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Experimental Chemotherapy with Combinations of Ergosterol Biosynthesis Inhibitors in Murine Models of Chagas' Disease

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We report the effects of ketoconazole and the bistriazole ICI 195,739 acting alone or in combination with the allylamine terbinafine (Lamisil) on murine models of Chagas' disease. Mice infected with 10^5 *Trypanosoma* (*Schizotrypanum*) *cruzi* blood trypomastigotes and treated orally with 30 mg of ketoconazole per kg of body weight per day for 7 days, starting at 24 h postinoculation, had 100% survival after 35 days, while controls (untreated) or animals that received 15 mg of ketoconazole or 100 mg of terbinafine per kg/day by the same route had 0% survival after the same period of time. However, all mice receiving the combination of 15 mg of ketoconazole plus 100 mg of terbinafine per kg/day survived for 35 days after infection; it was shown that the survival of the animals treated with this combination was statistically greater than that obtained with either drug acting alone and was indistinguishable from that observed with the high doses of ketoconazole, indicating a synergistic action of the drugs in vivo. However, most animals that survived after the 7-day treatments were not cured, as indicated by a delayed but persistent parasitemia. When the treatment was extended to 14 days, 100% survival was obtained 10 weeks after inoculation for mice treated with 30 mg of ketoconazole per kg/day and the combination of 15 mg of ketoconazole per kg/day plus 100 mg of terbinafine per kg/day, while two-thirds of the mice treated with 15 mg of ketoconazole per kg/day alone were alive after the 14-day treatment; controls or animals that received 100 mg of terbinafine per kg/day did not survive after 25 days. Parasitemia in all surviving mice was negative after 55 days but parasitological cure, as assessed by subinoculation of organs in naive animals, was predominant only in animals that received the combined drug treatment. We also investigated the bistriazole ICI 195,739 and found, as reported previously, that just 1 mg of the compound per kg/day administered orally for 5 days was enough to protect most mice from death 30 days after inoculation, but no parasitological cures were observed. However, in the protocol used in the present study, the protective activity of ICI 195,739 at suboptimal doses (0.5 mg/kg/day) could be enhanced when it was used in combination with terbinafine at doses of the allylamine that by themselves induced no significant protection. Survival of the mice was inversely correlated with the levels of parasitemia in all cases. Extension of the treatment period with the triazole to 15 days at 1 mg/kg/day afforded definitive protection against death, with parasitological cure being achieved in 50% of mice at 10 weeks postinoculation, but no enhancement of its activity at suboptimal doses was observed when it was used in combination with terbinafine during this extended observation period. Taken together, these results support the proposition that ketoconazole used in combination with terbinafine could be useful in the treatment of humans with Chagas' disease because it can promote parasitological cure without the need to resort to the use of high levels of the azole, which is known to interfere with hepatic function and steroid synthesis in the host. They also support the conclusions of previous in vitro studies which suggested that the triazole ICI 195,739 blocks the proliferation of *T. cruzi* by a mechanism which differs from those of classical ergosterol biosynthesis inhibitors.

