4-Amino Bis-Pyridinium Derivatives as Novel Antileishmanial Agents

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The antileishmanial activity of a series of bis-pyridinium derivatives that are analogues of pentamidine have been investigated, and all compounds assayed were found to display activity against promastigotes and intracellular amastigotes of *Leishmania donovani* and *Leishmania major*, with 50% effective concentrations (EC_{50}) lower than 1 μM in most cases. The majority of compounds showed similar behavior in both *Leishmania* species, being slightly more active against *L. major* amastigotes. However, compound VGP-106, 1’-(biphenyl-4,4’-diylmethylene)bis[4-(4-bromo-N-methylanilino)pyridinium] dibromide, exhibited significantly higher activity against *L. donovani* amastigotes (EC_{50}, 0.86 ± 0.46 μM) with a lower toxicity in THP-1 cells (EC_{50}, 206.54 ± 9.89 μM). As such, VGP-106 was chosen as a representative compound to further elucidate the mode of action of this family of inhibitors in promastigote forms of *L. donovani*. We have determined that uptake of VGP-106 in *Leishmania* is a temperature-independent process, suggesting that the compound crosses the parasite membrane by diffusion. Transmission electron microscopy analysis showed a severe mitochondrial swelling in parasites treated with compound VGP-106, which induces hyperpolarization of the mitochondrial membrane potential and a significant decrease of intracellular free ATP levels due to the inhibition of ATP synthesis. Additionally, we have confirmed that VGP-106 induces mitochondrial ROS production and an increase in intracellular Ca^{2+} levels. All these molecular events can activate the apoptotic process in *Leishmania*; however, propidium iodide assays gave no indication of DNA fragmentation. These results underline the potency of compound VGP-106, which may represent a new avenue for the development of novel antileishmanial compounds.

Leishmaniasis, a broad-spectrum disease caused by protozoan parasites of the genus *Leishmania*, is one of the world’s most neglected diseases, with 350 million people considered to be at risk of contracting leishmaniasis and more than 2 million new cases every year. Leishmaniasis has traditionally been classified into three main clinical forms, namely, visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL), which differ in terms of their immunopathologies and degrees of morbidity and mortality. *Leishmania donovani* causes the potentially fatal disease VL. In the absence of effective vaccines against leishmaniasis, the main means of controlling this disease is exclusively via chemotherapy. Current leishmaniasis treatments rely on a reduced arsenal of drugs, including pentavalent antimonials, amphotericin B, miltefosine, and paromomycin, all of which have drawbacks in terms of toxicity, efficacy, price, and inconvenient treatment schedules. To increase the therapeutic life span of these drugs and delay the emergence of resistance, the WHO has recommended combination therapy as a strategy to be implemented in clinical trials.

Pentamidine [1,5-bis(4-amidinophenoxy)pentane], which was first used for the treatment of sleeping sickness caused by *Trypanosoma brucei*, is currently a second-line drug for the treatment of visceral leishmaniasis. Pentamidine is actively transported into *Leishmania* promastigotes (1) and binds to nuclear and mitochondrial DNA (kinetoplasts), thereby hindering replication and transcription at the mitochondrial level (2). New diamidine and choline derivative dications have been developed recently in order to find new drugs with improved activity against leishmaniasis and lower toxicity (3–6).

We previously designed and synthesized a new set of bis-pyridinium compounds as inhibitors of the human choline kinase enzyme (7). This enzyme is a validated antitumor target, and all the above-mentioned compounds have shown a significant antiproliferative activity (7). Additionally, these compounds can be considered structural analogues of pentamidine in which the amine moieties, which are protonated at physiological pH, have been replaced by positively charged nitrogen atoms in a pyridinium ring. In view of this structural resemblance and with the intention of identifying potential antileishmanial drugs, we analyzed the antileishmanial activities of a series of bis-pyridinium derivatives. Compound VGP-106 was identified as a representative compound that displayed a potent antileishmanial activity against *L. donovani* intracellular amastigotes. As the least cytotoxic of the set of compounds assayed for THP-1 cells, it was selected to further elucidate their mechanism of action in this protozoan parasite.

