Activity of Bisnaphthalimidopropyl Derivatives against Trypanosoma brucei

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Current treatments for African trypanosomiasis are either toxic, costly, difficult to administer, or prone to elicit resistance. This study evaluated the activity of bisnaphthalimidopropyl (BNIP) derivatives against Trypanosoma brucei. BNIPDiaminobutane (BNIPDabut), the most active of these compounds, showed in vitro inhibition in the single-unit nanomolar range, similar to the activity in the reference drug pentamidine, and presented low toxicity and adequate metabolic stability. Additionally, using a murine model of acute infection and live imaging, a significant decrease in parasite load in BNIPDabut-treated mice was observed. However, cure was not achieved. BNIPDabut constitutes a new scaffold for antitrypanosomal drugs that deserves further consideration.

African trypanosomiasis is an infectious disease caused by parasites of the species Trypanosoma brucei. The parasite is transmitted by an insect vector, the tsetse fly (Glossina spp.). The disease is distributed mainly on the African continent, with distinct subspecies causing different forms of human disease. T. brucei gambiense produces a chronic form of infection that may last for years and was responsible for nearly 98% of trypanosomiasis cases in the past decade. The acute form is caused by T. brucei rhodesiense and usually kills the host within weeks, accounting for the remaining 2% of reported cases (1, 2).

Since vaccination remains elusive and vector control strategies are frequently insufficient, chemotherapy is still the most efficient option for controlling the disease (2–5). However, the drugs in current use have many drawbacks, mostly related to cost, effectiveness, toxicity, difficulty of administration, and appearance of resistance (6). Therefore, the development of new drugs is urgently needed.
Bisnaphthalimidopropyl (BNIP) derivatives have been shown to possess anticancer activity (7–11) and to have activity against a related trypanosomatid, *Leishmania infantum* (12–14). The potential activity of three BNIP derivatives previously synthesized (>96% pure [10, 11, 15]), namely, BNIPDiaminobutane (BNIPDabut), BNIPDiaminohexane (BNIPDahep), and BNIPDiaminoctane (BNIPDaoct) (Fig. 1A), against *T. brucei brucei* Lister 427 bloodstream forms (BSFs), was investigated. These derivatives were selected from a series of compounds on the basis of preliminary studies of bioavailability and in vitro and in vivo activity against *T. brucei* and *L. infantum* (12; our unpublished data). The in vitro antiparasitic activity was assayed using a resazurin assay, as previously described, with minor modifications (incubation with 10^9 parasites/well, in 200 μL [16]). All three BNIPs demonstrated a potent inhibitory effect on parasite growth, with a 50% inhibitory concentration (IC_{50}) within the nanomolar range (Fig. 1B; Table 1). BNIPDabut was the most active compound, with a mean IC_{50} (± standard deviation [SD]) of 2.4 ± 1.0 nM, similar to that of the reference drug pentamidine (mean IC_{50} ± SD, 2.9 ± 0.7 nM; Table 1). Since this class of compounds has been described as a group of inhibitors of the *L. infantum* Silent information regulator 2-related protein 1 (LiSir2rp1; GenBank accession number AAN39039.1) (15), we evaluated whether inhibition of the *T. brucei* orthologue *Tb* Sir2rp1 obtained by protein sequence alignment (Clone Manager 9, BLOSSUM 62 scoring matrix) might explain the differences observed (17). Moreover, no correlation between the enzymatic inhibition and activity toward *T. brucei* parasites was observed. To evaluate in vitro toxicity toward mammalian cells, all the compounds were studied with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT) in THP-1-derived macrophages and two primary cell cultures, rat cortical neurons and mouse hepatocytes (Table 2). The 50% cytotoxic concentration (CC_{50}) values for these molecules translate into selectivity indexes (SIs) higher than 100 (SI = CC_{50}/IC_{50}). All the BNIP derivatives exhibited a high SI, with BNIPDabut in particular being at least 800 times more selective toward *T. brucei* parasites. All the BNIPs had potency and selectivity that warranted additional characterization (Table 2). To further evaluate the potential toxic effects of BNIPs in host cells, a set of in vitro assays was performed in hepatocytes and neuronal primary cells. These assays evaluated different possible mechanisms of toxicity based on (i) reactive oxygen species determination (chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate [CM-H2DCFDA] probe, by high content analysis [HCA]), (ii) mitochondrial dysfunction (tetramethylrhodamine, methyl ester [TMRM] probe, by HCA), (iii) membrane integrity (lactate dehydrogenase quantification), (iv) apoptosis (caspase 3/7 activation), (v) either DNA damage for hepatocytes (H2AX antibody, by HCA) or neurite outgrowth for neurons (anti-tubulin III antibody, by HCA), (vi) cell viability as measured by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt probe], and (vii) Hoechst staining for nuclear detection. Nimesulide (400 μM) was included as a positive control and the vehicle as a neutral control (19–21). The relative percent deviation from the neutral control was quantified and assigned with a number from 0 to 5 (0 [0% to 20% deviation], 1 [20% to 40%], 2 [40% to 60%], 3 [60% to 100%], 4 [100% to 1,000%], 5 [>1,000% deviation]). The sum of these values was ranked posteriorly to create combined injury criteria that varied from no injury (0) to low injury (1 to <5), moderate injury (≥5 to <12), and high injury (≥12). All BNIPs showed a dose-dependent injury score close to that of pentamidine in both cell types (Fig. 2A and B). BNIPDabut had a toxicity profile indistinguishable from that of the reference drug pentamidine.

To infer metabolic stability, mouse microsomes were incubated over 45 min with each compound at 5 μM, and each drug was quantified by liquid chromatography-tandem mass spectrometry. Similarly to pentamidine, BNIPDabut was more stable than BNIPDahep and BNIPDaoct; 95% to 100% of the drug was not metabolized (Table 3). This high metabolic stability is an indicator that the molecule is not easily subjected to common inactivation or loss of potency by reactions catalyzed by liver enzymes and is kept intact in circulation for longer periods.

