Amiodarone Has Intrinsic Anti-*Trypanosoma cruzi* Activity and Acts Synergistically with Posaconazole†

Gustavo Benaim,[⊥,◇] John M. Sanders,[‖] Yael Garcia-Marchán,[◇] Claudia Colina,[‡] Renee Lira,[§] Aura R. Caldera,[§] Gilberto Payares,[◇] Cristina Sanoja,[◇] Juan Miguel Burgos,[#] Annette Leon-Rossell,[▽] Juan Luis Concepcion,[⊗] Alejandro G. Schijman,[#] Mariano Levin,[#,□] Eric Oldfield,[‖,▽] and Julio A. Urbina[§,*]

Laboratorio Química Biológica and Laboratorio de Permeabilidad Iónica, Instituto Venezolano de Investigaciones Científicas, Apartado 21927, Caracas 1020A, Venezuela, Instituto de Estudios Avanzados, Caracas, Venezuela, Instituto de Biologia Experimental, Universidad Central de Venezuela, Caracas, Venezuela, Lab.Biología Molecular de la Enfermedad de Chagas, INGEBI-CONICET, Buenos Aires, Argentina, Institut Cochin, Department Maladies Infectieuses, INSERM U567, Paris F-75014, France, Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, Department of Biophysics, University of Illinois at Urbana-Champaign, 607 South Mathews Avenue, Urbana, Illinois 61801, and Departamento de Biología, Universidad de Los Andes, Mérida, Venezuela

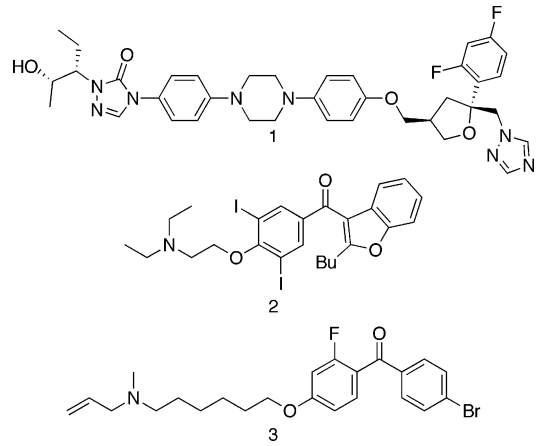
Received July 20, 2005

There is no effective treatment for the prevalent chronic form of Chagas' disease in Latin America. Its causative agent, the protozoan parasite *Trypanosoma cruzi*, has an essential requirement for ergosterol, and ergosterol biosynthesis inhibitors, such as the antifungal drug posaconazole, have potent trypanocidal activity. The antiarrhythmic compound amiodarone, frequently prescribed for the symptomatic treatment of Chagas' disease patients, has also recently been shown to have antifungal activity. We now show here for the first time that amiodarone has direct activity against *T. cruzi*, both in vitro and in vivo, and that it acts synergistically with posaconazole. We found that amiodarone, in addition to disrupting the parasites' Ca^{2+} homeostasis, also blocks ergosterol biosynthesis, and that posaconazole also affects Ca^{2+} homeostasis. These results provide logical explanations for the synergistic activity of amiodarone with azoles against *T. cruzi* and open up the possibility of novel, combination therapy approaches to the treatment of Chagas' disease using currently approved drugs.

Introduction

Chagas' disease remains the largest parasitic disease burden in Latin America, despite recent advances in the control of the transmission of the disease in some parts of the continent.[1] After the initial acute phase, which has a low (<10%) mortality and is often asymptomatic, a chronic condition establishes that can lead in 30−40% of cases to severe lesions of internal organs including Chagas' disease cardiomyopathy, which may result in cardiac arrhythmias, ventricular aneurysm, congestive heart failure, thromboembolism, and sudden death.[2] Specific treatments for this disease remain unsatisfactory due to limited efficacy, particularly in the prevalent chronic stage, and frequent deleterious side effects.[3] There is considerable interest in the development of new drugs for the treatment of Chagas' disease,[3] and in recent work it has been shown that compounds such as the sterol C14α demethylase inhibitor posaconazole ((2)-4-[4-[4-[4-[[(2*Rcis*)-5-(2,4-difluorophenyl)-tetrahydro-5-(1*H*-1,2,4-triazol-1-ylmethyl)furan-3-yl]methoxy]phenyl]-2,4-dihydro-2-[(*S*)-1-ethyl-2(*S*)-hydroxypropyl]-3*H*-1,2,4-triazol-3-one, **1**, Chart 1) can provide high percentages of parasitological cures in several animal models of acute and chronic Chagas' disease.[3−6] This compound, like many other azoles, blocks the biosynthesis of ergosterol, which is essential for parasite survival.[3]

Chart 1



There is, however, always interest in improving the efficacy of a given drug (and decreasing the likelihood of resistance) by using combination therapies. The K^+ and Ca^{2+} channel antagonist amiodarone (2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone, **2**, Chart 1) is the drug most frequently used to treat arrhythmias in chronic stage Chagas' disease patients with cardiac involvement.[2,7] In recent work, it has been shown that this compound has broad-spectrum antifungal activity,[8] mediated at least in part by disruption of Ca^{2+} homeostasis,[9,10] in addition to acting synergistically with the azole ergosterol biosynthesis inhibitors.[10,11] These results

† This article is dedicated to Dr. Harry Aquatella.

* To whom correspondence should be addressed. Tel: 58-212-5041660, Fax: 58-212-5041093, e-mail: jaurbina@ivic.ve.

§ Laboratorio Química Biológica, Instituto Venezolano de Investigaciones Científicas.

‡ Laboratorio de Permeabilidad Iónica, Instituto Venezolano de Investigaciones Científicas.

⊥ Instituto de Estudios Avanzados.

◇ Universidad Central de Venezuela.

INGEBI-CONICET.

□ Institut Cochin.

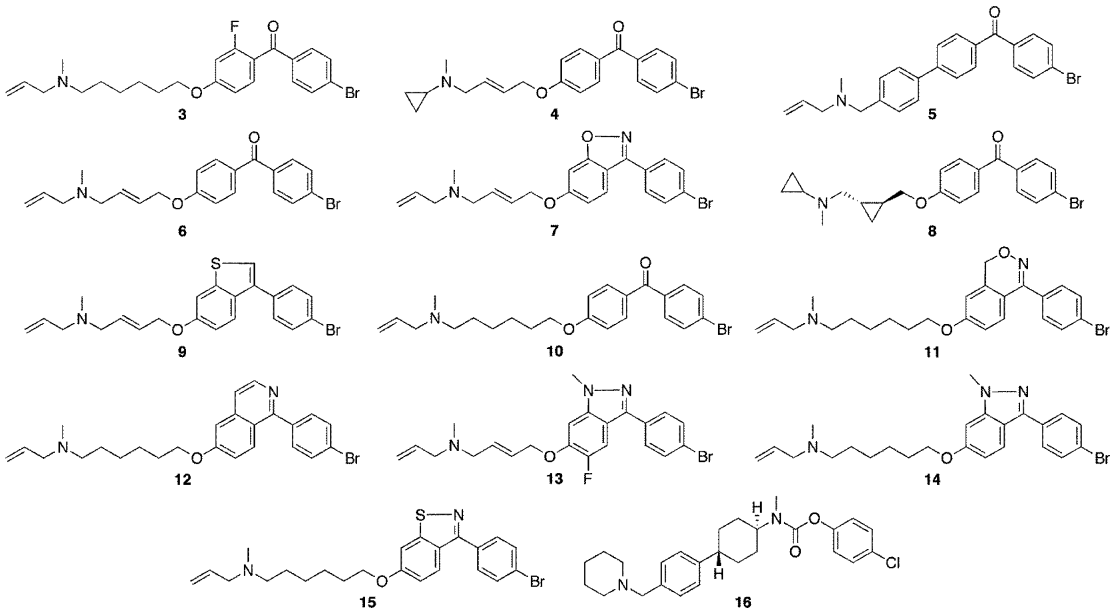
‖ Department of Chemistry, University of Illinois at Urbana-Champaign.

