A Thesis

entitled

Fragment Library Screening to Discover Selective Inhibitors of a Key Microbial Enzyme

by

Geng Gao

Submitted to the Graduate Faculty as partial fulfillment of the requirements for
the Master of Science degree in Chemistry

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The University of Toledo

December 2010
An Abstract of

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The aspartate biosynthetic pathway, essential in plants and in most bacteria and fungi, produces the amino acids threonine, lysine, methionine, and isoleucine. The second step in the aspartate biosynthetic pathway is catalyzed by aspartate-β-semialdehyde dehydrogenase (ASADH). To treat the growing number of multidrug resistant organisms a new protocol is required to develop novel antibiotics. Fragment library screening provides a better hit rate and requires fewer resources, and our understanding of the catalytic mechanism of ASADH allows drug development to target this essential enzyme. Custom fragment libraries have been assembled and screened against ASADHs isolated from three different microbes. The initial hits from the libraries have good ligand efficiency and selectivity despite the similarity of the structures of those ASADHs and the in virtually identical active sites. Further development of the hits showed that amino acid analogues have both binding and chiral selectivity for the bacterial enzyme forms. Benzophenone analogues selectively inhibit the Gram-negative ASADH, while the fungal form of ASADH is only inhibited by small haloacids and substituted aromatic acids. Structural studies of enzyme-inhibitor complex are being carried out to guide further development of more potent and selective inhibitors.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ASADH</td>
<td>Aspartate-β-semialdehyde dehydrogenase</td>
</tr>
<tr>
<td>ASA</td>
<td>Aspartate-β-semialdehyde</td>
</tr>
<tr>
<td>ca</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>CHES</td>
<td>N-Cyclo-2-aminoethanesulfonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>L. E.</td>
<td>Ligand efficiency</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino) ethanesulfonate</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethyleneglycol</td>
</tr>
<tr>
<td>$sp$</td>
<td>Streptococcus pneumoniae</td>
</tr>
<tr>
<td>$vc$</td>
<td>Vibrio cholerae</td>
</tr>
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</table>
Chapter 1: Introduction

1.1 Aspartate Biosynthetic Pathway

The aspartate biosynthetic pathway present in plants, bacteria and fungi leads to the production of four essential amino acids: lysine, threonine, methionine and isoleucine. Several important non-protein metabolic intermediates are also produced by this pathway, such as diaminopimelate used in cell wall biosynthesis and dihydrodipicolinate, a major component of bacterial spores. In addition, methionine is the precursor of S-adenosyl methionine, a methyl group donor, and several classes of quorum sensing molecules that trigger the release of virulence factors. Hence, malfunction of this pathway is lethal to microorganisms. Because this pathway is absent in mammals, irreversible inhibition of the pathway should be a good productive approach to develop novel antibacterial compounds. Below is the pathway scheme showing the intermediates and the final amino acid products.
Figure 1. Aspartate biosynthetic pathway scheme. Compounds in red are the four amino acids produced by the pathway.

There are several control steps regulating the pathway. Aspartokinase (AK) is the first enzyme to initiate the pathway, catalyzing the phosphorylation of aspartate. Several isoforms of this enzyme can be involved, depending on the different organisms, and each of the isoforms can be either a bifunctional or a monofunctional enzyme.\textsuperscript{1,6} Aspartate-β-semialdehyde dehydrogenase (ASADH) is the enzyme that catalyzes the dephosphorylation of β-aspartyl phosphate into aspartate-β-semialdehyde, with this step resulting in the first branch in the pathway.\textsuperscript{1} Homoserine dehydrogenase and homoserine kinase lead to the production of threonine and isoleucine at the last branch of the pathway, and those amino acid levels are regulating in the pathway by feedback to the aspartokinase-catalyzed initial reaction.\textsuperscript{7} Cyclization of aspartate-β-semialdehyde leads to two important metabolic intermediates and eventually to the amino acid lysine. Acylation of homoserine directs the pathway towards the synthesis of methionine.\textsuperscript{8}
1.2 Aspartate-β-semialdehyde Dehydrogenase

Aspartate-β-semialdehyde dehydrogenase leads to the production of aspartate-β-semialdehyde (ASA). Structural studies have identified the residues involved in binding the substrates at the active site of ASADH, including His 277, Arg 270, Asn 135, Arg 103, and Lys 246. Based on sequence homology, the ASADH family can be divided into three branches, with different forms of the enzyme present in Gram-negative, Gram-positive bacterial and Archaea/fungi. The structures of representative ASADHs from each of these branches have been determined in our laboratory despite the sequence differences, and the active site of each enzyme was found to be similar among these species.

1.2.1 *Streptococcus pneumoniae*-ASADH (sp-ASADH)

*Streptococcus pneumoniae* is a Gram-positive anaerobe and is the major cause of pneumonia. The structure of ASADH from *S. pneumoniae* was determined by Faehnle, et al. in 2006. Both the apo enzyme structure and the NADP-enzyme complex were solved. The overall structure of sp-ASADH is a homodimer, with each monomer having 13 β-strands and 8 α-helices folding into an N-terminal coenzyme binding domain and a C-terminal dimerization domain (Fig. 2). When the coenzyme NADP binds the N-terminal domain tends to rotate toward the C-terminal domain leading to a domain closure (Fig. 3).
Figure 2. Overall structure of *sp*-ASADH with NADP. The active site residues are shown in blue/orange sticks. The two monomers are colored grey and purple.
1.2.2 *Vibrio cholerae*-ASADH (vc-ASADH)

*Vibrio cholerae* is a gram-negative bacterium that causes cholera in humans. Blanco, et al. solved the structure of ASADH from this species in 2002, as well as a ternary complex included NADP and a covalently bound inhibitor S-methyl-L-cysteine. The *apo* enzyme is present as a dimer, with each monomer having a molecular weight of 37.4 KDa. The N-terminal domain of *vc*-ASADH provided the NADP binding site and the C-terminal domain provided the substrate binding pocket (Fig. 4). When NADP is bound, a number of conformational changes occurred in the active site, and these changes help to accommodate the substrate.
1.2.3 *Candida albicans*-ASADH (ca-ASADH)

Yeast species *Candida albicans* is a diploid fungus and is the agent of oral and genital infections in humans.\(^{11}\) The structure of ASADH from this fungus was recently solved by Arachea, et al. to moderately high resolution in a complex with its nucleotide cofactor presented.\(^{12}\) Overall, the enzyme is a homodimer, with each monomer containing an N-terminal coenzyme binding domain and a C-terminal dimerization domain (Fig. 5).
1.3 Fragment Library Screening

The pharmaceutical industry is developing “drug-like” molecules by high-throughput screening (HTS) of large compound libraries that target different clinically relevant pathways. Those molecules that show good affinity can be modified chemically, leading to improvements in the target affinity by several hundred fold. However, there are estimated to be about $10^{60}$ possible molecules containing up to 30 non-hydrogen atoms, making screening to cover all of these chemical diversities really impossible.\textsuperscript{13} When the molecular size decreases, then the number of possible molecules decreases exponentially. Therefore, it is theoretically more efficient to screen smaller molecules (or fragments) and then to expand them based on successful hits. The experimental support of this theory was provided by Nakamura and Abeles in 1985, however, difficulty remains in identifying and elaborating fragments with weak binding.

