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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 (GlucantimeTM or PentostamTM) 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 antileishmanial 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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Table of Contents

Page

Contents

1. INTRODUCTION:.....	5
2. KEYWORDS:	5
3. ACCOMPLISHMENTS:	5
4. IMPACT:	9
5. CHANGES AND PROBLEMS:.....	9
6. PRODUCTS.....	9
7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS	10
8. SPECIAL REPORTING REQUIREMENTS	11
9. APPENDICES: Scope of work	12

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:

What were the major goals of the project?

- 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 protocols to infect mouse with *L. (V.) peruviana* and *L. braziliensis* and evaluate 5 PEN/DNER for *in vivo* anti-leishmanial activity

What was accomplished under these goals?

This study has a one year delay in initiation due to contracting and invoicing issues. We initiated study activities on November 2015. We are reporting activities conducted from November 2015 to September 2016. The major accomplishments for this period are:

- A contract was successfully established between Asociacion Benefica PRISMA in Peru and USAMRAA to support the activities under this project.
- We identified and hired a full-time research assistant who will carry out laboratory activities for this project. Security and occupational health clearance have been completed
- Training of study personnel on *in vitro* culture of *Leishmania* promastigotes and flow cytometry has been completed
- Procurement of laboratory supplies has been completed for the first aim. Lab supplies and reagents were purchased in the United States and imported to Peru through a third party company. This includes a battery of standard anti-Leishmania drugs such as pentavalent and trivalent antimonial, paromomycin, miltefosin and amphotericin B, which will be used as positive control drugs for the assay.
- Optimization of the promastigote assay has been started. We have characterized the growth kinetics for one of our own parasites isolates *Leishmania (V.) braziliensis* strain MHOM/PER/90/LTB300 which we will use as internal control. The seeding concentration for the assay has been established at 1,000 promastigotes for 96 well plate formats with an incubation time of 72 hours. Additionally, the MTT assay was optimized to detect a range of live promastigotes from 2×10^6 down to 10^3 live promastigotes per well, establishing a wide dynamic range for the assay (See below Figures 1, 2 and 3).
- Using the promastigote assay, we determined the EC50 of *Leishmania (V.) braziliensis* strain MHOM/PER/90/LTB300 to be 2.1 in one experiment and 2.7 in a second experiment. This is in line with a fully susceptible parasite. We are in the process of obtaining 5-6 drug compounds from our collaborator Dr. Abhay Satoskar in order to test them using a panel of field isolates that we are currently selecting from our parasite repository.
- As for the amastigote assay, we are in the process of establishing a collaborative research agreement with the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) in Colombia. They have established a robust method to assess susceptibility of *Leishmania* parasites to major antimonial drugs. This method does not require preconditioning and have been shown to reliably estimate susceptibility using clinical isolates (Fernández O *et al*, 2012; Fernández OL *et al*, 2014). Our plan is to have our research assistant trained at the CIDEIM facilities in February 2017 and set up this assay in our lab at NAMRU-6.

- Over the next reporting period we plan to determine EC50 of the drug compounds from our partnering PI of this project and continue with training in Colombia for the amastigote assay. If we generate sufficient data, we plan to submit an abstract to the upcoming Military Health System Research Symposium (MHSRS) in 2017.

Figure 1. Calibration curve to estimate the number of live *L (V.) braziliensis* parasites based on optical density obtained in the MTT colorimetric assay

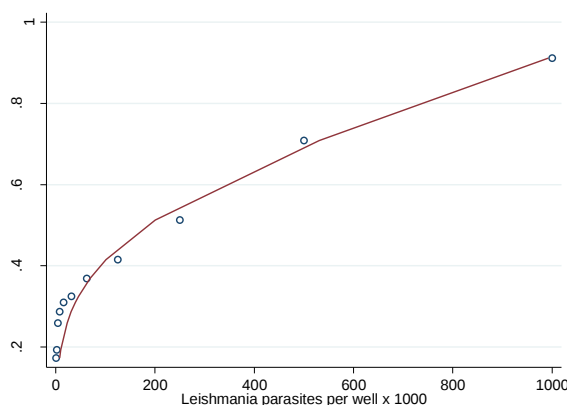


Figure 2. Optimization of incubation time and parasite seeding size for promastigote assay. 1,000 and 10,000 parasites in late log growth phase were seeded in each well of a 96 well plate and exposed to 2-fold dilutions of trivalent antimonial (0.39 to 200 $\mu\text{g}/\text{m}$). After 24, 48 or 72 hours, MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well and incubated for 2 hours. Plates were then centrifuged and supernatant discarded and pellet suspended in 200 microliters of DMSO and read at 650 nm. Plots show optical density versus SbIII dilutions in log scale 1,000 or 10,000 late log phase *L (V.) braziliensis*. The numbers on the top indicates each well in quadruplicates

