A combination of benznidazole and ketoconazole enhances efficacy of chemotherapy of experimental Chagas’ disease

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Despite the large number of patients infected with \textit{Trypanosoma cruzi}, there are no commercial drugs available with high efficacy for use in the clinical treatment of Chagas’ disease (American trypanosomiasis). As the prospects of the introduction of new compounds by the pharmaceutical industry are poor, alternative strategies are being designed to identify candidates among drugs already available on the market that could be used in combination to provide a synergic effect and improve the efficacy of chemotherapy. In this paper we investigated a possible synergic effect of drugs in mice inoculated with isolates of \textit{T. cruzi} susceptible (CL), moderately resistant (Y) and naturally resistant (Colombiana) to benznidazole and nifurtimox. Our data demonstrated that the combination of benznidazole with ketoconazole induced a synergic effect in mice infected with the CL and Y isolate of \textit{T. cruzi}. No differences were observed, however, in animals infected with the Colombiana isolate, suggesting that the synergic effect of benznidazole and ketoconazole is influenced by the isolate of parasite and that this could be important in further studies searching for useful combinations of drugs. Moreover, we observed that early treatment with ketoconazole could increase the cure rate in animals infected with the Y isolate. No positive effect, in relation to cure rate, was observed with the combination of benznidazole and ofloxacin. Our results re-emphasize the importance of identifying those compounds already on the market with synergic effects able to enhance the cure of \textit{T. cruzi} infection.

Introduction

Chagas’ disease is an endemic infection caused by the protozoan parasite \textit{Trypanosoma cruzi}, which is widely distributed in the Americas, extending from Mexico to Argentina, where 16–18 million people are infected and 90 million (25% of the population) are at risk of infection.\textsuperscript{1} Morbidity is relatively high, 17–30% of chronic chagasic patients displaying variable clinical manifestation including myocardopathy and gastrointestinal pathological dilatation.\textsuperscript{2,3}

The control of Chagas’ disease in Brazil for the past 10 years has been focused on intervention directed against vectors.\textsuperscript{4} Despite the success of vector control, with possible eradication of the vectorial transmission, there are still millions of infected people who will depend on medical care for at least the next 30 years.

In spite of the large number of infected patients, there are no commercially available drugs with high efficacy that can be used for mass and/or individual treatment. Specific chemotherapy with the two drugs available, benznidazole and nifurtimox, has been indicated for the treatment of the brief acute phase, with a cure rate of 50–70%.\textsuperscript{5} Prolonged follow-up studies with chronically infected patients, however, show a very low cure rate (8–20%) in patients treated for more than 10 years.\textsuperscript{5,7} Furthermore, both drugs can cause several side-effects including hypersensitivity reactions (dermatitis), peripheral neuritis, weight loss, gastrointestinal disturbances and peripheral polyneuropathy.\textsuperscript{5} In addition, differences in the susceptibility and natural resistance of a large number of \textit{T. cruzi} isolates to nitroderivatives has also been suggested as an important factor in the low rates of cure in treated chagasic patients.\textsuperscript{8–10} The improvement of currently available chemotherapy is thus a research priority in those countries where vectorial transmission has been controlled.

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The existence of T. cruzi isolates that are naturally resistant to benznidazole and nifurtimox has stimulated the search for alternative drugs suitable for the treatment of T. cruzi infection. Among the several compounds tested in vitro and in vivo against T. cruzi, it has been demonstrated that ketoconazole, an imidazole derivative highly effective in the treatment of topical and systemic fungal infection, is able to protect mice against fatal T. cruzi infection. Ketoconazole, however, failed to cure chronic fungal infection.13–15 Among the several compounds tested in vitro and in vivo against T. cruzi, it has been demonstrated that ketoconazole, an imidazole derivative highly effective in the treatment of topical and systemic fungal infection, is able to protect mice against fatal T. cruzi infection.13–15 Ketoconazole, however, failed to cure chronic phase human clinical and experimental Chagas’ disease.15 Some studies have also been performed to evaluate the effects of various drugs in vitro, with axenic as well as infected tissue culture, demonstrating that ofloxacin, a bacterial topoisomerase II inhibitor, is able to inhibit both proliferation and differentiation of T. cruzi in axenic cultures in vitro. No evidence, however, of in vivo activity of this drug has so far been provided.

