresponded to the maximal growth in TYI-S-33.

Seguro Social, Mexico City, Mexico) were axenically grown in TYI-S-33 medium supplemented with 17% (vol/vol) bovine serum (Biofluid, Rockville, Md.) (6). To increase the virulence of the amoeba strain, male Golden hamsters (*Mesocricetus auratus*) were inoculated by the intrahepatic route with 10⁶ amoebas and were killed 7 days later. An amoebic liver fragment was cut from the hamsters under sterile conditions and introduced into a tube containing Diamond medium sup-

plemented with serum, streptomycin (500 μg/ml), and penicillin (500 U/ml). After several transfers of the amoeba cultures in the presence of gradually decreasing antibiotic concentrations, the parasites were passaged six times through hamster liver before they were used (32). The original strain was donated by Ruy Pérez-Tamayo (Departamento de Medicina Experimental, Universidad Nacional Autónoma de México, Mexico City, Mexico).

Care of animals. For the C4 toxicity studies and induction of hepatic amoebiasis, all animals were handled according to the International Norms for Care and Use of Laboratory Animals (NOM 062-200-1999). All procedures were done at the Animal Care Unit of Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN; Mexico City, Mexico).

In vitro detection of carbamate antiamoebic activities. *E. histolytica* cultures of 48 h were adjusted to 10^4 cells/ml. Ten carbamates (Table 1) were dissolved in 0.2% (vol/vol) dimethyl sulfoxide (DMSO; Sigma), and each carbamate was tested by addition of the carbamate to cultures at concentrations ranging from 0.025 to 250 μg/ml and incubation at 37°C. Metronidazole (5 μg/ml) was the positive control for growth inhibition. The number of amoebas was measured at 3, 6, 9, 12, 24, 48, 72, and 96 h after cooling and centrifugation of the cultures at $500 \times g$ for 5 min at 4°C. The pellets were suspended in phosphate-buffered saline, and the amoebas were counted in a hemocytometer chamber. Viability was determined by trypan blue stain exclusion (8).

Mutagenicity of C4 in bacteria. Studies of the mutagenicity of C4 were performed as described by Maron and Ames (16) by using both the incorporation and the preincubation methods. These assays were performed with 0.5 ml of saline solution (SS) or 0.5 ml of the S9 fraction of rat liver (S9 mixture), bacteria, and different concentrations of C4. The S9 mixture was obtained from male Wistar rat liver induced with Aroclor 1254 (New England Nuclear, North Haven, Conn.) (16).

Plate incorporation test. *Salmonella enterica* serovar Typhimurium strains TA97, TA98, TA100, and TA102 were cultured for 16 h at 37°C on a shaking water bath at 90 rpm. In a sterile tube containing 2 ml of soft agar (at 45°C), the bacterial cultures (0.1 ml) were exposed to C4 at different final concentrations ranging from 3.12 to 400 μg/ml or to SS as a control. Afterwards, 2 ml of soft agar was added to each tube, the tube was vortexed for 3 s at low speed, and the tube contents were poured onto plates with Vogel-Bonner agar medium. The cultures were incubated at 37°C for 48 h.

Preincubation test. The preincubation test consisted of exposure of 0.1 ml of cultures (16 h) of *S. enterica* serovar Typhimurium strains TA97, TA98, TA100, and TA102 to C4 at different final concentrations ranging from 3.12 to 400 μg/ml or to SS. The cultures were incubated for 1 h with the C4 dilutions at 37°C on a shaking water bath at 90 rpm. Afterwards, 2 ml of soft agar was added to each tube, and the tube contents were poured onto plates with Vogel-Bonner agar medium. The cultures were incubated at 37°C for 48 h.

For both tests, the *Salmonella* histidine revertant (His$^+$) number was assessed with a Fisher colony counter. Positive controls of mutagenesis used for each strain (2AA, CP, ENNG, MC, and PA) were those recommended previously (16).

Cytotoxicity of C4. Hepatocytes were isolated from male Fischer 344 rats (weight, 180 to 200 g) by the collagenase perfusion method (19). Cells were cultured in Dulbecco-Vogt-modified Eagle's minimal essential medium (DMEM; Gibco, Grand Island, N.Y.) supplemented with 1.5 U of insulin and 7% (vol/vol) bovine serum (Equitech-Bio, Inc., Ingram, Tex.). Cell viability was measured by the trypan blue exclusion method, 800,000 cells were placed on each culture dish, and the culture dishes were incubated at 37°C for 1 h in a 9% CO$_2$ atmosphere to allow cell adherence. Afterwards, the medium was removed, C4 was added at different final concentrations ranging from 1.96 to 250 μg/ml in fresh medium, and the mixture was incubated for 4 h. The cells were then washed with DMEM and further incubated for 1 h with 0.4 mg of tetrazolium salt per ml in DMEM. Finally, the medium was removed and DMSO was added to dissolve the intracellular formazan that had formed. This supernatant was diluted in DMSO, and the optical density at 545 nm was read (21).

