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14. ABSTRACT

Arylimidamide compounds DB2002, DB766D, and DB1960A (DB766D and DB1960A are the same base compound with different salts) demonstrated in vitro potency as sole agents against the amastigote-macrophage form of a variety of *Leishmania* parasites known to cause cutaneous and visceral leishmaniasis (*L. major, L. guyanensis, L. panamensis, L. tropica, L. donovoni*, and *L. infantum*) yielding IC50 values ranging from 20 – 150 nM. No synergy was demonstrated with azole compounds in vitro against *L. major*. Initial in vivo testing of these compounds using an assay to detect inhibition of cutaneous infection induced by *L. major* showed DB766 and DB1960 merited further in vivo evaluation to assess the ability of these compounds to actually cure an established *L. major* lesion. The *L. major* -infected mice in the DB766 group (60 mpk, 10 days of treatment) showed a 40% reduction in ulcer size compared to the vehicle control group mean ulcer size. DB766 treated animals did show some evidence of toxicity at the 60 mpk dose, and any further experiments will need to be conducted at the 40 mpk level which will impact efficacy.

15. SUBJECT TERMS

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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 vitro potency of A1A compounds as sole agents was demonstrated in an amastigote macrophage assay[2] showing IC50s ranging from 20-150 nM against *L. major, L. tropica, L. guyanensis, L. panamensis, L. donovoni,* and *L. infantum.*

2) In vitro potency of posaconazole in an amastigote macrophage assay[2], the only azole to demonstrate activity in vitro against CL species, showed variable activity ranging from no activity observed against *L. panamensis* and *L. guyanensis* to modest activity against *L. tropica* to potent activity against *L. major*.

3) In vitro testing of toxicity of A1A compounds and posaconazole against RAW macrophages[2,4] showed IC50s ranging from 2.7 to 10.9 uM suggesting the potency observed of all compounds testing is specific in nature rather than simply overt toxicity against macrophages.

4). Combination testing[2,3] in vitro with A1A compounds combined with fluconazole and posaconazole against *L. major* showed no synergy; additive effects of posaconazole and A1A compounds against lesions caused by *L. major*, however, are likely.

5). Testing of posaconazole in vivo using a leishmania lesion suppression assay against *L. major* showed suppression of infection (method described in grant).

6). Testing in vivo with A1A compounds using a leishmania lesion suppression assay against *L. major* showed significant inhibition of infection by DB766 and DB1960

but not with DB2002 (DB766 and DB1960 are the same base compound with different salts).

7). Tests of DB766 and DB1960 in vivo using a leishmania lesion cure assay (method described in grant) showed a 40% reduction in lesion size compared to the group mean for the vehicle control 11 days post treatment. However, there was no statistically significant difference between group means between the vehicle control mice and the DB766D mice across all time points where ulcer sizes were measured.

8). Compound toxicity with DB766 was observed at the 60 mg/kg dose and a dose of 40 mg/kg was shown to be the maximum tolerated dose over time.

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

1a. Assess A1A-azole combinations in vitro against intracellular *Leishmania*.

In vitro potency testing[2,4] (see Appendix Table 1 for detailed data) of *L. major, L. tropica, L. panamensis, L. guyanensis*, showed A1A compounds were potent as sole agents. Previous testing with fluconazole, voriconazole, itraconazole, and ketoconazole showed no potency against *L. major, L. tropica, L. panamensis,* and *L. guyanensis*. Posaconazole is the only azole with in vitro potency demonstrated against CL species, and the potency is variable; while posaconazole is active against Old World CL species such as *L. major* and *L. tropica* it is not active against New World CL species such as *L. panamensis* and *L. guyanensis*. This data suggests that the use of posaconazole to treat CL may be effective in some species but not against CL lesions caused by New World infections. Three new A1A compounds have been synthesized by Dr. Boykin, and these compounds are on test as of December 2015.

In vitro toxicity testing of A1A compounds against RAW macrophages [2,4] (the species used for in vitro amastigote-macrophage testing) showed IC50s ranging from 2.7 uM for posaconazole to 4.9 uM for DB766/DB1960 to 10.9 uM for DB2002 (see Table 2) which supports the finding that the potency observed against *Leishmania* is specific in nature.

