Monocyclic Nitro-heteroaryl Nitrones with Dual Mechanism of Activation: Synthesis and Antileishmanial Activity

Juliana da Silva Pacheco1,4‡, Débora de Souza S. Costa2‡, Edédio Ferreira Cunha-Júnior1,5, Valter Viana Andrade-Neto1, Alan Fairlamb4, Susan Wylie4, Marília O. F. Goulart6, Danyelle C. Santos6, Thaissa L. Silva6, Marina A. Alves7, Paulo R. R. Costa2, Eduardo Caio Torres-Santos1*, Ayres G. Dias3*

1Laboratório de Bioquímica de Tripanosomatídeos, Instituto Oswaldo Cruz, FIOCRUZ, RJ, Brazil; 2Laboratório de Química Bioorgânica, Instituto de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, RJ, Brazil; 3Universidade do Estado do Rio de Janeiro, Centro de Tecnologia e Ciências, Departamento de Química Orgânica, RJ, Brazil; 4University of Dundee, School of Life Sciences, Division of Biological Chemistry and Drug Discovery, Dow Street, Dundee, DD1 5EH, Scotland, United Kingdom; 5Laboratório de Imunoparasitologia, Unidade Integrada de Pesquisa em Produtos Bioativos e Biocências, Campus UFRJ-Macae; 6Instituto de Química e Biotecnologia, Universidade Federal de Alagoas; 7Laboratório de Apoio ao Desenvolvimento Tecnológico, Universidade Federal do Rio de Janeiro, RJ, Brazil.
‡Equal contribution

ABSTRACT: New 5-nitro-furan nitrones (1a-c), 5-nitro-thiophen nitrones (2a-c) and 4-nitroaryl nitrone 4 were synthesized in one step, in high yields. Compounds 1a and 1b had the most potent leishmanicidal activity against intracellular amastigote forms of L. amazonensis and L. infantum (from 0.019 µM to 2.76 µM), with excellent selectivity (from 39 to 5673). The comparison of the leishmanicidal activity in promastigotes of wild type L. Donovani with those obtained in species overexpressing nitroreductases NRT1 or NRT2 shows that 1a,b are activated by both, a feature that could slow the development of resistance. The redox potential (E_redox) of these compounds obtained by cyclic voltammetry (0.57 V vs. Normal Hydrogen Electrode, for both compounds), compared to the other compounds shows that the reduction of the nitro group is modulated by the nitrones group. Formation of reactive oxygen species was not observed in promastigotes of L. infantum and murine macrophages. Compound 1b was administered orally to mice infected by Leishmania infantum and reduced the parasite load on the spleen by 76.6% and 95.0% with doses of 50 and 100mg/kg, respectively, administered twice a day, for five days. In the liver, the parasite load suppression was above 75% with either treatment.

1. Introduction

Leishmaniasis is among the seven most important tropical diseases, primarily affecting poor people in developing countries. Environmental changes such as deforestation and population migration contribute to its broad geographic distribution and increasing incidence. Cutaneous, mucocutaneous and visceral manifestations are the main clinical forms of this neglected disease, caused by Trypanosomatidae. According to the World Health Organization (WHO), cutaneous leishmaniasis is the most common form, with an estimated 0.6 million to 1 million new cases occurring annually. The visceral form is fatal if left untreated with estimated 50,000 to 90,000 new cases occurring each year.3

The chemotherapeutic potential of nitroheteroaromatic compounds for treating diseases caused by trypanosomatids was first reported in the 1950s. Nitrofurazone was reported as having activity against Trypanosoma brucei gambiensis followed by several other analogues in the following decades.3–9 However, severe toxicity issues limited their use in the clinic.10 Despite the fact that many nitro heteroaromatic compounds are still used today as therapeutics with acceptable safety profiles, the nitro group is regarded not only as a toxicological alert, but also as a no-go for many medicinal chemists. However, the knowledge of the role of specific and unique nitroreductases in their mode of action has brought nitro-compounds to the pipeline again.10–12 The role of nitro group in medicinal chemistry has been recently reviewed.13,14

Currently, the arsenal of antitypanosomal nitro-heteroaromatic drugs in clinical use is composed of benznidazole for treatment of the acute stage of Chagas Disease, nifurtimox for congenital Chagas’ disease and nifurtimoxin combination with efloinithine.
(NECT) for the late stage of Human African trypanosomiasis. These compounds are part of the list of essential medicines of WHO since 2009\textsuperscript{15,16} (Figure 1).