To determine the pharmacokinetics of BNIPDabut, a 10-mg/kg dose was administered to BALB/c mice by intravenous injection. Five minutes later, a concentration of 58 nM was achieved and, during the following 24 h, remained higher than 41 nM (data not shown); thus, the concentration was approximately eight times higher than the calculated CC_{50}.

Taking into consideration the previous results, we chose

<table>
<thead>
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<th>Compound</th>
<th>CC_{50} ± SD (μM) in:</th>
<th>Selectivity index in*:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>THP-1a</td>
<td>Hepatocytes</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>47.73 ± 3.32</td>
<td>18.21 ± 0.66</td>
</tr>
<tr>
<td>BNIPDabut</td>
<td>5.90 ± 0.40</td>
<td>9.19 ± 0.06</td>
</tr>
<tr>
<td>BNIPDahep</td>
<td>3.34 ± 0.11</td>
<td>4.23 ± 0.48</td>
</tr>
<tr>
<td>BNIPDaoct</td>
<td>3.88 ± 0.59</td>
<td>18.35 ± 4.58</td>
</tr>
</tbody>
</table>

*Selectivity index = CC_{50} cell line/IC_{50} T. brucei.
*a THP-1 derived macrophages.
BNIPDabut for in vivo efficacy studies. All the experiments involving animals were carried out in accordance with the Institute for Molecular and Cell Biology Animal Ethics Committees and the Portuguese and European Authorities for Animal Health guidelines. T. brucei brucei Lister 427 parasites were transfected with a construct kindly provided by M. Taylor, in which the red-shifted luciferase gene (PpyRE9h) is flanked by 5′VSG/3′tubulin (22). After transfection, clones were screened for bioluminescent signal, and the ones that expressed the highest levels were selected. Their in vitro growth was compared with that of wild-type parasites and was found to be similar (data not shown). In vitro detection limits were also analyzed for BSFs in a 96-well plate and determined to be approximately 2,500 cells (data not shown). Female BALB/c mice were inoculated intraperitoneally with 10^4 BSFs. Three days after infection, five female BALB/c mice were inoculated intra-

FIG 2 In vitro toxicity of BNIP derivatives. (A) Hepatotoxicity injury score. The score was calculated as the sum of individual scores obtained from a panel of in vitro cytotoxicity assays that include (i) measurement of reactive oxygen species (ROS) using CM-H2DCFDA and cell imaging by HCA, (ii) assessment of mitochondrial dysfunction measured by TMRM probe dynamics in cells and imaged by HCA, (iii) membrane integrity assay by lactate dehydrogenase quantification, (iv) DNA damage by imaging with H2AX antibody and HCA, (v) apoptosis by caspase 3/7 activation, (vi) Hoechst staining for nuclear detection, and (vii) cell viability by WST-8 probe. Nimesulide (400 μM), an approved drug with a mild toxicological profile, was included as a toxicity control. Individual scores were calculated on the basis of the relative percentage of deviation from the negative control quantified and assigned with a number from 0 to 5 (0 [0% to 20% deviation], 1 [20% to 40%], 2 [40% to 60%], 3 [60% to 100%], 4 [100% to 1,000%], or 5 [>1,000% deviation]). The data represent the mean sum of these values. (B) Neurotoxicity injury score. The score was calculated in a manner similar to that for the hepatotoxicity score, but instead of DNA damage by H2AX antibody, an assay to test neurite outgrowth as imaged with an anti-tubulin III antibody and HCA was performed. The data represent the mean sum of these values. Data are from 2 independent experiments.

TABLE 3 Mouse microsomal stability

<table>
<thead>
<tr>
<th>Compound</th>
<th>Metabolic stability (%)</th>
<th>In vitro intrinsic clearance (μl/min/mg protein)</th>
<th>Degradation non-NADPH dependent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentamidine</td>
<td>95–100</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>BNIPDabut</td>
<td>95–100</td>
<td>85</td>
<td>&lt;5</td>
</tr>
<tr>
<td>BNIPDahep</td>
<td>85</td>
<td>7</td>
<td>&lt;5</td>
</tr>
<tr>
<td>BNIPDact</td>
<td>64</td>
<td>20</td>
<td>9</td>
</tr>
</tbody>
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In conclusion, this work demonstrates that BNIPDabut has potent in vitro and in vivo antitrypanosomal activity with accept-
FIG 3 BNIPDabut in vivo efficacy against *T. brucei brucei*. (A) Schematic of the experimental design for evaluating the in vivo efficacy of BNIPDabut. (B) Mice were infected with $10^4$ luciferase-positive BSFs by intraperitoneal injection, and the different treatments were initiated 3 days after infection. Whole-mouse bioluminescence imaging was done on days 3, 4, 9, and 12 using an IVIS Lumina LT and after injection of 2.1 mg luciferin. The bioluminescence average radiance (photons per second per square centimeter per steradian [p/sec/cm²/sr]) of whole mice was quantified, and the mean ± standard deviation ($n = 4$) is shown by the bars. Parasitemia was determined with a hemocytometer, and the means ± standard deviations are represented by red dots. Red X’s represent the parasite concentration of the only animal in which parasites could be detected and quantified. The parasitemia detection limit was $5 \times 10^4$ parasites/ml. (C) Kaplan-Meyer survival curves of the infected mice treated with control and experimental doses of BNIPDabut. In panels B and C, the data are representative of 3 independent experiments.
able toxicity and high metabolic stability. However, chemical modifications are needed to improve its pharmacodynamic and/or pharmacokinetic properties.

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