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Redox-active dinitrodiphenylthioethers against *Leishmania*: Synthesis, structure–activity relationships and mechanism of action studies

Dawn A. Delfín *, Rachel E. Morgan, Xiaohua Zhu, Karl A. Werbovetz

The Ohio State University, College of Pharmacy, Division of Medicinal Chemistry and Pharmacognosy. 500 West 12th Avenue, Columbus, OH 43210, USA

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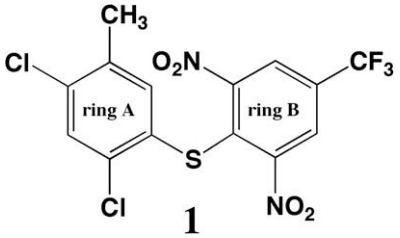
ABSTRACT

BTB 06237 (2-[(2,4-dichloro-5-methylphenyl)sulfanyl]-1,3-dinitro-5-(trifluoromethyl) benzene), a compound previously identified through QSAR pharmacophore development and a virtual screen of the Maybridge database, possesses potent and selective activity against *Leishmania* parasites. In the present study, several analogs of BTB 06237 were synthesized and analyzed for activity against *Leishmania* axenic amastigotes, their ability to reduce the level of parasitemia in peritoneal macrophages, and their ability to generate reactive oxygen species (ROS) in *L. donovani* promastigotes. It was found that an aromatic ring must be present in the position occupied by the 2,4-dichloro-5-methylphenyl group in the lead compound, but changing the functional groups generally has little effect on the antileishmanial potency. Alterations to the 1,3-dinitro-5-(trifluoromethyl)benzene ring have more influence on antiparasitic activity with two aromatic nitro groups and a third electron-withdrawing group being required. This structural requirement corresponds with redox potential, the ability to generate ROS in the parasites, and dissipation of the mitochondrial membrane potential. Finally, we used this collection of data to design a new antileishmanial compound with strong activity in vitro and improved properties as an antileishmanial candidate.

1. Introduction

Leishmaniasis is a collection of devastating tropical diseases caused by protozoan parasites of the *Leishmania* genus, affecting 12 million worldwide with 2 million new incidences occurring yearly.[1] The current treatments have a number of negative attributes including toxicity, expense, inconvenient/prohibitive routes of administration, and loss of effectiveness due to parasite resistance. New treatments must be investigated to stay ahead of these drawbacks, especially resistance.[2–4] To this end, we performed a quantitative structure–activity relationship study on a number of dinitroaniline sulfonamide compounds with known antileishmanial activity[5–7] and generated a pharmacophore. This pharmacophore was then used to perform a virtual screen of the Maybridge database of drug-like compounds, which led to the identification of BTB 06237 (2-[(2,4-dichloro-5-methylphenyl)sulfanyl]-1,3-dinitro-5-(trifluoromethyl)benzene, **1**), a compound with potent activity against *L. donovani* axenic amastigotes (IC_{50} = 0.52 ± 0.20 μM).[8] Initial investigations into the mechanism of action of this compound showed that it causes disruption of mitochondrial function and morphology.[8] This report describes the follow-up to the initial studies on this promising antileishmanial lead.

In order to learn more about the structure–activity relationships (SAR) around **1** against *Leishmania*, we obtained a series of analogs of this compound synthetically (13 compounds) or commercially (5 compounds). We then tested these analogs against axenic and intracellular amastigotes of *L. donovani* and β-lactamase-transfected *L. amazonensis*, respectively, in order to assess their antiparasitic activities. Aromatic nitro groups, as are present on compound **1**, are known to have significant biological redox activity leading to the potentially cytocidal production of ROS.[9–12] ROS production, in addition, could be correlated with disruption of mitochondria,[13] as was observed in our previous study.[8] We thus determined the likely effect of substituents on redox potentials of **1** and three other analogs, making connections between the antileishmanial activity, the production of reactive oxygen species (ROS) and dissipation of the mitochondrial membrane potential. The results of these SAR studies and mechanistic investigations are reported. Finally, the SAR data led us to design a novel compound, which was found to have promising antileishmanial activity in vitro and improved properties as an antileishmanial candidate.



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* Corresponding author. Tel.: +1 614 292 7475 fax: +1 614 292 4118.
E-mail address: delfin.1@osu.edu (D.A. Delfín).

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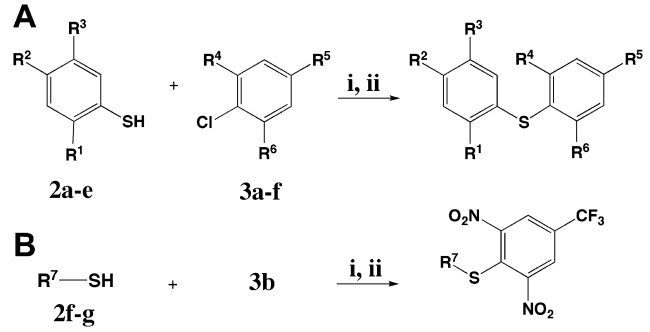
2. Results

2.1. Synthesis

The diarylthioether lead compound **1** consists of two aromatic rings: 'ring A,' the 2,4-dichloro-5-methyl substituted phenyl ring, and 'ring B,' the 2,6-dinitro-4-trifluoromethyl substituted phenyl ring. To synthesize analogs of **1**, we envisioned coupling a variety of thiols of ring A with a variety of aryl halides of ring B. Initial attempts at preparing these thioether compounds by uncatalyzed nucleophilic aromatic substitution were unsuccessful. One equivalent of each of the aromatic thiols (either **2a** or **2b**) and the aryl halides (either **3b**, iodobenzene or 1,3-dinitro-2-iodobenzene) with 3 or more mol equiv of KO*t*Bu as a base in anhydrous DMSO at 100 °C overnight were used in several separate attempts at uncatalyzed coupling. However, copper-catalyzed Ullmann condensation[14,15] as described in Scheme 1 was successful for preparing the desired compounds (1A for **4**–**7**, **12**, **16**–**19**, and **22**; 1B for **10**–**11**). We chose to synthesize most of these diaryl and alkyl–aryl thioether compounds utilizing readily available and inexpensive aryl chloride reagents to couple ring B with ring A (**3a**–**3f**) rather than the potentially higher-yielding aryl iodide reagents that were not commercially available.[14,15] The synthetic methodology was improved using neocuproine as the ligand in the reaction (as opposed to ethylene glycol), triethylamine as the base (as opposed to potassium *tert*-butoxide), and ethyl acetate as the solvent (as opposed to isopropanol or tetrahydrofuran). These reaction conditions provided the desired product in yields up to 59%. Yields may have been reduced due to the formation of disulfide from oxidation of the thiol starting material. Disulfide was recovered after every coupling reaction and we used an excess of thiol in an attempt to minimize this loss as much as possible.

In theory, electron-withdrawing groups on an aromatic electrophile should make it more reactive while electron-donating groups are deactivating in Ullmann condensation reactions. The opposite is true for an aromatic nucleophile. Our results were consistent with these established reactivity parameters.[14,15]

Compound **8** was obtained in a 47% yield by heating **7** to reflux for 4 h in 1:1 concentrated HBr in glacial acetic acid. Compound **21** was obtained in a 33% yield by an uncatalyzed nucleophilic aromatic substitution reaction between 2,4-dichloroaniline and **3b**, refluxing overnight in ethyl acetate containing triethylamine.



Scheme 1. Reagents and conditions: (i) compounds **2a**–**2g**, base (KO*t*Bu or TEA), 30 min; (ii) ligand (HO[CH_2]$_2$OH or neocuproine), CuII, compounds **3a**–**3f**, <80 °C, overnight; solvents: *i*PrOH, THF or EtOAc; 11–59% yield. Generally, using TEA, neocuproine and EtOAc gave higher reaction yields.

2.2. SAR part 1: in vitro activity against axenic and intracellular amastigotes and assessment of toxicity

The analogs were first tested in vitro against *L. donovani* axenic amastigotes. Compounds with potent activity (IC_{50} < 2 μM) were then evaluated against intracellular amastigotes of β-lactamase-transfected *L. amazonensis*[16] within murine peritoneal macrophages, and against Vero simian kidney cells to assess toxicity. Three classes of analogs were examined: ring A analogs where the substituents about ring A were altered or the aromatic ring was replaced with an alkyl group, ring B analogs where the substituents about ring B were altered, and linker analogs where the thioether linkage of **1** was changed to an ether or secondary amino linkage. The activities of these analogs are given in Table 1.

2.2.1. Ring A analogs (4–11)

From the data in Table 1, it is clear that an aromatic ring is required at the ring A position as the loss of aromaticity led to a significant decrease in activity (**10** and **11**). Interestingly, the presence of the sterically bulky cyclohexyl group makes **10** nearly three times more active than **11**, which contains an ethyl group at the corresponding position. Nevertheless, steric bulk is not the main factor for conveying activity in this series, as **10** is 70 times less active than **1**, demonstrating the importance of aromaticity at this position.