Combination testing [2,3] was conducted using posaconazole and fluconazole combined with DB2002 and DB766/DB1960 against *L. major* amastigote-macrophage forms and no synergy was observed (Figure 1, method is a modification of reference 2 using drug combination techniques outlined in reference 3). This assay was repeated, and the second repeat test demonstrated the same findings (data not shown). This data suggests the synergy observed using A1A compounds combined with azole compounds against *L. donovoni* may not be extensible to CL species (variability in potency of compounds and combinations of compounds against various species and even sub-

species of *Leishmania* causing VL and CL is quite common). The possibility remains that in vivo synergy may be observed that is not seen using in vitro testing, and also the additive effects of both compounds against CL may well provide lesion cures in certain species. Posaconazole has an IC50 of over 4 uM against *L. tropica* and no IC50 can be determined for posaconazole against *L. panamensis* and *L. guyanensis* (lack of activity argues against synergy in combination with A1A compounds as there is no appreciable activity of posaconazole against these parasites). This data suggests posaconazole's CL activity is species specific in nature. Drug combination of posaconazole and A1A compounds tested against *L. major* in vivo is the best opportunity to show an additive drug effect, and this assay is scheduled for 2nd Quarter FY2016.

3b. Compound Evaluations

In vivo testing 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 testing to suppress *L. major* infections with posaconazole were previously conducted in our laboratory last year (Figure 2, method described in detail in the grant application), and posaconazole treatment resulted in suppression of *L. major* infection. *L. major* lesion cure efficacy experiments using posaconazole as a sole agent and in combination with A1A compounds will be conducted in 2nd Quarter FY2016.

Subsequent in vivo testing was conducted to initially assess the potential of these compounds to inhibit infection by luciferase-expressing *Leishmania major* parasites using in vivo imaging (Figures 3 and 4, method described in detail in the grant application). As shown in Appendix 5, DB766 and DB1960 (which are the same base compounds in two different salts, DB766 is a hydrochloride salt and DB1960 is a mesylate salt) demonstrated activity in the lesion suppression screen that was

significantly different from the vehicle control group, however, the efficacy of DB2002 was not different from the vehicle control group.

The follow on in vivo assay was conducted using DB766 and DB1960 (DB766 and DB1960 are the same base compound; DB766 is a hydrochloride salt and DB1960 is a mesylate salt) to assess the efficacy of both compounds to actually cure an established *Leishmania major* lesion (Figures 5 and 6, method discussed in the grant application). Treatment with DB766 showed a 40% reduction in lesion size compared to the group mean for the vehicle control 11 days post treatment. However, there was no statistically significant difference between group means between the vehicle control mice and the DB766 mice across all time points where ulcer sizes were measured.

In addition, some toxicity with DB766 was observed at the 60 mg/kg dose which suggests further studies will need to be conducted at the 40 mg/kg dosing level. Toxicity is mainly determined based on weight loss but other signs of toxicity which are described in the main protocol are considered as well.

The kinetics of signal change in this experiment for DB766 are attributed to partial activity of this compound which initially reduced the parasite load which then increased exponentially after treatment was stopped. It is very unlikely this change in signal over time is due to drug resistance as none of these drugs have ever been used for treatment against the *Leishmania* parasites used to infect the mice. Decrease of luminescence signal on day 32 for DB766 is also likely not due to a failure of drug efficacy but to the appearance of dark ulcers that quench the luminescence signal. Signal quenching is another variable associated with in vivo imaging that must be taken into account when evaluating data.

With regards to the size of ulcer formation over time, DB1960 initially reduced parasite load in a statistically significant fashion which delayed ulcer formation when compared to the vehicle control group. The DB2002 treated group did not show any statistically significant differences in ulcer size compared to the vehicle control group.

A further in vivo evaluation of DB766 combined with posaconazole to achieve lesion cures will be conducted in the 2nd Quarter of FY2016. The maximum tolerable dose of both compounds dosed together has been determined, and DB766 will be dosed at 37.5 mg/kg combined with 75 mg/kg of posaconazole.