Figure 5. Ribbon structure of \textit{vc}-ASADH complexed with NADP. Two monomers are colored green and blue.\textsuperscript{12}
affinities and less specificity to the target protein. Regardless, this alternative approach has advantages over the traditional HTS because it requires fewer resources and typically has a much higher success rate. In addition, an identified fragment can be visualized when bound in the active site of target protein, with functional groups added to these core structures, guided by the structural studies to develop more potent molecules.

In a recent example, the (R)-enantiomer of an oral drug mexiletine was identified as a hit to the serine protease urokinase by fragment library screening. In spite of the weak potency, the molecule was optimized to a potent lead that had promising selectivity to different proteases compared with mexiletine itself using approaches based on the hit structure (Scheme 1).

![Scheme 1](image)

\[
\begin{align*}
IC_{50} & > 1 \text{ mM} \\
\text{L. E.} & < 0.31 \text{ kcal/mol}
\end{align*}
\]

\[
\begin{align*}
IC_{50} & = 0.072 \text{ μM} \\
\text{L. E.} & = 0.31 \text{ kcal/mol}
\end{align*}
\]

Scheme 1. Structural development from mexiletine into the lead compound. IC\text{50} is the half maximal inhibitory concentration.

Another successful example to develop a potent hit into a lead compound with more potency was done by researchers in Spain. 1,2,3,4-tetrahydroacridin-9-amine targeting acetylcholinesterase was found with a micromolar IC\text{50} and was further optimized into a lead compound that had a picomolar level IC\text{50} (Scheme 2).
IC\textsubscript{50} = 0.167 \mu M  \\
L. E. = 0.61 \text{kcal/mol}

\[
\begin{align*}
\text{IC}_{50} &= 0.00002 \mu M \text{ (20 pM)} \\
L. E. &= 0.41 \text{kcal/mol}
\end{align*}
\]

Scheme 2. Structural development from 1,2,3,4-tetrahydroacridin-9-amine into the lead compound.

In both cases, those initial compounds were identified based not on their weak binding affinities but on their good ligand efficiencies. The significance of ligand efficiency is discussed in the following section. On the other hand, traditional antibiotic development is focusing on broad-spectrum antibiotics. However, in the past few decades more narrow-spectrum antibiotics have been found that are selectively active against particular types of bacteria, such as Gram-positive or Gram-negative bacteria. Linezoid is a very good example of narrow-spectrum antibiotic, targeting infections caused by Gram-positive bacteria only and was first discovered by researchers at duPont\textsuperscript{18}. The selectivity of an antibiotic to specific microbial types could be an advantage for the treatment of bacterial infections because of the increase in drug resistance to broad-spectrum antibiotics. Also, selective antibiotics are potentially safer because only certain organisms are affected by selective antibiotics and any accompanied side effects would be reduced.
1.4 Binding Affinity and Kinetic Studies

Binding affinity is used to characterize the interaction of ligands with their binding sites. Higher binding affinity represents greater intermolecular forces between the ligand and the receptor. However, because of the smaller molecular size of the fragments, this criterion alone cannot be applied to assess fragment library screening. Instead, ligand efficiency (L.E.) is used, which is defined as binding energy per heavy atom, and is calculated by the following equation:\(^{19}\):

\[
\text{L.E. (ligand efficiency)} = -\frac{\Delta G}{N_{\text{non-hydrogen atoms}}}
\]

Equation 1. Calculation of ligand efficiency

Hopkins, et al. calculated that the acceptable ligand efficiency of a candidate compound should be greater than 0.29 kcal/mol per non-H atom.\(^{20}\) This would lead to an inhibition constant of less than 10 nM for a drug candidate with a MW of about 500. Therefore, the goal of this fragment library screening is to identify compounds that at least have an initial L.E. greater than 0.29 to continue lead compound development.

Kinetic studies of different ASADHs have been used to reveal how a compound from the library might inhibit the enzyme. Since this enzyme-catalyzed reaction requires two substrates, a compound that mimics the structure of either one of the substrates may compete with that substrate and bind to the active site either reversibly or irreversibly to inactivate the enzyme. Usually, irreversible inhibition means covalently modifying the enzyme, as was seen in a former study of the \(\nu c\)-ASADH inhibitor S-carbamoyl-L-cysteine, which showed thiolate group inactivation of the enzyme.\(^8\) In this study, we have focused on reversible inhibitors because thiol-containing drugs may not able to arrive at the target protein without encountering other targets in mammals.
Chapter 2. Materials and Methods

2.1 Materials

The 96 well screening plates, KCl, Na-Citrate, (NH₄)₂SO₄, K-phosphate were purchased from Fisher Scientific. CHES was purchased from Fisher Biotech. As for precipitate studies, PEG 400, PEG 2000 and PEG 3350 were purchased from Hampton Research and stored at 25 °C. Aspartate-ß-semialdehyde was synthesized as described and stored at -4 °C in 4M HCl due to its instability under neutral conditions. NADP and the compounds used in inhibitor screening and development were purchased from Sigma-Aldrich, Acros Organics or Alfa Aesar. The SPECTRAmax® 340PC Microplate Spectrophotometer used for library screening was purchased from Molecular Device, California. ASADHs from S. pneumoniae, V. cholerae and C. albicans were cloned, expressed and purified as previously described and then concentrated and stored in 50 mM Hepes (pH 7) with 1 mM EDTA and DTT at -20 °C.

2.2 Fragment Library Screening

The reduction reaction catalyzed by ASADH is shown in Scheme 3. Enzyme activities were measured by monitoring the reverse reaction using the increase of NADPH absorbance at 340 nm. ASADHs from Streptococcus pneumoniae, Vibrio cholerae and Candida albicans were each screened against the fragment libraries described in the following sections.
2.2.1 Assembling of Fragment Library

Two fragment libraries were assembled based on size, structural diversity and compound availability. These considerations included: (1) a fragment should have a maximum molecular weight of 300 Da, (2) good aqueous solubility, and (3) molecular diversity and chemical tractability.\textsuperscript{23-25} The water-soluble fragment library is composed of 384 compounds with an average molecular weight around 155 Da, with 90\% of the compounds having a molecular weight between 90 and 250 Da. The compounds were divided into four classes equally and prepared as 200 mM stock solutions:

1. \textbf{amino acids and derivatives}: natural and unnatural amino acids; N-derivatized and amino acid esters; halo- and phospho-amino acids; dipeptides.

2. \textbf{metabolites and analogues}: mono-, di- and tricarboxylic acids; halo acids; phosphorylated metabolites.