Chemotherapy for Chagas' disease remains unsatisfactory in terms of both the toxicities and the lack of effectiveness of the currently available drugs, nifurtimox and benznidazole, used to treat the disease, although there have been important advances in the study of the biochemistry and physiology of its causative agent, *Trypanosoma* (*Schizotrypanum*) *cruzi* (13, 21, 29). On the other hand, real breakthroughs have taken place in the chemotherapy of fungal infections in humans, animals, and plants with the introduction of broad-spectrum, orally active agents which specifically block the de novo biosynthesis of ergosterol of analogs, membrane components which are essential growth factors for these cells and which cannot be substituted by the host's cholesterol or phytosterol (3, 36, 37, 42, 48–50). The most important compounds in this group are the azoles, comprising imidazole and triazole derivatives, with ketoconazole being the reference compound, which acts at the level of C-14 demethylation of lanosterol (48–50), and the allylamines, fundamentally, naftifine and terbinafine (Lamisil), which block squalene epoxidase in fungi (3, 36, 37, 42). Trypanosomatid protozoa such as *T. cruzi* and the different species of *Leishmania* also have a strict requirement for ergosterol and related 4-desmethyl sterols, as shown in both in vitro and in vivo studies (4–11, 17, 19, 20, 22, 26–28, 30–32, 34, 43–47). However, although success has been claimed in the treatment of human cutaneous leishmaniasis with azoles (10, 47), the doses of ketoconazole reported to be effective for the prevention death from lethal *T. cruzi* infections in mice and for the promotion of parasitological cure (30–32, 34) are far greater than those that are known to produce hepatotoxicity and to block steroid hormone synthesis in humans (2, 41). We have recently found that the in vitro antiproliferative activity of ketoconazole against the epimastigote and intra-

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cellular amastigote stages of the parasite can be substantially potentiated by terbinafine, to the point that the use of these drugs at just 1 nM given in combination can completely prevent the infection of cultured vertebrate cells by the parasite or can eradicate the parasite from previously heavily infected cells (27, 28, 43). Because these concentrations are four orders of magnitude lower that those attained in the plasma of healthy human volunteers treated with well-tolerated therapeutic doses of the drugs (3, 12, 24, 35, 51), it was proposed (43) that this combination could also be effective in vivo.

A recently developed and highly promising compound is the bistriazole derivative ICI 195,739, which is also a potent antifungal agent that acts in these cells at the level of the C-14 demethylation of lanosterol (4, 11, 39). Ryley et al. (39) have also shown that this compound has a remarkable and specific activity against *T. cruzi* in vivo; our own in vitro studies have confirmed the very high level of susceptibility of *T. cruzi* proliferative stages toward this compound, and evidence for a dual mechanism of action against the parasite has been presented (28, 44).

Here we report the initial results of a study of experimental chemotherapy in murine models of Chagas' disease in which we used combinations of azoles (ketoconazole and ICI 195,739) with terbinafine.

MATERIALS AND METHODS

Strain. In all the experiments described here, the Y strain of *T. cruzi* was used; handling of live *T. cruzi* was done according to published guidelines (23).

Experimental chemotherapy. For comparative purposes, we followed as closely as possible the protocols described by McCabe et al. (30–32) for ketoconazole and those described by Ryley and colleagues (38, 39) for terbinafine and ICI 195,739. Briefly, groups of six to eight outbred NMRI albino female mice (weight, 25 to 30 g) were inoculated intraperitoneally with 10^5 blood trypomastigotes, and treatment was initiated 24 h later. The drugs were suspended in 2% methylcellulose containing 0.5% Tween 80 and were given by gavage; controls received the suspension only as a placebo. Treatment was given once daily for the periods indicated in the figure legends. Parasitemia was measured as described by Brener (13) by using tail blood. Hemocultures were carried out by inoculating 2 ml of liver infusion medium (15) with 0.4 ml of blood obtained from infected mice by cardiac puncture; microscopic examination of the cultures for the detection of proliferative epimastigote forms was done weekly for 6 weeks. Organs (spleen, heart, and liver) from surviving animals with negative parasitemia were minced individually in 1 ml of sterile phosphate-buffered saline supplemented with 10 mM D-glucose, and 0.4 ml of the suspension was inoculated into juvenile (weight, 15 to 20 g) animals.

