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Analogues of Marine Guanidine Alkaloids Are *in Vitro* Effective against *Trypanosoma cruzi* and Selectively Eliminate *Leishmania* (*L.*) *infantum* Intracellular Amastigotes

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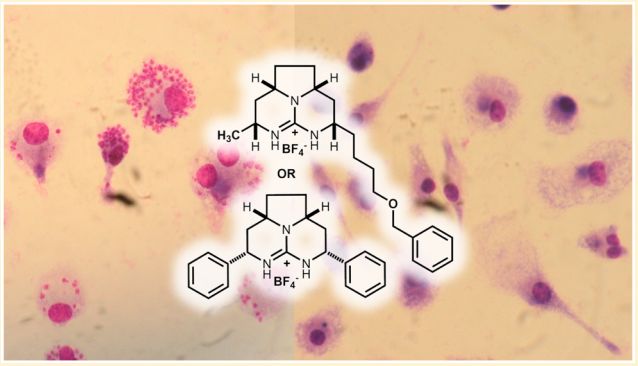
Supporting Information

ABSTRACT: Synthetic analogues of marine sponge guanidine alkaloids showed *in vitro* antiparasitic activity against *Leishmania* (*L.*) *infantum* and *Trypanosoma cruzi*. Guanidines **10** and **11** presented the highest selectivity index when tested against *Leishmania*. The antiparasitic activity of **10** and **11** was investigated in host cells and in parasites. Both compounds induced depolarization of mitochondrial membrane potential, upregulation of reactive oxygen species levels, and increased plasma membrane permeability in *Leishmania* parasites. Immunomodulatory assays suggested an NO-independent effect of guanidines **10** and **11** on macrophages. The same compounds also promoted anti-inflammatory activity in *L.* (*L.*) *infantum*-infected macrophages cocultivated with splenocytes, reducing the production of cytokines MCP-1 and IFN-γ. Guanidines **10** and **11** affect the bioenergetic metabolism of *Leishmania*, with selective elimination of parasites via a host-independent mechanism.



Considerable attention has been raised to address the effective cure of neglected tropical diseases (NTDs) in the past decade, which globally impact mainly economically disfavored nations. These infectious pathogenies, of which most have parasites as the causative agents, have spread and now affect populations in developed countries as well. Most drugs to treat NTDs were developed decades ago and show harmful, even deadly, adverse effects. Therefore, the search for new drugs or vaccines to treat human neglected diseases is a priority for the World Health Organization and other organizations.[1,2]

Two of the deadliest NTDs are leishmaniasis and Chagas disease. Leishmaniasis affects 12 million people in 98 countries mainly in Africa, Asia, and Latin America.[3,4] Two distinct human pathological conditions are observed for leishmaniasis, cutaneous and visceral. *Leishmania* (*L.*) *infantum* is the etiologic agent of visceral leishmaniasis (VL) in South America and southern Europe countries, while *Leishmania* (*L.*) *donovani* is in Asian and African countries. Visceral leishmaniasis promoted by *L.* (*L.*) *infantum* is fatal, with a mortality rate of 100% if untreated.[4] Leishmaniasis is included as a target disease by DNDi (Drugs for Neglected Diseases Initiative) and iOWH (Institute for One World Health).

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Table 1. Antileishmanial/Antitrypanosomal Activities and Mammalian Cytotoxicity of Guanidines and the Standard Drugs Benznidazole and Miltefosine

	T. cruzi trypomastigotes[a]	*L. (L.) infantum* promastigotes[b]	*L. (L.) infantum* amastigotes	NCTC cells	selectivity index[e]	
compound	IC_{50}[c] (μM) 95% CI[f]	IC_{50} (μM) 95% CI	IC_{50} (μM) 95% CI	CC_{50}[d] (μM) 95% CI	*Leishmania* amastigotes	*Trypanosoma cruzi* trypomastigotes
1	8 (7−9)	42 (36−49)	5 (2−14)	42 (35−51)	8	5
2	88 (65−119)	NA[g]	NA	>150	ND[h]	>2
3	49 (43−56)	NA	NA	>150	ND	>2
4	22 (21−24)	109 (97−123)	NA	>150	ND	>7
5	8 (8−8)	53 (45−64)	NA	100 (93−107)	ND	12
6	4 (2−9)	59 (47−74)	NA	84 (80−88)	ND	28
7	4 (4−7)	48 (39−61)	NA	57 (45−71)	ND	19
8	NA	NA	NA	>150	ND	ND
9	4 (4−5)	7 (4−10)	18 (15−21)	65 (58−73)	4	16
10	41 (36−47)	57 (50−65)	5 (4−7)	138 (115−164)	25	3
11	9 (8−10)	34 (30−39)	2 (1−4)	45 (42−49)	20	5
12	NA	NA	8 (6−12)	>150	>19	ND
13	NA	NA	NA	>150	ND	ND
14	35 (31−41)	9 (9−10)	19 (6−56)	116 (105−128)	6	3
15	79 (31−197)	57 (50−65)	24 (23−25)	70 (57−85)	3	0.9
16	69 (55−86)	47 (40−54)	NA	60 (46−79)	ND	0.9
17	0.9 (0.9−10)	NA	0.8 (0.3−2)	2 (2−3)	3	2
18	NA	NA	49 (34−72)	>150	>3.0	ND
benznidazole	16 (14−20)	ND	ND	>150	ND	>9
miltefosine	ND	16 (15−17)	17 (12−24)	122 (95−157)	7	ND

[a]Cell viability was determined using the resazurin assay. [b]Cell viability was determined using the MTT assay. [c]IC_{50}: 50% inhibitory concentration: concentration of compound required to reduce 50% of parasites. [d]CC_{50}: 50% cytotoxic concentration: concentration of compound that reduces the viability of mammalian cells by 50%. All data represent mean values for at least two separate experiments. [e]Selectivity index: ratio of CC_{50}/IC_{50}. [f]95% CI: 95% confidence interval. [g]NA: not active to the highest concentration of 100 μM. [h]ND: not determined.

