Spectrophotometric assay for dapE-encoded N-succinyl-L,L-di aminopimelic acid desuccinylase, a potential antibiotic target

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Introduction

Antibiotic resistance is a problem of utmost importance in the medical community. Because there have been very few breakthroughs toward new antibiotics in recent years, it is critical to explore new ways to inhibit bacterial proliferation and growth. The fact that many antibiotics operate on similar control mechanisms only intensifies the urgency to develop antibiotics with new mechanisms of inhibition to avoid resistance. There are several intrinsic differences between prokaryotic (bacterial) and eukaryotic (animal) cells. Unfortunately, the large majority of current antibiotics all take advantage of ribosomal differences which means some bacteria have evolved new ribosomal techniques to avoid being killed by several current antibiotics, thus the rise of antibiotic resistance. *N*-Succinyl-L,L-diaminopimelic acid desuccinylase enzyme (DapE) is an enzyme in the late stage of the *meso*-diaminopimelic/lysine biosynthetic pathway (**Figure 1**). DapE is a dimetalloenzyme that catalyzes the substrate *N*-succinyl-L,L-diaminopimelic acid (L,L-SDAP) to succinate and diaminopimelate (DAP) (**Scheme 1**)[3]. DapE activity results in production of *meso*-diaminopimelate (mDAP) from DAP which is ultimately converted to lysine. Lysine and mDAP are both essential components for peptidoglycan cell wall synthesis. This succinate enzymatic pathway is found in all Gram-negative and most Gram-positive bacteria.[1] Because of this, DapE has been identified as an attractive bacterial target.[3]

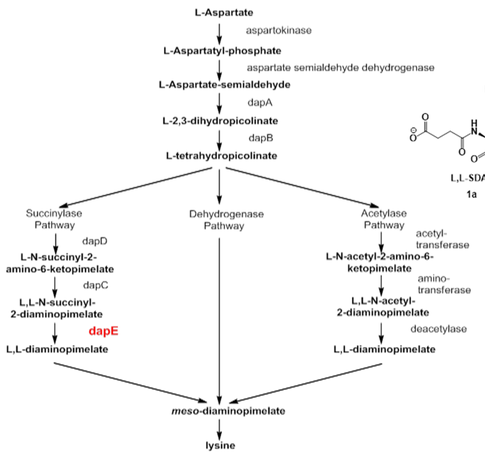
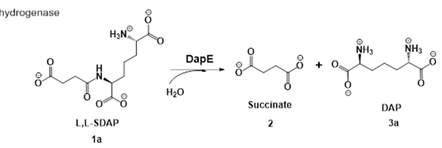
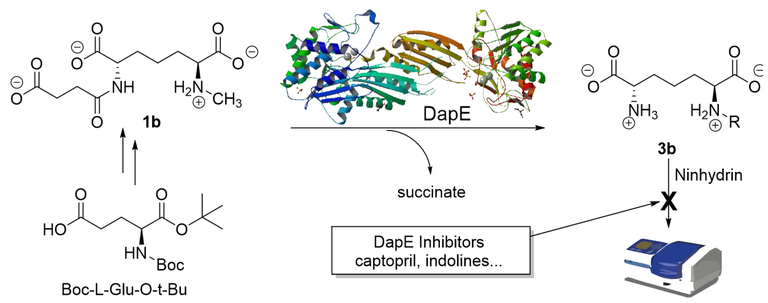


Figure 1. Bacterial mDAP/lysine synthetic pathway



Scheme 1.1 DapE enzymatic reaction



Scheme 1.2 Modified substrate *N*-methyl-SDAP synthesis and *N*-methyl-SDAP in assay

Abstract

The previously most widely used DapE assay monitors amide bond cleavage of the L,L-SDAP substrate spectrophotometrically at 225 nm.[1] This assay is straightforward and reliable for structurally simple inhibitors, but it cannot be used to test potential inhibitors that absorb strongly in the UV region, precluding its use in testing many preferred medicinal chemistry leads and analogs. Ninhydrin-based assays provide ease of application and reliability to detect primary amino groups such as that formed upon the hydrolysis of L,L-SDAP. We synthesized N^6-methylated and N^6-acetylated L,L-SDAP derivatives to prevent ninhydrin side reactions in the assay and also tested them as viable substrates for the *Haemophilus influenza* DapE (HiDapE) used in the assay. The data showed that the *N*-methylated derivative was both less reactive with ninhydrin and also was a better suited substrate for the HiDapE. In addition to the development of the assay itself we have also used a high throughput coupled screening to identify a number of lead HiTS (**Figure 2**) that show binding potential to DapE. These compounds will be tested as inhibitors themselves, while also being the basis for potential derivatives. The synthesis of analogs of these derivatives will be guided by structure activity relationships (SAR) as well as Lipinski's rule of five, a general guide for predicting orally activity of potential drugs.