Statistical analysis. The Kolmogorov-Smirnov test (14) for goodness of fit was used for all the statistical analyses of mortality curves in the present study. The mortality curves for the control (untreated) groups, which were highly reproducible, were used as the reference distribution function in each experiment; a one-side test was generally used because all treated groups had survival times equal to or greater than those of untreated controls, but two-sided tests were also used occasionally. The Kolmogorov-Smirnov test is preferred over the chi-square test for goodness of fit if the sample is small, because the Kolmogorov-Smirnov test is exact for small samples, while the chi-square test assumes

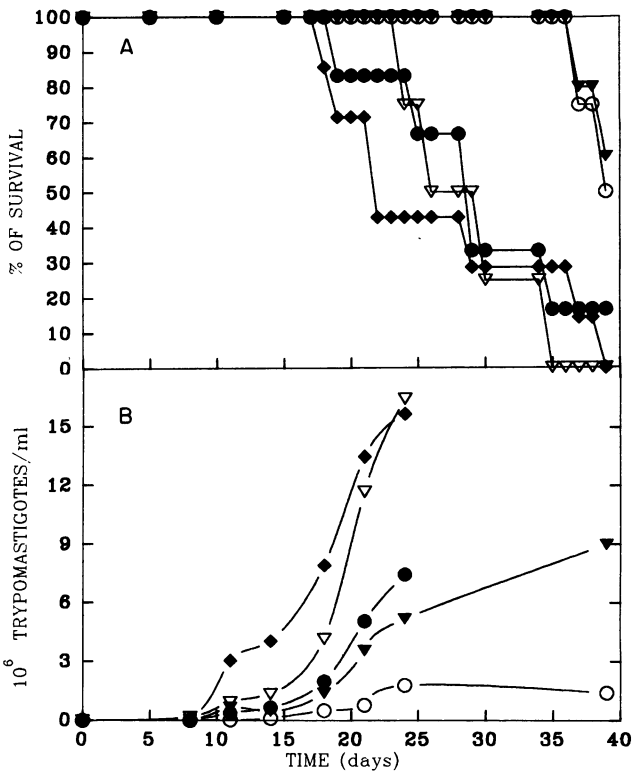

FIG. 1. Effects of ketoconazole and the combination of ketoconazole with terbinafine on survival of (A) or level of parasitemia in (B) mice infected with *T. cruzi* and treated for 7 days. Mice were inoculated intraperitoneally on day 0 with 10^5 blood trypomastigotes of the Y strain of *T. cruzi*; 24 h later oral treatment was initiated with the following drug regimens: controls (untreated) (◆), ketoconazole at 30 mg/kg/day (○), ketoconazole at 15 mg/kg/day (●), terbinafine at 100 mg/kg/day (▽), ketoconazole at 15 mg/kg/day plus terbinafine at 100 mg/kg/day (▼). Other experimental details are described in Materials and Methods.

that the number of observations (deaths) is large enough so that the chi-square distribution provides a good approximation of the distribution of the test statistics (14). Tests were carried out by using the StatWorks program package run on a Macintosh LC personal computer; statistical tables were consulted when needed.

Drugs. Terbinafine [SF-86-327; Lamisil; (*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphthalenemethanamine] (3, 37) was provided by A. Lindenmann and H. Stahelin, Sandoz, Ltd., through Luis Rodriguez, Sandoz de Venezuela, S.A.; ketoconazole {*cis*-1-acetyl-4-[-4[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl-methyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine} (50) was provided by Ingrid Straziota, Janssen Pharmaceutica, Caracas, Venezuela, and ICI 195,739 [(*R,S*)-2-(2,4-difluorophenyl)-1-(3-[(*z*)-4-(2,2,3,3-tetrafluoropropoxy)stiryl]-1,2,4-triazol-1-yl)-propan-2-ol] (11, 39) was kindly provided by John F. Ryley.

