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

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ABSTRACT: New 5-nitro-furannitrones (**1a-c**), 5-nitro-thiophennitrones (**2a-c**) and 4-nitroarylnitrone **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 nitrone 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 by76.6% 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 protozoan parasites belonging to the genus *Leishmania* (fam. *Trypanosomatidae*).[1,2] According to the World Health Organization (WHO), cutaneous leishmaniasis is the most common form, with an estimated 0.6 million to1 million new cases occurring annually. The visceral form is fatal if left untreated with anestimated 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 bruceigambiense*[4] followed by several other analogues in the following decades.[5–9] 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]

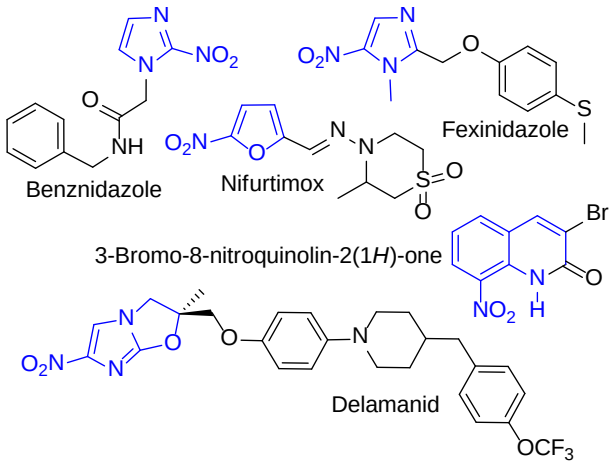


Figure 1.Nitroheteroarylcompounds with antiparasitic activity.

Currently, the arsenal of antitrypanosomal 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 eflornithine

(NECT) for the late stage of Human African trypanosomiasis. These compounds are part of the list of essential medicines of WHO since 2009[15,16] (Figure 1).

Regarding leishmaniasis, fexinidazole (Figure 1) was the first nitro-heteroaromatic compound to reach clinical trials[17,18], and its use was recently registered for sleeping sickness, as the first oral drug (DNDi, 2018[19]). The antitrypanosomal and leishmanicidal activities of 8-nitroquinolin-2-(1H)-one and derivatives was also reported (Pedron *et al*., 2018[20]). Delamanid, a bicyclic imidazole derivative approved for the treatment of drug resistant tuberculosis, showed potent activity against *Leishmania donovani in vitro* and *in vivo* and has been proposed for the oral therapy of visceral-leishmaniasis.[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)[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.[21]

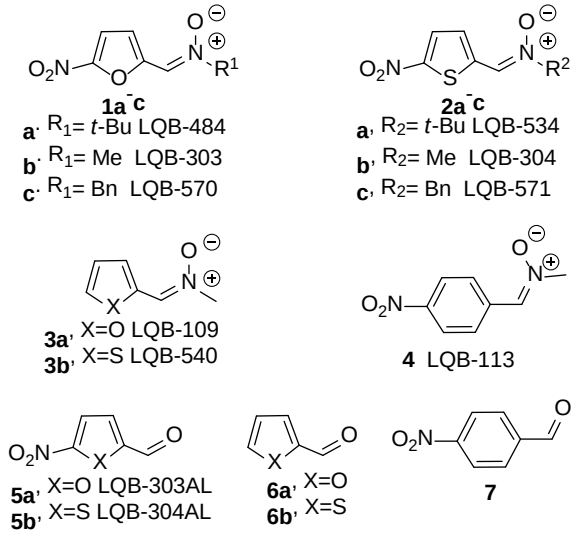


Figure 2. Nitro-heteroarylnitrones (**1**-**3**), **4** and aldehydes (**5**-**7**).

In this note we report the synthesis and leishmanicidal activity of the compounds shown in Figure 2. The importance of the new structural pattern bearing nitro and nitrone groups in the same molecule (**1a-c** and **2a-c**) is evidenced by comparing the potency and selectivity of these compounds with **3a** and **3b**. The bifunctional compound **4** was also studied to highlight the importance of the nitro-heteroaromatic moiety for the leishmanicidal activity. The nitro-aldehydes **5a**,**b** and **7**, used as starting materials to prepare the nitro-aryl nitrones, were also evaluated.

