Evaluation of Arylimidamides DB1955 and DB1960 as Candidates against Visceral Leishmaniasis and Chagas' Disease: In Vivo Efficacy, Acute Toxicity, Pharmacokinetics, and Toxicology…

ARTICLE *in* ANTIMICROBIAL AGENTS AND CHEMOTHERAPY · APRIL 2012

Impact Factor: 4.48 · DOI: 10.1128/AAC.06404-11 · Source: PubMed

Bioorganic & Medicinal Chemistry Letters 22 (2012) 6806–6810

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Antileishmanial bis-arylimidamides: DB766 analogs modified in the linker region and bis-arylimidamide structure–activity relationships

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ARTICLE INFO

Article history:
Available online 16 June 2012

In dedication to Professor Timothy Macdonald for his many contributions to the fields of Organic and Medicinal Chemistry

Keywords:
Leishmania
Drug discovery

ABSTRACT

Analogs of the lead antileishmanial bis-arylimidamide DB766 were prepared that possess unsymmetrical substitutions on the diphenylfuran linker, and an additional compound was synthesized that contains isopropoxy groups *meta* to the central furan. These agents all displayed nanomolar in vitro potency against intracellular *Leishmania* with selectivity indexes >100 compared to J774 macrophages. While the unsymmetrical analogs were toxic to mice when given ip at 30 mg/kg/day, the compound bearing the *meta* isopropoxy groups was well tolerated by mice and showed activity in a murine model of visceral leishmaniasis when administered ip at 30 mg/kg/day for five days.

Leishmaniasis is a disease that manifests in several forms, the two most common being a life-threatening visceral form and a cutaneous variety. Approximately one quarter of the estimated two million new cases of leishmaniasis occurring annually are of the visceral form, with the majority of these cases occurring on the Indian subcontinent, Sudan, and Brazil.[1] About three quarters of the new leishmaniasis cases are of the cutaneous variety, with most occurring in the Middle East, Afghanistan, Brazil, and Peru.[1] This spectrum of disease is caused by species of the genus *Leishmania*, a protozoan parasite. Regarding the worldwide burden inflicted by protozoan parasitic diseases, leishmaniasis is second only to malaria in terms of mortality and the total number of cases.[2,3] Although several classes of antimalarial drug candidates are in various stages of preclinical development or in clinical trials,[4] the same cannot be said for antileishmanial drug candidates. While the treatment of visceral leishmaniasis on the Indian subcontinent has improved over the last few years due to the registration of paromomycin[5] and the demonstration of the efficacy of single dose liposomal amphotericin B,[6] no new chemical entities are in clinical trials against leishmaniasis, and few new candidates are in the development pipeline.[7] Considering the limitations of the existing drugs, the dearth of new antileishmanial drug candidates requires renewed efforts to find small molecules capable of meeting the unmet needs in antileishmanial chemotherapy and a restored commitment to support these important drug discovery and development efforts.

As part of our efforts to identify new antileishmanial drug candidates, compounds were prepared inspired by diamidine antimicrobials that showed exceptional in vitro potency against intracellular *Leishmania*.[8] These molecules were originally termed reversed amidines because, unlike pentamidine and other diamidine antimicrobials, their imino group is bound to an anilino nitrogen instead of being directly attached to an aromatic ring. This compound class, now known by the more chemically descriptive name arylimidamides (AIAs), has produced numerous potent antileishmanial molecules,[8–10] including the hydrochloride salts DB766 and DB1852 (see Fig. 1 for structures) and their corresponding mesylate salts that were considered as candidates for preclinical development against visceral leishmaniasis.[11,12] All of the AIAs reported thus far are symmetrical bis-AIAs (compounds containing two terminal heteroaromatic groups). Those that advanced the farthest as antileishmanial candidates, DB766 and DB1852, possess isopropoxy and cyclopentyloxy groups *ortho* to the furan ring of the diphenylfuran linker. The goals of the present study are to examine the effects of (1) preparing unsymmetrical bis-AIAs related to DB766 and (2) positioning the isopropoxy groups of DB766 *meta* to the central furan of the linker.

