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Antiproliferative Effects and Mechanism of Action of ICI 195,739, a Novel Bis-Triazole Derivative, on Epimastigotes and Amastigotes of *Trypanosoma (Schizotrypanum) cruzi*

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The in vitro antiproliferative effects of ICI 195,739, a recently developed bis-triazole derivative (T. Boyle, D. J. Gilman, M. B. Gravestock, and J. M. Wardleworth, Ann. N.Y. Acad. Sci. 544:86–100, 1988; J. F. Ryley, S. McGregor, and R. G. Wilson, Ann. N.Y. Acad. Sci. 544:310–328, 1988), on epimastigotes and amastigotes of *Trypanosoma (Schizotrypanum) cruzi* and some aspects of its mechanism of action are described. Despite previous claims that triazole compounds act on susceptible organisms by essentially the same mechanism demonstrated for the imidazole compounds, i.e., by interfering with the synthesis of ergosterol at the level of the cytochrome P-450-dependent C-14 demethylation of lanosterol, our results indicate that ICI 195,739 acts on *T. cruzi* epimastigotes by a dual mechanism which involves blockade of ergosterol byosynthesis and a second, still-unidentified target whose alteration leads to immediate growth arrest. Although ICI 195,739 blocks ergosterol biosynthesis at the level of C-14 lanosterol demethylation, as shown by gas-liquid and thin-layer chromatography, growth arrest in ICI 195,739-treated cells is not related to the depletion of the endogenous ergosterol pool, contrary to what was previously found for ketoconazole, the reference compound among antifungal and antiprotozoal azole derivatives. Consistent with this observation is the fact that the concentration of ICI 195,739 required to inhibit de novo synthesis of ergosterol in epimastigotes by 50% is 60 nM, which is essentially identical to that previously found for ketoconazole under identical conditions, while the minimum concentration required to produce complete growth inhibition is 0.1 μM, which is 300 times lower than that of ketoconazole. With respect to the intracellular amastigote form proliferating inside vertebrate (Vero) cells, 10 nM is sufficient to eradicate the parasite completely in 96 h, with no effects on the host cells; this concentration is identical to that previously found for ketoconazole. Growth inhibition and morphological alterations induced by ketoconazole can be reversed by exogenously added ergosterol but not by cholesterol; for ICI 195,739, neither sterol is capable of reversing the drug effects. Contrary to what was observed for ketoconazole, the in vitro antiproliferative effects of ICI 195,739 on both forms of the parasite are not potentiated by the simultaneous presence of terbinafine, an allylamine which blocks ergosterol production by the parasite at a different level of the sterol biosynthetic pathway. These results, together with those of an accompanying study of the ultrastructural alterations induced by the drug, strongly support the notion that ICI 195,739 acts on *T. cruzi* by a novel combination of biochemical and cellular effects, which could explain its extraordinary potency in vivo against the parasite.

The azole derivatives have proven to be potent antifungal and antiprotozoal compounds which act via specific inhibition of the cytochrome P-450-dependent demethylation of lanosterol, a crucial step in the de novo biosynthesis of ergosterol and related C-24 methylated sterols, which have essential functions in these organisms and cannot be replaced by cholesterol (2, 4–7, 11, 13, 15, 16, 19, 21, 23, 25, 31, 33, 35, 40, 41). After the initial success of the imidazole derivatives, triazole compounds which display both increased activity and metabolic stability were introduced (26, 32, 36–39). In particular, fluconazole and itraconazole have been shown to be potent antiproliferative agents against *Leishmania* spp. (3, 8, 14) and *Trypanosoma (Schizotrypanum) cruzi*, the causative agent of Chagas' disease, both in vivo and in vitro (12, 20).

More recently, ICI 195,739, a novel 3′-styryl-substituted bistriazolyl tertiary alcohol, was developed (1, 9) and shown to have broad antifungal action and particularly high activity against *T. cruzi* when given orally in a murine model system (9, 30). Initial biochemical studies with fungi (1) have shown that this compound blocks ergosterol biosynthesis at the level of lanosterol demethylation, according to the accepted mechanism of other azole derivatives. However, the extraordinary potency and specificity of this compound against *T. cruzi* in vivo (30) could indicate particular biochemical targets for the drug or an increased sensitivity of the lanosterol demethylase to the triazole in this organism.

