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Potential Use of WR6026 as Prophylaxis against Transfusion-Transmitted American Trypanosomiasis

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Since transmission of Chagas' disease by the insect vector is under control in Brazil, transmission by blood transfusion is acquiring special relevance in areas where the disease is endemic and also in countries whose populations are free of infection but that are receiving immigrants from areas where the disease is endemic. Gentian violet, a phenylmethane dye, was the first agent used for the chemical prophylaxis of blood destined for transfusion. A concentration of 0.6 mmol of this dye per liter is effective at eliminating trypomastigotes from blood after 24 h of incubation at 4°C. It is the only effective trypanosomicidal agent available. In the search of alternate compounds, we examined a number of synthetic compounds. They were screened for their activities against blood trypomastigotes of the Y, CL, and B229 strains of *Trypanosoma cruzi* by using two or more dilutions of each compound. We found that compound Q45, a 6-methoxy-8(diethylaminohexylamino)lepidine dihydrochloride, was highly effective at clearing parasites from infected blood. Doses of 65 and 130 μg of this compound eliminated trypomastigotes from blood experimentally contaminated with *T. cruzi* parasites. These results indicate that Q45 is remarkably active against circulating trypomastigotes. Further studies evaluating Q45 as a prophylactic agent for preventing the transmission of *T. cruzi* by blood transfusion are of interest.

The protozoan parasite *Trypanosoma cruzi*, the agent of Chagas' disease, is endemic in many South American countries, where it is estimated that 16 million people are affected (32). In Brazil, transmission of *T. cruzi* by the insect vector is under control (26), and transmission by blood transfusion is acquiring special relevance. The transmission of *T. cruzi* by blood transfusion is also becoming a source of concern in countries whose populations are free of infection but that are receiving immigrants from areas where the disease is endemic (18, 27). In the 1950s, gentian violet, a phenylmethane dye, was demonstrated to be active against trypomastigotes present in blood destined for transfusion (21). A concentration of 0.6 mmol/liter eliminates all of the parasites after 24 h of incubation at 4°C (28). It is the only effective trypanosomicidal agent available. Despite its effectiveness, there are some restrictions to its use. It gives the blood a purple color and might stain the skin and mucosae of the recipients. A carcinogenic effect has been described in rodents, but it has not been proved in humans. The formation of microagglutination and rouleaux of erythrocytes are observed (30). New compounds with higher levels of activity and that are more safe are needed. The Expert Committee on Control of Chagas' Disease of the World Health Organization recommends that investigators search for new active compounds for the chemoprophylaxis of Chagas' disease. These can be either synthetic compounds or compounds obtained from natural products of plant origin. Our laboratory is actively searching for new compounds. Our approach is to search natural products from the Brazilian flora (10) and to examine products obtained by chemical synthesis. We report here the results obtained with a group of quinones, quinoline (synthetic compounds), and arthemisinine derivatives (semisynthetic).

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MATERIALS AND METHODS

T. cruzi. Trypomastigotes of the Y (25), CL (4), and B229 (8) strains of *T. cruzi* were used.

Mice. Adult male Swiss-Webster mice acutely or chronically infected with *T. cruzi* were used to obtain trypomastigotes. These were collected from acutely infected mice on day 7 for trypomastigotes of the Y strain and on day 14 after intraperitoneal (i.p.) inoculation for trypomastigotes of strains CL and B229. Chronically infected mice were obtained by treating mice infected i.p. with the Y strain with 5 mg of benznidazole per kg of body weight per day, which was administered orally for 1 week beginning 1 day after inoculation.

Compounds. The compounds used in the study are listed in Table 1. For the experiments, the compounds were dissolved in dimethyl sulfoxide (DMSO) and the working concentrations were prepared in tissue culture medium 199 (TC199) containing 10% heat-inactivated fetal bovine serum. Because of the excellent activity of compound Q45 in the initial screen and its limited supply, additional amounts of this compound were synthesized in our laboratory by the condensation of 8-amino-6-methoxylepidine with 6-diethylaminohexylbromide hydrobromide in the presence of triethylamine at 150°C (14). The 8-amino-6-methoxylepidine was obtained by reducing 6-methoxy-8-nitrolepidine with stannous chloride, granulated tin, and hydrochloric acid (7) (melting point, 86 to 87°C; melting point in the literature [31], 86.5 to 87.5°C; yield, 89%). The 8-nitro-6-methoxylepidine was prepared by treating *o*-nitro-*p*-methoxyaniline with 1,3,3-trimethoxybutane under modified Skraup reaction conditions (12) (melting point, 170°C; melting point in the literature [31], 169.5 to 171.5°C; yield, 25%). The 6-diethylaminohexylbromide hydrobromide was prepared by treating 6-diethylaminohexanol with 48% hydrobromic acid (12) (yield, 85%). The 6-diethylaminohexanol was obtained by reacting 6-chlorohexanol and diethylamine (31) (yield, 71%).