▽ Department of Biophysics, University of Illinois at Urbana-Champaign.

⊗ Universidad de Los Andes.

10.1021/jm050691f CCC: $33.50
Published on Web 01/05/2006

Chart 2



suggested the desirability of testing amiodarone against *T. cruzi*, alone and in combination with posaconazole.

Materials and Methods

Parasites. The EP stock of *T. cruzi* (a virulent strain isolated from a pediatric case in Carabobo State, Venezuela) was used in this study.[12] Handling of live *T. cruzi* was done according to established guidelines.[13]

Studies of in Vitro Antiproliferative Activity. The epimastigote form of the parasite was cultivated in liver infusion tryptose medium,[12] supplemented with 10% new born calf serum (Gibco) at 28 °C with strong (120 rpm) agitation. Cultures were initiated at a cell density of 2×10^6 epimastigotes mL^{-1}, and drugs were added at a cell density of $0.5-1.0 \times 10^7$ epimastigotes mL^{-1}. Cell densities were measured by using an electronic particle counter (model ZBI; Coulter Electronics Inc., Hialeah, FL) as well as by direct counting with a hemocytometer. Cell viability was followed by Trypan blue exclusion, using light microscopy. Amastigotes were cultured in Vero cells maintained in minimal essential medium supplemented with 1% fetal calf serum in a humidified atmosphere (95% air−5% CO_2) at 37 °C, as described previously.[5,6] Cells were infected with 10 tissue culture-derived trypomastigotes per cell for 2 h and then washed three times with phosphate-buffered saline (PBS) to remove nonadherent parasites. Fresh medium with and without drugs was added, and the cells were incubated for 96 h with a medium change at 48 h. The percent of infected cells and the numbers of parasites per cell were determined directly using light microscopy, and a statistical analysis of the results was carried out as described previously.[5,6] IC_{50} values were calculated by nonlinear regression, using the program GraFit (Erithacus Software Ltd, Surrey, UK). Fractional inhibitory concentrations (FIC) were calculated as described by Hallander et al.[14] Cytoplasmic free Ca^{2+} concentrations in control and drug-treated extracellular epimastigotes were determined by fluorimetric methods, using Fura-2, again as described previously.[15] Subcellular Ca^{2+} levels and mitochondrial membrane potentials were monitored on individual Vero cells infected with *T. cruzi* amastigotes by using time-scan confocal microscopy, as described in detail elsewhere.[16,17] Briefly, Vero cells heavily infected (72 h) with *T. cruzi* amastigotes were plated onto 22×40 mm glass coverslips (0.15 mm thickness) and incubated simultaneously with 10 μM cell-permeant Rhod-2 and 10 μg/mL Rhodamine-123 for 50 min at 37 °C in culture medium and then washed and incubated with Ringer's solution, with or without amiodarone. Under the conditions used,[17] fluorescence of Rhod-2 comes mainly from intracellular Ca^{2+}-rich compartments, like mitochondria, since its low affinity for Ca^{2+} limits its fluorescence in the Ca^{2+}-poor cytoplasm of the Vero cells or amastigotes. Rhodamine-123 is a mitochondrion-specific cationic dye, which distributes across the inner mitochondrial membranes strictly according to their membrane potential.[18]

Lipid Composition. Lipid analyses were carried out as described in the Supporting Information.

Enzymatic Assays. Native squalene synthase was isolated and purified from *T. cruzi* epimastigotes and assayed by a radiometric spot-wash assay as described before,[19] while pure recombinant farnesyl diphosphate synthase was assayed as described by Montalvetti et al.[20]

In Vivo Studies. In vivo studies were carried out by using the murine model of acute Chagas' disease described previously,[5,6] in which female NMRI−IVIC mice (20−25 g) were infected with 10^5 or 10^3 bloodstream trypomastigotes and drug treatment was started 24 h later. Treatments were given for 30 consecutive days at 20 mg kg^{-1} d^{-1} for posaconazole (30 doses) and/or at 50 mg kg^{-1} every other day (eod) for amiodarone (15 doses). Negative controls (i.e. untreated animals) received only the vehicle, while positive controls were treated with the anti-*T. cruzi* compound, nifurtimox, at 50 mg kg^{-1} d^{-1} for 30 days. Survival was followed daily and parasitemia weekly, the latter by direct microscopic examination. Animals were observed for 60 days postinfection, after which time parasitological cures were evaluated by using a combination of hemoculture, xenodiagnosis, and blood PCR tests.[5,6] For PCR, primers TcZ1 (5'-CGAGCTCTTGCCCACACGGGT-GCT-3') and TcZ2 (5'-CCTCCAAGCAGCGGATAGTTCAGG-3') were used to detect *T. cruzi* satellite DNA (TcZ DNA).[21]

Drugs. Posaconazole was provided by Dr. David Loebenberg (Schering Plough Research Institute, Kenilworth, NJ). Amiodarone hydrochloride was obtained from Sigma-Aldrich Company (St. Louis, MO).

Theoretical Calculations. A common feature pharmacophore model for human oxidosqualene cyclase was built by using the Catalyst 4.8 program (Accelrys, Inc., San Diego, CA). For the training set, we only considered compounds with activities within an order of magnitude of the most active compound reported previously (Chart 2).[22] Up to 255 best quality conformations were generated for each compound, with the exception of **3** (Ro48-8071) ([4′-(6-allylmethylaminohexyloxy)-2′-fluorophenyl]-(4-bromophenyl)-methanone, Chart 1), for which we used the X-ray crystallographic coordinates taken from PDB file 1W6J.[23] To ensure that Catalyst would only generate pharmacophores that fit the X-ray crystallographic structure of **3** from the protein/ligand complex, the