Regarding leishmaniasis, fexinidazole (Figure 1) was the first nitro-heteroaromatic compound to reach clinical trials\textsuperscript{17,18}, and its use was recently registered for sleeping sickness, as the first oral drug (DNDi, 2018\textsuperscript{19}). The antitrypanosomal and leishmanicidal use was recently registered for sleeping sickness, as the first oral therapy of visceral leishmaniasis.\textsuperscript{12}

All these compounds are pro-drugs and need to be activated by a nitroreductase. NTR1, a mitochondrial oxygen-insensitive nitroreductase, is known to transform monocyclic nitro-compounds such as nifurtimox into a hydroxylamine and an acyclic unsaturated nitrile, responsible for the biological effect. Wyllie, Fairlamb and co-workers identified a putative oxygen-sensitive NAD(P)H oxidase as another activating nitroreductase (NTR2)\textsuperscript{13} which preferentially reduces bicyclic nitro-compounds. Over expression of NTR2 in wild type parasites rendered cells hyper-sensitive to bicyclic nitro-heteroaromatic compounds, such as delamanid, but only marginally to the monocyclic nitro-heteroaromatic compounds, nifurtimox and fexinidazole sulfone, known to be activated by NTR1.\textsuperscript{21}

It is worth mentioning that leaving systems have a good tolerance for the nitroreductase function. For example, the detoxification of natural amines and enamines is accomplished by the oxidation to the corresponding nitroreductase metabolites.\textsuperscript{22,23} Also, nitrones have been detected as metabolites of several drugs and two in widespread use, chlorodiazepoxide\textsuperscript{24} and minoxidil\textsuperscript{25}, have this function in their chemical structure. In addition, nitrones are also important spin-scavengers, allowing the detection and identification of highly unstable free radical intermediates in the laboratory and in biological medium.\textsuperscript{26–28} Due to its presumed antioxidant properties PBN (α-phenyl-N-t-butyl-nitro) and some derivatives were evaluated as neuroprotectors in neurodegenerative diseases and some of them reached pre-clinical trials for the treatment of stroke.\textsuperscript{29–31} Arylnitrones were also reported to be protective against microvascular damage in ischemia/reperfusion in the ‘hamster cheek pouch’ assay.\textsuperscript{32–36} It has been suggested that the mechanism of biological action of these compounds is mainly related with their ability to modulate cytokine production.\textsuperscript{37–41}

2. Results and Discussion

2.1. Chemistry

Several methods are reported to prepare nitrones and we choose to use the protocol described by Dondoni and coworkers.\textsuperscript{42}

\begin{align*}
\text{Scheme 1. Synthesis of heteroaryl nitrones 1-3.} \\
\text{While the synthesis of N-t-butyl nitrones (1a and 2a) required the use of N-t-butylhydroxylamine hydrochloride and reflex in the presence of Et,N in CH}_2\text{Cl}_2 \text{ to go to completion, N-methyl and N-benzyl nitrones (1b; 2b; 3a,b and 4) were prepared in good yields by the reaction between the corresponding aromatic aldehydes (5 and 7) with less sterically hindered and less expensive N-methyl or N-benzylhydroxylamine hydrochloride in the presence of Et,N in CH}_2\text{Cl}_2 \text{ at room temperature. In all cases Z-geometry was observed in the resulting nitrones, as confirmed by NOE experiments. In addition, N-methyl nitrones can be prepared in the absence of solvent in few minutes when the reagents are macerated (SI).\textsuperscript{43,44} Nitro-heteroaryl nitrones 1a-c and 2a-c are described for the first time in this note.}
\end{align*}
2.2. In vitro activity against *L. infantum*, *L. amazonensis* and *L. donovani*

First, the synthesized compounds were screened for activity against promastigotes (insect stage) of the causative species of cutaneous (*L. amazonensis*) and visceral (*L. infantum*) leishmaniasis (Table 1). Next, active compounds from the first screening were evaluated against intracellular amastigotes of the same species. To assess antiparasitic selectivity, cytotoxicity was measured on murine peritoneal macrophages (Table 1, entry 1). Product 1a with 108 µM was the least toxic to macrophages with a similar CC<sub>50</sub> to miltefosine (96.5 µM), used as standard. Products 1b (CC<sub>50</sub> = 49.9 µM) and 2b (CC<sub>50</sub> = 24.2 µM) also showed low toxicity.