RESULTS

Figure 1A shows that ketoconazole given orally at 30 mg/kg of body weight per day for 7 days was able to protect from death mice infected with 10^5 *T. cruzi* blood trypomastigotes for up to 35 days after infection, while control animals had 0% survival after the same period; treatment

with ketoconazole at 15 mg/kg/day or terbinafine at 100 mg/kg/day did not prolong survival beyond that observed in controls. Statistical analysis of these data and those from two other independent experiments by the Kolmogorov-Smirnov goodness-of-fit test (see Materials and Methods) showed that there were no significant differences (one- or two-sided test, $P > 0.2$) between untreated animals and those treated with 15 mg of ketoconazole per kg/day or 100 mg of terbinafine per kg/day but a highly significant difference between untreated animals and those treated with 30 mg of ketoconazole per kg/day ($P < 0.01$). However, if infected animals were treated with the combination of ketoconazole at 15 mg/kg/day plus terbinafine at 100 mg/kg/day, again, 100% survival was obtained 35 days postinoculation and no significant statistical difference could be detected between this group and that treated with the high dosage of ketoconazole (30 mg/kg/day) ($P > 0.35$). The mortality curve for the group receiving this combination was significantly different from that for mice that received each drug alone ($P < 0.03$ when compared with the group receiving 15 mg of ketoconazole per kg/day and $P \leq 0.05$ when compared with animals receiving 100 mg of terbinafine per kg/day). It must be noted that the surviving mice in both of these treatment groups (ketoconazole at 30 mg/kg/day or the combination of ketoconazole at 15 mg/kg/day plus terbinafine at 100 mg/kg/day) were not free of the infection at the end of the observation period. Figure 1B shows that although the levels of parasitemia in these animals were much lower those in the animals in the three other experimental groups, there were significant numbers of circulating parasites at the end of the observation period and the numbers of parasites were stable or increasing. This was consistent with a decrease in the proportion of surviving animals (50%) 10 weeks after inoculation; however, this proportion of surviving animals was the same with surviving animals treated with a "protective" ketoconazole concentration or the combination (data not shown). Parasitological cure of surviving mice after this period, as assessed by subinoculation of organs in naive animals, was attained in only 25% of the mice treated with ketoconazole at 30 mg/kg/day, but all surviving animals treated with the drug combination were classified as cured by using this criterion in this particular experiment. In two other experiments, parasitological cure rates were 25 to 50% for animals that received ketoconazole alone at the high doses and 50 to 75% for those that received the combination.

Because the protection provided by the 7-day treatment was never complete, we extended the treatment period to 14 days. The results of a representative experiment are shown in Fig. 2; a second, independent experiment gave essentially the same results as those shown in Fig. 2. Animals treated with ketoconazole at 30 mg/kg/day or the combination of ketoconazole at 15 mg/kg/day with terbinafine at 100 mg/kg/day had 100% survival after 10 weeks, while all controls were dead by 30 days postinoculation (Fig. 2A); the mortality curves for the groups that received these treatments were compared with those for control animals, and highly significant differences were obtained ($P < 0.005$ in both cases), while no significant differences among the treated groups were obtained ($P > 0.2$). On the other hand, the mortality curve for animals treated with terbinafine alone at 100 mg/kg/day was indistinguishable from that for controls, while two-thirds of the animals that received ketoconazole alone at 15 mg/kg/day survived after the same time interval; in this case, the mortality curve was statistically well differentiated ($P < 0.01$) from that of untreated controls but was not significantly different from that for the groups that

