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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 vitro* and *in vivo*. 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*.

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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

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?

Objective 1: To optimize fermentation and scale-up manufacturing processes for high yield of CPZEN-45, including spray dried CPZEN-45 [Institute for Microbial Chemistry (IMC) and Research Triangle Institute (RTI)]

CPZEN-45 HCl is a semi-synthetic antibiotic produced from caprazamycins found in the fermentation broth of Streptomyces sp. MK730-62F2. During the **fermentation** of Streptomyces sp. MK730-62F2, multiple caprazamycins are produced, caprazamycin A, caprazamycin B, caprazamycin C, caprazamycin E caprazamycin F. For the production of CPZEN-45 HCl, the caprazamycins are converted to caprazene directly in the **fermentation** broth by acid hydrolysis and caprazene is then extracted from the fermentation broth by dichloromethane or methanol. Caprazene is then converted to CPZEN-45 HCl by a **synthetic process**.

Fermentation

Previous Progress: In Year 01 of this grant, IMC made significant progress in improving yields and successfully transferred the fermentation technology to Hisun. In order to continue to improve the productivity of caprazamycins, parent compounds of CPZEN-45, IMC applied the genetical technique called ZouA method to 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. During the six months covered by our semi-annual report (30 Sep 2021-30 Mar 2022), IMC has completed the whole genome sequence of Streptomyces strain MK-730-62F2 and two derivative strains and have analyzed them. More than 600 point mutations, including 312 non-synonymous mutations, were found in the derivative strain 218N bred by IMC and Meiji. Two additional non-synonymous mutations and a 3.2kb-deletion were found in the strain bred by Hisun from the 218N strain. Although it was difficult to identify the specific mutations which contributed to the higher productivity of caprazamycin in derivative strains because of the numerous mutations, some mutations found in the genes for transporters, putative gene clusters for the biosynthesis of secondary metabolites, and transcription regulators could result in higher productivity. In year 02, IMC conducted a comparative analysis of the titers, metabolites, and impurities between the production strain improved by Hisun in year 01 and the original production strain of IMC, based upon these results and those from their genomic analysis, IMC concluded that continuation of improved yield by genomic manipulation is not likely to yield meaningful results for the following reason. They found additional mutations in Hisun's strain which might be related to fermentation yield (deletion of a gene cluster involved in another metabolite), but caprazamycin productivity of Hisun's strain (1400 μg/ml) was inferior to IMC's strain (ca. 1900 μg/ml) in comparative studies performed by IMC under their laboratory conditions. Consequently, IMC recommended that improvement of fermentation yield such as breeding and optimization of culture condition should be performed for industrial scale with a new partner company for fermentation. In addition, 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 for clinical trials and commercialization and based upon IMC's assessment of Hisun's technical capabilities as for as further improving yield by improving the fermentation process, we decided to transfer the manufacturing of CPZEN-45 to another manufacturer. Therefore, we have been working to identify a fermentation Contract Manufacturing Organization (CMO) that could produce caprazene in compliance to cGMPs. As indicated in our Semi-Annual Report submitted

in April 2022, based on the strong expertise and experience in GMP fermentation, we had chosen the Abbvie North Chicago Fermentation Operations as the CMO for future cGMP production by fermentation of caprazene. Unfortunately, because of significant challenges Abbvie has encountered in their manufacturing of "baby formulas" in the past several months, our discussions with Abbvie have been put on hold. We are currently in negotiations with Biomar Microbial Technologies based in Leon Spain. Biomar specializes in the optimization and scale-up of laboratory methods into industrial processes. They have more than 25 years experience in growing a great range of microorganisms and over 15 years of experience in microbial fermentation manufacturing. They work on every stage of the process to optimize: the producing strain, the media, the seed strain, the fermentation parameters, the titer, the product recovery process, the batch sizes, and, of course, the associated costs. IMC will work with Biomar to facilitate the technology transfer and implementation of improvements to date.

Synthesis of CPZEN-45 HCL

The other major scope of research under Objective 1 has been led by RTI and has focused on developing an IND enabling, robust, scalable process to produce CPZEN-45 on a multi-kilo scale. During the Semi-Annual reporting period (30 Sep 2021-30 Mar 2022), RTI and Cambrex, the API manufacturer, focused on process improvement in the CPZEN-45 synthetic scheme from caprazene in order to obtain 1) improved conversion of intermediates and 2) removal of steps within the process which are not conducive to scale-up. Cambrex made successful efforts to improve the synthetic process through the modification of a number of different factors including, for example, changes to 1) reagent amounts/overages, 2) order of addition, 3) reaction, deprotection and extraction solvents, 4) ratios of reaction solvents, 5) pH and 6) temperature. The changes resulted in increased yields and improved purity at several intermediate steps. Scale-up of several of these intermediate steps were in progress at the close of the reporting period and the manufacturer was evaluating a salt conversion step to form the final product, CPZEN-45 monohydrochloride salt, through the use of resins or other chemical means. Analytically, Cambrex developed a rapid chromatography method (i.e. UPLC) to monitor reaction conversions during process development. They also initiated development of a stability indicating chromatography method for utilization in API routine, release, and stability testing.
During the current reporting period (01 Apr 2022-30 Oct 2022), Cambrex continued with efforts to improve the synthetic process as well as conduct scale up activities.

Process Improvement Activities

1) Established points of control for purity of the monohydrochloride salt

2) Evaluated various resin charge levels to form the monohydrochloride from the di-HCl salt intermediate.

3) Evaluated different solvents and conditions to optimize crystallization conditions of the monohydrochloride salt to improve the final purity of the drug substance

4) Evaluated the impact of solvent type/combinations, solvent volumes and reaction times for each step of the process

5) Attempted to isolate the di-HCl salt form as a possible intermediate stopping point. The salt was found to be deliquescent and therefore not able to be easily isolated as a

solid.

6) Evaluated workup conditions at the various steps

7) Evaluated hold point stability to assess whether reactions can be held under certain conditions and for what duration

8) Evaluated stability of the di-HCl salt for loss of chloride ion over time.