Chagas disease is caused by *Trypanosoma cruzi* as a potentially fatal disorder resulting in cardiomegaly and megacolon in about 30% of the patients.[5] Chagas disease remains one of the most severe public-health problems in 21 countries of Latin America, causing more than 7000 deaths per year without early and successful antiparasitic treatment. Over 25 million people are at risk of infection by *T. cruzi*, and about 7 million people are infected worldwide.[1] Chagas disease has spread into several European countries and Japan, probably due to population migration. Effective treatments for Chagas disease are urgently needed.[6]

Large drug screening campaigns to find new drugs to treat NTDs are currently being developed. New criteria for improving the success of clinical drug candidates with potential use in NTDs have been recently reviewed.[7] While most hit compounds for infectious diseases were discovered using phenotypic assays, subsequent optimization efforts need to establish the drug target.[7] Among various classes of natural and synthetic compounds that present antiparasitic activity, guanidines have shown to be of particular interest because of their potent activity and suitable pharmacokinetic profile.[8−11] On the basis of the findings recently reported by some of us showing potent and selective antileishmanial and antitrypanosomal activity for marine sponge-derived guanidine alkaloids,[11] herein we report the results of the investigation of 18 synthetic guanidines as antiparasitic agents against *Leishmania* and *Trypanosoma* parasites. The two most selective synthetic guanidines active against *L. (L.) infantum* were further investigated for the mechanism of action on the parasites and for their immunomodulatory activity.

RESULTS

Antileishmanial Activity and Antitrypanosomal Activity. *L. (L.) infantum* promastigotes were incubated for 48 h with guanidines **1**−**18**, and the viability of cells was determined by the MTT colorimetric assay. Compounds **1**−**18** showed IC_{50} values in the range between 6.6 and 110 μM. Intracellular *L. (L.) infantum* amastigotes were also treated with compounds **1**−**18**. The observed IC_{50} values were in the range between 0.8 and 49 μM. Miltefosine was used as a standard drug (IC_{50} of 16 μM). *T. cruzi* trypomastigotes were incubated with the same series of compounds for 24 h, and the cell viability was determined by the resazurin assay. Guanidines **1**−**18** displayed antitrypanosomal activity with IC_{50} values in the range between 0.9 and 88.5 μM. These results are presented in Table 1. Benznidazole was used as standard drug (IC_{50} of 16 μM) (Table 1).

Determination of the 50% Cytotoxic Concentration (CC_{50}) in Mammalian Cells. Aiming to evaluate the mammalian cytotoxicity of compounds **1**−**19**, NCTC cells (clone 929) were incubated for 48 h, and the cell viability was determined by the MTT colorimetric assay method. Guanidines **1**−**19** showed toxicity, with CC_{50} values in the range between 2.4 and >150 μM. Miltefosine and benznidazole were used as standard drugs and showed CC_{50} values of 122 and >150 μM, respectively (Table 1).

Immunomodulatory Potential of Compounds. Quantification of Nitric Oxide (NO). Guanidines **10** and **11** showed the highest selectivity index (25 and 20, respectively) as antileishmanial agents (Table 1, selectivity index) and were selected for further mechanism of action assays. Compounds **10** and **11** were incubated with peritoneal macrophages, and the

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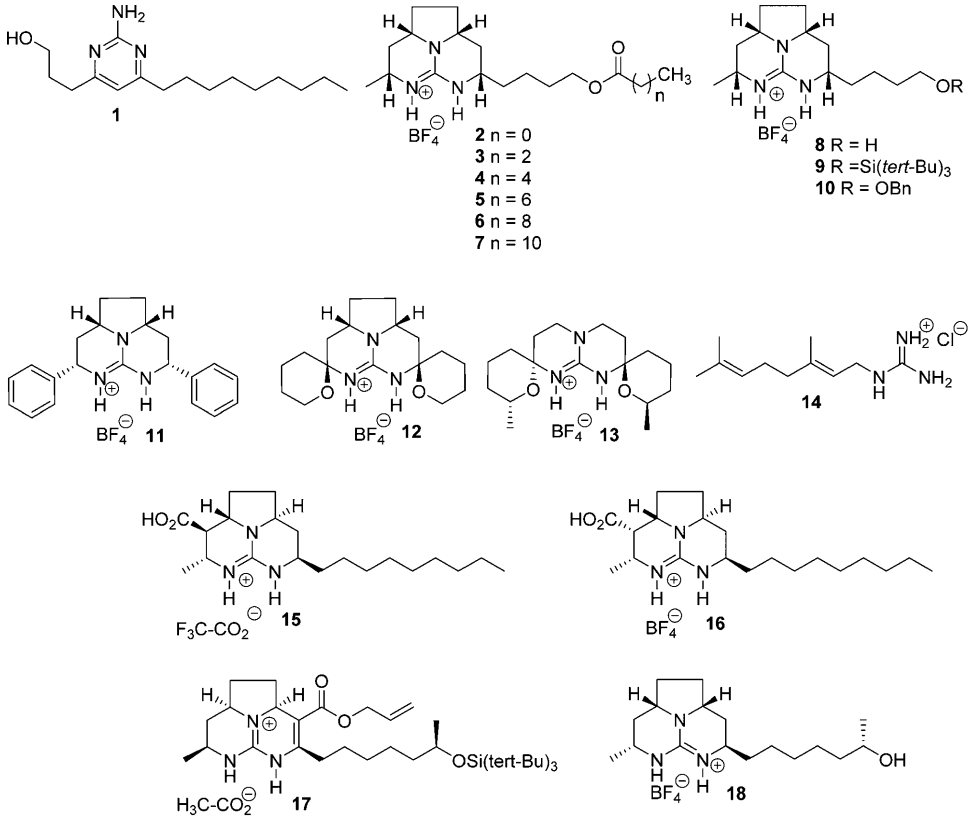