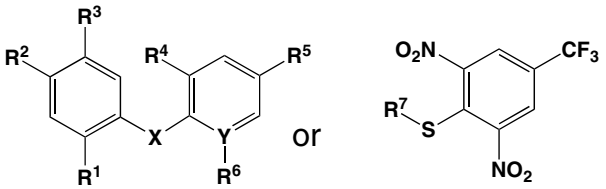
Among the aromatic ring A analogs, only compounds **4** and **6** have activity that is approximately equipotent to **1**; **5** and **7** are 3 times less potent and **8** and **9** are 10 and 14 times less active, respectively. However, the activity of these diphenyl thioethers is still quite high against *L. donovani* axenic amastigotes. All of the ring A analogs examined in the infected macrophage assay have activity against intracellular amastigotes in the micromolar range. With **1** and **6**, the potency decreases 6-fold going from the axenic amastigote assay to the infected macrophage assay, and **6** is very toxic to the macrophages themselves. With **4**, **5,** and **7**, the potency decreases 1.5- to 3-fold from the axenic amastigote assay to the intracellular assay, and **7** in particular has lower macrophage toxicity. Unfortunately, however, all of the ring A analogs were less selective than **1** for the parasites compared to Vero cells, indicating a greater potential for in vivo cytotoxicity is associated with these analogs.

2.2.2. Ring B analogs (12–19)

Although the substituents on ring A have little bearing on activity, ring B has stringent requirements that two nitro groups plus a third electron-withdrawing group (–CF_3 as in **1** and **16** or –CN as in **18**) must be present for a significant antileishmanial effect. A comparison of the activities of **1** and **16** indicate that the location of these three groups on ring B can be altered. Analogs that did not have two nitro groups (**13**–**15**, **19**) were inactive. Compound **14**, in which a pyridyl nitrogen replaces one of the nitro groups, is the only one of these mononitro and non-nitro compounds that retains a measure of activity against *L. donovani*. Compounds **12** and **17**, which do contain two nitro groups, are much less active than **1**. Notably, compound **17** possesses a weakly electron-*donating* group instead of an electron-*withdrawing* group and is completely inactive. Compound **12**, which instead bears an electronically-neutral hydrogen atom, retains weak activity against the parasites.

Compounds **16** and **18**, which are close to **1** in potency toward axenic amastigotes, are similarly active against intracellular amastigotes. However, they are less selective for the parasites compared to Vero cells.

Table 1
In vitro activities of compounds



Compound	R^1	R^2	R^3	R^4	R^5	R^6	X	Y	*L. donovani* IC_{50}[a] (μM)	Inf. Mφ IC_{50}[a] (μM)	Vero IC_{50}[a] (μM)
1	Cl	Cl	CH_3	NO_2	CF_3	NO_2	S	C	0.56 ± 0.12	3.4 ± 2.0[b]	9.7 ± 3.9
4	H	H	H	NO_2	CF_3	NO_2	S	C	0.96 ± 0.31	2.6 ± 1.7[c]	6.0 ± 0.2
5	H	H	CH_3	NO_2	CF_3	NO_2	S	C	1.6 ± 0.3	2.4 ± 0.8[c]	7.3 ± 1.1
6	Cl	Cl	H	NO_2	CF_3	NO_2	S	C	0.40 ± 0.10	2.6 ± 1.5[d]	4.3 ± 1.8
7	H	OCH_3	H	NO_2	CF_3	NO_2	S	C	1.9 ± 0.6	5.1 ± 2.8[b]	12 ± 2
8	H	OH	H	NO_2	CF_3	NO_2	S	C	3.3 ± 0.2	—	—
9	CO_2H	H	H	NO_2	CF_3	NO_2	S	C	7.3 ± 1.3	—	—
10	R^7 = cyclohexyl group								35 ± 13	—	—
11	R^7 = ethyl group								98 ± 24	—	—
12	Cl	Cl	CH_3	NO_2	H	NO_2	S	C	23 ± 6	—	—
13	Cl	Cl	CH_3	NO_2	CF_3	H	S	C	98 ± 7	—	—
14	Cl	Cl	CH_3	NO_2	CF_3	—	S	N	59 ± 8	—	—
15	Cl	Cl	CH_3	H	NO_2	H	S	C	88 ± 10	—	—
16	Cl	Cl	CH_3	NO_2	NO_2	CF_3	S	C	0.62 ± 0.44	1.8 ± 0.8[c]	5.2 ± 3.1
17	Cl	Cl	CH_3	NO_2	CH_3	NO_2	S	C	>200	—	—
18	Cl	Cl	CH_3	NO_2	CN	NO_2	S	C	1.0 ± 0.5	5.8 ± 2.3[b]	11 ± 1
19	Cl	Cl	CH_3	CF_3	CF_3	CF_3	S	C	100 ± 1	—	—
20	Cl	Cl	H	NO_2	CF_3	NO_2	O	C	3.4 ± 0.5	—	—
21	Cl	Cl	H	NO_2	CF_3	NO_2	NH	C	25 ± 3	—	—
Standards											
Pentamidine									2.2 ± 0.9	—	—
Amphotericin B									—	0.13 ± 0.08	—
Podophyllotoxin									—	—	0.023 ± 0.001

In the infected macrophage assay, these compounds were toxic to the macrophages at [b]13 μM, [c]6.3 μM, or [d]3.1 μM and above.
[a] Average value obtained in three or more independent experiments. Error reported is the standard deviation from the mean.

2.2.3. Linker analogs (20–21)

A thioether linkage (**6**) appears to be optimal with regard to antileishmanial activity. The ether-linked analog (**20**) is also active but is over 7-fold less potent than **6**. The presence of an amino linkage (**21**) renders the compound essentially inactive.

2.3. SAR part 2: antiparasitic activity correlates with estimated redox potential

To investigate whether the antileishmanial activity correlates with the nitro group redox activity of these compounds, we estimated the relative reduction potentials of dinitro compounds **1**, **12**, **17**, and **18** and compared this with their activities against axenic amastigotes. These compounds were chosen because they possess ring B substituents that would contribute to nitro group redox activities in different ways. Compound **1** (IC_{50} = 0.56 μM) possesses a $-CF_3$ group as the third electron-withdrawing group while **18** (IC_{50} = 1.0 μM) has a –CN group at that position. The third electron-withdrawing groups of **1** and **18** would theoretically bring the redox potential of the dinitro aromatic ring into a physiologically relevant range ($\geqslant -0.5$ mV).[9,13,17–19] Compound **12** (IC_{50} = 23 μM), on the other hand would be less likely to undergo redox reactions because it contains a hydrogen atom rather than a third electron-withdrawing group. Compound **17** (IC_{50} > 200 μM), with its weakly electron-*donating* methyl substituent, would, in theory, be even more difficult to reduce.

We used the Hammett constants (σ_p)[20] for the substituents present in these compounds (Table 2) to estimate the relative reduction potentials of the molecules compared to **12**. From this analysis, we can assume that the reduction potential of **1** and especially **18** would be higher than **12**, while that of **17** would be lower. The theoretical trend in order of (lowest to highest reduction po-

Table 2
Hammett constants for R^5 and IC_{50} values for compounds **1**, **12**, **17**, and **18**

Compound (–R^5=)	Average IC_{50}[a] (μM)	σ_p[b]
1 ($-CF_3$)	0.56	0.54
18 (–CN)	1.0	0.74
12 (–H)	23	0.00
17 ($-CH_3$)	>200	−0.14

[a] Data from Table 1.
[b] Values for σ_p are taken from Hansch et al.[20]

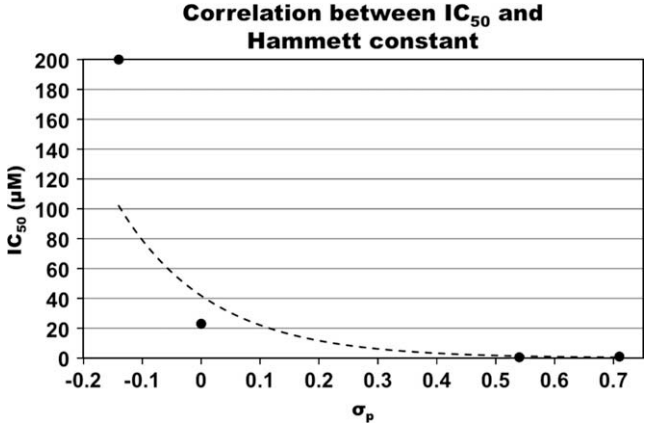


Figure 1. Correlation between σ_p values for the R^5 substituents of compounds **1**, **12**, **17**, and **18** and IC_{50} values. The IC_{50} value for **17** is assumed to be 200 μM. The dashed line represents the exponential trend line as determined in *Microsoft Excel*, r^2 = 0.90. Values for σ_p are taken from Hansch et al.[20]

tential) would be **17** < **12** < **1** < **18**. The σ_p values correlate strongly with antileishmanial activity, $r^2 = 0.90$ (Fig. 1). The correlation between IC_{50} and σ_p appears to be inverse-exponential where a higher σ_p value is correlated with a lower IC_{50} value (increased activity).

