AWARD NUMBER: W81XWH-18-1-0765

TITLE: Aerosol Delivery of CPZEN-45 for Treatment of Nontuberculous Mycobacterial (NTMs) Infections

PRINCIPAL INVESTIGATOR: Dr. Gail Cassell

CONTRACTING ORGANIZATION: PAI Lifesciences, Inc., Seattle, WA

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13. SUPPLEMENTARY NOTES

July 6,2021, PAI Life Sciences was notified that this grant had been transferred from the original organization, Infectious Disease Research Institute to PAI

14. ABSTRACT

This DOD Therapeutic Development Award is focused on a new antibiotic, CPZEN-45, discovered by our team for treatment of non-tuberculous mycobacterial infections (NTM) in patients with chronic obstructive pulmonary disease (COPD). We have two major objectives: **Objective 1**-To optimize fermentation and scale-up of manufacturing processes for high yield of CPZEN-45, including spray dried CPZEN-45. **Objective 2**- To further define and characterize *in vitro* efficacy of CPZEN-45 against additional species of NTMs recently isolated from VA patients with COPD. Specifically, our objective is to develop an efficacious regimen for COPD patients by screening multiple CPZEN-45 combinations with standard NTM compounds in human THP-1 cells using a checkerboard assay. **Synergy measurement by checkerboard analysis** will be used to determine the impact on potency of the combination of antibiotics in comparison to their individual activities. The optimized synergistic regimens will then be tested in COPD mouse and guinea pig efficacy models.

15. SUBJECT TERMS

Chronic Obstructive Pulmonary Disease, Veterans, CPZEN-45, Non-tuberculosis mycobacteria (NTM), NTM New Antibiotic Therapy, animal infection models, *M. avium*, *M. abscessus*.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC Chris Baker/grant officer
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

Non-tuberculous mycobacteria (NTM) are environmental bacteria found commonly in soil, water, and biofilms. Chronic lung disease is the most frequent disorder caused by NTM; moreover, NTM lung infections not uncommonly complicate individuals with chronic obstructive pulmonary disease (COPD, aka emphysema). The incidence and prevalence of NTM lung disease (NTM-LD) in the U.S. is increasing yearly and now surpasses that of tuberculosis (TB). Veterans are three times more likely to develop COPD and NTM infection than the general population. NTM-LD is often treated for at least 18-24 months with at least three and sometimes a four or more-drug regimen. Despite this intense regimen – reflecting the high resistance of NTM to available antibiotics – the long-term cure rate is at best ~50% as the relapse rate is high. Thus, new antibiotics are urgently needed. Members of our research team have discovered a new chemical entity, CPZEN-45, which has been shown to have a novel mechanism of action. It is considered highly promising because it has been shown: (i) to directly kill many pathogenic species of NTM (both drug sensitive and drug resistant), (ii) to have efficacy in laboratory animals experimentally infected with NTM, (iii) to possess an acceptable toxicity profile, and (iv) to be able to be delivered directly to the lungs as a dry powder. Before CPZEN-45 can be studied in patients with NTM-LD, we must do further pre-clinical work by making sure we can produce sufficient quantities of high quality CPZEN-45 as well as supply large amounts of the compound to do further testing in animals to further ensure efficacy and safety.

2. KEYWORDS:

Chronic Obstructive Pulmonary Disease, Veterans, CPZEN-45, Non-tuberculosis mycobacteria (NTM), animal infection models, NTM New Therapy, *M. avium, M. abscessus.*

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Goal 1: Optimize fermentation and scale-up of manufacturing processes for CPZEN-45, including spray dried CPZEN-45.

Goal 2: Define and characterize in vitro and in vivo efficacy of CPZEN-45 against NTM recently isolated from VA patients with COPD using our well characterized COPD mouse models and to evaluate CPZEN-45 inhaled therapy using a chronic NTM model in guinea pigs.

What was accomplished under these goals?

See **APPENDIX**

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

None.

What do you plan to do during the next reporting period to accomplish the goals?

