MLSCN Probe Summary

SOUTHERN RESEARCH MOLECULAR LIBRARIES SCREENING CENTER
The Drug Discovery Division of Southern Research

Screening Center: Southern Research Molecular Libraries Screening Center

PI of center: Gary A. Piazza, Ph.D.
Project Title: Screening for Inhibitors of the Mevalonate Pathway in *Streptococcus pneumoniae* 1: Inhibitors of Mevalonate Kinase (MK)
Grant Number: 1X01-MH-078936
Assay Submitter and Institution: Dr. Thomas Leyh, Albert Einstein College of Medicine
Assay or Pathway Target: *S. pneumoniae* MK in isoprenoid/steroid pathway
Probe PubChem Compound Identifier (CID): 16750003

Assay Provider Information

Specific Aim: To identify specific probes that inhibit the MK enzyme, the first of three enzymes in the mevalonate pathway of *S. pneumoniae*.

Significance: *S. pneumoniae* exacts an enormous toll on humanity. This pernicious organism kills approximately 3700 people per day, the majority of whom are children below the age of five (1). The populations at greatest risk for suffering life-threatening infections are the elderly and the young, immunosuppressed and diabetic individuals, and those with hematologic malignancies and renal failure (2). In the United States, on a per annum basis, the organism is estimated to cause 3000 cases of meningitis, 50,000 cases of bacterimia, 500,000 cases of pneumoniae, and 7 million episodes of acute otitis media (inner ear infection) that result in one million doctor visits and 5 billion dollars in related expenses (2).

Mankind has long suffered the debilitating consequences of *S. pneumoniae* infection. The organism was first isolated by Louis Pasteur in 1881 and was identified as a primary cause of lobular pneumonia soon thereafter (3). There are now more than 90 distinct serotypes (46 serogroups), each with a different propensity to cause human disease (4, 5). Previous battles with this organism have shown that our current strategies (vaccines and antibiotics) provide temporary solutions, rather than endpoints, for the problem. During the last century, multiple antibiotic classes were brought forward against the organism which responds, seemingly inevitably, using a combination of spontaneous mutagenesis, DNA transfer among related organisms, and amplification by positive selection, to circumvent the metabolic blockages set in place by the antibiotics (3, 4). Once an antibiotic-resistant strain emerges, it spreads quickly *via* colonization - pneumococci are found in the nasopharynx of 15% of well adults and up to 65% of children in child-care settings. It is generally agreed that successful containment of this organism requires constant vigilance (large-scale antibiotic-resistance surveillance efforts are underway at the Centers for Disease Control), the stringent use of antibiotics, and a pipeline of drugs and vaccines that enable us to respond quickly to changes in the streptococcal population. Unfortunately, the development of new antibiotics is no longer considered economically feasible by the pharmaceutical industry (6). Hence, our pipelines are drying up even as strains resistant to our last-line

antibiotics are beginning to appear. (7-11). The current pharmaco-economic landscape suggests that the financial burden for future antibiotic development will fall primarily on the shoulders of governmental funding agencies in economically advantaged countries. The threat of domestic and foreign strains of multiple-drug-resistant *S. pneumoniae* continues to mount, and is extremely serious (12-14).

Rationale: Recent work has uncovered a molecular Achille's heal in *S. pneumoniae* - mevalonate kinase, the first enzyme in the mevalonate pathway harbors an allosteric site that can be used to switch-off isoprenoid biosynthesis, which is essential for growth of the organism (15). *S. pneumoniae* mutants in which the mevalonate pathway has been inactivated are unable to survive in the mouse lung, and the levels of mevalonate in serum appear insufficient to support their growth (16, 17). These important biological studies validate the mevalonate pathway as a target for antimicrobial research.

The Mevalonate Pathway. The pathway is comprised of three consecutive reactions (see Fig. 1) that are catalyzed by the enzymes mevalonate kinase (MK), phosphomevalonate kinase (PMK) and diphosphomevalonate kinase decarboxylase (DPM-DC). The end-product of the pathway, isopentenyl diphosphate, is the 5-carbon building block used for the biosynthesis of isoprenoids, a diverse family of roughly 23,000 biologically active small molecules that includes cholesterol, steroid hormones, bile acids, electron transport carriers, carotenoids, vitamin A, Taxol, farnesyl diphosphate, and numerous other interesting compound classes. Given the ubiquity and metabolic significance of isoprenoids, it is not surprising that the mevalonate pathway is considered essential for the survival of organisms that require it for isoprenoid biosynthesis.