3. Accomplishments

d. Conclusions: Initial testing of 3 arylimidamide compounds have shown in vitro potency against an array of *Leishmania* species when tested as sole agents. Testing of azole compounds has shown variable potency of posaconazole against different *Leishmania* species (other azole compounds showed no potency in vitro). Combination testing of A1A compounds with fluconazole and posaconazole in vitro has shown no synergy against *L. major* (additive effects are likely given the activity of both

compounds). Further in vivo testing has demonstrated in vivo efficacy of arylimidamide compounds, however, complete cures of established lesions with DB766, the best compound tested, were not obtained. Further testing will be needed either with an azole compound to obtain additive activity or through testing different arylimidamide analogues. New A1A compounds have been synthesized, and they are on test at WRAIR to assess in vitro potency prior to assessing their in vivo activity.

4. IMPACT: The assessment of an initial set of A1A compounds showed efficacy in curing *L. major* lesions as sole agents, however, cures were not obtained. Subsequent testing of the most promising A1A compound, DB766, will be conducted in combination with posaconazole at WRAIR in the 2nd Quarter FY2016. New A1A compounds have been synthesized and they are on test for in vitro potency at WRAIR.

5. CHANGES/PROBLEMS: The statement of work was revised 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.

11. REFERENCES:

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4. Sharlow E, Leimgruber S, Murray S, Lira A, Sciotti R, Hickman M, Hudson T, Leed S, Caridha D, Barrios A, Close D, Grogl, M Lazo J. Auranofin Is an Apoptosis-Simulating Agent with in Vitro and in Vivo Anti-leishmanial Activity. ACS Chemical Biology, Dec 2013.

12. APPENDICES:

In vitro potency of A1A compounds and posaconazole *in vitro* against amastigote-macrophage forms of *L. donovoni, L. infantum, L. major, L. tropica, L. panamensis, and L. guyanensis.* Previous testing with fluconazole, ketoconazole, itraconazole, and voriconazole has shown no potency against CL species using this assay. The IC50 values are shown along with the R^2 value demonstrating the goodness of fit of the IC50 values calculated. DB766 and DB1060 reflect the same base compound with different salts. DB766 is a hydrochloride salt and DB1960 is a mesylate salt.

Table 1.

Species	Compound Tested	IC50 observed (uM)	R^2 Value Determined
L. donovoni	DB2002	0.17	0.88
	DB766/DB1960	0.01	0.85
	Posaconazole	0.85	0.80
L. infantum	DB2002	0.13	0.91
	DB766/DB1960	0.01	0.96
	Posaconazole	No IC50 shown	N/A
L. major	DB2002	0.02	0.96
	DB766/DB1960	0.04	0.98
	Posaconazole	0.21	0.92
L. tropica	DB2002	0.15	0.93
	DB766/DB1960	0.03	0.92
	Posaconazole	4.09	0.75
L. panamensis	DB2002	0.03	0.91
	DB766/DB1960	0.02	0.97
	Posaconazole	No IC50 shown	N/A
L. guyanensis	DB2002	0.04	0.90
	DB766/DB1960	0.01	0.95
	Posaconazole	No IC50 shown	N/A

Toxicity Testing of A1A compounds and posaconazole against RAW macrophages

Table 2

Compound Tested	IC50 observed (uM)	R^2 value determined
DB2002	10.86	0.97
DB766/DB1960	4.91	0.99
Posaconazole	2.73	0.99

Drug Combination testing of posaconazole and fluconazole with A1A compounds DB2002 and DB766/DB1960 against *L. major* in an amastigote-macrophage assay.