3. \textbf{carbohydrates and bases}: sugars, sugar acids and amino sugars; nucleobases.

4. \textbf{water soluble organics and aromatics}: 5- and 6-membered heterocyclic derivatives; amino alcohols; halo acids and amides; nitro- and hydroxybenzyl derivatives.

The DMSO-soluble fragment library also had 384 compounds with a stock
concentration at 400 mM. The average molecular weight of those compounds is about 160 Da and 90% of the compounds are between 90 and 240 Da. The compounds were also divided into four equal classes:

1. **benzene derivatives**: halo, cyano and hydroxybenzenes; benzaldehydes and benzoic acids.

2. **five-membered heterocycles**: oxygen, nitrogen and sulfur-containing heterocycles; halo, amino, carboxyl and methyl derivatives.

3. **six-membered heterocycles**: nitrogen and oxygen-containing heterocycles; halo, amino, nitro, hydroxy and carboxy derivatives.

4. **fused/multiple ring systems**: naphthalenes and indoles; quinines and benzyls; diphenyl and dibenzyl derivatives; cycloalkanes.

For each library one compound from each class was mixed together, resulting in 96 cocktails with the pH value adjusted to slightly basic for the cocktails in the soluble fragment library using either base or acid.

2.2.2 Preparation of Screening Environment

The screening was carried out in a reaction buffer composed of 120 mM CHES and 200 mM KCl, with the pH adjusted to 8.6 using 6 M KOH. The concentrations of NADP and ASA were set five times or higher of their $K_m$ values, respectively. The concentration of phosphate was set between six and twenty times of its $K_m$ value to maintain reasonable reproducibility. The $K_m$ and working concentrations of the substrates for ASADH from each organism are shown in Table 2.1.
Table 2.1. $K_m$ Values of ASADHs and Working Concentrations in Kinetic Screen

<table>
<thead>
<tr>
<th>Organism</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>Working concentrations (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASA</td>
<td>NADP</td>
<td>Phosphate</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>2$^a$</td>
<td>0.12</td>
<td>0.29</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>120$^b$</td>
<td>0.19</td>
<td>0.32</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>0.12$^c$</td>
<td>0.19</td>
<td>0.35</td>
</tr>
</tbody>
</table>

$^a$ Faehnle, et al. 9
$^b$ Moore, et al. 28
$^c$ Arachea, et al. 12

2.2.3 Kinetic Assay

A single row of reactions were carried out simultaneously on the 96-well plate by using the SPECTRAmax® 340PC Microplate Spectrophotometer. 139 µl of buffer and substrate (concentrations indicated in Table 2.1) solution were placed in each well, and then followed by 40 µl cocktail solution. 1 µl of ASA was added by a RAININ® electric pipettor and the reaction was triggered by addition of 20 µl enzyme solution. The final concentrations of enzymes were 10 µg/ml for sp-ASADH, 3 µg/ml for vc-ASADH and 40 µg/ml for ca-ASADH. 21 To minimize the variation between the reactions in different rows, the initial velocity of the reaction was compared to an internal standard by assuming the reaction with highest initial velocity rate in each row has no inhibitor present.

For the compounds in the DMSO-soluble fragment library, tolerance of the enzyme to DMSO was pre-screened. DMSO was added into the well, raising the v/v ratio gradually while the concentration of substrates and enzyme remained the same. The result
showed that the enzymes still retained 60% to 70% activity with the DMSO concentration up to 50%. Typical concentration of DMSO was set at 20% for DMSO soluble fragment library screening, conditions where these enzymes still possess around 80% of their original activities.

To classify the strength of these initial compounds, strong inhibition of ASADH is defined as less than 10% initial rate remaining as compared to the control reaction in the absence of inhibitors, 10% to 50% initial rate remaining is considered as moderate inhibition, higher than 50% initial rate remaining are considered as weak or no inhibition. Fragments showed only weak inhibition were not further pursued in this study.

2.3 Kinetic Studies

Cocktails that showed strong inhibitions were re-screened as individual compounds using the same protocol as describe above. Those compounds showed strong inhibition at 10 mM were further examined to quantitate their inhibitory strength. For the DMSO soluble fragment library, to minimize false-positives the spectrum of each compound was scanned from 300 nm to 500 nm. Those compounds (25 cocktails, with a total of 100 compounds) that precipitated in the wells or had strong absorption at 340 nm were adjusted either in a higher DMSO-containing condition or by decreasing their concentration to allow the enzyme assay to remain in a linear absorbance range.

2.3.1 Determination of Inhibition Constants

The strong inhibitors were screened using the same protocol as described (Section
2.2.3) but now varying the concentration of the inhibitors, typically by a serial dilution. The inhibition constants (K_i values) were then determined by Dixon Analysis initially assuming that the inhibitors were competing with ASA for binding to the enzyme because of their ASA-mimic structures. In some cases outlying data points were discarded based on a large percent error and the K_i values were re-determined. For example, when there are bubbles in the well the values determined by the plate reader would be significantly affected. In such case, these misreadings were discarded when calculating the K_i values).

2.3.2 Comparison of Inhibitor Efficiency

The binding affinities (K_i) of the inhibitors were replaced by the ligand efficiency values due to the reasons discussed in section 1.4. The ligand efficiencies for each of the inhibitors were calculated according to equation 1 after the K_i values were determined and the inhibition energies were calculated by using $-\Delta G = R \cdot T \ln K_i$.

2.4 Evaluation of Precipitants

To assist the planned structural studies of the enzyme-inhibitor complex and establish an environment in which the protein properties are unaffected, different precipitants used in structural studies were screened to test their effect on the enzymes. Several precipitants: (1) 20% PEG 3350, (2) 20% PEG2000, (3) 20% PEG400, (4) 1 M Na-Citrate or (5) 1 M (NH_4)_2SO_4 were added to the wells without changing the concentrations of the substrates or enzymes, and the initial velocity of the reactions were compared to an internal standard in the absence of precipitants.
Chapter 3: Results and Discussion

3.1 Fragment Library Screening

3.1.1 Cocktails and Comparison

Each of the libraries was initially screened against *S. pneumoniae-, V. cholerae- and C. albicans*-ASADHs as cocktails at 10 mM. There were 18 cocktails against *sp*-ASADH, 25 cocktails against *vc*-ASADH and another 18 cocktails against *ca*-ASADH identified from the soluble library as containing compounds that caused moderate to strong enzyme inhibition. Also, 39 cocktails against *sp*-ASADH, 34 cocktails against *vc*-ASADH and 37 cocktails against *ca*-ASADH were identified from the DMSO-soluble library. As described in Section 1, these ASADHs possess similar overall structures and high sequence homologies, but the cocktails showed inhibition of these enzymes quite differently even with the same conditions and substrates.