In this report we present the results of a study of the in vivo biological sensitivity of both proliferative stages of the parasite: epimastigotes, which proliferate in the digestive tract of the Reduviid vectors, and amastigotes, which thrive inside vertebrate cells. A biochemical study of the sensitivity of ergosterol biosynthesis of the epimastigote to this drug is also presented. The results, together with those of an accompanying ultrastructural study (18), suggest that in *T. cruzi* ICI 195,739 acts via a dual mechanism which involves blockade of ergosterol biosynthesis and a second, as yet unidentified target, whose alteration immediately blocks cell proliferation.

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MATERIALS AND METHODS

Both the EP and Y stocks of *T. cruzi* were used throughout this study. It was found that the susceptibilities of the two stocks to all ergosterol biosynthesis inhibitors tested were indistinguishable in the epimastigote and amastigote forms of the parasite (33); therefore, only the results with the EP stock are presented. The epimastigote form was cultivated in liver infusion-tryptose supplemented with 10% calf serum (10) at 28°C with strong agitation (120 rpm). Cultures were initiated with 2×10^6 epimastigotes per ml, and drugs were added when the cell density reached 10^7 epimastigotes per ml. Cell densities were measured with an electronic particle counter (model ZBI; Coulter Electronics Inc., Hialeah, Fla.) and by direct counting with a hemacytometer.

For analysis of drug effects on the sterol composition of epimastigotes, total lipids were extracted from control and drug-treated cells and fractionated as described previously (16). The neutral lipid fraction was saponified in 90% methanolic sodium hydroxide (0.3 M) for 90 min under reflux; the nonsaponifiable lipids were extracted with petroleum ether (bp 40 to 60°C) and dried in solution with anhydrous sodium sulfate. This fraction was analyzed by both gas-liquid chromatography (isothermic separation at 255°C in a 10-foot glass column packed with 3% OV-1 on Chromosorb W [100–200 mesh] with nitrogen as the carrier gas [20 ml/min]) and thin-layer chromatography (Merck 5721 silica gel plates, with heptane-isopropyl ether-glacial acetic acid [60:40:4] as the solvent [41]).

For the study of de novo synthesis of lipids, 0.025 μCi of [^{14}C]acetate (New England Nuclear; 55 mCi/mmol) was added together with the drug or carrier to the cultures; these were incubated for 48 h, and then the lipids were extracted, fractionated, and analyzed as described above (16). The radioactive fractions from the thin-layer chromatography were detected by autoradiography with Kodak XRP-5 plates, scraped off, and counted by liquid scintillation spectrometry in a LKB Rack-Beta counter, working at 80% efficiency for ^{14}C. To study the effect of exogenous sterols on the antiproliferative action of the drugs, they were added as Tween 80 suspensions to the growth medium together with the drug exactly as described by Nes et al. (22).

Amastigotes were cultivated in Vero cells maintained in minimal essential medium supplemented with 2% fetal calf serum in a humidified 95% air–5% CO_2 atmosphere at 37°C, as previously described (33). The cells were infected with a 20:1 ratio of tissue culture-derived trypomastigotes to Vero cells for 2 h and then washed three times with phosphate-buffered saline to remove nonadherent parasites; fresh medium with or without the drug was added, and the cells were incubated for various periods of time. The medium was changed every 48 h. Parasite proliferation was quantified by light microscopy as described previously (33).

ICI 195,739 [(*R,S*)-2-(2,4-difluorophenyl)-1-(3-[(*Z*)-4-(2,2,3,3-tetrafluoropropoxy)styryl]-1,2,4-triazol-1-yl)-3-(1,2,4-triazol-1-yl)-propan-2-ol] (9) was kindly provided by John F. Ryley, ICI Pharmaceuticals Division, Macclesfield, England; terbinafine [(*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphthalenemethanamine] (24) was provided by A. Lindenmann and H. Stähelin, Sandoz, Ltd., Switzerland, through Luis Rodriguez, Sandoz de Venezuela, S.A. Both drugs were added to the culture medium as dimethyl sulfoxide solutions; the final dimethyl sulfoxide concentration never exceeded 1% (vol/vol), and it had no effect by itself on the proliferation of parasites or on Vero cells. Ketoconazole (*cis*-1-acetyl-4-[-4[[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine) (15) was provided by John Russe, Janssen Pharmaceutica, Caracas, Venezuela; it was added to the culture medium as aqueous solutions titrated to pH 2.4 with HCl and sterilized by filtration through membrane filters (mean pore size, 0.22 μm; Millipore Corp., Bedford, Mass.).