In vivo model of ALA. Hamsters (*M. auratus*; age, 2 months; average weight, 100 g) were used for the in vivo model of ALA. They were caged individually and starved for 24 h before surgery. The animals were anesthetized with intraperitoneal sodium pentobarbital (Anestesal; Smith Kline, Mexico City, Mexico). ALA was induced by intrahepatic inoculation of 10^6 trophozoites in 0.2 ml of medium (*n* = 6 animals). The production of lesions at approximately 1.5 g per liver was standardized previously. Six groups of six animals each were used to test C4 as an amoebicide. The first two groups were treated with SS and DMSO, respectively, as negative controls; the third group was treated with metronidazole (5 mg/100 g of body weight) as a positive control for healing; and the other three groups were treated with C4 at 50, 75, and 100 mg/100 g of body weight, respectively. Treatments started 4 days after inoculation of the amoebas. Each hamster received 10 intraperitoneal injections of C4 as 1 injection every 3 days (72 h). The six groups were monitored daily for clinical signs, and at the end of the treatment all hamsters were killed. Sera from three random animals were analyzed for their liver enzymatic profiles. The characteristics of the hepatic lesions were recorded, and the livers were dissected and weighed. Representative injured fragments were collected and fixed with 10% paraformaldehyde in phosphate-buffered saline, and the sections were stained with hematoxylin-eosin (10, 14, 17, 30).

Liver enzymatic activities. A Beckman Synchron CX4 instrument was used to measure the levels of aspartate and alanine aminotransferases; amylase; total, direct, and indirect bilirubins; and total proteins in hamster sera.

Transmission electron microscopy. Amoebas (10^6 cells) treated with 125 or 250 μg of C4 per ml or with metronidazole (5 μg/ml) for 2 h at 37°C were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h with slow agitation. The samples were then centrifuged, embedded in Epoxy resin, and cut into 1-mm-thick sections. Semithin sections (thickness, 1 μm) were stained with toluidine blue. Thin sections (thickness, 60 to 90 nm) were stained with uranyl acetate and then lead citrate and examined with a JEOL910 transmission electron microscope (15).

Statistical analysis. All data represent the means of three independent experiments performed in triplicate. The results for the different times of incubation and the different concentrations of C4 in the in vitro studies were analyzed by analysis of variance (ANOVA).

RESULTS

In vitro studies. Of the 10 carbamic acid derivatives analyzed for their in vitro antiamoebic effects, 8 showed no significant effect at a concentration of 100 μg/ml when the effect was measured at 48 h (Table 1). C4 (LQM996; molecular weight, 199) was selected for the present study mainly due to its high degree of solubility in DMSO. Figure 1 shows the effects of different C4 concentrations on the growth of the amoebae through 96 h of culture. At 100 μg/ml, C4 was apparently amoebostatic until 72 h, when the growth of the cells in the culture began to decline. At that time, not only did the parasites not multiply but the number of amoebas diminished to half of the number inoculated; the rest of the parasites were apparently viable and capable of growing when the drug was removed. Compared with the control without drug, the level of growth inhibition due to the 100-μg/ml concentration of C4 was 97.88% (Table 1 and Fig. 1). However, at 72 h of treatment with 125 μg of C4 per ml, only one-third of the inoculated amoebas were found to be viable, but they were damaged, since they did not survive when the drug was removed. There was no significant difference between amoebic growth inhibition with 125 μg of C4 per ml and 5 μg of metronidazole per ml. C4 at a concentration of 250 μg/ml decreased the amount of parasites from that in the initial hours of culture.

Toxicity studies. Mutagenicity studies by the incorporation method showed that C4 at concentrations ranging from 3.12 to 400 μg/ml, with or without the S9 mixture, did not produce frame-shift mutations in *Salmonella* serovar Typhimurium His$^-$ strains TA97 and TA98. C4 did not induce mutations by base-pair substitutions in strain TA100, nor did it induce mutations due to oxidative damage in strain TA102 (data not shown). When the results of the incorporation test are negative, it has been suggested (16) that the samples must be analyzed by the preincubation test, which allows close contact of the possible mutagenic compound with sensitive bacteria for 1 h. By using this method, again, C4 was not mutagenic at concentrations ranging from 3.12 to 100 μg/ml; however, C4 was bactericidal at higher concentrations (Fig. 2A to D). Statistical analysis showed no significant difference between each of the C4 concentrations and the corresponding number of spontaneous revertants of the four *Salmonella* serovar Typhimurium strains.