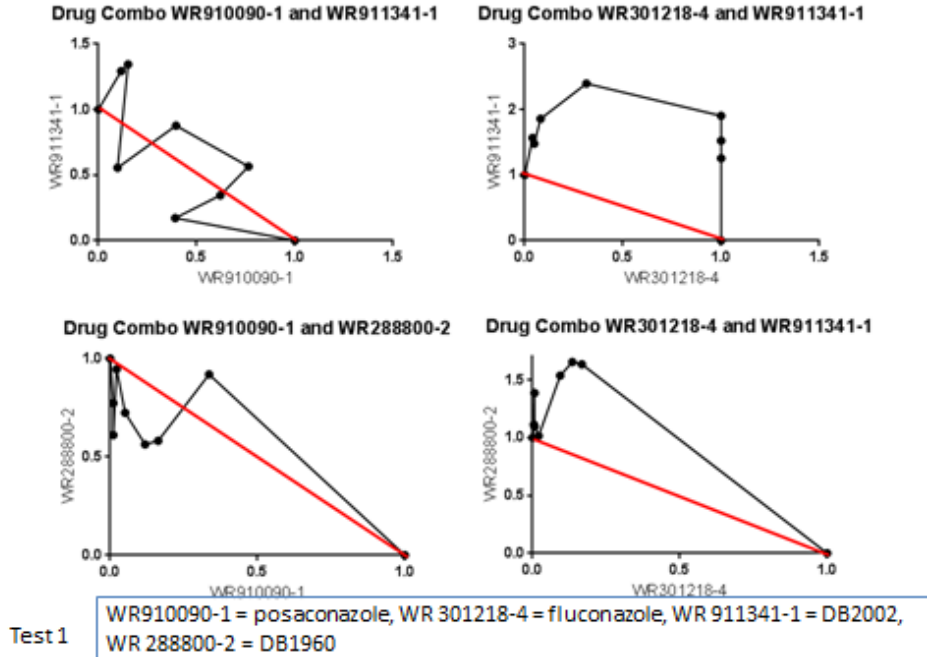

Figure 1: Drug combination study of DB2002, DB766/1960 combined with fluconazole and posaconazole in a constant ratio drug design with sum of fractional IC50s for drugs in different ratios on the x and y axes. Isobol curves (black line) falling below the red line suggest synergy, isobol curves on the red line suggest additive effects, and curves above the red line may show antagonism.