See **APPENDIX**

4. **IMPACT:**
What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report.

5. **CHANGES/PROBLEMS: Changes in approach and reasons for change**

Limited progress made since IDRI declared receivership November, 2019 and did not officially relinquish grant until December, 2020, and transfer of grant to PAI officially July 6. See **APPENDIX** for details about changes in approach and reasons for change.

Actual or anticipated problems or delays and actions or plans to resolve them

Problem: As a result of restructuring in IDRI due to receivership, Dr. Cassell, PI, departed IDRI December, 2019. However, the award was not officially relinquished until December, 2020. The steps necessary to relinquish the award in accordance with Washington State receivership court procedures took longer than anticipated. In addition, the transfer of the grant to PAI took longer than anticipated. **Actions to resolve**: With guidance provided by DOD Contract and Scientific Officers, the PI and PAI provided all necessary information for review and successful transfer of grant. See **APPENDIX** for details related to steps taken to continue to advance CPZEN development during the funding hiatus. Work originally proposed for years 02-03 has begun.

Changes that had a significant impact on expenditures

See APPENDIX

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

All animal protocols were resubmitted to DOD during grant transfer process. All were approved by IACUCs of Colorado State and Univ. North Carolina and by ACURO. There is no involvement of human subjects in this grant.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Dr. Gail Cassell
Project Role:	PD/PI
Contribution to Project:	Dr. Cassell coordinates all work with participating organizations, participates in experimental design, and reviews the results. See **APPENDIX** for roles and responsibilities of other investigators participating in the work.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

None.

What other organizations were involved as partners?

Subawards that **were** and **will be** active in project include RTI International, Colorado State University, University of North Carolina, the Denver Research Institute and the Institute for Microbial Chemistry. Subawards were terminated in December, 2019, as part of the relinquishment process. Subawards have been re-established with each except Denver Research Institute which will be paid on a fee for service basis going forward. A subaward has been established with a new partner, Stanford University. See **APPENDIX** for roles of each sub-awardee.

8. SPECIAL REPORTING REQUIREMENTS - QUAD CHART:

Aerosol Delivery of CPZEN-45 for Treatment of Non-Tuberculous Mycobacterial (NTMs) Infections

Log Number: PR171209
Award Number: W81XWH1810765

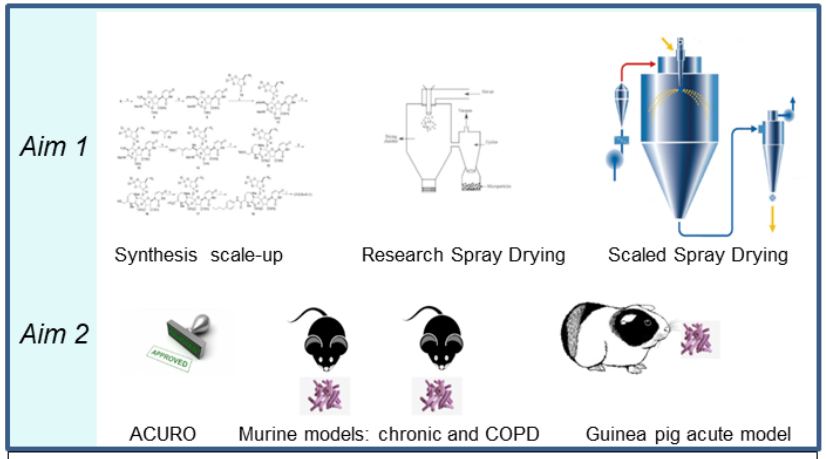
PI: Dr. Gail Cassell **Org:** PAI Life Sciences Inc. **PAI Award Amount:** $2,634,838.75

Study/Product Aim(s)

- Study Aim 1: Optimize fermentation and scale-up of manufacturing processes for CPZEN-45, including spray dried CPZEN-45.
- Study Aim 2: Define and characterize in vitro efficacy of CPZEN-45 against additional species of NTMs recently isolated from VA patients with COPD and to evaluate efficacy in our well characterized acute and chronic COPD mouse models and to evaluate CPZEN-45 Inhaled Therapy Using a Chronic NTM Model in guinea pigs

