

# Development of Therapeutics for *B. pseudomallei*

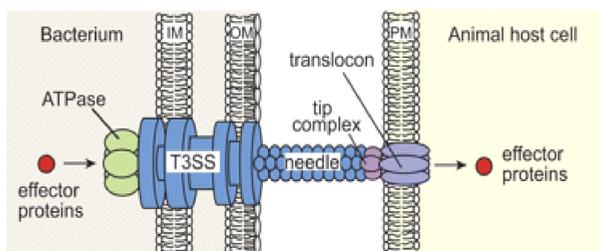
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## Overview

*Burkholderia pseudomallei*, a Gram-negative pathogen which infects humans and animals, is the causative agent of melioidosis. The bacterium is classified as a select agent by both the Centers for Disease Control and Prevention and U.S. Department of Agriculture, due to its severe course of infection, aerosol infectivity and worldwide availability for possible use as a warfare agent. Its survival and pathogenicity within a host requires the use of a type III secretion system (T3SS) to escape from endocytic vacuoles, ultimately leading to intra- and inter-cellular propagation. Mutations to the *B. pseudomallei* T3SS causes delayed vacuolar escape in macrophage cell lines and an attenuation of virulence following intranasal challenge in BALB/c mice.

The T3SS pathway is not active outside of animal infection, and therefore, represents a good target for developing therapeutics with a low probability of resistance development. Compounds have been developed that inhibit the T3SS of *B. pseudomallei* in a mixed bacterial - cell culture model. Inhibitory activity of compounds in this model was seen when delivered either pre- or post- infection. These compounds target the T3SS ATPase believed to be involved with removal of chaperone proteins from the T3SS effectors. The ATPase is inhibited by sterically blocking adenosine triphosphate (ATP) from binding in the Walker A motif. The structure of the ATP binding site in the T3SS is highly conserved among the various pathogens utilizing a T3SS. This presents the possibility for broad spectrum use of these compounds.

Through rational drug design, optimized compounds have been designed that are effective against *B. pseudomallei* *in vitro* at a delivered dosage of < 50 nM. In preparation for initial animal efficacy studies, these compounds were studied to determine its preliminary pharmacokinetic properties as well as *in vitro* ADME properties. A new drug that targets *B. pseudomallei* infections would greatly benefit the quality of care given to Warfighters as this bacterium possesses multiple drug resistance mechanisms causing delay in treatment with dangerous and potentially deadly consequences.



**Figure 1. Depiction of the T3SS Components.** The base of the needle is anchored in the inner and outer membrane of the bacterium. The needle extends into the host membrane. The ATPase is located at the base of the needle and has been hypothesized to function by removing a chaperone from the effector proteins. (Taken from Büttner D. and Sheng Yang He. 2009. Type III Protein Secretion in Plant Pathogenic Bacteria. *Plant Physiology* 150:1656-1664.)

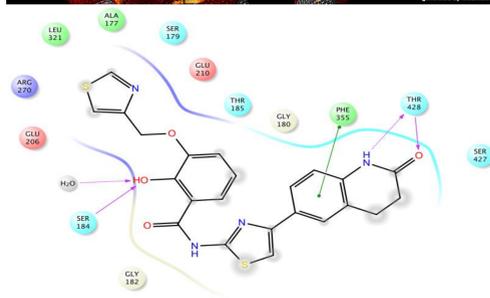
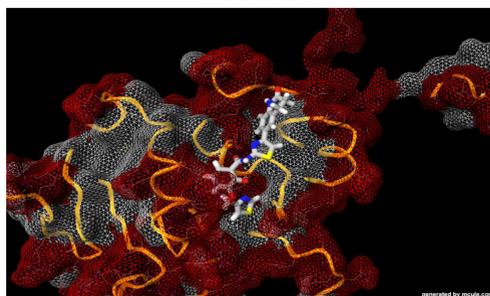
## Methods

**Bacterial agents and preparation:** Frozen stocks of *B. pseudomallei* 1026b were used to inoculate a 3 mL broth culture. The culture was grown overnight at 30° C and diluted immediately prior to infection 1:100 in warm DMEM containing 10% FBS supplemented with inhibitor library compounds dissolved in DMSO or 1% DMSO for the controls.

**Macrophage preparation:** J774.1 murine macrophages were maintained in DMEM containing 5% FBS and antibiotics. One day prior to infection, cells were harvested and seeded into a 96 well plate at a concentration of 1.6 x 10<sup>5</sup> cells/well in DMEM supplemented with 10% FBS w/o antibiotics. The macrophages were then incubated overnight at 37° C with 5.0% CO<sub>2</sub>.