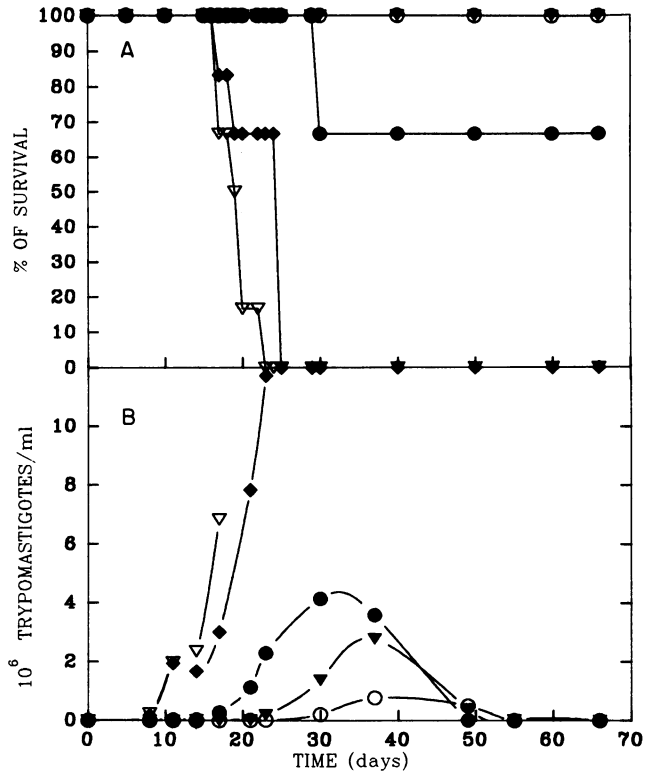

FIG. 2. Effects of ketoconazole and the combination of ketoconazole with terbinafine on the survival of (A) or level of parasitemia in (B) mice infected with *T. cruzi* and treated for 14 days. Mice were inoculated intraperitoneally at day 0 with 10^5 blood trypomastigotes of the Y strain of *T. cruzi*; 24 h later oral treatment was initiated with the following drug regimens: controls (untreated) (◆), ketoconazole at 30 mg/kg/day (○), ketoconazole at 15 mg/kg/day (●), terbinafine at 100 mg/kg/day (▽), ketoconazole at 15 mg/kg/day plus terbinafine at 100 mg/kg/day (▼). Other experimental details are described in Materials and Methods.

received high ketoconazole doses (30 mg/kg/day) or the combination. The level of parasitemia in the experimental groups treated with ketoconazole at 30 mg/kg/day or the combination of drugs rose slowly after a prolonged delay but was controlled in these animals and no circulating parasites were found 55 days postinoculation (Fig. 2B); the group that received 15 mg of ketoconazole per kg/day had a much shorter prepatent period and developed significantly higher levels of parasitemia than the first two groups. Surviving animals began to die at 80 days postinoculation for causes unrelated to *T. cruzi* infection; at day 87, subinoculation of the organs in naive mice and hemocultures were carried out for all three experimental groups. Although hemocultures were consistently negative for all groups, subinoculation of the organs indicated low levels of parasitological cure for both groups treated with ketoconazole alone (for mice treated with 15 or 30 mg of ketoconazole per kg/day, 33 and 25% of surviving animals, respectively, were cured), but all animals treated with the combination were found to be free of the infection. In a separate experiment, complete protection against death was again observed with both ketoconazole at 30 mg/kg/day or the combination of ketoconazole at 15 mg/kg/day plus terbinafine at 100 mg/kg/day, but the lack of parasitological cure was also found in both groups, albeit at a lower frequency in animals treated with the combination