Synergy between drugs can be a valuable way to improve treatment efficacy in several diseases. Using this approach, Urbina et al. demonstrated that the combination of ketoconazole with lovastatin has an anti-proliferative effect in vitro on T. cruzi epimastigote growth in LIT media and on amastigotes in tissue cultures. Unfortunately, these results were not observed in vivo when the same combination was used to treat infected mice. A synergetic effect of ketoconazole plus terbinafine and lovastatin, inhibitors of ergosterol biosynthesis, has also been demonstrated both in vitro and in a murine model Chagas’ disease. In view of the trypanosomicidal effect of ketoconazole and ofloxacin described previously, and the fact that these drugs are commercially available for human chemotherapy, we investigated in this study whether their combination with benznidazole could have a synergic effect and improve the effectiveness of treatment of Chagas’ disease.

Materials and methods

Drugs

Benznidazole (2-nitro-N-(phenylmethyl)-1H-imidazole-1-acetamide) is synthesized by F. Hoffman–La Roche, and commercially available in Brazil as ‘Rochagan’ (Produtos Roche Químicos e Farmacêuticos, Rio de Janeiro, Brazil). It has been used as a standard drug for human Chagas’ disease treatment in Brazil. Ketoconazole is a derived imidazole produced by Janssen Pharmaceutics and commercially available in Brazil as ‘Nizoral’ (Janssen-Cilag Farmacêutica, São José dos Campos, São Paulo, Brazil). Ofloxacin is a bacterial topoisomerase II inhibitor, synthetic quinolone synthesized by Janssen Pharmaceutics and commercially available in Brazil as ‘Ofloxan’ (Janssen Farmacêutica, São José dos Campos, São Paulo, Brazil).

Trypanosoma cruzi isolates

Three isolates of T. cruzi previously characterized by Filardi & Brener according to their susceptibility to benznidazole and nifurtimox in vivo were used; susceptible CL, moderately resistant Y and naturally resistant Colombiana. All isolates of T. cruzi were obtained as infected blood samples frozen in liquid nitrogen (as described by Filardi) from the T. cruzi cryobank at Laboratório de Doença de Chagas, Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte, MG, Brazil. All isolates were maintained in the laboratory by passages in Swiss–Webster female mice.

Infection and treatment schedules

Swiss–Webster female mice, 20–24 g, were obtained from the animal facility at Centro de Pesquisas René Rachou (FIOCRUZ). Animals were inoculated intraperitoneally with 1 x 10⁴ blood forms of T. cruzi. The inoculated animals were divided into 13 groups of 10–14 animals and received the various drug combinations. All compounds were suspended in distilled water using 4% gum arabic and each animal received 0.25 mL of drug suspension daily by gavage. Drug combinations consisted of benznidazole plus either ketoconazole or ofloxacin. Benznidazole was administrated in daily doses of 25, 50 or 100 mg/kg/day. One hour after benznidazole treatment, the second drug was administered at a dose of 120 mg/kg/day or 100 mg/kg/day for ketoconazole or ofloxacin, respectively. Treatment started immediately after the detection of patent parasitaemia (8–10 days after infection for Y isolate and 12–15 days after infection for CL and Colombiana isolates) and was administered for 20 consecutive days, for all drug combinations. One group of 11 animals received ketoconazole (120 mg/kg/day) starting 24 h after infection with Y isolate. Control groups consisted of untreated animals and those given each of the compounds alone.

Parasitaemia evaluation

Blood examination was performed daily to assess levels of parasitaemia, as described by Brener. Blood from the tail were transferred to a microscope slide, covered with a coverslip and examined for living flagellates by direct optical microscopy. Results were expressed as number of trypanomastigotes/μL blood. After treatment, parasitaemia was followed for different periods of time, depending on the previously described peak of parasitaemia for different isolates of T. cruzi (6 days for Y and 20 days for CL and Colombiana).

Test for cure assessment

Cure was assessed by using parasitological (haemoculture and xenodiagnosis) and serological (anti-live trypomastig-
Drug combinations for treatment of Chagas’ disease

Note antibodies) methods. Haemoculture was performed as described by Filardi & Brener and xenodiagnosis as described by Brener. Analysis of anti-live trypomastigote antibody was performed as described by Martins-Filho et al. Results were compiled as animals cured and those not cured. Animals were considered cured only when all tests showed negative results.

Statistical analysis
The \( \chi^2 \) analysis or Fisher’s Exact test was used to compare differences in cure rate between the groups receiving benznidazole, ketoconazole or ofloxacin alone with those receiving combinations of drugs. Differences were considered statistically significant at \( P < 0.05 \). All statistical analyses were carried out using Version 6 of Epi-Info software from the CDC.