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http://dx.doi.org/10.1016/j.bmcl.2012.06.037

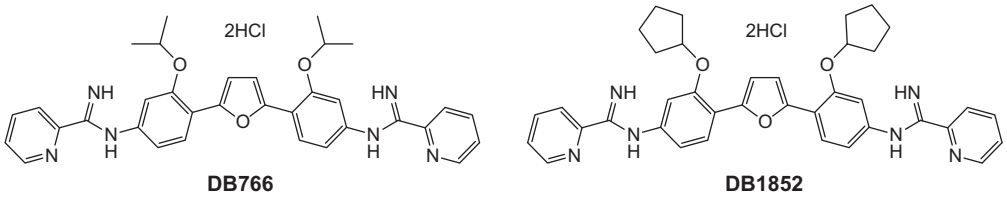
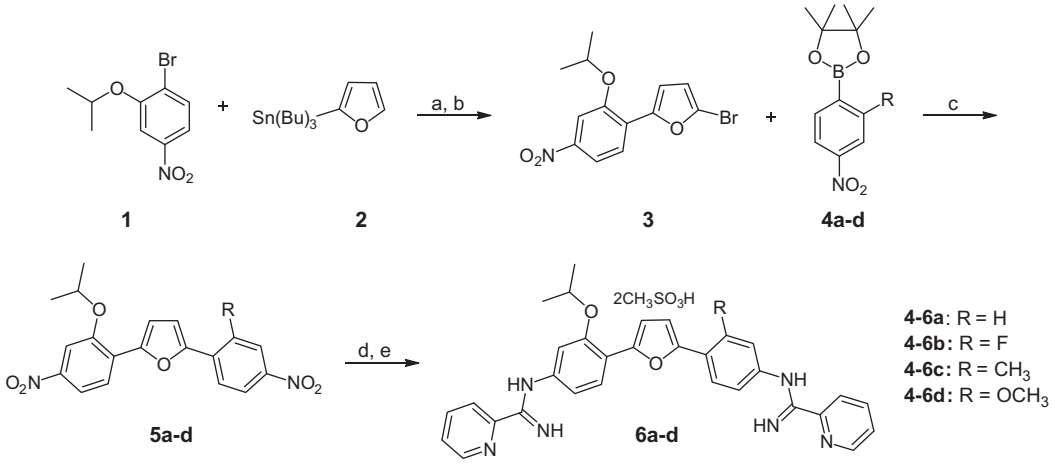


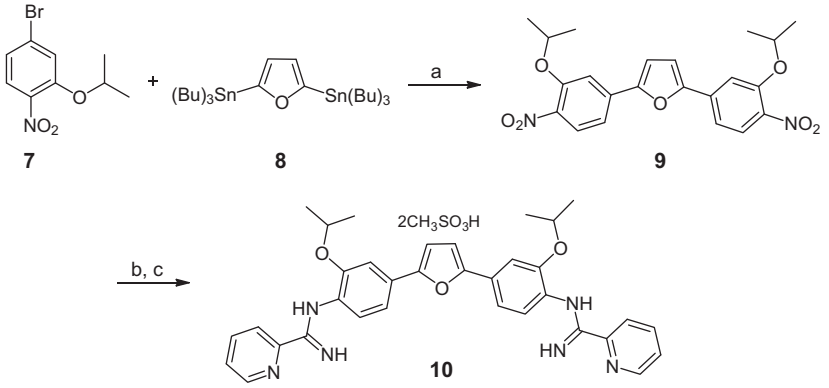
Figure 1. Structures of DB766 and DB1852.

For the preparation of unsymmetrical DB766 analogs (shown in Scheme 1), the synthesis of the key intermediate 1-bromo-2-isopropoxy-4-nitrobenzene (**1**) was achieved starting with the bromination of commercially available 2-amino-5-nitrophenol using sulfamic acid and sodium nitrite in aqueous HBr to form 2-bromo-5-nitrophenol in modest yield.[13] The phenolic group was then alkylated with 2-iodopropane in the presence of cesium carbonate/acetone to give **1** in 55% overall yield. Stille coupling between **1** and 2-(tributylstannyl)furan (**2**)[14] in the presence of catalytic $Pd(PPh_3)_4$ in anhydrous 1,4-dioxane followed by bromination of the intermediate with NBS in DMF afforded nitrophenylfuran **3** in good yield. Aryl boronate esters **4a–d** were obtained in low yields from the reaction of the corresponding commercially available 1-bromo-4-nitrobenzenes, bis(pinacolato)diboron, potassium acetate and catalytic $PdCl_2$(dppf) in DMSO.[15] Suzuki coupling between **3** and **4a–d** in the presence of catalytic $PdCl_2$(dppf) or $Pd(PPh_3)_4$ gave the desired unsymmetrical dinitrophenylfurans **5a–d** in excellent yields. Reduction of **5a–d** through catalytic hydrogenation yielded the corresponding diamino intermediates, which then reacted with *S*-(2-naphthylmethyl)-2-pyridylthioimidate hydrobromide in ethanol/acetonitrile to provide target compounds **6a–d**.[8,14]