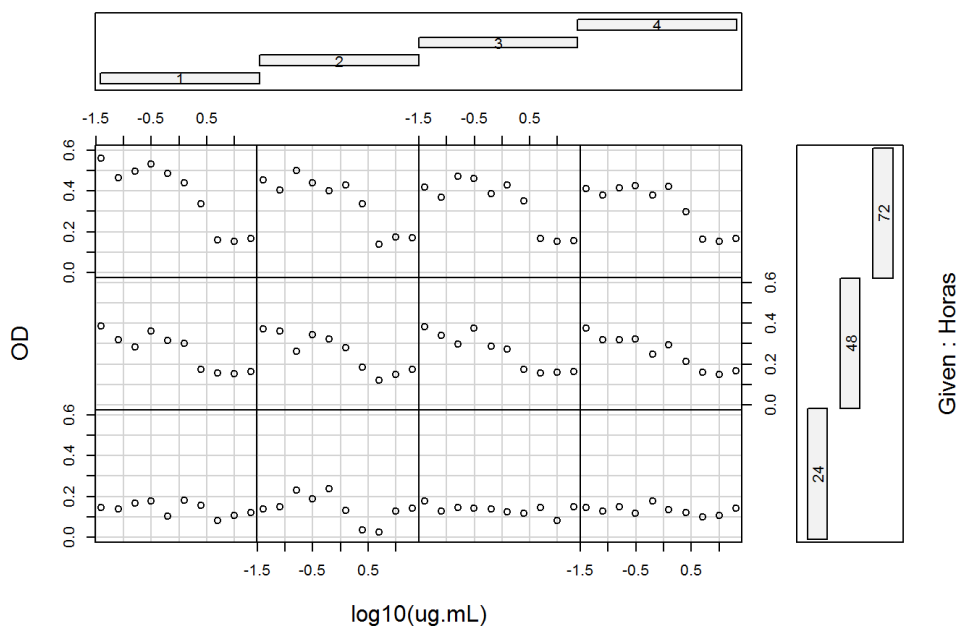
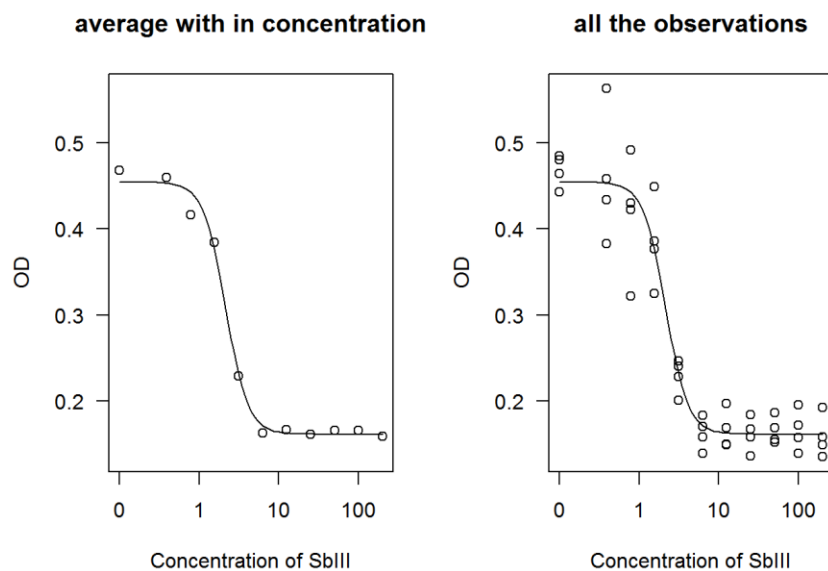


Figure 3. Calculation of the EC50 for *Leishmania (V.) braziliensis* for SbIII. Data generated in Figure 2 was fitted to a 4-parameter regression and the EC50 inferred from the estimated parameters. In two independent experiments EC50 was 2.7 and 2.1 $\mu\text{g}/\text{m}$ of SbIII equivalent