The antileishmanial activity in promastigotes of *L. amazonensis* shown in Table 1, entry 2). Products 1a (EC<sub>50</sub> = 1.99 µM), 1b (EC<sub>50</sub> = 9.37 µM), 2a (EC<sub>50</sub> = 11.32 µM), 2b (EC<sub>50</sub> = 9.72 µM) and 5b (EC<sub>50</sub> = 5.11 µM) were the most potent. Using a cut off of 10 µM we then assessed whether these products could inhibit the proliferation of intracellular amastigotes within macrophages. Compounds 1a (EC<sub>50</sub> = 2.76 µM), 1b (EC<sub>50</sub> = 1.07 µM) and 2b (EC<sub>50</sub> = 3.81 µM) were the most potent derivatives (Table 1, entry 3).

Table 1. IC<sub>50</sub> for leishmanicidal activity of nitro-heteroarylnitrones in *Leishmania* sp.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Cell</th>
<th>1a (µM)</th>
<th>1b</th>
<th>5a</th>
<th>2a</th>
<th>2b</th>
<th>5b</th>
<th>Miltefosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Macrophages CC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>107.8 ± 0.1</td>
<td>49.9 ± 0.8</td>
<td>27.8 ± 0.2</td>
<td>29.8 ± 1.9</td>
<td>24.2 ± 0.3</td>
<td>6.25 ± 0.43</td>
<td>96.5 ± 3.4</td>
</tr>
<tr>
<td>2</td>
<td>Promastigotes EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1.99 ± 0.06</td>
<td>9.37 ± 0.09</td>
<td>25.20 ± 2.65</td>
<td>11.32 ± 0.74</td>
<td>9.72 ± 3.90</td>
<td>5.11 ± 0.54</td>
<td>7.01 ± 0.98</td>
</tr>
<tr>
<td>3</td>
<td>Intracellular amastigotes EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>2.76 ± 0.84</td>
<td>1.07 ± 0.11</td>
<td>&gt;57.9</td>
<td>-</td>
<td>3.81 ± 1.84</td>
<td>&gt;11.54</td>
<td>3.4 ± 1.00</td>
</tr>
<tr>
<td>4</td>
<td>Selectivity Index (SI)**</td>
<td>39</td>
<td>47</td>
<td>&lt;0.5</td>
<td>-</td>
<td>6</td>
<td>&lt;0.5</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>Promastigotes EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.36 ± 0.10</td>
<td>0.88 ± 0.19</td>
<td>25.68 ± 5.44</td>
<td>13.26 ± 2.57</td>
<td>3.45 ± 0.52</td>
<td>1.39 ± 0.04</td>
<td>9.81 ± 0.43</td>
</tr>
<tr>
<td>6</td>
<td>Intracellular amastigotes EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.019 ± 0.01</td>
<td>0.169 ± 0.13</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.5 ± 0.40</td>
</tr>
<tr>
<td>7</td>
<td>Selectivity Index (SI)**</td>
<td>5670</td>
<td>295</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>193</td>
</tr>
</tbody>
</table>

*IC<sub>50</sub> and CC<sub>50</sub> values correspond to the average of three independent experiments conducted in triplicate ± standard error. N.D.- Not determined.