Results

Early treatment and the efficacy of treatment with ketoconazole
To determine whether the time between the infection and the beginning of treatment had any influence on the cure rate, we performed a parallel study in which animals infected with the Y isolate were submitted to different treatment schedules with ketoconazole. Infected animals received ketoconazole (120 mg/kg/day for 20 days) starting 24 h and 9 days after infection. We observed a 100% cure (11/11) for the animals treated 24 h after infection, but none of the animals (0/11) treated 9 days after infection were cured. These data demonstrated that the time span between the infection and the beginning of chemotherapy with ketoconazole is of fundamental importance in the effectiveness of treatment with ketoconazole.

Combination of benznidazole and ketoconazole
As ketoconazole was ineffective in animals infected with the Y isolate when therapy started 9 days after infection, the effect of ketoconazole combined with benznidazole was determined in continuing infections. For this purpose, animals infected with the CL, Y and Colombiana isolates of \( T. cruzi \) were treated immediately after the detection of patent parasitaemia, for all different drug combinations. The cure rate observed after treatment with benznidazole, ketoconazole and their combination are presented in Table I. The results demonstrate that in mice infected with the CL isolate a combination of benznidazole and ketoconazole resulted in a synergic effect, increasing the cure rate \( (P < 0.05) \). This effect can be clearly noted when comparing the percentage of cured animals after treatment with benznidazole (25 and 50 mg/kg/day) or ketoconazole (120 mg/kg/day) alone with those receiving the combination of benznidazole plus ketoconazole. Treatment with ketoconazole (120 mg/kg/day) alone or benznidazole

<table>
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<tr>
<th>Drug (mg/kg/day)</th>
<th>Isolates</th>
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<tr>
<td></td>
<td>CL</td>
</tr>
<tr>
<td>Untreated</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>BZ (25)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>BZ (25) + KZ (120)</td>
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</tr>
<tr>
<td>BZ (100)</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>BZ (100) + KZ (120)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>KZ (120)</td>
<td>0/9 (0)</td>
</tr>
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</table>

*Results are expressed as negative parasitological and serological tests/total number of animals tested, with cure rate, as a percentage, given in parentheses. Swiss–Webster female mice, 20–24 g, were inoculated with \( 10^4 \) \( T. cruzi \) blood forms. Treatment started after detection of patent parasitaemia (12–15 days after infection for CL and Colombiana isolate and 8–10 days after infection for Y isolate) and was administered for 20 consecutive days. Parasitological cure was assessed by haemoculture and xenodiagnosis and results were confirmed by serological test. Animals were considered cured when all three tests were negative.

Statistical differences \( (P < 0.05) \) between the groups receiving benznidazole or ketoconazole alone and those receiving combinations of drugs at the same dose.
(50 mg/kg/day) alone cured 0 or 9.1% of animals, respectively. In contrast, treatment with ketoconazole (120 mg/kg/day) plus benznidazole (25 or 50 mg/kg/day) cured 71.4 or 100%, respectively.

Treatment of animals infected with the Y isolate with the combination of benznidazole and ketoconazole also gave an increased cure rate in comparison with animals that received the drugs alone. Treatment with benznidazole alone (25 and 50 mg/kg/day) did not cure the infection, whereas benznidazole at 100 mg/kg/day cured 30.8% of the animals. Ketoconazole at 120 mg/kg/day when combined with benznidazole increased the cure rate to 38.5, 92.3 and 100%, respectively ($P < 0.05$), although ketoconazole alone cured only 9.1% of animals.

Although the combination of benznidazole (100 mg/kg/day) and ketoconazole (120 mg/kg/day) produced 30.8% cure in animals infected with the Colombiana isolate of *T. cruzi* in comparison with the treatment using the drugs alone, this difference was not statistically significant ($P > 0.05$).

Combination of benznidazole and ofloxacin

The cure rate observed after treatment with benznidazole and ofloxacin alone and combined is presented in Table II. No cure was observed when animals infected with CL, Y or Colombiana isolate were treated with ofloxacin alone at 100 mg/kg/day. We did not find any differences in the cure rate in animals treated with benznidazole plus ofloxacin in comparison with those treated with benznidazole alone for any of the isolates.

Levels of parasitaemia in animals treated with ofloxacin

The level of parasitaemia was investigated in animals infected with *T. cruzi* after chemotherapy with ofloxacin. The Figure shows the levels of parasitaemia in animals infected with CL, Y and Colombiana isolates of *T. cruzi* after treatment with ofloxacin. Parasitaemia was followed for different periods of time, depending on the peak of parasitaemia described previously for the *T. cruzi* isolates (6 days for Y, and 20 days for CL and Colombiana). By using this approach, we ensured that any *in vivo* trypanosomicidal effect of ofloxacin could be identified as a reduction in the high levels of parasitaemia observed in untreated animals. As observed in the Figure, despite its reported *in vitro* activity, ofloxacin did not have any trypanosomicidal effect *in vivo*. It is interesting to observe that in animals infected with Y isolate at day 2 after treatment, the level of parasitaemia was increased in the ofloxacin group in comparison with the untreated group. This result emphasizes the total lack of effect of ofloxacin as a trypanosomicidal drug for *in vivo* treatment.