2.4. SAR part 3: reduction potential corresponds to ROS generation and reduction of the mitochondrial membrane potential

Because of the clear correlation between the relative reduction potential of compounds **1**, **12**, **17,** and **18** and their antileishmanial activity, these compounds were assessed for their ability to generate ROS in the parasites. ROS generation is an understood consequence of redox activity in cells.[9,13,21,22] Induction of ROS in *L. donovani* promastigotes was measured by flow cytometric detection of the dye CM-H_2DCFDA, which enters into and remains within cells but becomes fluorescent only in the presence of ROS (especially peroxides).[23] Figure 2 shows that ROS-induced fluorescence levels increase 17-fold in *L. donovani* promastigotes treated with 250 μM of H_2O_2 for 24 h, which serves as a positive control. The parasites were also treated with the test compounds at 0.1, 1.0, 10 and 100 μM for 24 h.

Interestingly, **1** showed virtually no increase in ROS generation relative to controls at 0.1 and 1 μM, a very modest increase at 10 μM, and a dramatic increase at 100 μM. On the other hand, **18**, which was estimated to have greater redox potential, showed a dramatic increase in levels of ROS at both 10 and 100 μM. Thus, **18** is a better inducer of ROS than the parent compound **1** in the parasites even though their IC_{50} values against amastigotes are nearly identical (within experimental error). Supporting the hypothesis that compound **12** does not undergo redox activity to generate ROS in the parasites, there was no increase in fluorescence observed in parasites treated with any concentration of compound tested. Although **17** caused no increase in ROS generation at 0.1, 1, and 10 μM, it surprisingly showed a modest increase in fluorescence at 100 μM.

We previously demonstrated that **1** caused a concentration-dependent depolarization of the mitochondrial membrane potential ($\Delta\Psi_m$).[8] To determine if **18** is likewise able to dissipate $\Delta\Psi_m$ as a result of its ROS activity, and if ROS inactive compounds **12** and **17** are not, the $\Delta\Psi_m$ of *L. donovani* treated with test compounds for 24 h was measured using the dye rhodamine 123. Because ROS levels did not increase dramatically in parasites treated with less than 10 μM of the test compounds, 10 μM was therefore the lowest concentration used in this $\Delta\Psi_m$ assay. Figure 3 shows that treating the parasites with the uncoupler FCCP for 2 h at 100 μM (positive control) caused a reduction in the level of rhodamine 123 fluorescence by half compared to control parasites. In support of previous results, **1** is likewise able to reduce $\Delta\Psi_m$ in a concentration-dependent manner. At 100 μM, the reduction in fluorescence in parasites treated with **1** is 90%. Even at concentrations as low as 10 μM, **18** caused 90% reduction in $\Delta\Psi_m$. On the other hand, **12** and **17** were not able to reduce the parasites' $\Delta\Psi_m$ by more than ~20%. This is in accordance with their inability to generate ROS in the parasites.

Taken together, the data support the hypothesis that compounds **1** and **18** are active because their two nitro groups plus a third electron-withdrawing group allow biological redox activity to occur (i.e. they possess favorable reduction potentials), which leads to generation of ROS in the parasites and reduction of $\Delta\Psi_m$. Compounds **12** and **17**, which do not have favorable reduction potentials, do not have good antiparasitic activity, are not able to generate ROS in the parasites, and are not able to reduce $\Delta\Psi_m$. Figures 1 and 4 visually demonstrate how the IC_{50} values, reduction potentials, ROS levels, and reduction of $\Delta\Psi_m$ are correlated with one another.

2.5. Activity of the top candidate compound

In previous work, we showed that the lead compound **1** is inactive in vivo in a mouse model of visceral leishmaniasis despite its potency in vitro.[8] Although the cause of the lack of in vivo activity is still under investigation, we were suspect of the extreme lipophilicity of **1**. We used the software *ChemAxon* to estimate its log*P* (*c*log*P*) at 6.85, which is in general agreement with the published *c*log*P* value of 6.1[24] (in other words, extremely high). This value is significantly higher than the suggested upper limit for log*P* of ~5 for compounds with good bioavailability.[25] Therefore, we hoped to find amongst our active analogs a compound with a *c*log*P* value of approximately 5 or below in accordance with Lipinski's Rule of 5. The *c*log*P* values of the active compounds (IC_{50} < 2 μM) were similarly calculated in *ChemAxon* and are given in Table 3.

Combining the *c*log*P* values of the active analogs with the SAR data, the ideal compound would likely possess (1) a *p*-methoxy

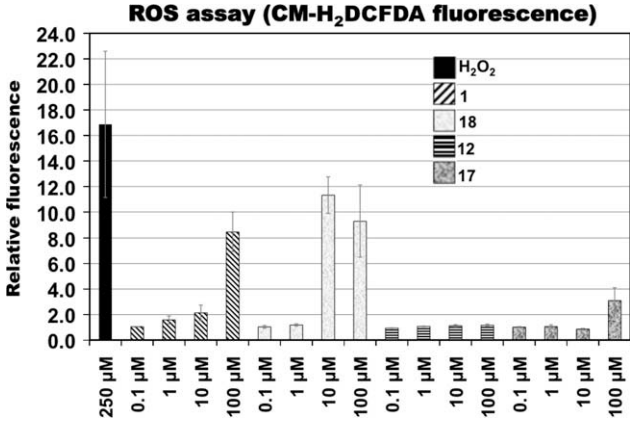


Figure 2. ROS levels of *L. donovani* promastigotes treated with test compounds. Parasites were incubated with compounds for 24 h ROS generation was and determined by the level of fluorescence from the oxidation of CM-H_2DCFDA in three separate experiments. Fluorescence levels of controls (parasites treated with 1% DMSO vehicle only) are normalized to 1.0 and the fluorescence relative to that value are shown for the other samples. Error bars represent the standard error of the mean values.

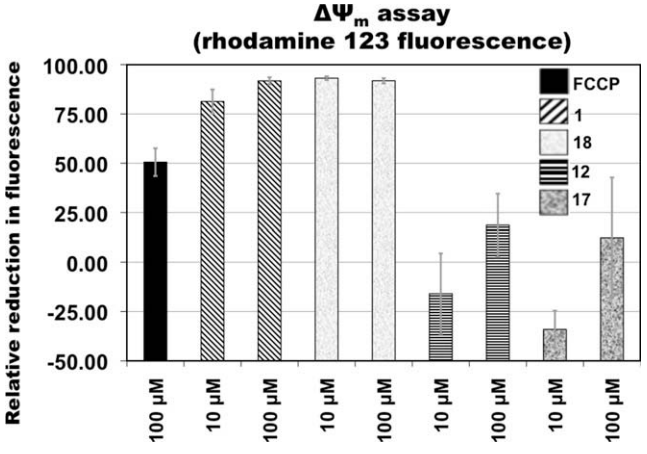


Figure 3. Reduction in mitochondrial membrane potentials of *L. donovani* promastigotes treated with test compounds. Parasites were incubated with compounds for 24 h, then mitochondrial membrane potentials were determined by the level of fluorescence of rhodamine 123 in three separate experiments. Fluorescence levels of controls (parasites treated with 1% DMSO vehicle only) are normalized to 0% and the % reduction in fluorescence relative to that value is shown for the other samples. Error bars represent the standard error of the mean values.

