Novobiocin Antagonism of Amastigotes of *Trypanosoma cruzi* Growing in Cell-Free Medium

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Inhibitors of the enzyme bacterial topoisomerase II (DNA gyrase) were evaluated for activity against *Trypanosoma cruzi* (Brazil strain), based on the theoretical need for a topoisomerase II in the replication of the kinetoplast DNA network. Novobiocin (500 µg/ml) antagonized amastigotes of *T. cruzi* growing in a cell-free medium at 37°C, as manifested by inhibition of multiplication, abnormal morphology of Giemsa-stained organisms, and delayed or absent growth of cells upon subculturing in a drug-free medium. In contrast, novobiocin (1,000 µg/ml) essentially had no effect on the multiplication and motility of epimastigotes growing in a cell-free medium at 27°C. This resistance of epimastigotes represented a difference in the physiology of this morphologic stage and not in the temperature of experimentation, because novobiocin inhibited multiplication of amastigotes at 27°C as well and accelerated transformation to epimastigotes. With *T. cruzi* growing within cultured human fibroblasts, novobiocin (200 µg/ml) markedly inhibited transformation of intracellular amastigotes to trypomastigotes. Clorobiocin, a structural analog of novobiocin and likewise an inhibitor of the B subunit of bacterial topoisomerase II, was five times more potent on a molar basis than novobiocin was in antagonism of amastigotes growing in a cell-free medium and did not antagonize epimastigotes. Coumermycin A1, another analog of novobiocin, and five 4-quinolone antibacterial agents, antagonists of the A subunit of bacterial topoisomerase II, inhibited neither amastigotes nor epimastigotes. These experiments indicate that novobiocin and clorobiocin represent a new structural class of drugs with activity against *T. cruzi*. Whether the mechanism of action of these drugs involves antagonism of a *T. cruzi* topoisomerase II or an unrelated target is yet to be determined.

Trypanosoma cruzi is the protozoan agent of Chagas' disease, an illness of considerable morbidity and mortality in South and Central America (37). There is no satisfactory chemotherapy for Chagas' disease (4, 27, 36). *T. cruzi* and related organisms contain a kinetoplast, a part of the mitochondrial structure of vital but largely unknown function (2, 12, 45). The kinetoplast contains thousands of DNA circles which are interlocked like links in a chain into a large fishnetlike network (2, 12, 45). The network in *T. cruzi* contains about 25 maxicircular DNA molecules, which are 33 kilobases in size and are the mitochondrial DNA of the cell; the remaining 25,000 DNA molecules, termed minicircles, are 1.44 kilobases in size, encode no known proteins, vary in sequence, and have no known function (2, 12, 45). During replication, minicircles separate from the network, replicate, and rejoin the network, which thus grows and then is cleaved into two networks, one for each daughter parasite cell (12). The enzyme topoisomerase II is known to separate and rejoin interlocked DNA circles in vitro (16, 28, 29, 33) and is needed for segregation of chromosomes in bacteria (46) and in yeasts (6, 48). Englund and Marini suggest that topoisomerase II may be necessary for replication of the kinetoplast DNA network (13). Type II topoisomerases from bacteria and certain eucaryotes, including the kinetoplast-containing organism *Crithidia fasciculata* (44), are inhibited in vitro by the antibiotic novobiocin (16, 18, 22, 26). The possibility that novobiocin might inhibit a putative topoisomerase II in the kinetoplast of *T. cruzi* led

us to test novobiocin and other inhibitors of bacterial topoisomerase II for antitrypanosomal activity.

T. cruzi has two morphologic stages in which the pathogen multiplies: the amastigote, the predominant stage within invaded mammalian cells, and the epimastigote, the major stage in the reduviid insect vector. The trypomastigote, which is the human bloodstream stage, and the metacyclic trypomastigote, which is the infectious stage in the insect vector, do not multiply. To evaluate novobiocin, we initially used a recently developed, convenient cell-free system for the continuous serial passage of *T. cruzi* amastigotes (by the morphologic definition of Hoare and Wallace [25]) in an axenic culture (39). Using this culture system, we found that novobiocin and an analog, clorobiocin (7), antagonized growth of amastigotes.