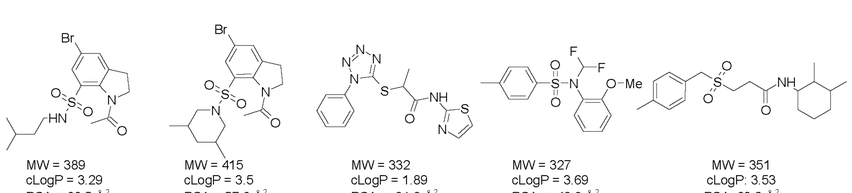


Figure 2. Five original hits for DapE inhibition

Assay Protocol

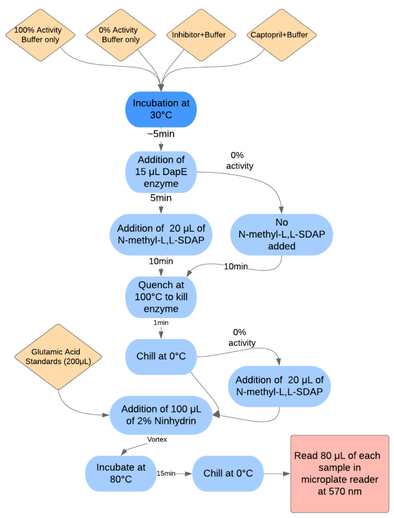
Buffer
The assay is run in 50 mM HEPES buffer at pH 7.5. This buffer tops off each reaction vial at 200 µL. Variable amounts per reaction are due to variable volumes of reagents added. All reactions are also run with 5% DMSO in solution due to solubility of some inhibitors.

Enzyme
We use the DapE enzyme isolated from *Haemophilus influenza* and it is run at roughly 9 nM in the assay.[1] Due to the delicacy of the active enzyme, mixing must be done via repetitive uptake and dispensing of solution gently in the pipette to avoid denaturing the enzyme.

Substrate
The substrate is an *N*-methylated SDAP derivative (*N*-methyl-L,L-SDAP) seen in **Scheme 2.1** and it is run at a 2 mM concentration in the assay[1].

Inhibitors
Inhibitors are usually prepared in a stock solution of 100% DMSO and kept at 0°C. They are diluted appropriately in a 50% DMSO/50% buffer solution so that after addition of 20 µL to the 200 µL reaction volume, all tubes will have the 5% DMSO solution mentioned above.[1] This does help with the solubility of some inhibitors, but DMSO has also been shown to activate the enzyme, making it a crucial variable to control.

Spectrophotometric Analysis
When the reaction is complete, ninhydrin reacts with cleaved substrate turning dark purple and the absorbance is measured at 570 nm. This means the darker wells have high activity and the lighter ones have low activity. Yellow arrow shows high activity and blue shows low activity in **Figure 3.**



Scheme 2. Assay workflow

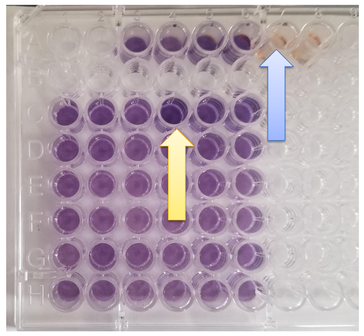


Figure 3. Sample plate

Inhibition Data Determined

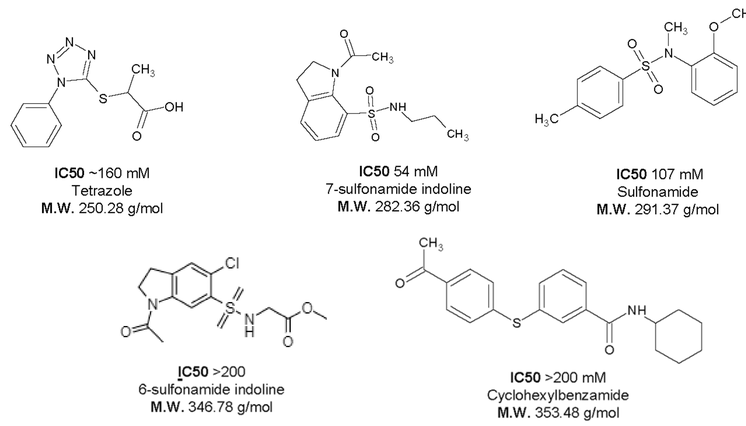


Figure 4. Five latest inhibitors tested with molecular weight (M.W.), IC_{50} (millimolar), and compound class