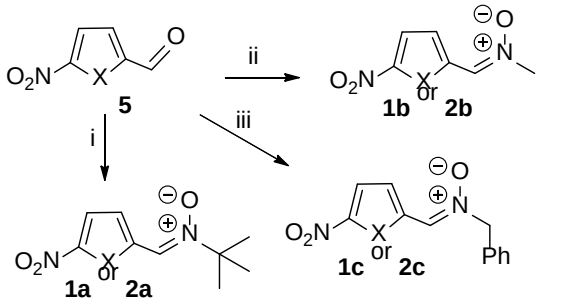
Promastigotes and amastigotes of *L. amazonensis* and *L. infantum* were used in our study. *Leishmania donovani* overexpressing NTR1 or NTR2 were also used and the results compared with those obtained with wild type.

It is worth mentioning that leaving systems have a good tolerance for the nitrone function. For example, the detoxification of natural amines and enamines is accomplished by the oxidation to the corresponding nitrone metabolites[22,23]. Also, nitrones have been detected as metabolites of several drugs and two in widespread use, chlorodiazepoxide[24]and minoxidil[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.[26–28] Due to its presumed antioxidant properties PBN (α-phenyl-*N*-*t*-butyl-nitrone) and some derivatives were evaluated as neuroprotectors in neurodegenerative diseases and some of them reached pre-clinical trials for the treatment of stroke.[29–31]Arylnitrones were also reported to be protective against microvascular damage in ischemia/reperfusion in the 'hamster cheek pouch' assay.[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.[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.[42]



i. *N*-*t*-BuBnNHOH.HCl, Na_2CO_3, TEA, CH_2Cl_2, reflux,1h
ii. *N*-MeNHOH.HCl, Na_2CO_3, TEA, CH_2Cl_2, r.t., 1h
iii. *N*-BnNHOH.HCl, Na_2CO_3, TEA, CH_2Cl_2, r.t., 1h

X=O **1a**, 90%; **1b**, 91%; **1c**, 80%
X=S **2a**, 72; **2b**,98%; **2c**, 85%

Scheme 1. Synthesis of heteroarylnitrones **1**-**3**.

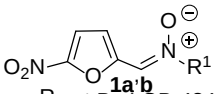
While the synthesis of *N*-*t*-butyl nitrones (**1a** and **2a**) required the use of *N*-*t*-butylhydroxylamine hydrochloride and reflux in the presence of Et_3N in CH_2Cl_2 to go to completion, *N*-methyl and *N*-benzylnitrones (**1b**,**c**; **2b**,**c**; **3a**,**b** and **4**) were prepared in good 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_3N, in CH_2Cl_2 at room temperature. In all cases *Z*-geometry was observed in the resulting nitrones, as confirmed by NOE experiments. In addition, *N*-methylnitrones can be prepared in the absence of solvent in few minutes when the reagents are macerated (SI).[43,44] Nitro-heteroarylnitrones **1a-c** and **2a-c** are described for the first time in this note.

2.2. *In vitro* activity against *L. infantum, L. amazonensis* and *L. donovani*

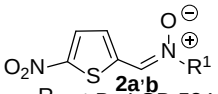
First, the synthesized compounds were screened for activity against promastigotes (insect stage) of the causative species of cutaneous (*L. amazonensis*) and visceral (*L. infantum*) leishmaniasis (Table 1). Next, active compounds from the first screening were evaluated against intracellular amastigotes ofthe 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_{50} to miltefosine (96.5 µM), used as standard. Products **1b** (CC_{50} = 49.9 µM) and **2b** (CC_{50} = 24.2 µM) also showed low toxicity.

The antileishmanial activity in promastigotes of *L. amazonensis*is shown in Table 1, entry 2). Products **1a** (EC_{50} = 1.99 µM), **1b** (EC_{50} = 9.37 µM), **2a** (EC_{50} = 11.32 µM), **2b** (EC_{50} = 9.72 µM) and **5b** (EC_{50} = 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_{50} = 2.76 µM), **1b** (EC_{50} = 1.07 µM) and **2b** (EC_{50} = 3.81 µM) were the most potent derivatives (Table 1, entry 3).

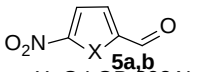
Table 1. IC_{50} for leishmanicidal activity ofnitro-heteroarylnitrones in *Leishmania sp*.



