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TITLE: Development of Novel Therapeutics for Neglected Tropical Disease  
Leishmaniasis

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**14. ABSTRACT**

Infections caused by protozoan parasites of the genus *Leishmania* include cutaneous (CL), mucosal (ML) and visceral leishmaniasis (VL). Over 12 million people are currently suffering from leishmaniasis, and approximately 2 million new cases occur annually, making it a major global health problem and WHO designated neglected tropical disease (NTD). Recently, CL has been seen in all branches of the US military and among DOD contractors returning from *Leishmania*-endemic countries such as Iraq and Afghanistan. Current widely used treatment for all forms of leishmaniasis including CL involves multiple injections of antimonial drugs (Glucantime<sup>TM</sup> or Pentostam<sup>TM</sup>) for 20 days or more. Therefore, this treatment has poor compliance, numerous adverse effects including death and is also not approved by the FDA therefore requiring use under IND in the US. Furthermore, in immunocompromised individuals antimonial treatment is associated with relapses. Other antileishmanial treatments currently under development do not offer new alternatives because they are either reformulations or combinations of existing drugs. Hence, there is pressing need for novel drugs for leishmaniasis. Our team is interested in discovering novel drugs to treat leishmaniasis from natural products. Work from our recently completed NIH-funded project has led to the discovery of antileishmanial molecules from the plant *Pentalinon andrieuxii*, which has been used by Mayan traditional healers for CL for many years. We have identified six sterols, including a novel sterol, pentalinosterol (PEN), with broad-spectrum activity against *Leishmania* species that cause CL and visceral leishmaniasis (VL). The synthesis of PEN has been established and methods for large scale synthesis of other active molecules are under development (PCT Int. App. WO 2012145734A1). Our preliminary studies show that synthetic PEN (sPEN) is safe and more potent than antimonials (SSG) in the treatment of CL and VL in animal models. We have also found that PEN exhibits immunomodulatory activity and promotes cellular immune responses required for leishmaniasis resolution. These findings indicate that PEN and other bioactive sterols as well as their derivatives could be novel broad-spectrum anti-leishmanial drugs. The goals of this 3 year project are to address the critical developmental need of lead optimization of analogues of two most promising compounds, PEN and DNER in the context of drug potency and specificity. Solubility and stability, key parameters in the development of a useful drug for this disease, will also be considered during the course of lead optimization and determine the mechanism(s) of antiparasitic action of bioactive analogues using a combination biochemical and in silico approaches. Aim 1 will comprise the synthesis of PEN and DNER analogues and screening their antileishmanial activity and toxicity using novel screening assays. Aim 2 will evaluate efficacies of active analogues in prevention and treatment of CL using an animal model. Aim 3 will determine the mechanisms of antiparasitic and/or immunomodulatory activities of active compounds using a combination biochemical and in silico approaches. These data will lay the foundation for advancing PEN and DNER analogues as novel drugs for leishmaniasis in humans.

**15. SUBJECT TERMS**

Cutaneous leishmaniasis, antileishmanial drugs, Pentalinosterol and 6,7-dihydronebridienone analogues

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## 1. INTRODUCTION:

The leishmaniasis comprise a number of diseases caused by obligate intracellular parasites of the genus *Leishmania*. More than 350 million people worldwide are at risk of contracting leishmaniasis. Cutaneous leishmaniasis (CL) is the most common form of infection, which manifests as localized skin lesions that may heal or become chronic, leading to significant tissue destruction and disfigurement. Other forms of infections are diffuse cutaneous leishmaniasis (DCL), mucosal leishmaniasis (ML), or potentially life-threatening visceral leishmaniasis (VL), which is characterized by dissemination of the parasites to the liver, spleen and bone marrow. Several drugs including pentavalent antimonials (Sb), Amphotericin B, miltefosine and paromomycin are used to treat leishmaniasis. However, all these drugs suffer from significant drawbacks, including the need for parenteral routes of administration, poor patient compliance due to long treatment lengths and toxicity, and/or high cost, which limits their use in disease endemic regions. In addition, the emergence of antimonial-resistant strains of VL is rapidly increasing worldwide. Therefore, there is a strong need for new anti-leishmanial drugs that are safe, affordable, and have broad-spectrum activity against different species of *Leishmania*, including Sb-resistant parasites.

