Award Number: W81XWH-14-2-0135

TITLE: Arylimidamide-Azole Combinations against Leishmaniasis

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REPORT DATE: September 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution is unlimited.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
September 2016	Annual	28 AUG 2015 – 27 AUG 2016

4. TITLE AND SUBTITLE

Arylimidamide-Azole Combinations against Leishmaniasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-2-0135

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Arylimidamide compound DB766D was tested in combination with posaconazole in combination based on the premise that the partial efficacy provided by each compound individually would provide cures when the combination was dosed together. At the maximum tolerable dose of the combined treatment, efficacy was observed, however, no cures of *L. major* lesions were observed. Three additional compounds, DB2342, DB2332, and DB2336 were tested for potency against *Leishmania* in vitro, and as all 3 were very potent, all 3 were tested in vivo for toxicity to determine the maximum tolerable dose and ultimately efficacy testing was conducted against *L. major* to suppress lesion formation. The maximum tolerable dose for all 3 compounds was determined, and lesion suppression testing was conducted in vivo at the maximum tolerable dose. None of the 3 compounds were shown to suppress lesion formation. Four additional phenoxyalkyl hybrid compounds were tested in vitro, and one compound, AA2-160, demonstrated potency in vitro against *Leishmania* species. This compound will be selected for further in vivo testing in Q1 Y3.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	9	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. **INTRODUCTION:** Existing oral treatments of visceral and cutaneous leishmaniasis have significant drawbacks to include serious side effects, variable efficacy, and expense. Intravenous treatment with liposomal amphotericin B (AmBisome) is expensive, lengthy, and impractical for deployed soldiers (treatment requires 21 days of intermittent IV therapy in a hospital setting). An inexpensive oral treatment for both visceral and cutaneous leishmaniasis that provides consistent efficacy against all species of *Leishmania* that infect man is a clear unmet need. This proposal is focused on a group of arylimidamide compounds which showed initial potency against visceral leishmaniasis in vitro and efficacy against visceral leishmaniasis in vivo. These compounds also showed interesting synergy with azoles which enhanced the efficacy of the arylimidamide compounds. The element of work performed at WRAIR encompasses the testing of these arylimidamide analogues against species of *Leishmania* that cause cutaneous leishmaniasis.

2. **KEYWORDS:** leishmaniasis, cutaneous, visceral, arylimidamide

3. **ACCOMPLISHMENTS:**
   a. Key Research Accomplishments

   1) *In vivo* lesion cure assessment[1] of a combination of DB766D and posaconazole against *L. major* was conducted at the maximum tolerable combined dose possible. While efficacy of the combination was noted, cures of established *L. major* lesions were not observed, and the efficacy of the combination was not superior to that of posaconazole alone.

   2) *In vitro* potency assessment of DB2342, DB2332, and DB2336 in an amastigote-macrophage assay were conducted[2] and all 3 were found to be potent against a broad array of *Leishmania* species.

   3) A dose rising experiment was conducted using DB2342, DB2332, and DB2336 to determine the maximum tolerable dose possible for follow-on efficacy testing[1].

   4. *In vivo* efficacy testing of DB2342, DD2332, and DB2336 was conducted using a lesion suppression assay[1] against *L. major* parasites at the maximum tolerable dose. Lesion suppression efficacy was not observed.

   5) *In vitro* testing of 4 new phenoxylalkyl A1A hybrids, AA2-119, AA2-120, AA2-128, and AA2-160, was conducted to determine potency against Leishmania strains in an amastigote-macrophage assay[2]. One compound, AA2-160 was shown to be potent in this assay. An in vivo assessment of this compound will be conducted in Q1 of Y3.

b. Major goal of the project: The major goal of this project is to develop an oral treatment for cutaneous and visceral leishmaniasis using A1A compounds combined with azole compounds.

c. Accomplishments in Support of the Statement of Work:

1b. Assess A1A-azole combinations in vivo against intracellular *Leishmania*.

Based on data derived during the first year of the grant, DB766D was tested in vivo combined with posaconazole at the maximum tolerable dose (37.5 mg/kg DB766D combined with 37.5 mg/kg posaconazole) in a cutaneous lesion cure assay[1] against *L. major* parasites consistent with the WRAIR Cutaneous Leishmaniasis Drug Discovery algorithm discussed in 3b below. As shown in Figure 1 in the Appendix, the progression of *L. major* lesion size over time is shown in BALB/c mice with established *L. major* lesions. The efficacy of the vehicle control, AmBisome (the positive control), DB766D alone, posaconazole alone, and the combination of DB766D and posaconazole together is shown. The efficacy of the combination was not shown to be superior in vivo to posaconazole by itself, and no cures of established *L. major* lesions were shown.