Bioassay. The initial screening of each compound was conducted with 2×10^5 bloodstream trypomastigotes of the Y strain per 100 μl of blood as described previously (10). Compounds showing activity in the initial screening were further evaluated by using strains Y, CL, and B229. Strain B229 was isolated from a human patient with chronic Chagas' disease, and strain CL was isolated from a naturally infected triatomine insect. To determine in vitro activity, each compound (at two times the molecular weight/100) was dissolved in 200 μl of DMSO plus TC199, and the solution was mixed with an equal volume of blood from acutely infected mice. Six dilutions of each compound from 25 to 250 μl were examined. Controls were tubes containing TC199 alone and DMSO plus TC199. The tubes were incubated for 24 h at 4°C. Thereafter, 5 μl of the suspension was

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TABLE 1. Synthetic compounds examined for their activities against the blood trypomastigote stage of *T. cruzi*

Compound	Chemical name	Mol wt
Q80	2-[*trans*-4-(4-Chlorophenyl)cyclohexyl]-3-hydroxyl-1,4-naphthoquinone	366.8
Q52	8-[(4-Amino-1-methylbutyl)amino]-2,6-dimethoxy-4-methyl-5-(3-trifluoromethylphenoxy)quinoline succinate	581.5
Q45	6-Methoxy-8-(diethylaminohexylamino)lepidine dihydrochloride (WR6026)	416.4
Q86	8-[(4-Amino-1-methylbutyl)amino]1-5-(1-hexyloxy)-6-methoxy 4-methylquinoline (DL-tartarate)	523.6
Q16	β-Arteether	312.4
Q66	Sodium artelinate	452.4
Q67	β-Artemether	298.3

examined microscopically for the presence of motile organisms, and 100 microscopic fields were examined at ×400 magnification. One hundred microliters of blood from the cutoff-negative tube (the tube with the lowest concentration of compound in which no motile trypomastigotes were observed) was then inoculated i.p. into each one of four Swiss-Webster mice. These mice were examined for patent parasitemia from day 7 to day 30 after inoculation. Blood from mice that did not develop patent parasitemia during this period was collected and used for hemocultures in liver infusion tryptose medium (5). In addition, serum from these mice was used to determine the presence of antibodies to *T. cruzi* by the indirect immunofluorescent-antibody assay (6). The lytic effects of the compounds on erythrocytes was determined by microscopy.

Compound Q45, which showed significant activity in the bioassays with the different strains of *T. cruzi*, was further investigated for its therapeutic activity. This was performed by treating mice infected i.p. with 5×10^3 trypomastigotes of the Y strain with 100 mg of the compound per kg/day for 20 days beginning on day 1 after infection. Mice infected and untreated were used as controls.

RESULTS

The results obtained with Q45 are provided in Table 2. At low concentrations, Q45 did not show any lysis and was remarkably active in eliminating trypomastigote forms from infected blood (Table 2). Compounds Q80, Q16, Q66, and Q67 did not show any activity even at the highest concentration examined. Q52 and Q86 caused blood coagulation, and lysis of erythrocytes was noted in tubes containing compound Q52. Compound Q45 caused lysis of erythrocytes at high concentrations. Further evaluation of Q45 both in vitro and in vivo (mice injected with treated blood after incubation of the blood for 24 h at 4°C) confirmed its excellent antitrypomastigote activity (Table 3). These results prompted experiments to evaluate the activity of Q45 against other strains of *T. cruzi*. The results are provided in Table 3. A concentration of 65 μg/ml eliminated trypomastigotes of each strain from contaminated blood. This was demonstrated by the absence of parasites after incubation of blood with Q45 for 24 h at 4°C and by the absence of circulating trypomastigotes in mice injected with contaminated and treated blood. This absence of parasites was confirmed by microscopy and hemocultures. In addition, sera from the mice injected with contaminated and treated blood did not develop antibodies to *T. cruzi*, as demonstrated by the indirect immunofluorescent-antibody assay.

Mice inoculated i.p. with trypomastigote forms of the Y strain of *T. cruzi* and treated with 100 mg of compound Q45 per kg/day for 20 days developed levels of parasitemia and mortality similar to those of the control group (data not shown).

TABLE 2. In vitro and in vivo activities of different concentrations of Q45 against trypomastigotes of the Y strain of *T. cruzi*

Drug and concn (μg/ml)	Activity[a]	
	In vitro[b]	In vivo[c]
Q45		
131.20	Neg	Neg
65.60	Neg	Neg
32.75	Neg	Pos
16.37	Pos	Pos
8.20	Pos	ND
Gentian violet (62.5)	Neg	Neg
DMSO + TC199	Pos	Pos
TC199	Pos	Pos

[a] Neg, active, absence of bloodstream trypomastigotes; Pos, inactive, presence of bloodstream trypomastigotes; ND, not determined.
[b] Blood was examined after incubation with Q45 for 24 h at 4°C.
[c] Blood was examined after i.p. inoculation into mice of blood treated with Q45 for 24 h at 4°C for from 7 to 30 days.

