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14. ABSTRACT

The threat of bioterrorism and the use of biological weapons against both military personnel and civilian populations has become an increasing concern for governments around the world. The 1984 Rajneeshee Salmonella attack, 2001 anthrax letter attacks, 2003 SARS outbreak, 2009 H1N1 swine flu pandemic, and the current US flu epidemic all illustrate our vulnerability to both deliberate and natural outbreaks of infectious disease and underscore the necessity of effective antimicrobial and antiviral therapeutics. The prevalence of antibiotic resistant strains and the ease by which antibiotic resistance can be engineered into bacteria further highlights the need for continued development of novel antibiotics against new bacterial targets. This research project directly addresses this need through the development of a broad spectrum inhibitor of the biothreat agents *Francisella tularensis* and *Yersinia pestis*.

During this period of performance, we have utilized our optimized assays with the *Y. pestis* MEP synthase and the *F. tularensis* MEP cytidylyltransferase to screen molecular libraries and identify effective inhibitors of both MEP synthase and MEP cytidylyltransferase.

15. SUBJECT TERMS
Enzymology, MEP pathway, bacteria, isoprene, drug discovery, antibiotics, screening, biothreat, inhibitors

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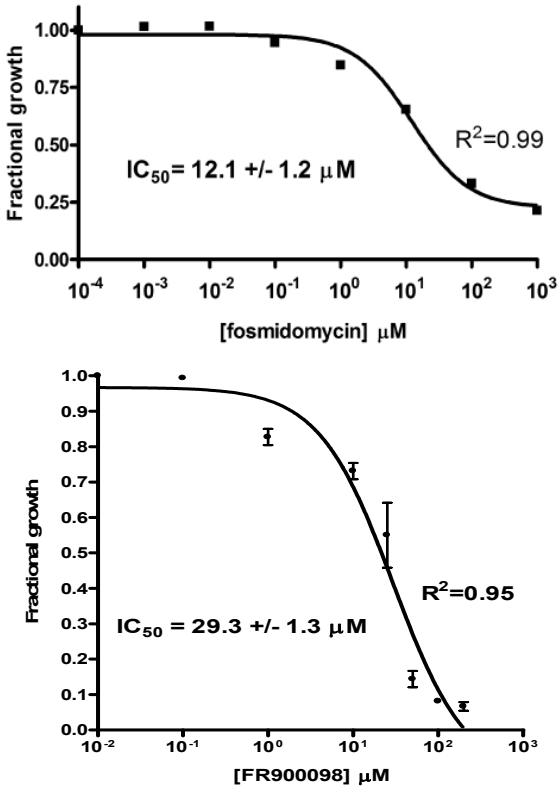
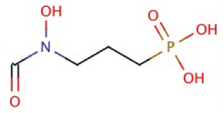
Introduction

The long term objective of this research is to identify and develop a broad spectrum inhibitor of *Francisella tularensis* and *Yersinia pestis*. The methylerythritol phosphate (MEP) biosynthetic pathway of *Francisella tularensis* and *Yersinia pestis* provide multiple enzymes that may be targeted for inhibitor development. This pathway is utilized by bacteria, apicomplexan protozoa, and plants for isoprenoid biosynthesis. Isoprenic compounds are vital for cellular processes such as electron transport, cell wall and membrane biosynthesis, and signal transduction. Despite their structural and functional diversity, all isoprenoids are derived from two building blocks, isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which originate from either the MEP pathway or the mevalonic acid (MVA) pathway depending on the organism. Humans acquire isoprenes through the nonhomologous MVA pathway, making enzymes in the MEP pathway very attractive targets for antimicrobial development.

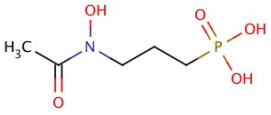
Body

We hypothesize that inhibitors of the MEP pathway in *Francisella tularensis* and *Yersinia pestis* will serve as effective antibiotics by blocking isoprene biosynthesis. In strong support of this hypothesis, we have demonstrated the dose-dependent inhibition of *F. tularensis* and *Y. pestis* growth *in vitro* using the compounds fosmidomycin and FR900098 (Figure 1).





Fosmidomycin



FR900098

Figure 1. Growth inhibition of *F. tularensis* (top) and *Y. pestis* (bottom) by the compounds fosmidomycin and FR900098 (these molecules inhibit MEP synthase, an enzyme in the MEP pathway). The structures of the compounds are shown.