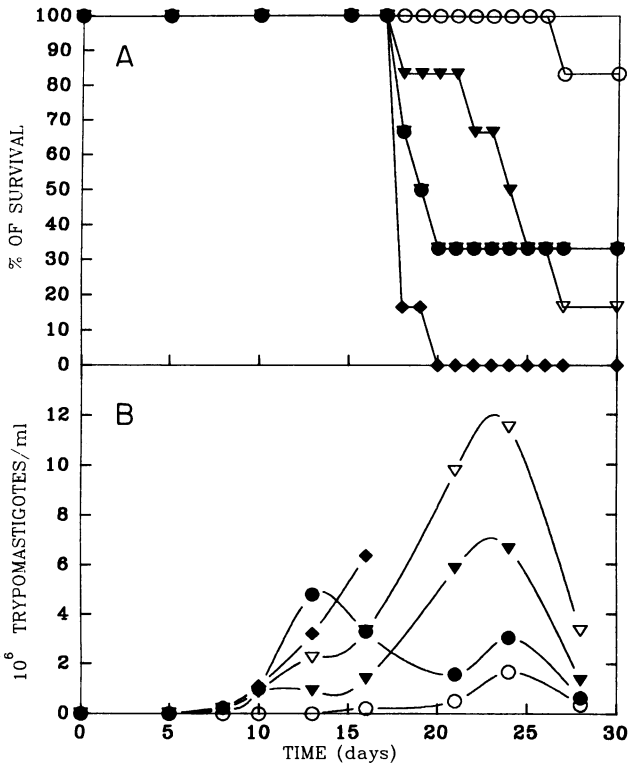

FIG. 3. Effects of ICI 195,739 and the combination of ICI 195,739 with terbinafine on the survival of (A) or level of parasitemia in (B) mice infected with *T. cruzi* and treated for 5 days. Mice were inoculated intraperitoneally at day 0 with 10^5 blood trypomastigotes of the Y strain of *T. cruzi*; 24 h later oral treatment was initiated with the following drug regimens: controls (untreated) (◆), ICI 195,739 at 1 mg/kg/day (○), ICI 195,739 at 0.5 mg/kg/day (●), terbinafine at 100 mg/kg/day (▽), ICI 195,739 at 0.5 mg/kg/day plus terbinafine at 100 mg/kg/day (▼). Other experimental details are described in Materials and Methods.

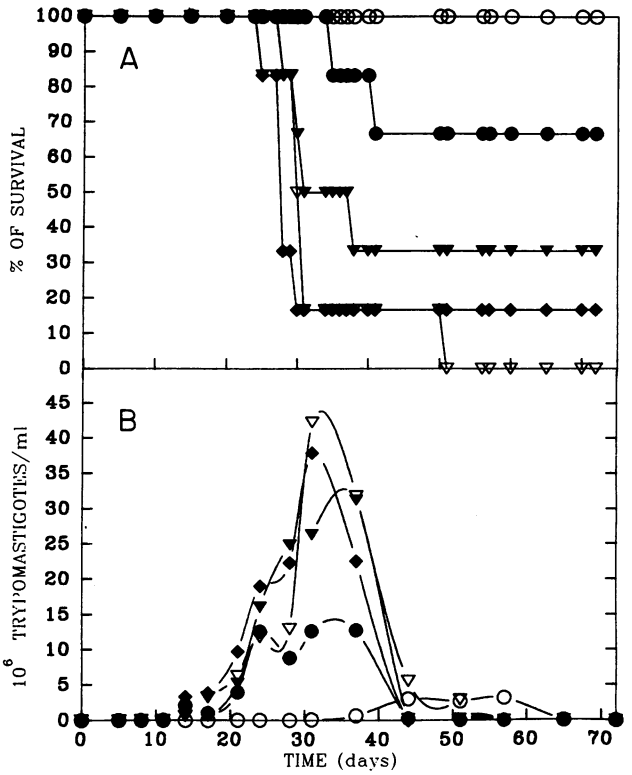

FIG. 4. Effects of ICI 195,739 and the combination of ICI 195,739 with terbinafine on the survival of (A) or level of parasitemia in (B) mice infected with *T. cruzi* and treated for 15 days. Mice were inoculated intraperitoneally at day 0 with 10^5 blood trypomastigotes of the Y strain of *T. cruzi*; 24 h later oral treatment was initiated with the following drug regimens: controls (untreated) (◆), ICI 195,739 at 1 mg/kg/day (○), ICI 195,739 at 0.5 mg/kg/day (●), terbinafine at 100 mg/kg/day (▽), ICI 195,739 at 0.5 mg/kg/day plus terbinafine at 100 mg/kg/day (▼). Other experimental details are described in Materials and Methods.

(50% for animals treated with ketoconazole alone at 30 mg/kg/day and 66% for animals treated with the combination of ketoconazole at 15 mg/kg/day plus terbinafine at 100 mg/kg/day).