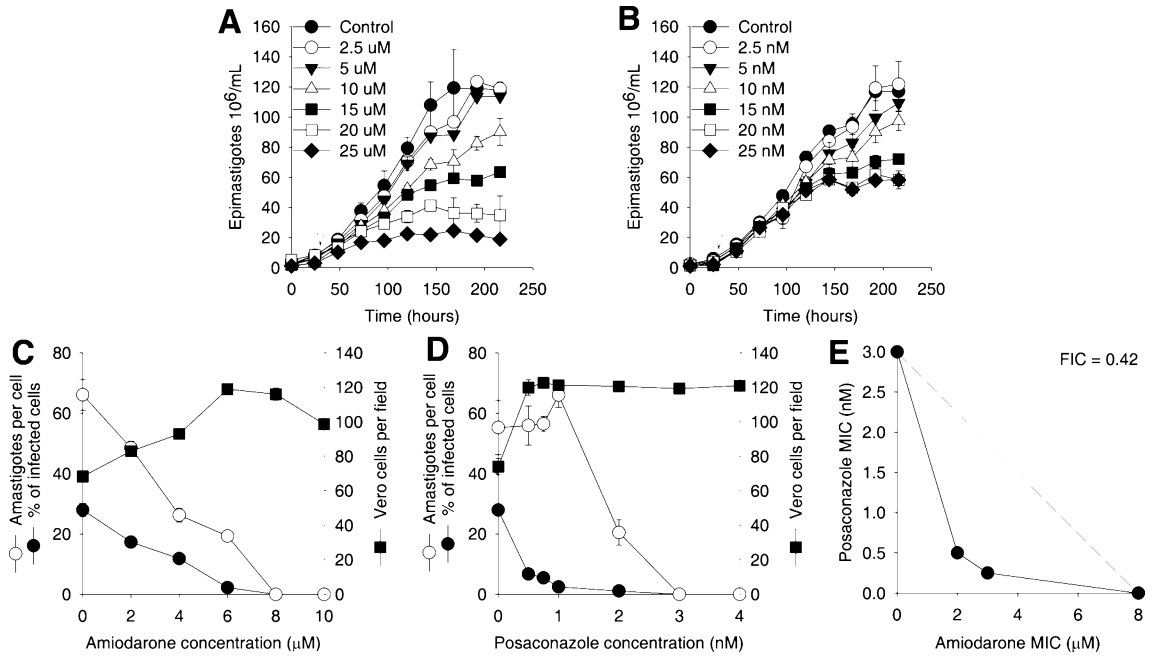


Figure 1. Antiproliferative activities of amiodarone and posaconazole against *Trypanosoma cruzi* in vitro. Effects of amiodarone (A) and posaconazole (B) on the growth of *T. cruzi* extracellular epimastigotes. Experiments were carried out in triplicate and each bar represents one standard deviation. Effects of amiodarone (C) and posaconazole (D) on the proliferation of *T. cruzi* amastigotes cultured inside Vero cells at 37 °C. Shown are the percentage of infected cells (●), the number of amastigotes per cell (■), and the number of Vero cells per field (■) after 96 h of incubation, as a function of drug concentration. Experiments were carried out in quadruplicate and each bar represents one standard deviation. (E) Isobologram illustrating the combined effects of amiodarone and posaconazole on *T. cruzi* intracellular amastigotes. The drug concentrations (alone or in combination) that led to the complete eradication of the intracellular parasites after 96 h are shown. The broken line corresponds to the predicted positions of the experimental points for simple additivity. A fractional inhibitory concentration (FIC) of 0.42 was calculated according to Hallander et al.[14]

ligand was assigned a principal value of two, whereas all other compounds were assigned principal values of one. By assigning only **3** a principal value of two, only the X-ray structure of **3** can influence the geometries of pharmacophores output by the program; the other training set compounds are merely required to adopt a geometry consistent with the output pharmacophores. This provides structural information, such that the mapped conformations of a given inhibitor might better approximate the actual conformations in the enzyme. Additional details are provided in the Supporting Information. Autodock 3.05[24] was run with default parameters with the exceptions of ga_num_evals, which was set to 2 500 000 (default is 250 000), and ga_run, which was set to 100 (default is 10). Docking and cross-docking experiments with 1W6J and 1W6K gave similar results. For more details, see the Supporting Information.

Results and Discussion

We show in Figure 1 the effects of amiodarone and posaconazole on the proliferation of *T. cruzi* in vitro. Amiodarone (**2**) had a clear, dose-dependent effect on proliferation of the epimastigote (extracellular) stages, with a minimal inhibitory concentration of 20 μM and an IC_{50} of 9 μM (Figure 1A), while for posaconazole the corresponding values were 20 and 14 nM (Figure 1B). Against the clinically relevant intracellular amastigote form of the parasite, both drugs were even more potent. Amiodarone had minimal inhibitory concentration and IC_{50} values of 8 and 2.7 μM (Figure 1C), while for posaconazole (**1**), the corresponding values were 3 and 0.25 nM (Figure 1D). These results indicate that amiodarone has activity against both proliferative stages of *T. cruzi* comparable to its in vitro antifungal activity.[8,10,11] To explore this similarity in more depth, we investigated the effects of amiodarone and posaconazole in combination against *T. cruzi*, since synergistic effects between amiodarone and various other azole drugs have been reported in several yeasts and fungi. We used a checkerboard technique and the method of Hallander et al.[14] and obtained a fractional inhibitory concentration value of 0.42 (Figure 1E), indicating strong synergism.

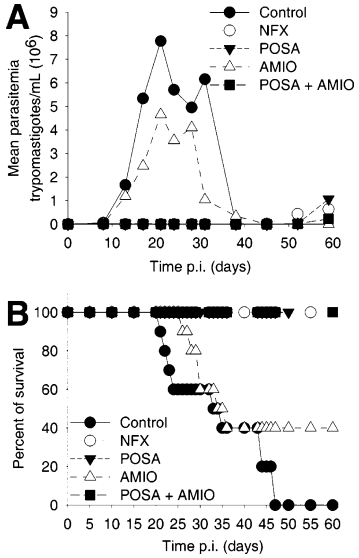


Figure 2. Activities of amiodarone (AMIO), posaconazole (POSA), and nifurtimox (NFX) in a murine model of acute Chagas' disease. NMRI-IVIC mice were inoculated with 10^5 bloodstream form trypomastigotes of the Y strain and treatment started 24 h later. All drugs were given orally for 30 consecutive days at the following doses: AMIO, 50 mg kg^{-1} every other day (eod); POSA and NFX at 50 mg kg^{-1} d^{-1}.

Next, we investigated if the anti-*T. cruzi* activity of amiodarone detected in vitro could also be observed in vivo. In a preliminary study, NMRI mice were infected with a relatively high inoculum (10^5 trypomastigotes/mouse), which led to a fulminant acute infection as previously described,[5,6] oral drug

Table 1. Effects of Amiodarone and Posaconazole in a Murine Model of Acute Chagas' Disease. 10^3 Trypomastigotes Mouse^{-1}, Y Strain, 86 days pi[a]

treatment conditions	survival	negative parasitological tests[b]
no treatment (control)	8/10	3/8
amiodarone, 50 mg kg^{-1}, eod, 15 doses	6/10	0/6
posaconazole, 20 mg kg^{-1}d^{-1}, 30 doses	10/10	6/10
amiodarone, 50 mg kg^{-1}, eod, 15 doses + posaconazole, 20 mg kg^{-1} d^{-1}, 30 doses	10/10	8/10
nifurtimox, 50 mg kg^{-1} d^{-1}, 30 doses	8/10	4/8

[a] Female NMRI-IVIC mice (20−25 g) were infected with 10^3 bloodstream trypomastigotes and drug treatment started 24 h later, at the doses and frequencies indicated. [b] Hemoculture, xenodiagnosis, and TcZ DNA PCR; see Materials and Methods.