From the selectivity index (SI) between CC<sub>50</sub> in macrophages and EC<sub>50</sub> in intracellular amastigotes of *L. amazonensis*, 1a with an SI of 39 and 1b with an SI of 47(Table 1, entry 4) were identified as the best meeting DNDi’s target product profile of > 100-fold selectivity (Katsuno et al., 2015). At this point, product 2b (SI of 6) was disregarded. Likewise, nitroaldehydes 5a and 5b showed no selectivity (Table 1, entry 1). Compound 4 (not shown) was inactive, unveiling the importance of the heterocyclic aromatic moiety for antileishmanial activity. Nitrones 3a and 3b were inactive against macrophages (CC<sub>50</sub> ≥100 µM) as well as *L. amazonensis* promastigotes due to the lack of the nitro group at C5 in the heteroaromatic ring. As an outcome of this first screening against *L. amazonensis*, it is clear that both groups (nitro and nitrone) are required in the heterocyclic scaffold for a potent biological activity and low toxicity. The second screening with *L. infantum* promastigotes (Table 1, entry 5) revealed that the products 1a (EC<sub>50</sub> = 0.36 µM), 1b (EC<sub>50</sub> = 0.88 µM), 2b (EC<sub>50</sub> = 3.45 µM) and 5a (EC<sub>50</sub> = 1.39 µM) were preferentially more potent on this visceral species in comparison with *L. amazonensis*. Further studies with products 1a and 1b against intracellular amastigotes revealed the potency in the nanomolar range for 1a (EC<sub>50</sub> = 0.019 µM) and 1b (EC<sub>50</sub> = 0.169 µM) which are, respectively, 14-fold and 6-fold more potent for intracellular amastigotes of *L. infantum* than of *L. amazonensis* (Table 1, entry 5). Together with their cytotoxicity profile, a selectivity index (SI) of 5,760 obtained for product 1a...
and 295 for product 1b make both of them very promising hit compounds for antileishmanial drug discovery (Table 1, entry 6). The produg profiles of these compounds were determined comparing results obtained with promastigotes of wild type L. donovani with those obtained with genetically modified promastigotes of L. donovani over expressing the nitroreductase 1 (NTR1high) or nitroreductase 2 (NTR2high) genes (Table 2). Miltefosine, currently used in the treatment of leishmaniasis, nifurtimox, a commercial nitro-drug mainly activated by NTR112 and delamanid, a bicyclic nitro drug activated by NTR221, were used as reference drugs standards. Table 2, entry 1 shows that 1a is almost equally strongly activated by LdNTR1 (17 times) and LdNTR2 (21 times) while 1b is even more activated (38 times) than nifurtimox (17 times) by LdNTR1 but less activated by LdNTR2 (6.1 times) than 1a (21 times) and delamanid (12.5 times). In contrast, 1c with a bulky benzyl substitution was less sensitive to both types of activation.

Thiophene derivative 2a presented a similar profile of 1a being equally activated by LdNTR1 and LdNTR2 (17 and 18 times, respectively). However, 2b was less sensitive to activation by both nitroreductases (4.6 and 7 times) than 1b. As observed with 1c, 2c was also less sensitive to the bioactivation by both nitroreductases.

We evaluated how the nitroderivatives would act on peritoneal macrophages and on L. infantum, regarding the production of ROS, initially labelling parasites with 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), using antimycobiotics as positive ROS-production standard.46 Figure 2a displays the effect of our compounds on the production of ROS in L. amazonensis promastigotes. Interestingly, nitroheteroaryl nitrones 1a,b and 2a,b did not induce the production of important amounts of ROS when compared with untreated parasites.

**Table 2.** IC50 for leishmanicidal activity of LQB’s nitrones in Leishmania donovani overexpressing NTR1 or NTR2.

<table>
<thead>
<tr>
<th>Entry</th>
<th>L. donovani</th>
<th>1a</th>
<th>1b</th>
<th>1c</th>
<th>2a</th>
<th>2b</th>
<th>2c</th>
<th>Reference Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild Promastigotes EC50 (µM)</td>
<td>0.87 ± 0.026</td>
<td>1.16 ± 0.005</td>
<td>0.99 ± 0.036</td>
<td>4.55 ± 0.149</td>
<td>1.12 ± 0.042</td>
<td>0.41 ± 0.011</td>
<td>Nifurtimox 1.89 ± 0.052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1a</td>
<td>1b</td>
<td>1c</td>
<td>2a</td>
<td>2b</td>
<td>2c</td>
<td>Delamanid 0.0015 ± 0.00003</td>
</tr>
<tr>
<td>2</td>
<td>NTR1high Promastigotes EC50 (µM)</td>
<td>0.05 ± 0.001</td>
<td>0.03 ± 0.001</td>
<td>1.22 ± 0.006</td>
<td>0.27 ± 0.009</td>
<td>0.24 ± 0.043</td>
<td>0.30 ± 0.019</td>
<td>Nifurtimox 0.11 ± 0.002</td>
</tr>
<tr>
<td>3</td>
<td>Ratio WT/ NTR1high</td>
<td>17</td>
<td>38</td>
<td>0.81</td>
<td>17</td>
<td>4.6</td>
<td>1.3</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>NTR2high Promastigotes EC50 (µM)</td>
<td>0.04 ± 0.005</td>
<td>0.19 ± 0.030</td>
<td>1.33 ± 0.037</td>
<td>0.31 ± 0.023</td>
<td>0.25 ± 0.021</td>
<td>0.76 ± 0.013</td>
<td>Delamanid 0.00012 ± 0.000001</td>
</tr>
<tr>
<td>5</td>
<td>Ratio WT/ NTR2high</td>
<td>21</td>
<td>6.1</td>
<td>0.74</td>
<td>18</td>
<td>4.5</td>
<td>0.53</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* IC50 values correspond to the average of three independent experiments conducted in triplicate ± standard error.