4. IMPACT:

Nothing to report

5. CHANGES AND PROBLEMS:

- This project has been delayed due to contractual and invoicing issues. The award notification was issued in early April 2014. After 6 months of negotiation, the contract between USAMRAA and Asociacion Benefica PRISMA was signed. There were additional delays related to receiving the funds and working out the invoicing system. PRISMA received funds for the first year of the study in November 2015. Since then we have made some progress on the administrative and scientific aspects of this study which are described in section 3.
- For procurement of laboratory supplies that are specific to this project we have experienced difficulties and delays due to importation issues. These supplies need to be purchased in the United States and the Peruvian government requires importation permits for each of these items which can take up to 90 days. We have now a mechanism to import reagents for this project with a time-around time of approximately 40-60 days
- Another challenge has been the development of the amastigote assay. Most amastigote assays developed to date are conducted with standard parasites strains which have predictable growth rate and high macrophage infection rates. Very few investigators have used clinical isolates collected from the field to develop drug susceptibility assays. The reason is that clinical isolates have different phenotypic features (eg. different growth rates, resetting formation, variable infection rates in host cells, etc) that makes standardization difficult. Some researchers have used complex preconditioning of promastigotes to increased infection rates of field isolates to macrophages (da Luz *et al*, 2009). A research team from CIDEIM in Colombia has established a robust assay to estimate the susceptibility of *Leishmania spp* parasites to various drugs used in humans for the treatment of cutaneous leishmaniasis (Fernández O *et al*, 2012; Fernández OL *et al*, 2014).. We are currently in conversation to establish a research collaboration agreement to be able to receive training. The idea is that we will adapt their assay at NAMRU-6 with their support and also that they serve as external quality control center for our own assays developed as part of this project.

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 aspect of the project, including award negotiation, budget allocation and contracting issues.
Funding Support:	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
Name:	Maxy de los Santos, Ph.D
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	not available
Nearest person month worked:	0.5
Contribution to Project:	Dr De los Santos received his doctoral degree from Universidad de Madrid in Spain. He has extensive research experience in molecular biology and parasitological techniques and has over 15 years of experience working with Leishmania. He is our consultant for all experiments involving parasite manipulation during this project
Funding Support:	Dr. Baldeviano is U.S. government employee. No salary is provided for him under 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

We have are in discussions to establish a research collaboration with

Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM)

Av. La Maria #19-225

Cali, Valle del Cauca, Colombia

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES: 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		
Kickoff Coordination Meeting of participating institutions	1	Dr. Satoskar	Dr. Baldeviano
Major Task 1.1: Synthesis of PEN and DNER analogues			
Subtask 1.1.1: Synthesis of PEN analogues will be carried out through modification of two key functional groups (10-15 analogs to be tested) Participating teams:			
Subtask 1.1.2: Synthesis of DNER analogues will be carried out through modification of C17 side chain (8 analogs) Participating teams: - Team A (Drs. Fuchs and Kinghorn Labs will oversee			

Subtask 1.1.3: 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	
Major Task 1.2: Evaluation of microbicidal activity using standard laboratory strains			
Subtask 1.2.1: Determination of IC50 of compounds using			
Subtask 1.2.2: Determination of IC50 of compounds using			
Subtask 1.2.3: Determination of toxicity of compounds using eukaryotic cell lines	8	Dr. Satoskar	
<i>Milestone #2: Library of 5-10 PEN and DNER analogue compounds with data on in vitro activity against standard laboratory strains of Leishmania</i>	5	Dr. Satoskar	
Major Task 1.3: Evaluation of microbicidal activity using clinical isolates			
Subtask 1.3.1: Determination of IC50 of compounds using <i>L (V.) peruviana</i> and <i>L (V.) brasiliensis</i> promastigote cultures from clinical isolates 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)			
Subtask 1.3.2: Determination of IC50 of compounds using <i>L (V.) peruviana</i> and <i>L (V.) brasiliensis</i> amastigote cultures from clinical isolates 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)			
<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.

	Timeline		
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Major Task 2.1: Development of topical formulations of PEN (PEN-A) and DNER (DNER-A) analogues			
Subtask 2.1.1: First step Topical formulation of PEN-As (Bachelder Lab)			
Major Task 2.2: Evaluate the efficacies of PEN and DNER analogues in preventing development of CL using <i>L. major</i> and <i>L. mexicana</i> models			
Subtask 2.2.1: First step: Efficacy of PEN-A in prevention of CL			
Major Task 2.3: Evaluation of efficacies of PEN and DNER analogues in the treatment of CL using <i>L. major</i> and <i>L. Mexicana</i> models			
Subtask 2.3.1: First step: Efficacy of PEN-A in treatment of CL Second step: Efficacy of DNER-A in treatment of CL			
Major Task 2.4: 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			
Subtask 2.4.1: Amend ongoing IACUC protocol to infect mouse with <i>L(V.) peruviana</i> and <i>L. braziliensis</i> Subtask 2.4.2: Efficacy screening of 5-6 PEN and DNER analogue compounds	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

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