Table 2. Effects of Amiodarone and Posaconazole on the Free Cytoplasmic Ca^{2+} Concentration of *Trypanosoma cruzi* Epimastigotes[a]

measurement conditions[a]	control	posaconazole[b]	posaconazole[c]
2 mM Ca^{2+}	95 ± 35 (n = 15)	86 ± 27 (n = 8)	461 ± 64 (n = 9)
2 mM Ca^{2+} + amiodarone[d]	174 ± 39 (n = 6)	193 ± 39 (n = 4)	668 ± 50 (n = 4)
0 mM Ca^{2+}	99 ± 11 (n = 4)	97± 15 (n = 4)	480 ± 40 (n = 4)
0 mM Ca^{2+} + amiodarone[d]	193 ± 26 (n = 4)	131 ±11 (n = 4)	611 ± 77 (n = 4)

[a] Free cytoplasmic calcium concentrations were determined using fluorimetric methods with Fura-2, as described in previously.[15] Concentrations are expressed in nM. [b] Posaconazole treatment; 12.5 nM for 48 h. [c] Posaconazole treatment; 12.5 nM for 96 h. [d] Amiodarone concentration; 12.5 μM.

Table 3. Free Sterols Present in *Trypanosoma cruzi* Epimastigotes (EP stock) Grown in the Absence or Presence of Posaconazole, Amiodarone, or Their Combination[a]

sterol[c]	control	posaconazole[a]	amiodarone[b]	posaconazole[a]+ amiodarone[b]
exogenous cholesterol	31.2	46.1	63.4	78.8
		Endogenous, 14-Desmethyl:		
ergosterol	15.3	5.1	5.1	n.d.[d]
24-ethyl-5,7,22-cholesta-trien-3β-ol	15.4	12.2	6.7	n.d.
ergosta-8,24(24′)-dien-3β-ol	6.5	13.7	10.2	n.d.
ergosta-5,7-dien-3β-ol	9.5	3.6	4.1	n.d.
ergosta-5,7,24(24′)-trien-3β-ol	8.5	n.d.	n.d.	n.d.
ergosta-7,24(24′)-dien-3β-ol	7.6	3.2	3.0	n.d.
24-ethyl-5,7-cholesta-dien-3β-ol	6.0	7.5	7.5	n.d.
		Endogenous, 14-Methyl:		
24-methylene-dihydrolanosterol	n.d.	3.2	n.d.	16.9
lanosterol	n.d.	5.5	n.d.	4.3

[a] Posaconazole concentration = 12.5 nM. [b] Amiodarone concentration = 12.5 μM. [c] Free sterols were isolated and purified from whole cells grown in the absence or presence of the indicated drug concentrations for 96 h; they were analyzed by high-resolution gas−liquid chromatography coupled with mass spectrometry, as described in Materials and Methods. [d] n.d., not detected.

treatment was started 24 h post-inoculation (pi) and given for 30 days with amiodarone (50 mg kg^{-1} every other day, eod), posaconazole (20 mg kg^{-1} d^{-1}), amiodarone plus posaconazole, nifurtimox (50 mg kg^{-1} d^{-1}), or no drug. As can be seen in Figure 2, treatment of infected animals with amiodarone alone reduced parasitemia (Figure 2A), increased survival 60 days pi (Figure 2B; 0% for untreated controls vs 40% for amiodarone-treated animals) and, when given in combination with posaconazole, delayed the development of parasitemia when compared with the all other treatment groups (Figure 2A). In a second model, mice were infected with a lower inoculum (10^3 trypomastigotes/mouse) and treated with the same therapeutic schemes used for the first infection model. Survival and parasitological cures, 86 days pi, are presented in Table 1. Most (8/10) animals treated with the combination tested negative to hemoculture, xenodiagnosis, and blood PCR for nuclear satellite (TcZ) DNA, indicating very low parasite burdens (<50 trypomastigotes/mL[21]), versus 6/10 for those treated with posaconazole alone and 4/8 animals treated with the conventional treatment, nifurtimox. These results demonstrate that amiodarone has in vivo anti-*T. cruzi* activity and support further in vivo studies of this drug as an antiparasitic agent, used alone or in combination.

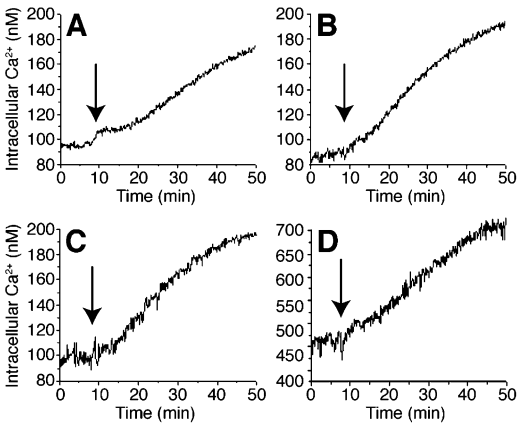


Figure 3. Effects of amiodarone (AMIO) and posaconazole (POSA) on Ca^{2+} homeostasis in *T. cruzi* epimastigotes. Epimastigotes, grown in the absence or presence of 12.5 nM POSA, were incubated in the presence of the cell permeant dye Fura-2 for 45 min. Cytoplasmic free Ca^{2+} concentrations were determined fluorimetrically, as described elsewhere. (A) Effects of 12.5 μM AMIO on cytoplasmic free Ca^{2+} concentration in control (untreated) cells incubated with 2 mM external free Ca^{2+}. (B) Effects of amiodarone (12.5 μM) on cytoplasmic free Ca^{2+} concentration in control (untreated) cells incubated in the presence of 1 mM external EGTA. (C) Effects of 12.5 μM AMIO on cytoplasmic free Ca^{2+} concentration in cells grown in the presence of 12.5 nM POSA for 48 h, incubated with 2 mM external free Ca^{2+}. (D) Effects of 12.5 μM AMIO on cytoplasmic free Ca^{2+} concentration in cells grown in the presence of 12.5 nM POSA for 96 h, incubated with 2 mM external free Ca^{2+}. Arrows indicate the time of addition of AMIO.