**MATERIALS AND METHODS**

**Chemical compounds.** The synthesis of choline kinase inhibitors has been described previously (7). The compounds tested (see Table S1 in the supplemental material) were dissolved in dimethyl sulfoxide (DMSO) at 10 mM. Carbamoylcyanine p-trifluoromethoxyphenylhydrazone (FCCP), carbonyl cyanide m-chlorophenylhydrazone (CCCP), amphotericin B, 4’,6-diamidino-
2-phenylindole dilactate (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA), ethylene glycol-bis(2-aminoethyl)ether)-N,N,N',N'-tetraacetic acid (EGTA), propidium iodide (PI), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). MitoFusin was purchased from Aeta Zentaris (Frankfurt, Germany). Fluor-4-AM, Pluronic F-127, Sytox green, bis-(1,3-dibutylbarbituric acid)/trimethine oxonol (DiBAC_4(3)), Mitosox red, and JC-1 were purchased from Invitrogen (Carlsbad, CA). All the chemicals were of the highest quality available.

**Leishmania cell lines and cultures.** Promastigotes of the reference strains *Leishmania donovani* (MHOM/IND/80/D88) and *Leishmania major* (MHOM/IL/80/Friedlin) for VL and CL, respectively, were grown at 28°C in RPMI 1640-modified medium (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (iFBS) (Invitrogen) (8). Human myelomonocytic cell line (THP-1) culture and determination of cellular toxicity. THP-1 cells were grown at 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% iFBS, 2 mM glutamate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells, at 3 × 10^6/well in 96-well plates, were differentiated to macrophages with 20 ng/ml of PMA treatment for 48 h followed by 24 h of culture in fresh medium (10). The cellular toxicity of all compounds was determined using the colorimetric MTT-based assay (9), as described previously for *Leishmania* promastigotes, except for the incubation temperature, which was 37°C in this case.

**Susceptibility analysis in intracellular *Leishmania* amastigotes.** To determine the susceptibility of intracellular *Leishmania* amastigotes to these compounds, macrophage-differentiated THP-1 cells were infected at a macrophage/parasite ratio of 1:10. Infected-cell cultures were maintained at 37°C with 5% CO_2 at different compound concentrations in RPMI 1640 medium plus 10% iFBS. After 72 h, macrophages were fixed for 20 min at 4°C with 2.5% paraformaldehyde in phosphate-buffered saline (PBS): 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 130 mM NaCl, and 2.6 mM KCL adjusted to pH 7) and permeabilized with 0.1% Triton X-100 in PBS for 30 min. Intracellular parasites were detected by nuclear staining with DAPI (Invitrogen). The percent infection and the mean number of amastigotes from infected macrophages were determined in 200 macrophages well.

**DNA constructs and transfection.** *CEK* (choline/ethanolamine kinase) and *EK* (ethanolamine kinase) from *L. donovani* (GeneDB *L. donovani*, accession codes LdBPK_351480.1 and LdBPK_271330.1, respectively) were isolated from the genomic DNA of *L. donovani* by PCR using sense and antisense primers 5'-atgattataATGTAGTGTTGCAATTC (lowercase indicates nucleotides added for gene cloning) and 5'-atgattattTCAGATAAGCTTGTTGTCCTC for *CEK* and 5'-atcggggtGTGGTGCAATTTCGCTATG and 5'-atgattattTCACAGGTCTCCTGACACG for *EK*. Restriction sites (underlined) were added for subsequent cloning. The resulting fragments were cloned into the Leishmania expression vector pXG-Hyg (11) and sequenced. *L. donovani* promastigotes were transfected with pXG-Hyg-CEK or pXG-Hyg-EK by electroporation in 2-mm-/H9262 with DAPI (Invitrogen) (8). The membrane potential-sensitive probe DiBAC_4(3) was used as an internal control.