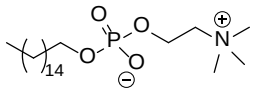
a. R_1= *t*-Bu LQB-484
b. R_1= Me LQB-303



a. R_1= *t*-Bu LQB-534
b. R_1= Me LQB-304



a. X=O LQB-303AL
b. X=S LQB-304AL



Miltefosine

Entry	Cell	1a	1b	5a	2a	2b	5b	Miltefosine
1	**Macrophages**CC_{50} (µM)	107.8 ± 0.1	49.9 ± 0.8	27.8 ± 0.2	29.8 ± 1.9	24.2 ± 0.3	6.25 ± 0.43	96.5 ± 3.4
	L. amazonensis							
2	PromastigotesEC_{50} (µM)	1.99 ± 0.06	9.37 ± 0.09	25.20 ± 2.65	11.32 ± 0.74	9.72 ± 3.90	5.11 ± 0.54	7.01 ± 0.98
3	Intracellular amastigotesEC_{50} (µM)	2.76 ± 0.84	1.07 ± 0.11	> 57.9	-	3.81 ± 1.84	> 11.54	3.4 ± 1.00
4	Selectivity Index (SI)**	39	47	<0.5	-	6	<0.5	28
	L. infantum							
5	PromastigotesEC_{50} (µM)	0.36 ± 0.10	0.88 ± 0.19	25.68 ± 5.44	13.26 ± 2.57	3.45 ± 0.52	1.39 ± 0.04	9.81 ± 0.43
6	Intracellular amastigotesEC_{50}(µM)	0.019 ± 0.01	0.169 ± 0.13	ND	ND	ND	ND	0.5 ± 0.40
7	Selectivity Index (SI)**	5670	295	-	-	-	-	193

* IC_{50} and CC_{50} values correspond to the average of three independent experiments conducted in triplicate ± standard error. N.D.- Not determined.

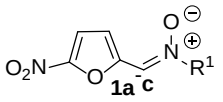
From the selectivity index (SI) between CC_{50} in macrophages and EC_{50} in intracellular amastigotes of *L. amazonensis*, **1a** with an SI of 39 and **1b** with an SI of 47(Table 1, entry 4) were identified as the best meeting DNDi's target product profile of > 100-fold selectivity (Katsuno *et al*., 2015[45]). 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_{50}>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_{50} = 0.36 µM), **1b** (EC_{50} = 0.88 µM), **2b** (EC_{50} = 3.45 µM) and **5a** (EC_{50} = 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_{50} = 0.019 µM) and **1b** (EC_{50} = 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 cytoxicity profile, a selectivity index (SI) of 5,760 obtained for product **1a**

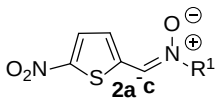
and 295 for product **1b** make both of them very promising hit compounds for antileishmanial drug discovery (Table 1, entry 6).
The prodrug profiles of these compounds were determined comparing results obtained with promastigotes of wild type *L. donovani* with those obtained with genetically modified promastigotes of *L. donovani* over expressing the nitroreductase 1 ($NTR1^{high}$) or nitroreductase 2 ($NTR2^{high}$) genes (Table 2). Miltefosine, currently used in the treatment of leishmaniasis, nifurtimox, a commercial nitro-drug mainly activated by NTR1[12] and delamanid, a bicyclic nitro drug activated by NTR2[21], 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 (H_2DCFDA), using antimycin A or nifurtimox as positive ROS-production standard.[46]
Figure 2a displays the effect of our compounds on the production of ROS in *L. amazonensis* promastigotes. Interestingly, nitroheteroarylnitrones **1a**,**b** and **2a**,**b** did not induce the production of important amounts of ROS when compared with untreated parasites.

Table 2. $IC_{50}$for leishmanicidal activity of LQB's nitrones in *Leishmania donovani* overexpressing NTR1 or NTR2.