To probe the molecular basis of the antiparasitic activity of amiodarone and its synergism with posaconazole, we tested the effects of both drugs, alone and in combination, on the calcium homeostasis of both epimastigotes (Figure 3A−D, Table 2) and intracellular amastigotes (Figure 4A,B). Amiodarone at 12.5 μM induced a rapid increase in free cytoplasmic Ca^{2+} levels in epimastigotes (Figure 3A, Table 2), as determined by Fura-2 fluorescence.[15] The increase was due to drug-induced Ca^{2+} release from intracellular compartments, since the same effect was observed in the absence of external Ca^{2+} (Figure 3B, Table 2). Cells exposed to 12.5 nM posaconazole for 48 h gave results similar to control cells (Figure 3C, Table 2), but cells exposed to posaconazole for 96 h had basal Ca^{2+} cytoplasmic levels 4.5-fold higher than did control cells (Figure 3D, Table 2). Moreover, amiodarone was able to induce a further increase in these levels, which reached values 7-fold higher than those of the control cells (Figure 3D, Table 2). We were also able to detect selective effects of amiodarone on Ca^{2+} homeostasis in intracellular *T. cruzi* amastigotes: Figure 4A,B show that when heavily infected Vero cells were exposed to amiodarone, there was a rapid release of Ca^{2+} from the parasite's mitochondria, as detected by Rhod-2 fluorescence[17] (in red). There was also a collapse of the mitochondrial membrane potential, as dem-

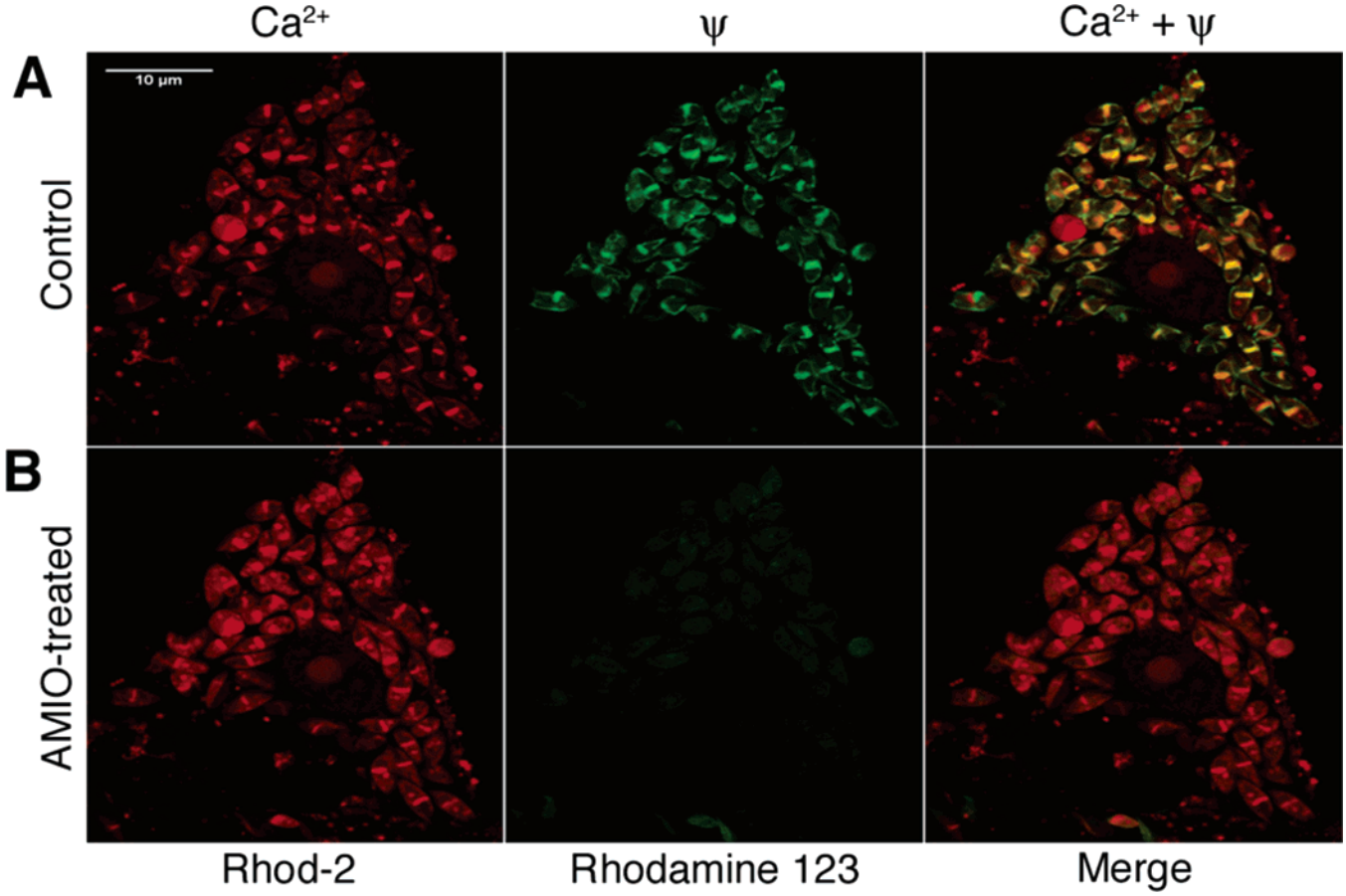


Figure 4. Effects of amiodarone (AMIO) on Ca^{2+} homeostasis in *T. cruzi* amastigotes; Vero cells infected with *T. cruzi* were double-labeled with Rhod-2 (red) and Rhodamine 123 (green) to visualize mitochondrial free Ca^{2+} concentrations and transmembrane electrical potentials, respectively. (A) Control cells, (B) cells treated with 12.5 μM AMIO for 20 min. Note the drastic AMIO-induced reduction of the mitochondrial membrane potential and the corresponding increase in the parasites' free cytoplasmic Ca^{2+} concentration. These effects were not observed in the host cells.

onstrated by a decrease in Rhodamine 123 fluorescence[18] (in green), associated with the increase in the parasites' $[Ca^{2+}]_{cyt}$. However, no effects were seen on the host cells' free cytoplasmic Ca^{2+} levels. Such results contrast with those previously obtained in yeast, where it was shown that amiodarone disrupted Ca^{2+} homeostasis largely by inducing a rapid influx of this ion from the extracellular milieu, mediated by caffeine-sensitive MID-1 channels.[9,10] In any case, the results in Figures 3 and 4 and Table 2 clearly indicate that *both* amiodarone and posaconazole can disrupt Ca^{2+} homeostasis in *T. cruzi*, a fact that could explain their antiproliferative synergism against *T. cruzi*. However, this may not be the only origin of the synergistic effects of these drugs, since it is possible that amiodarone also interferes with sterol biosynthesis, based on the fact that other Ca^{2+}-channel blockers are known to inhibit sterol isomerases and yeast growth.[25,26]

We show in Table 3 the results of an experiment designed to test this possibility, in which we compared the sterols present in *T. cruzi* epimastigotes treated with amiodarone, posaconazole, or their combination, with those present in control (no drug treatment) cells. In cells treated with 12.5 nM posaconazole, we observed the expected[5,27] reduction of the parasite's endogenous 14-desmethyl sterols and a concomitant accumulation of 14-methyl sterols (lanosterol and eburicol), Table 3, Figure 5. However, in cells treated with 12.5 μM amiodarone, there was also a significant reduction in the overall levels of endogenous sterols, but no accumulation of sterol intermediates, indicating inhibition of de novo sterol synthesis at a prelanosterol level (Table 3, Figure 5). When cells were treated with the combination of amiodarone *plus* posaconazole, it was found that the (exogenous) cholesterol level increased to nearly 80% of total sterols, with no detectable levels of the parasite's normal 14-desmethyl sterols (Table 3). These findings clearly demonstrate that amiodarone has a previously unknown effect on sterol biosynthesis, providing a second logical basis for its synergism with posaconazole. Since no accumulation of squalene was observed, amiodarone does not target squalene epoxidase (Figure 5). There was some inhibitory activity on native *T. cruzi* squalene synthase (SQS),[19] albeit with low potency: the IC_{50} values were 12 and 8 μM against microsomal and glycosomal SQS, respectively (data not shown). Likewise, there was no inhibition of a recombinant farnesyl diphosphate synthase[20] at 50 μM amiodarone (data not shown). On the other hand, a TLC analysis of the neutral lipids from amiodarone-treated cells showed accumulation of a compound having an R_f value corresponding to that of squalene epoxide, indicating inhibition at the level of oxidosqualene cyclase (OSC, see Figure 5).