**Transmission electron microscopy (TEM) in *L. donovani* parasites.** ATP was measured using a CellTiter-Glo luminescence assay, which generates a luminescent signal proportional to the amount of ATP present, as described previously (13). Briefly, promastigotes (4 × 10^4/ml) were incubated at 28°C in culture medium for 24 and 48 h and then washed and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) overnight at 4°C. After fixation, the cell suspensions were washed in the same buffer and then postfixed in a mixture of 1% phosphate-buffered osmium tetroxide and 1% potassium ferrocyanide for 1 h prior to dehydration in a graded ethanol series and infiltration and embedding in EMBed 812 resin (EMS). Thin sections were cut and mounted on 300-mesh copper grids before staining with uranyl acetate and lead citrate (12). The samples were viewed using a Zeiss Libra 120 Plus TEM.

**Analysis of mitochondrial membrane potential (ΔΨ_m) in *L. donovani* parasites.** Changes in ΔΨ_m were measured by flow cytometry using the JC-1 fluorescent marker, as described previously (14). Briefly, parasites (1 × 10^7 parasites/ml) were incubated with 0.5 or 1 μM VGP-106 for 24 and 48 h at 28°C in culture medium, then washed twice, resuspended in PBS, and further incubated at 28°C with 5 μM JC-1 for 10 min in HEPES-buffered saline (HBS) (21 mM HEPES, 0.7 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, and 6 mM glucose; pH 7). Parasite aliquots (2 × 10^6/ml) were incubated at 28°C in culture medium for 24 and 48 h with 0.5 and 1 μM VGP-106. A 25-μl aliquot of each sample was then transferred to a 96-well plate, mixed with the same volume of CellTiter-Glo, and incubated in the dark for 10 min. The resulting bioluminescence was measured using an Infinite F200 microplate reader (Tecan Austria GmbH, Austria).

**Determination of plasma membrane depolarization in *L. donovani* parasites.** Plasma membrane permeabilization in *L. donovani* promastigotes. Sytox green dye was used to assess plasma membrane integrity, as described previously (15). Briefly, parasites (1 × 10^7 parasites/ml) were treated with 10 or 30 μM VGP-106 for 1 and 3 h at 28°C in HBS, washed twice with HBS, and then incubated with 2 μM Sytox green (final concentration) for 15 min at 28°C. The parasites were subsequently transferred into a 96-well microplate (100 μl/well), and the fluorescence due to binding of the dye to intracellular nucleic acids was recorded using an Infinite F200 microplate reader. The control for maximum fluorescence was obtained by addition of 0.05% Triton X-100.