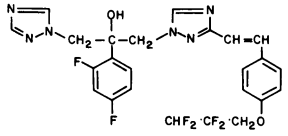

FIG. 1. Molecular structure of ICI 195,739 (9).

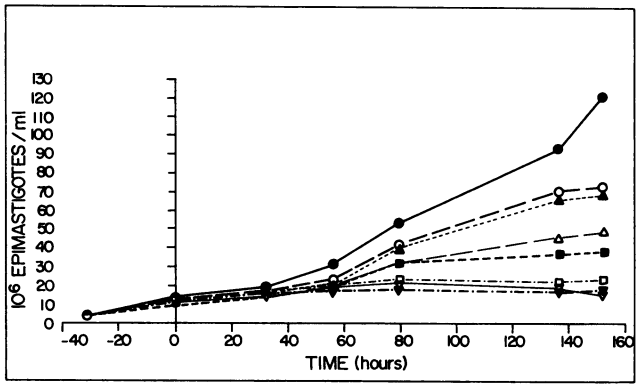

FIG. 2. Effects of ICI 195,739 on the proliferation of *T. cruzi* epimastigotes. Epimastigotes were cultivated in liver infusion-tryptose medium as described in Materials and Methods. Drug concentrations: ●, 0 M; ○, 10^{-9} M; ▲, 3×10^{-9} M; △, 10^{-8} M; ■, 3×10^{-8} M; □, 10^{-7} M; ▼, 3×10^{-7} M; ▽, 10^{-6} M. Time zero corresponds to the moment of addition of the drug.

RESULTS

The effects of the bis-triazole derivative ICI 195,739 (Fig. 1) on the in vitro proliferation of *T. cruzi* epimastigotes in liver infusion-tryptose medium are presented in Fig. 2. The drug significantly slowed the growth of the parasites at concentrations as low as 3 nM and blocked it completely at 0.1 μM, a concentration 300 times lower than those deter-

TABLE 1. Lack of synergism between ICI 195,739 and terbinafine against *T. cruzi* epimastigotes[a]

Drug(s)	Concn (M)	% Inhibition of growth[b]
ICI 195,739	10^{-9}	65
	10^{-8}	34
Terbinafine	10^{-7}	48
	10^{-6}	32
ICI 195,739 + terbinafine	$10^{-9} + 10^{-7}$	35
	$10^{-8} + 10^{-7}$	24
	$10^{-9} + 10^{-6}$	29
	$10^{-8} + 10^{-6}$	21

[a] Epimastigotes were cultivated in liver infusion-tryptose medium at 28°C as described in Materials and Methods.

[b] Growth was calculated as [(cell density 152 h after drug addition)/(cell density at time of drug addition)] − 1. Percent inhibition of growth was calculated as [(growth in drug-treated cultures)/(growth in control cultures)] × 100.

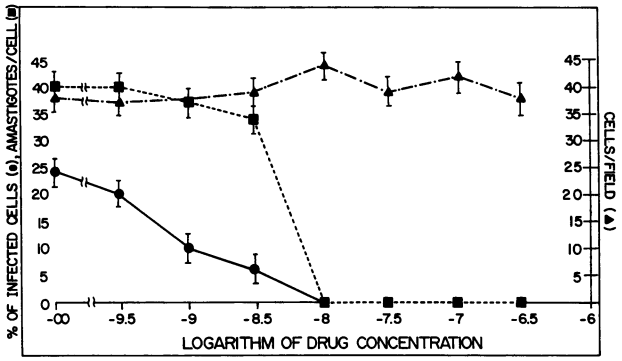

FIG. 3. Concentration dependence of the effects of ICI 195,739 on the proliferation of intracellular *T. cruzi* amastigotes and Vero cells. Symbols: ●, percent infected cells; ■, number of amastigotes per cell; ▲, number of Vero cells per field after 96 h as a function of ICI 195,739 concentration. Vero cells were infected with *T. cruzi* as described in Materials and Methods. Bars represent 1 standard deviation.

mined previously for ketoconazole and terbinafine (33). The cells treated with this concentration of ICI 195,739 remained viable for 96 h, but after this period of time morphological alterations and cell lysis began to take place. When we investigated the effects of the combined action of ICI 195,739 and terbinafine on these cells, based on the synergism observed previously between allylamine and ketoconazole under the same conditions (33), some indication of synergism was found at very low concentrations, but at higher concentrations (still below the MICs of both drugs), the effects were at most additive (Table 1).