NO content was evaluated after 24 h. Both compounds revealed no capacity to upregulate NO production (Figure 1). Bacterial lipopolysaccharide (LPS) was used as the positive control.

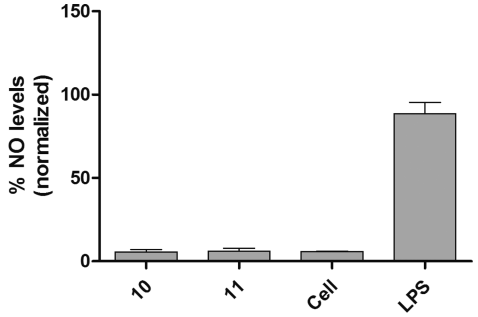

Figure 1. Evaluation of nitric oxide levels of peritoneal macrophages incubated with compounds **10** and **11** at the respective IC_{50} values (intracellular amastigote assay = 5 and 2 μM, respectively) for 24 h. The Griess reaction was used to measure the concentration of nitrite (NO_2^-). *$p < 0.05$.

Cellular Immune Response of *L.* (*L.*) *infantum*-Infected Macrophages Cocultivated with Splenocytes. Noninfected and *L.* (*L.*) *infantum*-infected macrophages were treated for 48 h with guanidines **10** and **11** and were cocultivated with or without splenocytes. The production of cytokines MCP-1, IL-6, IL-10, TNF, and IFN-γ was detected by flow cytometry analysis. Compound **10** decreased the production of cytokines MCP-1 ($p < 0.05$) and IFN-γ ($p < 0.05$) in both noninfected and *L.* (*L.*) *infantum*-infected macrophages. The same result was observed when macrophages were cocultivated with or without splenocytes. A similar downregulation effect ($p < 0.001$) of MCP-1 was also observed in splenocytes incubated with guanidines **10** and **11**. However, compounds **10** and **11** did not promote alteration of cytokines TNF, IL-6, and IL-10 levels (data not shown). The production of cytokine IFN-γ was negatively modulated by **10** and **11** in noninfected as well as in *L.* (*L.*) *infantum*-infected macrophages cocultivated with splenocytes (Figure 2).

Evaluation of Lethal Action of Guanidines 10 and 11 on *L.* (*L.*) *infantum*. Permeability of Plasma Membrane. Guanidines **10** and **11** (57 and 34 μM, respectively) promoted a slight alteration in plasma membrane permeability of *L.* (*L.*) *infantum* promastigotes, measured using the fluorescent probe SYTOX Green. Compounds **10** and **11** promoted approximately 25% and 10% higher fluorescence intensity (Figure 3) after 60 min when compared to untreated parasites ($p < 0.05$) (Figure 3). Triton X-100 was used for normalization as 100% permeabilization.

Evaluation of Mitochondrial Membrane Potential. Alteration of *L.* (*L.*) *infantum* mitochondrial membrane potential was investigated in the presence of **10** and **11** (57 and 34 μM, respectively). Promastigotes were incubated with both compounds separately for 60 min, and the mitochondrial membrane potential was monitored using the fluorescent probe rhodamine 123. Both compounds induced a significant reduction ($p < 0.001$) of the fluorescence intensity of rhodamine 123 when compared to untreated *L.* (*L.*) *infantum* promastigotes. The 70% depolarization intensity caused by both compounds was similar to the level observed with the positive control sodium azide (Figure 4).

Reactive Oxygen Species (ROS) Regulation. Production of ROS by *L.* (*L.*) *infantum* promastigotes in the presence of guanidines **10** and **11** was determined using the fluorescent probe H_2DCF-DA. After 120 min of incubation, guanidine **10** promoted significant alteration in *L.* (*L.*) *infantum* ROS levels ($p < 0.001$), resulting in a 3-fold higher content than the