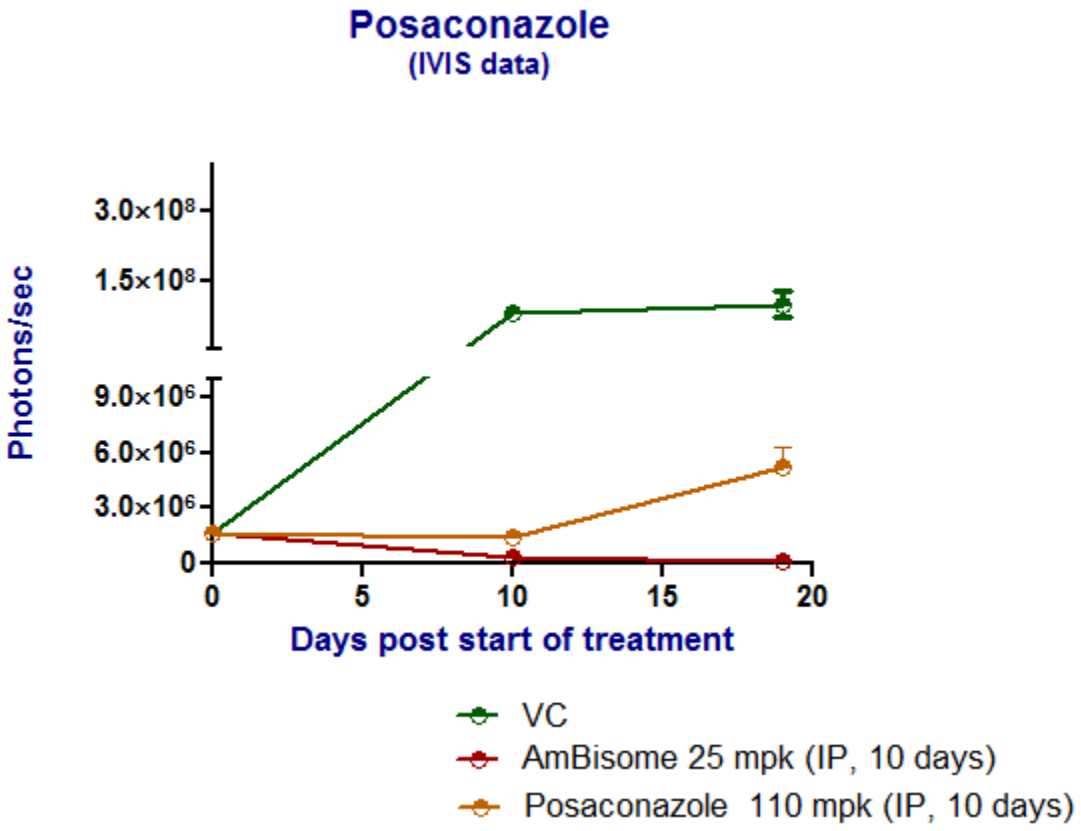

Figure 2. Progression of luminescence signal in BALB/c mice infected with $1X10^7$ stationary phase *L. major* promastigotes and treated IP with 110 mg/kg posaconazole. Bars represent means ± SEMs for a total of 5 BALB/c mice. In vivo imaging results for leishmania suppression screen (MLS assay) utilize luciferase expressing *Leishmania major* parasites. Endpoints for this in vivo test include parasite signal in photons/second in the vehicle and treated groups (which reflects the parasite burden over time) and the size of the lesions in the vehicle and treated groups.