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MATERIALS AND METHODS

Chemicals. Drugs were kindly provided by the following sources: novobiocin monosodium salt by The Upjohn Co., Kalamazoo, Mich.; clorobiocin by May and Baker, Ltd., Dagenham, England; coumermycin A1 by Bristol Laboratories, Syracuse, N.Y.; oxolinic acid by Warner-Lambert

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Research Institute, Morris Plains, N.J.; norfloxacin by the Merck Institute for Therapeutic Research, Rahway, N.J.; ofloxacin by Ortho Pharmaceutical Corp., Raritan, N.J.; ciprofloxacin by Miles Laboratories, Inc., West Haven, Conn.; and nifurtimox by Bayer Argentina S.A., Buenos Aires, Argentina. Novobiocin was also purchased from Sigma Chemical Co., St. Louis, Mo. Nalidixic acid was purchased from Calbiochem-Behring, La Jolla, Calif. For stock solutions, novobiocin (10 mg/ml) was dissolved in phosphate-buffered saline and sterilized by filtration with 0.45- μ m-pore membrane filters (Millipore Corp., Bedford, Mass.). Clorobiocin (20 mg/ml) was dissolved in 95% ethanol. Coumermycin A1 (10 mg/ml) was dissolved in dimethyl sulfoxide. Nalidixic acid (10 mg/ml), oxolinic acid (10 mg/ml), and ciprofloxacin (2 mg/ml) were each dissolved in 0.1 N NaOH. Norfloxacin (1 mg/ml) and ofloxacin (1 mg/ml) were each dissolved in 0.02 N NaOH.

Organisms and culture systems. The Brazil strain of *T. cruzi* (C15C2 clone, twice cloned from the parent stock) (42) was used for all experiments.

Parasites were maintained by serial passage in a cell-free medium as amastigotes at 37°C (39) and as epimastigotes at 27°C. Amastigotes growing at 27°C were obtained by transfer of amastigotes growing at 37 to 27°C. The F94 medium (42) used was modified by a decrease in riboflavin to a final concentration of 2 μ g/ml. Serial passage was in 6.0-ml cultures in either glass (16 by 125 mm) or polystyrene tubes (15 ml; Corning Glass Works, Corning, N.Y.) or in 3.0-ml cultures in polypropylene tubes (5-ml Cryotubes; A/S Nunc, Roskilde, Denmark); the type of tube used did not significantly influence our data.

For experiments with *T. cruzi* growing within cultured human cells, fibroblasts (2×10^5 cells per cover slip) from human embryonic lungs were grown to confluency on cover slips in Leighton tubes at 35°C (40). Fibroblasts were infected with amastigotes (5×10^5 per cover slip) from cell-free cultures. A cycle of intracellular parasite growth was completed in 5 to 7 days.

Cell morphology was assessed by staining with a Wright-Giemsa method (38).

Experimental protocols. To test drugs for antitrypanosomal activity in axenic cultures, we used two protocols. In protocol 1 (39), logarithmically growing organisms were harvested, counted in a Neubauer hemacytometer, smeared and stained, and diluted in fresh medium to 1×10^6 to 2×10^6 organisms per ml. Aliquots (6.0 ml) of the fresh suspension were distributed into glass culture tubes. After incubation for 1 day, two cultures were harvested, and the organisms were counted and stained. A drug or diluent was then added to equal numbers of the remaining tubes, and incubation was continued. Two drug-treated and two drug-free cultures were harvested daily to assay for drug effect.

In protocol 2, organisms were diluted with the medium to 1×10^6 to 2×10^6 per ml and distributed into culture tubes (6.0 ml in a polystyrene tube or 3.0 ml in a polypropylene tube). A drug in different amounts was added to each culture, and diluent was added to the control culture. After reincubation of the cultures, aliquots were removed from each tube twice daily for experiments at 37°C and once daily for experiments at 27°C to assay for drug effect.