However, nitroaldehyde 5b, but not 5a, was able to trigger ROS in a dose and time-dependent manner. The production of ROS in murine peritoneal macrophages follows the same trend (Figure 2b). For nitroaldehyde 5b an increased ROS production was observed and can be correlated with its high toxicity.

Next, we decided to evaluate the ROS production using a mixture of compounds 5b and 1a (Figures 2a). These compounds were combined in a 1:1 molar ratio and incubated with the parasite or murine macrophages at the EC50 concentration, respectively. 0.88 µM and 16.25 µM. It is noteworthy that the generation of ROS was controlled during the 4h of incubation, confirming the antioxidant activity of 1a. These results are in agreement with the known radical scavenging capacity of the nitrone functional group47, present in the structure of 1a. It is worth noting that the aldehyde group also stabilizes the nitro anion radical formed (see Eo, infra); however, the aldehyde group is electrophilic and can combine with endogenous amino groups to exert toxicity.
Figure 2. Treatment with nitro-heteroarylnitrones generate ROS in *L. amazonensis* and mammalian cells. *L. amazonensis* promastigotes treated with nitro-heterocyclic compounds for a 4 h kinetic; concentration of the nitro-heterocyclic compounds was based on the anti-promastigote EC50. b) Murine macrophages incubated for 4h with 0.5 x EC50, EC50 and 2 x EC50 nitro-heterocyclic compounds, concentrations based on anti-promastigote assay. ROS generation was quantified using H2DCFDA (Molecular Probes). Results are presented as means ± SD; n=3; p<0.05, **p<0.01 and ***p<0.001, (Untreated) x (treated); #p<0.001, (5a x (1b+5a).

3. Mechanistic considerations

The antiparasitic mechanism of action of nitroaromatic compounds at the molecular level is not yet fully understood, but certainly involves activation through the reduction of the nitro group. Taking in account the structural similarity between 1a,b and nifurtimox, we considered different mechanisms for the antiparasitic activity of this compound, which have been proposed by several groups. In *T. cruzi* the one electron reduction to the corresponding nitro anion radical followed by recycling nifurtimox at the expense of superoxide formation was discarded (Figure 3a, left), based on the fact that ROS production was only observed in doses of nifurtimox much higher than the therapeutic dose. More recently, it has been suggested that nifurtimox is preferentially reduced by NTR1 through a sequential two-electron transfer pathway, leading to the corresponding nitroso and hydroxylamine derivatives (Figure 3a). An acyclic olenin conjugated with two electron withdrawing groups (strong 1,4-addition acceptor) is also formed from this hydroxylamine (highlighted in blue). These metabolites play a role in the antiparasitic activity and toxicity of nifurtimox. Reduction of 1a,b by *LdNRT1* (Figure 3b) could lead to similar metabolites observed for nifurtimox, although we have no experimental evidence to support this proposal. As shown in Figure 2, except for 5a, we also did not observe oxidative stress in our experiments.

On the other hand, nifurtimox is only marginally activated by NTR2, the new nitroreductase discovered by Wyllie and coworkers. NTR2 reduce preferentially bicyclic nitro compounds, but the mechanism of this reduction via one or two electron reduction pathways is still not clear.

While nifurtimox is a good structural model for the activation of our nitro-nitrones by NTR1, in delamanid and other bicyclic nitro-heteroaryl compounds activated by NTR2 the heterocyclic moiety is quite different and so, we do not have a structural model for the reduction pathway involving our compounds and NTR2. However, a one electron route suggested in Figure 3b seems a good working hypothesis. This pathway can be estimated by cyclic voltammetry, which allows to measure the redox potential (E°) of a studied system based on a one electron transfer pathway.