Approach

New antibiotics for Non-Tuberculosis Mycobacterial Lung Disease (NTM-LD) are urgently needed. We have discovered a new chemical entity, CPZEN-45, which has is highly promising since it: (i) directly kills many pathogenic species of NTM (both drug sensitive and drug resistant), (ii) has *in vivo* efficacy, and (iii) possess an acceptable toxicity profile, and (iv) can be delivered directly to the lungs as a dry-powder. Before CPZEN-45 can be studied in patients with NTM-LD, we must do further pre-clinical work - making sure we can produce sufficient quantities of high quality CPZEN-45 as well as supply large amounts of the compound to do further testing in animals to further ensure efficacy and safety.



Accomplishments: Aim 1: CPZEN-45 drug synthesis scaled to 500 g;
Aim 2: ACURO approvals for both models received

Timeline and Cost

Activities CY	18-20	21	22
Aim1: Scale up / Task 1: Improve drug substance yield			
Aim1: Scale up / Task 2: Transfer spray drying			
Aim2: Define and characterize efficacy of CPZEN-45 / Task 1: ACURO review			
Aim2: Define and characterize efficacy of CPZEN-45 / Task 2: in vivo Models			
Estimated Budget ($K)	**$638k**	**$600k**	**$2034k**

Updated: November 4th, 2021

Goals/Milestones

CY18 Goals – Synthesis Scale-up and Animal Modeling
☑ CPZEN-45 scaled to 500 g
☑ ACURO approval received
CY21 Goal – Spray Drying Scale-up and Murine Testing
☐ Research spray dried lots for guinea pig and mouse models
☑ Begin transfer to spray-dry manufacturer
☑ Begin murine testing
CY22 Goal – Manufacturing and Animal Modeling
☑ Complete scale-up manufacturing
☑ Complete murine testing
☑ Complete guinea pig testing

Comments/Challenges/Issues/Concerns
- N/A

Budget Expenditure to Date
Projected Expenditure: $601,988.50
Actual Expenditure: $638,186.25

AWARD CHART Continued:

PR171209: Aerosol Delivery of CPZEN-45 for Treatment of Nontuberculous Mycobacterial (NTMs) Infections

PI: DR. GAIL CASSELL, INFECTIOUS DISEASE RESEARCH INSTITUTE

Budget: $3,273,025.00 **Topic Area:** Antimicrobial Resistance **Mechanism:** W81XWH-17-PRMRP-TTDA

Research Area: Chemotherapy/Pharmacotherapy (803), Drug Resistance / Multidrug Resistance (804) **Award Status:** 9/30/18 – 9/29/21

Study Goals:

To improve the clinical outcome for patients with nontuberculous mycobacterial (NTM) lung disease by further development of a new antibiotic, CPZEN-45.

Specific Aims:

1) Optimization of fermentation and scaling up manufacturing to support IND enabling activities;
2) Evaluation of efficacy in a more relevant animal model of chronic obstructive pulmonary disease with a chronic NTM lung infection,
3) To identify the most optimal combination of drugs for eradicating NTM in this model, and to prove efficacy by administration of CPZEN- 45 by aerosol.

Key Accomplishments:

Publications: None
Patents: None
Funding Obtained: None

APPENDIX

The Infectious Disease Research Institute (IDRI), initial recipient of the Award, entered receivership at the end of 2019. The initial process of transferring both the Principal Investigator, Dr. Gail Cassell, and this project to another institution, PAI Life Sciences (PAI), began in November 2019. The intent by IDRI to relinquish the award was communicated in a letter to Kevin R. Moore, grants Officer, U.S. Army Medical Research Acquisition Activity 20 December, 2020. The reason for the transfer was that IDRI had to significantly downsize their operations due to financial constraints. The nature of the receivership delayed the process of transfer which took longer than was anticipated and was not officially completed until July 6, 2021. The overall progress to date on the grant is briefly summarized below.