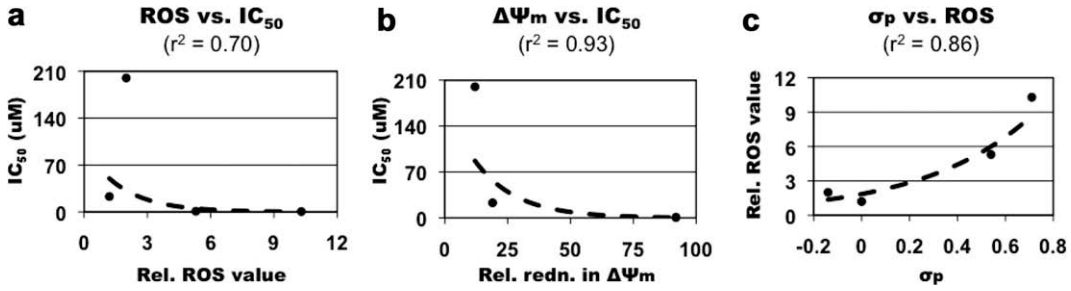
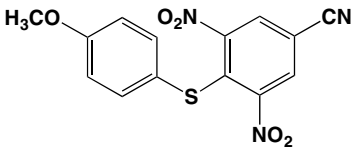


Figure 4. Correlations between the various assay parameters, supporting the hypothesis that activity in *Leishmania* is related to (a) ROS generation, (b) reduction of the $\Delta\Psi_m$, and (c) estimated reduction potential. Average values were used as the specific data points. In (a) and (b), the IC_{50} value of **17** is assumed to be 200 μM. The dashed lines represent the exponential trend line of the data as determined in *Excel*, with the r^2 values given in parentheses.

group on ring A which is still active against the parasites, with low Vero cell toxicity (see **7** in Table 1) but improved *c*log*P* value relative to **1** at 5.09; (2) the two necessary nitro groups on ring B plus a cyano group analogous to **18**, which is active against the parasites (Table 2) and improves the *c*log*P* value relative to **1** at 5.78. Additionally, the cyano analog **18** appears to possess more redox activity than **1**. The structure of this rationally designed compound **22** is shown below. The *c*log*P* of **22** at 4.02 is acceptable with regard to Lipinski's rules. As a result, we synthesized this compound and tested it against the parasites in vitro and in vivo. The results of these assays are given in Table 4 and are compared to the data for **1**. In vitro, **22** is essentially equipotent to **1**. When **22** was given in vivo to *L. donovani*-infected BALB/c mice at 25 mg/kg/day for 5 days, it caused a 28% decrease in parasitemia on average, with activity just reaching statistical significance compared to vehicle-treated mice ($p = 0.05$). Comparatively, **1** did not reduce the parasitemia in the mice even at 50 mg/kg/day.[8]



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3. Discussion

The SAR study showed that in this class of compounds based on **1**, ring A must be aromatic but may carry nearly any functional group and still remain active. Thus, appending certain functional groups to this ring may be a good way to modulate the chemical properties of the compounds to improve bioavailability without a significant reduction in antiparasitic activity. However, it must be noted that very polar and negatively-charged groups appear to decrease the activity more than 10-fold. The effects of heteroaromatic rings and multi-ring systems at this position are worthy of exploration.

Ring B is the 'business end' of these compounds *via* its redox activity. Close associations between antiparasitic activity, reduction potential, and ROS generation support this conclusion about the function of ring B and explain the requirement for two nitro groups and a third electron-withdrawing group. Considering all of the data together, including microscopy of the parasite mitochondrion described in our previous publication,[8] we propose the following as a likely mechanism of action of these compounds: the molecule enters the parasite and, if the compound possesses two nitro groups and a third electron-withdrawing group, it undergoes reduction to the nitro anion radical form. We describe the proposed mechanism of nitro anion radical formation and subsequent redox cycling in Scheme 2. During the subsequent redox cycling, ROS are generated in the parasites and disruption of the mitochondrial membrane potential occurs along with overall mitochondrial dilation and breakdown.[8] Ultimately, the parasites die. The compounds, especially **1** and **22**, appear to be more selective in their redox activities against the parasites than against mammalian cells. However, these data do not indicate whether the generation of ROS causes the mitochondrial breakdown or if a direct mitochondrial effect leads to the generation of ROS. A time-course study delineating the precise mechanistic order of the observed effects is needed to further dissect the relationship between ROS formation and mitochondrial disruption. Furthermore, it would be useful to investigate whether these compounds bind to a specific target within the parasites. Several nitroheterocyclic compounds have been shown to act as inhibitors of trypanothione reductase and as inducers of redox cycling (or 'subversive substrates') in the presence of both trypanothione reductase and lipoamide dehydrogenase.[28,29] It would be logical to focus on the action of these active dintroaromatics against such enzymes as well.

Though the requirements for two nitro groups and a third electron-withdrawing group are rather stringent, the effects of electron-withdrawing groups other than trifluoromethyl and cyano should be investigated in a future series of analogs; such groups may show additional potential for modulating the chemical prop-

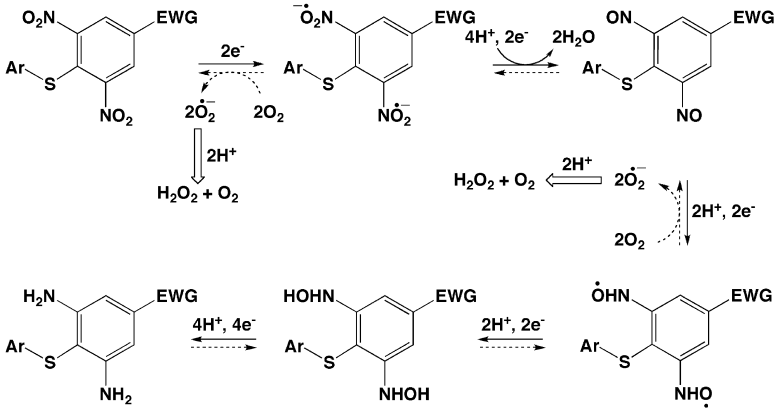
Table 3
*c*log*P* values of the compounds with potent activity against *L. donovani*

	*c*log*P*
1	6.85
4	5.34
5	5.81
6	6.38
7	5.09
16	6.85
18	5.78

Table 4
Antileishmanial activity data for **1** and **22**

	L. donovani IC_{50}[a] (μM)	Inf. Mφ IC_{50}[a] (μM)	Vero cells IC_{50}[a] (μM)	BALB/c mice % reduction in infection[c]
1	0.56 ± 0.12	3.4 ± 2.0[b]	9.7 ± 3.9	−2% ± 1%[d]
22	0.67 ± 0.24	2.6 ± 1.2[b]	11 ± 2.9	28% ± 13%
Miltefosine	—	—	—	41% ± 13%

[a] Average value obtained in three or more independent experiments. Error reported is the standard deviation from the mean.
[b] In the infected macrophage assay, these compounds were toxic to the macrophages at 13 μM and above.
[c] Average value obtained from 4 or 5 mice and is compared to vehicle-treated controls. Error reported is the standard deviation from the mean. Compound doses (given i.p.): **1** at 50 mg/kg/day for 5 days, **22** at 25 mg/kg/day for 5 days, miltefosine at 3 mg/kg/day for 5 days.
[d] Data taken from Delfín, et al., *J. Med. Chem.*, **2006**, *49*, 4196–4207.[2]



Scheme 2. Proposed redox cycling of nitroaromatic molecules[9–11,13,26,27] as applied to this series of compounds. 'Ar' represents an aromatic ring and 'EWG' represents an electron-withdrawing group. The solid arrows indicate the reduction reactions, leading to amino groups, while the dotted arrows show the reverse oxidation reactions. During the oxidation reactions, the ROS superoxide radical anion ($O_2^{\bullet -}$) is formed. Superoxide dismutase may then convert $O_2^{\bullet -}$ to the ROS H_2O_2 (open arrows). Both nitro groups may or may not undergo redox cycling and may not be simultaneously reduced/oxidized.

erties of these compounds for improved bioavailability. Finally, although the ether and thioether linkages between rings A and B are not equivalent with regards to activity, the ether compound is still somewhat active. There are several possibilities for the linker effect, including (1) a hydrogen bond acceptor may be required at the linker atom to interact with a hydrogen bond donor at a yet unknown binding site; the sulfur and oxygen linkers can serve as hydrogen bond acceptors while the NH linker in **21** is more likely to act as a hydrogen bond donor; (2) the minimum energy conformation of the thioether may allow for more efficient binding to the target than with the ether or anilino compound; or (3) the larger and more polarizable thioether may confer ideal electronic, steric, and/or hydrophobic features to the molecules.

The SAR data, along with an analysis of the *c*log*P* values of the active compounds, led us to synthesize **22** as an antileishmanial agent with promising in vitro and measurable in vivo activity. This is an improvement over **1** in that **1** caused no reduction in parasite burden in *Leishmania*-infected mice, but **22** caused a 28% reduction. The improved in vivo activity of **22** compared to **1** may be due to the decreased lipophilicity of this compound. However, the in vivo activity of **22** is admittedly lower than we hoped and may be due to other factors such as metabolic inactivation. Additional studies on this novel antileishmanial lead compound will hopefully lead to analogs with improved potency in vitro as well as in vivo.