The drug was more effective against the intracellular amastigote form of the parasite proliferating inside Vero cells than against the epimastigotes, as found with other ergosterol biosynthesis inhibitors (33). The concentration required to reduce the number of infected cells to 50% of the number in the control (untreated) cultures was 1 nM, while the parasites were totally eradicated with ICI 195,739 at 10 nM (Fig. 3). On the other hand, no effects of the bis-triazole derivative on the proliferation of Vero cells were observed up to 1 μM. Again, the combination of ICI 195,739 with terbinafine had only additive effects on the amastigotes (Table 2), in contrast to the strong synergism observed in the same system with the combination of terbinafine and ketoconazole (33).

To investigate the basis of these differences, we compared

TABLE 2. Lack of synergism between ICI 195,739 and terbinafine against *T. cruzi* amastigotes[a]

Drug(s)	Concn (M)	% of cells infected	No. of amastigotes/cell
None (control)		20.4 ± 0.8	36.3 ± 1.3
ICI 195,739	10^{-10}	17.2 ± 2.1	30.6 ± 3.4
	10^{-9}	9.1 ± 1.4	37.3 ± 9.8
Terbinafine	10^{-8}	14.6 ± 1.8	35.2 ± 8.6
	10^{-7}	8.7 ± 1.4	29.4 ± 2.0
ICI 195,739 + terbinafine	$10^{-10} + 10^{-8}$	11.0 ± 0.9	39.6 ± 9.5
	$10^{-9} + 10^{-8}$	10.5 ± 2.0	29.2 ± 2.4
	$10^{-10} + 10^{-7}$	10.9 ± 0.7	29.4 ± 1.8
	$10^{-9} + 10^{-7}$	9.5 ± 1.8	32.6 ± 2.2

[a] Amastigotes were cultivated in Vero cells at 37°C as described in Materials and Methods.

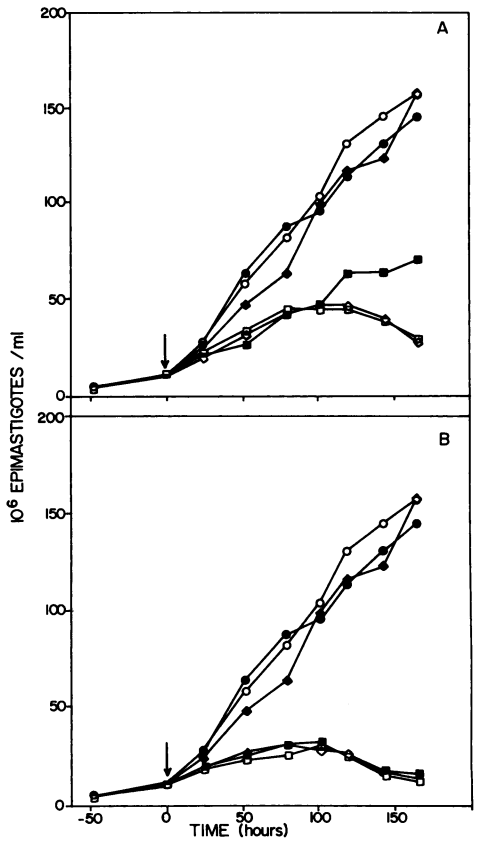

FIG. 4. Influence of exogenous sterols on the antiproliferative effects of ketoconazole (A) and ICI 195,739 (B) on *T. cruzi* epimastigotes. Epimastigotes were cultivated in liver infusion-tryptose medium as described in Materials and Methods. Symbols: ○, no additions; ◆, ergosterol (10 μg/ml); ●, cholesterol (10 μg/ml); ◇, 1 μM ketoconazole (A) or 0.1 μM ICI 195,739 (B); ■, 1 μM ketoconazole (A) or 0.1 μM ICI 195,739 (B) plus ergosterol (10 μg/ml); □, 1 μM ketoconazole (A) or 0.1 μM ICI 195,739 (B) plus cholesterol (10 μg/ml). Time zero (arrow) corresponds to the moment of addition of the drug and/or sterol.

the effects of exogenous sterols on the antiproliferative action and morphological effects induced by the two azoles on the epimastigotes. Exogenous cholesterol or ergosterol added to the growth medium as Tween 80 suspensions to give a final sterol concentration of 10 μM had no effect on the proliferation of the epimastigotes by themselves (Fig. 4A); however, ergosterol but not cholesterol partially reversed the effect of ketoconazole on the growth rate of the cells and completely abolished the ketoconazole-induced cell lysis observed after 120 h of incubation with the drug, which is caused by alterations in the permeability properties of the cell membrane (34) and massive swelling of the mitochondrion (17) associated with depletion of the preformed ergosterol pool (16). Direct microscopic observation of the cells (not shown) confirmed that only ergosterol abolished the gross morphological alterations induced by the imidazole derivative, associated with mitochondrial swelling. In contrast, neither of the two sterols had any effect on the growth inhibition and cell lysis induced by ICI 195,739 (Fig. 4B), a fact also confirmed by direct microscopic observation of the cells (not shown).