SUMMARY OF ACHIEVEMENTS RELATIVE TO ORIGINAL SOW October 1, 2018 to September 30, 2019 [For Detailed Progress See Annual Report Year 01]:

Specific Aim 1: Optimize fermentation and scale-up of manufacturing processes. PROJECT LEADERS and SITES [PROJECT LEADERS – G. Cassell and D. Carter, Site 1-
PAI Life Sciences Inc.; T. Zhu, Site 2- Hisun Pharmaceuticals; M. Shibasaki, Y. Ishizaki, and K. Yamazaki, Site 2A, Institute for Microbial Chemistry (IMC); D. Stevens and A. Hickey, Site 4 - Research Triangle Institute (RTI)]

Major Task 1 – Improve drug substance yield to reduce cost. Our major accomplishment in Year 01 has been to improve the yield of CPZEN-45 and to successfully transfer manufacturing processes for CPZEN-45 from IMC to Hisun Pharmaceuticals, our manufacturing partner at that time. IMC performed the production of 4 batches (500g total) of CPZEN-45 from Caprazene, starting material. Caprazene was supplied from the mixture of caprazamycins A-G (CPZs) obtained by fermentation by Hisun. The general and synthesis of caprazene from CPZs yielded 500 g of highly pure CPZEN-45 with demonstrated activity against well characterized, standard strains of NTM. Based upon the HPLC and MIC analyses, we can state unequivocally that the transfer of technology for manufacturing of CPZEN-45 were successfully transferred from IMC to HISUN. In order to continue to improve the productivity of caprazamycins which are parent compounds of CPZEN-45, IMC applied the genetical technique called ZouA method to a caprazamycins producing strain Streptomyces sp. MK730-62F2. This method enables one to drastically increase the copy number of a biosynthetic gene cluster of certain antibiotics. By using this technique, IMC succeeded to enhance the copy number of caprazamycin biosynthetic gene cluster to 30 copies/genome and the resulting strain produced 5 times higher concentrations of caprazamycins than the parent strain. **Milestones Achieved- Drug Product Manufacturing improvements 60% completed and technology successfully transferred to Hisun.**

Major Task 2- Transfer spray-drying method to Contract Manufacturing Organization, produce drug product for animal studies and develop drug product processes for GMP scale-up: No progress made in Year 01. Studies were not to begin until Year 2.

Specific Aim 2 - Define and characterize *in vitro* and *in vivo* efficacy of CPZEN-45 against clinical NTM isolates PROJECT LEADERS and SITES [G. Cassell, Site 1-PAI Life Sciences Inc.; D. Ordway, Site 3- Colorado State University (CSU); E. Chan, Site 3A-Denver Veterans Administration and National Jewish Hospital; D. Stevens and A. Hickey, Site 4 -RTI; M. Braunstein, University of North Carolina.

Major Task 3 - Quantify CPZEN-45 activity against a representative panel of recent clinical isolates: Collect and speciate NTM clinical isolates from VA COPD patients - **100% completed**; Characterize phenotypically and genotypically clinical isolates – all clinical isolates phenotypically characterized and whole genome sequencing **completed on 30% of clinical isolates**; Determine i*n vitro* susceptibility of clinical isolates to CPZEN-45 – **100% completed**; Establish optimal combination for treatment regimen by checkerboard titration – 100% completed once but must be replicated. **Milestone Achieved: Recent clinical isolates from COPD patients collected and characterized phenotypically and over half of isolates characterized genotypically.** Determination of optimal combination for in *vivo* treatment regimen by checkerboard titration 50% completed. Specifically, Dr. E. Chan at the Denver VA, has obtained 30 isolates from VA/UCH (Colorado) and ten isolates were obtained from VA (NYU) for the CPZEN-45 study. No patient information is associated with the isolates. MALDI-TOF MS was used to identify isolates. NTM strains were from COPD patients infected with *M. abscessus* (mixed rough/smooth), *M. massiliense* (mixed rough/smooth) and *M. bolletii*. Dr. Ordway has grown the NTM strains to high titers, bottled, frozen and quantified the bacterial colony forming units (CFU). She has completed THP-1 cell checkerboard MICs against *M. abscessus* 103 showing CPZEN-45 was synergistic with improved MICs when combined with Clofazimine, Amakacin, Vancomycin, Cefepime, and Rifampicin). Dr. M. Strong of NJH has established the phylogenetic tree of clinical isolates of NTM, using results of whole genome sequencing of the first10 isolates from VA patients. Samples were sequenced on the MiSeq (2x300bp), data processed by genomic and phylogenomic analysis. The read coverage was adequate for all isolates (35x to 116x). All 5 MAB isolates are confirmed as *M. abscessus* subspecies *abscessus* based on this analysis. The isolates are phylogenetically diverse members of *M. abscessus* subspecies *abscessus*. All 5 MAC isolates are confirmed as *M. avium* based on this analysis, and are phylogenetically diverse.