3b. Compound Evaluations

In vitro testing of 3 arylimidamide molecules, DB2336, DB2342, and DB2332, was conducted in an amastigote-macrophage assay[2] against an array of *Leishmania* parasites. As shown in Table 1, all 3 arylimidamide molecules were potent in this assay. Accordingly, all 3 were assessed in vivo initially for toxicity testing using a small group of BALB/c animals to determine the maximum tolerable dose. The maximum tolerable doses for each molecular are shown in Table 2, and based on this data, efficacy testing was planned in a lesion suppression assay against *L. major* parasites.

In vivo testing of DB2336, DB2342, and DB2332, was conducted in accordance with the WRAIR Cutaneous Leishmaniasis Drug Discovery Algorithm[1] (this is also described in the grant application) which involves testing of compounds with demonstrated potency and metabolic stability in vitro followed by in vivo testing of intraperitoneally dosed compounds for efficacy in a leishmania lesion suppression assay in immune-permissive BALBC/c mice (BALB/c mice are immune-permissive for Leishmania infection due to an imbalanced Th1/Th2 ratio) against *L. major* followed by testing of IP-dosed compounds for lesion cure against *L. major* in BALB/c mice. Compounds that have shown curative efficacy in Tier 1 are then progressed to a higher tier of study for lesion cure using oral dosing in BALB/c (Tier 2). Orally dosed compounds capable of curing lesions in BALB/c mice that pass Tier 2 testing are then assessed further through testing in immunocompetent Syrian Golden Hamsters (Tier 3). Compounds that have progressed to Tier 3 are then assessed for preclinical studies using Ames testing, hERG assays, in vitro micronucleus assays, drug-drug interaction studies, liver enzyme

induction assays, etc. Candidate drugs that survive this testing battery are deemed suitable for consideration for clinical testing in man.

In vivo efficacy testing of DB2336, DB2342, and DB2332 was conducted using a lesion suppression assay as previously described against luciferase-expressing *L. major* parasites[1]. As shown in Figure 2, none of the 3 compounds tested showed efficacy in this assay.

Further *in vitro* testing of 4 different phenoyxalkyl hybrid molecules (AA2-119, AA2-120, AA2-128 and AA2-160) was conducted in an amastigote-macrophage assay[2] against an array of *Leishmania* parasites. As shown in Table 3, 1 of the 4 compounds, AA2-160, showed potency against *Leishmania* parasites. This compound will be selected for further in vivo testing in Q1 of Year 3.

3. Accomplishments: The efficacy of 3 arylimidamide compounds was assessed in vivo using a leishmania suppression assay directed against luciferase expressing *L. major* parasites. As shown in table 2.

d. Conclusions: The combination of the best arylimidamide compound tested to date, DB766D, and posaconazole, the only azole with activity against cutaneous *Leishmaniasis* in our model, showed efficacy against established *L. major* lesions[1], however, the efficacy observed was no greater than posaconazole as a single agent. Further testing of 3 arylimidamide molecules, DB2336, DB2342, and DB2332, in vivo using a *L. major* lesion suppression assay[1]. Further in vitro testing of 4 phenoxyalkyl molecules in vitro showed 1 compound, AA2-160, with potency against *L. major* in an amastigote-macrophage assay. In vivo testing of this compound will take place in Q1 of Year 3.

4. IMPACT: The search for an orally bioavailable arylimidamide analogue with efficacy against cutaneous *Leishmania* species continues. In vitro and in vivo testing of multiple analogues has not yet identified a winning compound capable of curing *L. major* lesions. One compound, DB766D, showed efficacy but has not shown cures. Other analogues have shown potency in vitro, but no efficacy in vivo. Additional testing in Q1 of Year 3 will begin against a representative phenoxyalklyl compound, AA2-160, for efficacy. Additional analogues will be tested in vitro and in vivo as they are created.