To test this hypothesis, the Couch lab at George Mason University is collaborating with Walter Reed Army Institute of Research (WRAIR) in the screening of compound diversity libraries using enzyme-based assays for lead inhibitor discovery, evaluation of lead inhibitors in microbial growth assays, determining X-ray crystal structures of the MEP pathway enzymes MEP synthase and MEP cytidylyltransferase in complex with inhibitors, and using this information to design and synthesize novel broad spectrum antibacterials.

MEP Synthase (IspC)

During this period of effort, we used a combination of affinity column chromatography, mass spectrometry, and enzymology to definitively identify the active component in e29, the natural product inhibitor described in previous reports. A manuscript detailing this research is currently in preparation. As this inhibitor is the first of its kind, able to allosterically inhibit the enzyme, crystal screens with the *Yersinia pestis* MEP Synthase in complex with the inhibitor are presently underway.

As depicted in the Figure below, we have also performed a high-throughput screen of MEP Synthase using a commercially purchased molecular library (LOPAC library). Due to its wide dynamic range of activity, the primary screen was performed with the *Y. pestis* MEP Synthase. To ensure broad spectrum activity, a secondary screen was then performed with purified

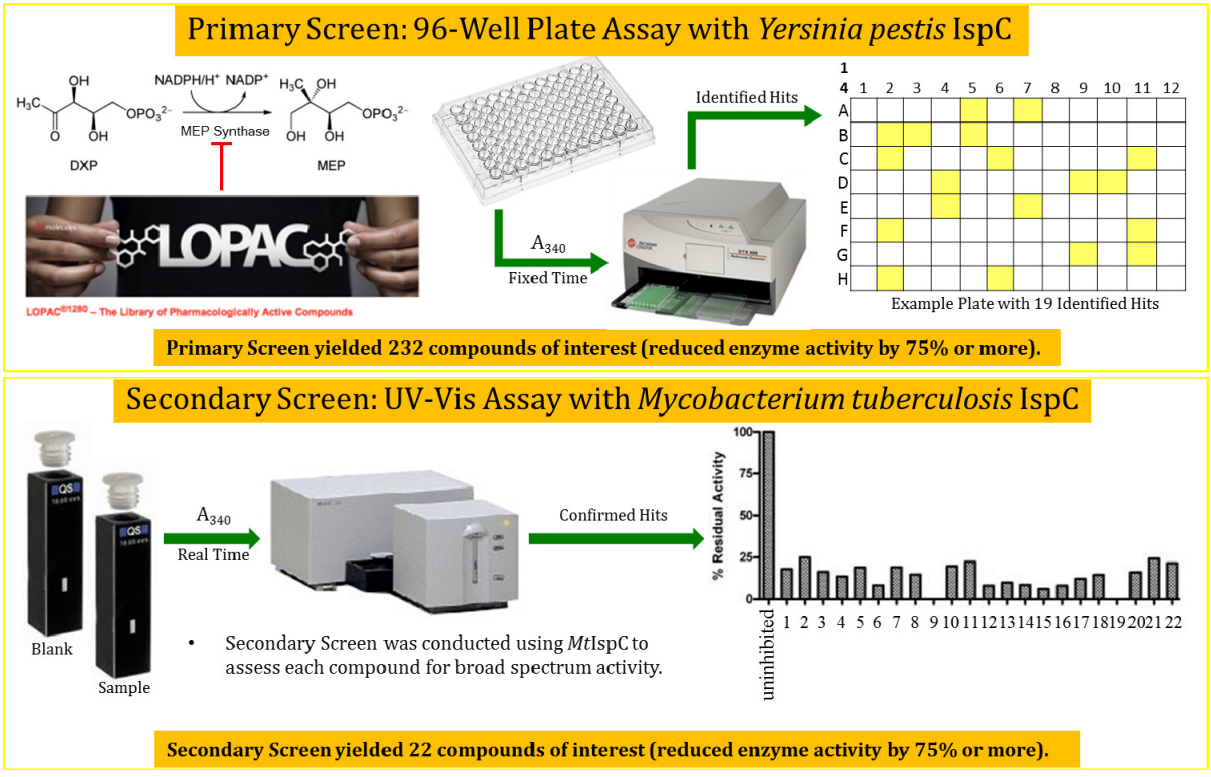


Figure 2. Schematic of the approach to performing the high-throughput screen with IspC.

recombinant *Mycobacterium tuberculosis* MEP Synthase and the 232 hits identified in the primary screen. To eliminate false positives (compounds that have innate absorption at 340 nm, thereby interfering with the NADPH signal (ΔA_{340})), a tertiary screen was then performed, using an assay coupling MEP Synthase with MEP Cytidylyltransferase (IspD), as illustrated in Figure 3. The seven molecules demonstrating inhibition in both the single and coupled assays are considered hit compounds. Bacterial growth inhibition assays with these top seven compounds are currently underway. Screening of a second commercially available molecular library is also underway.