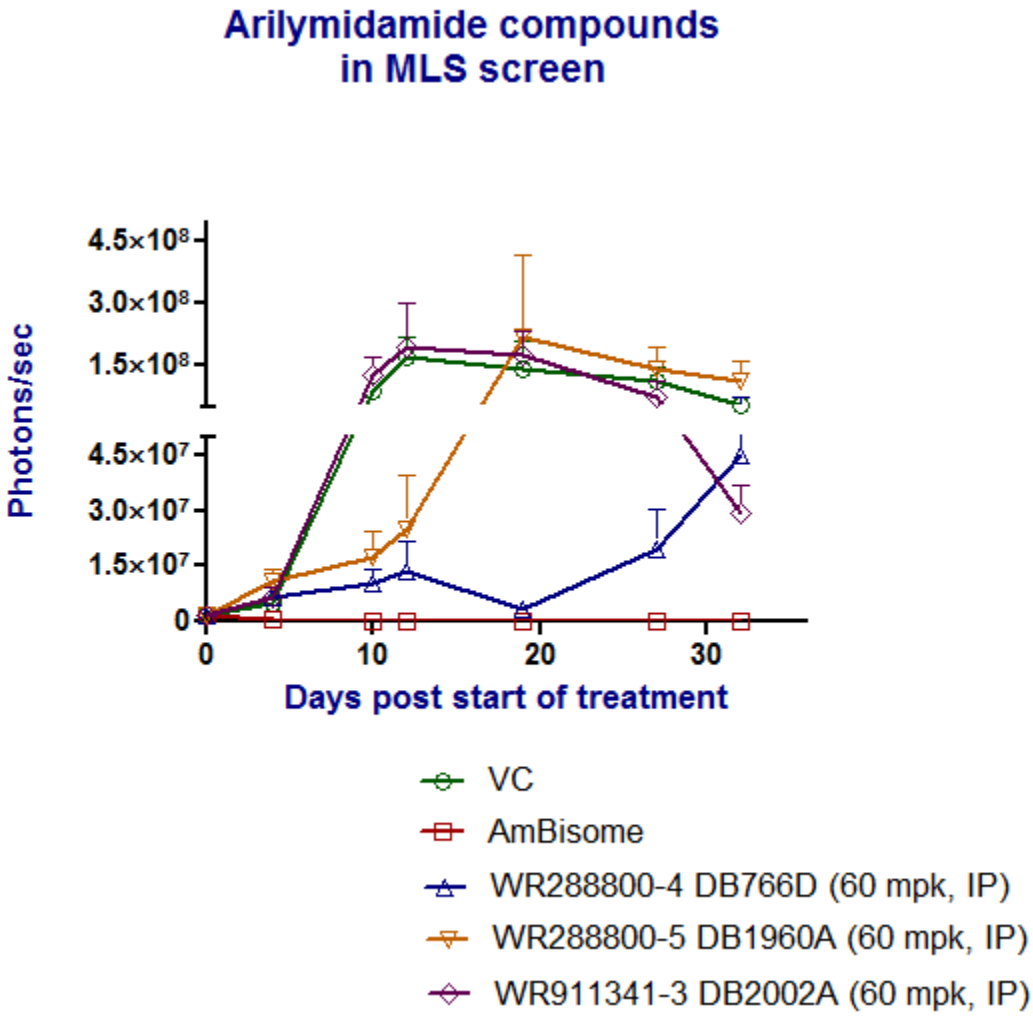

Figure 3: Progression of luminescence signal in BALB/c mice infected with $1X10^7$ stationary phase *L. major* promastigotes and treated IP with 60 mg/kg WR911341-3 (DB2002A), WR288800-4 (DB766D), and WR288800-5(DB1960A). Bars represent means ± SEMs for a total of 5 BALB/c mice. In vivo imaging results for leishmania suppression screen (MLS assay) using luciferase expressing *Leishmania major* parasites. Endpoints for this in vivo test include parasite signal in photons/second in the vehicle and treated groups (which reflects the parasite burden over time) and the size of the lesions in the vehicle and treated groups. The positive control (liposomal amphotericin B or AmBisome) was administered IP at 25 mg/kg.