For subcultures in drug-free medium, parasites were counted and diluted to 10^3 /ml of medium. A culture sample (2 ml) was then placed over a slant of modified NNN medium agar (38) and incubated at 27°C. These conditions were chosen to permit growth of either amastigotes or epimastigotes. Periodically the cultures were agitated, and 7 μ l was

examined under a cover slip for the presence of organisms. Observation of one or more parasites was scored as positive growth. An inoculum of 10^3 organisms for a subculture was selected on the basis of experiments which demonstrated that organisms not exposed to a drug could be detected after 4 to 10 days of growth at concentrations between 10^4 and 10^5 organisms per ml.

For experiments with *T. cruzi* growing within cultured human fibroblasts, fibroblasts were grown to confluency on cover slips. Seven days later, two-thirds of the cover slips were infected with amastigotes grown in cell-free medium. At this time or 2 days later, novobiocin was added to the uninfected cultures (to assay for toxicity of the drug on the fibroblasts) and to one half of the infected cultures. Diluent was added to the other half of the infected cultures. Duplicate cover slips from each of the three limbs of the experiment were removed daily, and the cells were stained to evaluate for drug effect.

RESULTS

Effect of novobiocin on *T. cruzi* in cell-free medium. Novobiocin minimally inhibited multiplication of epimastigotes at 27°C in cell-free medium at 500 μ g/ml (Fig. 1A) and 1,000 μ g/ml (data not shown). Similarly, an effect of the drug on motility of epimastigotes could not be detected, with >95% of the drug-exposed organisms remaining actively motile. In contrast, novobiocin inhibited multiplication of amastigotes in the cell-free medium at 37°C in a dose-dependent fashion (Fig. 1B and Table 1). Thus, 125 μ g of novobiocin per ml minimally inhibited, 250 μ g/ml partially inhibited, and 500 μ g/ml completely inhibited multiplication of amastigotes. At concentrations of ≥ 200 μ g/ml, novobiocin also caused morphologic abnormalities (crenated and pyknotic cells, ghost cells, or cells with bizarre morphology) and delayed or inhibited growth of organisms diluted in the cell-free medium after 48 h of drug exposure (Table 1). A lower drug concentration (100 μ g of novobiocin per ml) did not alter multiplication, morphology, or growth on the subculture in drug-free medium (data not shown). These morphologic and subculture data indicate that novobiocin (≥ 200 μ g/ml) crippled or killed amastigotes and was not just reversibly inhibiting the organisms.

The cell-free medium used to grow the amastigotes contained streptomycin (100 μ g/ml) and penicillin (100 μ g/ml) to reduce bacterial contamination. High concentrations of streptomycin antagonize growth of *Leishmania tarentolae* (50). To exclude the possibility that antagonism of *T. cruzi* represented an effect of novobiocin in concert with streptomycin or penicillin, we tested novobiocin in medium lacking these two antibiotics. The inhibition of amastigotes by novobiocin was unchanged (data not shown).

The spectral properties of novobiocin are altered by magnesium, suggesting binding of this cation by the drug (5). To exclude the possibility that the antagonistic effect of novobiocin resulted from limitation of magnesium in medium secondary to interaction with the drug, the experiments were repeated with medium containing a fivefold increased concentration of magnesium. No change in the antagonistic effect of novobiocin was detected (data not shown).

Novobiocin antagonized amastigotes at 37°C but not epimastigotes at 27°C. This difference might have resulted from differences in the morphologic stages of the parasites or in the temperatures of experimentation. To evaluate these possibilities, we studied the effect of novobiocin on amastigotes growing in cell-free medium at 27°C. We have observed that upon transfer of amastigotes growing in the

FIG. 1. Effect of novobiocin on *T. cruzi* growing as epimastigotes in cell-free medium at 27°C (A) and as amastigotes at 37°C (B). Aliquots were removed serially from cultures for cell counts at the indicated intervals. Symbols for (A): ●, 0 µg of novobiocin per ml; ○, 125 µg/ml. Symbols for (B): ●, 0 µg of novobiocin per ml; ○, 125 µg/ml; ■, 250 µg/ml; □, 500 µg/ml. Each point represents an average of two counts for epimastigotes and four counts for amastigotes. Bars indicate the ranges of values.