9) Evaluated of water content in relation to the performance of the resin (i.e., dry versus wet resin comparison)

10) Performed solution stability of the final product and the stability impact of oven drying the final product

11) Evaluated resin stability/shelf life after initial use

12) Utilized UPLC and qNMR methods to assist with assessing completion of reactions and purity of reaction products.

Scale-Up Related Activities

13) Continued scale-up at each stage of the process including:

a. Isolation of BOC-CPZEN-45

b. BOC deprotection to form CPZEN-45 di-HCl

c. Treatment of di-HCL CPZEN-45 to form CPZEN-45 HCl (final product) and recrystallization

14) Evaluated a capacity model to determine if the scale up batch could be increased from the planned 500 gram batch to an 800 gram batch. This evaluation indicated scale up to the 800 gram scale could be completed in the same equipment and at no additional cost.

15) Finalized the evaluation at the 50 gram scale in early August.

16) Established testing attributes for the scale up batch (800 grams; non-GMP)

17) Established a plan to isolate a quantity of reference standard from the 800 gram batch with further purification and establish the testing requirements to certify the standard batch.

Analytical Development

Cambrex established and optimized a purity, assay and impurity method by evaluating different solution gradients and through evaluation of a different ion pairing reagent. These changes allowed for better peak separation and sharpness which met USP guidelines. During a pre-validation evaluation of the method, results of LOQ, accuracy, linearity, specificity, and method precision were satisfactory and a protocol was established to begin a full qualification. In addition, protocols for method verification for water content and method qualification for chloride content were established.

OBJECTIVE 2: Define and characterize *in vitro* and *in vivo* efficacy of CPZEN-45 against clinical NTM isolates [Colorado State University (CSU), University of North Carolina (UNC), RTI, Stanford University, National Jewish Health and IMC)

- **Colorado State University (CSU)**: **Change in Subaward Principal Investigator (PI)**

Since the beginning of our DOD Therapeutic Award, Dr. Diane Ordway, had been the PI of our Colorado State University (CSU) Sub-Award which is focused on Objective 2. On March 15, 2022, Kelly Bergeron, Research Administrator, Officer of Sponsored Programs CSU requested that due to Dr. Ordway's resignation of her CSU faculty position related to her health status that she be replaced as Sub-Award PI by Dr. Mercedes Gonzalez-Juarrero. Dr. Gonzalez-Juarrero is exceptionally qualified to carry on the *in vitro* and *in vivo* efficacy programs as originally outlined. However, regardless of the excellent expertise of our new CSU collaborators, **the** change in personnel has resulted in a significant delay of our in *vitro* and *in vivo* work to be performed by CSU. It has required time to familiarize the new investigators and their technicians with our previous and proposed work. In addition, critical animal experiments have been delayed related to submission of amendments in CSU's major mouse experimentation (Amendments submitted to CSU IACUC for change in CSU PI plus protocol amended to include intrapulmonary delivery of CPZEN-45 to mice). ACURO approval received July 14, 2022. Mouse model characterization studies begun. First comprehensive mouse efficacy results are anticipated 30 Jan 2023.

Due to the fact that the proposed guinea pig studies were to be based upon data obtained by CSU and performed with RTI (Dr. Hickey) and UNC scientists (Dr. Braunstein), the start of the guinea pig studies also was delayed. In efforts to establish stable infection of guinea pigs with nontuberculous mycobacteria, Dr. Braunstein used a whole-body aerosol chamber (Madison chamber). Male 300g Hartley guinea pigs were infected with a clinical isolate of *Mycobacterium abscessus* strain A (provided by Dr. Diane Ordway CSU) by the aerosol route. One day post-aerosol exposure, a group of four animals were necropsied and the entirety of their lung homogenate plated to determine the bacterial burden delivered to the lung. At one day post-infection, the mean bacterial burden in the lungs was ~$4x10^3$CFU. By Day 27, the lung bacterial burden had cleared.

During the current reporting period (01 Apr 2022-30 Oct 2022) the NTM strain used was switched, as were the route of delivery [from aerosol to intratracheal (IT) instillation], and infectious dose delivered to guinea pigs. The new strain used is a clinical isolate *M. abscessus* 103 (provided by Mercedes Gonzalez Juarrero CSU). Upon receiving this strain growth curve experiments were used to establish its doubling time. The Braunstein group also confirmed the strain is sensitive to CPZEN using a resazurin microtiter assay (REMA) and serial dilutions of CPZEN (not shown). In April 2022, they practiced the IT protocol and in July 2022 they performed an IT infection experiment with the assistance of Dr. Victoria Baxter (Department of Compartment Medicine, UNC). Male 300g Hartley guinea pigs were anaesthetized and a 0.1ml volume of syringe passaged *M. abscessus* 103 was administered by IT delivery. One day post-aerosol exposure a group of four animals were necropsied and the entirety of their lung homogenate plated to determine the bacterial burden. At one day post-infection, the mean bacterial burden in the lungs was ~1.7x10^5CFU. This result indicates the starting infectious dose was increased nearly 2 logs from the past experiment. By Day 25, 2 animals had CFU below the limit of detection and two animals had CFU at ~1 x10^2CFU resulting in a mean of 4.4 x10^1 lung CFU in this group of animals (Figure 1). Thus, even with a higher starting infectious dose to the lungs and a different strain of *M. abscessus* the animals were clearing the infection. Most recently, Braustein's lab has infected guinea pigs with *M. avium* clinical strain 104 currently being used in CSU's mouse experiments. This NTM is the most commonly isolated from patients with COPD and appears to be more pathogenic and able to persist longer in mice compared to *M. abscessus* strains. Although at Day 15 in guinea pigs, there was a significant drop in lung CFU, it was relatively modest (Figure 2).