5. **CHANGES/PROBLEMS:** The statement of work was revised in Year 1 to reflect in vitro and in vivo testing in accordance with the WRAIR CL Drug Discovery Algorithm[1]. This algorithm provides a cohesive, disciplined, resource-sparing, peer-reviewed method of progressing compounds in vitro and in vivo against Old World and New World *Leishmania* species causing cutaneous leishmaniasis. Given the vagaries of leishmania drug development, higher tier testing of compounds against a particular schedule simply to fulfill a statement of work is not warranted; compound efficacy in lower tier testing must drive the test schedule for higher tier testing.

6. **PRODUCTS:** no product developed yet.

7. **PARTICIPANTS:** collaborations under this grant include investigators at Ohio State University, Georgia State University, the University of South Florida, and the University of Kansas.

8. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:** Nothing to report.

9. **INVENTIONS, PATENTS AND LICENSES:** Nothing to report.

10. **REFERENCES:**

1. Grogl, M., Hickman, M. Ellis, W. Hudson, T. Lazo, J., Sharlow, E., Johnson, J., Berman, J., and Sciotti, R. Review: Drug Discovery Algorithm for Cutaneous Leishmaniasis. Am J Trop Hyg 88(2), pp. 216-221, 2013.
2. Khraiwesh, Mozna, Leed, Susan, Roncal, Norma, Johnson, Jacob, Sciotti, Richard, Smith, Philip, Read, Lisa, Paris, Robert, Hickman, Mark and Grogl, Max. Antileishmanial Activity of Compounds Derived from the Medicines for Malaria Venture Open Access Box Against Intracellular Leishmania major Amastigotes. American Journal of Tropical Medicine and Hygiene, published online 26 October 2015.

11. **APPENDICES:**

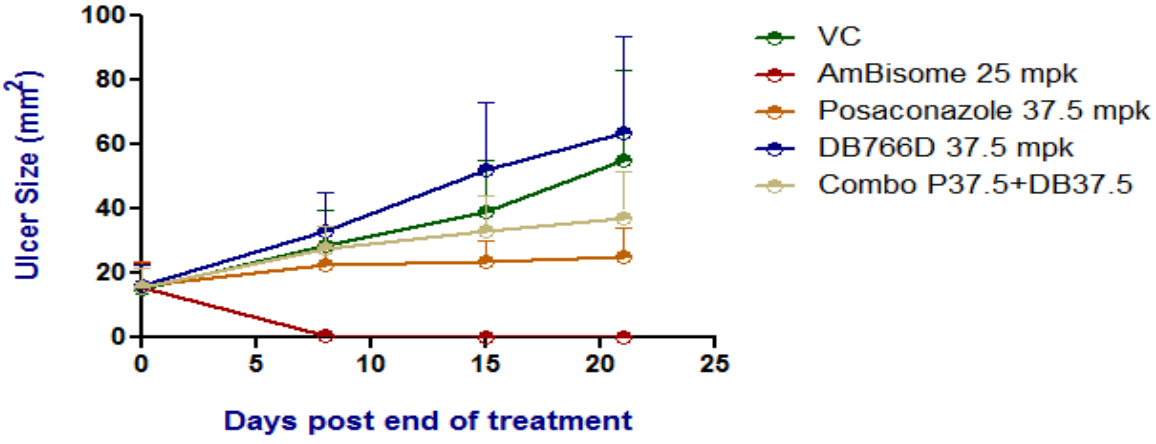

Figure 1: An *in vivo* assessment of the efficacy of DB766D (37.5 mpk) combined with posaconazole (37.5 mpk) was conducted using a lesion cure assay[1] against *L. major.* Progression of ulcer sizes in BALB/c mice infected with $1X10^7$ stationary phase *L. major* promastigotes and treated PO with 37.5 mg/kg DB766D and 37.5 mg/kg posaconazole are shown. Bars represent means ± SEMs for a total of 5 BALB/c mice. The combination of the two compounds was more efficacious than the vehicle, however, no lesion cures were obtained similar to those shown after administration of AmBisome. The combination of DB766D and posaconazole was not superior in efficacy to that of posaconazole alone.

Compound	*L. major* IC50 (nM)	*L. donovoni* IC50 (nM)	*L. guyanensis* IC50 (nM)	*L. panamensis* IC50 (nM)	*L. tropica* IC50 (nM)	*L. mexicana* IC50 (nM)
DB2336	180	80	340	520	240	150
DB2342	90	60	70	130	70	40
DB2332	180	70	120	110	100	90

Table 1: The potency of DB2336, DB2342, and DB2332 was assessed in vitro using an amastigote-macrophage assay against *L. major, L. infantum, L. guyanensis, L. panamensis, L. tropica,* and *L. mexicana*. All 3 compounds were shown to be potent with IC50s ranging from 40-520 nM.