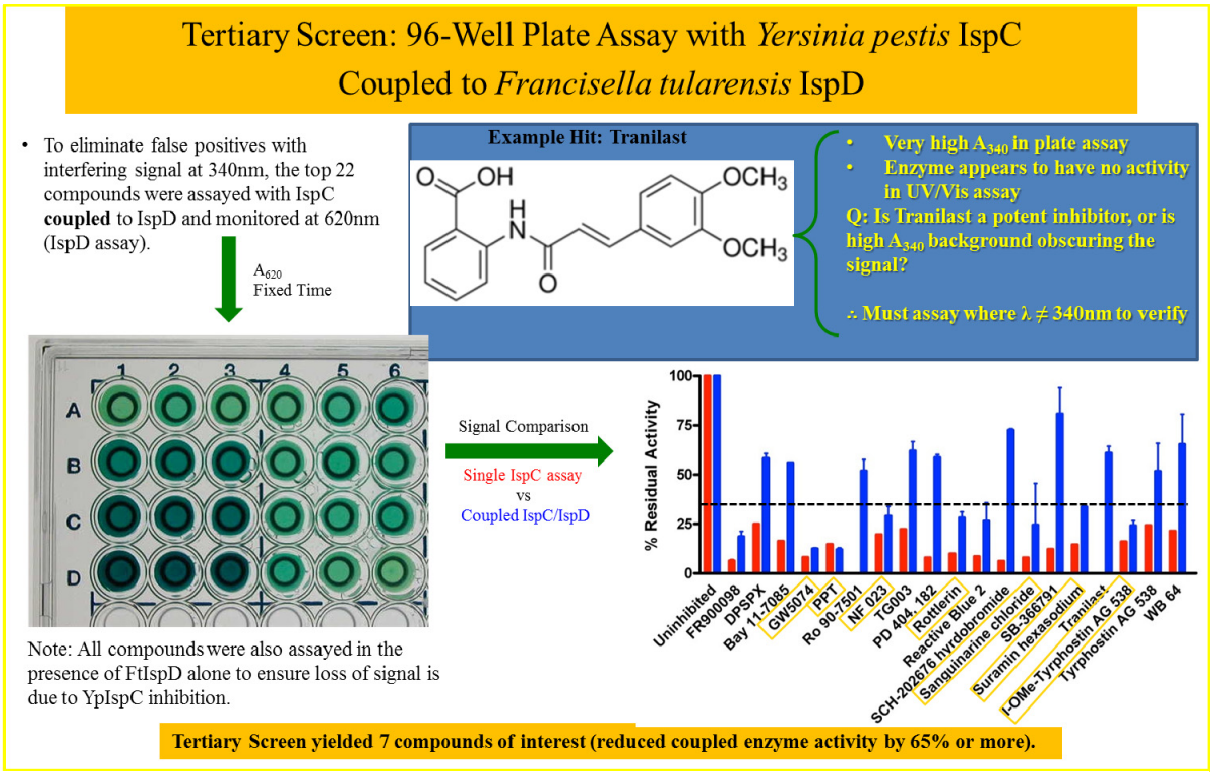
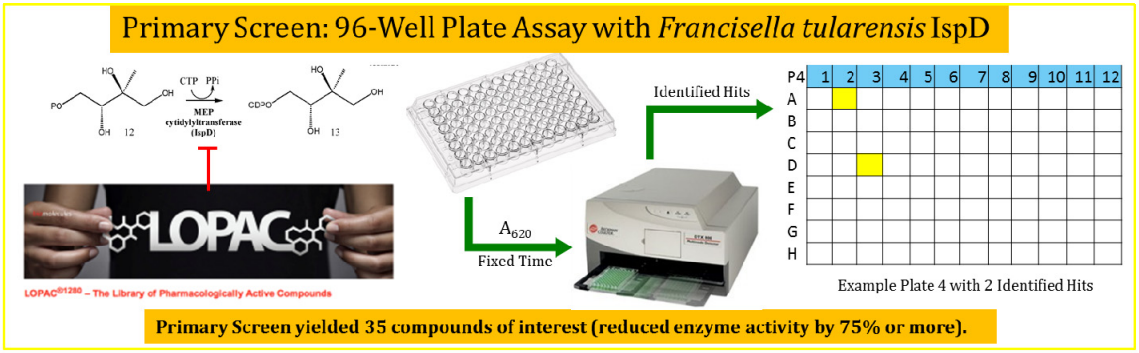


Figure 3. Schematic of the approach to performing the tertiary screen with IspC.