Future Compounds

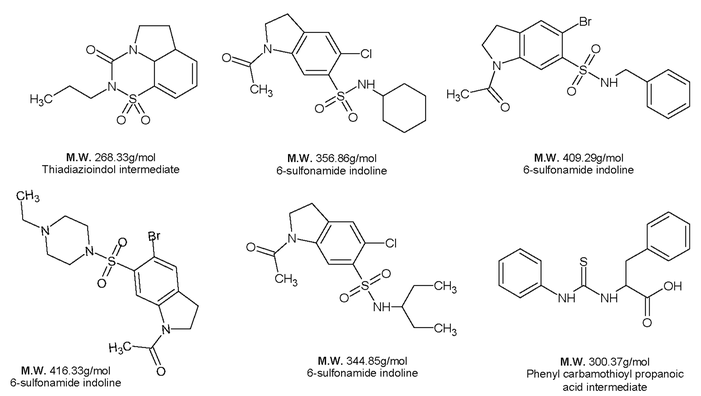


Figure 5. Compounds to be tested

Sulfate Inhibition

Activation of DapE with DMSO
When looking at our 1.3Å crystal structure (PDB 5uej) with our collaborators, it was observed that there are two sulfates bound in each active site. This prompted us to note that in the substrate bound structure, two of the carboxylates attached to the substrate are located in the same place. We hypothesized that sulfate also is a competitive inhibitor of the enzyme in addition to our small molecule inhibitors. We then decided to determine the IC50 of lithium sulfate using the same assay outlined above. The results and logarithmic inhibition curve are displayed below in **Figure 6.** The IC50 for sulfate was determined to be 31 ± 13 mM.

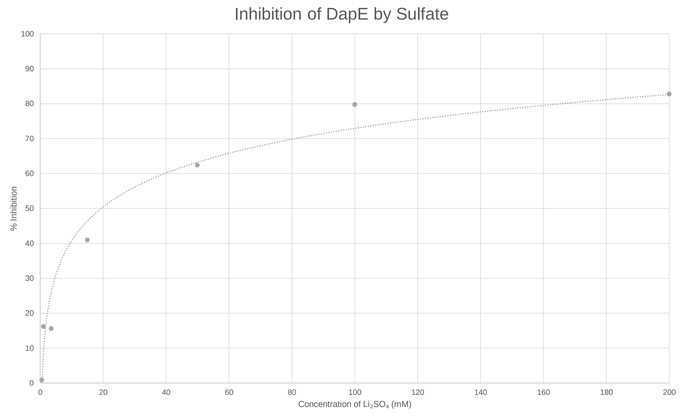


Figure 6 Enzymatic inhibition by Lithium Sulfate

Targeted Molecular Dynamics
The results of this prompted our group to begin investigating the distances between these two sulfates as the enzyme goes through closure. We began this by investigating enzymatic conformations in VMD using targeted molecular dynamics (TMD). This involves applying a force to certain atoms in computer software with the intent of reaching a target conformation. As we suspected the two sulfates bound in the active site grow significantly closer as force is applied. This has led us to begin the next leg of our research.

Bisulfonates
Following our investigation into sulfate as an inhibitor and our collaborators work with TMD, we have decided to synthesize several bisulfonates with variable length alkyl chains connecting them. We hope to measure the IC50 of each of these chains but ultimately publish a number of inhibitor bound crystal structures which will give us insight into how the enzyme closes. The varying lengths will in theory pull the enzyme closed to varying extends which will give snapshots into enzymatic closure.

Future Plans

Primarily, we will be focused on obtaining IC50 data for the rest of our library of compounds, including those listed earlier, which will help to direct the synthesis of future compounds. We will also be investigating the inhibition potential of bis-sulfonates as we predict that the sulfonate groups will bind tightly to the active site, based on the demonstrated inhibition by anionic sulfate. In addition, we will be working with collaborators in molecular dynamics to investigate the activation seen by low concentrations of inhibitors.

Citations

1.Heath TK, Lutz MR, Jr., Reidl CT, Guzman ER, Herbert CA, Nocek BP, et al. (2018) Practical spectrophotometric assay for the dapE-encoded *N*-succinyl-L,L-diaminopimelic acid desuccinylase, a potential antibiotic target. PLoS ONE 13(4): e0196010. https://doi.org/10.1371/journal. pone.0196010
2. Nocek, B., Heath, T., Becker, D., Joachimiak, A., Anderson, W.F., Center for Structural Genomics of Infectious Diseases (CSGID) . 1.30 A crystal structure of DapE enzyme from *Neisseria meningitidis* MC58.
3. Gillner DM, Becker DP, Holz RC. Lysine biosynthesis in bacteria: a metallodesuccinylase as a potential antimicrobial target. *J. Biol. Inorg. Chem.* 2013; 18: 155–163. https://doi.org/10.1007/s00775-012-0965-1 PMID: 23223968.

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