Liver cells contain an efficient battery of enzymes that detoxify xenobiotics. The effect of C4 on cultures of primary liver
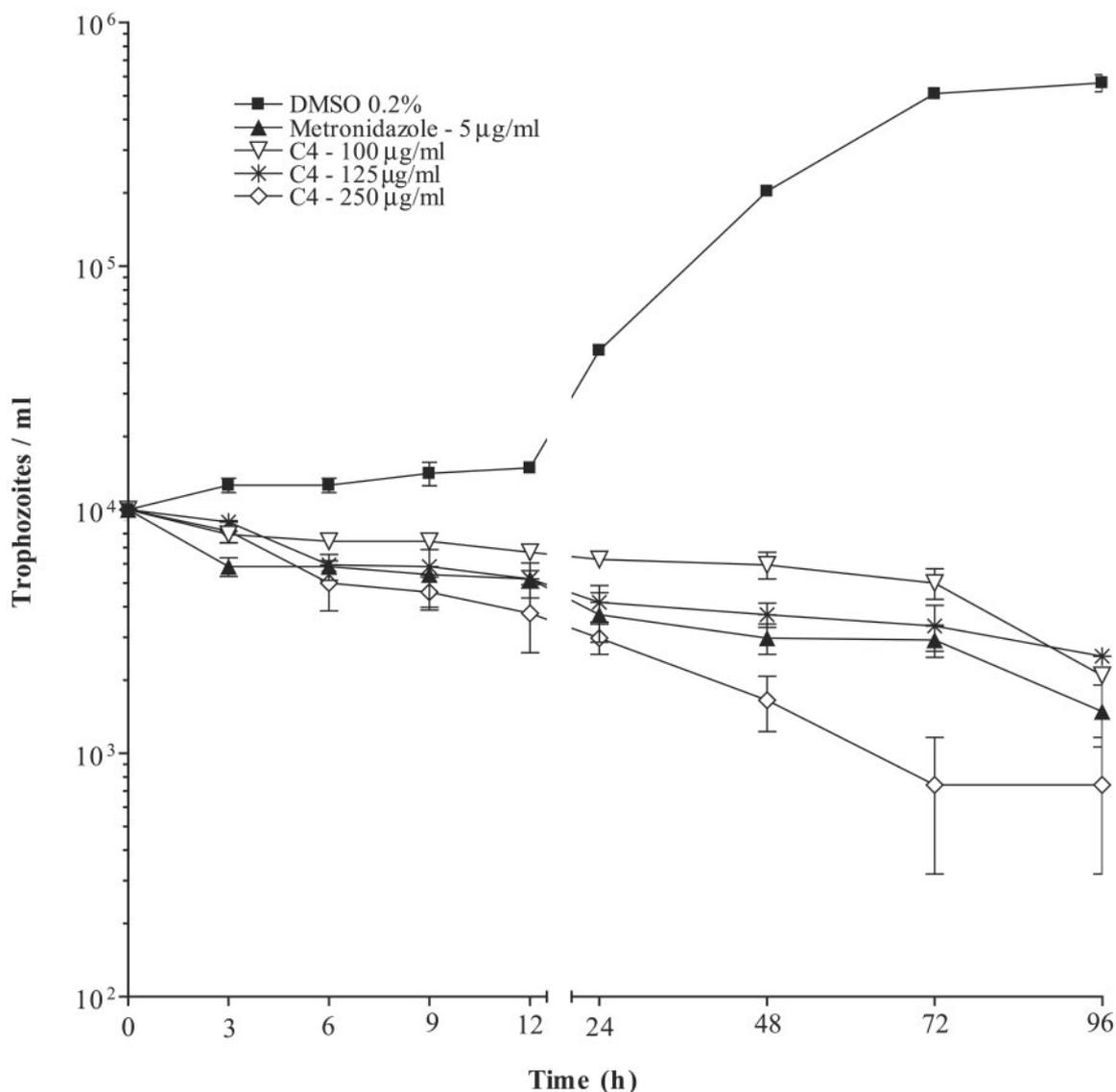
FIG. 1. Kinetics of growth inhibition of *E. histolytica* trophozoites treated with different concentrations of C4. Strain HM-1:IMSS (10^4 cells/ml) was incubated in medium TYI-S-33 at 37°C for 96 h with 100, 125, or 250 μg of C4 per ml dissolved in 0.2% DMSO. Samples were taken out at the indicated times, and the amoebas were counted in a Neubauer chamber. Viability determination was done by exclusion of trypan blue dye. Negative controls were amoebas in DMSO. Positive controls were amoebas treated with 5 μg of metronidazole per ml. The results represent the means and standard deviations of triplicate experiments.

cells from Fischer 344 rats was tested by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)–formazan method. Figure 3 shows that 125 μg of C4 per ml was moderately toxic for hepatocytes, which had a rate of survival of 77.4% compared with that for nontreated hepatocytes. The results for the hepatocytes treated with lower concentrations were not statistically significantly different from those for hepatocytes treated with DMEM or DMSO only.