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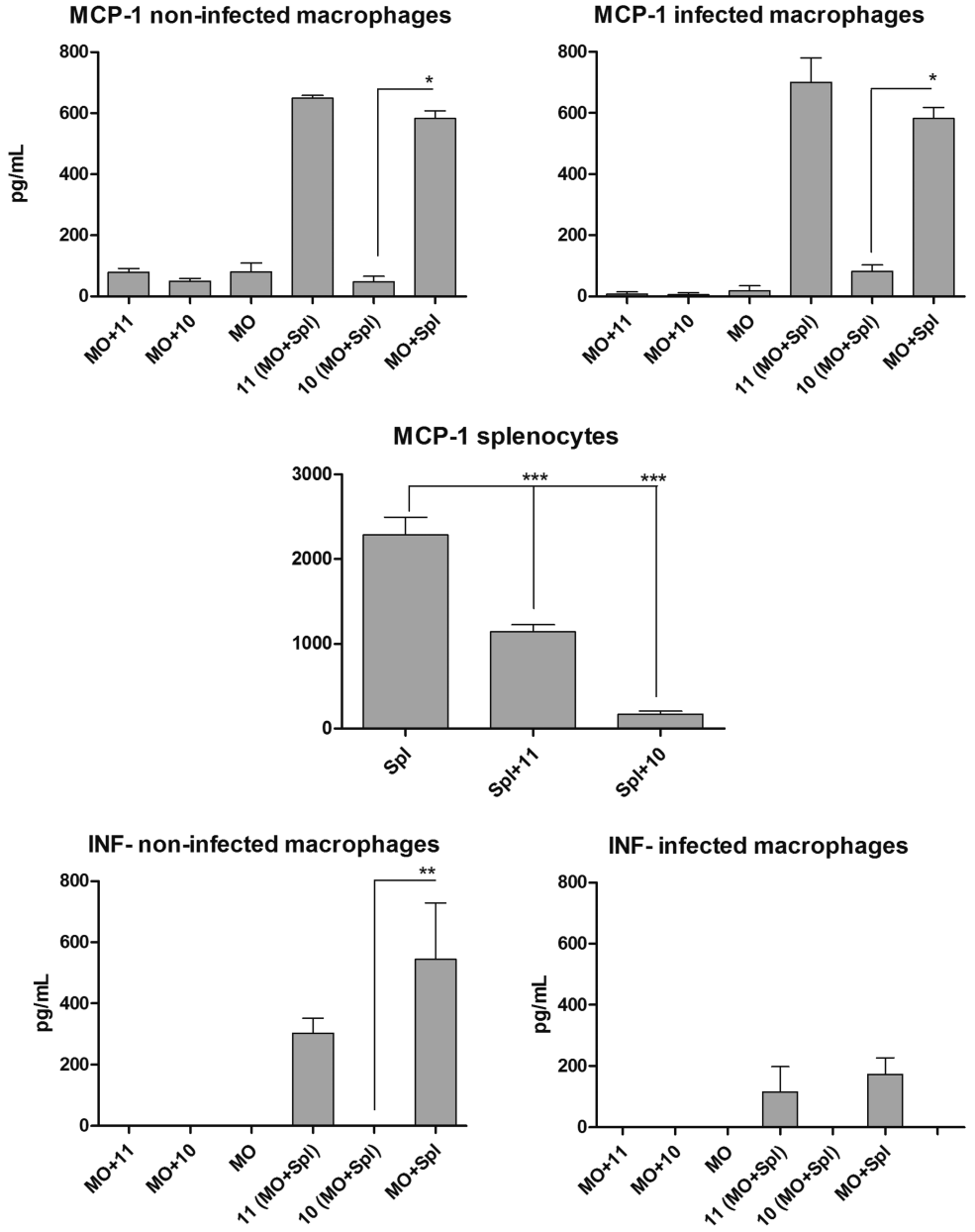

Figure 2. Immunomodulatory effect of compounds **10** (30 μM) and **11** (15 μM) on the production of MCP-1 and IFN-γ by uninfected macrophages, lymphocytes (LY), and *L.* (*L.*) *infantum*-infected macrophages cocultivated or not with lymphocytes. Results are expressed in pg/mL. Cytokines were measured with a CBA (cytometric bead array). *Escherichia coli* lipopolysaccharide for macrophages and concanavalin A (ConA) for lymphocytes (1 μM) were used as controls. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

sodium azide positive control. Compound **11** also promoted alteration of ROS levels in *L.* (*L.*) *infantum*, 60% higher than in untreated parasites ($p < 0.05$) (Figure 5).

DISCUSSION

Several guanidine compounds have been evaluated as antiparasitic compounds and showed significant *in vitro* antileishmanial and antitrypanosomal activity when compared to standard drugs.[11−18] On the basis of our recent discovery of a series of alkaloids from the sponge *Monanchora arbuscula*, active against *L.* (*L.*) *infantum* and *T. cruzi* parasites,[11] we evaluated an additional series of 18 synthetic guanidine derivatives against *L.* (*L.*) *infantum* and *T. cruzi*.

Among 18 guanidine compounds tested against *T. cruzi* trypomastigotes, 14 (77%) showed activity below 100 μM, of which compounds **1**, **5**, **6**, **7**, **9**, **11**, and **17** presented IC_{50} values below 10 μM. Guanidines **5**, **6**, **7**, and **9** showed low cytotoxicity and the highest selectivity indexes (SI), between 12 and 28. Guanidine **17** was approximately 17-fold more effective than the standard drug benznidazole. Guanidines **6** and **7** were about 4-fold more effective, presenting a trypanocidal activity observed by the lack of mitochondrial activity measured by resazurin. Other guanidine compounds were tested against *T. cruzi* and showed potent activity, in the range between 1 and 10 μM,[13] similarly as observed for compounds **1**, **5**, **6**, **7**, **9**, **11** and **17**. Compound **17** displayed potent antitrypanosomal activity, with an IC_{50} value of 0.9 μM. However, the selectivity index was below 10 and therefore does not meet an important criterion for the hit stage.