There were also a number of cocktails which showed only moderate inhibition to each form of the enzyme. In presence of these cocktails, the initial velocity of the reaction retains from 30% to 50% catalytic activity compared to that of the external standard. Similarly, cocktails that were able to retain over 60% of the initial velocity compared with the external standard were defined as weak inhibition cocktails. Figure 6 shows examples of the strength of the different degrees of inhibition in a graphic way.
Figure 6. Inhibition strength of different cocktails during screening. The lettered curves show different inhibition strengths of the cocktails as: A, external standard (no inhibition). B, weak inhibition. C, moderate inhibition. D, false positive and E, strong inhibition.

As shown in Figure 7, out of the soluble library only 4 of the cocktails had good inhibitory effects against all three enzyme forms, and 11 cocktails had inhibition against two of the three enzymes. Surprisingly, 28 cocktails showed inhibition against only one enzyme form despite the highly conserved active site geometry among the members of the ASADH family.

The DMSO-soluble library showed 28 inhibitory cocktails against all three forms of enzyme, 13 cocktails against two forms and 12 inhibited only one form of the target enzymes (Fig. 8). The significant increase of the number of cocktails that inhibit all forms of the enzyme from this DMSO-soluble library was probably caused by the colored aromatic compound which caused strongly absorb at the monitoring wavelength range. This issue was resolved in further screen by decreasing the concentrations of the individual compounds.
Figure 7. Inhibition pattern of the soluble fragment library. The library was screened against *Vibrio cholerae* (vc-ASADH), *Streptococcus pneumoniae* (sp-ASADH) and *Candida albicans* (ca-ASADH). Red dots represent cocktails showed good inhibition against all enzyme forms, orange dots represent cocktails showed good inhibition against 2 enzyme forms and yellow dots represent cocktails showed good inhibition against only 1 form of enzymes.
Figure 8. Inhibition pattern of the DMSO fragment library. This library was screened against *Vibrio cholerae* (vc-ASADH), *Streptococcus pneumoniae* (sp-ASADH) and *Candida albicans* (ca-ASADH). Color scheme is the same as in Figure 7.

In summary, 41 cocktails (with a total of 164 compounds) from the soluble library and 53 cocktails (with a total of 214 compounds) from the DMSO-soluble library were selected to proceed to the further screen as individual compounds. For example, cocktails 12, 77, 80 and 96 from the water soluble library showed strong inhibition against all three enzyme forms. The individual compounds from each of these cocktails (Table 3.1) were each tested separately to identify the inhibitory component.
Table 3.1. Components of Representative Water Soluble Library Cocktails

<table>
<thead>
<tr>
<th>Cocktail</th>
<th>Components</th>
</tr>
</thead>
</table>
| 12       | aminomethane sulfonate  
D-arabonic acid δ-lactone  
5-amino-3-methylisoxazole  
L-2-chlorosuccinate       |
| 77       | α-methyl-DL-serine  
phosphoenol-pyruvate  
4-phenylimidazole  
maleimide                |
| 80       | 4-nitrophenyl- phosphate  
pyrrole-2-carboxylate  
propionic acid  
N, N-bis(phosphonomethyl)glycine |
| 96       | L-valine  
urea  
L-xylose  
vanillin               |

3.1.2 Individual Compounds and Comparison

The cocktails that were selected for further screening were then screened as separate compounds as described above. As expected, many of the compounds from the DMSO-soluble library had strong absorption at 340 nm that produced false-positive results in the cocktail screen. A number of these compounds were eliminated as possible inhibitors when the concentrations were decreased to a level (2-5 mM) where the change of NADPH was measurable, because the absorbance dropped rapidly when the concentrations of the compounds were decreased and the reaction time courses suggested that those compounds were no longer inhibitory.

As a summary, 10 compounds from each library (hit rate 2.6%) were identified as good inhibitors against one or two forms of the enzyme, respectively. 8 compounds were identified as sp-ASADH inhibitors, 14 compounds inhibited vc-ASADH and 5 compounds were found to be ca-ASADH inhibitors. Surprisingly, none of the compounds
showed good inhibition against all 3 forms of enzyme, while 7 out of these 20 compounds had inhibition against both bacterial forms of the enzyme.

3.2 Inhibitor Identification

To measure the potency of each inhibitor, compounds that showed good inhibition at low millimolar range were screened in a single row with serial dilution and $K_i$ values were calculated as described in section 2. For instance, 3-bromopyruvate was first tested at 20 mM and still strongly inhibits the enzyme at 0.1 mM. Therefore, it was tested against the ca-ASADH with a serial dilution starting from 0.2 mM and ended at 0.0031 mM. Figure 9 shows the different inhibitions of 3-bromopyruvate against ca-ASADH in a serial dilution assay. When the initial velocities of the reactions in presence of different concentrations of inhibitors are acquired, $1/\text{velocity}$ is plotted as a function of the concentration of the inhibitor and the $K_i$ can be read from the plot (Fig. 10). The adjusted $K_i$ values were determined by a fit to the Dixon equation using an Enzyme Kinetics Program package adapted from those by Cleland. The experiments were repeated at least twice to maintain the reproducibility. In table 3.1, different threshold concentrations of the non-inhibitors are present because ca-ASADH has a relatively low $k_{cat}$ value so that reproducible data cannot be obtained unless those inhibitors are tested at a lower concentration.
Figure 9. Time courses of 3-bromopyruvate inhibition against ca-ASADH. Top curve represents the external standard in the absence of inhibitor, while the curves below represent the increase of NADPH in the presence of 0.0031, 0.0063, 0.0125, 0.025, 0.05, 0.1 and 0.2 mM 3-bromopyruvate, respectively.

Figure 10. Dixon plot for determination of inhibition constant of N-iodosuccinimide.
Table 3.2. ASA Dehydrogenase Inhibitors Identified from Fragment Library Screening