Compounds possessing aromatic nitro groups are often red-flagged as not being viable drug development candidates because their redox activity could lead to host toxicity. However, several nitroaromatic compounds are in clinical use. For example, nifurtimox and benznidazole are used to treat Chagas disease, which is caused by the related kinetoplastid parasite *Trypanosoma cruzi*.[10,30] Additionally, many nitroheterocycles are under laboratory investigation against a variety of kinetoplastids because of their encouraging potential as treatments against these parasites.[27,31] The ROS-generating ability of nitroamomatic compounds is useful because kinetoplastid parasites are particularly sensitive to oxidative stress.[32] On the other hand, these drugs are considered to be toxic. Therefore, novel nitroaromatic therapeutic agents should have as large a selectivity index as possible to avoid some of the toxicity issues of the current nitroaromatic drugs. Our current lead compounds **1** and **22** have moderate, but respectable, selectivity towards parasites over mammalian cells. It is possible that additional SAR efforts may result in compounds with improved selectivities. In vivo selectivity may improve after a full analysis of the ADMET properties of this class of compounds followed by a rational design of additional drug candidates. Another viable option for active but potentially toxic nitroaromatic drugs is to use combination therapy. The minimum effective dose of the compounds could be reduced if combined with other antiparasitic drugs. Sanz et al. recently published their studies on *T. cruzi* superoxide dismutase (SOD) inhibitory compounds.[33] SOD is a major antioxidant enzyme in kinetoplastids. A potent SOD inhibitor, in synergy with a pro-oxidant compound such as the active analogs reported here, could conceivably lead to safe yet effective treatments for diseases caused by kinetoplastid parasites. Already in clinical use is a nifurtimox/eflornithine combination to extend the useful life of the latter drug in the treatment of human African trypanosomiasis.[34,35] With further investigation, a similar outcome might be possible for the potent antiparasitic agents described in this report.

4. Conclusions

We demonstrated that the nitroaromatic compounds reported here are redox-active agents that increase ROS generation in *Leishmania* parasites and dissipate the mitochondrial membrane potential. Additionally, while our previous ligand-based drug discovery efforts led to **1**, a potent new antileishmanial compound, in this study we were able to attain modest in vivo activity in this class of compounds using SAR analysis. The resulting lead compound, **22**, is thus a starting point for efforts toward developing a new series of molecules to treat leishmaniasis. More research on **22** and its analogs is warranted, including additional SAR and metabolic analyses.

5. Experimental

5.1. General

All commercially available solvents and reagents were used without further purification. Reagents for synthesis were purchased from Sigma–Aldrich (St. Louis, MO), except: concentrated sulfuric acid, ethylene glycol, triethylamine (Mallinckrodt, Phillipsburg, NJ); 2-chloro-1,3-dinitrobenzene, 2-chloro-1,3-dinitro-5-methylbenzene (Alfa Aesar, Ward Hill, MA); and 2,4-dichloroaniline (Acros, Geel, Belgium). Analogs **9**, **13**–**15**, and **20** were purchased from Maybridge (Cornwall, England) and were used without further purification in antileishmanial assays. Reagents used for the biological assays were: CellTiter Reagent (Promega, Madison, WI); CM-H_2DCFDA, Modified Eagle's Medium—alpha modification

(Invitrogen, Carlsbad, CA); miltefosine (Cayman Chemical Co, Ann Arbor, MI); and nitrocefin (Thermo Fisher Scientific, Waltham, MA). Proton (^{1}H) NMR spectra were generated at 300 MHz on a Bruker Avance 300 spectrometer (Billerica, MA) in acetone-d_6, methanol-d_4 or chloroform-*d*. Chemical shifts are expressed in ∂ units (ppm) relative to tetramethylsilane. Carbon-13 (^{13}C) NMR spectra were generated at 75 MHz on the same instrument. High-resolution mass spectra (HRMS) were obtained on a Micromass LCT electrospray ionization spectrometer (Waters Corp., Milford, MA). Elemental analysis (anal.) was performed at Atlantic Microlab (Norcross, GA). Melting points (mp) were taken on a Thomas Hoover capillary melting point apparatus (Philadelphia, PA) and are uncorrected. Spectrophotometry was performed with a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA) and the data was analyzed using *SoftMax Pro* software (Amersham Biosciences, Piscataway, NJ). Flow cytometry was performed on a Becton Dickson FACSCalibur instrument (Ruthersford, NJ) and the data were analyzed using FlowJo software (TreeStar, Ashland, OR).

5.2. 2,4-Dichloro-5-methylbenzenethiol/bis(2,4-dichloro-5-methylbenzene) disulfide (2a)

To chlorosulfonic acid (20 ml), 2,4-dichlorotoluene (1.25 g, 7.74 mmol) was added dropwise with stirring. After 3 h of heating at 60 °C followed by cooling to room temperature, the reaction mixture was added dropwise to a 1 L beaker of crushed ice. The precipitate formed was collected by extraction three times with 100 ml ethyl acetate, giving 1.51 g of 2,4-dichloro-5-methylbenzenesulfonyl chloride as an off-white solid, 75% yield, mp 65–70 °C (lit. 67–72 °C).[36–38] ^{1}H NMR (methanol-d_4) ∂ 2.49 (3H, s), 7.88 (1H, s), and 8.14 (1H, s), consistent with the ^{1}H NMR reported in the literature.[38] HRMS $[M+Na]^+$ calcd 280.8974, obsd 280.8971. To provide sufficient material of this intermediate for the synthesis of ring B analogs, 50 ml of chlorosulfonic acid and 12.5 g of 2,4-dichlorotoluene (77.4 mmol) were reacted as described above. The precipitate formed during workup was collected by suction filtration and washed with a 50% saturated sodium bicarbonate solution (100 ml). The product was dried under vacuum for several days, giving ~11 g (54% crude yield) of a white solid with the same R_f as the small-scale product on TLC (silica gel, eluent 50% dichloromethane/hexanes). It was used in the subsequent reaction without further purification. To a flask containing crushed ice (250 ml), sulfonyl chloride (10 g, 38.5 mmol) was added. Concentrated sulfuric acid (20 ml) was then added dropwise via addition funnel. While maintaining the reaction temperature at −10 to 0 °C, zinc dust (20 g, 0.3 mol) was added portionwise. After 1 h of stirring at −10 to 0 °C, the reaction was allowed warm to room temperature. The reaction was then heated to 100 °C for 6 h. After cooling to room temperature the resulting white precipitate was collected by suction filtration and dried under vacuum for several days, giving 12.8 g of a white solid, 86% yield, mp 119–124 °C (lit. 85 °C[38] or 95–97 °C[39] for thiol, 123–125 °C[40] for disulfide). The melting point, ^{1}H NMR [(methanol-d_4) ∂ 2.29 (6H, s), 7.36 (2H, s) and 7.38 (2H, s)] and mass spectrometry ($[M+Na]^+$ 406.9) data were consistent with oxidation of the product to the disulfide form.[38] ^{13}C NMR (chloroform-*d*) ∂ 17.98, 128.84, 129.01, 130.91, 131.08, 135.30, 137.03. In order to reduce the disulfide to the thiol for subsequent reactions, zinc-mediated reduction of the disulfide was repeated as described above on the product no more than 24 h prior to its use.

5.3. Coupling reactions

Ullmann condensation methods were used to synthesize analogs that were not commercially available. In general, aromatic or aliphatic thiols were coupled with chloro- or iodo-containing substituted aryl compounds. Thiols at 1 mole equivalent (mol equiv) were deprotonated using 2 mol equiv of a base. Ligand-coordinated copper (I) iodide (at 0.2 or 2 mol equiv of ligand and 0.2 mol equiv of CuI) was then added under an inert atmosphere (Ar or N_2). Finally, aryl halides were added at 0.8–1.5 mol equiv Reactions were performed at reflux in 10–20 ml of solvent overnight under an inert atmosphere. During work up, the product was partitioned between chloroform and 50% brine solution. The aqueous layer was extracted with additional chloroform, the organic layer was filtered through sodium sulfate or magnesium sulfate, and then the solvent was removed in vacuo to yield crude compound.

5.3.1. 1,3-Dinitro-2-phenylsulfanyl-5-(trifluoromethyl)benzene (4)

Thiophenol **2b** (165 mg, 1.5 mmol) was coupled to 4-chloro-3,5-dinitrobenzotrifluoride **3b** (271 mg, 1.0 mmol) using KO*t*Bu (224 mg, 2.0 mmol), ethylene glycol (125 mg, 2.0 mmol), and copper(I) iodide (38 mg, 0.2 mmol) in isopropanol (10 ml). The product was purified by silica gel column chromatography (eluent 0–15% chloroform in hexanes) and preparative thin-layer chromatography (eluent 50% chloroform in hexanes), giving 120 mg of a yellow solid, 35% yield, mp 101–104 °C (lit. 105–106 °C).[41] ^{1}H NMR (acetone-d_6) ∂ 7.34–7.46 (5H, m) and 8.61 (2H, s) (lit. 7.36 (m) and 8.73 (q, J = 0.7 Hz).[42] ^{13}C NMR (acetone-d_6) ∂ 123.43 (q, J_{CF} = 273.2 Hz), 126.63 (q, J_{CF} = 3.8), 130.55, 130.92, 131.63 (q, J_{CF} = 33.7 Hz), 132.21, 132.31, 133.38, 154.22. HRMS $[M+Na]^+$ calcd 366.9976, obsd 367.0000. Anal. ($C_{13}H_7F_3N_2O_4S$) calcd C 45.35%, H 2.05%, N 8.14%; obsd C 45.33%, H 2.14%, N 7.99%.

