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Novel arylalkylamine compounds exhibits potent selective antiparasitic activity against *Leishmania major*

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Abstract

Leishmania major (*L. major*) is a protozoan parasite causal agent of Leishmaniasis. It is estimated that 12 million people are currently infected and around 2 million infections occur each year. Current treatments suffer of high toxicity for the patient, low efficacy toward the parasite, high cost, and are losing effectiveness due to parasite resistance. Discovering novel small molecule with high specificity/selectivity and drug-like properties for anti-leishmanial activity remains a significant challenge. The purpose of this study is to communicate the design and synthesis strategies of novel chemical compounds based of the arylalkylamine scaffold with selective toxicity towards *L. major* and less toxicity to human cells *in vitro*. Here, we have developed a structure activity relationship (SAR) study of arylalkylamine AA1 in order to study their anti-parasitic effect in *L. major*. Overall, 27 arylalkylamine compounds derived from AA1 were synthesized and purified by silica gel column chromatography. The purity of each analog was confirmed by spectroscopic methods (¹H, ¹³C NMR and LC/MS). Among these analogs, the compound AA9 showed the best toxic activity on *L. major* (LD₅₀ = 3.34 μM), which represents a 9 fold higher lethality as compared with its parental AA1 (Fer-1) compound (LD₅₀ = 28.75 μM). In addition, AA9 showed no significant toxicity at 80 μM on U20S Human Osteoblasts, Raw 264.7 Macrophages or intraperitoneal macrophages. In summary, our combined SAR study and biological evaluation data of AA1-AA27 compounds allow the identification of novel arylalkylamine compound AA9 that exhibits potent cytotoxicity against *L. major* promastigote with minimum toxic effect on human cells.

Keywords

Leishmania major; Amphotericin B; arylalkylamine compounds; mammalian cells

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Supplementary data

Supplementary (bioassays, protocols, synthesis of analogs and their ¹H NMR and LC–MS) data associated with this article can be found, in supplementary information file.

Leishmaniasis caused by protozoan parasites *Leishmania major*, affect millions of people worldwide.¹ This disease is among the World Health Organization's Neglected Tropical Diseases (NTD) list.² It has a wide range of clinical symptoms and over 20 species and subspecies of *Leishmania* that can infect humans, causing three different diseases: visceral (VL), cutaneous (CL), or mucocutaneous leishmaniasis (MCL).³ This infection is encountered in tropical and subtropical regions of the world. Approximately 350 million people are at risk in 88 countries around the world.⁴ An estimated 12 million people are currently infected and around 2 million infections occur each year.⁵ Currently, there is no human vaccine available to prevent or treat these parasitic infections despite numerous efforts.⁶ Several drugs were utilized to cure Leishmaniasis; for example, the chemotherapeutic pentavalent antimonial drugs (Meglumine antimoniate and Stibogluconate, Scheme 1.) are still used for treatment.⁷ Nevertheless, this treatment requires daily injections for 28 days and recently an increase of therapeutic failure cases were reported.^{8,9} The 2-(hexadecyloxy-oxido-phosphoryl)oxylethyl-trimethyl-azanium (miltefosine, Scheme 1.) was reported to be the first oral drug and showed low toxicity in the treatment of leishmaniasis, though there is no evidence that miltefosine is better than meglumine antimony.^{10, 11, 12} On the other hand, the polyene antibiotic amphotericin B (Scheme 1.) is the most extensively used drugs for this disease.^{13,14} Recently, Wyllie and co-workers reported that the 1-methyl-2-((4-(methylthio)phenoxy)methyl)-5-nitro-1H-imidazole (fexinidazole, Scheme 1.), a drug currently in phase 1 clinical trials for treating African trypanosomiasis, displays potential for treating visceral leishmaniasis.¹⁵ The absence of highly efficient and potent new drugs or vaccines to control *L. major* makes the search for active chemotherapeutic agents an "urgent priority" in parasitic research.^{16,17} Maldonado and co-workers reported that the cationic steroid antibiotics (ceragenins) containing the sterol backbone bearing amino acids were effective compounds with submicromolar potency against *L. major* promastigotes.¹⁸ The same research group described the synthesis and the high biological activity of new ruthenium(II) complexes of ketoconazole against *L. major* nontoxic to human or murine normal cells.^{19,20} A recent study suggested that biological compounds that involve the production of reactive oxygen species (ROS) may induce cell death in various leishmaniasis.^{21,22} Almeida-Amaral and co-workers reported that the flavonol quercetin and the epigallocatechin gallate (Scheme 1.), known potent antioxidants, induced death in *Leishmania amazonensis* and *braziliensis* respectively.^{21,22} Recently, Stockwell and co-workers reported the discovery of an arylalkylamine AA1-type compound (Fer-1) that was shown to be a potent and specific inhibitor of a unique form of non-apoptotic, iron-dependent, oxidative cell death, known as ferroptosis.^{23,24} The Fer-1 compound inhibits the lipid ROS production through an antioxidant mechanism. Here we demonstrate, for the first time, that the arylalkylamines AA1-AA27 compounds with drug-like properties, particularly the compound AA9, are lethal to the *L. major* promastigotes parasites. Using the high-content imaging assay, we indicated that compound AA9 inhibits the proliferation of *L. major* amastigotes propagated in macrophages. Our mechanistic investigation showed that AA9 mediate the cell death through the production of ROS.

