Candimine-Induced Cell Death of the Amitochondriate Parasite *Trichomonas vaginalis*

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Candimine (**1**), an alkaloid from the bulbs of *Hippeastrum morelianum*, was found to be cytotoxic for the amitochondriate parasite *Trichomonas vaginalis.* Candimine (**1**) induced cell death with an unprecedented group of effects that failed to fulfill the criteria for apoptosis and apoptosis-like death already reported in trichomonads. Arrest of the parasite cell cycle, and morphologic and ultrastructural alterations, including marked cytoplasmic vacuolization, were induced by **1**. The present findings suggest some similarities to paraptotic cell death, described for multicellular organisms. This study contributes to both a better understanding of the biological effects of **1** and *T. vaginalis* cell biology.

Trichomonas vaginalis is a unicellular eukaryotic protist that parasitizes the human genitourinary tract and causes trichomonosis, recognized as the most prevalent nonviral sexually transmitted disease (STD).[1] The clinical presentation is mainly problematic in women, where it may be asymptomatic or cause severe vaginitis and cervicitis.[2] The complexity of trichomonosis pathogenesis is illustrated by the interaction of the parasite with human cells, tissues, and the immune system.[3] Another important aspect of trichomonosis is the association between *T. vaginalis* infection and an increased risk of transmission of other STDs, including the human immunodeficiency virus.[4] Besides the significance of the parasite as an etiologic agent, unusual cellular features make *T. vaginalis* a suitable alternative model microorganism for the study of cell death.[5] *T. vaginalis* does not contain mitochondria, but possesses another type of membrane-bound organelle called a hydrogenosome, which is able to produce H_2.[6] The mitochondrion is recognized as a "death signal integrator" in multicellular organisms and in all mitochondriated protozoa, since the pathways leading to programmed cell death (PCD) are localized in this organelle.[7] The potential implication of hydrogenosomes in *T. vaginalis* cell death has been called into question and is not yet understood. Previous studies demonstrated that unicellular parasites devoid of mitochondria, such as *T. vaginalis*, undergo a form of cell death resembling apoptosis, the most frequent form of PCD.[8,9] However, despite these contributions many questions remain unanswered, and efforts have continued in the search for promising cytotoxic natural product compounds, especially among the alkaloids.

Candimine (**1**) is the major constituent of the bulbs of *Hippeastrum morelianum* Lem. (Amaryllidaceae). The first and, so far, only isolation of this unusual lactone alkaloid occurred in 1962,[10] from *H. candidum.* A limited knowledge of the biological properties of **1** led to an interest in investigating this homolycorine-type alkaloid in more detail. Recently, our group has revealed that **1** inhibits extracellular nucleotide hydrolysis in *T. vaginalis*, which could be relevant in increasing susceptibility of this parasite to the host immune response.[11] The present study aimed to investigate the cytotoxicity of **1** against *T. vaginalis* by several approaches, in order to identify the mechanism of action concerned.

Results and Discussion

Effect of Candimine (1) on the Viability of *T. vaginalis* Trophozoites. In the first set of experiments, the cytotoxicity of **1** against a fresh clinical isolate of *T. vaginalis* was investigated by constructing a dose−response curve (Figure 1A). The vehicle used for alkaloid solubilization did not exhibit any cytotoxic effect, while the positive control, metronidazole (50 μM), was cytotoxic against 100% of the trophozoites (data not shown). The alkaloid **1** was cytotoxic against trichomonads (from 2.5 μM to 1 mM), but this activity was not dose-dependent. The effect on the fresh clinical isolate was dependent on the inoculum, with a higher cytotoxic activity exhibited against low densities of trophozoites, while against long-term growth isolate, the cytotoxicity was maintained independent of the inoculum (Figure 1B and C). This profile was preserved in subsequent experiments to determine the kinetic growth curve: a low inoculum (5.0×10^4 trophozoites/mL; Figure 2B) was more sensitive to candimine than a higher inoculum (1.0×10^5 trophozoites/mL; Figure 2A). In order to investigate the persistence of the cytotoxic effect, 12 h- and 24 h-treated trophozoites were centrifuged, and the candimine-treated pellet was inoculated in a clean medium, without alkaloid. Untreated trophozoites were submitted to the same procedure to compare the effect. The treated parasites developed a kinetic growth curve similar to the control (parasites grown without **1**), and under both conditions a pronounced growth peak was observed at 48 h, 12 h later than the original growth kinetics of parasites not inoculated in a clean culture medium (Figure 3). Furthermore the cytotoxic effect was found to be reversible, which suggests that the presence of alkaloid is essential to maintain the cytotoxicity.