The synthesis of a DB766 analog containing isopropoxy groups *meta* to the furan attachment point (shown in Scheme 2) required the preparation of key intermediate 4-iodo-2-isopropoxy-1-nitrobenzene (**7**). Toward this end, 3-iodophenol was nitrated with sodium nitrite in glacial acetic acid to form 5-iodo-2-nitrophenol in low yield.[16] The alkylation of the phenolic group was carried out under conditions similar to those described above to afford **7** in almost quantitative yield. 2,5-Bis(tri-*n*-butylstannyl)furan (**8**) was prepared from furan via lithiation with *n*-butyllithium and



Scheme 1. Reagents and conditions: (a) 1,4-dioxane, $Pd(PPh_3)_4$, reflux, overnight (68–85%); (b) DMF, NBS, rt, overnight (72–83%); (c) DMSO, KOAc, $PdCl_2$(dppf), 100 °C, overnight or $Pd(PPh_3)_4$, Na_2CO_3, toluene/MeOH/water, reflux, overnight (56–69%); (d) H_2, Pd/C, EtOAc, EtOH (85–96%); (e) (i) *S*-(2-naphthylmethyl)-2-pyridylthioimidate hydrobromide, EtOH/MeCN, rt; (ii) CH_3SO_3H, CH_2Cl_2 (41–55%).



Scheme 2. Reagents and conditions: (a) 1,4-dioxane, $Pd(PPh_3)_4$, reflux, overnight (41–55%); (b) H_2, Pd/C, EtOAc, EtOH; (c) (i) *S*-(2-naphthylmethyl)-2-pyridylthioimidate hydrobromide, EtOH/MeCN, rt; (ii) CH_3SO_3H, CH_2Cl_2 (55%).

Table 1
In vitro biological activity (nM) of AIAs[a]

Compound	IC_{50} versus intracellular *L. donovani*	IC_{50} versus intracellular *L. amazonensis*	IC_{50} versus J774 macrophages
6a	5.3 ± 1.2	93 ± 28	11,000 ± 1000
6b	150 ± 30	190 ± 120	27,000 ± 5000
6c	21 ± 18	49 ± 19	13,000 ± 3000
6d	7.0 ± 1.7	110 ± 50	14,000 ± 2000
10	26 ± 14	290 ± 80	10,000 ± 2000
DB766	36 ± 5[b]	87 ± 15[b]	Not tested
Amphotericin B	45 ± 8	120 ± 30	Not tested
podophyllotoxin	Not tested	Not tested	18 ± 14

[a] Mean ± standard deviation of at least three determinations.
[b] Values taken from Ref. 11.

subsequent treatment with tri-*n*-butyltin chloride as previously described.[17] Stille coupling between **7** and **8** provided the corresponding 2,5-bis(4-nitrophenyl)furan **9**, which was reduced by catalytic hydrogenation to yield the corresponding diamine. This diamine then reacted with two equivalents of *S*-(2-naphthylmethyl)-2-pyridylthioimidate hydrobromide in ethanol/acetonitrile to yield target compound **10**.

Compounds **6a–d** and **10** were evaluated for their activity against both *L. donovani* and *L. amazonensis* intracellular amastigotes (please see Table 1) using methods that have been described previously.[18,19] Each of these target compounds displayed IC_{50} values in the nanomolar range against *Leishmania* parasites. The in vitro potency of these molecules was similar to that of amphotericin B, the clinical antileishmanial drug exhibiting the greatest in vitro activity, and was also in the range of that reported for the AIA lead compound DB766 (IC_{50} values of 36 and 87 nM against *L. donovani* and *L. amazonensis* intracellular amastigotes, respectively).[11] Consistent with our previous observations for other AIAs, the target compounds were generally more potent against intracellular *L. donovani* than against *L. amazonensis*.[10,11] Comparing the four unsymmetrical AIAs (**6a–d**), the steric bulk of the R group did not have a major effect on potency, as both compounds **6a** and **6d** displayed single digit nanomolar IC_{50} values against intracellular *L. donovani* and possessed IC_{50} values ~100 nM against *L. amazonensis*. The electronics of the R group appeared to have a greater influence on potency, as fluorinated congener **6b** was the least active in this series against both *Leishmania* species. Symmetrical AIA **10** displayed an IC_{50} value against *L. donovani* similar to that reported for DB766 and in the range of the IC_{50} values observed for **6a–d**. Compound **10** was less active than DB766 and the unsymmetrical AIAs against *L. amazonensis*, however. Dose–response curves for **6a** and **10** in the intracellular *Leishmania* assays are given in Figure 2. In terms of mammalian cell toxicity, compounds **6a–d** and **10** exhibited IC_{50} values from 10,000 to 27,000 nM against J774 macrophages, providing in vitro selectivity indexes (IC_{50} vs J774/IC_{50} vs *Leishmania*) of 180–2075 against *L. donovani* and 115–265 against *L. amazonensis*.