Discovering small molecule with drug-like properties (highly lethal to parasite, highly soluble and less toxic to human cells) remains a significant challenge. We have previously

reported the design and synthesis of Pictet-Spengler condensation derivatives (tetrahydro- β -varboline) that exhibit a specific cell death in cancer cells.²⁵ The purpose of this study is to communicate the design and synthesis strategies of novel chemical compounds with selective toxicity towards *L. major* and less toxicity to human cells *in vitro*. We therefore developed potent small molecules based on the arylalkylamine AA1 (Fer-1) scaffold. This serves towards creating novel molecules with better antiparasitic activity and cytotoxicity. We modified our previously reported synthesis to create 27 arylalkylamine (AA1-AA27) compounds (Scheme 2).

First, various esters (**2**) were prepared with a good yield (66–85% yield) starting from commercially available acids [4-chloro-3-nitrobenzoic acid (**1a**), 4-chloro-2-fluoro-5-nitrobenzoic acid (**1b**) and 6-chloro-5-nitronicotinic acid (**1c**)] and alcohols [ethanol, dodecanol and morpholinoethanol] using dicyclohexylcarbodiimide (DCC) and dimethylamine pyridine (DMAP) coupling reagents. Second, a nucleophilic aromatic substitution reaction (S_NAr) between the prepared esters (**2**) and cyclohexylamine in the presence of potassium carbonate in DMSO at 60 °C provided 68–90% yield of the nitro derivatives (**3**). Third, hydrogenolysis of the nitro derivatives (**3**) provided the desired aniline derivatives at 70–94% yield. Finally, the aniline of the latter compounds was reacted through either reductive amination condition in the presence of sodium triacetoxyborohydride in the presence of arylaldehydes or acylation reaction to generate the arylalkylamine derivatives (**4**) at 58–82% yield. All the compounds were purified using column chromatography on silica gel and characterized using nuclear magnetic resonance (NMR) and liquid chromatography coupled mass spectrometry (LC/MS) (see supporting information).

In order to evaluate the efficacy of the arylalkylamine AA1 (Fer-1) as potential anti-parasite scaffold, we first tested its lethality in *L. major* as a lead compound. Our evaluation of AA1 compound for 72 h showed modest activity in *L. major* strain Friedlin clone V1 Luciferase promastigotes (LD₅₀ = 28.75 μ M) compared to the known positive controls treated with Amphotericin B (at 25 μ M) (Figure. 1).