Ultrastructural Changes in *T. vaginalis* Induced by Candimine (1). An ultrastructural study of the parasite in the presence of **1** revealed striking morphological alterations, although the cell membrane remained intact in all parasites observed. Scanning electron microscopy (SEM) of the trophozoites treated with **1** showed loss of the original shape of the parasites with some fissures on the surface after 6 and 24 h of treatment (Figures S1B−F, Supporting Information), indicating also that this damage apparently

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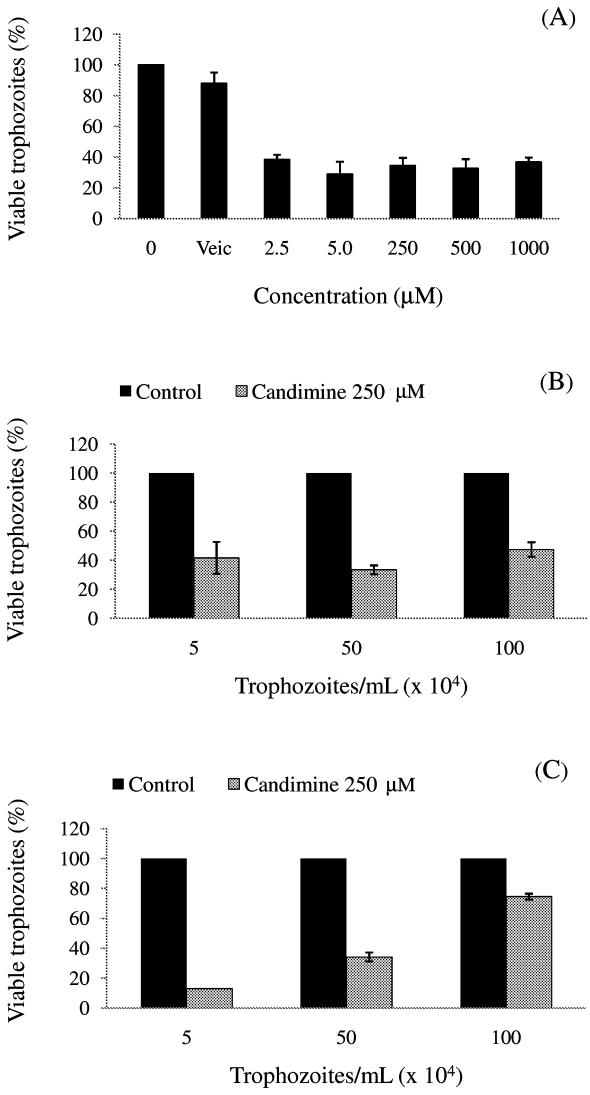

Figure 1. Effect of candimine (**1**) on the number of viable trophozoites of *T. vaginalis*. (A) Dose−response curve of **1** (from 2.5 to 1000 μM) against 5.0×10^5 TV-VP60 trophozoites/mL, after 24 h. Cytotoxicity of candimine (250 μM) against (B) a long-term growth (30236) and (C) a fresh clinical (TV-VP60) isolate, at 5.0×10^4, 5.0×10^5, and 1.0×10^6 trophozoites/mL, after 24 h.