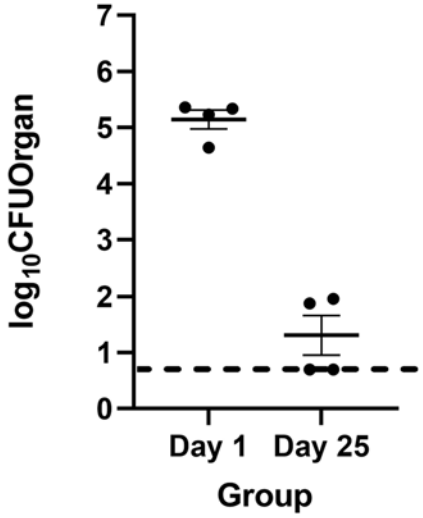

Fig 1. Groups of male Hartley guinea pigs (n=4) were with placed under anesthesia and infected by intratracheal instillation with ~5x10^6 CFU of *M. abscessus* 103. At days 1 and 25 post-infection, groups of mice were sacrificed and CFU in lung homogenate determined by plating on 7H10 Middlebrook agar. Data is presented as mean with standard error.

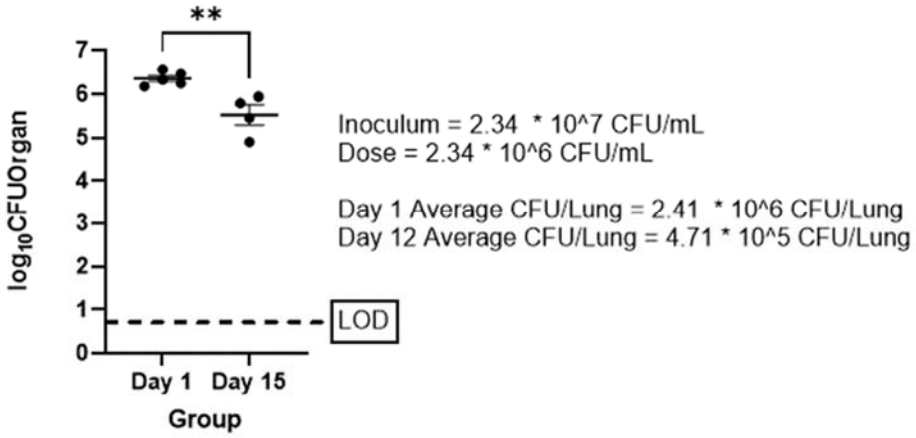

Fig. 2*: M. avium* (MAC104) guinea pig infection Day 1 vs. Day 15

- **Stanford University: Mechanism of CPZEN-45 and Interaction with Other Anti-mycobacterial Drugs**

Lynette Cegelski's laboratory has been developing and applying a new approach to directly detect and define molecular level changes in mycobacterial cell wall composition during treatment with CPZEN-45. More specifically, during Semi-Annual Reporting Period (30 Sep 2021-30 Mar 2022), her laboratory introduced a solid-state NMR platform to define mycobacterial cell wall composition in purified cell wall samples and in whole cell samples. This approach is uniquely enabling to define compositional changes in such insoluble and heterogeneous systems which pose a challenge to analysis by conventional methods. The approach offers advantages over solution-based chemical analyses that require degradative solubilization protocols that can result in chemical changes and misses insoluble material that is not liberated during digestions. By detecting all the carbons in the sample at once, relative changes to cell wall components can also be observed. The sample preparation protocols, including nutrient medium selection, antibiotic treatment conditions, and treatment times were developed alongside susceptibility and antibacterial combination evaluations to investigate CPZEN-45 against different non-tuberculous mycobacteria (NTMs). In order to assign changes to isolated cell wall spectra and to changes in spectra of whole cell samples, the group isolated or is isolating individual cell wall components (mycolic acids, peptidoglycan and arabinogalactan) and obtaining their NMR spectra. The compositional and architectural complexity of the mycobacterial cell envelope distinguishes species of the mycobacteria from other prokaryotes such as *B. subtilis*. The inner segment of the cell wall consists of peptidoglycan (PG) covalently attached to arabinogalactan (AG) through a phosphodiester bond by a unique phosphoryl-N-acetylglucosaminosyl-rhamnosyl linkage. Beyond this, the AG is esterified by long-chain (C70-C90) mycolic acids (MA). These three components (PG, AG, and MA) comprise the core of the cell wall and are referred to, collectively, as the mAGP complex. By way of short background, the direct demonstration of changes in cell wall composition will validate and/or provide additional molecular detail for how cell wall composition is influenced by CPZEN-45. Early biochemical experiments conducted by IMC to explore CPZEN-45 mode of action were performed in the Gram-positive model organism, *B. subtilis*, and demonstrated that the primary target of CPZEN-45 in *B. subtilis* is TagO, an enzyme involved in teichoic acid (TA) biosynthesis. IMC later showed in mycobacteria that CPZEN-45 inhibits WecA (a TagO ortholog), involved in AG synthesis. Consequently, CPZEN-45 is expected to cause depletion of teichoic acids (TA) in *B. subtilis* and AG in mycobacteria. Dr. Cegelski's lab has developed whole-cell solid-state NMR methodology in order to investigate the influence of CPZEN-45 on the actual cell wall that is produced by bacteria after CPZEN-45 treatment. This could reveal whether CPZEN-45 may have additional activity in its mechanism of action. For example, TagO catalyzes the first committed step in the synthesis of TA in *B. subtilis*. However, inhibition of the early stages in TA synthesis is not by itself lethal for cells. Later stages of TA synthesis are considered lethal, possibly due to accumulation of toxic intermediates in the cell or depletion of cellular pools of undecaprenyl phosphate, intermediate which is used for both TA and PG synthesis. Though CPZEN-45 has been shown to inhibit WecA, IMC also has shown it has some activity towards MraY, which is involved in the early stage of PG biosynthesis. The Cegelski lab has sought to identify how the cell wall composition changes as a result of CPZEN-45 treatment in order to provide atomic-level compositional parameters to understand the influence of CPZEN-45 on cells and aberrant cell wall produced during treatment.