While AA1 compound has a modest activity in *L. major* (Figure. 1), we were surprised to observe that AA1 is not cytotoxic in mammalian cells (LLC-MK2) at the highest concentration of 240 μ M (Figure. 1). Encouraged by the cytotoxicity data of AA1, we decided to develop a SAR study of AA1 compound. We designed and synthesized 26 additional (AA2-AA27) analogs of AA1 (Scheme 2) to evaluate their lethality specifically in *L. major* promastigotes. The lethality data (LD₅₀) of the AA1 and analogs are shown in Tables 1 and 2.

The SAR study of AA1 compounds and their lethality data in *L. major* allow us to drive the following conclusions: First, the nitro analog AA2 was not potent (Table 1). Second, the dodecyl and morpholinoethyl esters (AA5 and AA7, Table 1) did not improve the potency. Third, modification on the N-cyclohexyl (AA3 and AA6, Table 1) as well on the benzene ring (AA4 and AA8 Table 1) did not improve the potency of AA1. Fourth, reductive amination reaction between AA1 and various aryl aldehydes generated additional analogs (AA9-AA24, Tables 1 and 2). Among these analogs, the compound AA9 (Table 1) showed

the best activity in *L. major* ($LD_{50} = 3.34 \mu M$) which represent a higher lethality (up to 9 fold) than the lead molecule (AA1) (Figure. 2).

In addition, no significant cytotoxicity of compound AA9 was shown at a concentration up to $80 \mu M$ in U20S Human Osteoblasts, Raw 264.7 Macrophages or intraperitoneal macrophages (Figure. 2). Fifth, the carbamate AA25 (Table 2) was not potent which confirm the importance of the NH-benzyl moiety. Finally, the di-benzyl AA26 and the acid AA27 analogs (Table 2) showed no improvement of the potency. Based on our SAR study, the NH-benzyl moiety (a hydrophobic moiety) of the AA9 compound seems very crucial for the potency. We then, evaluated the AA9 compound in the intracellular proliferation of *L. major* using the well-established high-content imaging assay.^{28,29} The effect of AA9 was determined against the intracellular proliferation of *L. major* amastigotes propagated in macrophages. First, starch activated intraperitoneal macrophages were placed in a 96 well microplate. After 24 h, macrophages were infected with *L. major* metacyclic promastigote, at a density of 10 parasites per 1 cell, followed by the treatment with AA9 for 48 h. The cells were fixed and stained with annexin-V 488 and DAPI. The number of infected cells and amastigotes were determined by HCIA, using BD pathway bio-imager microplate confocal microscope. As observed in Figure. 3A, compound AA9 successfully inhibit 80% of the intracellular proliferation of the parasite at a concentration of $5 \mu M$ as compared with negative control treated with 1% DMSO. To confirm the efficacy of the AA9, we compared its efficacy with the known Amphotericin B (Amp B, $25 \mu M$) used as a positive control (Figure. 3B).

We hypothesized, based on the recent study^{21,22} that AA9 compound may induce cell death in L-Major through the production of ROS. We then investigated the intracellular ROS in L-major after treatment with AA9 compound. To do so, we assessed the reactive oxygen species produced within the cells including superoxide, hydrogen peroxide, and other reactive oxygen intermediates in *L. major* promastigotes cells. Cells were incubated with AA9 for 24 h at $3 \mu M$. After incubation period, H_2DCFDA was added and the cells were incubated for an additional 20 min at $37^\circ C$. Fluorescence intensity was evaluated using a fluorescent microplate reader. The levels of ROS in AA9 ($3 \mu M$) treated cells after 300 min increased by 1.75-fold, compared with vehicle control 1% DMSO (Figure. 4). Moreover, AA9 produces intracellular ROS in the parasite in a dose and time dependent manner, with a similar pattern that positive control treated with hydrogen peroxide (Figure. 4).