We next investigated the triazole derivative ICI 195,739 and found, as reported previously by Ryley et al. (39), that with just 1 mg of the compound per kg/day given orally for 5 days, most (five of six) mice were protected from death at 30 days postinoculation, while no untreated controls survived after that period of time (Fig. 3A); the mortality curves for these two groups were statistically very different ($P < 0.01$). For animals treated with 100 mg of terbinafine per kg/day or 0.5 mg of ICI 195,739 per kg/day, mortality curves were not significantly different from those for untreated controls ($P > 0.1$). For the group treated with the combination of 100 mg of terbinafine per kg/day plus 0.5 mg of ICI 195,739 per kg/day, survival was significantly improved when compared with those of controls ($P < 0.01$) or the groups treated with the drugs alone ($P < 0.05$), but it was also significantly inferior to that of the group that received ICI 195,739 alone at 1 mg/kg/day ($P < 0.05$). Parasitemia was quite effectively controlled in the group that received the triazole at 1 mg/kg/day (Fig. 3B) and was essentially suppressed after 30 days, while the groups that received 0.5 mg of the same drug per kg/day or 100 mg of terbinafine per kg/day developed parasitemia at levels comparable to those in controls in the first 2 weeks. The group that received the combination of ICI 195,739 at 0.5 mg/kg/day plus terbinafine at 100 mg/kg/day developed a much milder parasitemia than the groups that received the drugs alone in the first 2 weeks of the infection, accounting for the increased survival; at later times, a second wave of parasitemia developed in these animals, but it was controlled by the surviving animals at the end of the observation period. A second, independent experiment produced the same results. Extending the treatment period to 15 days led to the complete protection from death for those animals that received 1 mg of the triazole per kg/day for up to 10 weeks postinoculation (Fig. 4A), when five of six untreated animals and all of those receiving terbinafine at 100 mg/kg/day were dead; two of three of those animals that received 0.5 mg/kg/day survived after the same period. The mortality curves for the animals that received the triazole treatments were found to have very significant statistical differences compared with those for untreated animals or those that received the allylamine alone ($P < 0.003$ for 1 mg/kg/day and $P \leq 0.005$ for 0.5 mg/kg/day). On the other hand, survival of the group that received the combination of 0.5 mg of ICI 195,739 per kg/day plus terbinafine at 100 mg/kg/day was statistically inferior to that of the group that received the triazole alone ($P < 0.01$), indicating that the

dominant factor providing protection over this extended observation period was the triazole. As before, the mean levels of parasitemias (Fig. 4B) inversely correlated with survival frequencies in all cases. Parasitological cure in the surviving animals was found in 50% of the cases in all groups by subinoculation of organs into naive animals; hemocultures gave consistently lower scores for animals with active infections.

DISCUSSION

The results obtained in the present study with ketoconazole or terbinafine given alone are in good agreement with those reported previously by McCabe et al. (30) and Ryley et al. (39) in similar murine models of Chagas' disease. The fact that two drug dosages which afford no protection when used alone (ketoconazole at 15 mg/kg/day or terbinafine at 100 mg/kg/day) can give complete protection against death up to 35 days postinoculation if given in combination (Fig. 1A) indicates that they have a synergistic action in vivo. In the present study we used the accepted definition of synergism (40, 43, 44), that is, an effect produced by a combination of components which is greater than the sum of the effects produced by each of the components alone. Although parasitological cure was not achieved in all surviving animals in the present study, even by using the drug combination for 14 days, the fact that parasite eradication was consistently observed in at least 50% of the animals subjected to treatment with the drug combination is a significant improvement over the results reported by McCabe et al. (32), who found that mice treated with up to 60 mg of ketoconazole per kg/day for 9 weeks did not attain parasitological cure, and only some of those treated with 120 mg/kg/day had an indication of parasitological cure. Because terbinafine alone was not able to give any significant protection even in the 14-day treatment (Fig. 2), our results obtained with the extended treatments again indicate that the two drugs have synergistic activities, which could reduce the dosage level of ketoconazole and the length of the ketoconazole treatment required for both protection from death and parasitological cure. The lack of activity of orally administered terbinafine against *T. cruzi* infections in mice has also been found in other murine models of human infections involving organisms susceptible to the drug in vitro (16, 25, 33) and may reflect the rapid metabolism of this drug by rodents (24); in other vertebrates with less active metabolisms, this situation could be improved, as indicated by the much better systemic antifungal activity of the drug in the guinea pig (33). The fact remains, however, that the residual activity of the drug in mice is enough to potentiate the effects of ketoconazole.