Major Task 4 - Evaluate efficacy in animal models: Evaluation of CPZEN-45 in COPD NTM murine models, and aerosol efficacy in guinea pig model were not begun in Year 01.

SUMMARY OF <u>REVISED</u> SOW – July 6, 2021 to September 30, 2022

There are **<u>no</u>** significant changes in our overall specific aims or major tasks for this award. However, due to the unanticipated long delay in the transfer of the award to PAI, we made adjustments in the roles and responsibilities of our partners in order to continue to advance progress toward clinical development of CPZEN-45 and to enhance our chances for success. This has been, in part, made possible by an NIAID NIH award: "Development of Inhaled CPZEN-45; 03/01/2019-02/28/2023; (note: reported to DOD in Year 01 Annual Report). The NIH grant is to develop CPZEN-45 for **tuberculosis**, specifically to fund cGMP tox studies and document preparation to support a pre-IND meeting with FDA. There is **<u>no</u>** duplication with the goals of the current DOD grant (detailed information to document lack of overlap was submitted to DOD during the grant transfer process). In contrast, the two are synergistic. The SOW for the remaining DOD award period is summarized below with emphasis placed upon the SOW for Sub-Awards with changes in their roles and responsibilities.

Institute Microbial Chemistry – No Changes in SOW

Although IMC has made significant progress in improving yields of CPZEN-45 and successfully transferred the technology to Hisun, in the coming months, IMC will continue to explore more efficient purification methods for Caprazamycins and better synthetic methods for CPZEN-45 with the aim of reducing the manufacturing cost of CPZEN-45. Specifically, they will continue to improve caprazamycins-producing bacteria and increase their productivity by examining conventional methods and new genetic perspectives. They will analyze the entire genome of several caprazamycins-producing bacteria and examine the regulatory mechanism of biosynthesis. IMC will conduct a comparative analysis of the titers, metabolites, and impurities between the production strain improved by Hisun and the original production strain of IMC, and if possible, genetic analysis of the strains. IMC will perform genome sequencing of MK730-62F2 strain and construct plasmids for induction of zouA-RsA and RsB with certain drug resistant genes. They will continue to elucidate the mechanism of action of CPZEN-45 on NTM. This will include the elucidation of drug resistance mechanisms. Finally, they will also continue to study methods suitable for industrial production for the extraction and purification of caprazamycins and the synthesis of CPZEN-45 and will transfer this knowledge and skills to new manufacturing partners.

