Ultrastructural Alterations Induced by ICI 195,739, a Bis-Triazole Derivative with Strong Antiproliferative Action against Trypanosoma (Schizotrypanum) cruzi

KEYLA LAZARDI,1 JULIO A. URBINA,1,* AND WANDERLEY DE SOUZA2

Centro de Biologia Celular, Escuela de Biologia, Facultad de Ciencias, Universidad Central de Venezuela, Apartado 47860, Los Chaguaramos, Caracas 1041, Venezuela,1 and Instituto de Biofisica “Carlos Chagas Filho”, Centro de Ciencias da Saude, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21949, Brazil2

Received 21 September 1990/Accepted 11 January 1991

The ultrastructural alterations induced in vitro by ICI 195,739, a recently developed bis-triazole derivative with potent antiproliferative effects on Trypanosoma (Schizotrypanum) cruzi, are reported. On epimastigotes, the triazole at its minimum growth-inhibitory concentration (0.1 μM) produced immediately (within 24 h) gross alterations in the organization of the cytoplasm and the appearance of large electron-dense granules; at this time, many cells were binucleated, indicating a blockade in cytokinesis. At later times (120 h), mitochondrial swelling, a characteristic effect reported previously for the dioxolane-imidazole ketoconazole when the preformed ergosterol pool is depleted, was the predominant effect and led to cell lysis. In amastigotes proliferating in Vero cells, the drug at 10 nM produced mitochondrial swelling, autophagic vacuoles, and massive alterations of the plasma membrane, leading to complete parasite destruction after 96 h of incubation of the infected monolayers with the drug. The results support previous conclusions that ICI 195,739 has a dual mechanism of action against T. cruzi, involving blockade of ergosterol biosynthesis and a direct effect on cell division which cannot be reversed by addition of exogenous ergosterol.

We have recently been involved in the study of the biochemical, biophysical, and ultrastructural basis of the antiproliferative effects of ergosterol biosynthesis inhibitors on Trypanosoma (Schizotrypanum) cruzi, the causative agent of Chagas’ disease (18, 31, 33). We have shown that ketoconazole, a dioxolane-imidazole derivative with potent antifungal and antiprotozoal activity which acts by inhibiting ergosterol biosynthesis at the level of the cytochrome P-450-dependent demethylation of lanosterol (2, 4-7, 14, 16, 18, 20, 22, 23, 25, 30-36), induces cell lysis in T. cruzi not as a consequence of accumulation of the di- and trimethylated precursors of the sterol but as a result of depletion of the preformed ergosterol pool, which leads to irreversible alteration of the plasma membrane and the kinetoplast-mitochondrion complex (19, 31, 33). Parallel studies with terbinafine, an allylamine derivative (24, 26-28), indicated that this drug affects the growth of T. cruzi, probably via the accumulation of a toxic metabolite, and potentiates the action of ketoconazole (19, 31).

Compared with the imidazole compounds, triazole derivatives such as fluconazole and itraconazole have shown superior activity against T. cruzi (13, 21) and the related protozoan parasites of the genus Leishmania (3, 8, 15). Of particular interest is the recent report of a novel bis-triazole derivative, ICI 195,739, with potent antifungal and antiprotozoal effects, particularly against T. cruzi (1, 9, 29). In a parallel study (32), we have shown that ICI 195,739 is 300 times more potent than ketoconazole against the epimastigote (culture) form but has comparable activity against the (intracellular) amastigote stage. We also found that the drug has a dual mechanism of action, involving blockade of ergosterol biosynthesis at the level of lanosterol demethylation, a mechanism shared with ketoconazole, and a direct effect on cell division, probably related to the accumulation of a toxic metabolite. In this report we present the results of a detailed study of the ultrastructural alterations induced by ICI 195,739 on both proliferative stages of T. cruzi. The results support the proposition of a dual mechanism of action for this drug.