Structurally, amiodarone has considerable similarities to other known OSC inhibitors (including those known to inhibit *T. cruzi* growth[28]), as shown for example by comparison with the benchmark OSC inhibitor **3**. Both are halogenated bisaryl ketones with cationic oxymethylene side chains, and they have similar sizes. To investigate these similarities in more depth, we investigated the structural features key to OSC inhibition in a series of 14 potent OSC inhibitors[22] (Chart 2), using pharmacophore modeling with the Catalyst 4.8 program (Accelrys Inc., San Diego, CA).

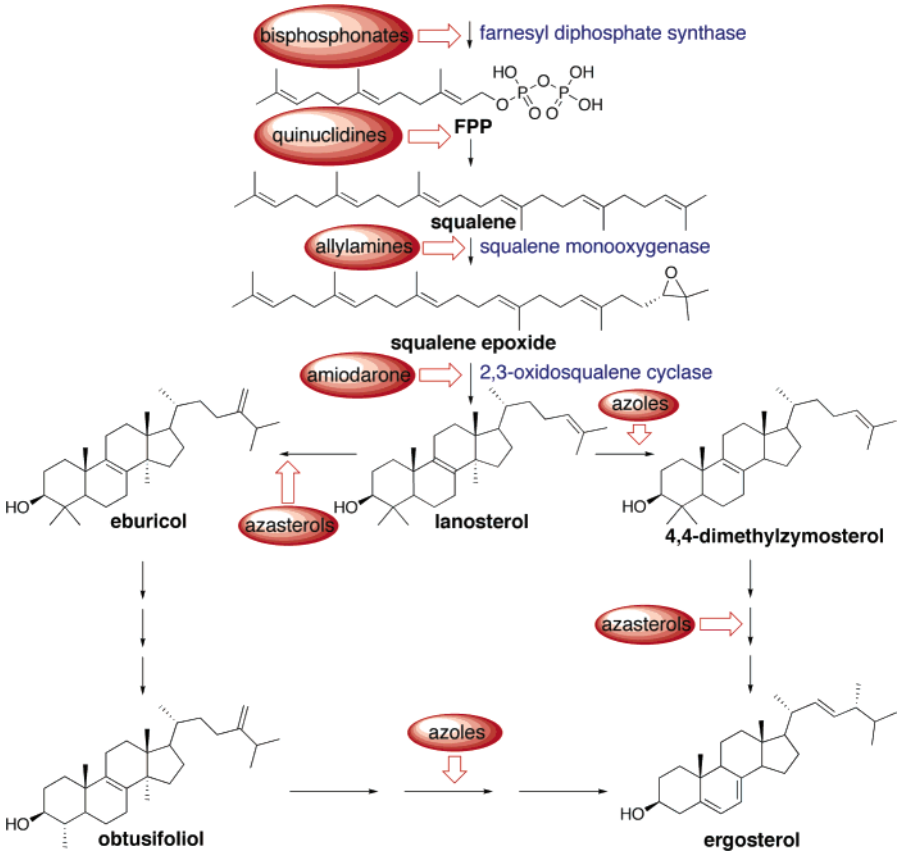


Figure 5. Sterol structures and sites of action of different inhibitors of ergosterol biosynthesis.

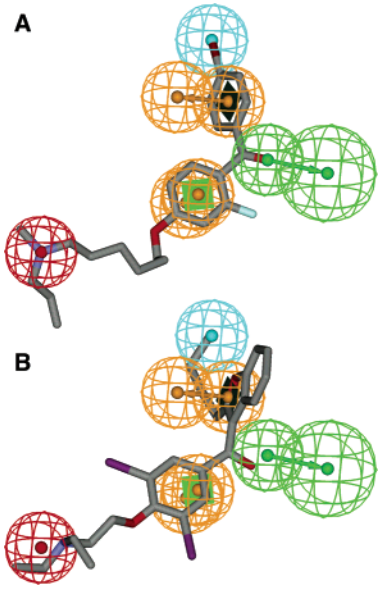


Figure 6. Pharmacophore model for oxidosqualene cyclase with (A) the X-ray crystallographic structure of **3** from 1W6J and (B) the best fit conformation of **2.** Pharmacophore features are: positive charge (red), aromatic ring (orange), hydrogen bond acceptor (green), and hydrophobic (cyan).

The results (Figure 6A) indicated the presence of five features common to the most active OSC inhibitors: a positive charge feature (red), two aromatic ring features (orange), a hydrogen bond acceptor feature (green), and a hydrophobic feature (cyan). These features are all present in amiodarone, although amiodarone does not fit the pharmacophore ideally (Figure 6B), which would result in activity lower than that of the most potent inhibitors. We also used the Autodock 3.05 program[24] to determine whether amiodarone could fit into the sterol/inhibitor binding site in OSC. By way of controls, we first docked lanosterol and **3** into the active site of OSC (Figure 7A,B), where we found rms deviations of 0.98 Å and 0.97 Å from the crystallographic results, for lanosterol and Ro48-8071, respectively. Then, we docked amiodarone, obtaining the top five structures shown in Figure 7C, which are clearly similar to those found with **3**. Moreover, we found that many features of the pharmacophore model are associated with intermolecular interactions with the OSC protein, again providing support for amiodarone's effect on ergosterol biosynthesis. These facts indicate that amiodarone could represent a novel class of OSC inhibitor, a fact which may also help explain its antifungal activity.[8]

Of course, care needs to be exercised when considering combination therapy in humans. In this respect the US Federal Drug Administration has recently (March, 2004) added the following paragraph to the Cordarone (amiodarone HCl 200 mg tablets) label: 'Fluoroquinolones, macrolide antibiotics, and azoles are known to cause QTc prolongation. There have been reports of QTc prolongation, with or without TdP, in patients taking amiodarone when fluoroquinolones, macrolide antibiotics, or azoles were administered concomitantly, see WARNINGS, Worsened Arrhythmia'. However, studies in healthy volunteers have shown than posaconazole, in contrast with other azole derivatives, has no significant effects on the QTc interval,[29,30] a fact which could probably allow its concomitant administration with amiodarone. Nevertheless, caution should be used, as both compounds are substrates of cytochrome P450 3A4 (CYP3A4).