Hisun – SOW Changes

Following IMC's successful transfer to Hisun of manufacturing technology of CPZEN-45, Hisun manufactured 2.4 kg of CPZEN-45 which has been used/ or will be used for the following: Optimization of spray-drying conditions; GLP rat and dog repeat dose toxicology studies; and genetic toxicology studies. In 2020-21, Hisun produced two additional batches of caprazamycins (2 kg paid for by the NIH R01, and 14 kg paid for by supplemental funds from NIAID). However, Hisun currently does not have the capacity to establish GMP processes for production of GMP CPZEN-45. In order to insure timely and consistent GMP CPZEN-45 manufacturing, we have decided to transfer the manufacturing of CPZEN-45 to a U.S. manufacturer. This will ensure that we have a source of GMP drug for clinical trials and commercialization. Therefore, ~ 4 kg of the caprazymicins previously produced by Hisun will be used by Cambrex (see detailed proposal in the RTI budget justification submitted to DOD in the grant transfer process) to do the synthetic optimization, scale up processes, analytical development, production of GMP CPZEN-45 for clinical studies and ICH stability studies. The process development improvements will be focused on removing class 3 solvents and eliminating steps that are not easily scalable (chromatography, etc.). Significant analytical activities are also proposed, including the qualification of a reference standard, method development for assay and impurities, forced degradation studies and additional assays to support the characterization requirements for GMP manufacturing.

RTI – SOW Changes

The original DOD proposal awarded to IDRI included funds to initiate scale-up of the CPZEN-45 spray drying processes as a step towards final aerosol product development. In the period during which there was a hiatus in work on the DoD proposal, brought about by the institutional changes at IDRI and waiting for the transfer of the DOD grant to PAI, we have made substantial progress in drug product development with the NIH R01. Also, during this period, we have focused on establishing sufficient drug supply to support the cGMP drug product for release testing for the IND submission (late 2022), Phase I (2023) and potentially Phase II clinical studies. Since the analytical work leading to GMP manufacturing processes is a critical element of the drug product

development, we will use some of the unexpended DOD funds for the analytical work to be performed by Cambrex as well as staff to assist preparation of the necessary documents for the NTM IND meeting. We consider this critical to finalizing the NTM IND submission to FDA and moving to clinical supply and hopefully expediting obtaining the clinical data to support use of CPZEN-45 to treat patients with chronic NTM infections. We anticipate that the timing of a pre-IND meeting to discuss the development of CPZEN-45 for NTM will be held in May, 2022 after GMP manufacturing processes have been developed and completion of the remaining rat and dog GMP toxicity studies. CPZEN-45 has already received FDA orphan drug designation for tuberculosis and the pre-IND meeting for TB was held in April, 2021. In this pre-IND meeting for TB, we received guidance on the manufacturing process and our GLP toxicology study design and proposed doses. This information will also support an IND for an NTM indication.

While waiting on transfer of the DOD award to PAI, RTI has been able to use funding from their NIH R01 grant, to develop analytical methods and scale-up processes for spray drying of CPZEN-45. These methods and processes have been transferred to **Crititech**, a contract research organization located in North Carolina. This work was originally going to be performed by PAI in collaboration with Recipharm. Evaluation of aerosol performance with the cyclohaler will now be conducted over the next 15 months by PAI, RTI, and Crititech and materials packaged in capsules. Over the next 12 months final, released drug product will be placed on a formal stability program.

In the coming 6 mos, CPZEN-45 will be spray dried by Crititech for guinea pig efficacy studies. Dr. Miriam Braunstein of the University of North Carolina (UNC) will evaluate the efficacy of this CPZEN-45 formulation in a guinea pig (GP) model. The GP is ideal to assess efficacy as it is a commonly used small animal model for preclinical drug studies related to COPD and other mycobacterial infections. The GP model is also ideally suited for testing inhaled drugs, as planned for this project.

Specifically, UNC will establish a guinea pig model of NTM infection using *Mycobacterium abscessus* for the project. Development of the model at UNC will involve four experiments that will include guinea pigs that are first treated to model COPD and then infected with *M. abscessus*. The model development will enable the selection of time points for initiation of drug treatment and for the necropsy endpoints for the future efficacy experiments. Once the model is established, UNC will test the efficacy of dry powder inhaled formulations of CPZEN-45 in the guinea pig model of *M. abscessus* infection in two independent replicate experiments. An efficacious formulation will be defined as one for which a statistically significant level of bacterial reduction is observed in the lungs of drug treated animals compared to untreated groups.