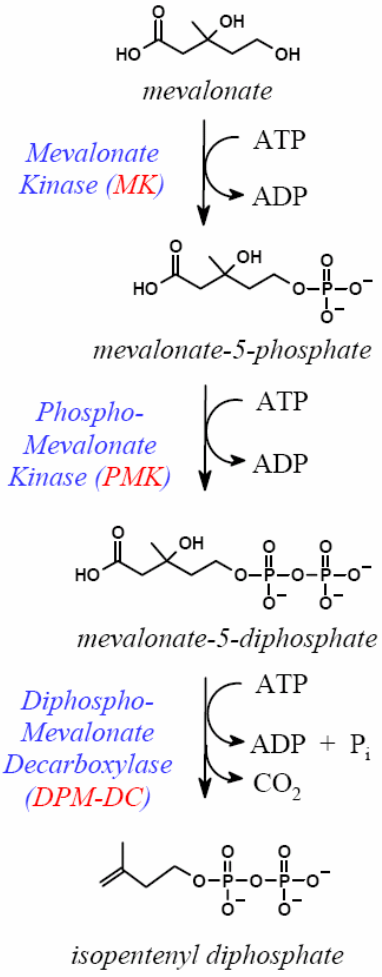


Fig. 1. The Mevalonate Pathway

The screening assay. Because they are all kinases, the turnover of each of the enzymes in the mevalonate pathway can be monitored using the same, robust assay – the pyruvate kinase (PK) lactate dehydrogenase (LDH) assay (Figure 2). In all cases, the reactions proceed with little sign of product inhibition until essentially all of the available substrate is converted to product. Among the advantages of this assay are: ***i***, ATP is regenerated from ADP (which avoids both substrate, ATP, depletion and product, ADP, inhibition); ***ii***, the extremely favorable energetics of the PK and LDH reactions place a large, favorable thermodynamic bias on the primary kinase reaction; ***iii***, the oxidation of NADH is monitored readily either by a change in absorbance (= 6.22 mM * NADH ε -1 cm-1, ~ 0) or fluorescence (λ * NAD ε ex = 339 nm , λem = 460 nm).

Screening Center Information

Assay Implementation and Screening

- **PubChem Bioassay Name:** Screening for Inhibitors of the Mevalonate Pathway in Streptococcus Pneumoniae - MK.

- **PubChem Bioassay AIDs**: 555, 557, 1000

- **Primary Assay Description (PubChem)**: Mevalonate kinase (MK; E.C. 2.7.1.36) catalyzes the ATP-dependent conversion of (R)-mevalonate to ADP plus (R)-5-phosphomevalonate. The activity of MK was measured spectrophotometrically by coupling the formation of ADP to the reactions of pyruvate kinase and lactate dehydrogenase. The rate of ADP formation was quantitated by the reduction of absorbance (OD340) due to the oxidation of NADH to NAD by lactate dehydrogenase. A kinetic assay was chosen to minimize interference by compounds that absorbed at 340 nm.

  The NIH small molecule repository was initially screened at 10 μM for MK inhibitory activity. Three hundred and twenty compounds that displayed the highest %inhibition were scored as hits and re-tested at ten concentrations ranging from 0.2 to 100 μM. To confirm that the hits specifically inhibited MK and not other coupling enzymes used in the assay, the hits were tested in parallel in a counter-screen that contained only these 'coupling enzymes' in the absence of MK.

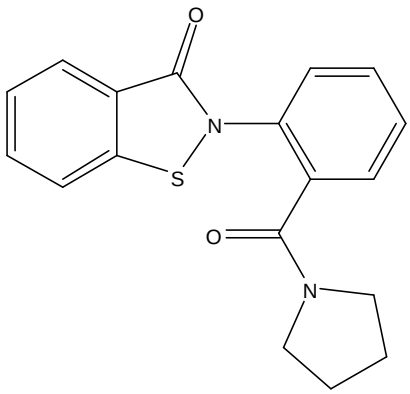
- **Center Summary of Primary Screen:**
  A total of 65,241 compounds were initially screened at a final concentration of 10 μM. Z' scores for this assay were consistently greater than 0.75 demonstrating that this is a very robust assay. The mean inhibition of the compound wells was 5.35% with a standard deviation of 5.20%. Inhibition values of greater than 20.95% were outside the calculated noise of the assay defined by $\bar{x}$ + 3σ.

  Three hundred and twenty compounds that had the highest %inhibition were scored as hits. These hits were then tested in dose response at ten concentrations, ranging from 100 μM to 0.196 μM. To confirm that the compounds were specifically inhibiting MK and not one of the other enzymes in the assay, the hits were tested in parallel in an assay that contained only these 'coupling enzymes'. Eighty-one compounds were determined to specifically inhibit MK. IC_{50} values ranged from 0.5 - >100 μM.