Conclusions. The results described above are of interest for many reasons. First and foremost, we find that amiodarone, a drug currently in use for the symptomatic treatment of arrhythmias in many Chagas' disease patients, has an unexpected, direct

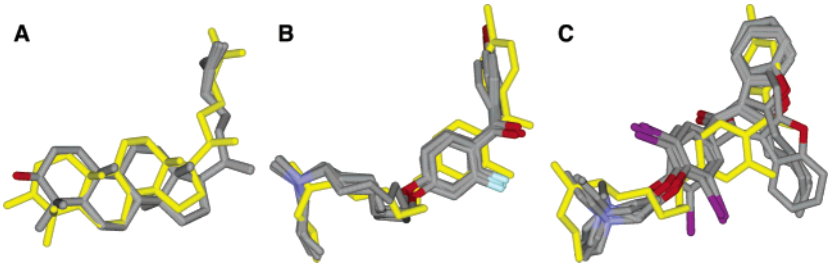


Figure 7. Molecular docking results based on oxidosqualene synthase structures (PDB files 1W6J and 1W6K). (A) Five top-scoring docked lanosterol structures together with its X-ray structure (in yellow); rms deviation = 0.98 Å. (B) Five top-scoring docked **3** structures together with its X-ray structure (in yellow); rms deviation = 0.97 Å. (C) Five top-scoring docked amiodarone structures together with the X-ray structure of **3** (in yellow).

anti-*T. cruzi* activity and that this activity is potentiated by the ergosterol biosynthesis inhibitor posaconazole. Amiodarone disrupts Ca^{2+} homeostasis in *T. cruzi* by inducing the release of this ion from intracellular stores, specifically from the single giant mitochondrion present in those cells. This action is, however, parasite-specific, since it was not observed in the mammalian host cells, which grew normally under amiodarone levels that led to loss of parasite viability within 24 h. We also find that posaconazole affects Ca^{2+} homeostasis, and that amiodarone augments this effect. Disruption of Ca^{2+} homeostasis in posaconazole-treated *T. cruzi* epimastigotes and amastigotes is probably related to structural alterations of the parasite's mitochondrion that are observed in cells depleted of endogenous sterols upon prolonged exposure to ergosterol biosynthesis inhibitors.[31,32] In addition, we find that amiodarone blocks sterol biosynthesis in *T. cruzi*, and that posaconazole potentiates this effect as well. Thus, the combined effects of two mutually reinforcing molecular mechanisms, disruption of the parasite's Ca^{2+} homeostasis and blockade of de novo ergosterol biosynthesis, could account for the antiproliferative and synergistic effects of the two drugs against this parasite. Moreover, we find that amiodarone has direct in vivo activity in murine models of Chagas' disease. Taken together, these results suggest that antiarrhythmia treatment of Chagas' disease patients with amiodarone could have the added benefit of reducing the patient's parasite load and enhancing the efficacy of specific antiparasitic agents.

Finally, the observation that amiodarone has direct broad-spectrum antifungal activity and that amiodarone/azole combinations are synergistic strongly suggests that similar growth inhibition mechanisms operate in yeasts and fungi, in addition to *T. cruzi*, and that further development of such combination therapies may be of broad general interest.

Acknowledgment. This work received financial support from the Howard Hughes Medical Institute (Grants 55000620 to J.A.U and 55003682 to M.J.L.), the Instituto Venezolano de Investigaciones Científicas (IVIC), Fondo Nacional para la Ciencia y Tecnología, Venezuela (S1-1999000058 and G-2001000637, to G.B.), the Consejo de Desarrollo Científico y Humanístico, Universidad Central de Venezuela (PI-03-10-4798-05, to G.B), by the United States Public Health Service (GM-65307, to E.O.), by an award from the American Heart Association, Greater Midwest Affiliate (to J.M.S.), by a NIH Training Grant (GM-08276 to A.L.-R.), by the UNICEF/World Bank/UNDP/World Health Organization Program on Research and Training in Tropical Diseases (TDR, Grant A 20285 to A.S.) by the Fundación Bunge y Born (to A.S.) and the Fundación Nacional de Ciencia y Tecnología, Argentina (to M.J.L.). M.J.L. is International Professor of a Chaire Internationale de Recherche Blaise Pascal, Fondation Ecole Normale Superieure, Region Ile de France, Paris, France (October 2005−October 2006). The technical assistance of Hector Rojas, Margarita Bisio, Carlos Rodrigues, and Imeria Nuñez Odreman is gratefully acknowledged.

Supporting Information Available: Additional experimental and theoretical details. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) W. H. O. Control of Chagas Disease. *Tech. Rep. Ser.* **2002**, *905*, 1−109.
(2) Rassi, A., Jr.; Rassi, A.; Little, W. C. Chagas' heart disease. *Clin. Cardiol.* **2000**, *23*, 883−889.
(3) Urbina, J. A.; Docampo, R. Specific Chemotherapy of Chagas disease: controversies and advances. *Trends Parasitol.* **2003**, *19*, 495−501.
(4) Molina, J.; Martins-Filho, O.; Brener, Z.; Romanha, A. J.; Loebenberg, D.; Urbina, J. A. Activity of the Triazole Derivative SCH 56592 (Posaconazole) Against Drug-Resistant Strains of the Protozoan Parasite *Trypanosoma (Schizotrypanum) cruzi* in Immunocompetent and Immunosuppressed Murine Hosts. *Antimicrob. Agents Chemother.* **2000**, *44*, 150−155.
(5) Urbina, J. A.; Payares, G.; Contreras, L. M.; Liendo, A.; Sanoja, C.; Molina, J.; Piras, M. M.; Piras, R.; Perez, N.; Wincker, P.; Loebenberg, D. Antiproliferative Effects and Mechanism of Action of SCH 56592 against *Trypanosoma (Schizotrypanum) cruzi*: In Vitro and In Vivo Studies. *Antimicrob. Agents Chemother.* **1998**, *42*, 1771−1777.
(6) Urbina, J. A.; Payares, G.; Molina, J.; Sanoja, C.; Liendo, A.; Lazardi, K.; Piras, M. M.; Piras, R.; Perez, N.; Wincker, P.; Ryley, J. F. Cure of Short- and Long-Term Experimental Chagas Disease using D0870. *Science* **1996**, *273*, 969−971.
(7) Mason, J. W. Amiodarone. *N. Engl. J. Med.* **1987**, *316*, 455−466.
(8) Courchesne, W. E. Characterization of a novel, broad-based fungicidal activity for the antiarrhythmic drug amiodarone. *J. Pharmacol. Exp. Ther.* **2002**, *300*, 195−199.
(9) Courchesne, W. E.; Ozturk, S. Amiodarone induces a caffeine-inhibited, MID1-dependent rise in free cytoplasmic calcium in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **2003**, *47*, 223−234.
(10) Gupta, S. S.; Ton, V.-K.; Beaudry, V.; Rulli, S.; Cunningham, K.; Rao, R. Antifungal activity of amiodarone is mediated by disruption of calcium homeostasis. *J. Biol. Chem.* **2003**, *278*, 28831−28839.
(11) Afeltra, J.; Vitale, R. G.; Mouton, J. W.; Verweij, P. E. Potent Synergistic In Vitro Interaction between Nonantimicrobial Membrane-Active Compounds and Itraconazole against Clinical Isolates of *Aspergillus fumigatus* Resistant to Itraconazole. *Antimicrob. Agents Chemother.* **2004**, *48*, 1335−1343.
(12) de Maio, A.; Urbina, J. A. *Trypanosoma (Schizotrypanum) cruzi*: terminal oxidases in exponential and stationary growth phase emipastigotes cultured in vitro. *Acta. Cient. Venez.* **1984**, *35*, 136−141.
(13) Hudson, L.; Grover, F.; Gutteridge, W. E.; Klein, R. A.; Peters, W.; Neal, R. A.; Miles, M. A.; Scott, M. T.; Nourish, R.; Ager, B. P. Suggested guidelines for work with live *Trypanosoma cruzi*. *Trans. R. Soc. Trop. Med. Hyg.* **1983**, *77*, 416−419.
(14) Hallander, H. O.; Dornbush, K.; Gezelius, L.; Jacobson, K.; Karlsson, I. Synergism between aminoglycosides and cephalosporin with antipseudomonal activity: interaction index and killing curve method. *Antimicrob. Agents Chemother.* **1982**, *22*, 743−752.
(15) Benaim, G.; Bermudez, R.; Urbina, J. A. Ca^{2+} transport in isolated mitochondrial vesicles from *Leishmania braziliensis* promastigotes. *Mol. Biochem. Parasitol.* **1990**, *39*, 61−68.