**Infection assay:** The cells were removed from the incubator and the media was aspirated off. The warm bacterial/inhibitor solution was added to each well. The plate was then incubated to 2 hours at 37° C with 5.0% CO<sub>2</sub>. Following the incubation, the bacterial/inhibitor solution was removed from each well and the wells were washed with warm PBS. The macrophages were then incubated in DMEM supplemented with 10% FBS and containing inhibitor compounds/DMSO and kanamycin (to kill extracellular bacteria), and incubated for 3 hours at 37° C with 5.0% CO<sub>2</sub>. Following this incubation, the media was aspirated off and the cells were washed with warm PBS. Ice cold 0.25% Triton X100 was added to the wells in order to facilitate the release of the macrophages from the wells. The solutions were then vortexed to lyse the macrophages and release the intracellular bacteria. The samples were then enumerated by serial dilutions and plating onto growth media. The plates were then counted.

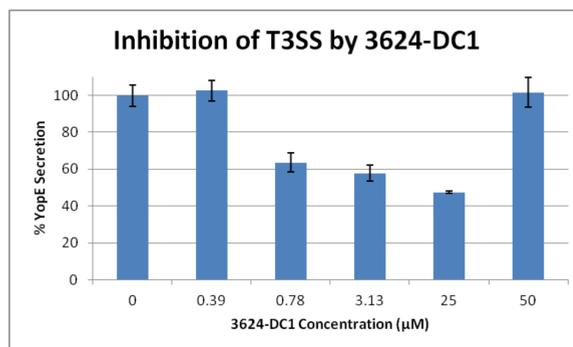
## Results



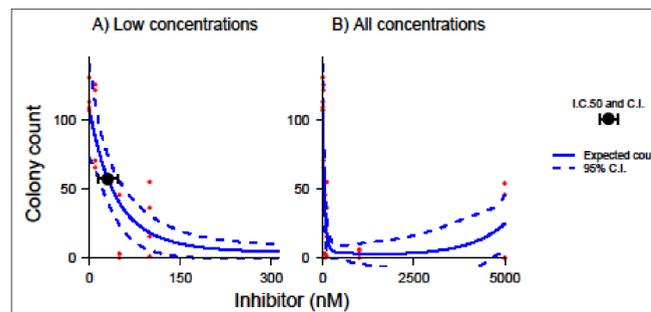
**Figure 2. Docking Analysis of 3624-DC1.** Compound 3624-DC1 was modeled docked with T3SS ATPase EscN, a crystallized homolog of SpaL. **Top**) 3624-DC1 blocks the ATP binding site of the ATPase. The head of the molecule sits outside of the binding pocket while the tail sits into the pocket to block the binding of ATP. **Bottom**) Interaction diagrams of the 3624-DC1-EscN docking model were generated to identify the amino acid residues involved with binding. The *in silico* modeling suggest that 3 hydrogen bonds and 1  $\pi$ - $\pi$  bond is formed during the interaction.

Compound	MMGBS	Docking score	MW	Donor HB	Acceptor HB	logP	o/w
3624-DC1	-66.018797	-4.890598	478.54	2	8.5	3.827	
3624	-59.18134	-4.200674	462.54	2	8.75	3.841	

**Figure 3. Calculated Properties of 3624-DC1.** The Schrödinger QikProp software was utilized to predict the binding strength, Lipinski rule of five properties, and theoretical binding strength for 3624-DC1 its parent compound, 3624. Both compounds were shown to comply with the "druglikeness" set forth by Lipinski. However, the modification of the parent compound increased its theoretical binding strength and proposed solubility. In laboratory testing, both of these theoretical results were confirmed; 3624-DC1 was more active and solubilized easier.

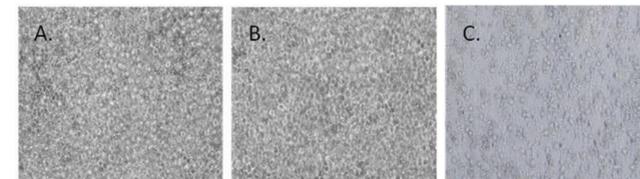


**Figure 4. Inhibition of T3SS by 3624-DC1.** The ability of 3624-DC1 to inhibit Type-III Secretion was tested in a *Yersinia pestis* model. The bacteria was grown at 26C in BHI broth initial and then brought up to 37C to mimic infection of an animal. EGTA was then added to artificially induce activation of the T3SS system. The media was filter sterilized and analyzed by ELISA for the presence of YopE, a T3SS effector molecule. The addition of 3624-DC1 suppressed the secretion of YopE indicating activity.