Probe Optimization

- Chemical name of probe compound: 2-[2-(pyrrolidine-1-carbonyl)phenyl]-1,2-benzisothiazol-3-one

- Chemical Structure (incl stereochem if known):



- Describe mode of action for biological activity of probe:
  This probe has an EC_{50} versus *S. pneumoniae* MK of 510 nM.

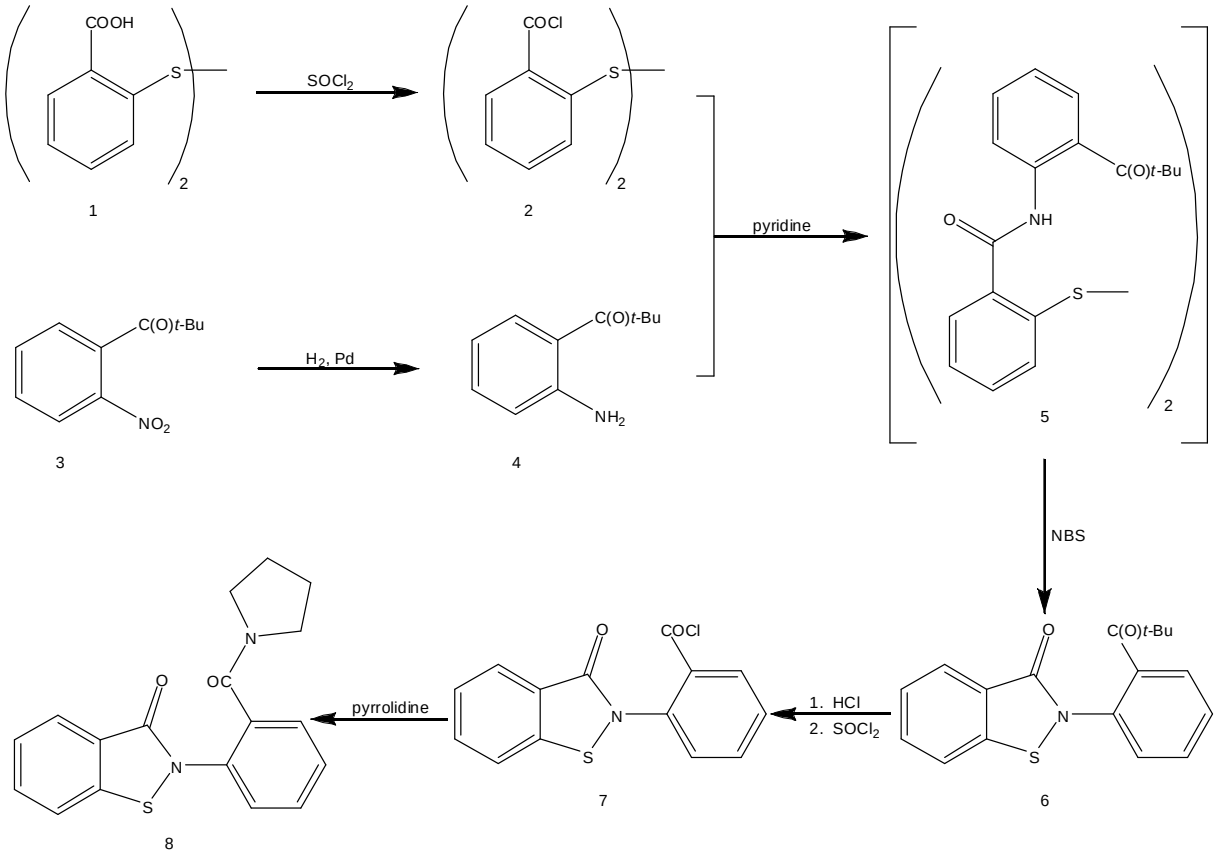
- Has this compound been provided to the MLSMR?:
  It has been approved and bar-coded vials have been requested from DPI for submission of this compound and analogs.

- Description of secondary screens used to optimize probe structure:
  Probe optimization relied primarily upon in-house measurement of EC_{50}s versus MK, while concurrently monitiring EC_{50}s versus the related enzyme, diphosphomevalonate decarboxylase (DPMDC) to ensure specificity. Currently, K_is and additional data are being collected in the assay provider's laboratory.