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Kᵢ values (mM)</th>
<th>Selectivity a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sp-ASADH</td>
<td>vc-ASADH</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.036±0.004</td>
<td>0.00023±0.00006</td>
</tr>
<tr>
<td>L-cysteine ethyl ester</td>
<td>0.85±0.22</td>
<td>0.00043±0.00011</td>
</tr>
<tr>
<td>L-cysteine sulfinate</td>
<td>1.3±0.4</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>L-homocystine</td>
<td>&gt;200 b</td>
<td>0.47±0.10</td>
</tr>
<tr>
<td>S-carbamoyl-L-cysteine</td>
<td>&gt;200 b</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>D-glucosaminic acid</td>
<td>&gt;200 b</td>
<td>3.2±0.6</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.46±0.14</td>
<td>0.57±0.07</td>
</tr>
<tr>
<td>D-2,3-diaminopropionate</td>
<td>0.27±0.05 c</td>
<td>0.47±0.08 c</td>
</tr>
<tr>
<td>3-bromopyruvate</td>
<td>&gt;200 b</td>
<td>&gt;200 b</td>
</tr>
<tr>
<td>Maleimide</td>
<td>&gt;200 b</td>
<td>&gt;200 b</td>
</tr>
<tr>
<td>N-iodosuccinimide</td>
<td>0.006±0.002</td>
<td>0.016±0.004</td>
</tr>
<tr>
<td>Pyridoxal-5-phosphate</td>
<td>0.14±0.04</td>
<td>&gt;200 b</td>
</tr>
<tr>
<td>4-hydroxy benzophenone</td>
<td>2.4±0.42</td>
<td>0.33±0.05</td>
</tr>
<tr>
<td>5-chloro-2-nitrobenzaldehyde</td>
<td>&gt;10 b</td>
<td>0.53±0.17</td>
</tr>
<tr>
<td>2,6-dibromoquinone chlorimide</td>
<td>&gt;10 b</td>
<td>0.99±0.11</td>
</tr>
<tr>
<td>2-chloro-3',4'-dihydroxyacetophenone</td>
<td>&gt;10 b</td>
<td>0.12±0.015</td>
</tr>
<tr>
<td>1,2-naphthoquinone-4-sulfonate</td>
<td>&gt;10 b</td>
<td>0.37±0.14</td>
</tr>
<tr>
<td>(bromomethyl)cyclohexane</td>
<td>&gt;20 b</td>
<td>&gt;20 b</td>
</tr>
<tr>
<td>2-methoxy-4-nitroaniline</td>
<td>&gt;20 b</td>
<td>&gt;20 b</td>
</tr>
<tr>
<td>3,4-dihydroxyphenylacetate</td>
<td>&gt;20 b</td>
<td>&gt;20 b</td>
</tr>
</tbody>
</table>

a ratio of Kᵢ values against representative gram-positive, gram-negative and fungal ASADHs
b no inhibition was observed; the Kᵢ is estimated to be at least 10 times greater the highest concentration examined
c adjusted for inhibition by only the D-isomer

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3.2.1 sp-ASADH Inhibitors

Of those 20 new inhibitors that were identified, 8 compounds show either very good or moderate inhibition against sp-ASADH. L-cystine, L-cysteine ethyl ester and L-cysteine sulfinate show a broad range of $K_i$ values (Table 3.2) but the inhibition mechanism is likely to be modification of the active site cysteine of the enzyme as discussed previously. However, even though it has a similar structure, L-cysteine sulfinate was found to be relatively weakly inhibiting sp-ASADH while L-cystine appears to be very strong. D-2,3-diaminopropionate has a potency against the two bacterial enzyme forms that is stronger than propionate itself, but only by a factor of two. N-iodosuccinimide shows a very good potency against both bacterial enzymes, but maleimide which has a very similar structure does not show any inhibition even at a high concentration (20 mM). Pyridoxal-5-phosphate is also very potent against sp-ASADH, but the structure suggests that this compound is likely to bind at the NADP binding site instead competing against ASA, because of its similarity to NADP.

3.2.2 vc-ASADH Inhibitors

As expected, the five thiol-containing compounds (Table 3.2) were found to be vc-ASADH inactivators because of the reason that was discussed in section 1.4. Propionate and D-2, 3-diaminopropionate show comparable potency and they are likely to inhibit the enzyme in the same way compared with their inhibition of sp-ASADH. Also, N-iodosuccinimide shows good potency while maleimide was not inhibiting, and D-glucosaminate was found to be a moderate inhibitor of vc-ASADH. So far, most of the bacterial ASADH inhibitors are amino acids, suggesting that this family could be a good
candidate for further development of the inhibitors.

There are five compounds that show decent $K_i$ values against this Gram negative enzyme form in the DMSO-soluble library (Table 3.2); they are either halo-derivatives (5-chloro-2-nitrobenzaldehyde, 2,6-dibromoquinone chlorimide and 2-chloro-3’,4’-dihydroxyacetophenone) or di-benzene structures (4-hydroxybenzophenone and 1,2-naphthoquinone-4-sulfonate). Some analogue developments have been examined for these two series of structurally related compounds and the results are described in below.

3.2.3 ca-ASADH Inhibitors

Unlike the bacterial ASADH inhibitors, only five compounds were found to be inhibitors of the fungal enzyme ca-ASADH. All of these compounds possess ring structures except for 3-bromopyruvate. A surprising result is that N-iodosuccinimide, which is a potent inhibitor against bacterial ASADHs, does not show any inhibition against ca-ASADH. However, maleimide is a good ca-ASADH inhibitor, with this discrepancy suggesting that different binding interactions were recognized between the bacterial ASADHs and fungal ASADH because of the similar active site of these three enzyme forms. Bromomethyl cyclohexane ($K_i = 0.25$ mM) only has one electronegative group (Br-) but it showed a potency more than twice that of 3,4-dihydroxyphenylacetate ($K_i = 0.63$ mM) which has a greater number of potential hydrogen-bonding groups.
3.2.4 Selectivity of Inhibitors

Despite the expectation of covalent enzyme inactivation (discussed in Section 1.4), these cysteine family inhibitors still showed significantly different selectivity against bacterial ASADHs. For instance, S-carbamoyl-L-cysteine is more than 1500-fold more potent against \textit{vc}-ASADH than against \textit{sp}-ASADH, while L-cysteine sulfinate possesses only 5-fold greater potency against \textit{vc}-ASADH than against \textit{sp}-ASADH. Yet \textit{ca}-ASADH was not inhibited by any of these cysteine derivatives, suggesting a difference in the reactivity of the active site cysteine nucleophile. For the five \textit{ca}-ASADH inhibitors, none of them inhibits either of the bacterial ASADHs, indicating a different inhibition mechanism between the bacterial and fungal forms of ASADH. Except for maleimide and N-iodosuccinimide discussed in the previous section, among the bacterial ASADH inhibitors, pyridoxal-5-phosphate was found to be the only \textit{sp}-ASADH selective inhibitor with 1400-fold higher potency than against \textit{vc}-ASADH. Except for D-glucosamate, the rest of the \textit{vc}-ASADH selective inhibitors are not showing the same level of selectivity when compared to pyridoxal-5-phosphate, but still have at least 8-fold greater potency than against \textit{sp}-ASADH.

An interesting result provided by structural studies was that the racemic 2,3-diaminopropionate was found to inhibit the enzymes through only the D-isomer (Pavlovsky, unpublished result), thereby the \( K_i \) values of this inhibitor were adjusted by half due to the racemic mixture used in fragment library screening. Overall, the inhibitors that have been identified from fragment library screening show selectivity not only to enzyme forms but also isomers. However, in the initial cocktail screening, a few cocktails showed apparent inhibition to all three enzyme forms even though other false positives
have been eliminated because of strong absorbance. Furthermore, none of the individual compounds showed inhibition to either enzyme form although their original cocktails showed inhibition as mentioned above. Based on these discrepancies, several of these cocktails were tested in every possible combination of either two or three of the component compounds and none of the combinations was found to inhibit the enzyme. There is no clear explanation for this result until now, but it is reasonable to eliminate those cocktails experimentally because the individual compounds were determined as non-inhibitors.