Whole-cell NMR spectroscopy platform to identify cell wall compositional changes in whole cell samples

The ^{13}C cell wall and whole cell NMR platform was first developed by the Cegelski lab with *M. smegmatis* (as a commonly used model organism) and *B. subtilis* (as a Gram-positive organism which has been used extensively in previous work with CPZEN-45). During the period (30 Sep 2021- Mar 30 2022- 30), interfaced with susceptibility and antibacterial combination evaluations to investigate

CPZEN-45 against NTM, Cegelski's lab performed the first whole cell NMR experiments with *M. avium*. They compared the effect of CPZEN-45 treatment with the effect of control inhibitors, with known modes of action. Tunicamycin inhibits TagO and the production of teichoic acid (TA) in *B. subtilis* and inhibits WecA and the production of arabinogalactan (AG) in mycobacteria. Vancomycin inhibits PG synthesis in both bacteria, and ethambutol targets enzymes in the later stages of AG (and lipoarabinomannan) biosynthesis in mycobacteria. Importantly, carbons of WTA/AG and PG are distinguishable even in the whole cell spectra, which allows them to compare each component with respect to treatment with different cell-wall targeting antibiotics.

The ^{13}C CPMAS spectra of *B. subtilis* treated with CPZEN-45 revealed spectral differences corresponding to changes in cell wall composition. In *B. subtilis*, CPZEN-45 treatment resulted in a decrease in the polysaccharide carbons between 60–80 ppm and the anomeric carbon region between 93-104 ppm. This is consistent with reduction of WTA content in the cell wall (based on Cegelski's published work in *Staphylococcus aureus*). Complementary to this, the ^{13}C NMR spectrum of *B. subtilis* treated with tunicamycin, known to inhibit WTA in *B. subtilis*, exhibited similar reductions as observed for CPZEN-45 treated cells. Moreover, CPZEN-45 and tunicamycin treated spectra were distinct from the spectrum of whole cells treated with vancomycin, which inhibits the PG component of the cell wall. This supports the mechanism of CPZEN-45 working primarily on TagO in *B. subtilis*.

CPZEN-45 activity in M. smegmatis and C. glutamicum by whole cell NMR

The ^{13}C CPMAS spectra of *M. smegmatis* treated with the control antibiotic vancomycin showed expected decrease in the anomeric signal centered at 101 ppm and in the polysaccharide region around 72 ppm. This is consistent with inhibition of PG synthesis by vancomycin. The spectrum of ethambutol treated *M. smegmatis* whole cells was consistent with inhibition of AG, as indicated by the decrease in the anomeric carbon intensity around 107 ppm and the polysaccharide region between 60-80 ppm. The influence of CPZEN-45 on *M. smegmatis* depended on the CPZEN-45 treatment concentration. At lower concentration, the spectra revealed expected changes associated with AG reduction, similar to that of ethambutol (results from current reporting period) and consistent with the mode of action of WecA inhibition. At higher concentration, the carbon changes show reductions in the both the AG and PG sugar carbons, similar to treatment with vancomycin. This could indicate that at high concentrations, CPZEN-45 has activity on PG in mycobacteria and targets more than just AG through inhibition of WecA. This could be attributed to additional activity on MraY, for example, which has been observed by IMC *in vitro* by CPZEN-45 at higher concentration. Alternatively, depletion of PG could result from aberrant synthesis of AG, which would use up the cell's resources of undecaprenyl phosphate. In *C. glutamicum*, treatment with CPZEN-45 indicated inhibition of primarily AG, consistent with inhibition of WecA. The CPZEN-45 treated spectrum was similar to the spectrum of *C. glutamicum* treated with ethambutol and distinct to the spectrum of vancomycin treated whole cells. The *M. smegmatis* and *C. glutamicum* comparison could indicate that CPZEN-45 has a different effect depending on the organism or concentration or could reflect the difference in the susceptibility of different bacteria to CPZEN-45 (*M. smegmatis* and *C. glutamicum* MIC for CPZEN-45 are 8 and 1 μg/mL, respectively).

CPZEN activity in slow-growing M. avium - antimicrobial susceptibility

CPZEN-45 is especially effective against slow growing mycobacteria, including *M. avium 25291*, *M. intracellulare* JCM 3684 and *M. tuberculosis* H37R and MDR with MIC values between 0.2 and 6.25 μg/mL. Cegelski's lab confirmed by the broth microdilution method that CPZEN-45 is very effective against *M. avium 25291* and *M. avium 700898* strains obtained from ATCC (MIC values 1.0 and 0.5 μg/mL, respectively). Combination of vancomycin and CPZEN-45 was synergistic in two rapidly growing NTM, *M. smegmatis* and a clinical isolate of *M. mucogenicum/M. phocaicum*.

CPZEN activity in slow-growing M. avium by whole cell NMR

During the current reporting period (01 Apr 2022 – 30 Oct 2022) the nutrient medium and growth conditions, including growth time points at which to introduce CPZEN-45 treatment, were optimized

for the preparation of whole cell samples using two reference *M. avium* strains (ATCC 700898 and 25291). There were differences in growth kinetics and overall cell densities achieved by the two strains in Middlebrook medium formulations, wherein *M. avium* 25291 grew more slowly and less well than 700898 in several media conditions. Cegelski's lab performed solid-state NMR experiments on several *M. avium* samples and observed anticipated changes in whole cell composition as cells were harvested from different growth stages. Each *M. avium* sample corresponds to cells harvested from a total of 1.2 L of cells. As a slow growing mycobacterium, cells are grown for almost 2 days to reach exponential phase and are grown for longer than 5 days for later stationary phase samples. Antibiotic treated cell densities are documented to enable comparison with matched control (untreated) samples of similar cell density. Using ethambutol as a control antibiotic, the Cegelski lab successfully achieved optimal growth parameters to enable sensitivity to detect changes in cell wall composition in the intact whole-cell spectra. Furthermore, they discovered that changes to the whole cell spectra of *M. avium* treated with CPZEN-45 are similar to that of ethambutol. This experiment was repeated and forms the basis for future targeted experiments with *M. avium* and CPZEN-45. At intermediate and high doses of CPZEN-45 (corresponding to 5X, 10X and 20X the MIC values), they observed larger reductions in spectral regions attributed to both PG and AG in the whole cell spectra. The higher dose may be leading to additional activity, such as inhibiting MraY. Alternatively, the inhibition of WecA and the production of PG with fewer AG linkages may have an influence in reducing PG production. Interestingly, the mycolic acid and mycomembrane contributions are comparable in all CPZEN-45 treated samples.