**Measurement of ROS production in *L. donovani* parasites.** The membrane potential-sensitive probe DiBAC_4(3) was used to measure potential changes. Thus, parasites (1 × 10^7 parasites/ml) were incubated with and without 30 μM VGP-106 for 3 h in HBS at 28°C and then treated with 2 μM DiBAC_4(3) for 10 min at 28°C, as described previously (15). Parasites treated with a 10 μM concentration of the depolarizing agent CCCP for 15 min were used as controls. DiBAC_4(3) fluorescence was analyzed by flow cytometry.
TABLE 1 Antileishmanial activitya and toxicity in THP-1b cells of bis-pyridinium compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (µM) (SI)c</th>
<th>Promastigotes</th>
<th>Amastigotes</th>
<th>THP-1 EC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. major</td>
<td>L. donovani</td>
<td>L. major</td>
<td>L. donovani</td>
</tr>
<tr>
<td>VGP-106</td>
<td>21.55 ± 3.72</td>
<td>0.36 ± 0.09</td>
<td>13.07 ± 6.30 (15.8)</td>
<td>0.86 ± 0.46 (240.2)</td>
</tr>
<tr>
<td>VGP-114</td>
<td>0.47 ± 0.04</td>
<td>0.61 ± 0.09</td>
<td>0.10 ± 0.03 (1000.6)</td>
<td>0.85 ± 0.04 (117.7)</td>
</tr>
<tr>
<td>VGP-118</td>
<td>29.15 ± 5.73</td>
<td>0.65 ± 0.19</td>
<td>6.21 ± 1.02 (2.4)</td>
<td>0.18 ± 0.03 (85.3)</td>
</tr>
<tr>
<td>VGP-130</td>
<td>0.50 ± 0.07</td>
<td>0.73 ± 0.11</td>
<td>0.09 ± 0.02 (903.7)</td>
<td>2.02 ± 0.05 (40.3)</td>
</tr>
<tr>
<td>VGP-138</td>
<td>0.74 ± 0.19</td>
<td>2.11 ± 0.48</td>
<td>0.30 ± 0.16 (586.8)</td>
<td>4.01 ± 0.43 (43.9)</td>
</tr>
<tr>
<td>VGP-146</td>
<td>0.21 ± 0.06</td>
<td>0.33 ± 0.07</td>
<td>0.10 ± 0.04 (156.1)</td>
<td>0.42 ± 0.01 (37.2)</td>
</tr>
<tr>
<td>VGP-150</td>
<td>0.36 ± 0.11</td>
<td>0.77 ± 0.04</td>
<td>0.09 ± 0.03 (267)</td>
<td>0.55 ± 0.16 (43.7)</td>
</tr>
<tr>
<td>VGP-162</td>
<td>0.40 ± 0.08</td>
<td>0.35 ± 0.02</td>
<td>0.37 ± 0.03 (29.6)</td>
<td>1.00 ± 0.08 (11.0)</td>
</tr>
<tr>
<td>VGP-174</td>
<td>1.70 ± 0.01</td>
<td>0.34 ± 0.03</td>
<td>0.41 ± 0.05 (6.1)</td>
<td>0.86 ± 0.03 (2.8)</td>
</tr>
<tr>
<td>VGP-182</td>
<td>2.51 ± 0.01</td>
<td>0.92 ± 0.2</td>
<td>0.42 ± 0.12 (11.2)</td>
<td>0.52 ± 0.12 (9.1)</td>
</tr>
<tr>
<td>AmB</td>
<td>0.32 ± 0.02</td>
<td>0.21 ± 0.01</td>
<td>0.24 ± 0.01 (59.7)</td>
<td>0.28 ± 0.13 (51.1)</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>16.65 ± 1.23</td>
<td>6.60 ± 1.57</td>
<td>10.61 ± 0.89 (2.5)</td>
<td>0.88 ± 0.14 (30.5)</td>
</tr>
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</table>

a Parasites were grown as described in Materials and Methods for 72 h at 28°C (promastigotes) or 37°C (intracellular amastigotes) in the presence of increasing concentrations of compounds. Data are means ± standard deviations of three independent determinations.
b THP-1 cells were grown as described in Materials and Methods for 72 h at 37°C, in the presence of increasing concentrations of compounds. Cell viability was determined using an MTT-based assay. Amphotericin B (AmB) and miltefosine were used as the standard antileishmanial agents. Data are means ± standard deviations from three independent experiments.
c Selectivity indexes (SI) were calculated by dividing the EC50 for THP-1 by that for intracellular amastigotes.

RESULTS

Cytotoxicity of choline kinase inhibitors against Leishmania lines. The antileishmanial activities of 10 choline kinase inhibitors (see Table S1 in the supplemental material) reported previously (7) were evaluated against promastigotes and intracellular amastigotes of *L. donovani* and *L. major* in order to identify potential candidates for further optimization as antileishmanial drugs. The results are shown in Table 1; miltefosine and amphotericin B were used as the reference antileishmanial drugs. Most assayed compounds exhibit a specific high activity against promastigotes and intracellular amastigotes of *L. major*, with EC50s for amastigotes of between 0.09 and 0.42 µM, except for compounds VGP-106 and VGP-118 (EC50 13.07 and 6.21 µM, respectively). With regard to *L. donovani*, all assayed compounds displayed EC50s for promastigotes below 1 µM, except compound VGP-138 (EC50 2.11 µM). Although these values were slightly higher for intracellular amastigotes, they were similar to those of the antileishmanial drug miltefosine.

Our analysis of the effect on THP-1 cells showed that bis-pyridinium derivatives (VGP-106, VGP-114, VGP-118, VGP-130, and VGP-138) are less cytotoxic than the bis-quinolinium counterparts (VGP-146, VGP-150, VGP-162, VGP-174, and VGP-182) (Table 1), with a higher selectivity index (ratio between EC50 THP-1 and EC50 in intracellular amastigotes) than miltefosine (Table 1).