Further insight into the differences between the mecha-

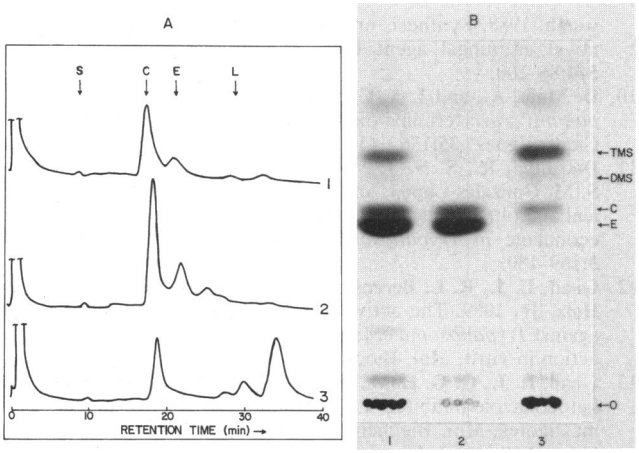

FIG. 5. Gas-liquid (A) and thin-layer (B) chromatographs of the sterol fraction of *T. cruzi* epimastigotes. Curves and lanes: 1, epimastigotes incubated in the presence of 0.1 μM ICI 195,739 for 120 h; 2, control (untreated) epimastigotes; 3, epimastigotes incubated in the presence of 1 μM ketoconazole for 120 h. (A) Arrows indicate the retention times for squalene (S), cholesterol (C), ergosterol (E), and lanosterol (L). (B) Arrows indicate origin (O), ergosterol (E), cholesterol (C), dimethyl sterols (DMS), and trimethyl sterols (TMS). Lipids were extracted, fractionated, and analyzed as described in Materials and Methods.

nisms of action of the two azoles was obtained by investigating their effects on sterol content and de novo synthesis of sterols in epimastigotes. Figure 5A shows the gas-liquid chromatograph of nonsaponifiable neutral lipids obtained from control cells (center) and from growth-arrested cells incubated for 120 h with 0.1 μM ICI 195,739 (top) or 1 μM ketoconazole (bottom). By peak integration and calibration, it was found that in the control cells the main sterols were cholesterol (46.8%; this sterol is taken up from the growth medium [see below and reference 16]) and ergosterollike 4-desmethyl sterols, previously identified as ergosterol, ergosta-5,7-dien-3β-ol, 24-ethyl-cholesta-5,7,22-trien-3β-ol, and 24-ethylcholesta-5,7-dien-3β-ol (2,12), which constituted 44.2%; and lanosterol and related trimethyl sterols (9%). In cells incubated with 1 μM ketoconazole for 120 h, growth inhibition coincided, as reported previously (16), with the almost complete depletion of the ergosterollike 4-desmethyl sterols, which now amounted to 9.6% of the total; exogenous cholesterol constituted 30.1%, while the majority of the sterols were trimethyl sterols (lanosterol and 24-methylenedihydrolanosterol [2, 12]), which added up to 60.3%. In contrast, growth-inhibited epimastigotes incubated for 120 h with 0.1 μM ICI-195,739 contained, besides cholesterol (59.6%), large amounts of ergosterollike 4-desmethyl sterols (25%) and increased amounts of trimethyl sterols (15%). These results were confirmed qualitatively by thin-layer chromatography (Fig. 5B), which separates the lipids by adsoption rather than by partition, as in gas-liquid chromatography.

When we investigated the de novo synthesis of sterols, we found that although ICI 195,739 blocked the incorporation of [^{14}C]acetate in the ergosterollike 4-desmethyl sterol fraction separated by thin-layer chromatography (see Materials and Methods) and proportionally increased the labeling of the trimethyl sterol fraction, the concentration of the drug required to inhibit incorporation of the precursor into the ergosterol fraction by 50% was 60 nM, which is essentially identical to that found previously for ketoconazole (50 nM [16]). This concentration is also very similar to that previously reported to inhibit ergosterol biosynthesis by intact *Candida albicans* cells (38 nM [1]). Under no conditions was ^{14}C labeling of the cholesterol fraction found, as reported previously (2, 16).