TABLE 3. Results of tests to determine activity of Q45 against blood trypomastigotes of strains Y, CL, and B229 of *T. cruzi* obtained from acutely or chronically infected mice

Concn examined (μg/ml)	Activity[a]			
	In vitro incubation[b]	Mouse inoculation[c]	Hemoculture[d]	IFA test
260	Neg	Neg	Neg	Neg
130	Neg	Neg	Neg	Neg
65	Neg	Neg	Neg	Neg
32.5	Neg	Pos	Pos	Pos
16.25	Neg	Pos	Pos	Pos

[a] Neg, absence of parasites or antibodies to *T. cruzi*; Pos, presence of parasites or antibodies; IFA test, indirect immunofluorescent-antibody test.
[b] Blood was examined for motility after incubation of test compound for 24 h at 4°C.
[c] Blood from mice inoculated with blood treated with Q45 for 24 h at 4°C for from 7 to 45 days was examined.
[d] Blood was cultured 30 to 45 days after inoculation of mice with blood treated with Q45 for 24 h at 4°C and was examined after 20 days of incubation at 26 to 28°C.

DISCUSSION

Of the compounds examined in the present study, only compound Q45 showed significant activity against the blood form of *T. cruzi*. This compound was first synthesized and studied by scientists from the Walter Reed Army Institute of Research under the code number WR6026 (3, 16, 17, 29). The compound is in the public domain and was synthesized in our laboratory to provide sufficient quantities for our studies. The bioavailabilities and pharmacokinetics of intravenous and oral doses of this compound were studied in beagle dogs (13). In addition, tests conducted by the Walter Reed Army Institute of Research with healthy male subjects with oral doses of up to 60 mg revealed no significant drug-related symptoms and no physical or laboratory abnormalities (22). WR6026 was effective for the treatment of visceral leishmaniasis in 16 patients at a dosage of 0.75 to 1.00 mg/kg/day. The therapy was associated with minimal toxicity; adverse effects included gastrointestinal distress, headache, and methemoglobinemia (24). Previously, WR6026 was shown to be inactive against *T. cruzi* infection in mice (16). We confirmed this inactivity when mice inoculated with the Y strain of *T. cruzi* and treated with compound WR6026 for 20 days developed levels of parasitemia and mortality similar to those in infected and untreated mice (data not shown). WR6026 showed activity against *T. cruzi* trypomastigotes in blood after 2 h of incubation at 37°C but was not active at 25°C (11).

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Of interest was the fact that compound Q80, an hydroxynaphthoquinone that has been shown to be highly active against tachyzoites and cysts of *Toxoplasma gondii* (1, 2), was completely inactive against *T. cruzi* under the conditions used in the present study. Compound Q52, a quinoline succinate, was also inactive, possibly because of its poor solubility. The insolubility of some compounds, particularly Q80, Q86, and Q52, may have caused them to have little or no activity against trypomastigotes. Compounds Q16, Q66, and Q67 are arthemisinine derivatives which have been shown to be active in the treatment of malaria (9, 15, 19), but they did not show any activity against *T. cruzi.*

Trypanosomicidal activity was demonstrated with 8-aminoquinoline (compound Q86), a compound analog of primaquine that was used to treat human malaria during the World War II. Clinical trials are being conducted in Brazil and other countries to evaluate lepidine (compound Q45) for the treatment of leishmaniasis, which is caused by *Leishmania donovani*. Brazilian researchers and researchers from the Walter Reed Army Institute of Research are performing those studies.

A recommendation of the Second Annual Meeting of Applied Research on Chagas' Disease (23) states that "the group stressed the need for research on new drugs and procedures for the chemoprophylaxis of transfusional Chagas' disease, mainly in order to reduce the time of exposure required for blood sterilization. An exposition time of two hours or less must be pursued for those small services in which blood storage is difficult, as well as for those emergency situations when blood transfusion requires immediate actions" (23).

We feel that the use of the lepidine (compound Q45) may show some advantages over the use of gentian violet. It does not change the color of the transfusion blood and does not stain mucous membranes. It is well tolerated by humans; adverse effects were not detected in human volunteers (20, 22). Moreover, Q45 was effective at concentrations similar to those at which gentian violet is effective, and it did not have any noticeable deleterious effect on erythrocytes. The high level of antitrypanosomicidal activity and low level of toxicity of lepidine suggest that further studies evaluating its potential use as a chemoprophylactic agent for preventing the transmission of *T. cruzi* by blood transfusion are of interest.

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