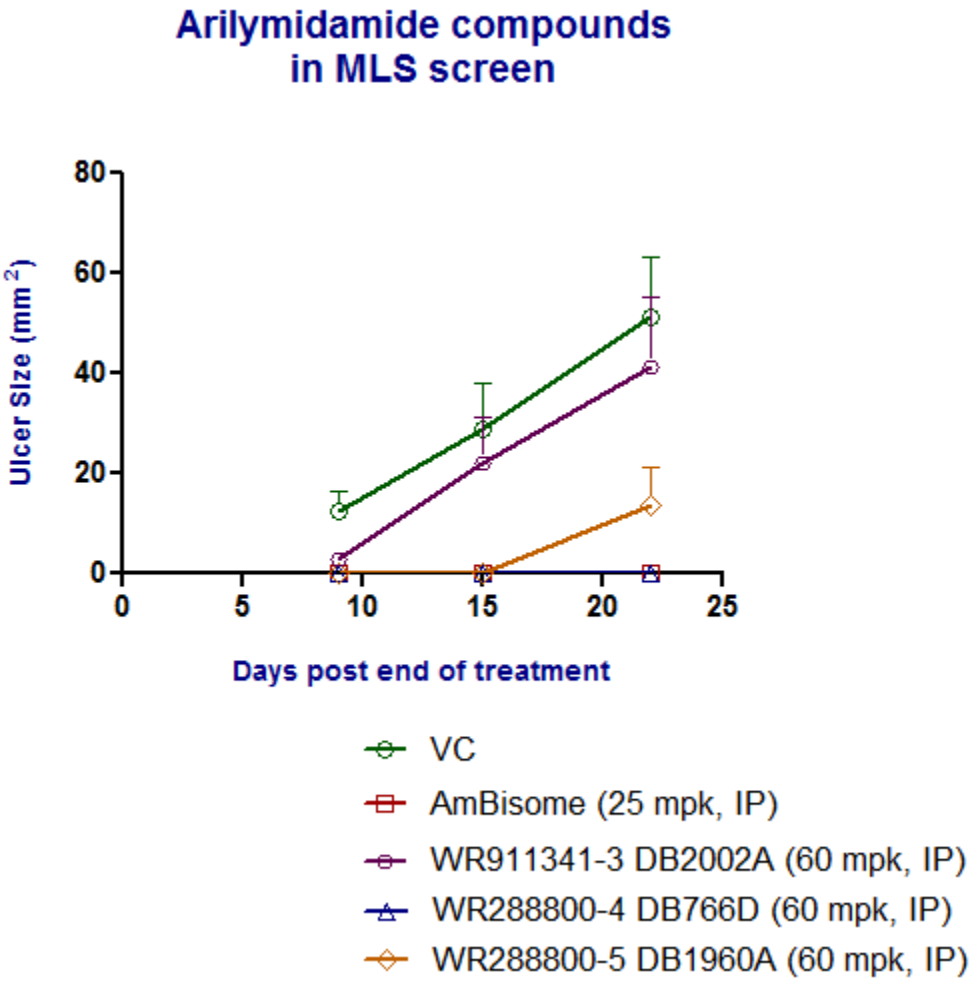

Figure 4: Progression of ulcer size in BALB/c mice infected with $1X10^7$ stationary phase *L. major* promastigotes and treated IP with 60 mg/kg WR911341-3 (DB2002A), WR288800-4 (DB766D), and WR288800-5(DB1960A). Bars represent means ± SEMs for a total of 5 BALB/c mice.

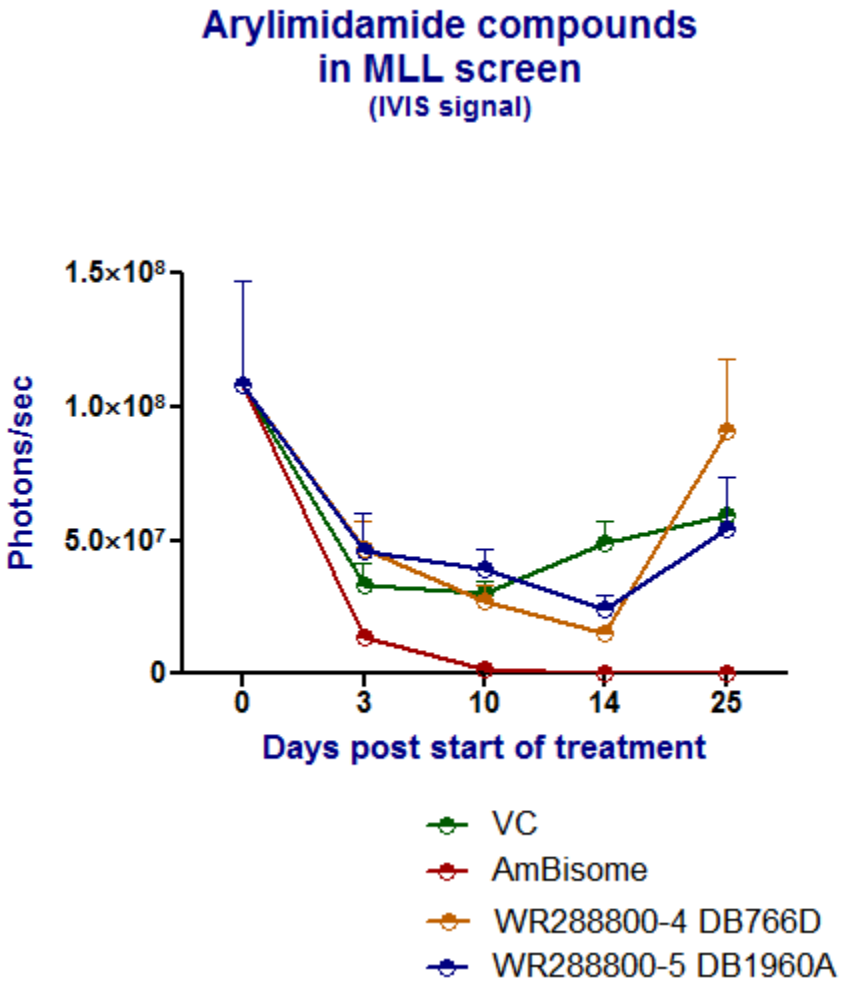

Figure 5: Progression of luminescence signal in BALB/c mice infected with 1X10^7 stationary phase *L. major* promastigotes and treated PO with 60 mg/kg WR288800-4 (DB766D), and WR288800-5(DB1960A). Bars represent means ± SEMs for a total of 5 BALB/c mice. In vivo imaging results for leishmania lesion cure (MLL assay) using luciferase expressing Leishmania major parasites. The endpoint for this in vivo test is the size of the lesions in the vehicle and treated groups. Luminescence signal is used as a means for evaluating the trend of the parasite load at the infection site. The positive control (AmBisome) was administered IP at 25 mg/kg