cell-free medium at 37 to 27°C, the parasites continue to multiply as amastigotes, although epimastigotes may grow out after various numbers of passages. Novobiocin (500 µg/ml) was found to inhibit completely the multiplication of amastigotes at 27°C. Additionally, in four of five experiments, epimastigotes became the predominant stage in 5 to 7 days in cultures of drug-exposed but not drug-free organisms. In three experiments for which quantitative analyses were possible, calculations indicated that the increase in titer of epimastigotes occurred more rapidly in the presence of novobiocin in comparison with the drug-free control, suggesting that novobiocin had accelerated transformation of some amastigotes to epimastigotes. For example, in one experiment, 1.8×10^6 parasites (of which 0.7% were epimastigotes) per ml were exposed to 1,000 µg of novobiocin per ml. Later (65 h), the titer had increased to 2.5×10^6 parasites per ml, of which 68% were epimastigotes. In the drug-free control culture, after 65 h, the titer had increased to 8.1×10^6 parasites per ml, of which 3% were epimastigotes. The generation time of epimastigotes in cell-free medium at 27°C was unaffected by novobiocin and was 20 h in the presence of 1,000 µg of the drug per ml. It was thus possible to calculate $[(1.8 \times 10^6)(0.007)(2^{65/20})/(2.5 \times$

$10^6)(0.68) = 0.07]$ that only 7% of the epimastigotes in the culture of amastigotes exposed to novobiocin for 65 h could be generated by multiplication of the epimastigotes present when the drug was initially added. The remaining 93% were derived at least in part from amastigotes that had transformed to epimastigotes. A similar calculation for the drug-free culture revealed that 54% of the epimastigotes were not derived from epimastigotes present at the beginning of the experiment; this is consistent with some transformation in the absence of the drug. We interpret these experiments to indicate that the resistance of epimastigotes to novobiocin represents a difference in the physiology of morphologic stages and not in the temperatures of experimentation and that high drug concentrations seem to accelerate transformation of amastigotes to epimastigotes.

Effect of novobiocin analogs, 4-quinolone antibacterial agents, and nifurtimox on *T. cruzi* growing in cell-free medium. Clorobiocin (7), an analog of novobiocin, is also an antagonist of the B subunit of bacterial topoisomerase II (14). In cell-free medium at 37°C, 25 µg of clorobiocin per ml minimally inhibited, 50 µg/ml mostly inhibited, and 100 µg/ml completely inhibited multiplication of amastigotes (data not shown). Clorobiocin also induced morphologic abnormalities in amastigotes similar to those induced by novobiocin and prolonged or prevented growth of organisms diluted into drug-free medium after 48 h of exposure to 100 µg of clorobiocin per ml (data not shown). Thus, clorobiocin (molecular weight, 695) was five times more potent on a molar basis than novobiocin (molecular weight, 612). Clorobiocin, like novobiocin, minimally inhibited multiplication of epimastigotes and did not alter motility. Coumermycin A1 (50 µg/ml) (20), another analog of novobiocin, antagonized neither amastigotes nor epimastigotes growing in cell-free medium.

Nalidixic acid (200 µg/ml), oxolinic acid (100 µg/ml), norfloxacin (40 µg/ml), ofloxacin (100 µg/ml), and ciprofloxacin (40 µg/ml), all antagonists of the A subunit of bacterial topoisomerase II (17, 47; D. C. Hooper, J. S. Wolfson, K. S. Souza, G. L. McHugh, and M. N. Swartz, unpublished data), were evaluated and found to have no activity against either amastigotes or epimastigotes growing in cell-free medium.

TABLE 1. Effect of novobiocin on *T. cruzi* amastigotes growing in cell-free medium

Novobiocin concn (µg/ml) ^a	Organism multiplication (fold) ^b	Cell morphology (% normal) ^c	Drug-free broth subcultures (no. positive/no. inoculated) on day:				
			4	7	11	14	21
0	3	95	4/4				
200	<1	83	0/4	0/4	1/4	2/4	2/4
500	<1	73	0/4	0/4	0/4	0/4	1/4
1,000	<1	0	0/4	0/4	0/4	0/4	0/4

^a Two 6-ml cultures were harvested for each drug concentration at 48 h of exposure; novobiocin was added after 24 h of growth.