If the simple explanation (dual activity) for Cegelski's NMR results are correct, and CPZEN-45 does target WecA and then also has activity against MraY, that could explain difficulty in obtaining resistance. CPZEN-45 could be exhibiting a dual mode of action in one compound rather than needing two, which always enhances the effectiveness of an antibiotic. Also related: perhaps only at very low concentrations (much lower than MIC) would it then be possible to obtain WecA mutants that are resistant.

- **Mechanism of Action of CPZEN-45 and Need to Evaluate Potential of Clinical Isolates to Develop Resistance: National Jewish Health (Whole Genome Sequencing), CSU and IMC**

  During the current reporting period, IMC cloned the *WecA* ortholog gene of *Mycobacterium tuberculosis* (*Mtb*), *M. avium*, and *Bacillus subtilis* (*TagO* gene) into a *M. smegmatis* strain which lacks its own ortholog using an *E. coli*–Mycobacteria shuttle vector. They also tried to introduce a mutated (Ile243Ser) *TagO* gene of *B. subtilis* which contributes to the resistance to CPZEN-45, but they could not achieve it. This probably indicates that the mutated TagO enzyme does not function well enough for the proliferation of *M. smegmatis* cells even though the wild-type TagO does. Note that cloning the TagO and its mutant were performed within the past 3 months. IMC has also continued their efforts to obtain WecA mutants resistant to CPZEN-45 using a continuous subculture of *M. smegmatis* transformants mentioned above. Error-prone DNA amplification using Phi29 polymerase is also in progress. Note that the mutation frequency in the error-prone PCR was analyzed within the past 3 months and it remains in process.

  Michael Strong's laboratory for Computational and Molecular Genomics at National Jewish Health in collaboration with CSU has been actively working to further characterize the clinical NTM isolates that are being tested by CSU, UNC, and Stanford. As reported in our Annual 2021 report and recent 2022 Semi-Annual Report, to date, Dr. Strong's research group has sequenced the genomes of twenty NTM isolates for this project, including eight *M. abscessus* subspecies

abscessus, nine *M. avium*, two *M. chimaera*, and one similar to *M. intracellulare*. Phylogenetic analysis, following whole genome sequencing revealed significant phylogenetic diversity among the *M. abscessus* subspecies *abscessus* and *M. avium isolates*, including three *M. abscessus* subspecies *abscessus* isolates that are within the dominant circulating clone. This analysis will help us interrogate whether there are intra-species genetic variations that impact efficacy of CPZEN-45.

As previously reported, Dr. Strong's group has also initiated research to investigate sequence variation among the CPZEN-45 drug target, WecA, including examining nucleotide and protein sequence variation in clinically relevant NTM species. WecA is present in *Mtb*, *M. avium* complex MAC species, *M. abscessus*, and other rapidly growing mycobacteria (RGM). Comparisons to the *Mtb* WecA protein reveals that the sequence identity of MAC species compared to *Mtb* ranges from 91% to 95%, whereas the sequence identity of the three subspecies of *M. abscessus* to *Mtb* is closer to 78%. This sequence variation, particularly if located at or near proposed drug activity or binding sites, would likely influence affinity and activity of CPZEN-45.

Since submission of our 2021 Annual Report and especially since submission of our 2022 Semi-Annual Report, in addition to inter-species variation among the CPZEN-45 drug target WecA, the Strong group has also investigated intra-species protein and genetic variation of WecA. A genomic analysis of **1300 drug resistant clinical *Mtb*** isolates revealed minimal intra-species WecA variation, including only 4 unique non-synonymous mutations: A276V (3 isolates), H343Y (3 isolates), G165S (1 isolate), and V357G (3 isolates) and only 4 unique synonymous mutations, including I159I (2 isolates), T44T (1 isolate), D371D (1 isolate), and A358A (1 isolate).

The Strong group has also interrogated the intra-species and intra-subspecies genetic variation of WecA in clinically relevant NTM species, including the analysis of over **1500 NTM genomes (921 clinical isolates of *M. avium,* the most common NTM infection in patients with chronic lung disease)** that have been sequenced by Dr. Strong's research group and are banked at National Jewish Health and a collection of publicly available NTM genomic data. Among their findings, they identified 25 unique variable sites detected within 1207 *M. abscessus* WecA genes, 29 variable sites within 921 *M. avium* WecA genes, and 196 variable sites within 892 *M. intracellulare* WecA genes analyzed. The frequency of mutations at variable sites across the WecA gene, compared to reference genomes, ranges from 0.1% (T209G in 1 out of 1207 isolates) to 22% (A225G in 269 out of 1207 isolates) in *M. abscessus*, 0.1% (A170G in 1 out of 921 isolates) to 31% (G1063A in 289 out of 921 isolates) in *M. avium*, and 0.1% (C585T in 1 out of 892 isolates) to 50% (T396C in 450 out of 892 isolates) in *M. intracellulare*. Based on their analysis of 921 clinical *M. avium* WecA genes, and their inter-species comparisons of slowly growing clinical mycobacteria, we hypothesize that since the intra-species genetic variation is minimal in the WecA gene (**only 29 positions within the entire *M. avium* WecA gene have any variability**) and that there is high sequence conservation (**above 90%**) among clinically relevant slowly growing mycobacterial WecA protein sequences, including *M. avium*, **we hypothesize that WecA may be an ideal drug target with minimal resistance potential**, and that selective pressures on the essential function of WecA may limit drug resistance related mutations This hypothesis is also supported by recent work of others who have confirmed the essentiality of WecA's encoding gene in *Mtb* by demonstrating that transcriptional silencing of WecA is bactericidal *in vitro* and in macrophages (Husziar, et al., Antimicrob. Agents Chemother. 2017.e01310-17). In addition, we have found that there are certain regions of the *M. avium* WecA protein that have 100% conservation across all analyzed slowly growing and rapidly growing clinically relevant mycobacteria, suggesting that **these regions of the protein sequence may have high selective pressures <u>not</u> to mutate**, and if these are near the CPZEN-45 binding site then that may explain why resistant mutants have not

been identified to date and why we believe that **WecA may be an ideal drug target, with minimal CPZEN-45 resistance potential** and why IMC and some of our other colleagues have tried unsuccessfully to isolate experimentally induced CPZEN-45 resistant mutants of *Mtb*.