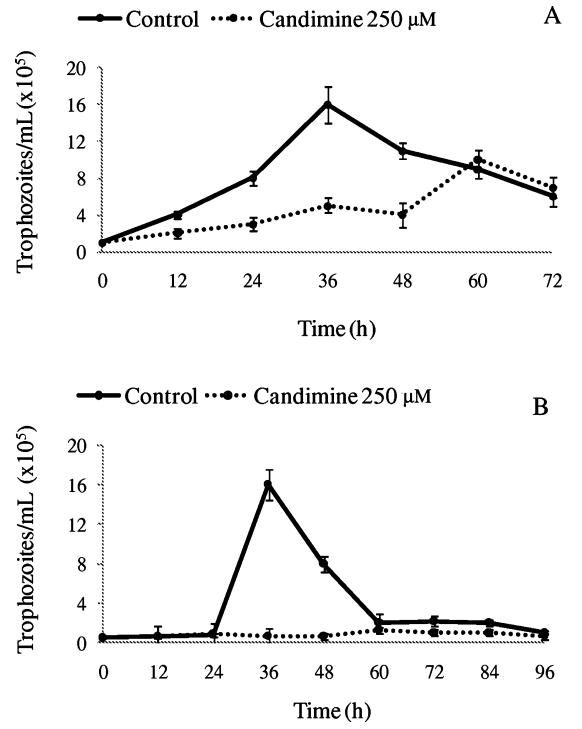

Figure 2. Kinetic growth curve of *T. vaginalis* (TV-VP60) treated with candimine (**1**) (250 μM). (A) Initial inoculum of 1.0×10^5 trophozoites/mL. (B) Initial inoculum of 5.0×10^4 trophozoites/mL.

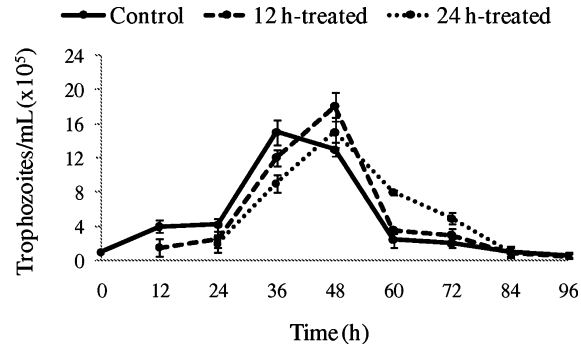

Figure 3. Kinetic growth curve of *T. vaginalis* (TV-VP60) previously treated for 12 and 24 h with candimine (250 μM) after inoculation in culture medium without candimine (**1**).

is time-dependent. Pseudocysts at the flagella internalization stage (Figure S1F, Supporting Information) could be observed after 24 h, as seen with colchicine treatment.[12] Transmission electronic microscopy (TEM) showed additional important features of both 6 h- (Figure S2, Supporting Information) and 24 h-treated trophozoites (Figure S3, Supporting Information). For example, the appearance of numerous vacuoles was evident in treated trophozoites. An intense cytoplasmic vacuolization was observed in several parasites with large peripheral vacuoles between the plasma membrane and the cytoplasm (Figures S2B−F and S3B−H, Supporting Information). A second type of vacuole was also seen, which was striated and located close to the nucleus (Figures S2F, S3D, and S3H, Supporting Information), exhibiting internal membranous structures (Figures S2D, S3E, and S3F, Supporting Information) similar to those observed in mebendazole-treated parasites[13] and containing myelin-like figures[14] (Figures S3C and S3G, Supporting Information). Large vacuoles containing microtubules, such as those seen in paclitaxel- and colchicine-treated trophozoites, were not found,[15] but, otherwise, some vacuoles showing autophagic features, similar to cellular material on degradation, were noted (Figures S2C and S3D-G, Supporting Information). After treatment with **1** for 6 and 24 h, modifications in size, shape, and intracellular localization of hydrogenosomes were apparent. Double membranes were seen in close contact with some hydrogenosomes (Figures S2B−D and S3E−G, Supporting Information), suggesting autophagy. Additionally, some of them were in the process of division, especially by segmentation. These results suggest a possible effect of **1** on the hydrogenosomes, and the simultaneous processes of autophagy and cell division observed could be cellular survival instincts. Additionally, the Golgi apparatus was not disturbed by **1** after 6 h, when a complete golgikinesis was observed by TEM (Figure S2E, Supporting Information). In contrast, in 24 h-treated trophozoites, this organelle could not be identified in the degenerated cytoplasm matrix. The key finding was that **1** did not induce the formation of multinucleated cells, as seen after paclitaxel and nocodazole treatment,[16] with the nucleus remaining intact and displaying an elongated, abnormal shape (Figures S2F, S3D and S3H, Supporting Information). The endoplasmic reticulum was seen undisturbed around the nucleus, forming the outer membrane of the nuclear envelope.[17]