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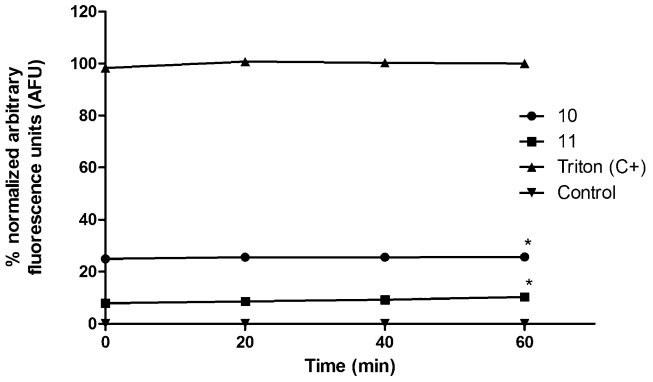

Figure 3. Permeability of *L. (L.) infantum* plasma membrane incubated with compounds **10** (57 μM) and **11** (34 μM), assessed with the vital dye SYTOX Green. Promastigotes were treated with Triton X-100 and used as 100% permeabilization. A control group (untreated) was also included. Fluorescence was determined using a fluorimetric microplate reader (FilterMax F5 multi-mode microplate reader) with excitation and emission wavelengths of 485 and 520 nm, respectively. *$p < 0.05$.

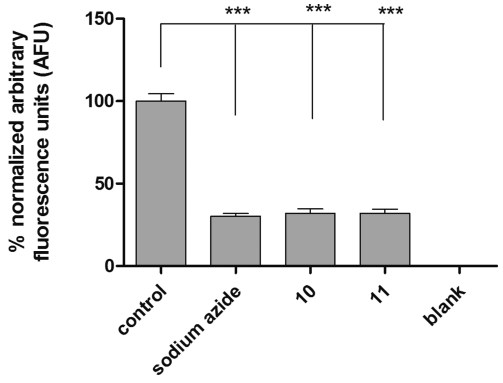

Figure 4. Evaluation of the mitochondrial membrane potential of *L. (L.) infantum* promastigotes incubated with compounds **10** (57 μM) and **11** (34 μM). Untreated parasites were also incubated with rhodamine 123 (control group). Fluorescence was determined using a fluorimetric microplate reader (FilterMax F5 multi-mode microplate reader) with excitation and emission wavelengths of 488 and 525 nm, respectively. Sodium azide (10 mM) was used as positive control. ***$p < 0.001$.

Guanidine compounds isolated as natural products and synthetic derivatives have also shown potent antileishmanial activity.[14−18] Eleven (61%) out of 18 synthetic guanidines herein evaluated displayed antileishmanial activity against promastigotes, nine of which were active against *L. (L.) infantum* intracellular amastigotes below 100 μM. Five of these displayed IC_{50} values below 10 μM (**1**, **10**, **11**, **12**, and **18**). Considering the recommendations of the DNDi for antileishmanial agents,[19] an $IC_{50} < 10$ μM and SI ≥ 10 are determinant features for antileishmanial compounds at the hit stage.[19,20] Guanidines **10** and **11** presented SIs of 25 and 20, respectively, and were selected for investigation of the lethal mechanism of action against *Leishmania* parasites.

Due to the complexity of intracellular amastigote defenses, an antileishmanial suitable hit compound needs to specifically target the parasite that lives in the acidic parasitophorous vacuole inside the macrophage.[21] Guanidines **10** and **11** showed more potent antiparasitic activity against intracellular amastigotes than to extracellular promastigotes. In order to investigate if the antileishmanial activity of **10** and **11** against

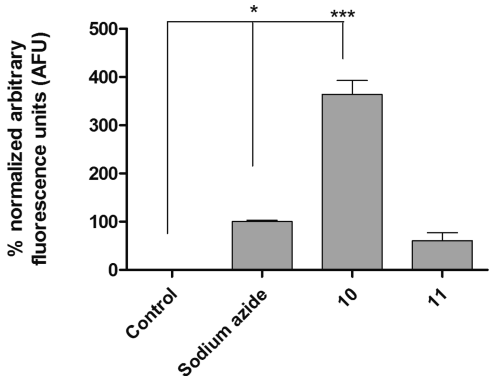

Figure 5. Evaluation of reactive oxygen species levels in *L. (L.) infantum* promastigotes incubated with compounds **10** (57 μM) and **11** (34 μM). The parasites were incubated with the probe H_2DCf-DA, and the fluorescence intensity was detected using a fluorimetric microplate reader (FilterMax F5 multi-mode microplate reader) with excitation and emission wavelengths of 485 and 520 nm, respectively. Sodium azide (10 mM) was used as positive control. *$p < 0.05$, ***$p < 0.001$.

intracellular amastigotes could be ascribed to an immunomodulatory effect in host cells, we evaluated cytokine production by macrophages in the presence of guanidines **10** and **11**. This approach enables differentiating compounds that are directly active in the parasite from those that exert an antiparasitic effect by an immunomodulatory response.[22]