3.3 Inhibitor Development

Several inhibitors identified from this fragment library screening were selected for further inhibitor development (highlighted in Table 3.2) based on their selectivity, potency and ligand efficiency. These inhibitors are classified as: amino acids and benzophenones for the bacterial ASADH inhibitors and halo-acids and phenyl-acids as fungal ASADH inhibitors. The calculation of ligand efficiency is described below taking 2-aminoadipic acid as an example:

There are 11 non-hydrogen atoms in 2-aminoadipic acid based on its chemical formula: \( \text{OOC}-(\text{CH}_2)_3\text{CH(NH}_3^+)\text{COO}^- \). The binding energy can be obtained by \(-\Delta G = R \cdot T \cdot \ln K_i\) \((K_i = 0.00047 \text{ M, } R = 1.987 \text{ kcal·K/mol, } T = 298\text{K}).\) For this inhibitor the \(\Delta G\) value is \(-4.95\) kcal/mol. Finally, the ligand efficiency of 2-aminoadipic acid can be obtained by dividing this value by the number of non-hydrogen atoms in the inhibitor to give: \(-\Delta G/11 = 0.45\) kcal/mol per non-H atoms.
3.3.1 Amino Acid Inhibitors of Bacterial ASADHs

A series of modifications and derivatizations were examined for the potent compound D-2,3-diaminopropionate ($K_i = 0.27$ mM) based on modeling studies, and this set of compounds were screened against both $sp$- and $vc$-ASADH, with the results shown in Table 3.3.

Among those compounds, mono-aminocarboxylates were tested with chain lengths up to six carbons, but only the 4- and 6-carbon derivatives (2-aminobutyrate and 5-aminocaproate) show moderate inhibition against $sp$-ASADH with good L.E. values (Table 3.3). 5-aminocaproate is also an inhibitor of $vc$-ASADH along with 2-aminoadipate. For 2-aminobutyrate, only the L-isomer was found to be an inhibitor of $sp$-ASADH. When one of the amino groups is removed from the parent compound, the resulting compounds still have good inhibition with chiral selectivity (D-2-aminopropionate and D-3-aminopropionate) against $sp$-ASADH, but are no longer inhibitory against $vc$-ASADH. Clearly these two derivatives are not as potent as the parent compound, which confirm the involvement of both amino groups in binding activities. Homologues of diaminocarboxylates were tested from 4- to 7-carbon structures, and only 2,4-diaminobutyrate and 2,5-diaminopentanoate show inhibition against both enzyme forms. Again, these two inhibitors were found to inhibit the enzyme with the L-isomer only. There is no evidence yet from crystal studies to prove which isomer was mainly involved in 5-aminocaproate and 3-aminopropionate inhibition, but it will be interesting if these inhibitors were found to inhibit the enzyme through different isomers because the enzyme requires L-ASA and a D-isomer inhibitor would potentially require an unknown binding orientation.
Table 3.3. Amino Acid Inhibitors for Bacterial ASADHs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Chemical formula</th>
<th>sp-ASADH</th>
<th>vc-ASADH</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Kᵢ (mM)</td>
<td>L. E.</td>
<td>Kᵢ (mM)</td>
</tr>
<tr>
<td>D-2,3-diaminopropionate</td>
<td>CH₂(NH₂)CH(NH₂)COO⁻</td>
<td>0.26±0.05</td>
<td>0.70</td>
<td>0.46±0.07</td>
</tr>
<tr>
<td>D-2-aminopropionate</td>
<td>CH₂CH(NH₂)COO⁻</td>
<td>0.84±0.09</td>
<td>0.70</td>
<td>&gt; 20 a</td>
</tr>
<tr>
<td>D-2-aminopropionate</td>
<td>CH₂CH(NH₂)COO⁻</td>
<td>0.75±0.06</td>
<td>0.61</td>
<td>&gt; 20 a</td>
</tr>
<tr>
<td>L-2,4-diaminobutyrate</td>
<td>CH₂(NH₂)CH₂CH(NH₂)COO⁻</td>
<td>0.81±0.11</td>
<td>0.53</td>
<td>0.14±0.04</td>
</tr>
<tr>
<td>L-2,5-diaminopentanoate</td>
<td>CH₂(NH₂)(CH₂)₂CH(NH₂)COO⁻</td>
<td>0.75±0.08</td>
<td>0.48</td>
<td>0.59±0.09</td>
</tr>
<tr>
<td>DL-5-aminocaproate</td>
<td>H₂N(CH₂)₃COO⁻</td>
<td>0.41±0.06</td>
<td>0.58</td>
<td>0.56±0.08</td>
</tr>
<tr>
<td>D-glutamate</td>
<td>'OOC(CH₂)₂CH(NH⁺)COO⁻</td>
<td>0.087±0.014</td>
<td>0.56</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>trans-3-hexenedioate</td>
<td>'OOC-CH₂CH=CHCH₂COO⁻</td>
<td>5.2±1.3</td>
<td>0.31</td>
<td>0.24±0.05</td>
</tr>
<tr>
<td>3-nitropropionate</td>
<td>'O₂N(CH₂)₂COO⁻</td>
<td>0.24±0.06</td>
<td>0.62</td>
<td>0.39±0.03</td>
</tr>
<tr>
<td>2-amino-4-phosphonobutyrate</td>
<td>'O₃P-(CH₂)₂CH(NH⁺)COO⁻</td>
<td>&gt; 20 a</td>
<td>&lt; 0.21</td>
<td>1.56±0.28</td>
</tr>
<tr>
<td>2-amino-3-phosphonopropionate</td>
<td>'O₂P-CH₂CH(NH₃⁺)COO⁻</td>
<td>&gt; 20 a</td>
<td>&lt; 0.23</td>
<td>1.53±0.26</td>
</tr>
<tr>
<td>D-aspartate</td>
<td>'OOC-CH₂CH(NH₃⁺)COO⁻</td>
<td>&gt; 20 a</td>
<td>&lt; 0.26</td>
<td>&gt; 20 a</td>
</tr>
<tr>
<td>L-aspartate</td>
<td>'OOC-CH₂CH(NH₃⁺)COO⁻</td>
<td>&gt; 20 a</td>
<td>&lt; 0.26</td>
<td>&gt; 20 a</td>
</tr>
<tr>
<td>D-2-aminoadipate</td>
<td>'OOC-(CH₂)₂CH(NH⁺)COO⁻</td>
<td>&gt; 20 a</td>
<td>&lt; 0.21</td>
<td>0.47±0.09</td>
</tr>
<tr>
<td>L-glutamate</td>
<td>CH₃-CH₂CH(NH₃⁺)COO⁻</td>
<td>0.31±0.07</td>
<td>0.60</td>
<td>&gt; 20 a</td>
</tr>
<tr>
<td>DL-2-aminobutyrate</td>
<td>'OOC-(CH₂)₂CH(NH₃⁺)COO⁻</td>
<td>0.88±0.09</td>
<td>0.60</td>
<td>&gt; 20 a</td>
</tr>
</tbody>
</table>