5.3.2. 2-[(3-Methylphenyl)sulfanyl]-1,3-dinitro-5-trifluoromethyl benzene (5)

m-Thiocresol **2c** (500 mg, 4.0 mmol) was coupled to 4-chloro-3,5-dinitrobenzotrifluoride **3b** (1.08 g, 4.0 mmol) using KO*t*Bu (898 mg, 8.0 mmol), ethylene glycol (496 mg, 8.0 mmol), and copper(I) iodide (152 mg, 0.8 mmol) in isopropanol (10 ml). The product was purified by silica gel column chromatography (eluent 0–20% chloroform in hexanes) yielding 362 mg of semi-pure material. A 25 mg sample was further purified by three consecutive elutions on a single plate of C_{18} reverse-phase preparative TLC (eluent 4:2:1 water:methanol:acetone). From this 14 mg (56% of total mass used) of pure compound (a yellow solid) was obtained, mp 88–92 °C. This would correspond to a 13% overall yield if all of the available material was similarly purified. ^{1}H NMR (acetone-d_6) ∂ 2.28 (3H, s), 7.19–7.29 (4H, m) and 8.59 (2H, d, J = 0.6 Hz). ^{13}C NMR (acetone-d_6) ∂ 20.18, 123.19 (q, J_{CF} = 270.9 Hz), 125.37 (q, J_{CF} = 3.7), 129.11, 129.46, 130.05, 130.18 (q, J_{CF} = 34.2 Hz), 130.57, 131.19, 132.38, 139.67, 152.85. HRMS $[M+Na]^+$ calcd 381.0133, obsd 381.0135. Anal. ($C_{14}H_9F_3N_2O_4S$) calcd C 46.93%, H 2.53%, N 7.82%; obsd C 46.89%, H 2.46%, N 7.73%.

5.3.3. 2-[(2,4-Dichlorophenyl)sulfanyl]-1,3-dinitro-5-(trifluoromethyl)benzene (6)

2,4-Dichlorothiophenol **2d** (358 mg, 2 mmol) was coupled to 4-chloro-3,5-dinitrobenzotrifluoride **3b** (460 mg, 1.7 mmol) using triethylamine (405 mg, 4 mmol), neocuproine (83 mg, 0.4 mmol), and copper(I) iodide (76 mg, 0.4 mmol) in ethyl acetate (10 ml). The product was purified by silica gel column chromatography (eluent 0–20% dichloromethane in hexanes), giving 164 mg of a yellow solid, 23% yield, mp 103–107 °C. ^{1}H NMR (acetone-d_6) ∂ 7.41 (1H, dd, J = 2.2 and 8.8 Hz), 7.54 (1H, d, J = 8.8 Hz), 7.64 (1H, d, J = 2.2 Hz) and 8.69 (2H, d, J = 0.7 Hz). ^{13}C NMR (acetone-d_6) ∂ 122.07 (q, J_{CF} = 272.8 Hz), 125.67 (q, J_{CF} = 3.8 Hz), 128.38, 128.58, 128.95, 129.99, 131.24 (q, J_{CF} = 35.9 Hz), 135.14, 135.78, 136.39, 153.04. HRMS $[M+Na]^+$ calcd 434.9197, obsd 434.9193. Anal. ($C_{13}H_5Cl_2F_3N_2O_4S$) calcd C 37.79%, H 1.22%, N 6.78%; obsd C 37.93%, H 1.22%, N 6.76%.

5.3.4. 4-{[2,6-Dinitro-4-(trifluoromethyl)phenyl]sulfanyl}phenyl methyl ether (7)

4-Methoxybenzenethiol **2e** (505 mg, 3.6 mmol) was coupled to 4-chloro-3,5-dinitrobenzotrifluoride **3b** (812 mg, 3.0 mmol) using triethylamine (607 mg, 6 mmol), neocuproine (125 mg, 0.6 mmol), and copper(I) iodide (114 mg, 0.6 mmol) in ethyl acetate (20 ml). The product was purified by reverse-phase C18-silica gel column chromatography (eluent 50–90% acetone in water), giving 458 mg of a yellow solid, 41% yield, mp 125–127 °C. ^{1}H NMR (acetone-d_6) ∂ 3.83 (3H, s), 6.91–6.96 (2H, m, AA' of an AA'XX' system), 7.37–7.43 (2H, m, XX' of an AA'XX' system), 8.54 (2H, d, J = 0.6 Hz). ^{13}C NMR (acetone-d_6) ∂ 54.97, 115.21, 120.68, 122.21 (q, J_{CF} = 272.3 Hz), 125.27 (q, J_{CF} = 3.8 Hz), 129.54 (q, J_{CF} = 35.5 Hz), 133.36, 135.25, 152.40, 161.09. HRMS [M+H]$^+$ calcd 375.0262, obsd 375.0269. Anal. ($C_{14}H_9F_3N_2O_5S$) calcd C 44.92%, H 2.42%, N 7.48%; obsd C 44.97%, H 2.40%, N 7.44%.

5.3.5. 2-Cyclohexylsulfanyl-1,3-dinitro-5-(trifluoromethyl)-benzene (10)

Cyclohexylmercaptan **2h** (116 mg, 1.0 mmol) was coupled to 4-chloro-3,5-dinitrobenzotrifluoride **3b** (271 mg, 1.0 mmol) using KO*t*Bu (224 mg, 2.0 mmol), ethylene glycol (125 mg, 2.0 mmol, Mallinckrodt), and copper(I) iodide (38 mg, 0.2 mmol) in isopropanol (10 ml). The product was purified by silica gel column chromatography (eluent 100% hexanes), giving an orange–yellow solid, 11% yield, mp 60–64 °C. ^{1}H NMR (acetone-d_6) ∂ 1.16–1.43 (6H, m), 1.59 (1H, m), 1.78 (4H, m) and 8.58 (2H, d, J = 0.6 Hz). ^{13}C NMR ∂ (acetone-d_6) 24.96, 25.54, 33.23, 49.83, 122.10 (q, J_{CF} = 272.8), 123.50 (q, J_{CF} = 3.8), 125.57, 132.14 (q, J_{CF} = 35.8 Hz), 155.61. HRMS [M+Na]$^+$ calcd 373.0446, obsd 373.0448. Anal. ($C_{13}H_{13}F_3N_2O_4S$) calcd C 44.57%, H 3.74%, N 8.00%; obsd C 45.01%, H 3.81%, N 7.98%.

5.3.6. 2-Ethylsulfanyl-1,3-dinitro-5-(trifluoromethyl)benzene (11)

In this coupling method, the ligand was neocuproine at 0.2 mol equiv, the base was KO*t*Bu at 2 mol equiv and the solvent was tetrahydrofuran (THF). Ethanethiol **2i** (839 mg, 11 mmol) was coupled to 4-chloro-3,5-dinitrobenzotrifluoride **3b** (271 mg, 1.0 mmol) using KO*t*Bu (224 mg, 2.0 mmol), neocuproine (42 mg, 0.2 mmol), and copper(I) iodide (38.1 mg, 0.2 mmol) in anhydrous THF (10 ml). The product was purified by silica gel column chromatography (eluent 100% hexanes), giving 59 mg of a yellow solid, 20% yield, mp 88–90 °C (lit. 89–90 °C).[43] ^{1}H NMR (acetone-d_6) ∂ 1.24 (3H, t, J = 7.4 Hz), 3.03 (2H, q, J = 7.4 Hz) and 8.61 (2H, d, J = 0.6 Hz). ^{13}C NMR (acetone-d_6) ∂ 13.84, 30.87, 122.15 (q, J_{CF} = 272.5 Hz), 123.91 (q, J_{CF} = 3.8 Hz), 127.74, 131.57 (q, J_{CF} = 35.5 Hz), 155.06. HRMS [M+Na]$^+$ calcd 318.9976, obsd 318.9967. Anal. ($C_9H_7F_3N_2O_4S$) calcd C 36.49%, H 2.38%, N 9.46%; obsd C 36.94%, H 2.43%, N 9.39%.

5.3.7. 2-[(2,4-Dichloro-5-methylphenyl)sulfanyl]-1,3-dinitrobenzene (12)

2,4-Dichloro-5-methylthiophenol **2a** (368 mg, 2 mmol) was coupled to 2-chloro-1,3-dinitrobenzene **3a** (344 mg, 1.7 mmol) using triethylamine (405 mg, 4 mmol), neocuproine (83 mg, 0.4 mmol), and copper(I) iodide (76 mg, 0.4 mmol) in ethyl acetate (20 ml). The product was purified by silica gel column chromatography (eluent 0–30% dichloromethane in hexanes), recrystallization from hexanes, reverse-phase C_{18}-silica gel column chromatography (eluent 0–50% acetone in water), and recrystallization from dichloromethane, giving 213 mg of a yellow solid, 35% yield, mp 181–184 °C. ^{1}H NMR (acetone-d_6) ∂ 2.29 (3H, t, J = 0.4 Hz), 7.33 (1H, d, J = 0.4 Hz), 7.53 (1H, s), 7.98 (1H, dd, J = 8.1 Hz) and 8.29 (2H, d, J = 8.1 Hz). ^{13}C NMR (acetone-d_6) ∂ 18.46, 122.21, 128.37, 129.79, 129.83, 131.18, 132.61, 134.72, 134.98, 136.24, 153.34. HRMS [M+Na]$^+$ calcd 380.9480, obsd 380.9469. Anal. ($C_{13}H_8Cl_2N_2O_4S$) calcd C 43.47%, H 2.24%, N 7.80%; obsd C 43.57%, H 2.16%, N 7.76%.