![Image](image_url)

Figure 3. Activation of nifurtimox and nitro-heteroarylnitrones 1a,b by nitroreductases of leishmania.

Pedron and coworkers reported that in 8-nitroquinolin-2-(1H)-ones (Figure 4a) the antiparasitic activity in *L. infantum* could be correlated with the redox potential measured by cyclic voltammetry and only compounds with values above -0.6V vs NHE, in aprotic medium, presented antileishmanial activity. An intramolecular hydrogen bond involving the nitro group and the...
N-H at the lactam moiety in 8-nitroquinolone and the formation of an aromatic oxanion is responsible for the important increase in the redox potential from -0.84 V in 8-nitroquinoline to -0.54 V. Nevertheless, these 8-nitroquinolinol-2-(1H)-ones are activated by NTR1 (2e transfer) and, despite that, the leishmanicidal activity could be correlated with the redox potential (E°), based on a monoelectronic transfer process.

In Figure 4b we show that the nitrone group can stabilize the resulting nitro-anion radical formed from 4 by resonance through the aromatic ring and E° value increase from -0.85 V in nitrobenzene to -0.65 V. So, 4 is reduced easier than nitrobenzene. Interestingly, the aldehyde group in p-nitrobenzaldehyde 7 stabilizes still better the radical anion (E° -0.55 V). A similar trend was observed in nitro-nitrones 1a,1b and 2a,2b, having E° in the range of -0.56 V to -0.59 V (4, E° -0.65 V) showing a slight dependence on the nature of the heterocyclic (O × S) and the substituent at the nitrogen atom. The aldehyde group in 5a is also capable of stabilizing the anion radical better than nitrones (E° -0.38 V). However, while nitrone group is in general not toxic, the aldehyde group is electrophilic and can react with important bioamines, explaining the higher toxicity of aldehydes over nitrones.

However, the data in Figure 4 also show that an appropriate E° value is a necessary condition to be a substrate of these enzymes, but other factors must be considered concerning the antiparasitic activity such as the molecular recognition between substrate and enzyme. For example, based on E°, 4 could be reduced by NTRs, but this compound doesn’t have leishmanicidal activity. Another point to be considered is the eventual formation of acyclic strong Michael acceptors, which have been correlated with the antileishmanial effect. In 4, a ring opening would involve the cleavage of a strong C-C bond with the destruction of the high stabilized aromatic ring and this pathway seems unfavorable when compared with 1a,1b. We could also speculate that these nitro-furan nitrones are more prone to undergo this pathway than nitro-thiopheninitrones (2a,2b), once thiophens are more aromatic than furans.

4. In vitro pharmacokinetic and physicochemical properties of compound 1b.

Drug candidates frequently fail at different stages of drug discovery and development. Wang and Urban reported that the most prominent cause of failure is poor pharmacokinetic properties.

Initially, in silico characteristics were evaluated by the pkCSM tool. Compound 1b showed compliance with the rule of 5 and solubility in aqueous medium > 10µM (confirmed experimentally). It did not show any toxicity characteristic, except for the Ames test, which is expected to give positive because of the presence of the nitro group, but without significance to humans (SI).

Since 1b has a potent leishmanicidal activity, excellent bioselectivity and, in addition, is the less expensive to synthesize among the nitro-heteroarylnitrones, we performed a preliminary evaluation of its pharmacokinetic parameters in vitro (SI); a summary of these results is shown in Figure 5. Compound 1b showed chemical stability at pH 7, without degradation greater than 25%; however, at pH 2, almost 75% of the compound was degraded within 6 h. This compound showed good metabolic stability evaluated in human microsomal fraction S9 and reasonable mouse plasma stability (Figure 5). These data provide vital information since the desirable candidate for leishmaniasis must be administered orally.

The low stability in acidic pH could possibly be minimized through appropriate formulation. Our initial solution, however, was the co-administration with omeprazole.

5. Efficacy in experimental Visceral Leishmaniasis.

As a proof of concept, we evaluated the effectiveness of compound 1b administered orally in mice infected with L. infantum (Figure 6). The animals were treated every 12 h for five consecutive days. As a strategy to minimize the possible degradation of compound 1b in the stomach, a proton pump inhibitor was administered orally 1h before treatment (omeprazole). Sima et al., demonstrated that the administration of 20mg/kg of omeprazole (ip) raises the stomach pH in rats from 3.5 to 6.7.
Importantly, omeprazole does not present leishmanicidal activity, as shown in Figures 6a and 6b.