(16) Mendoza, M.; Mijares, A.; Rojas, H.; Colina, C.; Cervino, V.; DiPolo, R.; Benaim, G. Evaluation of the presence of a thapsigargin-sensitive calcium store in trypanosomatids using *Trypanosoma evansi* as a model. *J. Parasitol.* **2004**, *90*, 1181−1183.
(17) Colina, C.; Flores, A.; Rojas, H.; Acosta, A.; Castillo, C.; Garrido Mdel, R.; Israel, A.; DiPolo, R.; Benaim, G. Ceramide increase cytoplasmic Ca^{2+} concentration in Jurkat T cells by liberation of calcium from intracellular stores and activation of a store-operated calcium channel. *Arch. Biochem. Biophys.* **2005**, *436*, 333−345.
(18) Nicholls, D. G.; Ward, M. W. Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. *Trends Neurosci.* **2000**, *23*, 166−174.
(19) Urbina, J. A.; Concepcion, J. L.; Rangel, S.; Visbal, G.; Lira, R. Squalene synthase as a chemotherapeutic target in *Trypanosoma cruzi* and *Leismania mexicana*. *Mol. Biochem. Parasitol.* **2002**, *125*, 35−45.
(20) Montalvetti, A.; Bailey, B. N.; Martin, M. B.; Severin, G. W.; Oldfield, E.; Docampo, R. Bisphosphonates are potent inhibitors of *Trypanosoma cruzi* farnesyl pyrophosphate synthase. *J. Biol. Chem.* **2001**, *276*, 33930−33937.
(21) Schijman, A. G.; Vigliano, C.; Burgos, J.; Favaloro, R.; Perrone, S.; Laguens, R.; Levin, M. J. Early diagnosis of recurrence of *Trypanosoma cruzi* infection by polymerase chain reaction after heart transplantation of a chronic Chagas' heart disease patient. *J. Heart Lung Transplant.* **2000**, *19*, 1114−1117.
(22) Lenhart, A.; Reinert, D. J.; Aebi, J. D.; Dehmlow, H.; Morand, O. H.; Schulz, G. E. Binding structures and potencies of oxidosqualene cyclase inhibitors with the homologous squalene-hopene cyclase. *J. Med. Chem.* **2003**, *46*, 2083−2092.
(23) Thoma, R.; Schulz-Gasch, T.; D′Arcy, B.; Benz, J.; Aebi, J.; Dehmlow, H.; Hennig, M.; Stihle, M.; Ruf, A. Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase. *Nature* **2004**, *432*, 118−122.
(24) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* **1998**, *19*, 1639−1662.
(25) Krajewska-Kulak, E.; Niczyporuk, W. Effects of the combination of ketoconazole and calcium channel antagonists against *Candida albicans* in vitro. *Arzneim. Forsch.* **1993**, *43*, 782−783.
(26) Moebius, F. F.; Reiter, R. J.; Bermoser, K.; Glossmann, H.; Cho, S. Y.; Paik, Y. K. Pharmacological analysis of sterol delta8-delta7 isomerase proteins with [3H]ifenprodil. *Mol. Pharmacol.* **1998**, *54*, 591−598.
(27) Munayyer, H. K.; Mann, P. A.; Chau, A. S.; Yarosh-Tomaine, T.; Greene, J. R.; Hare, R. S.; Heimark, L.; Palermo, R. E.; Loebenberg, D.; McNicholas, P. M. Posaconazole is a potent inhibitor of sterol 14alpha-demethylation in yeasts and molds. *Antimicrob. Agents Chemother.* **2004**, *48*, 3690−3696.
(28) Hinshaw, J. C.; Suh, D. Y.; Garnier, P.; Buckner, F. S.; Eastman, R. T.; Matsuda, S. P.; Joubert, B. M.; Coppens, I.; Joiner, K. A.; Merali, S.; Nash, T. E.; Prestwich, G. D. Oxidosqualene cyclase inhibitors as antimicrobial agents. *J. Med. Chem.* **2003**, *46*, 4240−4243.
(29) Sansone, A.; Courtney, R.; L., M.; Calzetta, A.; Soni, P.; Joseph, D.; Zhang, M.; Laughlin, M. Posaconazole Has No Clinically Significant Effect on QTc Interval in Healthy Volunteers. Presented at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICCAC), Washington, DC, Oct 30−Nov 2, 2004, Poster A1100.
(30) Courtney, R.; Pai, S.; Laughlin, M.; Lim, J.; Batra, V. Pharmacokinetics, safety and tolerability of oral posaconazole administered in single and multiple doses in healthy adults. *Antimicrob. Agents Chemother.* **2003**, *47*, 2788−2795.
(31) Lazardi, K.; Urbina, J. A.; DeSouza, W. Ultrastructural alterations induced by two ergosterol biosynthesis inhibitors, ketoconazole and terbinafine, on epimastigotes and amastigotes of *Trypanosoma (Schizotrypanum) cruzi*. *Antimicrob. Agents Chemother.* **1990**, *34*, 2097−2105.
(32) Lazardi, K.; Urbina, J. A.; DeSouza, W. Ultrastructural alterations induced by ICI 195,739, a bis triazole with strong antiproliferative action against *Trypanosoma (Schizotrypanum) cruzi*. *Antimicrob. Agents Chemother.* **1991**, *35*, 736−740.

JM050691F