^b Increase in number of organisms in culture as a multiple of the concentration at 24 h of growth. Values are the averages of determinations from duplicate cultures.

^c Wright-Giemsa stain. Average of determinations from duplicate cultures rounded to the nearest percent.

FIG. 2. Effect of novobiocin (200 $\mu\text{g/ml}$) on *T. cruzi* growing within cultured fibroblasts. Novobiocin was added 48 h after infection to one culture (A), and diluent was added to the other culture (B). Photomicrographs (magnification, $\times 400$) were taken 120 h after infection. Arrows identify nests of intracellular amastigotes (in panel A, rounded forms) and of intracellular trypomastigotes (in panel B, tapered forms).

Nifurtimox, a drug used to treat human disease caused by *T. cruzi* (4, 27, 36), partially inhibited the multiplication of both amastigotes and epimastigotes growing in cell-free medium at 1 $\mu\text{g/ml}$ and completely inhibited multiplication at 2.5 $\mu\text{g/ml}$.

Effect of novobiocin on *T. cruzi* growing within cultured fibroblasts. Cultured human fibroblasts were exposed to novobiocin (200 $\mu\text{g/ml}$) 2 days after infection with *T. cruzi*. *T. cruzi* multiplied intracellularly as amastigotes but did not transform to trypomastigotes late in the intracellular cycle, a change which occurred normally in the drug-free control (Table 2 and Fig. 2). When novobiocin (200 $\mu\text{g/ml}$) was added at the time of infection of fibroblasts with *T. cruzi*, transformation of amastigotes to trypomastigotes again was not seen (data not shown). In addition, in drug-treated, noninfected fibroblasts, there was a suggestion of drug toxicity to the fibroblasts, in that cells appeared somewhat thin and extended and eventually some cell dropout occurred.

DISCUSSION

Based on the theoretical need for type II topoisomerases in the replication of the kinetoplast DNA network, we

TABLE 2. Effect of novobiocin (200 $\mu\text{g/ml}$) on transformation of *T. cruzi* amastigotes into trypomastigotes within cultured human fibroblasts

Day of infection	% of fibroblasts infected with intracellular trypomastigotes ^a	
	Novobiocin treated ^b	Drug free
1	<1	<1
3	<1	<1
4	<1	87
5	<1	66
7	<1	57

^a Average of determinations from duplicate specimens in which 100 infected cells per cover slip were analyzed.

^b Novobiocin was added 2 days after infection of tissue cells with *T. cruzi*.

evaluated antagonists of bacterial topoisomerase II in an attempt to identify agents with antitrypanosomal activity. Novobiocin and the structurally related compound clorobiocin antagonized *T. cruzi* amastigotes growing in cell-free medium at 37°C, as manifested by inhibition of multiplication, abnormal morphology of Giemsa-stained organisms, and delayed or absent growth of drug-exposed cells upon subculture in drug-free medium. In contrast, these drugs minimally antagonized multiplication and had no effect on motility of epimastigotes growing in the cell-free medium at 27°C. The reason for this resistance of epimastigotes to a drug, such as impermeability to a drug or an altered or absent drug target, is not known. This resistance does reflect a difference in the physiology of this morphologic stage and not in the temperature of experimentation, because novobiocin inhibited multiplication of amastigotes at 27°C and accelerated transformation to epimastigotes. We interpret these experiments to indicate that novobiocin and clorobiocin represent a new structural class of drugs with activity against *T. cruzi* amastigotes growing in cell-free medium.

Coumermycin A1, an analog of novobiocin and likewise an inhibitor of the B subunit of bacterial topoisomerase II, and five 4-quinolone antibacterial agents, inhibitors of the A subunit of bacterial topoisomerase II, antagonized neither amastigotes nor epimastigotes growing in the cell-free medium.

For *T. cruzi* growing within cultured human fibroblasts, novobiocin inhibited the transformation of amastigotes to trypomastigotes. In addition, multiplication of intracellular amastigotes was depressed by 200 μg of novobiocin per ml (S. C. Pan and P. G. Pate, unpublished data). We could not determine whether the interference in the intracellular life cycle of *T. cruzi* by novobiocin represented toxicity of the drug directly to the parasite or whether the interference was secondary to toxicity of the drug to host fibroblasts.