Immunomodulators have been investigated as drugs for the clinical treatment of leishmaniasis, but with limited efficacy when administered alone.[23,24] Guanidines **10** and **11** caused no upregulation of NO in macrophages. Flow cytometry analysis indicated that **10** and **11** significantly suppressed the production of IFN-γ and MCP-1 cytokines in the host cell, causing no significant alteration of TNF, IL-6, and IL-10 levels. Cytokines play different roles during a *Leishmania* infection in macrophages. In *L. (L.) infantum*-infected human macrophages, treatment with monocyte chemotactic protein chemokine (MCP-1) enhances NO production and the antileishmanial activity.[25] MCP-1 is a leukocyte activator, related to the production of proinflammatory cytokines and microbicidal molecules. It promotes NO release and increased parasite killing by *T. cruzi*-infected macrophages. Upregulation of cytokine IL-6 has been associated with a lethal outcome of the disease, preceding death in patients with visceral leishmaniasis.[26] High levels of IFN-γ, IL-10, and IL-6 are associated with human visceral leishmaniasis and with leishmaniasis persistence.[27,28]

In addition to the downregulation of inflammatory cytokines induced by guanidines **10** and **11** in *Leishmania*-infected macrophages cocultived with splenocytes, both compounds promoted the death of intracellular amastigotes. Attenuation of immune response by immunomodulators, such as pentoxyfylline associated with pentavalent antimony, decreases tissue inflammation in patients, leading to a curative therapy associated with the decrease of TNF and IFN-γ levels.[29] These results suggest that guanidines **10** and **11** exert antileishmanial activity by a direct effect on parasites, promoting anti-inflammatory modulation in host cells, which might contribute to a beneficial prognosis of the disease.

Because the antileishmanial activity of guanidines **10** and **11** was associated with other mechanisms than merely macrophage activation, we investigated possible intracellular targets in

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Leishmania. Damage to the plasma membrane and mitochondria was then evaluated using different fluorescent probes, as mitochondria are unique machinery in protozoan parasites and have been shown as a potential target for antiparasitic drugs.[30−33] The permeability of the *L.* (*L.*) *infantum* plasma membrane was observed using green-fluorescent nuclear and chromosome counter stain, which enters live cells and exhibits >500-fold fluorescence enhancement after binding nucleic acids.[34] Both guanidines **10** and **11** increased fluorescence levels, indicating a slight membrane permeabilization within 60 min of incubation.

The mitochondrial membrane potential is crucial for ATP generation in the respiratory chain.[33] In contrast to mammalian cells, where the presence of multiple mitochondria ensures compensation for functionally impaired ones, trypanosomatids present a single mitochondrion.[30] We investigated the effects of **10** and **11** on *Leishmania* mitochondria at initial contact. Both compounds **10** and **11** induced rapid depolarization of the mitochondrial membrane potential to levels similar to the positive control. Amphotericin B, used in the treatment of leishmaniasis, also increases permeability of the *Leishmania* plasma membrane, followed by a rapid decrease in the mitochondrial membrane potential.[35] Paromomycin significantly decreases the mitochondrial membrane potential of *Leishmania*, indicating that this organelle might be the ultimate drug target.[31,32]

Leishmania parasites present various defense mechanisms to cope with oxidative stress, including expression of antioxidant enzymes such as trypanothione,[36] superoxide dismutase,[37] peroxidases,[38] trypanothione S-transferase,[39] and 6-phosphogluconate dehydrogenase.[40] These enzymes, including iron superoxide dismutase and peroxiredoxin, are located in the mitochondria.[41] During oxidative stress, excessive amounts of ROS are produced in the mitochondria. These oxidant components, including superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorite (OCl^-), and peroxynitrite ($ONOO^-$), are reactive signaling chemicals that accumulate under pathological conditions and lead to oxidative stress caused by dysfunction of the *Leishmania* mitochondrial respiratory chain.[42] Guanidine **10** was the most effective compound at enhancing ROS levels in *Leishmania*, while **11** showed a similar effect but to a lesser extent when compared to the positive control (sodium azide). Our results are in agreement with the mechanism of action (MoA) of other guanidines in protozoa. The antimalarial guanidine proguanil collapse the mitochondrial membrane potential of the parasite *Plasmodium*, the etiologic agent of malaria.[43] Considering similar effects in mitochondria, it seems probable that the guanidines herein investigated affect the respiratory system of *Leishmania*, leading to parasite death. This outcome may be a useful strategy to kill protozoans, as these microorganisms present a single mitochondrion. The capacity of these compounds to induce parasite death in the intracellular form without host cell activation is another significant outcome, important to provide new drug candidates useful to treat immunodeficient patients.

A series of 18 guanidines tested against *T. cruzi* and *L.* (*L.*) *infantum* parasites provided an elevated number of selective hits, with IC_{50} values below 10 μM. The two most active antileishmanial guanidines, compounds **10** and **11**, induce depolarization of the mitochondrial membrane potential, promoting a strong alteration of ROS levels in *Leishmania* parasites. Despite an effective detoxification system of these parasites, if not rapidly scavenged, the accumulated ROS are able to induce strong cellular damage. This effect might contribute to oxidative stress induced by guanidines **10** and **11**, leading to *L.* (*L.*) *infantum* elimination. A computational study was performed to predict pan-assay interference compounds (PAINS), but none of the compounds were predicted as PAINS (Supporting Information). The *in silico* studies, the *in vitro* potency of guanidines **10** and **11** (<10 μM), a selectivity index > 10, and the respective MoA against *Leishmania* parasites without affecting host cells allow us to select these compounds as possible hits for future investigations. Further experiments are in progress in order to gather information about ADMET properties and *in vivo* efficacy of compounds **10** and **11**. Results will be reported in due time.