Analysis of cell size and granularity by flow cytometry revealed important features in 6 h- and 24 h-treated trophozoites. Regarding

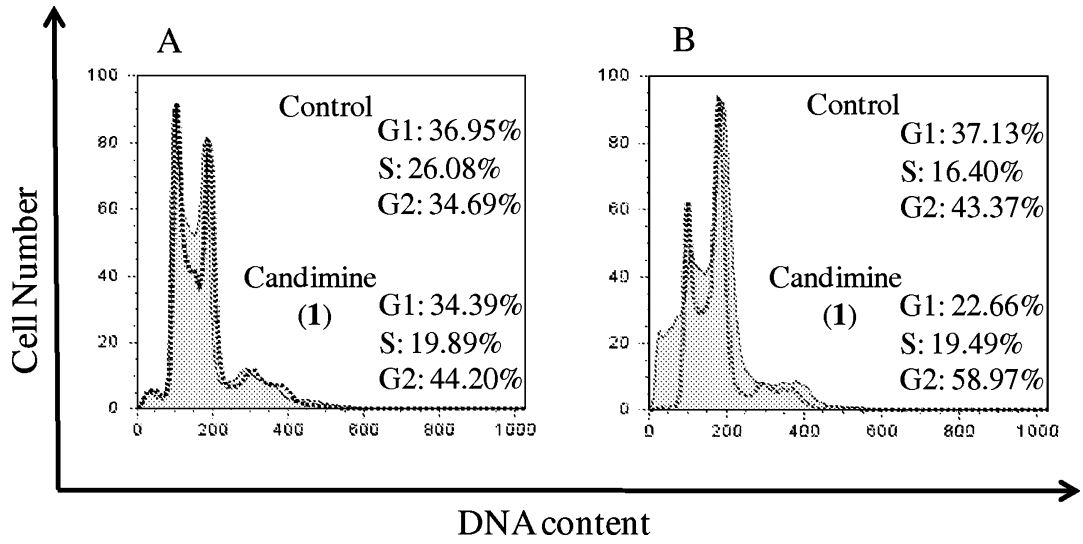

Figure 4. Effect of candimine (**1**) (250 μM) on the cell cycle of *T. vaginalis*: (A) 6 h treatment and (B) 24 h treatment (TV-VP60 isolate). Untreated trophozoites (solid line) and candimine-treated trophozoites (dotted line).

the cell granularity, considered as the vacuolization rate, 6 h-treated trophozoites presented less vacuolization than 24 h-treated parasites (Figures S4A and S4B, Supporting Information). On the other hand, the size of the trophozoites was not altered by **1** (Figures S5A and S5B, Supporting Information). Since determinant morphologic features of apoptotic cell death, such as apoptotic bodies, were not identified in candimine-treated *T. vaginalis*, two other hallmarks of apoptosis were additionally investigated, as described below.

Effect of Candimine (1) on DNA Fragmentation, Phosphatidylserine Exposure, and Cell Cycle in *T. vaginalis*. The results indicated that **1** does not induce phosphatidylserine (PS) exposure (Figures S6A and S6B, Supporting Information). The 6 h- and 24 h-treated trophozoites demonstrated the same electrophoretic profile as controls (untreated parasites), with no fragmentation being induced by **1** (Figure S7, Supporting Information). Additionally, **1** inhibited the *T. vaginalis* cell cycle in the G2 phase, after 24 h (Figure 4B), and this effect was more pronounced than after 6 h (Figure 4A). This result is in agreement with the kinetic growth curve, since inhibition of cell division reflects a reduced number of trophozoites.