IMC has also tried to isolate a *M. avium* strain resistant to CPZEN-45 using the type strain ATCC 25291 but they have not been able to do so. Indeed, the mutation rate was less than5x10^-10 at most. Also, IMC has screened over 30 clinical isolates of *M. avium* collected over a period of three decades from different geographic locations and have identified **no** CPZEN resistant strains. The possibility of drug resistance arising during treatment was recently addressed by one of us (Hickey of RTI) by plating tissue homogenate on media containing 20 µg/mL CPZEN-45, **which is 12X the minimum inhibitory concentration (MIC)** for *Mtb* strain H37Rv2. No resistant *Mtb* were recovered following **4 weeks** of CPZEN-45 treatment of animals. This could possibly suggest that development of resistance of NTM and *Mtb* to CPZEN-45 *in vivo* may indeed be infrequent. This is why we believe that **WecA may be an ideal drug target, with minimal CPZEN-45 resistance potential**. If confirmed, this would add to the attractiveness of CPZEN-45 as a new therapy for mycobacterial infections including those due to *Mtb* as well as NTM.

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?

The work to be performed during the next reporting period will remain focused upon our original two objectives and therefore do not differ from our original SOW: **Objective 1**- To optimize fermentation and scale-up 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 and to determine impact upon potency of the combination of antibiotics in comparison to their individual activities. The most optimal synergistic regimens will then be tested in chronic mouse and guinea pig efficacy models.

Objective 1: To optimize fermentation and scale-up manufacturing processes for high yield of CPZEN-45, including spray dried CPZEN-45 [Institute for Microbial Chemistry (IMC) and Research Triangle Institute (RTI)]

Our highest priority will be to identify a GMP fermentation manufacturing partner. We will continue negotiations with Biomar Microbiologic Technologies. If an agreement can be reached, IMC will work with Biomar to facilitate the technology transfer and implementation of improvements to date. RTI will continue to work with Cambrex, the API manufacturer to complete the following activities regarding the process improvement in the CPZEN-45 synthetic scheme from caprazene in order to obtain 1) improved conversion of intermediates and 2) removal of steps within:

1) Manufacture of an 800 gram batch of is in progress and is expected to be completed during the next reporting period. The batch will be tested and released for use for preparation of CPZEN-45 spray dried drug product. The API will also be set on stability.
2) A reference standard will be qualified. A portion of the 800 gram batch will be isolated and further purified prior to qualification.
3) Planning, staging, execution of the cGMP campaign including setting of the batch on stability.
4) Complete additional analytical method development and validation, where required.
5) Establishment of specifications for starting materials, finish products and intermediates, where required.

OBJECTIVE 2: Define and characterize *in vitro* and *in vivo* efficacy of CPZEN-45 against clinical NTM isolates and to further define the mechanism of action of CPZEN-45 [Colorado State University (CSU), University of North Carolina (UNC), RTI, Stanford University, and National Jewish Health and IMC)

It is critical that we complete our originally proposed *in vitro* and *in vivo* studies if we are to advance the clinical development of CPZEN-45. We will continue evaluating the *in vitro* activity of CPZEN-45 drug susceptibility against drug-susceptible laboratory strains, laboratory strains with known patterns of resistance, and our well characterized clinical isolates representing different geographical regions using standardized methods in our mouse and guinea pig models. We will also determine the mycobacterial burden and sterilizing activity of CPZEN-45 alone and in combination with other antibacterial drugs using our mouse and guinea pig models. In particular, Dr. Braunstein's laboratory at UNC will continue to work towards our goal of establishing a guinea pig model of *M. abscessus* infection that will allow us to evaluate the efficacy of inhaled CPZEN-45. As a new approach to establish stable NTM infection, they will perform an experiment where they repeat the IT instillation protocol with *M. abscessus* 103 strain a total of three times (separated over a 5 day period). For the necropsy time points, they will plate organ homogenates at Days 1, 10, and 25. Although our goal has been to establish a chronic model of infection, if necessary, we can utilize an acute model of infection in guinea pigs that lasts for a shorter period of time. The rationale for testing repeated exposure is that it may better mimic the exposure encountered by individuals who develop NTM disease and it and it may influence adaption of the infecting *M. abscessus* and establishment of disease. In support of this repeat delivery approach, CSU has demonstrated the benefit of repeated *M. abscessus* IT infections for establishing sustained murine infection.

As an alternate strategy, UNC will change the NTM strain being used from *M. abscessus* to the *M. avium* 104 strain (provided by Mercedes Gonzalez Juarrero CSU). As this NTM appears to be more pathogenic and able to persist longer in mice compared to *M. abscessus* strains, we will infect guinea pigs by the IT route with *M. avium* 104 and determine the lung CFU burden at Days 1, 10, and 25

Dr. Cegelski and her team plan to perform solid-state NMR experiments with the *M. avium* 104 strain (provided by Mercedes Gonzalez Juarrero CSU), being used by other members in the collaborative team. They will also examine the influence of altering ethambutol dosing on whole cell NMR results reporting on cell wall composition. In particular, they may similarly see a reduction in peptidoglycan that could indicate the observations for CPZEN-45 is compatible with a cellular response to reduced PG-AG linkages. They will also examine the influence of combination treatment of CPZEN-45 with vancomycin or other cell wall inhibitors. They have identified antibiotic synergy between CPZEN-45 and vancomycin and synergy between CPZEN-45 and isoniazid and will examine the compositional changes detected by solid-state NMR analysis to reveal the nature of the synergy in altering the cell wall in mycobacteria. **This work is essential to identifying an optimal drug regimen for testing *in vivo* in both the mouse and guinea pig models.**