Our team is interested in discovering novel drugs to treat leishmaniasis from natural products. Work on an ongoing NIH-funded project (AI092624; A. Satoskar, PI, A.D. Kinghorn, Co-PI) has led to the discovery of antileishmanial molecules from the plant *Pentalinon andrieuxii*, which has been used by Mayan traditional healers to successfully treat CL for many years. We have identified six sterols, including 6,7-Dihydroneeridienone (DNER) as well as a novel sterol, pentalinosterol (PEN) and its closely related structural analogue cholest-4-en-3-one (C3ONE), with broad-spectrum activity against *Leishmania* species that cause CL and VL. The synthesis of PEN has been established and methods for large scale synthesis of other active molecules are under development (PCT Int. App. WO 2012145734A1). Our preliminary studies show that synthetic PEN (sPEN) is safe and more potent than antimonials for the treatment of CL and VL in animal models. We have also found that PEN exhibits immunomodulatory activity and promotes cellular immune responses required for leishmaniasis resolution. These findings indicate that PEN and other bioactive sterols as well as their derivatives could be novel broad-spectrum anti-leishmanial drugs. The goals of this 3 year project are to: 1) synthesize and evaluate analogues of two most promising compounds, PEN and DNER; 2) use *in vitro* high throughput screening assays and an animal model of visceral leishmaniasis to explore structure-activity relationships and optimize the physicochemical properties of this natural product for advancing these classes as a potential therapeutic agents for the treatment of leishmaniasis; and 3) determine the mechanism(s) of antiparasitic action of bioactive analogues using a combination biochemical and *in silico* approaches. These studies will address the critical developmental need of lead optimization in the context of drug potency and specificity. Solubility and stability, key parameters in the development of a useful drug for this disease, will also be considered during the course of lead optimization.

## 2. KEYWORDS:

Cutaneous leishmaniasis, antileishmanial drugs, Pentalinosterol and 6,7-dihydroneeridienone analogues

## 3. ACCOMPLISHMENTS:

The major goal of this study (NAMRU-6 component) was:

- i) To optimize promastigotes and amastigote *in vitro* assays to measure the susceptibility of *Leishmania* clinical isolates to standard anti-leishmanial drugs
- ii) To assess 5 PEN/DNER compounds for anti-leishmania activity using the promastigote assay previously optimized and clinical isolates of *L. (V.) peruviana* and *L. (V.) braziliensis*
- iii) To assess 5 PEN/DNER compounds for anti-leishmania activity using the amastigote assay previously optimized and clinical isolates of *L. peruviana* and *L. braziliensis*

- iv) To amend ongoing IACUC protocol to infect mouse with *L(V.) peruviana* and *L braziliensis* and evaluate 5 PEN/DNER for *in vivo* anti-leishmanial activity

Some administrative and logistic aspects have been or are in the process of being fulfilled:

- A contract was successfully established between Asociacion Benefica PRISMA and USAMRAA to support the activities for this project.
- We identified and hired a full-time research technician contractor who will conduct laboratory activities for this project
- The research technician received training at NAMRU-6 on *in vitro* culture of *Leishmania* promastigotes and flow cytometry
- Procurement of laboratory supplies and reagents have started in order to complete assay optimization

Over the next reporting period we plan to complete with assay optimization in order to generate validation data to demonstrate that our assay is able to measure *in vitro* susceptibility of *Leishmania spp.* to anti-leishmanial drug and compounds

#### 4. IMPACT:

Nothing to report

#### 5. CHANGES AND PROBLEMS:

This project is delayed due to contractual and invoicing issues. The award notification was issued in early April 2014. It took 6 months for the contract between USAMRAA and Asociacion Benefica PRISMA to be signed. Additionally, there were delays related to receiving the funds and working out the invoicing system. PRISMA received funds for the first year of the study in late October 2015. Since then we have made some progress on the administrative aspects of the study. We have hired a contractor laboratory technician who will perform all laboratory and logistic activities for NAMRU-6 as outlined in the scope of work (appendix). The contractor received training on general laboratory procedures, *Leishmania* culturing techniques, and flow cytometry assay, all of which will be useful for assay optimization. We have begun to procure supplies in order to initiate assay optimization for the *in vitro* part of the study.