In summary, we demonstrated for the first time that arylalkylamine type-compounds possess anti-*Leishmania* activity with no toxicity to human cells. 27 arylalkylamine compounds derived from AA1 (Fer-1) were synthesized and purified by silica gel column chromatography. The purity of each analog was confirmed by spectroscopic methods (1H , ^{13}C NMR and LC/MS). The lead optimized compound AA9 showed the best toxic activity on *L. major* ($LD_{50} = 3.34 \mu M$), which represents a 9 fold higher lethality as compared with its parental AA1 (Fer-1) compound ($LD_{50} = 28.75 \mu M$) (Table 3). In contrast, AA9 was not significantly toxic at $80 \mu M$ on U20S Human Osteoblasts, Raw 264.7 Macrophages or intraperitoneal macrophages (Table 3). We are currently developing the synthesis of additional analogs with drug-like properties (e.g., better solubility and selective potency) for

mechanism evaluation and *in vivo* efficacy study in BALB/c mice infected with transgenic *L. major*.

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26. Antiparasitic activity of AA1 compound in *L. major* Luciferase promastigotes for 72 h. Cytotoxicity evaluation of AA1 after 96 h incubation in Monkey kidney cells (LLC-MK2).
27. Antiparasitic activity of AA9 evaluated in *L. major* Luciferase promastigotes for 72 h. Cytotoxicity evaluation of AA9 compound in U2OS Human Osteoblasts and Raw 264.7 Macrophages treated with AA9 for 96 h. Intraperitoneal Macrophages (IP ϕ) treated with AA9 for 48 h.
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30. A) Number of infected cells with 5 or more amastigotes per macrophage. After 48 h of treatment, compound AA9 inhibited the intracellular proliferation of *L. major* inside its host. Negative control treated with 1% DMSO. Positive control treated with Amp B at 25 μ M. B). Representative pictures of macrophages infected with *L. major* amastigotes and treated with negative (1% DMSO) and positive (Amp B at 25 μ M) controls, and AA9 at 5 μ M respectively.
31. Evaluation of reactive oxygen species (ROS) in *L. major* promastigotes treated for 24 h with AA9 at 3 μ M. Negative controls treated with 1% DMSO and Medium only. Positive control treated with Hydrogen Peroxide at 100 μ M. As observed, the production of intracellular ROS in the parasite occurs in a dose and time dependent manner, with similar values as the positive control treated with hydrogen peroxide.
32. IC50: Median inhibitory concentration. LD50: Median lethal dosage, calculated with 95% confidence interval. TI: Therapeutic index.

Figure 1.
Evaluation of the antiparasitic activity and cytotoxicity of AA1 compound.²⁶

Figure 2.
Evaluation of antiparasitic activity and cytotoxicity of the lead compound AA9.²⁷

Figure 3.
Proliferation assay of the lead compound AA9.³⁰

Figure 4. Evaluation of reactive oxygen species (ROS) in *L. major* promastigotes treated with the lead compound AA9.³¹

Scheme 1.

Chemical structures of known anti-leishmania compounds.

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Table 1

LD50 of arylalkylamine (AA1) and analogs (AA2-AA16).

Name/Structure	LD50(μM)
Fer-1 (AA1)	28.75
AA2	>10
AA3	>10
AA4	>10
AA5	>10
AA6	>10
AA7	>10
AA8	>10
AA9	3.34
AA10	>10
AA11	>10

Name/Structure	LD50(μM)
AA12	>10
AA13	>10
AA14	>10
AA15	>10
AA16	>10

Table 2

LD50 of arylalkylamine AA9 and analogs (AA17-AA27).

Name/Structure	LD50(μ M)
AA9	3.34
AA17	>10
AA18	>10
AA19	4.98
AA20	>10
AA21	4.92
AA22	9.49
AA23	>10
AA24	>10
AA25	>10
AA26	>10

Name/Structure	LD50(μM)
AA27	>10

Table 3Antiparasitic activity and cytotoxicity of compounds AA1 and AA9.³²

Compound	Mammalian cells IC ₅₀ (μM)					<i>L. major</i> LD ₅₀ (μM)
	U20S Human Osteoblasts	LLC-MK ₂	Raw 264.7 Macrophages	IPφ	Promastigotes LD ₅₀ (μM)[TI]	
AA1	N/A	>240	N/A	N/A	28.75 [8.35]	
AA9	>80	N/A	>80	>80	3.34 [23.95]	