In vivo studies. ALA was induced in hamsters by intrahepatic injection of trophozoites from a virulent strain of *E. histolytica* (30). Figure 4A (bar and photo) shows the liver damage produced 4 days after administration of the parasites; the lesion constituted an average of 23.3% of the total weight of the hamster liver (initial control without treatment and

solvents in order to confirm the virulence of the strain). Figure 4B and C (bars and photos) shows that liver abscesses constituting 80 and 82% of the total liver weight developed at day 34 in 6 of 12 animals that survived when they were treated only with SS and DMSO, respectively; the animals showed bulky abdomens, loss of weight, dyspnea, and lethargy. Figure 4D (bar and photo) shows the results for the hamster liver after the administration of 10 doses of metronidazole; all animals survived the treatment with this drug. Administration of 50, 75 or 100 mg of C4 per 100 g of body weight did not produce apparent damage to the animals (data not shown). These doses decreased the sizes of the liver lesions in animals infected with parasites by 23, 84, and 94%, respectively (Fig. 4E to G [bars and photos], respectively). However, one of six hamsters with
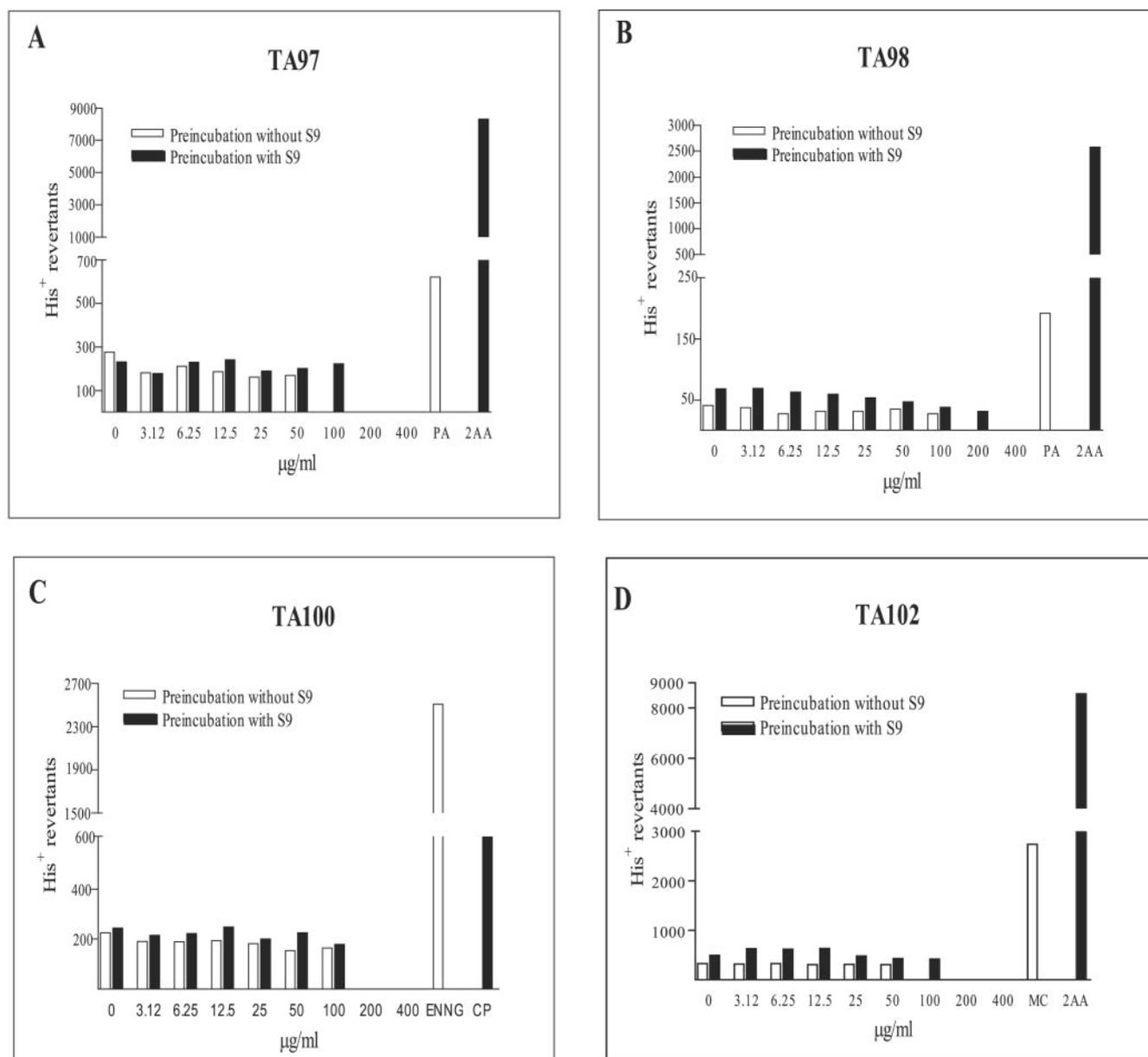
FIG. 2. Mutagenicity of the C4 carbamic acid derivative for *Salmonella* serovar Typhimurium His$^-$ strains. Positive controls (final concentration) were PA (82 µg/ml), 2AA (16 µg/ml), CP (820 µg/ml), ENNG (8.2 µg/ml), and MC (0.04 µg/ml). (A and B) Strains TA97 and TA98, respectively, used for detection of frame-shift mutations; (C) strain TA100, used for determination of base-pair substitutions; (D) strain TA102, used for determination of damage due to free radicals. Average rates of reversion for spontaneous revertants ± standard deviations were as follows: TA97, 251.3 ± 26.0; TA98, 40.0 ± 2.6; TA100, 223.0 ± 18.6; TA102, 335.6 ± 19.8. Data represent the means of three experiments, with each experiment performed in triplicate.