IMC will continue to focus on attempts to induce CPZEN-45 mutant strains. In the coming months, the Strong group will start interrogating whether protein structural models could be used to advance our understanding of the impact of WecA variation on drug activity. Indeed, with alpha fold and protein prediction algorithms, this approach will be invaluable in further understanding differences

in NTM clinical isolate responsiveness to CPZEN-45. Dr. Strong's laboratory, will investigate whether the highly conserved regions of WecA could be near a putative binding site of CPZEN-45, in order to better explain the intriguing observation of limited resistance to CPZEN-45 among mycobacteria. **If confirmed, our ultimate goal to improve the outcome in patients with NTM chronic lung disease will become more of a reality.**

Since 2008, our team has worked on CPZEN-45 to develop it for treatment of patients with multi-drug resistant tuberculosis. We have achieved critical milestones in characterizing the physical properties of CPZEN-45, that support its further development including defining its PK properties and toxicology profile *in vitro* and *in vivo* in multiple animal species. Therefore, we have begun related translational activities. A Type B "pre-IND" meeting for TB was held with the FDA. This meeting went very well with positive comments from the agency. We have applied for and received orphan drug designation from the FDA for CPZEN-45 therapy of drug resistant TB. We are currently drafting the Investigational New Drug application required for our *Mtb phase l safety and tolerability trial in healthy adults.*

We can leverage what is learned from our Phase 1 trial for TB to move seamlessly into efficacy studies in humans as part of an independent clinical development program for NTM. ***Importantly, April 21, 2022 we were notified by FDA (by the Senior Regulatory Health Project Manager of Anti-Infective Group 2) that CPZEN-45 for treatment of NTM would not require a new IND but rather we can add a new protocol for the new indication to the existing IND if the investigational product is for the exact same product (same formulation) and the indications are under the jurisdiction of the same Division.***

While GMP compound and chronic GLP toxicology studies will not be required for the phase l trial, per the FDA for the phase ll trial for NTM they are absolute requirements. This cannot be accomplished without the work remaining in the current Therapeutic Award from DOD. Our continuing work will allow us to further optimize and standardize the manufacturing of caprazene under cGMP standards required for obtaining cGMP toxicology data to support a ph ll NTM efficacy trial and to obtain the additional *in vitro* and *in vivo* MIC data on NTM to support the necessary IND.

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

Continuing challenges and delays in progress are mainly attributed to the delay in institutional transition of the ORIGINAL award. The Infectious Disease Research Institute, initial recipient of this DOD Therapeutic Award, entered receivership January 2020 (PI notified by IDRI leadership, November 1, 2019, ie only 13 months after the beginning of the Award). The complex nature of the receivership and the delayed process of receivership took much longer than was anticipated. This led to no work being performed until the official notification that the grant had been successfully transferred to PAI Life Sciences, July 6, 2021. This hiatus in funding combined with loss of personnel and finalizing new Sub-awards has precluded our being able to accomplish our proposed work according to the original timelines.

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

Problem:**Colorado State University (CSU): Change in Subaward Principal Investigator (PI)**
Since the beginning of our DOD Therapeutic Award, Dr. Diane Ordway, had been the PI of our Colorado State University (CSU) Sub-Award which is focused on Objective 2. On March 15, 2022, Kelly Bergeron, Research Administrator, Officer of Sponsored Programs CSU requested that due to Dr. Ordway's resignation of her CSU faculty position related to her health status that she be replaced as Sub-Award PI by Dr. Mercedes Gonzalez-Juarrero. Dr. Gonzalez-Juarrero is exceptionally qualified to carry on the *in vitro* and *in vivo* efficacy programs as originally outlined. However, regardless of the excellent expertise of our new CSU collaborators, **the change in personnel has resulted in a significant delay of our in *vitro* and *in vivo* work to be performed by CSU**. It has required time to familiarize the new investigators and their technicians with our previous and proposed work. In addition, critical animal experiments have been delayed related to submission of amendments in CSU's major mouse experimentation.

Actions to resolve: We have now received ACURO approval to proceed but we are just now beginning to start the first animal experiments. Due to the fact that the proposed guinea pig studies were to be based upon data obtained by CSU and performed with RTI (Dr. Hickey) and UNC scientists (Dr. Braustein), the start of the guinea pig studies also was delayed thus requiring additional time to perform those studies.

Problem: Stanford University Delays in Progress Due to Health and Climate Issues
As described in our last Semi-Annual Report and also the request for the NCE, Dr. Cegelski's lab will complete whole-cell NMR experiments with *M. avium* and will isolate cell walls from CPZEN-45 treated cells. We have also identified antibiotic synergy between CPZEN-45 and vancomycin and synergy between CPZEN-45 and isoniazid and will examine the compositional changes detected by solid-state NMR analysis to reveal the nature of the synergy in altering the cell wall in mycobacteria. However there has been a delay in progress due to **two** major circumstances: 1) Specifically, their group experienced continual intermittent delays throughout the past year due to **COVID**-related disruptions for personnel, specifically including quarantine/isolation requirements for infected individuals and close contacts and required isolation days associated with travel (domestic and international). They dealt with these challenges as best they could, with progress described above and in their past reports. This situation has now improved significantly. 2) In addition, Stanford University experienced a long 5-day power outage in June 2022 due to the Edgewood fire that disabled a main power line from the Pacific Gas and Electric power plant, which had long delays in repairing due to fire hazards at the site. All of Dr. Cegelski's laboratory equipment was shut down for 6 days, and they lost experiments with mycobacteria that were in progress and were delayed in starting new experiments due to major schedule changes and working to repair equipment that was damaged. In addition, one of their NMR spectrometers was affected (power supply damaged).

Changes that had a significant impact on expenditures

See above section on problems and delays

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

SUBMITTED TO AND APPROVED BY:

- IACUC approval September 24, 2020; Expiration September 23, 2023
- ACURO approval on April 28, 2021;

STATUS:

- The IACUC and ACURO protocols were approved.
- .

Amendments Submitted to Colorado State University (CSU) IACUC:

- Amendment 1280 – Change in CSU PI
- IACUC approval April 13, 2022
- Continuing Review Date: September 23, 2022
- Amendment 1280 – Protocol amended to include intrapulmonary delivery of CPZEN-45 to mice
- IACUC approval May 11, 2022; IACUC approval expires 09/23/2023

STATUS:

- Both amendments approved by IACUC
- Amendments submitted to ACURO – approval granted as of 07/12/2022 (see approval letter in Appendix)

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?