a. R_1= *t*-Bu LQB-484
b. R_1= Me LQB-303
c. R_1 = Bn LQB-570



a. R_1= *t*-Bu LQB-534
b. R_1= Me LQB-304
c. R_1 = Bn LQB-571

Entry	*L.donovani*	1a	1b	1c	2a	2b	2c	Reference Drugs
1	Wild Promastigotes EC_{50} (µM)	0.87 ± 0.026	1.16 ± 0.005	0.99 ± 0.036	4.55 ± 0.149	1.12 ± 0.042	0.41 ± 0.011	Nifurtimox 1.89 ± 0.052 Delamanid 0.0015 ± 0.00003
2	$NTR1^{high}$ Promastigotes EC_{50} (µM)	0.05 ± 0.001	0.03 ± 0.001	1.22 ± 0.006	0.27 ± 0.009	0.24 ± 0.043	0.30 ± 0.019	Nifurtimox 0.11 ± 0.002
3	Ratio WT/ $NTR1^{high}$	17	38	0.81	17	4.6	1.3	17
4	$NTR2^{high}$ Promastigotes EC_{50} (µM)	0.04 ± 0.005	0.19 ± 0.030	1.33 ± 0.037	0.31 ± 0.023	0.25 ± 0.021	0.76 ± 0.013	Delamanid 0.00012 ± 0.00001
5	Ratio WT/ $NTR2^{high}$	21	6.1	0.74	18	4.5	0.53	12.5

* IC_{50} 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 EC_{50} 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 group[47], present in the structure of **1a**. It is worth noting that thealdehyde group also stabilizes the nitro anion radical formed (see E°, *infra*); however, the aldehyde group is electrophilic and can combine with endogenous amino groups to exert toxicity.

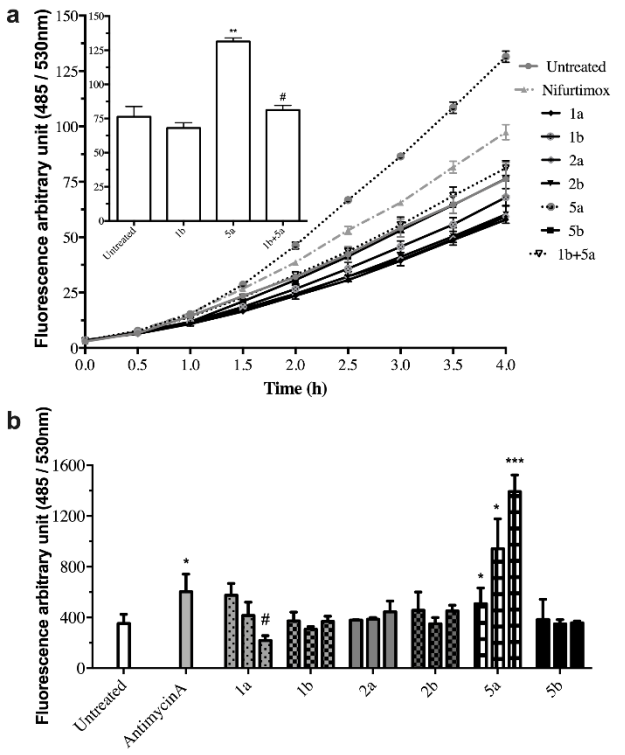


Figure 2. Treatment with nitro-heteroarylnitrones generate ROS in *L.amazonensis* and mammalian cells) *L. amazonensis* promastigotes treated with nitro-heterocyclic compoundsfor a 4 h kinetic; concentration of the nitro-heterocyclic compounds was based on the anti-promastigote EC_{50}. b) Murine macrophages incubated for 4h with 0.5 x EC_{50}, EC_{50} and 2 x EC_{50} nitro-heterocyclic compounds, concentrations based on anti-promastigote assay. ROS generation was quantified using H_2DCFDA (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.[11,48–50] 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.[39] More recently, it has been suggested that nifurtimox is preferentially reduced by NTR1[11,21,48] through a sequential two-electron transfer pathway, leading to the corresponding nitroso and hydroxylamine derivatives (Figure 3a). An acyclic olefin 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.[10,14,48] Reduction of **1a**,**b** by *Ld*NTR1 (Figure 3b) could lead to similar metabolites observed for nifurtimox, although we have no experimental evidence tosupport 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.[13,21]

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[51], which allows to measure the redox potential (E^o) of a studied system based on a one electron transfer pathway.