Nalidixic acid (250 $\mu\text{g/ml}$) inhibits the trypanosomatid *C. fasciculata* growing on agar (19) but not *T. cruzi* growing in cell-free medium. The reason for this difference likely relates

to differences in the physiologies of the organisms, the methods of cultivation, or both.

The experiments presented here are the first to use a system for continuous serial passage of amastigotes in cell-free medium to evaluate compounds for an antagonistic effect on *T. cruzi*. This system is unconventional in that intracellular stages are grown extracellularly. We believe that this system is unlikely to be greatly misrepresentative, however, because the extracellular amastigotes are indistinguishable in ultrastructure from amastigotes growing within mammalian cells (41), are infectious to fibroblasts (40), cause chronic subclinical infections in white mice (40), and are antagonized by nifurtimox at concentrations which antagonize *T. cruzi* growing in infected humans and animals (32) and within infected cultured mammalian cells (9, 21, 23, 34). Cell-free systems for amastigote (rather than epimastigote) cultivation (39, 49) seem preferable for studies designed to be relevant to growth of the parasite in infected mammals. As has been discussed in detail by others (3, 27), all experimental systems—including systems for cell-free cultivation, growth within cultured eucaryotic cells, and animal models—have specific advantages and disadvantages for studies of drug effects on a pathogen.

Differences in the ability of a drug to antagonize insect stages as compared with intracellular stages of *T. cruzi* and *Leishmania* species have been previously observed (1, 30, 31), but it was difficult to determine whether the differences related to differences in stages or in environments (extracellular versus intracellular). By use of *T. cruzi* epimastigotes and amastigotes growing in cell-free medium at 27°C, it was possible to determine that differences in morphologic stages contributed to the different susceptibilities of epimastigotes and amastigotes to novobiocin.

It seems unlikely that novobiocin itself will be useful in the chemotherapy of Chagas' disease for the following reasons. Concentrations of novobiocin that antagonize *T. cruzi* amastigotes growing in cell-free medium and within cultured human fibroblasts are at the limits of achievable, nontoxic drug levels in the sera of patients (15). Novobiocin given at one-fifth the 50% lethal dose to mice infected with *T. cruzi* Y does not prolong life-spans in comparison with those of untreated, infected animals (10). Intraperitoneal injections of novobiocin also do not prolong the life-span of mice lethally infected with *T. cruzi* H510 (P. G. Pate, J. S. Wolfson, S. C. Pan, and M. N. Swartz, unpublished data). Finally, *T. cruzi* Sonya bloodstream stages isolated from mice and suspended in Krebs-Ringer glucose do not decrease in number or alter in morphology when exposed for 24 h at 4°C to novobiocin (600 µg/ml) (24). Whether clorobiocin or another of the many analogs of novobiocin (20) might be efficacious is not known and seems worthy of investigation.

The mechanism by which novobiocin inhibits amastigotes growing in cell-free medium is not known. Novobiocin and its analogs in moderate concentrations inhibit enzymes other than type II topoisomerases, including DNA polymerases (11, 35) and aminoacyl-tRNA synthetases (51), and thus the target of novobiocin in *T. cruzi* might be unrelated to topoisomerase II. Novobiocin (200 µg/ml), however, inhibits topoisomerase II purified from the trypanosomatid *C. fasciculata* (44). Novobiocin (500 µg/ml) also inhibits a catenating enzyme partially purified from *T. cruzi* Tehuantepec epimastigotes (8, 43), and this or another *T. cruzi* topoisomerase seems a plausible site of drug action.

A topoisomerase II drug target might be located in the nucleus, the kinetoplast, or both. Particularly enticing would be a location in the kinetoplast, because this vital organelle

is not present in mammalian cells and its replication presumably depends upon topoisomerase II activity. Thus, probes which might identify the kinetoplast as a susceptible target are a legitimate objective of study. To establish whether novobiocin might be such a probe, further reasonable steps would be to identify drug-specific alterations in the kinetoplast and to isolate a topoisomerase II from *T. cruzi* to determine its site of origin and sensitivity to novobiocin.

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