MEP Cytidylyltransferase (IspD)

As illustrated in Figure 4, we have also performed a high-throughput screen with the *Francisella tularensis* MEP Cytidylyltransferase. As the enzyme assay is coupled to the activity of pyrophosphatase, a tertiary screen was performed with the latter enzyme alone to assure the hit compounds are selective for IspD (Figure 5). Bacterial growth inhibition assays with these top five compounds are currently underway. Screening of a second commercially available molecular library is also underway.



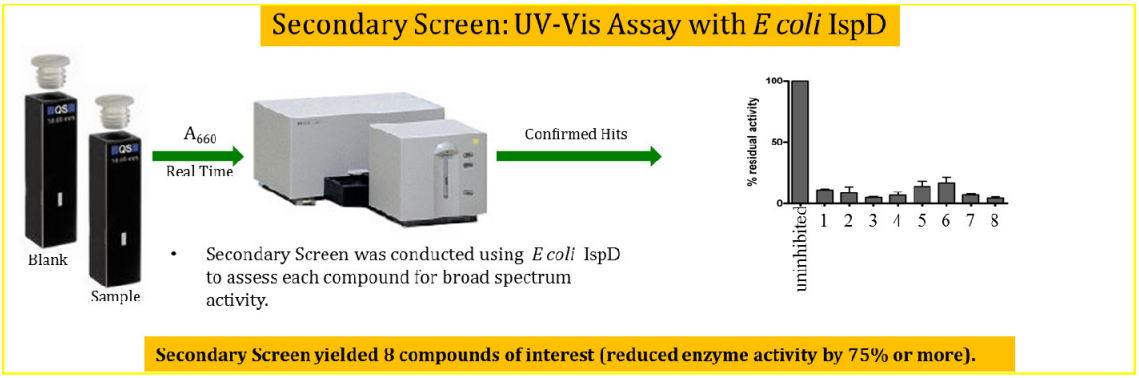


Figure 4. Schematic of the approach to performing the high-throughput screen with IspD.

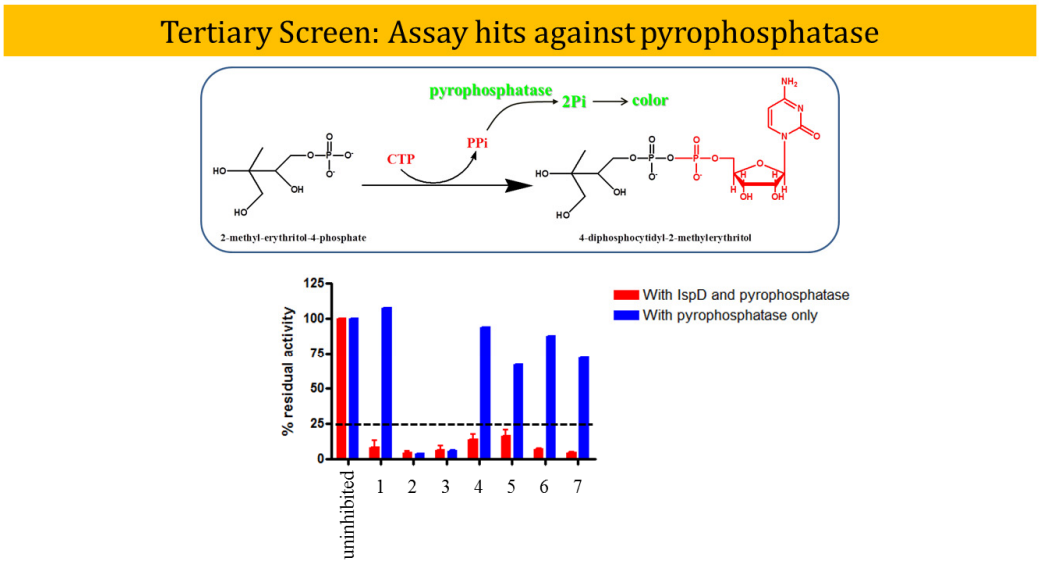


Figure 5. Schematic of the approach to performing the tertiary screen with pyrophosphatase and IspD.