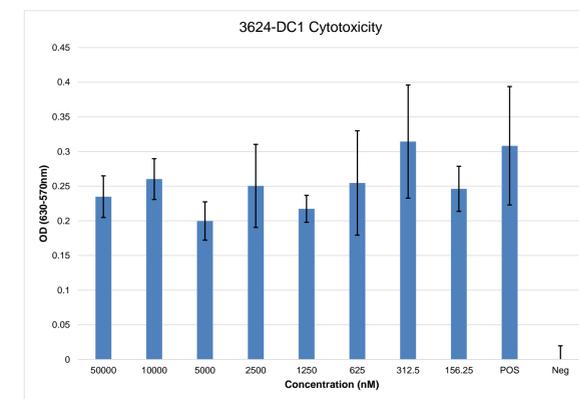


**Figure 5. Inhibition of Burkholderia pseudomallei infection in a Cell Culture Model.** J774.1 macrophages were infected with various strains of *B. pseudomallei* with various concentrations of 3624-DC1 as described in the methods section. The statistical analysis of the plate counts was used to determine the EC<sub>50</sub> against each strain. The data demonstrate that the compound was active against multiple strains of the bacteria utilizing differing resistant mechanisms suggesting the 3624-DC1 is able to circumvent the mechanisms.

<i>B. pseudomallei</i> strain	EC <sub>50</sub> (nM)
1026B	12.82
K96243	44.39
406e	68.02
MSHR5885	64.41
HBPU10134A	34.96



**Figure 6. The Effects of Inhibitor Compounds Added Post-Infection.** J774.1a mouse macrophages were infected with *Burkholderia* sp. With an efflux resistance for 2 hours (1B and 1C) prior to being overlaid with agarose containing kanamycin media with and without inhibitor. After 24 hours, the inhibitor-treated cells appear to remain confluent similar to the non-infected control (1A). The untreated cells show significant lysis and pathogenesis.



**Figure 7. Cytotoxicity of 3624-DC1.** To determine what, if any, level of cytotoxicity compound 3624-DC1 had an MTT assay was performed using VERO cells. The cells were grown overnight in the presence of various concentrations of 3624-DC1. 10% DMSO was used as the cytotoxic control. The following day the MTT reaction was performed as per the manufacturer's instructions. All the way up to 50,000 nM the compound was shown to not have significant cytotoxic effects.

Route of Administration	Observed C <sub>max</sub> (ng/mL)	Observed C <sub>max</sub> /Dose (ng/mL/kg)	Observed T <sub>max</sub> (hr)	Terminal Elimination Half-Life (hr)	Clearance (mL/hr/kg)	Volume of Distribution (mL/kg)	AUC <sub>last</sub> (hr*ng/mL)	AUC <sub>∞</sub> (hr*ng/mL)	AUC <sub>∞</sub> /Dose (hr*ng/mL/kg)
IP - 1 mg/kg	1030	1030	0.5	1.02	380	557	2620	2630	2630

**Figure 8. Preliminary Pharmacokinetic Properties 3624-DC1.** 3624-DC1 was administered to mice via intraperitoneal (IP) to determine the compounds properties in an animal model. 3624-DC1 was rapidly absorbed after IP administration with an observed T<sub>max</sub> of 30 minutes. However, due to the similarity of the 15 and 30 minute concentrations, the T<sub>max</sub> may actually be earlier than the observed 30 minute value. As dosage was 1 mg/kg, the compound had approximately 100% bioavailability. The elimination half-life was determined to be 1.02 hours.

## Discussion

*Burkholderia pseudomallei* is a biological warfare agent that processes a number of naturally occurring antibiotic resistance mechanisms. Studies have shown that knocking out the T3SS virulence mechanism makes that bacteria avirulent. Compound 3624-DC1 has been developed to target the ATPase of the *B. pseudomallei* T3SS. The compound was shown to inactivate the T3SS permitting the destruction of the bacteria by immune system cells. It was even shown to be effective against multi-drug resistant (MDR) *B. pseudomallei* strain 1026b expressing both the BpeAB-OprB and AmrAB-OprA-mediated efflux systems.

This inhibition was shown to both a pre-infection and post-infection model. The (EC<sub>50</sub>) in a mammalian macrophage cell culture model of 66 ng/mL with a 95% CI of 36 to 119 ng/mL against *B. pseudomallei* strain 1026b, this compares favorably with published Minimum Inhibitory Concentrations (MIC) of Ciprofloxacin, Doxycycline, and Tetracycline (~500 ng/mL) against the same strain. Previously, we have demonstrated that the parent compound, 3624, was effective against other T3SS utilizing bacteria such as Shigella, Salmonella, and EPEC suggesting the broad usefulness of the compound. The safety index (CC<sub>50</sub>/EC<sub>50</sub>) for 3624-DC1 in J774.1a mouse macrophages was shown to be >300 as no toxicity was seen at the highest tested concentration of 50 µM while the compound had an EC<sub>50</sub> of ~13 nM.

The pK data demonstrates that the compound has a 100% bioavailability when given via IP. The pK data also suggest that the compound is non-toxic to animals and is cleared from the system by the liver. Current efforts are ongoing to further optimize the compound in preparation for pre-IND studies. Creating a drug against *B. pseudomallei* that is able to circumvent MDR will increase the quality of care for the Warfighter.

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