Cell death is traditionally classified in two forms: necrosis, which is a passive, uncontrolled form of death, and programmed cell death (PCD), a highly regulated process with defined cellular pathways.[18] Apoptosis is characterized by early chromatin condensation, followed by fragmentation of the nucleus, with or without DNA fragmentation, little or no ultrastructural modification of cytoplasmic organelles, and plasma membrane blebbing, with the membrane remaining intact, and it is usually mediated by a mechanism that involves caspases.[19] In multicellular organisms and in all protozoa harboring mitochondria, this organelle plays a central role in both caspase-dependent and -independent pathways, producing or taking up various molecules that are essential for the execution of PCD.[20] In amitochondriate organisms such as trichomonads, this death machinery is supposed to be absent, and the mechanisms that lead to cell demise remain to be elucidated. Previous studies demonstrated that *T. vaginalis* undergoes a form of apoptosis-like cell death[8] with some morphologic aspects similar to paraptosis.[9] Paraptosis is defined as a nonapoptotic PCD that fails to fulfill the criteria for apoptosis and is morphologically characterized by large cytoplasmic vacuoles and uncleaved, nearly normal chromatin.[9]

As per the Nomenclature Committee on Cell Death (NCCD),[19] the effects of candimine (**1**) on *T. vaginalis* trophozoites reflect the characteristics of dying cells since few effectively dead cells could be seen by microscopy, suggesting that the cells are already dead and have disappeared from the culture. The present study identified new features of *T. vaginalis* cell death since **1** caused arrest of the parasite cell cycle, altered morphologic and ultrastructural features, did not induce the formation of apoptotic bodies, left the cell membrane intact, and did not cause exposure of PS residues nor DNA fragmentation. Thus, candimine-induced cell death in *T. vaginalis* fails to fulfill the criteria for apoptosis and instead exhibits features similar to those of paraptosis. It could be suggested that since small numbers of cytoplasmic double-membrane vesicles were found containing degraded material, the autophagic phenomenon is not responsible for candimine-induced cell death. In fact, the importance of autophagy as a death effector mechanism remains open to question.[21] Autophagy may be responsible for an acceleration of cell death that would still occur in its absence, or it may account for a fraction of the cell death that occurs in response to a certain stimulus.[22,23] Additional molecular studies are clearly necessary to determine whether the cell death mechanism activated by **1** is an unknown form of PCD, considering that the dominant cell death phenotype is determined by the relative speed of the available death programs. In other words, although characteristics of several death pathways may be displayed, only the fastest and most effective death pathway is evident.[24] Therefore, the present study provides relevant biological data regarding candimine (**1**) and represents a starting point for additional studies on homolycorine-type alkaloids.

Experimental Section

General Experimental Procedures. SEM images were recorded using a JEOL 6060 scanning electron microscope, and TEM images were obtained with a JEOL 1210 apparatus. Flow cytometry analysis was carried out using a FACSCalibur flow cytometer and analyzed by FlowJo software. The melting point was measured on a Wagner and Munz Kofler hotbench apparatus. The UV spectrum was obtained on a Agilent 8453 UV−vis spectrophotometer. The 1D ^{1}H and 2D NMR spectra were recorded on a Varian VXR-500 spectrometer (500 MHz), and 1D ^{13}C (100 MHz) NMR spectra were obtained on a Varian Mercury-400 spectrometer (400 MHz). The 1D and 2D NMR spectra were measured at 25 °C using $CDCl_3$, and the chemical shifts are given on the δ scale and were referenced to residual $CHCl_3$ (δ_H 7.26 and δ_C 77.23). The mass spectrum was recorded on a Agilent MSD 5975 Inert XL mass spectrometer. The purity of **1** was checked by HPLC, with the analysis being carried out on a Waters Alliance 2690 analytical chromatograph with a Polar-RP C_{18} column (150 mm × 3.9 mm; Phenomenex). The mobile phase consisted of a linear gradient (50:50 [v/v] methanol−water for 25 min), and a photodiode array (PDA, Waters) detector was employed.

Plant Material. *Hippeastrum morelianum*, a native species from the south of Brazil, was collected in March 2005 at Atibaia City, in

São Paulo, Brazil. The species was identified by Julie Dutilh from the University of Campinas (UNICAMP), São Paulo, Brazil. A voucher specimen is deposited in the herbarium at UNICAMP, Brazil, under reference number UCE 14351.