ALA died after treatment with seven doses of the highest concentration of C4. At the end of the treatment with 100 mg of C4 per 100 g of body weight in five animals, we observed a yellow point in the lesion that corresponded to healing tissue. Histopathological analysis revealed the regeneration of hepatic tissue, such as new vessels and hepatocytes, foamy macrophages, and fibers (17). A representative ALA lesion for each C4 treatment is shown in Fig. 4E to G. None of the groups of animals presented with critical clinical signs during treatment with C4.

Histological analysis of the livers from hamsters treated only

with C4 (*n* = 6 animals treated with each dose) showed that the liver parenchyma had normal hepatocytes with apparently characteristic nuclei and sinusoidal cells; thus, this compound did not damage the liver (Fig. 4H shows the histology of the liver of a hamster treated with 100 mg/100 g of body weight), and abscess healing by treatment with C4 showed a clear dose-response.

Hepatic function tests. Drug metabolism and transport data for the livers of hamsters treated with C4 were expressed as the enzymatic activity profile of the organ. In the control hamsters the levels of all enzymes tested except amylase were similar to
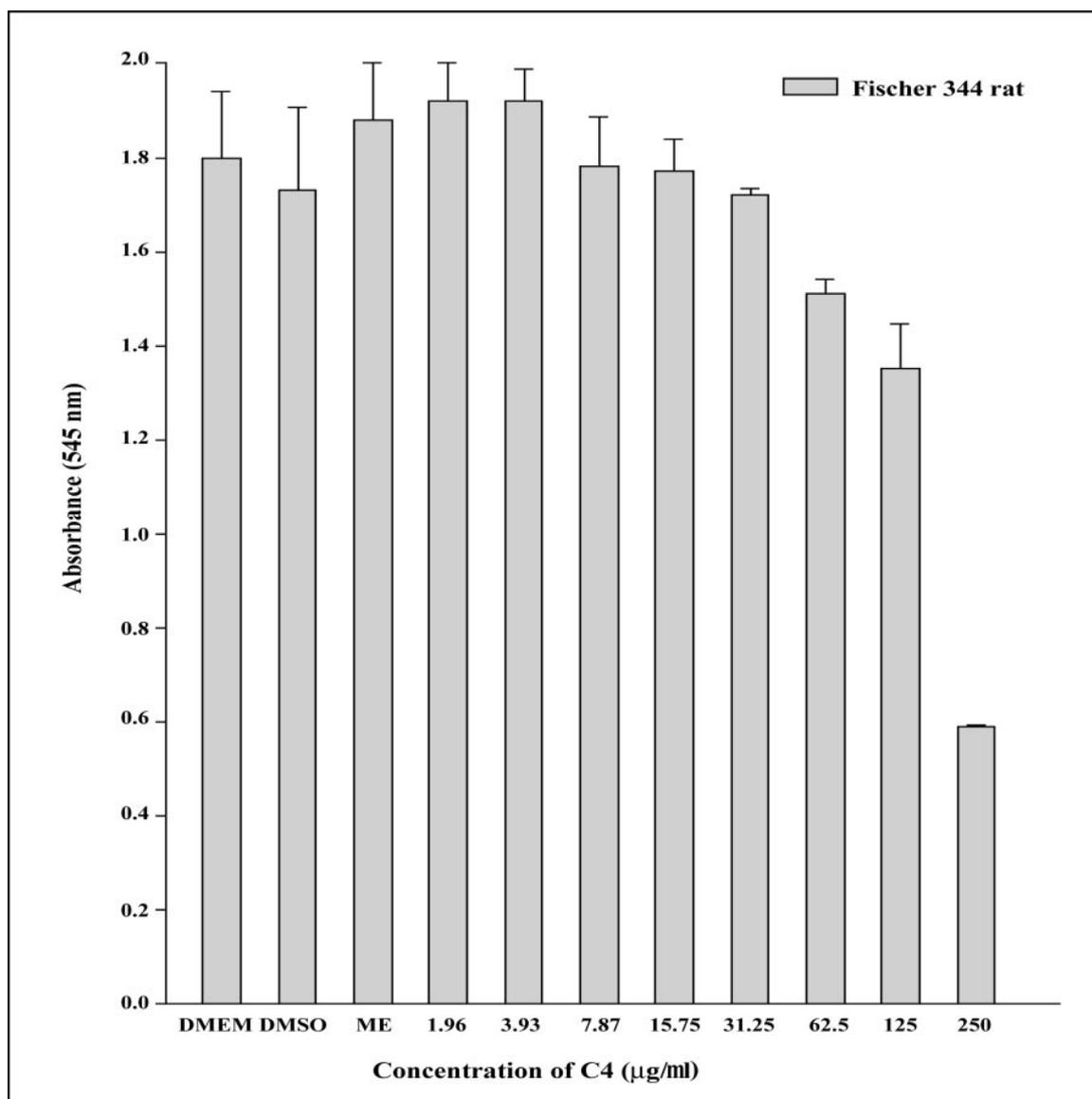
FIG. 3. Toxicity of the C4 carbamic acid derivative for primary cultures of 344 Fischer rat liver cells. Hepatocytes were exposed to C4 by the collagenase perfusion method. Three replicates of 8×10^5 cells were incubated for 4 h at 37°C in a 9% CO_2 atmosphere with C4 at final concentrations of 