CSU – No Changes <u>But</u> Enhanced Chances for Success by Application of New Expertise and New Technology

The **first goal** of the work to be performed by Dr. Diane Ordway of Colorado State University (CSU) study is to quantify bacterial burden and organ pathology CPZEN-45 combination treatment activity against a representative panel of 10 carefully selected clinical isolates of *Mycobacterium avium complex* group strains in an **acute** mouse infection model of COPD. The isolates will be selected from 30 recent phenotypically characterized, clinical isolates provided by

Dr. Ed Chan from the Denver Veterans Administration Hospital and the Denver Research Institute. The whole genome of these 30 isolates and genomic variation in the WecA gene (target of CPZEN-45) will be sequenced by Dr. Michael Strong at the National Jewish Hospital. CSU **synergy measurement by checkerboard analysis** will be used to determine the impact on potency of the combination of antibiotics in comparison to their individual activities. The **second goal** of the work to be performed by CSU is to quantify bacterial burden and organ pathology CPZEN-45 combination treatment activity against a representative panel of 10 clinical isolates of *Mycobacterium avium complex* group strains in a **chronic** mouse infection model of COPD. The optimal combination of antibiotics will also be determined by Dr. Lynette Cegelski, Department of Chemistry, Stanford University using a new solid state NMR technique recently established by Dr. Cegelski's team. Finally, an additional study will be to investigate the antimycobacterial activity of new CPZEN-45 **regimens** identified by checkerboard titration and NMR technologies against 5 studies of *M. abscessus* and 5 studies of *M. avium* by infecting SCID and Kramnick mice to evaluate mouse bacterial strain virulence.

Stanford University

Investigators at IMC have previously shown that CPZEN-45 inhibits the first step in the synthesis of the mycobacterial cell wall core, the Achilles heel of *M. tuberculosis*. To date, no other WecA inhibitor has been launched clinically, nevertheless the mAGP complex is the principal structure of the cell wall and thus essential for the growth of *M. tuberculosis*. Thus, it is not surprising that CPZEN-45 is cidal and active against both rapidly and slow growing *M. tuberculosis*. More recently the IMC investigators have shown CPZEN-45 may also target the final step in the biogenesis of the cell wall core of Mycobacterium spp. CSU and collaborators have demonstrated that genetic knockout of one of the genes responsible for the ligation/covalent attachment of the AG-linker unit to peptidoglycan renders mycobacteria even more susceptible to CPZEN-45 than to CPZEN-45 alone. Since the chemical mechanisms of the WecA reaction and this final ligation are related, the ligation mechanism itself may be susceptible to CPZEN-45, and, moreover, clearly then combinations of CPZEN-45 plus antibiotics like **vancomycin** or beta-lactams should be one of the most powerful combined regimens for all mycobacteria. If proven to be true, this new combination regimen could have a major impact on treatment of both TB and NTM. In the first year of our DOD therapeutic award grant, Diane Ordway has shown by checkerboard titrations that **vancomycin** has an additive effect in combination with CPZEN-45.

Independently, over the past two years, Dr. Lynette Cegelski and her team at Stanford have developed 13C cross polarization magic angle spinning solid-state nuclear magnetic resonance (NMR) spectra of intact cell walls that have been uniquely valuable in dissecting the atomic and molecular details of antibiotics whose modes of action were not clear from biochemical experiments alone. Since the official grant transfer to PAI, Dr. Cegelski and her team have obtained 13C "whole-cell NMR data" which showed that CPZEN-treated cells had a reduction in peaks that looked similar to vancomycin-treated cells. Since vanco inhibits peptidoglycan, that would result in an inhibition also of arabinogalactan that gets attached to it, the expectation would be that whole-cell NMR data for CPZEN, inhibiting arabinogalactan production, would look similar to vancomycin-treated cells. They compared these with other inhibitors that give very different results (ethambutol and tunicamycin). They have succeeded with the cell wall isolation, so now they will isolate the cell walls from the antibiotic-treated cells to see the effect

more selectively by only looking at carbons associated with mAGP and at 15N NMR. They have prepared the conditions which involved determining the best OD to add antibiotic at and the number of hours to grow cells following treatment to ensure the impact of antibiotic treatment on actual cell wall composition can be observed.