Discussion

Despite extensive studies in the past to search for alternative drugs for use in the treatment of Chagas’ disease, treatment of human cases is still restricted to the use of two nitroheterocyclic drugs, benznidazole and nifurtimox.

In this paper we investigated a possible synergic effect of benznidazole with ketoconazole or ofloxacin in mice.

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</tr>
<tr>
<td>BZ (25)</td>
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<td>BZ (100) + OFL (100)</td>
<td>11/12 (91.7)</td>
</tr>
<tr>
<td>OFL (100)</td>
<td>0/12 (0)</td>
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Results are expressed as negative parasitological and serological tests/total number of animals tested, with cure rate, as a percentage, given in parentheses. Swiss–Webster female mice, 20–24 g, were inoculated with $10^6$ *T. cruzi* blood forms. Treatment started after detection of patent parasitaemia (12–15 days after infection for CL and Colombiana isolates and 8–10 days after infection for Y isolate) and was administered for 20 consecutive days. Parasitological cure was assessed by haemoculture and xenodiagnosis and results were confirmed by serological test. Animals were considered cured when all three tests were negative.
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It is interesting to note that our data show that ketoconazole alone was not enough to cure animals infected with the Y isolate of *T. cruzi*, when treatment was started 9 days after infection; however, when dosing started 24 h after infection, animals were cured. These data are in agreement with those of McCabe *et al.*,14 who demonstrated that although mice infected with four different isolates of *T. cruzi* can be markedly protected from death by early treatment with ketoconazole, the therapeutic effectiveness was dependent on the delay between the infection and the beginning of treatment. They showed that the results can range from 100 to 0% survival from day 5 to day 9 after infection. These results and ours emphasize the necessity of early treatment of Chagas’ disease.

We have also investigated here a possible synergic effect produced by a combination of benznidazole and ofloxacin. Unfortunately, our data did not demonstrate any positive effect with such a combination, despite the results of Gonzales-Perdomo *et al.*,16 who showed that an inhibitor of bacterial topoisomerase II inhibits the proliferation and differentiation of *T. cruzi* in axenic cultures in vitro, as well as in infected tissue culture. Moreover, we found that treatment with ofloxacin increased the parasitaemia in animals infected with the Y isolate. A similar result in animals infected with the Y isolate has been reported by Brener *et al.*,15 who evaluated the effect of lovastatin combination with ketoconazole.

It is interesting to observe that the Colombiana isolate, which is naturally resistant to benznidazole, was not susceptible to any of the drug combinations tested here. Despite several studies investigating the mechanisms of drug resistance in different *T. cruzi* isolates, the mode of resistance of the Colombiana isolate is still unknown.

In conclusion, our results confirmed the importance of identifying among compounds already marketed those with synergic effects that are able to enhance the cure of Chagas’ disease. This approach may help avoid the high costs and time-consuming research on toxicity and bioavailability of drugs for human consumption, the ultimate stage of the drug development process. Moreover, considering that several publications have shown the inefficacy of benznidazole and nifurtimox in treatment of Chagas’ disease, our results support the use of combinations of benznidazole and ketoconazole in clinical trials, mainly in those cases of Chagas’ disease when diagnosis is confirmed shortly after infection. The use of benznidazole and ketoconazole in humans could contribute to an increase in therapeutic efficacy, even though their use in isolation does not give the expected results.13 Finally, as suggested previously by Andrade *et al.*,9 the fact that particular isolates of *T. cruzi* each have their own level of susceptibility to certain drugs further supports the strategy of using different drug combinations to improve chemotherapy success in different geographical regions.

**Figure.** Effect of ofloxacin treatment on levels of parasitaemia in animals infected with (a) CL, (b) Y and (c) Colombiana isolates of *Trypanosoma cruzi*. Results are expressed as mean number of trypomastigotes/5 μL of tail blood for animals treated with ofloxacin at 100 mg/kg/day (continuous line without symbols), benznidazole at 100 mg/kg/day (●), as well as for untreated control (○). Swiss–Webster female mice, 20–24 g, were inoculated with 10⁴ *T. cruzi* blood forms. Treatment started after detection of patent parasitaemia (12–15 days after infection for CL and Colombiana isolate and 8–10 days after infection for Y isolate) and was administered for 20 consecutive days.

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