EXPERIMENTAL SECTION

Chemicals. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue; MTT), resazurin (AlamarBlue), SYTOX Green, rhodamine 123, and H_2DCFDA were purchased from Molecular Probes. Giemsa stain, DMSO, and MeOH were obtained from Merck. RPMI-1640 medium, M-199 medium, Hank's balanced salts, phosphate-buffered saline (PBS), trypan blue stain, and miltefosine were obtained from Sigma. Other reagents were purchased from Sigma-Aldrich. The cytometric beads array (CBA, mouse inflammation kit, Biosciences) was from Becton Dixon.

Synthesis of Substrate Compounds. Guanidines **1**−**18** were tested as antileishmanial and antitrypanosomal compounds. Pyrimidine **1**, tricyclic guanidines **2**−**11** and **15**−**18**, tetracyclic guanidine **13**, and pentacyclic guanidine **12** were prepared as previously described.[17,44−54] The naturally occurring guanidine alkaloid nitensidine D (**14**)[50] was prepared as its hydrochloride salt in 53% yield by the reaction of geranylamine with 1*H*-pyrazole-1-carboxamidine hydrochloride (see Supporting Information).

General Bioassay Procedures. Golden hamsters and BALB/c mice were obtained by the animal breeding facility at the Adolfo Lutz Institute-SP, Brazil. The animals were maintained in sterilized cages under a controlled environment and received water and food *ad libitum*. Animal procedures were performed with the approval of the Research Ethics Commission (project number CEUA IAL/Pasteur 02/2011), in agreement with the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

Parasites and Mammalian Cell Maintenance. *L.* (*L.*) *infantum* (MHOM/BR/1972/LD) was maintained in golden hamsters (*Mesocricetus auratus*) up to approximately 60−70 days postinfection. Promastigotes were maintained in M-199 medium supplemented with 10% fetal calf serum (FBS) and 0.25% hemin at 24 °C. Amastigotes were obtained from the spleen of previously infected hamsters and purified by differential centrifugation. Macrophages were collected from the peritoneal cavity of BALB/c mice by washing them with RPMI-1640 medium supplemented with 10% FBS and were maintained at 37 °C in a 5% CO_2-humidified incubator. Trypomastigotes of *T. cruzi* (Y strain) were maintained in Rhesus-monkey kidney cells (LLC-MK2-ATCC CCL 7), cultivated in RPMI-1640 medium supplemented with 2% FBS at 37 °C in a 5% CO_2-humidified incubator. The murine conjunctive cells (NCTC clone 929, ATCC) were maintained in RPMI-1640 supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. NCTC-clone 929 cells were cultivated in M-199 medium, supplemented with 10% FBS at 37 °C in a 5% CO_2-humidified incubator.

Determination of the 50% Inhibitory Concentration (IC_{50}). Promastigotes in late growth phase (nonstationary at 3×10^7/mL, passage 5) were counted in a hemocytometer chamber and seeded at 1×10^6/well, with a final volume of 150 μL. The compounds were dissolved in DMSO and diluted in M-199 medium in 96-well microplates, with the highest concentration of 100 μM for 48 h at 24 °C. The parasite viability was determined using the MTT colorimetric assay.[55] The optical density was read at 570 nm (FilterMax F5 multimode microplate reader, Molecular Devices) using control wells

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without drugs (100% viability) and without cells (blank). The control group consisted of promastigotes incubated with 0.5% DMSO. Miltefosine was used as a standard drug. Compounds were tested to the highest concentration of 100 μM and were reported as NA (not active) when the IC_{50} value was above this concentration. For amastigotes, Peritoneal macrophages were collected from the peritoneal cavity of BALB/c mice, and the macrophages were seeded at 1×10^5/well for 24 h in a 16-well slide. Amastigotes were prepared as described previously in a 1:10 ratio of macrophages to amastigotes for 24 h at 37 °C in a 5% CO_2-humidified incubator. The compounds were incubated with infected macrophages for 120 h. Miltefosine was used as standard drug. Subsequently, the cells were fixed with MeOH, stained with Giemsa, and observed using a light microscope. The parasite burden was determined by the number of infected macrophages out of 400 cells.[20] Compounds were tested to the highest concentration of 100 μM and were reported as NA when the IC_{50} value was above this concentration.

Trypomastigotes of *T. cruzi*. Free trypomastigotes were counted in a hemocytometer chamber and seeded at 1×10^6 cells per well in 96-well microplates. The compounds were diluted in RPMI-1640 medium and incubated in highest concentrations to 150 μM for 24 h at 37 °C in a 5% CO_2-humidified incubator. The parasite viability was determined using resazurin (0.011% in PBS).[56] The optical density was read at 570 nm using control wells without drugs (100% viability) and without cells (blank). The control group consisted of trypomastigotes incubated with 0.5% DMSO. Benznidazole was used as a standard drug. Compounds were tested to the highest concentration of 100 μM and were reported as NA when the IC_{50} value was above this concentration.