Considering the outstanding potency of these AIAs against intracellular *Leishmania* and their in vitro selectivity against intracellular parasites, **6a–d** and **10** were evaluated for toxicity to female BALB/c mice to determine an appropriate dose for in vivo antileishmanial efficacy assays (all animal protocols used in the course of this work were approved by the Institutional Animal Care and Use Committee of The Ohio State University; **6a–d** and **10** were dissolved in water for in vivo administration). Compounds **6a–d** were toxic to the mice when administered at an ip dose of 30 mg/kg/day. Mice given a single 30 mg/kg ip dose of **6a** died within 24 h of compound administration; necropsy of these animals revealed signs of severe toxicity to the gastrointestinal tract (black and swollen stomach, intestine and colon). Animals receiving **6b–d** were euthanized after either the second or third dose of compound due to hyperactivity and tremors. Because of the high toxicity of these compounds at the 30 mg/kg dose and because the lead compound DB766 required a 30 mg/kg ip dose to show good in vivo antileishmanial efficacy, compounds **6a–d** were not tested at lower doses. Compound **10**, on the other hand, was well tolerated when given ip at 30 mg/kg/

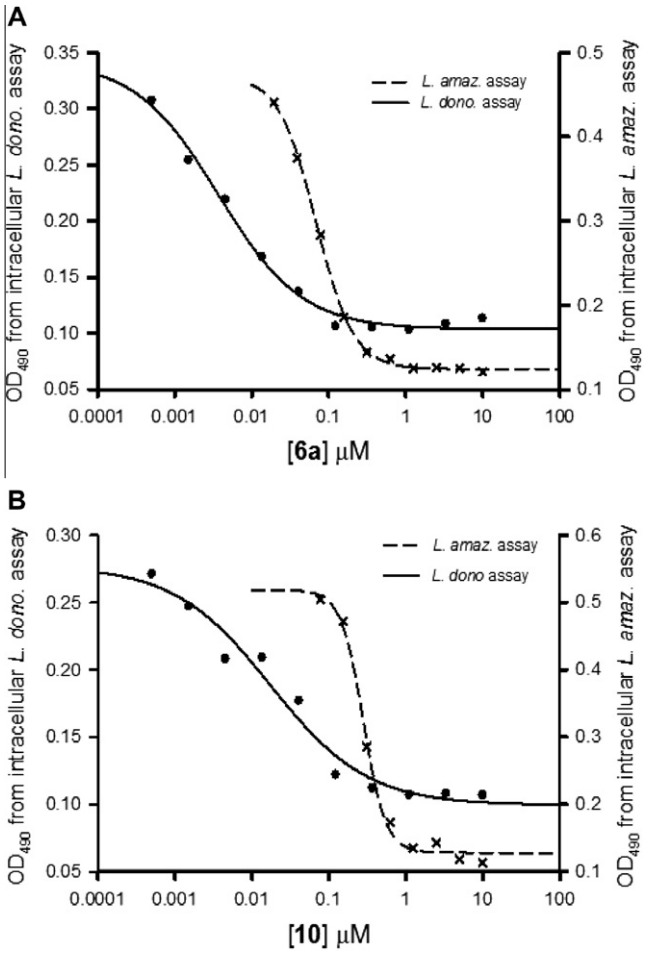


Figure 2. Dose–response curves showing the susceptibility of intracellular *Leishmania* to **6a** and **10**. Experiments pictured above were performed in triplicate as indicated in Table 1, with the results of a representative experiment shown. *Panel A*, susceptibility of *L. donovani* and *L. amazonensis* to **6a**. In the *L. donovani* assay, the uninfected control average absorbance was 0.11 and the infected control average absorbance was 0.28. In the *L. amazonensis* assay, the uninfected control average absorbance was 0.11 and the infected control average absorbance was 0.53. *Panel B*, susceptibility of *L. donovani* and *L. amazonensis* to **10**. In the *L. donovani* assay, the uninfected control average absorbance was 0.11 and the infected control absorbance average was 0.28. In the *L. amazonensis* assay, the uninfected control average absorbance was 0.13 and the infected control average absorbance was 0.54.