5.3.8. 2-[(2,4-Dichloro-5-methylphenyl)sulfanyl]-1,5-dinitro-3-(trifluoromethyl)benzene (16)

2,4-Dichloro-5-methylthiophenol **2a** (267 mg, 1.4 mmol) was coupled to 2-chloro-3,5-dinitrobenzotrifluoride **3c** (322 mg, 1.2 mmol) using triethylamine (283 mg, 2.8 mmol), neocuproine (58 mg, 0.3 mmol), and copper(I) iodide (53 mg, 0.3 mmol) in ethyl acetate (20 ml). The product was purified by silica gel column chromatography (eluent 0–20% dichloromethane and hexanes) and reverse-phase C_{18}-silica gel column chromatography (eluent 25–75% acetone and water), giving 300 mg of a yellow solid, 59% yield, mp 126–130 °C. ^{1}H NMR (acetone-d_6) ∂ 2.24 (3H, s), 7.13 (1H, s), 7.59 (1H, s), 8.91 (1H, d, J = 2.6 Hz), 9.06 (1H, d, J = 2.6 Hz). ^{13}C NMR (acetone-d_6) ∂ 18.45, 122.01 (q, J = 274.5 Hz), 123.61, 125.13 (q, J = 5.9 Hz), 129.74, 130.43 (d, J = 1.1 Hz), 131.02, 131.92, 132.80, 134.53, 135.67 (q, J = 32.5 Hz), 136.51, 148.37, 154.99. HRMS [M+Na]$^+$ calcd 448.9353, obsd 448.9356. Anal. ($C_{14}H_7Cl_2F_3N_2O_4S$) calcd C 39.36%, H 1.65%, N 6.56%; obsd C 39.63%, H 1.69%, N 6.48%.

5.3.9. 2-[(2,4-Dichloro-5-methylphenyl)sulfanyl]-1,3-dinitro-5-methylbenzene (17)

2,4-Dichloro-5-methylthiophenol **2a** (368 mg, 1.9 mmol) was coupled to 2-chloro-1,3-dinitro-5-methylbenzene **3d** (367 mg, 1.7 mmol) using triethylamine (405 mg, 4 mmol), neocuproine (83 mg, 0.4 mmol), and copper(I) iodide (76 mg, 0.4 mmol) in ethyl acetate (20 ml). The product was purified by silica gel column chromatography (eluent 0–25% dichloromethane in hexanes), recrystallization from hexanes, reverse-phase C_{18}-silica gel column chromatography (eluent 25–100% acetone/water), and a second recrystallization from hexanes, giving 178 mg of a yellow solid, 28% yield, mp 177–178 °C. ^{1}H NMR (acetone-d_6) ∂ 2.27 (3H, m), 2.63 (3H, t, J = 0.7 Hz), 7.23 (1H, m, J = 0.8 Hz), 7.52 (1H, m, J = 0.3 Hz), 8.12 (2H, d, J = 0.7 Hz). ^{13}C NMR (acetone-d_6) ∂ 18.50, 20.00, 117.52, 128.50, 129.70, 130.56, 131.87, 133.83, 134.44, 136.10, 143.64, 153.59. HRMS [M+Na]$^+$ calcd 394.9636, obsd 394.9639. Anal. ($C_{14}H_{10}Cl_2N_2O_4S$) calcd C 45.05%, H 2.70%, N 7.51%; obsd C 45.11%, H 2.67%, N 7.42%.

5.3.10. 4-[(2,4-Dichloro-5-methylphenyl)sulfanyl]-3,5-dinitrobenzonitrile (18)

2,4-Dichloro-5-methylthiophenol **2a** (194 mg, 1 mmol) was coupled to 4-chloro-3,5-benzonitrile **3e** (191 mg, 0.84 mmol) using triethylamine (203 mg, 2 mmol), neocuproine (42 mg, 0.2 mmol), and copper(I) iodide (38 mg, 0.2 mmol) in ethyl acetate (10 ml). The product was purified by silica gel column chromatography (eluent 0–50% chloroform in hexanes) and recrystallization from hexanes, giving 133 mg of a yellow solid, 41% yield, mp 195 °C. ^{1}H NMR (acetone-d_6) ∂ 2.31 (3H, s), 7.56 (1H, d, J = 0.6 Hz), 7.59 (1H, s), and 8.78 (2H, s). ^{13}C NMR (acetone-d_6) ∂ 18.38, 113.06, 114.82, 127.81, 130.14, 130.56, 132.08, 133.98, 136.25, 136.35, 136.64, (the signal for the nitro-containing carbons on ring B, expected between 150 and 160 ppm, is too small to be distinguished from noise). HRMS [M+Na]$^+$ 405.9432, obsd 405.9435. Anal. ($C_{14}H_7Cl_2N_3O_4S$) calcd C 43.77%, H 1.84%, N 10.94%; obsd C 43.54%, H 1.82%, N 10.76%.

5.3.11. 2-[(2,4-Dichloro-5-methylphenyl)sulfanyl]-1,3,5-tris(trifluoromethyl)benzene (19)

2,4-Dichloro-5-methylthiophenol **2a** (368 mg, 1.9 mmol) was coupled to 2,4,6-tris(trifluoromethyl)chlorobenzene **3f** (538 mg, 1.7 mmol) using triethylamine (405 mg, 4 mmol), neocuproine (83 mg, 0.4 mmol), and copper(I) iodide (76 mg, 0.4 mmol) in ethyl acetate (20 ml). The product was purified by silica gel column chromatography (eluent 100% hexanes), recrystallization from hexanes and reverse-phase C_{18}-silica gel column chromatography twice (eluent 0–75% then 50–90% acetone/water), giving 90 mg of a white

solid, 11% yield, mp 87–89 °C. ^{1}H NMR (acetone-d_6) ∂ 2.11 (3H, s), 6.53 (1H, s), 7.55 (1H, s) and 8.60 (2H, s). ^{13}C NMR (acetone-d_6) ∂ 18.46, 122.25, 122.39 (q, J_{CF} = 274.5), 122.67 (q, J_{CF} = 272.6), 127.36, 128.84, 129.06 (m), 131.92, 132.92, 133.11 (q, J_{CF} = 30.7), 135.37, 136.13, 138.73 (q, J_{CF} = 30.9). Anal. ($C_{16}H_7Cl_2F_9S$) calcd C 40.61%, H 1.49%, N 0.00%; obsd C 40.87%, H 1.50%, N 0.00%. While the other physical data for **19** are consistent with its proposed structure, this compound's peak was not observed using HRMS.

5.3.12. 4-[(3,5-Dinitrobenzonitrile)sulfanyl] phenyl methyl ether (22)

4-Methoxybenzenethiol **2e** (108 mg, 0.77 mmol) was coupled to 4-chloro-3,5-dinitrobenzonitrile **3e** (150 mg, 0.65 mmol) using triethylamine (155 mg, 1.54 mmol), neocuproine (32 mg, 0.15 mmol), and copper(I) iodide (30 mg, 0.15 mmol) in ethyl acetate (8 ml). The product was purified by silica gel column chromatography (eluent 0–70% chloroform in hexanes) and recrystallization from hexanes and dichloromethane, giving 125 mg of a yellow solid, 57% yield, mp 194–196 °C. ^{1}H NMR (DMSO-d_6) ∂ 3.76 (3H, s), 6.86–6.95 (2H, m, AA' of an AA'XX' system), 7.30–7.37 (2H, m, XX' of an AA'XX' system), 8.81 (2H, s). ^{13}C NMR (DMSO-d_6) ∂ 55.39, 110.98, 115.23, 115.35, 121.17 132.21, 133.78, 135.02, 151.23, 160.41. HRMS $[M+Na]^+$ calcd 354.0161, obsd 354.0146. Anal. ($C_{14}H_9N_3O_5S$) calcd C 50.76%, H 2.74%, N 12.68%; obsd C 50.51%, H 2.69%, N 12.47%.