- Center comments on chemistry strategy leading to probe identification:

As always when appropriate analogs are available, chemistry began with purchase of a limited set of commercial benzothiazolones related to the initial hit to gather preliminary structure-activity (SAR) data. Notably, the initial hit bore an *N*-phenylsulfonamide substituent rather than the *N*-phenylcarboxamide moiety found in the final probe. We made an early strategic decision to prepare and test the corresponding *N*-phenylcarboxamide analog of the original hit, since there were many more commercially available starting materials for preparation of these compounds compared to the sulfonamide analogs. The carboxamides proved quite active, so in the end a series of 35 *N*-phenylcarboxamides was prepared and tested, resulting in identification of the current probe.

- Detailed synthetic pathway for making probe:



Experimental:
Synthesis of **2**:
A suspension of **1** (15 g) in of $SOCl_2$ (50 mL) was refluxed overnight to afford a clear solution, which was distilled under vacuum to give **2** as a solid that was used in the next step without further purification.

Synthesis of **4**:
2-Nitrobenzoyl chloride (25g) was treated with anhydrous *t*-butyl alcohol (40 mL) in anhydrous pyridine (40 mL). The solution was stirred for 1h, then evaporated to give an oil which was then dissolved in ethyl acetate (300 mL) and washed with water (200mL x 2). Removal of solvent gave **3** as an oil. Compound **3** was dissolved in ethanol (150 mL) and acetic acid (5 mL). Pd on carbon catalyst (0.5g, 5%) was added to the solution, which was then hydrogenated at 50 psi overnight. TLC showed a new fluorescent spot under UV. The solvent was removed, and the residue was recrystallized from ethyl ether/hexane to give **4** as a colorless solid. The yields were relatively poor for this *ortho*- series compared to the corresponding *meta*- and *para*-substituted analogs.

Synthesis of **6**:
A mixture of **2** (8.5g, 25 mmole) and **4** (9.5g, 50 mmole) in anhydrous pyridine (60 mL) was stirred for 1 h. The solution was poured into ice-waterand the sticky gum collected and treated with warm toluene. The precipitate was collected by filtration to give **5** as a colorless solid (5.5g, 33.6%). The filtrate was evaporated, the residue dried as a foam containing additional **5**. Treatment of **5** (5.5g) in anhydrous pyridine and chloroform with *N*-bromosuccinimide (5.0g in 20 ml of dioxane) gave **6**, which was purified by column chromatography on silica gel (4.25g, 78%).

Synthesis of **7**:
Compound **6** (4.5g) was dissolved in ethanol (50 mL) containing concentrated HCl (4 mL). The solution was stirred for 2 h, then concentrated. The residue was dried *in vacuo* for 2 hours, after which 40 mL of $SOCl_2$ was added. The mixture was refluxed until a clear solution was obtained (generally overnight for the *ortho*- series). The solution was evaporated to afford a solid, which was used for the next step without further purification.

Synthesis of **8**:
To a 25 mL round-bottomed flask containing anhydrous chloroform (7 mL), imidazole (2.2 mmole), and **7** (0.52 mmole), pyrrolidine (0.75 mmole) was added. The solution was stirred under an atmosphere of argon overnight, whereupon 0.3 mL of acetic acid was added. After 4 min the mixture was evaporated. Crude **8** was purified using a silica gel column eluted with hexane/ethyl acetate, giving **8** as a colorless solid.

- Center summary of probe properties (solubility, absorbance, fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:

  There are no unusual properties known for this compound in terms of fluorescence, reactivity, toxicity, though limited toxicity data is described below. The probe should be useful for inhibition of the first step in the bacterial isoprenoid biogenetic pathway without inhibition of the subsequent enzymatic steps.

Appendices:

Comparative data on probe, similar compound structures and prior probes:
As this study began, the Leyh laboratory had ascertained that a proprietary, charged, fluorinated mevalonate analog was an inhibitor of this enzyme. However, the charged phosphate moiety reduced cellualr uptake, rendering it unsuitable for many studies. In addition, the chemical structure of this compound is unknown to us. Other analogs of phosphomevalonate would be expected to inhibit the enzyme as well, but would suffer the same shortcomings.

Comparative data showing probe specificity for target:
The Leyh laboratory is currently isolating the human homolog of this bacterial MK, which will provide useful information about the true specificity of this probe. That data may not be available for some time. We have demonstrated in-house that the probe has antibacterial activity against *S. pneumoniae*, and also against *Enterococcus faecalis*, but not againsy *Staphylococcus aureus*. Additionally, while many of the benzisothiazolones that we prepared possess dual inhibitory activity against both MK and DPMDC, particularly in the para-substituted series (see corresponding probe report), this probe is inactive versus DPMDC.

We have also tested the probe for cytotoxicity in A549 cells. In that system, this compound had an TC_{50} of 13.7 μM, ~27-fold higher than its IC_{50} against the bacterial enzyme and suggesting some specificity.

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