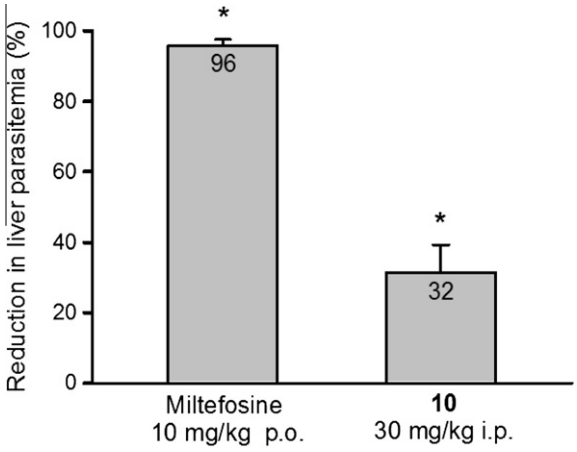


Figure 3. Efficacy of **10** in a murine model of visceral leishmaniasis. Compound **10** and the antileishmanial standard drug miltefosine were administered in five daily doses to *L. donovani*-infected BALB/c mice as indicated in the text, then animals were euthanized and liver parasitemia was determined microscopically. Data are presented as the percentage reduction of liver parasitemia compared to infected, untreated control animals. Bars and error bars indicate the means and standard deviations, respectively, of groups of four animals. $^{*}P$ <0.05 compared to untreated control.

day for five days. This dosing regimen was thus selected for the evaluation of **10** in a murine model of visceral leishmaniasis (Fig. 3). Antileishmanial efficacy was assessed by established methods in which *L. donovani*-infected BALB/c mice were treated with test compound or vehicle daily for five days starting one week post infection and liver parasitemia was assessed microscopically two weeks post infection.[19] Compound **10** reduced the liver parasite burden by 32%, whereas the standard antileishmanial drug miltefosine decreased liver parasitemia by 96% when administered orally at a dose of 10 mg/kg/day × 5 (Fig. 3). A previous study showed that the lead AIA DB766 reduced liver parasitemia by 63% when given ip at a dose of 30 mg/kg/day × 5.[11]

AIAs display outstanding in vitro activity against intracellular *Leishmania* as demonstrated in previous work[8–11] and as shown in Table 1. We are unaware of another structural class of molecules that contains as many compounds possessing nanomolar potency against intracellular *Leishmania*, although phospholipid analogs,[20] arylisoquinolinium salts,[21] and cyanines[18,22] also exhibit mid-nanomolar in vitro potency against *Leishmania* within host macrophages. Given the range of bis-AIAs that have been prepared and tested for activity against intracellular *Leishmania* in vitro, a detailed structure–activity relationship can now be outlined for these compounds (Fig. 4). Regarding terminal groups containing a six membered aromatic ring, 2-pyridyl groups are preferred over phenyl,[8] 2-pyrimidinyl, or 2-pyrazinyl groups,[9] and substitution of the 2-pyridyl terminal group at the *para* position with a halogen (but not a methoxy) group reduces potency.[9] For AIAs with a 5-membered heteroaromatic ring as the terminal group, compounds containing 5-imidazolyl and 2-pyrrolyl rings were at least an order of magnitude less active than the corresponding 2-pyridyl compound, while the 4-(2-methylthiazolyl) compound retained the in vitro (but not in vivo) activity of the corresponding 2-pyridyl congener.[9] Regarding the linker, a bis-AIA with a phenyl linker was inactive against intracellular *Leishmania*, but bis-AIAs bearing 4,4′-biphenyl and 4,4′-diphenyl ether linkers were as potent as the corresponding bis-AIAs containing a 4,4′-diphenylfuran linker.[10] Concerning substitution on the diphenylfuran linker, inclusion of halogen, alkyl or alkoxy groups on the phenyl rings improves potency compared to the diphenylfuran derivative lacking such substitutions.[8,9] The alkoxy groups on the phenyl rings can be quite large, as the cyclopentyloxy-containing compound DB1852 retains mid-nanomolar in vitro potency against intracellular *Leishmania*,[9,12] but the benzyloxy derivative was 3-fold and sixfold less potent than DB1852 and DB766, respectively.[9] Our present study indicates that bis-AIAs bearing unsymmetrical substitutions on the diphenylfuran linker retain the nanomolar antileishmanial potency of their symmetrical bis-AIA counterparts (Table 1).