1.96 to 250 μg/ml in fresh medium and were then incubated with a tetrazolium salt for 1 h at 37°C. Three independent experiments were done. Bars indicate standard deviations. Metronidazole (ME) was used at concentration of 5 μg/ml and was the positive control. DMSO (0.2%) and DMEM were used as negative controls. ANOVA was used to compare the means for the treated and the negative control cultures. ∗, significantly different ($P < 0.001$) from the results for the controls.

the human reference values. Treatment with neither metronidazole nor C4 produced changes in enzyme levels compared with those in the control animals. Also, C4 did not alter the liver function in animals with hepatic abscesses treated with this carbamate (data not shown).

Transmission electron microscopy of *E. histolytica* exposed to C4. *E. histolytica* trophozoites untreated and treated with DMSO showed normal cytoplasmic vacuoles and nuclei with typical chromatin arrangements. Parasites exposed to metronidazole showed structural changes; were of various sizes, mostly spherical, and highly vacuolated; and contained increased amounts of glycogen. The level of membrane damage was approximately 30% in amoebas treated with 125 μg of C4

per ml, and the cells were vacuolated. Most amoebas in cultures treated with 250 μg of C4 per ml were killed by the action of C4; the remainder were round with scarce pseudopodia and displayed atypical irregular nuclei, abundant vacuolization, and changes in the plasma membrane and arrangements of intracellular material as result of cellular damage (data not shown).