MATERIALS AND METHODS

The Y stock of T. cruzi was used throughout this study. This stock displays the same susceptibility to the growth-inhibitory effects of ketoconazole and terbinafine as the EP stock studied previously (31, 32). The epimastigotes (equivalent to the form that develops in the Reduvid vector) were cultivated in liver infusion-trypomastigotes supplemented with 10% calf serum (10) at 28°C with strong agitation (120 rpm); the 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 determined by direct counting with a hemacytometer.

Amastigotes were cultivated in Vero cells maintained in minimal essential medium supplemented with 2% fetal calf serum in a humidified 95% air–5% CO2 atmosphere at 37°C, as described previously (31). The cells were infected with a 10:1 ratio of tissue culture-derived trypomastigotes to Vero cells for 24 h; the medium was then changed, and incubation was continued for 96 h, with medium changes every 48 h. At this point, 80% of the cells were infected and the number of amastigotes per cell varied between 60 and 100. The drugs were added to the medium, and the cells were further incubated for various periods, with medium changes every 48 h.

For electron microscopy studies, the epimastigotes were centrifuged at 1,000 × g for 10 min, collected, and fixed in 0.1 M sodium phosphate buffer–3.5% saccharose–2.5% glutaraldehyde–4% paraformaldehyde, pH 7.3, for 2 h. Amas-
FIG. 1. (A) General view of an untreated epimastigote form of *T. cruzi*, showing the kinetoplast (k), extension of the mitochondrion (arrow), and the nucleus (n). Magnification, ×22,000. (B to D) Epimastigotes treated with 0.1 μM ICI 195,739 for 48 h. Note condensation of chromatin (B), appearance of electron-dense structures (asterisk in panel C), and binucleated cell (D). Magnification: (B and C) ×14,500; (D) ×11,000. (E and F) Epimastigotes incubated in the presence of 0.1 μM ICI 195,739 for 120 h. Note intense swelling of the mitochondrion (asterisks). Magnification: (E) ×14,000; (F) ×10,000.
FIG. 2. Effect of ICI 195,739 (10 mM) on amastigotes of T. cruzi proliferating inside Vero cells in vitro. Various levels of cytoplasmatic vacuolization (v), appearance of autophagic vacuoles (asterisk in panel B), and membrane lesions (arrows) are evident. The cells were incubated for 48 h (A and C), 72 h (B), or 96 h (D) with the drug. Magnification: (A to D) ×11,500.

tigote-infected monolayers were fixed under the same conditions and then scraped off with a rubber spatula. Fixed cells were washed with 0.1 M sodium phosphate buffer–3.5% saccharose, pH 7.3, and postfixed in 1% OsO4 containing 1 mM CaCl2 and 0.8% potassium ferricyanide for 2 h in the dark. Dehydration was carried out in ethanol; samples were embedded in Epon, and ultrathin sections were obtained with an LKB Ultratome III (LKB Instruments Co., Rockville, Md.). The ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 100CX electron microscope.

ICI 195,739 \((R,S)-2-(2,4\text{-difluorophenyl})-1-(3-[(Z)-4-(2,2,3,3\text{-tetrafluoropropoxy})styryl]-1,2,4\text{-triazol-1-yl}-3-(1,2,4\text{-triazol-1-yl})\text{-propan-2-ol}\) (9) was kindly provided by John F.
Ryley, ICI Pharmaceuticals Division, Macclesfield, England. The drug was added 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 Vero cells.

RESULTS AND DISCUSSION

Figure 1A shows the characteristic ultrastructural features of normal epimastigote stages of T. cruzi, which can be readily cultivated axenically in vitro and are equivalent to those found in the intestinal tract of the Reduviid vectors of the parasite: elongated shape supported by an array of subpellicular microtubules, anterior flagellum, flagellar pocket, and a single giant mitochondrion, which branches through the cell and contains a large condensation of mitochondrial DNA, called the kinetoplast (11). Epimastigotes treated with the minimum growth-inhibitory concentration of ICI 195,739 (0.1 μM) displayed almost immediate ultrastructural alterations consisting of abnormal condensation of chromatin (Fig. 1B), the appearance of very large electron-dense deposits in the cytoplasm (Fig. 1C), and many binucleated cells, indicating arrest at cytokinesis (Fig. 1D). However, after 120 h of incubation with the drug, a further ultrastructural alteration developed: a large swelling of the mitochondrion, with loss of the inner membrane and the matrix’s electron density, which eventually led to the cell’s lysis (Fig. 1E and F). This last effect is identical to that observed previously by us in epimastigotes incubated with low (1 μM) concentrations of ketoconazole after the ergosterol pool was depleted (18, 19, 31). This confirms that in T. cruzi, azole-induced ergosterol depletion leads to an irreversible alteration of mitochondrial structure and function as well as alterations of the plasma membrane (33) which underlie the loss of cell viability.