Of the considerable number of bis-AIAs possessing potent in vitro activity against intracellular *Leishmania*, only derivatives bearing relatively large alkoxy substituents on a diphenylfuran linker such as DB766, DB1852, and compound **10** are relatively well tolerated in mice. In a previous report, we examined the in vivo properties of both DB766 and DB745, a compound identical in structure to DB766 except that the isopropoxy groups were replaced by smaller ethoxy groups.[11] DB745 displayed in vitro and in vivo antileishmanial efficacy similar to DB766 but was significantly more toxic to mice, despite the fact that comparable doses of these compounds resulted in significantly higher exposure for DB766. Considering that the in vivo toxicity data for compounds **6a**–**6d**, like DB745, fit our structure–toxicity relationship outlined above, we do not believe that it is justified to sacrifice more animals to measure the pharmacokinetics of these toxic compounds. Like the lead compound DB766, its regioisomer **10** displayed no overt toxicity to mice when given ip at 30 mg/kg/day × 5, but was less effective than DB766 at reducing the liver parasite burden in *L. donovani*-infected mice (Fig. 3). Previous work showed that micromolar levels of DB766 and related AIAs accumulate in the livers of mice after a single dose.[11,12] If this is also the case for **10**, sufficient compound should be present in the liver to eradicate the parasites (Fig. 2B). Within hepatic tissue, *L. donovani* reside within Kupffer cells, the macrophages of the liver. Since Kupffer cells comprise only 2% of the liver cell population[23] and AIA levels have not been measured in this cell type, it is possible that **10** and other AIAs do not reach sufficient concentrations within the parasites to eliminate infection. Testing this hypothesis will require the isolation of Kupffer cells from animals dosed with an AIA and the determination of compound levels within these cells.

Despite the potency of the AIAs against intracellular *Leishmania* and the favorable distribution of members of this class of compounds, we have thus far been unable to identify an AIA with the combination of safety and efficacy required to progress to preclinical

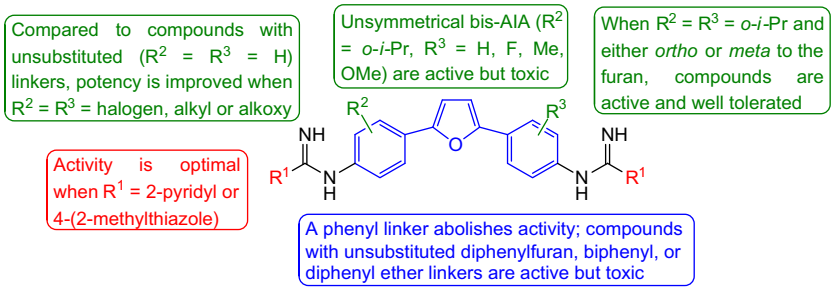


Figure 4. Structure–activity map for AIAs against intracellular *Leishmania*.

development against leishmaniasis. Thus far, our published work has focused on bis-AIAs (compounds containing two terminal heteroaromatic groups). Initial studies indicate that mono-AIAs also show activity against intracellular *Leishmania* and no overt toxicity to mice. Further work concerning the antileishmanial activity of mono-AIAs will be reported in due course. Mechanism of action studies are also in progress to identify the molecular target or targets of the AIAs in *Leishmania*. If a specific target can be found for the AIAs, it may be possible to design inhibitors of this target that retain the antileishmanial potency of the AIAs while achieving greater in vivo antileishmanial efficacy and an acceptable toxicity profile. This may be the best strategy to capitalize on the outstanding in vitro antileishmanial potency of the AIAs.

Acknowledgment

This work was supported by the Bill and Melinda Gates Foundation. The sponsor had no role in designing the study, in data collection/analysis, or in the decision to publish this work.

Supplementary data

Supplementary data (experimental procedures for the synthesis of all new compounds, along with the complete chemical characterizations for these molecules) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.06.037.

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