DISCUSSION

Synthetic ethyl and methyl phenylcarbamates are used, for example, as pesticides and insecticides (2). According to their chemical structures and theoretical studies of their qualitative structure-activity relationships, some of these compounds have
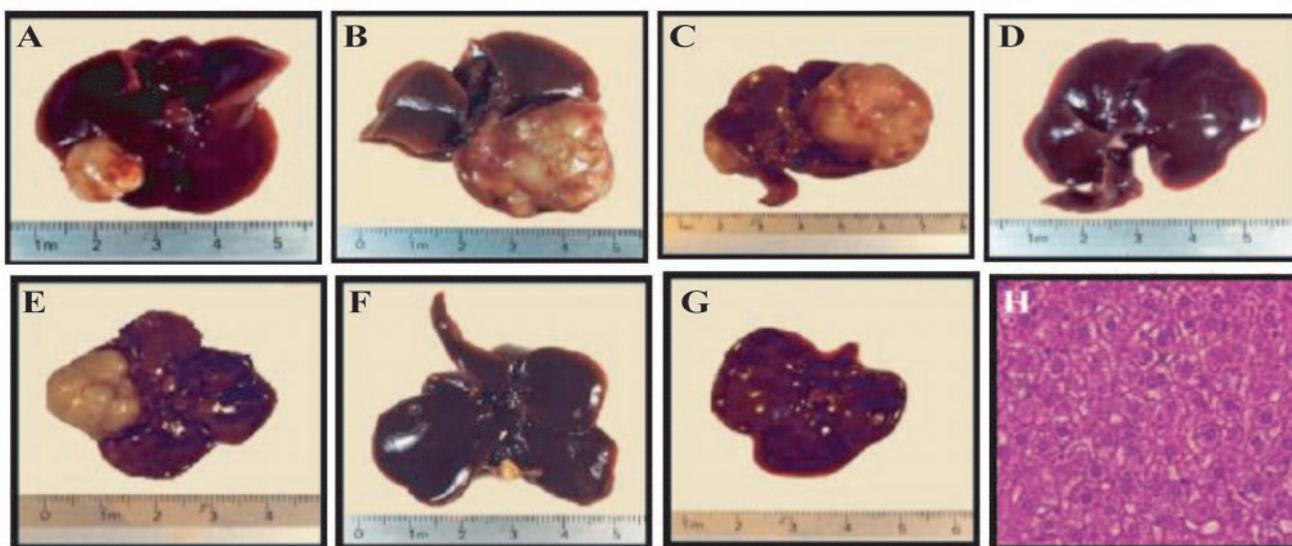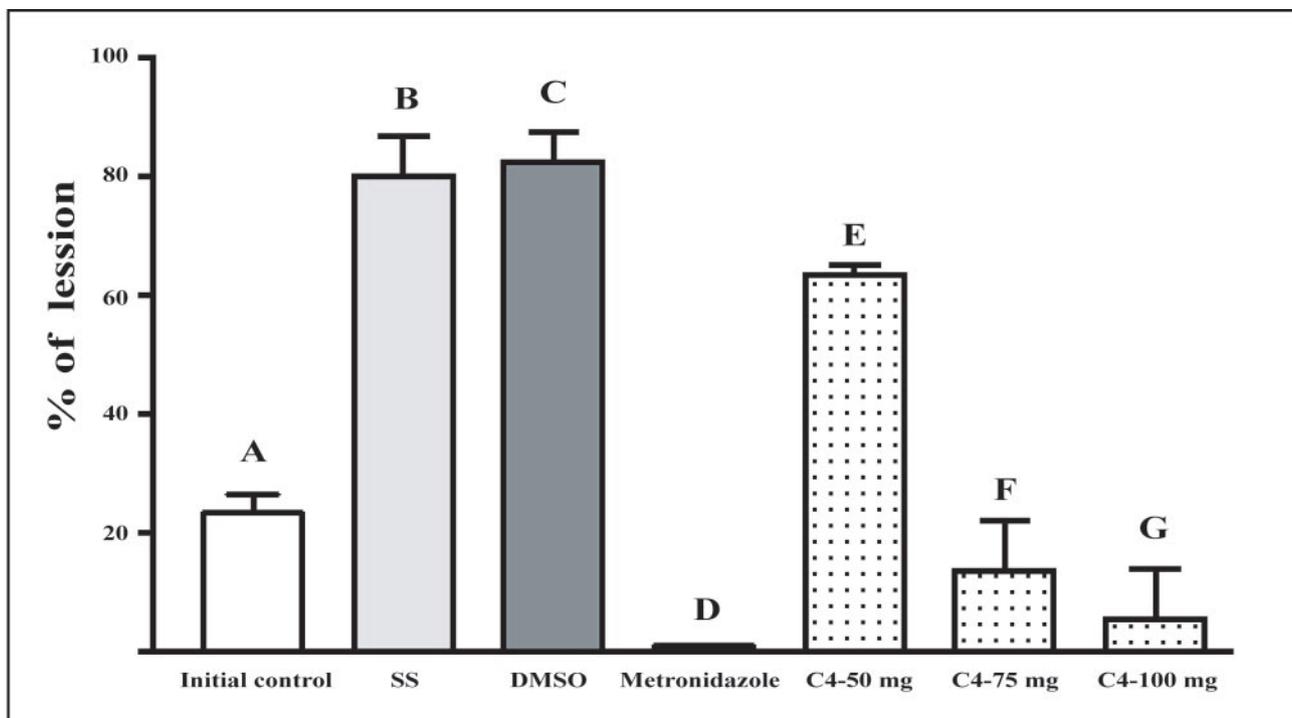
FIG. 4. Percentage of damaged liver in hamsters exposed to the carbamate C4. Abscesses were induced by intrahepatic inoculation of 10^6 trophozoites in 0.2 ml of medium. Treatments started 4 days after infection, when the abscess reached an average of 23% of the total liver weight (A). Intraperitoneal injections of C4 were given every 3 days up to 10 times. Animals were treated with saline solution (B), DMSO (C), 5 mg of metronidazole per 100 g of body weight (D), 50 mg of C4 per 100 g of body weight (E), 75 mg of C4 per 100 g of body weight (F), or 100 mg of C4 per 100 g of body weight (G). (H) Histopathology of hamster liver treated only with 100 mg of C4. Each group represents the average value for six animals, and bars indicate standard deviations. ANOVA was used to compare means between the C4 and the SS or DMSO treatments. *, significantly different ($P < 0.001$) from the results for the controls.