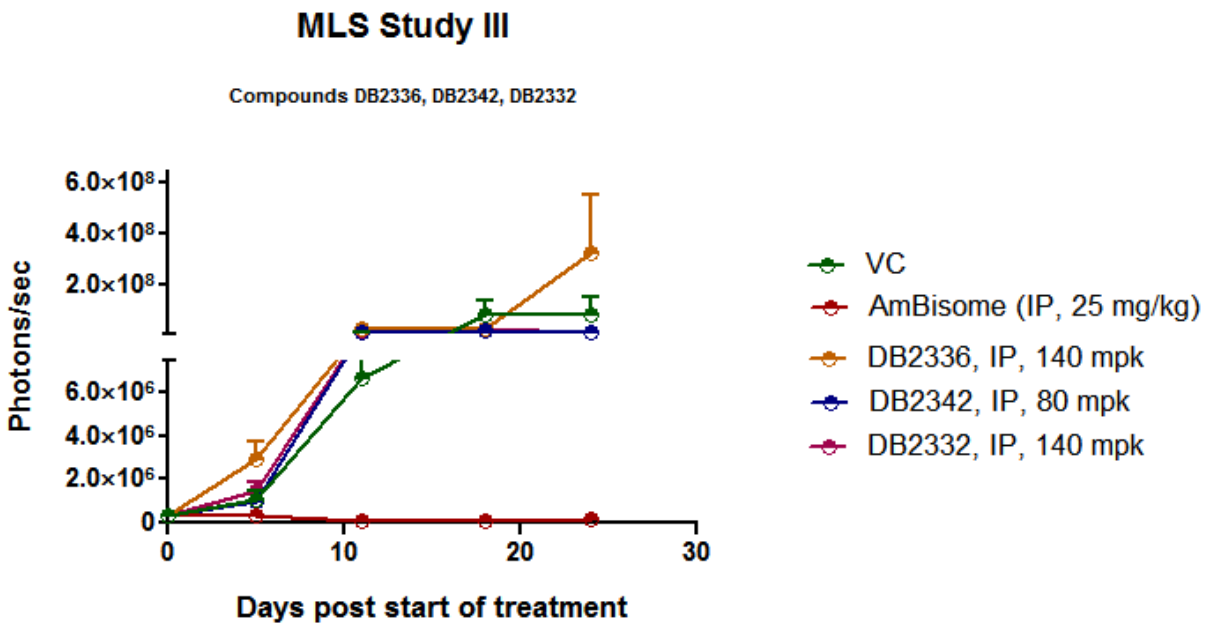

Figure 2: An *in vivo* assessment of the efficacy of DB2336 (140 mpk) , DB2342 (80 mpk) and DB2332 (140 mpk) administered intraperitoneally was conducted using a lesion suppression assay[1] against *L. major* parasites. Parasite proliferation was assessed by measuring the photons/sec of 1 x 10^7 stationary phase luciferase expressing *L. major* parasites in BALB/c mice using an In Vivo Imaging System. Bars represent means ± SEMs for a total of 5 BALB/c mice. The efficacy of all 3 test compounds was not superior to the vehicle control.

Compound Tested	Toxic Dose	Maximum Tolerable Dose Determined for Follow-On Efficacy Testing
DB 2342	160 mg/kg	80 mg/kg
DB2332	Nontoxic at max dose tested of 160 mg/kg	140 mg/kg
DB2336	Nontoxic at max dose tested of 160 mg/kg	140 mg/kg

Table 2: Toxicity testing was conducted at a range of doses of 20, 50, and 160 mg/kg in small groups of 2 BALB/c mice. 2 of the 3 compounds (DB2332 and DB2336) showed no toxicity at the highest dose tested while DB2342 showed toxicity at the highest dose tested, 160 mg/kg.

Compound	Target Strain	IC50 (ng/ml)
AA2-119	*L. major*	445.5
AA2-120	*L. major*	673.6
AA2-128	*L. major*	916.7
AA2-160	*L. major*	146

Table 3: The potency of DB2336, DB2342, and DB2332 was assessed in vitro using an amastigote-macrophage assay[2] against *L. major*. One of the 4 compounds tested, AA2-160, showed potency in this assay. This compound will be selected for toxicity testing and *in vivo* efficacy testing in Q1 of Year 3.