5.4. Other synthetic methods

5.4.1. 4-(2,6-Dinitro-4-trifluoromethyl-phenylsulfanyl)-phenol (8)

Compound **7** (75 mg, 0.2 mmol) was suspended in a 1:1 solution of concentrated HBr and glacial acetic acid (10 ml) and heated to reflux for 4 h. The pH of the cooled reaction mixture was adjusted to 4–5 with saturated $NaHCO_3$ solution. Ethyl acetate (100 ml) was added and the product was partitioned into the organic layer. The product was purified by silica gel column chromatography (eluent 0–10% ethyl acetate in hexanes), giving 34 mg of a yellow solid, yield 47%, mp 176–180 °C. ^{1}H NMR (acetone-d_6) ∂ 6.81–6.86 (2H, m, AA' of an AA'XX' system), 7.29–7.34 (2H, m, XX' of an AA'XX' system), 8.52 (2H, d, J = 0.4 Hz). ^{13}C NMR (acetone-d_6) ∂ 116.86, 119.27, 122.36 (q, J_{CF} = 263.1 Hz), 125.47 (q, J_{CF} = 4.5 Hz), 129.51 (q, J_{CF} = 36.8 Hz), 133.91, 135.73 (m, J = 1.7 Hz), 152.47, 159.35. HRMS $[M+Na]^+$ calcd 382.9925, obsd 382.9899. Anal. ($C_{13}H_7F_3N_2O_5S$) calcd C 43.34%, H 1.96%, N 7.78%; obsd C 43.07%, H 1.86%, N 7.76%.

5.4.2. (2,4-Dichlorophenyl)-(2,6-dinitro-4-trifluoromethyl-phenyl)amine (21)

2,4-Dichloraniline (162 mg, 1.0 mmol) was dissolved in ethyl acetate (10 ml) under N_2 (g). Triethylamine (202 mg, 2.0 mmol) and 4-chloro-3,5-dinitrobenzotrifluoride **3b** (271 mg, 1.0 mmol) were added and the reaction mixture was heated to reflux overnight. 50% brine solution (100 ml) and ethyl acetate (100 ml) were added to the cooled reaction mixture. The product was partitioned into the organic layer and further extracted from the aqueous layer twice with 50 ml ethyl acetate. The solvent was removed from the combined extracts in vacuo. The product was purified by silica gel column chromatography (eluent 0–4% ethyl acetate in hexanes) and by recrystallization from hexanes, giving 51 mg of a yellow solid, yield 33%, mp 149 °C. ^{1}H NMR (acetone-d_6) ∂ 7.28 (1H, d, J = 8.7 Hz), 7.33 (1H, dd, J = 2.1 and 8.7 Hz), 7.66 (1H, d, J = 2.1 Hz), 8.68 (2H, s), and 9.72 (1H, s). HRMS $[M+Na]^+$ calcd 417.9585, obsd 417.9557. Anal. ($C_{13}H_6Cl_2F_3N_3O_4$) calcd C 39.42%, H 1.53%, N 10.61%; obsd C 39.47%, H 1.50%, N 10.44%.

5.5. Axenic amastigote assay

Leishmania donovani axenic amastigotes (WHO designation MHOM/SD/62/1S-CL2$_D$) were adapted from promastigotes and maintained in amastigote medium (as previously described[6]) at 37 °C. 6×10^4 cells in a final volume of 60 µl were plated in each well of a 96-well plate except negative controls. Compounds were added to the appropriate wells and 2-fold dilutions permitted a range of concentrations to be examined for each compound. Cells were incubated at 37 °C for 3 days in a humidified environment containing 5% CO_2. Cell proliferation was determined by a colorimetric assay using the tetrazolium dye-based CellTiter reagent.[44] Several hours after addition of 12 µl of dye to each well of the 96-well plate, the absorbance was observed at 490 nm. IC_{50} values were calculated using the SoftMax Pro software using the dose-response equation $y = [(a - d)/(1 + (x/c)^b)] + d$ where x = drug concentration, y = Abs_{490}, a = upper asymptote of a four-parameter curve, b = slope, c = IC_{50}, and d = lower asymptote. Each compound was tested in three or more separate experiments.

5.6. Infected macrophage assay

Macrophage production was induced in CD-1 mice weighing 31–34 g at 8–12 weeks by i.p. administration of 2% w/v starch in phosphate-buffered saline (PBS). After 24 h, the mice were sacrificed. Ten milliliters of macrophage medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 50 units/ml penicillin, and 50 µg/ml streptomycin) was injected into the peritoneal cavity of the mice, agitated by massaging, then removed to extract macrophages. The pooled peritoneal extracts were centrifuged at 350g for 5 min at 4 °C. The supernatant was removed and the pellet was resuspended in 1 mL medium. The concentration of viable cells was determined by trypan blue staining. The cells were plated in a 96-well plate in 100 µl of medium containing 1×10^6 cells/ml and the cells were allowed to adhere overnight at 37 °C. The wells were washed with 100 µl of Hank's balanced salt solution (HBSS), then 100 µl of medium containing *L. amazonensis* transfected with β-lactamase at 5×10^6 cells/ml was added. After infection was allowed to occur overnight at 34 °C, the wells were washed with HBSS as before, then compounds were added to the appropriate wells. Twofold dilutions permitted a range of concentrations to be examined for each compound. The final volume in each well was 200 µl. After 72 h of incubation at 34 °C, the medium was removed from each well, washing with 200 µl PBS. 100 µl of a lysis solution of 0.1% Triton-X 100 in PBS containing 100 µM of the indicator dye nitrocefin was added to each well. The plate was incubated at 37 °C for 4 h, then the absorbance of each well determined at 490 nm. IC_{50} values were calculated as in the axenic amastigote assay.

5.7. Vero cell toxicity assay

Vero cells (ATCC, Rockville, MD) were maintained in minimum essential medium, alpha modification containing Glutamax-I and supplemented with 10% HI-FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. The cells were treated with test compounds similarly to the axenic amastigote assay above, except that 1×10^3 cells in a final volume of 100 µl were plated in each well of a 96-well plate. The CellTiter reagent, at 20 µl per well, was also used to assay cell growth as in the axenic amastigote assay.

5.8. Reactive oxygen species assay

Leishmania donovani promastigotes were maintained in promastigote medium.[8] Parasites (1×10^7) were collected from log-phase cell culture by centrifugation at 1500g for 15 min at room temperature. A dye solution containing 10 µg/ml CM-H_2DCFDA in promastigote medium lacking FBS was prepared, and the parasites were suspended in 10 ml of this dye solution. The parasites were incubated for 60 min at 25 °C to accumulate the dye. The parasites

were then centrifuged as before and resuspended to 1×10^6 cells/ml in 10 ml promastigote medium. Cell culture (500 μl) was placed in each well of a 24-well plate. Test compounds were added to the wells in a vehicle of 1% DMSO and the cells were incubated as before for 24 h. Then, the cells were transferred to microcentrifuge tubes and centrifuged at 3000g for 15 min. The supernatant was removed and the cells were resuspended in 1 ml of PBS. Fluorescence intensity in 10,000 individual cells was measured by flow cytometry.

5.9. Mitochondrial membrane potential assay

Leishmania donovani (1×10^7) promastigotes were collected from confluent cell culture by centrifugation at 1500g for 15 min at room temperature. The parasites were then centrifuged as before and resuspended to 1×10^6 cells/ml in 10 ml promastigote medium. Cell culture (500 μl) was placed in each well of a 24-well plate. Test compounds were added to the wells in a vehicle of 1% DMSO and the cells were incubated as before for 24 h, except for FCCP, with which the cells were incubated for 2 h. The cells were then transferred to microcentrifuge tubes and centrifuged at 3000g for 15 min. A dye solution containing 1 μM of rhodamine 123 was made and the parasites were incubated in 500 μl of this solution for 1 h. The cells were recentrifuged, the supernatant was removed and the cells were resuspended in 1 ml of PBS. Flow cytometry was performed as above.

5.10. *c*log*P* determination

The free online program *ChemAxon/Marvin* (http://www.chemaxon.com) was used to calculate *c*log*P* values. The compound structures were drawn using a *Java* applet.

5.11. In vivo antileishmanial assay

Six to eight-week-old female BALB/c mice (~20 g) were infected intravenously via the tail vein with 5×10^7 *Leishmania donovani* LV82 promastigotes (obtained by transforming amastigotes derived from an infected hamster spleen) and randomly sorted into groups of 4. Treatment was started 7 days post-infection with intraperitoneal injections of either 10 ml/kg PBS, 10 ml/kg vehicle (10% DMSO, 70% cremophor/ethanol (3:1) and 20% PBS), 25 mg/kg **22** dissolved in the DMSO/cremophor/ethanol vehicle described above, or 3 mg/kg miltefosine dissolved in PBS. Animals were dosed intraperitoneally once daily for five continuous days. On day 14, all mice were sacrificed, their livers and spleens were removed and weighed, and liver smear slides were prepared from a cut surface. Smears were methanol fixed and Geimsa stained. Parasitemia was determined by counting the number of amastigotes per 1000 liver cells times the liver weight (in mg). Body weight was determined pre- and post-treatment as a gross indicator of toxicity.

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