DISCUSSION

The accepted paradigm for the mechanism of action of antimycotic and antiprotozoal azoles, including both imidazole and triazole derivatives, is the inhibition of cytochrome P-450-dependent demethylation of lanosterol, which in turns lead to cellular alterations due to ergosterol depletion and the accumulation of methylated precursors (2, 4–7, 11, 13, 15, 16, 19, 21, 23, 25, 26, 31–38, 40, 41); this mechanism has received direct support by the correlation which has been established between the antiproliferative action of these drugs, their effect on the cell's sterol composition, and their in vitro interaction with the cyotochrome P-450 isoenzymes of susceptible organisms (39). Supporting evidence for this mechanism in the action of ICI 195,739 on fungi has also been presented previously (1). However, the results of the present study indicate that the mechanism of action of the bis-triazole derivative ICI 195,739 on the proliferative stages of *T. cruzi* involves a dual effect on the biochemical machinery of these cells, including the classical blockade of the C-14 demethylation of lanosterol, which contributes to the delayed cell lysis produced by the drug and a second, as yet unidentified, target which is involved in the immediate growth arrest induced by the drug at concentrations equal to or above 0.1 μM. Several lines of evidence support this contention.

The minimum growth-inhibitory concentration of ICI 195,739 against epimastigotes is 300 times lower than that of ketoconazole, while the concentration required to inhibit ergosterollike 4-desmethyl sterol synthesis by both drugs is the same; this demonstrates that for ICI 195,739, primary growth inhibition is not causally related to the blockade of sterol biosynthesis.

Complete growth arrest of the epimastigotes by low (≥0.1 μM) concentrations of ICI 195,739 is not correlated with the depletion of ergosterollike 4-desmethyl sterols, contrary to the growth inhibition induced by similar (<1 μM) concentrations of ketoconazole which only takes place after complete depletion of the preformed pool of this compound (16) (Fig. 5).

Growth inhibition and eventual cell lysis induced in the epimastigotes by ketoconazole can be reversed by the addition of exogenous ergosterol but not cholesterol, demonstrating that the loss of cell viability produced by this drug is due to an essential and specific requirement for ergosterol or ergosterollike sterols by these cells. Conversely, the growth inhibition produced by ICI 195,739 cannot be reversed by cholesterol or ergosterol, demonstrating that in this case growth blockade is not related to ergosterol depletion.

The antiproliferative action of ICI 195,739 on both epimastigotes and amastigotes of *T. cruzi* is not potentiated by the allylamine terbinafine, a compound with strong antimycotic and antiprotozoal activity which acts on a different point of the ergosterol biosynthesis pathway (24, 27–29, 33) and was previously shown to potentiate extraordinarily the in vitro effects of ketoconazole on *T. cruzi* (33). As synergism is usually found when drugs which potentiate each other's effects act on different points of the same biosynthetic pathway or on different biosynthetic pathways which

contribute to the same final end product (see reference 33 and references therein), the lack of synergism of ICI 195,739 and terbinafine is a further indication that the primary effect of the triazole leading to growth arrest is not related to the ergosterol biosynthesis pathway and is most probably related to the accumulation of a toxic compound.

In support of this hypothesis, an accompanying ultrastructural study (18) revealed that ICI 195,739 at its MIC (0.1 μM) induces, immediately after contact with the parasite, the appearance of large electron-dense bodies and many binucleated cells, indicating arrest at cytokinesis. The electron-dense bodies could contain the putative toxic compound responsible for the blockade of cytokinesis, but the chemical nature of this compound is still unknown. The same study showed that after 120 h, when the ergosterol content of these cells was partially depleted (Fig. 5), a large swelling of the mitochondrion was observed, a characteristic effect found in epimastigotes and amastigotes depleted of ergosterol by incubation with ketoconazole (17), which can be reversed by exogenous addition of the sterol. This shows the second effect of ICI 195,739 on *T. cruzi*, clearly demonstrating its interference with the ergosterol biosynthesis pathway, which nevertheless is not responsible for primary growth arrest.

In summary, the results of the present in vitro study indicate that the bis-triazole derivative ICI 195,739 acts on *T. cruzi* by a dual mechanism, which could explain its extraordinary potency in vivo (30). Further studies on the specific target responsible for the initial growth arrest, as it seems to be specific to the parasite and essential for its proliferation, are in progress.

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