been predicted to be antibiotics or antiparasitic agents, but these properties have not been demonstrated (3). In this context, we evaluated 10 carbamates to determine their possible amoebicidal capacities. Among these compounds, we selected the two compounds with the highest levels of antiamoebic activity, and on the basis of its solubility, C4 was selected for further studies. Table 1 shows that the percentage of growth

inhibition of *E. histolytica* trophozoites was different according to the functional group in the carbamic chemical structure.

Several procedures can be used to test the antiamoebic activities of drugs in vitro, but none of them is free of criticism. First, in axenic cultures *E. histolytica* trophozoites exist under growth conditions completely different from those that exist when they invade host tissues; inside the host, immune defense

mechanisms, bacteria, and other factors affect multiplication and attack of the target site. Second, there are different opinions about the best container to be used to culture amoeba; microplates, dishes, or glass tubes can be used for culture, but we chose glass tubes, since these provide a better quantitative recovery of amoebas, can be used for long periods of testing, and also produce an adequate proportion of amoeba per amount of medium that does not interfere with the logarithmic growth phase. Third, the drug effect is influenced by the size of the amoeba inoculum, growth phase, and the period of amoeba exposure to the drug (8, 22). For C4, the use of an inoculum of 10^4 amoebas and the time of amoeba contact with the drug were important, since they allowed us to determine over a short period of time the amoebostatic versus the amoebicidal effect.

By using a wide range of C4 concentrations, we found that it was amoebicidal for in vitro growth at a concentration greater than 100 μg/ml. Another important characteristic was that, at this concentration, C4 did not exhibit mutagenic activity by the Ames test when it was incubated with bacteria, which is in contrast to the data reported for metronidazole, the drug currently used for the treatment of invasive amoebiasis, as well as for other antiparasitic drugs (1, 5, 7, 33).

The use of MTT in rat liver cell cultures is a good indicator of various dehydrogenases and mitochondrial function and, thus, is a good cellular toxicity marker (31). With this tool we demonstrated that a concentration of 125 μg/ml was moderately toxic for Fischer 344 rat liver cells. This variety of rat liver cells was selected because of its high degree of susceptibility to all kinds of carcinogens.

We used an experimental model of ALA in hamsters to determine the possible curative effect of C4 on amoebiasis in vivo. This model provides a hepatic abscess of approximately 20% of the total organ weight after 4 days of intrahepatic inoculation of trophozoites. The results of previous studies of the effect of metronidazole on ALA in hamsters, together with the information obtained in this work about the in vitro effects of metronidazole and C4, were the bases for choosing the dose of C4 to be used in our in vivo studies (10, 14, 17). C4 inhibited growth of the ALA. Furthermore, the functions of several liver enzymes were unaltered during C4 administration.

The results of the present study show that the carbamate derivative C4 is not genotoxic to bacteria and did not alter hepatic enzyme function. This compound was also capable of diminishing ALA development in the hamster model. However, the effectiveness of C4 could be increased and its toxicity could be decreased if it were used in combination with silica beads or divalent cations or if it were administered in liposomal formulations (20, 23, 29). Also, we believe that several exposures of amoebas to C4 (such as during in vivo treatments) could diminish the effective dose of the carbamate required. On the other hand, we have observed synergistic effects between C4 and metronidazole, which should possibly improve the activities of both drugs. Thus, C4 could be considered for evaluation in future pharmacological studies as an antiamoebic candidate that could eventually be used for the treatment of human amoebiasis. We are studying the mechanism of action of C4 against *E. histolytica* trophozoites.

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