Key Research Accomplishments

- Optimization of MEP synthase and MEP cytidylyltransferase assays for HTS.
- Screening of molecular libraries for inhibitors of MEP synthase and MEP cytidylyltransferase.
- Identification of a previously unknown allosteric site on the MEP synthase enzyme from *F. tularensis*, *Y. pestis*, and *M. tuberculosis*.
- On-demand production and delivery of recombinant proteins to WRAIR for X-ray crystallography.

Reportable Outcomes

Haymond A, Johny C, Dowdy T, Schweibenz B, Villarroel K, Young R, Mantooth CJ, Patel T, Bases J, Jose GS, Jackson ER, Dowd CS, and **Couch RD**. Kinetic Characterization and Allosteric Inhibition of the Yersinia pestis 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase (MEP Synthase). PLoS One. 2014 Aug 29;9(8):e106243.

Chofor, R., Risseeuw, M.D., Pouyez, J., Johny, C., Wouters, J., Dowd, C.S., Couch, R.D., Van Calenbergh, S. Synthetic Fosmidomycin Analogues with Altered Chelating Moieties Do Not Inhibit 1-Deoxy-D-xylulose 5-phosphate Reductoisomerase or *Plasmodium falciparum* Growth *In Vitro*. Molecules. 2014; 19(2):2571-2587.

Jackson, E.R., San Jose, G., Brothers, R.C., Edelstein, E.K., Sheldon, Z., Haymond, A., Johny, C., Boshoff, H.I., Couch, R.D., and Dowd, C.S., The effect of chain length and unsaturation on Mtb Dxr inhibition and antitubercular killing activity of FR900098 analogs. Bioorganic & Medicinal Chemistry Letters, 2014 Jan 15;24(2):649-53.

- Funds for this project are used to support a lab technician (Ms. Chinchu Johny) and a graduate student (Ms. Amanda Haymond).

Conclusion

In summary, during this fiscal period, we have optimized assays with the *Y. pestis* MEP synthase and the *F. tularensis* MEP cytidylyltransferase for use in HTS. The screening of a commercially available library has identified several hit molecules for each of these enzymes. We have also identified the natural product allosteric inhibitor of MEP synthase.

During the next fiscal period, we will focus on screening a second large and chemically diverse molecular library for additional inhibitors of MEP synthase and MEP cytidylyltransferase.

References

1. Jawaid S, Seidle H, Zhou W, Abdirahman H, Abadeer M, et al. (2009) Kinetic characterization and phosphoregulation of the Francisella tularensis 1-deoxy-D-xylulose 5-phosphate reductoisomerase (MEP synthase). PLoS ONE 4: e8288. doi:10.1371/journal.pone.0008288.

2. Tsang A, Seidle H, Jawaid S, Zhou W, Smith C, et al. (2011) Francisella tularensis 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase: kinetic characterization and phosphoregulation. PLoS ONE 6: e20884. doi:10.1371/journal.pone.0020884.

3. Zhang, Chung, Oldenburg (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 4: 67–73.

4. Henriksson LM, Unge T, Carlsson J, Aqvist J, Mowbray SL, et al. (2007) Structures of Mycobacterium tuberculosis 1-deoxy-D-xylulose-5-phosphate reductoisomerase provide new insights into catalysis. J Biol Chem 282: 19905–19916. doi:10.1074/jbc.M701935200.

5. Björkelid C, Bergfors T, Unge T, Mowbray SL, Jones TA (2012) Structural studies on Mycobacterium tuberculosis DXR in complex with the antibiotic FR-900098. Acta Crystallographica Section D 68: 134–143. doi:10.1107/S0907444911052231.

6. San Jose G, Jackson ER, Uh E, Johny C, Haymond A, et al. (2013) Design of Potential Bisubstrate Inhibitors against Mycobacterium tuberculosis (Mtb) 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase (Dxr)-Evidence of a Novel Binding Mode. Medchemcomm 4: 1099–1104. doi:10.1039/C3MD00085K.

7. Koppisch AT, Fox DT, Blagg BSJ, Poulter CD (2002) E. coli MEP synthase: steady-state kinetic analysis and substrate binding. Biochemistry 41: 236–243.