Pilot tests of MIC values where they compared control inhibitors with CPZEN, indicate that CPZEN is really effective at inhibiting *M. avium* which confirms previous results obtained by Dr. Ordway plus they observed synergy when testing vancomycin plus CPZEN together against NTM. Over the coming months, the Stanford team will perform comprehensive solid-state NMR analysis of clinical isolates of NTM also utilized by Dr. Ordway at CSU. The results of the studies at CSU and Stanford will allow us to select the most optimal treatment regimen of antibiotics to be utilized in the proposed *in vivo* efficacy studies. The Stanford group will perform the ^{13}C and ^{15}N whole-cell and cell-wall NMR experiments as detailed in their recent publication (Romaniuk JAH and Cegelski L. Peptidoglycan and Teichoic Acid Levels and Alterations in *S. aureus* by Cell-Wall and Whole-Cell NMR. *Biochemistry* (2018) 57, 3966-3975) and as reviewed recently in Cell Chemical Biology (Brown AR, Gordon RA, Hyland SN, Siegrist MS, Grimes CL. Chemical Biology Tools for Examining the Bacterial Cell Wall. Cell Chemical Biology (2020) 27, 1052-1062).

SPECIFIC STATEMENT OF WORK AND ORGANIZATIONS AND INVESTIGATORS TO BE INVOLVED

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Site 6: Stanford University
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Specific Aim 1: Optimize fermentation and scale-up of manufacturing processes for high yield of CPZEN-45, including spray dried CPZEN-45.	**Timeline(Month)**	**Site 1**	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**
Major Task 1 – Improve drug substance yield to reduce costs and manufacture for animal studies							
Improve fermentation and purification with improved CPZEN-45 strain	18		Shibasaki				
Manufacture non-GMP batch for rodent efficacy studies	Complete						
Manufacture non-GMP drug substance for guinea pig	Complete						
Optimize process development and analytical methods for GMP manufacturing	9				Dr. Moody/ Cambrex		
Milestones Achieved- Manufacturing improvements completed, reducing cost and providing drug substance for in vivo studies							
Major Task 2- Transfer spray-drying method to CMO, produce							

drug product for animal studies and develop drug product processes for GMP scale-up							
Develop analytical method and scale-up processes for spray-drying	Complete						
Spray-dry CPZEN-45 for guinea pig efficacy studies	6				Dr. Hickey/ Dr. Carter/ Crititech		
Evaluation of aerosol performance with cyclohaler	15				Dr. Hickey/ Dr. Carter/ Crititech		
Drug product stability evaluation	12				Dr. Hickey/ Dr. Carter/ Crititech		
Milestones Achieved: Spray-drying for in vivo studies complete, method transfer for scale-up and drug product stability completed							
Specific Aim 2- Further define and characterize *in vitro* efficacy of CPZEN-45 against additional species of NTMs from VA patients with COPD. Evaluate efficacy in characterized acute and chronic COPD mouse models. Evaluate CPZEN-45 Inhaled Therapy Using a Chronic							

NTM Model in Guinea Pigs							
Major Task 3							
Further define and characterize *in vitro* efficacy of CPZEN-45 against additional species of NTMs from VA patients with COPD.				Ordway,Chan, Strong			Cegelski
ACURO review of mouse study protocols	1			Dr. Ordway			
Evaluation of CPZEN-45 in LP-SCID chronic NTM infection model	9			Drs. Ordway/ Chan			
Evaluation of CPZEN-45 combination in COPD NTM mouse model	9			Drs. Ordway/ Chan			
ACURO review of guinea pig study protocols	1				Dr. Hickey	Dr. Braunstein	
Evaluate CPZEN-45 in guinea pig model of acute NTM infection	15				Dr. Hickey	Dr. Braunstein	
Milestones Achieved: Efficacy studies in mice and guinea pigs complete	18						