#### 6. PRODUCTS:

Nothing to report

#### 7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

The following individuals have worked on this project:

Name:	G. Christian Baldeviano, Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	not available
Nearest person month worked:	1
Contribution to Project:	Dr. Baldeviano has so far overseen all administrative

Funding Support:	aspect of the project, including award negotiation, budget allocation and contracting issues. Dr. Baldeviano is U.S. government employee.
Name:	Carmen Lucas, ScM
Project Role:	Research Manager
Researcher Identifier (e.g. ORCID ID):	not available
Nearest person month worked:	1
Contribution to Project:	Mrs. Lucas has so far provided logistical support for laboratory research including placing orders, tracking packages, coordinating custom liberation of imported items. Additionally, she manages the laboratory of Parasitology coordinating all research activities
Funding Support:	Mrs. Lucas is U.S. government employee.
Name:	Lucy Espinoza, BS.
Project Role:	Research technician
Researcher Identifier (e.g. ORCID ID):	not available
Nearest person month worked:	12
Contribution to Project:	Ms. Espinoza has so far received training on all laboratory procedures needed to complete this project. Additionally, she is in charge of keeping updated the inventory of all materials and supplies that is being purchased for this project
Funding Support:	Ms. Espinoza is a PRISMA full time employee who was hired to complete this project

The following organizations are partners for this project:

Asociacion Benefica PRISMA  
Calle Carlos Gonzáles N°251 Urbanización Maranga, Lima 32 - Perú

Ohio State University  
281 W. Lane Ave.  
Columbus, Ohio 43210

## 8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

## 9. APPENDICES: Scope of work

## SOW

The following SOW describes the breakdown of the proposed work as well as the key personnel involved and the location of the study sites. This study involves two study sites:

**Site 1: The Ohio State University**, 320 W 10th Ave, Columbus, OH 43210. Ohio State is the primary organization conducting this study. Dr Satoskar, the initiating PI for this application, and his research team will oversee all activities related to the synthesis of PEN and DNER analogues as well as initial screening using standard laboratory strains. Dr. Satoskar's team will be in charge of selecting the most promising analogues and ship them to NAMRU-6 for *in vitro* testing with clinical *Leishmania* isolates. In addition, Dr. Satoskar team will conduct *in vivo* studies to test the efficacy of a selected subset of compounds using the mouse model of cutaneous leishmaniasis. Finally, Dr. Satoskar's team will conduct a series of mechanistic experiments to elucidate the mode of actions of the leading drug candidates identified in the previous experiments.

**Site 2: Naval Medical Research Unit No. Six (NAMRU-6)**, Venezuela Avenue block 36, Callao 2, Peru.

NAMRU-6 is the collaborative institution for this study. Dr. Baldeviano, the partnering PI, and his team will oversee all aspects of *in vitro* testing of selected PEN and DNER analogues using a variety of *L. (V.) peruviana* and *L. (V.) braziliensis* clinical isolates collected from endemic areas. In addition, NAMRU-6 will conduct

**Aim 1: Synthesize and screen PEN and DNER analogues for their antiparasitic activity and toxicity against different *Leishmania* species that cause CL, including patient isolates from s endemic regions.**

	Timeline (months)	Site 1	Site 2
Kickoff Coordination Meeting of participating institutions	1	Dr. Satoskar	Dr. Baldeviano
<b>Major Task 1.1: Synthesis of PEN and DNER analogues</b>			
<b>Subtask 1.1.1:</b> Synthesis of PEN analogues will be carried out through modification of two key functional groups (10-15 analogs to be tested) Participating teams: <ul style="list-style-type: none"> <li>• Team A (Drs. Fuchs and Kinghorn Labs will oversee component design)</li> <li>• Team B (Site 1 core facility; fee for service):</li> </ul>	1-5	Dr. Satoskar	
<b>Subtask 1.1.2:</b> Synthesis of DNER analogues will be carried out through modification of C17 side chain (8 analogs) Participating teams: <ul style="list-style-type: none"> <li>• Team A (Drs. Fuchs and Kinghorn Labs will oversee component design)</li> <li>• Team B (Site 1 core facility; fee for service):</li> </ul>	1-4	Dr. Satoskar	
<b>Subtask 1.1.3:</b> Generation of hybrid sterols. First step Fuchs/Kinghorn Labs Second step Fuchs/Kinghorn Labs	5-6	Dr. Satoskar	
<i>Milestone #1: Library of 20 PEN and DNER analogue compounds to be tested in vitro.</i>	5	Dr. Satoskar	
<b>Major Task 1.2:</b> Evaluation of microbicidal activity using standard laboratory strains <b>(Total mice needed =150)</b>			