However, the mechanisms of growth arrest of the two drugs at low concentrations (0.1 to 1 μM) are clearly different. Whereas with ketoconazole at this concentration growth stops only after complete depletion of the ergosterol pool and most probably as a consequence of the mitochondrial and plasma membrane alterations (secondary trypanocidal effect [18, 19, 31, 33]), with ICI 135,739 at the same concentrations growth stops almost immediately, long before ergosterol pool depletion and the onset of mitochondrial alterations (primary trypanocidal effect [32]). To produce a primary trypanocidal effect with ketoconazole a concentration of 30 μM has to be used, i.e., 300 times greater than the concentration of ICI 195,739 (18, 31). This suggests that the chromatin alterations and the accumulation of large amounts of electron-dense material induced by the triazole but not by ketoconazole are involved in the arrest in cytokinesis of the large majority of the treated cells. This result supports the proposition advanced by us that the triazole could have a second biochemical target in these cells, apart from its demonstrated effect on ergosterol biosynthesis at the level of C-14 demethylation of lanosterol (1, 32). Although this second target has not yet been identified, support for the hypothesis of a dual mechanism of action comes from our studies on the biochemical effects of ICI 195,739 on T. cruzi epimastigotes (32).

Finally, the ultrastructural alterations observed in epimastigotes treated with ICI 195,739 bear no resemblance to those reported for two other imidazole derivatives, miconazole and econazole, which caused the disappearance of nuclear chromatin, vacuolization, and decrease in electron density of the cytoplasm (12).

Concerning the effects on amastigotes proliferating inside Vero cells, Fig. 2 shows typical lesions observed in cells with 10 nM ICI 195,739, which include typical mitochondrial alterations, intense vacuolization, autophagic vacuoles, and massive alterations of the plasma membrane, leading to a complete breakdown of the parasite’s cells. The infection was eradicated, as determined by both light and electron microscopy, after 96 h of incubation with the drug, starting with 80% infected cells and 60 to 100 amastigotes per cell. These alterations resembled those observed with ketoconazole in the same system; however, the effects were more drastic and appeared earlier than those observed with the imidazole (19). This correlates well with the increased potency of the triazole in vivo (29). On the other hand, the effects of the triazole on T. cruzi amastigotes differ from those reported for ketoconazole on Leishmania tropica amastigotes proliferating inside human macrophages (17), which include increased cytoplasmic density and uneven distribution of cytoplasmic ribosomes, although autophagic vacuoles are also found.

In conclusion, the ultrastructural effects produced by ICI 195,739 on T. cruzi epimastigotes support the notion that the drug has a dual mechanism of action: one affecting nuclear structure and cytokinesis, which produces immediate growth arrest, and another, probably mediated by the azole-induced ergosterol depletion, which produces massive alterations of the mitochondrion and plasma membrane and produces cell lysis. Only this last effect is shared with ketoconazole. In amastigotes, the effects of the drug are similar to but more rapid than those observed with ketoconazole and seem to be mediated by a general breakdown of the membranous subcellular structures.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Marlene Cazaza and Sebastião Cruz.

This work was supported by a Scientific Exchange Grant by the Consejo de Desarrollo Científico y Humanístico, Universidad Central de Venezuela (to J.U.), and from Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Conselho de Pesquisa da Universidade Federal do Rio de Janeiro (to W.S.).

REFERENCES