a no inhibition observed at 2 mM, Kᵢ was estimated to be at least 10 times greater.
To expand this series, mono-amino carboxylates with phosphono groups and amino-dicarboxylates were also tested. The glutamate (5 carbons) isomers show significant different \( K_i \) values, with the L-isomer only inhibiting \( sp \)-ASADH. The phosphono mono-amino carboxylates were found to only inhibit \( vc \)-ASADH and to do so with only moderate potency. Furthermore, a 6-carbon dicarboxylate (\( trans \)-3-hexenedioic acid) and a nitro analogue (3-nitro-propionate) were also examined and they each show moderate \( K_i \) values. 3-nitro-propionate possesses a better L. E. value because of its small size, but the hexenedioic acid shows better selectivity between the Gram-positive and Gram-negative enzyme forms. Another surprising result was that neither D-aspartate nor L-aspartate was found to inhibit either of the two enzymes, despite being only one carbon short that the glutamate inhibitors. These results suggest both steric and geometric factors are playing a role in inhibitor binding. Overall, the development of these amino acid inhibitors suggests a 4- to 6-carbon dicarboxylate with at least one amino group as the obtained core structure (Scheme 4).

![Scheme 4. Minimal inhibitory amino acid pharmacophore (n = 0-3, m = 1-2).](image)

3.3.2 Benzophenone Inhibitors of \( vc \)-ASADH

4-hydroxybenzophenone was selected as a lead compound for \( vc \)-ASADH inhibitor development because of its weak affinity for \( sp \)-ASADH and non-inhibitory properties towards the fungal ASADH. A series of 4-hydroxybenzophenone analogues were tested and the kinetic results are shown in Table 3.4.
Table 3.4 Benzophenone Inhibitors for Gram-negative ASADH

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>X&lt;sup&gt;b&lt;/sup&gt;</th>
<th>R&lt;sup&gt;b&lt;/sup&gt;</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; values (mM)</th>
<th>L. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-hydroxybenzophenone</td>
<td>O</td>
<td>OH</td>
<td>0.33±0.05</td>
<td>0.32</td>
</tr>
<tr>
<td>Benzophenone</td>
<td>O</td>
<td>----</td>
<td>&gt; 20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.16</td>
</tr>
<tr>
<td>4-aminobenzophenone</td>
<td>O</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>&gt; 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.18</td>
</tr>
<tr>
<td>4-benzoyl benzoate</td>
<td>O</td>
<td>COO&lt;sup&gt;−&lt;/sup&gt;</td>
<td>1.1±0.3</td>
<td>0.24</td>
</tr>
<tr>
<td>2-benzoyl benzoate</td>
<td>O</td>
<td>COO&lt;sup&gt;−&lt;/sup&gt;</td>
<td>2.5±0.8</td>
<td>0.21</td>
</tr>
<tr>
<td>2, 2′, 4, 4′-tetrahydroxybenzophenone</td>
<td>O</td>
<td>(OH)&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.041±0.007</td>
<td>0.33</td>
</tr>
<tr>
<td>benzophenone imine</td>
<td>NH</td>
<td>----</td>
<td>0.37±0.08</td>
<td>0.34</td>
</tr>
<tr>
<td>benzophenone hydrazone</td>
<td>N-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>----</td>
<td>29±6</td>
<td>0.14</td>
</tr>
<tr>
<td>benzophenone oxime</td>
<td>N-OH</td>
<td>----</td>
<td>26±4</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<sup>a</sup> inhibitor strongly absorbs at 340 nm, K<sub>i</sub> was estimated at least 10 times that of the measurable concentration.

<sup>b</sup> parent compound:

![Chemical Structure](attachment:image.png)

The parent compound (benzophenone) did not show any inhibition at 2 mM and the change of absorbance of NADPH required to follow the reaction was not measurable at higher inhibitor concentrations because of its absorbance. Addition of an amino group at the 4′-position encountered the same issue, and the K<sub>i</sub> value was estimated to be at least 10 mM. Addition of carboxyl group at the 2′ or 4′-position showed moderate inhibition and the absorbance of these inhibitors did not interfere with the enzyme activity assay. Consequently, hydroxyl groups were added to the 2′ and 4′-position of each benzene ring and the resulting compound (2,2′,4,4′-tetrahydroxy benzophenone) was examined and showed reasonably good potency (more than 10-fold stronger than the parent compound) and good L. E. value. When replacing the central carbonyl group with either imine,
hydroxyl or amine groups, different potencies were observed: benzophenone imine showed good $K_i$ value and L. E suggesting that a hydrogen bond donor group at that position is necessary, while both benzophenone hydrazone and benzophenone oxime had much weaker inhibition. These results suggested that properly placed derivatives on the aromatic rings would bring the possibility of developing more potent inhibitors.

3.3.3 Organic Acid Inhibitors of *ca*-ASADH

An intriguing result from fragment library screening was that the fungal enzyme inhibitors did not show any inhibition of the bacterial ASADHs, while inhibition could not be detected for any of the bacterial enzyme inhibitors when examining against *ca*-ASADH (Table 3.2). Since *ca*-ASADH has a similar overall structure to those of bacterial enzymes, this selectivity profile would be a good point to track with subsequent structural studies that might establish a structural basis for this selectivity.

3-bromopyruvate shows good inhibition and high ligand efficiency against *ca*-ASADH (Table 3.5), while other halo-derivatives (fluorine and iodine) also show decent $K_i$ values. Propionates carrying a halogen at 3’-position (3-bromopropionate and 3-fluoropropionate) also showed good potencies. A set of control experiments with these two inhibitors were carried out by examining these compounds against bacterial ASADHs and no detectable inhibitions were observed. This result further suggested that there is a different binding pocket or mode of interaction in fungal ASADH that is unlike the bacterial ASADHs, because 3-aminopropionate was identified as an inhibitor ($K_i = 1.49$ mM) of *sp*-ASADH (Table 3.3). Shorter acids with halo- derivatives (chloroacetate and bromoacetate) acted as moderate inhibitors of *ca*-ASADH while adding two fluorines
to chloroacetate (chlorodifluoroacetate) increases the potency of this compound to a sub-millimolar $K_i$ range (Table 3.5). This entire set of halo acids showed extremely high L. E. values against this fugal ASADH.

3,4-dihydroxyphenylacetate has a low millimolar $K_i$ and a L. E. greater than 0.3. Derivatives at the 3’-position of the parent compound (phenylacetate) produced several inhibitors (3-hydroxyphenylacetate and 3-aminophenylacetate) with comparable potency and slightly better L. E. values (Table 3.5). However, derivatizing the 4’-position produced weaker inhibitors (4-nitrophenylacetate and 4-aminophenylacetate), and either an extended carboxyl chain (phenyllactate) or the addition of an amino group at the end of side chain (phenylglycine) resulted in the complete loss of inhibition.