PARTICIPANTS

(Updated 30 December, 2022)

Site 1: PAI
1616 East Lake Ave E
Seattle, Suite 250, WA 98102
PI: Gail Cassell, PhD;
gail.cassell@pailifesciences.com ;
206-714-2724; 317-332-7374

Site 2: Institute for Microbial Chemistry
4-23 Kamiosaki, Shinagawa-ku
Tokyo 141-0021, Japan
Partnering PI: Masakatsu Shibasaki, Director of IMC;
mshibasa@bikaken.or.jp
03-3447-7779

Site 3: Colorado State University
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Fort Collins, CO 80523-1601
Partnering PI: Mercedes Gonzalez-Juarrero
Mercedes.gonzalex-juarrero@colostate.edu ; 970-491-7306

Site 4: RTI International
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Partnering PI: Diana Severynse-Stevens, dianastevens@rti.org ; 919-541-5903

Site 5: University of North Carolina
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Chapel Hill, NC 27599-7290
Partnering PI: Miriam Braunstein
Braunstein@med.unc.edu ; 919-966-5051

Site 6: Stanford University
Department of Chemistry
Mudd Bldg., Rm 121
333 Campus Drive
Stanford, CA 94305
Partnering PI: Lynette Cegelski
cegelski@stanford.edu
650-725-3527

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

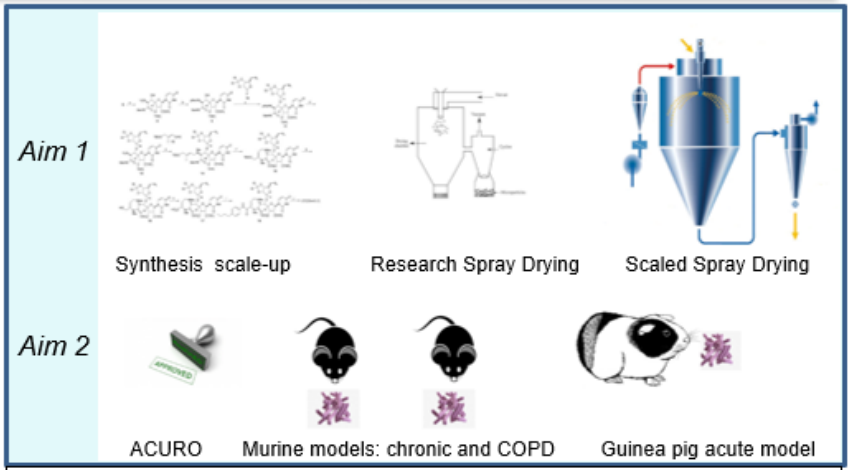
PI: Dr. Gail Cassell **Org:** PAI Life Sciences Inc. **PAI Award Amount:** $2,634,838.75

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: IMC/Biomar scale-up; Cambrex manufacturing and release
Aim 2: Guinea pig results, new murine results

Timeline and Cost

Activities CY	18-20	21-22	23
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**	**$1,126k**	**$1,308k**

Updated: Nov 30th, 2022

Goals/Milestones

CY22 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

CY23 Goal – Manufacturing and Animal Modeling
❑ Complete scale-up manufacturing
❑ Complete murine testing
❑ Complete guinea pig testing

Comments/Challenges/Issues/Concerns

- Due to the prior awardee being in Receivership, some delays have occurred for PAI to get the project efficiently moving again.

Budget Expenditure to Date
Projected Expenditure: $2,434,216
Actual Expenditure: $1,126,136

9. APPENDIX

DEPARTMENT OF THE ARMY

HEADQUARTERS, U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
810 SCHREIDER STREET
FORT DETRICK, MD 21702-5000
July 14, 2022

Director, Office of Research Protections
Animal Care and Use Review Office (ACURO)

Subject: Approval of Proposal Number Proposal Number PR171209, Award Number W81XWH-18-1-0765 entitled, "Aerosol Delivery of CPZEN-45 for Treatment of Nontuberculous Mycobacterial (NTMs) Infections"

Dr. Gail Cassell, PhD
Pai Life Sciences, Inc.
Seattle, WA, US

Dear Cassell:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Conducted and Supported Research and Training"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"

In accordance with the above references, the amendment to ACURO protocol PR171209.e003 entitled, "Aerosol Delivery of CPZEN-45 for Treatment of non-tuberculous mycobacterial(NTMs)," IACUC protocol number 1280, Protocol Principal Investigator Mercedes Gonzalez-Juarrero, is approved by the ACURO as of 07/12/2022 for the use of mice and will remain so until its modification, expiration or cancellation. This protocol amendment was approved by the Colorado State University IACUC on 5/11/2022; IACUC approval expires 09/23/2023.

Required Actions:

A. Submit to ACURO for review and approval prior to implementing:

• IACUC-approved de novo reviews of the protocol

• IACUC-approved significant changes to this protocol (see guidance document)

B. Notify ACURO within 5 business days of any of the following:

• Any noncompliance, suspensions or adverse events (see guidance document)

• Receipt of notification that the institution is under investigation by USDA

• AAALAC, International accreditation status change

For further assistance, please contact ACURO at , FAX (301) 619-4165, or via e-mail: usarmy.detrick.medcom-usamrmc.other.acuro@mail.mil.

NOTE: Do not construe this correspondence as approval for any contract funding. Only the Contracting Officer or Grant Officer can authorize expenditure of funds. It is recommended that you contact the appropriate Contract Specialist or Contracting Officer regarding the expenditure of funds for your project.

Sincerely,

Krinon Moccia, DVM, MPH, DACLAM
LTC, VC, USA
Director, Animal Care and Use
Review Office

Copies Furnished:
Dr. Lon V. Kendall
Dr. Diane Ordway
Karen Dobos
Corrine Lindstadt
Colorado State University IACUC
Mercedes Gonzalez-Juarrero
Dr. Patricia Young
Darrick Carter