VGP-106 (1,1′-[(biphenyl-4,4′-dilmethylene)bis[4-(4-bromo-N-methylamino)pyridinium] dibromide] (Fig. 1) was chosen as a representative compound to further investigate the mechanism of action of this new family of compounds. This compound showed strong activity against *L. donovani* and was the least cytotoxic of the set of compounds assayed for THP-1 cells.

Drug susceptibility assay of *L. donovani* lines overexpressing CEK or EK. Considering that all the above compounds were initially designed for inhibition of human choline kinase (ChK) (7), we decided to study whether there is a correlation between their ChK inhibitory activity and antileishmanial activities. The Leish-
mania genome includes two genes homologous to human Chok, namely, the genes for choline/ethanolamine kinase (CEK) and ethanolamine kinase (EK). The corresponding proteins can be overexpressed in L. donovani promastigotes by transfecting the parasites with a plasmid carrying the Leishmania CEK or EK genes. Expression levels were tested by reverse transcription-PCR (RT-PCR) using GADPH as an expression control (Fig. 2). The susceptibility of transfected parasites to compound VGP-106 was determined in both promastigotes and intracellular amastigotes. As can be seen from Table 2, there are no significant differences between the EC50 for parasites overexpressing CEK or EK enzymes and those for control parasites. These results suggest that the mechanism of action of this compound in Leishmania is independent of the aforementioned enzymes. If this were not the case, overexpression of these enzymes would have resulted in an increased resistance to the inhibitory effect of the compound, and therefore an increase in the EC50.

**Uptake of VGP-106 in Leishmania lines.** To determine whether there were any differences in the uptake mechanism of VGP-106 between Leishmania lines that could explain differences in susceptibility to this compound, a spectrofluorometric assay taking advantage of the intrinsic fluorescence of this compound was carried out. A concentration of 100 μM VGP-106 allowed us to obtain significant fluorescence levels for experiments of drug uptake at different short time points. As can be seen from Fig. 3A, VGP-106 uptake by the two Leishmania species reached saturation very quickly, and no significant differences in VGP-106 accumulation at 28°C and 4°C were observed (Fig. 3B), suggesting that uptake occurs by diffusion. Additionally, VGP-106 accumulation in L. donovani is only 18% higher than that in L. major, which does not explain the observed differences in susceptibility.

**Effect of VGP-106 on the ultrastructural morphology of L. donovani.** The ultrastructural effect of VGP-106 in L. donovani promastigotes was studied by TEM. Parasites incubated with 0.5 μM for 24 or 48 h showed an intense swelling of the mitochondrion with loss of cristae and with no apparent kinetoplast alteration (Fig. 4C and E). This change was more evident at 1 μM, when the mitochondrion appeared as a huge vacuole covering a large fraction of the intracellular space. However, no further alterations were observed in other organelles or in the plasma mem-

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**TABLE 2** Susceptibility to VGP-106 of L. donovani lines overexpressing CEK or EK

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>EC50 (μM) Promastigotes</th>
<th>Amastigotes</th>
</tr>
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<tbody>
<tr>
<td>pXG</td>
<td>0.36 ± 0.09</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>pXG-CEK</td>
<td>0.36 ± 0.09</td>
<td>0.42 ± 0.05</td>
</tr>
<tr>
<td>pXG-EK</td>
<td>0.43 ± 0.05</td>
<td>0.33 ± 0.03</td>
</tr>
</tbody>
</table>

*Control (pXG) and transfected (pXG-CEK and pXG-EK) parasites were grown as described in Materials and Methods for 72 h at 28°C (promastigotes) or 37°C (intracellular amastigotes) in the presence of increasing concentrations of compound. Data are means ± SD of three independent determinations.
brane of the parasites. Furthermore, cell debris appeared after incubation with 1 μM V9262 MAT48h (Fig. 4F), suggesting a necrotic process.