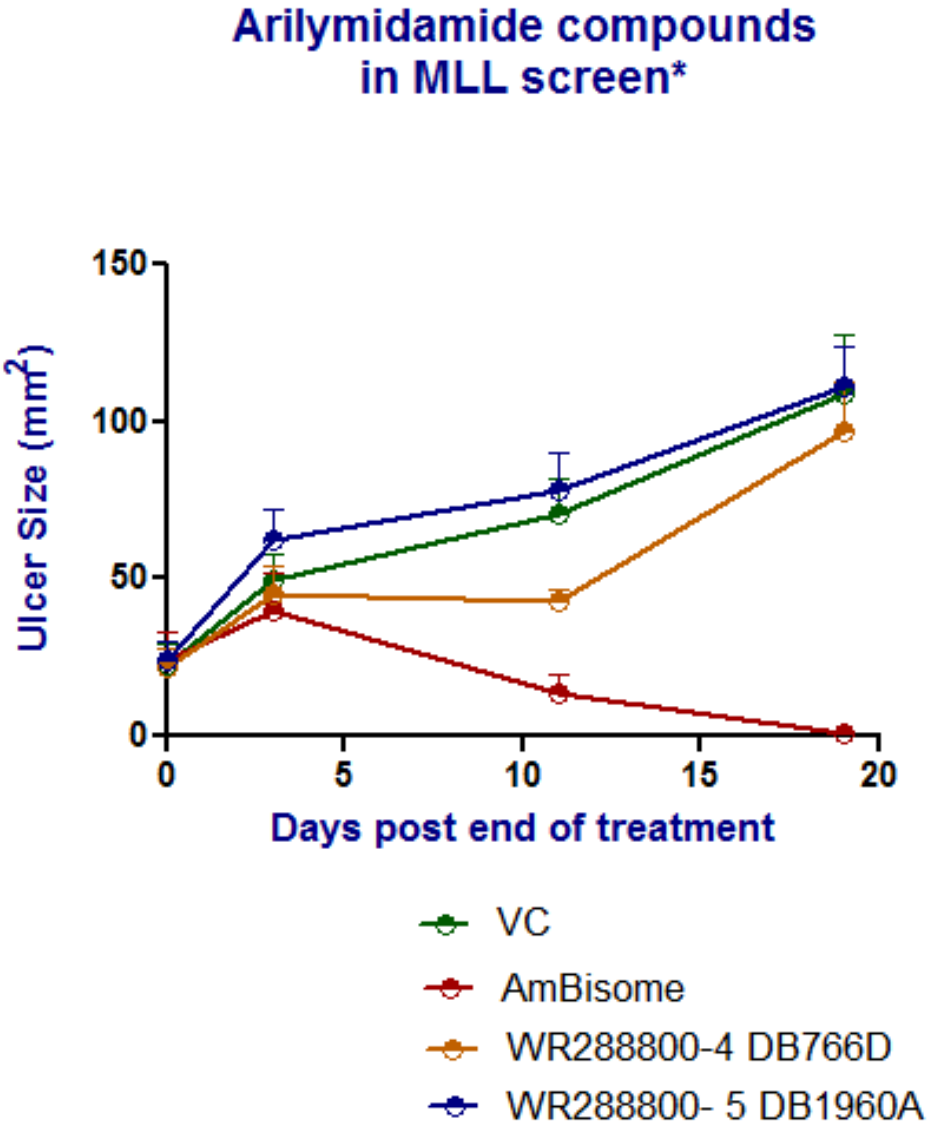

Figure 6: Progression of ulcer sizes in BALB/c mice infected with $1X10^7$ stationary phase *L. major* promastigotes and treated PO with 60 mg/kg WR288800-4 (DB766D), and WR288800-5(DB1960A). Bars represent means ± SEMs for a total of 5 BALB/c mice.