Treatment with 1b showed a reduction in parasite load on the spleen of 76.6% and 95.0% with doses of 50 and 100mg / kg, respectively, being 2 to 3 times less potent than miltefosine. In the liver, parasite suppression was above 75% at the three doses used, without the difference in efficiency between them, being as active as miltefosine. The liver is one of the major sites for parasite burden and after 5 days of treatment, the results for compound 1b meet the criteria for a lead that should be profiled for further development. It is worth to note that 1a and 2a have in common a bulky group (t-butyl) attached to the nitrogen atom while in 1b and 2b a small methyl group occupies this place.

6. Conclusion

Nitro-heteroarylnitrone 1a and 1b are non-chiral compounds prepared in one step, in excellent yields, from commercially available 5-nitrofurfural, 5-nitro-2-formyl-thiophene and N-t-BuNH2 or N-MeNH2. The prodruk profile of these compounds is unusual as they are activated by both NtNRT1 and NtNRT2 in L. donovani. Reliance on a single enzyme for prodruk activation may leave drugs such as nifurtimox and fexinidazole vulnerable to the emergence of drug resistance, thus the activation by both NRT1 and NTR2 at the same time is a promising option to overcome this problem. The examples showed in this work, are to the best of our knowledge, the first in which monocyclic nitro-compounds are markedly activated by NTR2. A criteria list proposed by Katsuno and co-workers to define antileishmanial hit compounds includes novel structure, easy synthesis (up to 5 reaction steps), IC50 < 10 μM (against intracellular L. donovani) and selectivity index > 100 (in comparison with mammalian cells). Of the tested nitro-heteroarylnitrone, compounds 1a and 1b meet all these criteria.

Efficacy in an experimental model was demonstrated with > 70% reduction in liver parasite burden after at most 5 doses at 50mg per kg delivered orally once or twice per day. Altogether, these results indicate that the strategy of joining nitro and nitro compound in the same scaffold was successful and suggest that the study with compounds 1a and 1b should be extended to assays in other animal models. New patterns of substitution at the nitrogen atom can be used to modulate the chemical stability and selectivity for nitroreductases.

6. REFERENCES


(51) Hillard, E. A.; de Abreu, F. C.; Ferreira, D. C. M.; Janou, G.; Goulart, M. O. F.; Amatore, C. Electrochemical Parameters and Techniques in Drug Devel-


ASSOCIATED CONTENT
Supporting Information
(Word Style “TE_Supporting_Information”). A listing of the contents of each file supplied as Supporting Information should be included. For instructions on what should be included in the Supporting Information as well as how to prepare this material for publication, refer to the journal’s Instructions for Authors.

SYNOPSIS TOC (Word Style “SN_Synopsis_TOC”). If you are submitting your paper to a journal that requires a synopsis graphic and/or synopsis paragraph, see the In

The Supporting Information is available free of charge on the ACS Publications website.

brief description (file type, i.e., PDF)
brief description (file type, i.e., PDF)

AUTHOR INFORMATION
Corresponding Author
* (Word Style “FA_Corresponding_Author_Footnote”). Give contact information for the author(s) to whom correspondence should be addressed.

Present Addresses
†If an author’s address is different than the one given in the affiliation line, this information may be included here.

Author Contributions
The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally. (match statement to author names with a symbol)

Funding Sources
Any funds used to support the research of the manuscript should be placed here (per journal style).

Notes
Any additional relevant notes should be placed here.

ACKNOWLEDGMENT
(Word Style “TD_Acknowledgments”). Generally the last paragraph of the paper is the place to acknowledge people (dedications), places, and financing (you may state grant numbers and sponsors here). Follow the journal’s guidelines on what to include in the Acknowledgement section.

ABBREVIATIONS
CCR2, CC chemokine receptor 2; CCL2, CC chemokine ligand 2; CCR5, CC chemokine receptor 5; TLC, thin layer chromatography..

REFERENCES
(Word Style “TF_References_Section”). References are placed at the end of the manuscript. Authors are responsible for the accuracy and completeness of all references. Examples of the recommended formats for the various reference types can be found at http://pubs.acs.org/page/4authors/index.html. Detailed information on reference style can be found in The ACS Style Guide, available from Oxford Press.