Extraction and Isolation. Fresh bulbs (2 kg) were extracted five times with ethanol at room temperature over a period of 10 days. The resulting extracts were combined and concentrated under vacuum at 40 °C. The ethanolic extract was acidified with a 10% HCl solution and exhaustively extracted with $CHCl_3$, to give extract A. The acidic solution was adjusted to pH 10 with 25% NH_4OH and extracted with $CHCl_3$ to give extract B. This extract was separated by vacuum-liquid chromatography on silica gel, eluted with acetone, dichloromethane, *n*-butanol, ethyl acetate, and methanol. Chromatography (CH_3Cl-CH_3OH, 100:0 to 10:90) on silica gel of the butanolic fraction afforded **1** (110 mg). Candimine (**1**) exhibited at least 98% purity, as assessed by HPLC-PDA, and showed the following: mp 209−211 °C; UV (MeOH) λ_{max} (log ε) 234 (4.45), 276 (3.92), 309 (3.58) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.68 (1H, s, J = 1.0 Hz, H-10), 6.03 (2H, s, OC*H*$_2$O), 5.63−5.66 (1H, m, H-3), 4.50 (1H, d, J = 1.5 Hz, H-1), 4.37 (1H, d, J = 1.5 Hz, H-2), 4.13 (3H, s, OC*H*$_3$), 3.15−3.19 (1H, m, H-12α), 2.85 (1H, d, J = 9.5 Hz, H-10b), 2.63 (1H, d, J = 9.5 Hz, H-4a), 2.41−4.44 (2H, m, H-11), 2.24−2.29 (1H, m, H-12*β*), 2.09 (3H, s, NC*H*$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 161.7 (C, C-6), 152.8 (C, C-9), 145.7 (C, C-7), 145.5 (C, C-4), 140.4 (C, C-10a), 137.1 (C, C-8), 118.4 (CH, C-3), 111.0 (C, C-6a), 103.6 (CH, C-10), 102.1 (CH_2, O*C*H$_2$O), 81.0 (CH, C-1), 66.9 (CH, C-2), 66.6 (CH, C-4a), 60.8 (CH_3, O*C*H$_3$), 56.2 (CH_2, C-12), 43.6 (CH_3, N*C*H$_3$), 41.2 (CH, C-10b), 28.0 (CH_2, C-11); EIMS *m*/*z* 345 [M$^+$], (<1), 190 (1), 162 (2), 134 (1), 125 (100), 96 (35).

For cytotoxicity assays a candimine (**1**) stock solution (10 mM) was prepared by solubilization of the crystalline powder in distilled water, followed by pH adjustment with HCl and NaOH to a final pH of 6.0, to guarantee a limpid and homogeneous solution. Subsequently, the stock solution was sterilized by filtration (0.22 μm) and diluted to lower concentration solutions.

***Trichomonas vaginalis* Cultures.** *T. vaginalis* isolates 30236 (from the American Type Culture Collection) and TV-VP60[25] were used in this study. The fresh clinical isolate was obtained from epidemiological surveys in Porto Alegre, Rio Grande do Sul, Brazil. Trichomonads were cultured axenically in trypticase−yeast extract−maltose (TYM) medium[26] (pH 6.0), supplemented with 10% (v/v) heat-inactivated adult bovine serum, and incubated at 37 °C (±0.5). Organisms in the logarithmic phase of growth and exhibiting motility and normal morphology were harvested, centrifuged, and resuspended in fresh TYM medium for cytotoxicity assays. All experiments were performed in triplicate and with at least four independent cultures (n = 4).