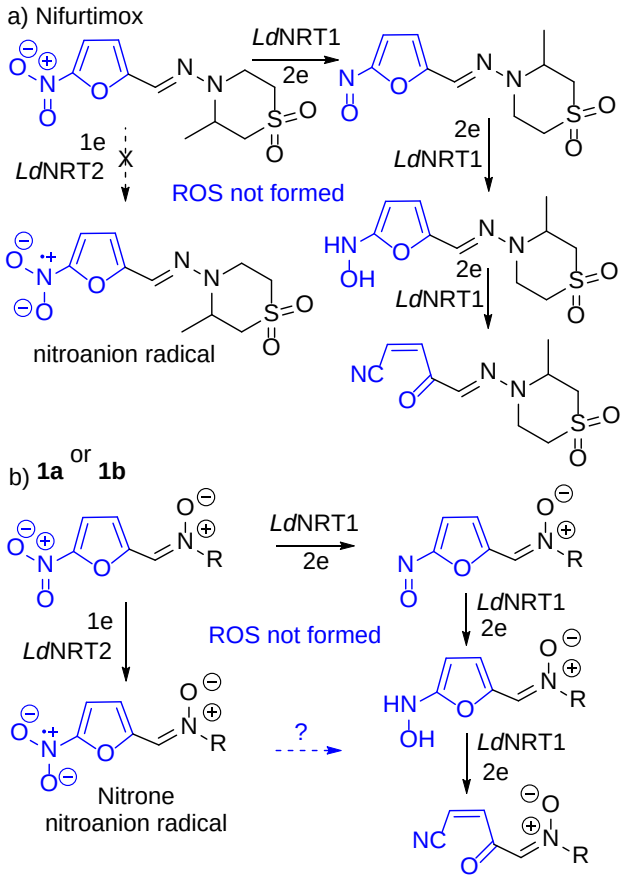


Figure 3. Activation of nifurtimox and nitro-heteroarylnitrones **1a**,**b** by nitroreductases of leishmania.

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.[52] 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 oxyanion is responsible for the important increase in the redox potential from -0.84 V in 8-nitroquinoline to -0.54 V. Nevertheless, these 8-nitroquinolin-2-(1H)-ones are activated by NTR1 (2e transfer) and, despite that, the leishmanicidal activity could be correlated with the redox potential (E^o), based on a monoelectronic transfer process.

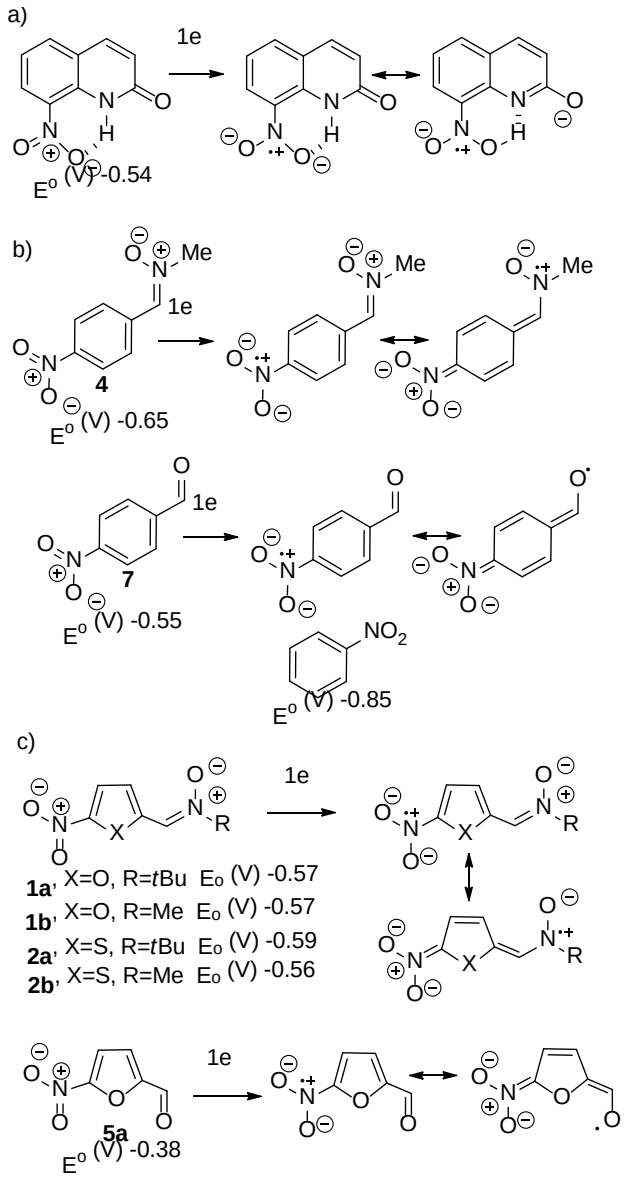


Figure 4. Stabilization of anion radicals resulting from 1e nitro reduction and redox potential of some nitro aromatic compounds measured by cyclic voltammetry in DMSO.