A comparison of the results of studies on the therapeutic actions of azoles and allylamines in experimental systemic mycoses and in human patients shows that effective doses in murine models are at least one order of magnitude greater (on a weight basis) than those required in humans, probably because of the more active drug catabolism in the former (3, 24). Thus, the fact that the results presented here indicate that a dosage of ketoconazole of ≤30 mg/kg/day, when combined with terbinafine, can provide complete protection against death and parasitological cure in a significant number of mice could suggest that well-tolerated doses of the azole in humans (200 to 400 mg/day, corresponding to 3 to 6 mg/kg of body weight per day) in combination with the allyalmine could afford a similar level of protection. In any case, as indicated in the Introduction, the serum and plasma drug levels attained in both volunteers and patients after administration of safe doses of these drugs (3, 12, 24, 35, 51) are four orders of magnitude greater than those previously found by us to be sufficient to eradicate amastigote infections in vitro (27, 43), indicating that there is a strong possibility that adequate dosing in vivo could lead to appropriate levels of both drugs in infected tissues.

The high level of in vivo activity of the bistriazole ICI 195,739, which confers to infected mice almost complete protection against death when it is used at a dosage of just 1 mg/kg/day in short-term (5- to 15-day) treatments (Fig. 3 and 4), agrees with the activity originally reported by Ryley et al. (39) and in our own work in vitro with this compound (28, 44), which indicated that ICI 195,739 is significantly more active than ketoconazole against *T. cruzi*. However, in our in vitro studies, we found no mutual potentiation of ICI 195,739 and terbinafine when the two drugs were used together against the epimastigotes or the clinically relevant amastigote form (28, 44); this fact was explained by a dual mechanism of action of this drug, which acts not only at the level of ergosterol biosynthesis but also on cell division. Although the in vivo results of the short-term (5 days) treatments consistently indicated potentiation of the triazole's action by the allylamine, particularly at the level of the circulating parasites (Fig. 3B), the long-term outcomes resulting from prolonged treatments (Fig. 4) indicated that the dominant factor on survival and parasitological cure was the activity of the triazole, and the activities of the combinations of the triazole with terbinafine were simply additive or slightly antagonistic. These results agree with the in vitro results mentioned above and with the proposition that the extraordinary potency of ICI 195,739 against *T. cruzi* is the result of a dual mechanism of action.

In conclusion, the results of the present study support the notion that combinations of azoles with other ergosterol biosynthesis inhibitors acting at different points of the biosynthetic pathway could be useful in the treatment of human Chagas' disease because they could allow the use of lower levels of those compounds, and particularly lower levels of ketoconazole, which has well-known toxicity problems when used at dosages of greater than 400 mg/day (2, 41). This proposition is further supported by the results of our recent studies on the antiproliferative effects of combinations of ketoconazole with lovastatin, a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (1), against *T. cruzi* both in vitro and in vivo (45). Finally, the in vivo results obtained with ICI 195,739 in the murine model described here are consistent with our previous interpretation that the special activity of this compound against *T. cruzi* is due to a dual mechanism of action, which is not restricted to sterol biosynthesis inhibition; in such a case, potentiation by other sterol biosynthesis inhibitors that act at different points of the pathway is not necessarily expected.

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