VGP-106 induces hyperpolarization of ΔΨm and decreases intracellular ATP levels in L. donovani. To determine the effects of VGP-106 on mitochondrial function, we studied the variation in ΔΨm (Fig. 5). Parasites incubated with 0.5 and 1 μM VGP-106 for 24 and 48 h showed a significant increase in the JC-1 red/green fluorescence ratio compared with untreated parasites, indicating that this compound induces hyperpolarization of ΔΨm. The uncoupling reagent FCCP (10 μM, 10 min) was used as a control for fully depolarized promastigotes. As ΔΨm is essential for mito-

FIG 4 Ultrastructural effect of VGP-106 in L. donovani promastigotes by transmission electron microscopy (TEM). Ultrathin sections of control promastigotes of L. donovani (A and B) or promastigotes treated with 0.5 and 1 μM VGP-106 for 24 h (C and E) and 48 h (D and F) are shown. Mitochondria (M), kinetoplasts (K), and nuclei (N) are indicated. Scale bars: 1 μm (A, C, and E) or 2 μm (B, D, and F).
Mitochondrial ATP synthesis, the intracellular ATP level was measured using a CellTiter-Glo luminescence assay, which generates a luminescent signal proportional to the amount of ATP. As observed, VGP-106 significantly reduces the intracellular ATP level in *L. donovani* promastigotes after treatment with either 0.5 or 1 μM VGP-106 for 24 h (Fig. 6).

VGP-106 does not affect the plasma membrane integrity of *L. donovani*. To assess whether VGP-106 induces plasma membrane permeabilization, we first monitored the entry of the vital dye Sytox green into the cytoplasm of *L. donovani* promastigotes previously treated with VGP-106. The assays at the highest concentration (30 μM, 3 h) produce only 20% of the fluorescence increase obtained with 0.05% Triton X-100, which was used as a control that induces full permeabilization (Fig. 7A). Additionally, depolarization of the plasma membrane was measured using DiBAC4(3) as a fluorescence probe. After treatment with 30 μM VGP-106 for 3 h, no change was observed in the plasma membrane potential (Fig. 7B), confirming that this compound does not affect the plasma membrane integrity of parasites.

VGP-106 increases mitochondrial ROS production and cytosolic Ca2+ levels in *L. donovani*. The modification of ΔΨm induced in *Leishmania* by a variety of drug treatments has been associated with the production of reactive oxygen species (ROS), which induce damage to the components of the electron transport chain, disrupt mitochondrial function, decrease cellular ATP levels, and produce cell death (17). The generation of mitochondrial ROS was measured using the cell probe Mitosox red, which selectively targets mitochondria and is oxidized by local superoxide. As can be seen from Fig. 8, VGP-106 produces a concentration- and time-dependent increase in ROS levels.

Mitochondrial damage is associated with both ROS production and variations in intracellular calcium homeostasis.
tigotes treated with 10 and 30 μM VGP-106 showed higher cytosolic Ca\(^{2+}\) levels than untreated control parasites (Fig. 9A). To ascertain the source of the Ca\(^{2+}\) responsible for this effect, the experiment was repeated in the presence of EGTA to rule out the entry of external Ca\(^{2+}\) (Fig. 9B). Under these conditions, the fluorescence was significantly reduced, suggesting that VGP-106 induces an increase in the entry of external Ca\(^{2+}\).

**Effect of VGP-106 on the *L. donovani* cell cycle.** Since the elevation in cytosolic Ca\(^{2+}\), mitochondrial dysfunction, ROS generation, and reduction of intracellular ATP levels are all distinctive events during the progression of an apoptosis-like process, we examined whether VGP-106 produces DNA fragmentation, a key feature of apoptosis. We determined the hypodiploid DNA content in parasites by monitoring PI fluorescence using flow cytometry. After incubation of parasites with 10 and 30 μM VGP-106 during 24 and 48 h, there were no significant differences in the percentage of parasites with DNA in the sub-G\(_1\) phase with respect to untreated parasites (Fig. 10), indicating that there is no genomic DNA fragmentation. Therefore, our findings are consistent with nonprogrammed cell death.