In Figure 4b we show thatthe nitrone group canstabilize the resulting nitro-anion radical formed from **4** by resonance through the aromatic ring and E^o 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^o -0.55 V). A similar trend was observed in nitro-nitrones **1a**,**b** and **2a**,**b**, having E^o in the range of -0.56 V to -0.59 V (**4**, E^o 0.65 V) showing a slight dependence on the nature of the heterocyclic (O x 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^o -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^o 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^o, **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**,**b**. We could also speculate that these nitro-furan nitronesare more prone to undergo this pathway than nitro-thiophenenitrones (**2a**,**b**), 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.[56] 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).[56]

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.[54] 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.[55] Im-

portantly, omeprazole does not present leishmanicidal activity, as shown in Figures 6a and 6b.

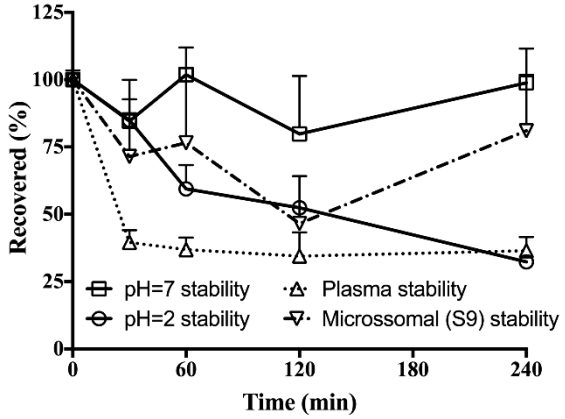


Figure 5. Chemical, microsomal and plasmatic stability of compound **1b**.

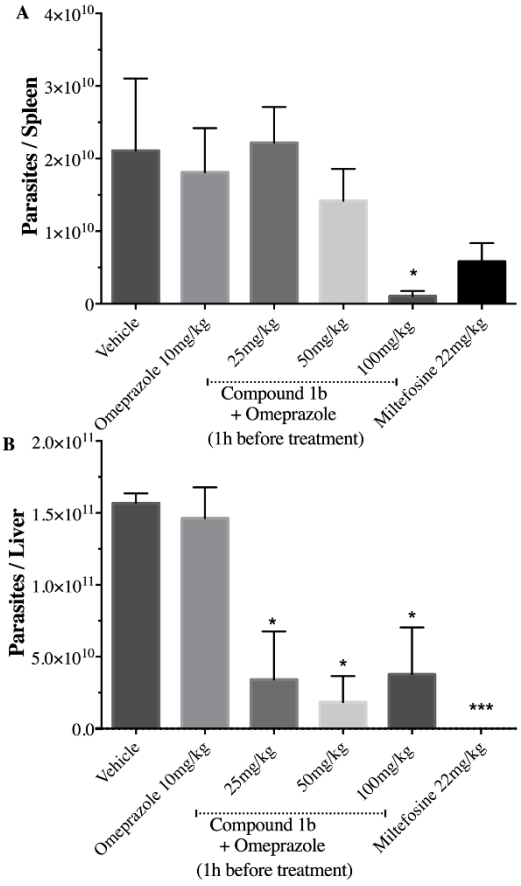


Figure 6. Efficacy of **1b** prototype in an experimental model of visceral leishmaniasis. BALB/c mice (n=5) were infected intraperitoneally with 1x10^8 *L. infantum* promastigotes. Seven days after infection, treatment with oral omeprazole 10mg was started 1 h before treatment with **1b** derivative at concentrations of 25, 50 and 100mg/kg twice a day, orally. Control groups were treated with 22mg/kg of miltefosine twice a day and vehicle. The parasitic load in the spleen (a) and liver (b) was estimated by a limiting dilution test. *p<0.05 and *** p<0.001 treated groups vs omeprazole group.

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-heteroaylnitrones**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*-BuNHOH or *N*-MeNHOH. The prodrug profile of these compounds is unusual asthey are activated by both *Ld*NRT1 and *Ld*NRT2 in *L. donovani*. Reliance on a single enzyme for prodrug 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 [45] to define antileishmanial hit compounds includes novel structure, easy synthesis (up to 5 reaction steps), IC_{50} < 10 μM (against intracellular *L. donovani*) and selectivity index > 100 (in comparison with mammalian cells). Of the tested nitro-heteroarylnitrones, 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 nitrone 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.

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ABBREVIATIONS

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

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