<b>Subtask 1.2.1:</b> Determination of IC50 of compounds using <i>Leishmania</i> promastigote cultures	7-9	Dr. Satoskar	
<b>Subtask 1.2.2:</b> Determination of IC50 of compounds using <i>Leishmania</i> amastigote cultures	7-9	Dr. Satoskar	
<b>Subtask 1.2.3:</b> Determination of toxicity of compounds using eukaryotic cell lines	8	Dr. Satoskar	
<i>Milestone #2: Library of 5-10 PEN and DNER analogue</i>	5	Dr. Satoskar	
<i>compounds with data on in vitro activity against standard laboratory strains of Leishmania</i>			
<b>Major Task 1.3:</b> Evaluation of microbicidal activity using clinical isolates			
<b>Subtask 1.3.1:</b> Determination of IC50 of compounds using <i>L. (V.) peruviana</i> and <i>L. (V.) brasiliensis</i> promastigote cultures from clinical isolates <ul style="list-style-type: none"> <li>• Selection of 20 geographically diverse isolates of <i>L. (V.) peruviana</i> and <i>L. (V.) brasiliensis</i> with high and low IC50 to antimonial drugs (sodium stibogluconate)</li> <li>• Determine IC50 on 20 clinical isolates for each compound (n=10)</li> </ul>	8-12		Dr. Baldeviano
<b>Subtask 1.3.2:</b> Determination of IC50 of compounds using <i>L. (V.) peruviana</i> and <i>L. (V.) brasiliensis</i> amastigote cultures from clinical isolates <ul style="list-style-type: none"> <li>• Selection of 20 geographically diverse isolates of <i>L. (V.) peruviana</i> and <i>L. (V.) brasiliensis</i> with high and low IC50 to antimonial drugs (sodium stibogluconate)</li> <li>• Determine IC50 on 20 clinical isolates for each compound (n=10)</li> </ul>	8-12		Dr. Baldeviano
<i>Milestone #3: Library of 5-6 PEN and DNER analogue compounds with accepted cidal activity in promastigote and amastigote model on 20 different clinical isolates</i>	12	Dr. Satoskar	Dr. Baldeviano

**Aim 2: Evaluate the efficacy of active PEN and DNER analogues for the treatment of leishmaniasis using an animal model of CL.**

	<b>Timeline (months)</b>	<b>Site 1</b>	<b>Site 2</b>
<b>Major Task 2.1:</b> Development of topical formulations of PEN (PEN-A) and DNER (DNER-A) analogues			
<b>Subtask 2.1.1:</b> <ul style="list-style-type: none"> <li>• First step Topical formulation of PEN-As (Bachelder Lab)</li> <li>• Second step Topical formulation of PEN-As (Bachelder Lab)</li> </ul>	12-15	Dr. Satoskar	
<b>Major Task 2.2:</b> Evaluate the efficacies of PEN and DNER analogues in preventing development of CL using <i>L. major</i> and <i>L. mexicana</i> models <b>(Total mice needed= 480 mice)</b>			
<b>Subtask 2.2.1:</b> <ul style="list-style-type: none"> <li>First step: Efficacy of PEN-A in prevention of CL</li> <li>Second step: Efficacy of DNER-A in prevention of CL</li> </ul>	16-24	Dr. Satoskar	

<b>Major Task 2.3:</b> Evaluation of efficacies of PEN and DNER analogues in the treatment of CL using <i>L. major</i> and <i>L. Mexicana</i> models <b>(Total mice needed=480 mice)</b>			
<b>Subtask 2.3.1:</b> First step: Efficacy of PEN-A in treatment of CL Second step: Efficacy of DNER-A in treatment of CL	17-24	Dr. Satoskar	
<b>Major Task 2.4:</b> Evaluation of efficacies of PEN and DNER analogues in the treatment of CL using <i>L(V.) peruviana</i> and <i>L. braziliensis</i> mouse model in Peru			
<b>Subtask 2.4.1:</b> Amend ongoing IACUC protocol to infect mouse with <i>L(V.) peruviana</i> and <i>L. braziliensis</i> <b>Subtask 2.4.2:</b> Efficacy screening of 5-6 PEN and DNER analogue compounds <b>(Total mice needed= 130 mice)</b>	12-14		Dr. Baldeviano
<i>Milestone #4: Library of 1-4 PEN and DNER analogue compounds with in vivo efficacy data on old and new world Leishmania species causing CL</i>	24	Dr. Satoskar	Dr. Baldeviano