**DISCUSSION**

Numerous dicationic compounds have been tested as antileishmanial drugs over the past few years (3–6). In this work, we determined the antileishmanial effect of a set of human choline kinase inhibitors with a bis-pyridinium-type structure (see Table S1 in the supplemental material) and provide a first insight into the antileishmanial mechanism of action of a promising lead compound.

All compounds assayed displayed antileishmanial activity against promastigotes and intracellular amastigotes of both *L. donovani* and *L. major*, with EC\(_{50}\)s lower than 1 μM in most cases. Most compounds showed similar behavior in both *Leishmania* species, being slightly more active against *L. major* amastigotes. Nevertheless, compounds VGP-106 and VGP-118, which have a 4-(4-bromo-N-methylaniline)pyridinium group as the cationic head, exhibit significantly higher activity against *L. donovani* intracellular amastigotes.

VGP-106 was chosen as a representative compound in order to further elucidate the uptake and mode of action of this family of inhibitors. As a result, we have determined that the uptake of VGP-106 in *Leishmania* promastigotes quickly reached saturation and is a temperature-independent process, thereby suggesting that the compound crosses the parasite membrane by diffusion. An interesting observation was the differences in drug accumulation between *L. donovani* and *L. major* lines and the fact that these dissimilarities do not explain the different susceptibilities of *Leishmania* promastigotes and intracellular amastigotes to VGP-106; a similar situation has been described previously for sitamaquine (18). Considering that VGP-106 inhibits human ChoK (7), we decided to determine whether this set of bisquaternary derivatives produce parasite death by inhibiting homologous *Leishmania* enzymes. Several symmetrical biscationic compounds have previously demonstrated antimalarial activity resulting from inhibition of phosphatidylcholine biosynthesis (19). As described for eukaryotic cells, phosphatidylcholine is the main phospholipid of the plasma membrane in *Leishmania*, and there are two enzymes homologous to human ChoK in *Leishmania*: EK and CEK. In the VGP-106 susceptibility assay, results for *Leishmania* parasites overexpressing CEK and EK were similar to those for control parasites, meaning that these two enzymes are not implicated in the mode of action of this compound in *Leishmania*. This is in line with the fact that *Leishmania* is not auxotrophic for choline or ethanolamine (20), unlike *Plasmodium*, where ChoK inhibition leads to death of the parasite (21). Our results are consistent with
those obtained by other authors for *Trypanosoma brucei*, indicating no changes in phospholipid metabolism after treatment with bissacetic choline-derived analogues (6).

The TEM images showed an extremely swollen mitochondrion for parasites treated with compound VGP-106 compared to control parasites. In contrast, other organelles and the plasma membrane mostly appear intact, suggesting that this compound induces mitochondrial dysfunction in the parasites. The *Leishmania* mitochondrion is the target for a wide variety of leishmanicidal drugs, including some in clinical use, such as pentamidine (22) and miltefosine (23), and others at different stages of development, such as sitamaquine (15), tafenoquine (24), chalcones (25), and histatin 5 (26). Our study of the effect of VGP-106 on mitochondrial function under the same assay conditions showed a hyperpolarization of the mitochondrial membrane potential and a significant decrease of intracellular free ATP levels due to the inhibition of ATP synthesis. These findings suggest that VGP-106 may accumulate in the mitochondrion, thereby altering the mitochondrial function, as described previously for other positively charged compounds, such as dequalinium (27) and pentamidine (28), and other diamidine derivatives (29). In contrast to the observed hyperpolarization in $\Delta \Psi_m$ induced by VGP-106, pentamidine, sitamaquine, and tafenoquine induce $\Delta \Psi_m$ depolarization in *Leishmania* (15, 24, 30), whereas camptothecin induces an initial hyperpolarization followed by a depolarization of mitochondrial *L. donovani* (31). Leishmanial F$_0$F$_1$ ATPase plays a key role in increasing the mitochondrial membrane potential, which may depend on glycolytic ATP use by F$_0$,F$_1$ ATPase in the reverse mode, as described for hyperpolarization in mammalian cells (31), for which it has been proved that hyperpolarization of the inner mitochondrial membrane leads to ROS production (32). Our experimental assays with the probe Mitosox red confirmed that VGP-106 induces mitochondrial ROS production in a time- and concentration-dependent manner. This ROS production triggers an increase in intracellular Ca$^{2+}$ and subsequent steps in the apoptotic process in *Leishmania*. Interestingly, the Ca$^{2+}$ pools involved may vary according to the stimulus. Thus, whereas oxidative stress induced by H$_2$O$_2$ involves mobilization from intra- and extracellular Ca$^{2+}$ pools (33), only the intracellular pool is implicated after complex II poisoning with thenoyltrifluoroacetone plus pentamidine (34). An increase in cytosolic calcium homeostasis is known to be an essential initial event in cell death (35) and is related to ROS production and mitochondrial dysfunction. Although VGP-106 increases intracellular Ca$^{2+}$ levels due to Ca$^{2+}$ entry from the external medium, the question of whether the entry of external Ca$^{2+}$ is due to unspecific and transitory membrane permeabilization by VGP-106 or to effects on Ca$^{2+}$ channels remains unanswered. The entry of external Ca$^{2+}$ in *Leishmania* apoptosis induced by external H$_2$O$_2$ (33), camptothecin (31), and curcumin (36) and mediated by specific channels activated by ROS or its derived metabolites inside the cells (37) was described previously.