Cytotoxicity in Mammalian Cells. NCTC cells were counted in a hemocytometer chamber, seeded at 6×10^4/well, and incubated in highest concentrations to 150 μM for 48 h at 37 °C in a 5% CO_2-humidified incubator. The cell viability was determined using the MTT assay.[55] Miltefosine was used as standard drug. The selectivity index was determined using the relationship CC_{50} against NCTC/IC_{50} against parasites.

Evaluation of Plasma Membrane Permeability. Late growth-phase (nonstationary at 3×10^7/mL, passage 7) promastigotes were washed in PBS, seeded at 2×10^6/well, and incubated with 1 μM SYTOX Green for 15 min at 24 °C.[34] The compounds **10** and **11** were added at their respective IC_{50} values (57 and 34 μM, respectively), and the fluorescence was measured every 20 min for 60 min total. The maximum permeabilization was obtained with 0.1% Triton X-100. Fluorescence intensity was determined using a fluorimetric microplate reader (FilterMax F5 multi-mode microplate reader, Molecular Devices) with excitation and emission wavelengths of 485 and 520 nm, respectively. The following internal controls were used in the evaluation: (i) the background fluorescence of test compounds, (ii) the possible interference of DMSO, (iii) untreated promastigotes, and (iv) medium without any cells. Samples were tested in triplicate.[57]

Evaluation of Mitochondrial Membrane Potential. The change in mitochondrial membrane potential in intact promastigotes was estimated by measuring rhodamine-123 accumulation.[58] Parasites (2×10^6 promastigotes/mL) were resuspended in Hank's balanced salt solution−glucose (HBSS-Glc) and incubated with **10** and **11** at the respective IC_{50} values (57 and 34 μM, respectively) for 60 min at 25 °C. Subsequently parasites were incubated with rhodamine-123 for 10 min (0.3 μg/mL, 5 min, 37 °C), washed by centrifugation, and resuspended in HBSS-Glc, and the fluorescence was determined using a fluorimetric microplate reader with excitation and emission wavelengths of 485 and 520 nm, respectively. Sodium azide (10 mM) was used as the positive control.[59] The following internal controls were used in the evaluation: (i) the background fluorescence of test compound, (ii) the possible interference of DMSO, (iii) untreated promastigotes, and (iv) medium without any cells. Samples were tested in triplicate.

Detection of ROS. Promastigotes (2×10^6/well) were washed with HBSS and incubated with **10** and **11** at the respective IC_{50} values (57 and 34 μM, respectively) for 60 min. H_2DCFDA was added (5 μM), and the cells were incubated for 15 min. Fluorescence intensity was evaluated using a fluorimetric microplate reader with excitation and emission wavelengths of 485 and 520 nm, respectively. Sodium azide (10 mM) was used as the positive control.[60−62] The following internal controls were used in the evaluation: (i) the background fluorescence of test compounds, (ii) the possible interference of DMSO, (iii) untreated promastigotes, and (iv) medium without any cells. Samples were tested in triplicate.

Quantification of Nitric Oxide. NO content was measured in the culture supernatants from peritoneal macrophages treated with compounds **10** and **11** at the respective IC_{50} values (intracellular amastigote assay = 5 and 2 μM, respectively) for 24 h and analyzed by Griess assay.[63] Results obtained were extrapolated from a standard curve prepared with $NaNO_2$ at different concentrations (0 to 400 μM). Bacterial LPS (1 μg/mL) was used as a positive control. Samples were tested in triplicate.

Cellular Immune Response Assay. Peritoneal Macrophages Cocultived with Splenocytes and *in Vitro* Infection. Peritoneal macrophages were isolated from BALB/c mice and seeded into the 24-well plate at 1.5×10^5 cells/well. After 24 h *L.* (*L.*) *infantum* amastigotes were used to infect the cells at a 1:10 ratio (macrophage/amastigotes).[20] After 24 h infected macrophages were cocultivated with splenocytes at a concentration of 6:1. Preparations were obtained by crushing spleens in PBS (pH 7.2). Subsequently, splenocyte suspensions were washed twice with PBS and erythrocytes were lysed with ammonium-chloride-potassium (ACK) buffer (0.15 M NH_4Cl; 10 mM $KHCO_3$; 0.1 M Na_2EDTA) for 5 min. After washing two times with PBS by centrifugation at 4 °C for 10 min, the cells were resuspended in RPMI 1640 medium and 10% heat-inactivated FBS. The viability of the cells used in the experiments was always higher than 85%, as measured by trypan blue exclusion. After 48 h with compounds **10** (30 μM) and **11** (15 μM), supernatants were collected to perform the cytokine assay. Proteins levels were determined using an inflammatory CBA kit according to the manufacturer's instructions. The concentrations of the released mediators were measured: interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemotactic protein-1 chemokine (MCP-1), interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin-12p70 (IL-12p70) using flow cytometry. Sham-treated macrophages infected with *L.* (*L.*) *infantum* were used as controls.

Statistical Analysis. The results are represented as the mean and standard deviation of replicate samples from at least two independent assays. The IC_{50} values were calculated using sigmoidal dose−response curves using GraphPad Prism 5.0 software. The 95% confidence interval is included in parentheses with the analyses. The *t* test was used for significance testing ($p < 0.05$).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00256.

Procedure for preparation of compound **14**; ^{1}H and ^{13}C NMR spectra for compounds **1**−**14** (not previously published) and **16**−**18**; PAINS analysis for guanidines (PDF)

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Notes

The authors declare no competing financial interest.

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