Cytotoxicity Assay. The cytotoxicity of candimine (**1**) against *T. vaginalis* was determined in vitro. Parasites were counted with a hemocytometer and adjusted to an adequate cellular density for each test, from 5.0×10^4 to 1.0×10^6 trophozoites/mL, in TYM medium. Candimine (**1**) was added to this incubation system at final concentrations of 2.5, 5.0, 50, 100, 250, 500, and 1000 μM. Three controls were carried out: parasites only; a test with parasites and the vehicle for solubilization of the alkaloid; and a test with metronidazole (50 μM) as the positive control. Results were expressed as the percentage of living organisms compared to control parasites after 24 h of incubation, considering motility, normal morphology, and exclusion of trypan blue dye (0.2%). The kinetic growth curves were obtained with TV-VP60 isolates, with inocula of 5.0×10^4 and 1.0×10^5 trophozoites/mL, in the presence and absence of 250 μM **1**, in TYM medium. The results were expressed as the percentage of living organisms compared to control parasites, counted every 12 h, considering motility and normal morphology.

Scanning Electron Microscopy (SEM). *T. vaginalis* trophozoites from the 30236 isolate, at 5.0×10^5 trophozoites/mL, were incubated with candimine (**1**) (250 μM) for 6 and 24 h. Parasites were (i) harvested by centrifugation; (ii) fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2); (iii) postfixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2); (iv) dehydrated in acetone; and (v) critical-point-dried using CO_2. The samples were coated with gold−palladium and observed in the electron microscope.

Transmission Electron Microscopy (TEM). *T. vaginalis* trophozoites from the 30236 isolate, at 5.0×10^5 trophozoites/mL, were incubated with candimine (250 μM) for 6 and 24 h. Parasites were (i) harvested by centrifugation; (ii) fixed with 2.5% glutaraldehyde and 4.0% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2); (iii) postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2); (iv) dehydrated in acetone; and (v) embedded in EmBed. Ultrathin sections were stained with uranyl acetate and lead citrate before being observed in the electron microscope.

Gel Electrophoresis for Detection of DNA Laddering. DNA from *T. vaginalis* (1.0×10^6 trophozoites/mL) treated with candimine (**1**), at 250 μM for 6 and 24 h, was isolated using the Wizard SV Genomic DNA purification system (Promega, Madison, WI), according to the manufacturer's instructions. The purity of extracted DNA was assessed by measuring absorption spectra at λ = 260/280 nm, and the DNA concentration was further determined. The extracted DNA was loaded onto a 2% agarose gel stained with 10× GelRed (Invitrogen, Carlsbad, CA), with a 1 kB DNA ladder (GibcoBRL) as a molecular size marker. After electrophoresis, the gel was viewed under UV illumination and photographed.

Flow Cytometry. Parasites, at 5.0×10^5 trophozoites/mL, were treated or not with candimine (**1**) (250 μM) for 6 and 24 h and then washed twice with phosphate-buffered saline (PBS) before resuspending in 1× binding buffer at a concentration of 1.0×10^6 trophozoites/mL. A 100 μL amount of this suspension was transferred to a 5.0 mL culture tube, and 5.0 μL of Annexin V FITC and 5.0 μL of PI were added. The cells were gently vortexed and incubated for 15 min at 25 °C, in the dark. Then 400 μL of 1× binding buffer was added to each tube and analyzed by flow cytometry within 1 h. The following controls were used: (i) unstained cells; (ii) cells stained with Annexin V FITC (no PI), and (ii) cells stained with PI. A total of 20 000 events were registered. All reagents used were from the Annexin V FITC apoptosis detection kit (BD Pharmingen).

The parasites, at 5.0×10^5 trophozoites/mL, were treated or not with **1** for 6 and 24 h, harvested, and washed three times with PBS. Cells were then fixed with 70% cold ethanol at 4 °C for 45 min. After that, cells were incubated with 100 μg/mL RNase A, at 37 °C for 15 min, and then stained with 50 μg/mL PI (Sigma) at room temperature for 30 min and kept away from the light. Cycling cells were measured with a FACSCalibur flow cytometer, with the acquisition of 50 000 events.

Statistical Analysis. Results were expressed as means ± SD. Statistical analysis was conducted with Student's t test or one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Statistical significance was considered at $p < 0.05$.

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Supporting Information Available: MET and MEV images of trophozoites treated with candimine (**1**), additional flow cytometry histograms showing the effect of **1** on the size and granularity of *T. vaginalis*, a representative graphic expressing the annexin V results, and an agarose gel presenting the DNA fragmentation of the trophozoites after treatment with **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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