All these molecular events activate programmed cell death with DNA nicking and fragmentation as the final outcome, both of which are characteristic of an apoptosis-like death in *Leishmania*. To determine whether VGP-106 produces apoptosis-like cell death, its effect on the cell cycle has been studied. However, the assays with PI indicate no signs of DNA fragmentation, contrary to its proven effect on the cell cycle. DNA fragmentation was quantified by measuring the percentage of cells in the sub-G$_1$ DNA region. The DNA content degradation profiles of promastigotes were determined by flow cytometry and PI staining. Parasites were incubated without (control) or with 10 and 30 $\mu$M VGP-106 for 24 h (A) and 48 h (B) and then loaded with PI, as described in Materials and Methods. The distribution of DNA content was analyzed by flow cytometry. Histograms are representative of three independent experiments, with 10,000 parasites analyzed per group.

**FIG 10** Effect of VGP-106 on the *L. donovani* cell cycle. DNA fragmentation was quantified by measuring the percentage of cells in the sub-G$_1$ DNA region. The DNA content degradation profiles of promastigotes were determined by flow cytometry and PI staining. Parasites were incubated without (control) or with 10 and 30 $\mu$M VGP-106 for 24 h (A) and 48 h (B) and then loaded with PI, as described in Materials and Methods. The distribution of DNA content was analyzed by flow cytometry. Histograms are representative of three independent experiments, with 10,000 parasites analyzed per group.
to the results obtained with pentamidine that binds to DNA (2). These results, together with the cell debris observed by TEM, confirm nonprogrammed cell death or necrosis. Recently, similar results were obtained with the iron chelator 2,2-dipyridyl, which leads to a multifactorial response in Leishmania braziliensis that results in cellular collapse, with a marked mitochondrial impairment and subsequent cell death not associated with DNA fragmentation (38).

In conclusion, our studies suggest that VGP-106 inhibits mitochondrial functionality, thereby inducing a rapid drop in intracellular ATP levels in Leishmania. At the same time, the increase in mitochondrial ROS production and elevation of intracellular Ca$^{2+}$ leads to hyperpolarization of ΔΨ$m$. Taken together, these biological events induced by VGP-106 trigger necrosis in Leishmania. The findings for VGP-106 reported herein support the view that this drug shows potency against Leishmania with sufficient activity for further in vivo studies. Preliminary in vitro experiments with VGP-106 using hamsters infected with L. infantum by oral or intraperitoneal administration showed no oral activity or high toxicity, respectively. Additional experiments are necessary for (i) evaluation of oral bioavailability, (ii) determination of the intrinsic toxicity, (iii) new experiments by intraperitoneal administration using lower dose of compound and increase number of days and intervals of treatment, and (iv) new routes of administration. Additionally, these studies may serve as a guide for the future design of VGP-106 analogues that are more specific and have higher antileishmanial activity.

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