Table 3.5. Halo Acid and Aromatic Acid Inhibitors of ca-ASADH

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (mM)</th>
<th>L. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-bromopyruvate</td>
<td>0.34±0.07</td>
<td>0.68</td>
</tr>
<tr>
<td>3-fluoropyruvate</td>
<td>0.14±0.02</td>
<td>0.76</td>
</tr>
<tr>
<td>3-iodopyruvate</td>
<td>0.18±0.02</td>
<td>0.73</td>
</tr>
<tr>
<td>3-bromopropionate</td>
<td>0.64±0.05</td>
<td>0.73</td>
</tr>
<tr>
<td>3-chloropropionate</td>
<td>0.24±0.01</td>
<td>0.83</td>
</tr>
<tr>
<td>chloroacetate</td>
<td>4.6±0.62</td>
<td>0.64</td>
</tr>
<tr>
<td>bromoacetate</td>
<td>3.2±0.57</td>
<td>0.68</td>
</tr>
<tr>
<td>chlorodifluoroacetate</td>
<td>0.59±0.05</td>
<td>0.63</td>
</tr>
<tr>
<td>3,4-dihydroxyphenylacetate</td>
<td>0.63±0.14</td>
<td>0.37</td>
</tr>
<tr>
<td>phenylacetate</td>
<td>0.83±0.28</td>
<td>0.42</td>
</tr>
<tr>
<td>4-nitrophenylacetate</td>
<td>3.63±0.71</td>
<td>0.33</td>
</tr>
<tr>
<td>phenyllactate</td>
<td>&gt; 200 a</td>
<td>&lt; 0.09</td>
</tr>
<tr>
<td>4-hydroxyphenylpyruvate</td>
<td>&gt; 200 a</td>
<td>&lt; 0.07</td>
</tr>
<tr>
<td>4-aminophenylacetate</td>
<td>2.17±0.24</td>
<td>0.26</td>
</tr>
<tr>
<td>phenylglycine</td>
<td>&gt; 200 a</td>
<td>&lt; 0.09</td>
</tr>
<tr>
<td>3-phenylpropionate</td>
<td>0.63±0.08</td>
<td>0.40</td>
</tr>
<tr>
<td>3-hydroxyphenylacetate</td>
<td>0.49±0.15</td>
<td>0.41</td>
</tr>
<tr>
<td>3-aminophenylacetate</td>
<td>0.61±0.13</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*a no inhibition observed at 20 mM, $K_i$ was estimated to be at least 10 times greater.
3.3.4 Model Guided Inhibitors of Bacterial ASADHs

Based on the structural studies of several inhibitors bound to \( sp\)-ASADH (Pavlovsky, unpublished results), compounds that are capable of better filling into the active site of the enzyme were tested, with the kinetic results shown in Table 3.6.

### Table 3.6. Model Guided Inhibitors of Bacterial ASADHs

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>( sp)-ASADH</th>
<th>( vc)-ASADH</th>
<th>selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_i ) (mM)</td>
<td>L. E.</td>
<td>( K_i ) (mM)</td>
</tr>
<tr>
<td>terephthalate</td>
<td>5.21± 1.17</td>
<td>0.26</td>
<td>0.33± 0.08</td>
</tr>
<tr>
<td>2-aminoteraphthalate</td>
<td>0.78± 0.16</td>
<td>0.33</td>
<td>3.39± 0.68</td>
</tr>
<tr>
<td>cyclohexane-1, 4-dicarboxylate</td>
<td>0.45± 0.16</td>
<td>0.38</td>
<td>0.99± 0.31</td>
</tr>
<tr>
<td>4-sulfobenzoate</td>
<td>2.69± 1.56</td>
<td>0.27</td>
<td>&gt; 100 (^a)</td>
</tr>
<tr>
<td>3, 7-naphthalene disulfonate</td>
<td>&gt; 100 (^a)</td>
<td>&lt; 0.08</td>
<td>&gt; 100 (^a)</td>
</tr>
<tr>
<td>1, 5-naphthalene disulfonate</td>
<td>&gt; 100 (^a)</td>
<td>&lt; 0.08</td>
<td>&gt; 100 (^a)</td>
</tr>
<tr>
<td>1, 3, 6-naphthalene trisulfonate</td>
<td>4.03± 0.66</td>
<td>0.13</td>
<td>&gt; 100 (^a)</td>
</tr>
<tr>
<td>2-naphthalene sulfonate</td>
<td>3.71± 0.59</td>
<td>0.22</td>
<td>2.24± 0.47</td>
</tr>
</tbody>
</table>

\(^a\) no inhibition observed at 10 mM, \( K_i \) was estimated to be at least 10 times greater.

Those compounds were composed of 1 or 2 six-membered rings with extended acid side chains that could possibly interact with the arginine and lysine side chains at the active site of the enzyme. Cyclohexane-1,4-dicarboxylate showed sub-millimolar \( K_i \) values with both \( sp\)- and \( vc\)-ASADH as well as good ligand efficiencies. However, when the cyclohexane ring was replaced with a 6-membered aromatic ring (terephthalate), the compound showed more than 10-fold weaker inhibition against \( sp\)-ASADH but 3-fold stronger against \( vc\)-ASADH, leading to a 16:1 selectivity for the Gram-negative enzyme form. When an amino group was added to the 2'-position, the derivative (2-amino
terephthalate) became a strong inhibitor against sp-ASADH and a weak inhibitor of vc-ASADH with a nearly 70-fold reversal in selectivity. This discrepancy caused by the introduction of a single amino group suggests that more structural evidence is needed to understand the nature of these inhibitors’ interaction at the active site of ASADHs. Comparisons among 1,3,6-naphthalene trisulfonate, 4-sulfobenzoate and 2-naphthalene sulfonate suggested that it is quite likely only one of the sulfonate groups can interact with the residues at the active site for this set of naphthalene sulfonate analogues. The corresponding naphthalene disulfonates are not inhibitors of either enzyme form.

3.4 Effects of Precipitants

Structural information of the active site bound with inhibitors is critical to confirm the screening and model results. Parallel structural studies using X-ray diffraction of single crystals of cocrystallized ASADH with inhibitors are in progress and several structures with inhibitors bound at the active site have been solved (Pavlovsky, unpublished results). However, many of the inhibitors identified by the kinetic study have not yet been validated by structural studies and a few inhibitors (e.g. 2-aminoadipate) show inhibition against one enzyme form by kinetic study but were found to bind with a different enzyme form by structural studies. Because of the differences between these two studies, and the demand of structural studies for the profiles of the inhibitors, we decided to screen the inhibitors that did not show any binding to the crystals under the conditions that are as close as possible to the structural study based on following factors: the structural study is using cofactor and inhibitor to validate ternary complexes with the enzyme, while the kinetic study is testing how much the inhibitor interferes with the
binding of substrates and enzyme. Therefore, it is not possible for structural study to use both substrates during crystallization. Crystallization also requires the presence of precipitants to induce crystal growth, and these precipitants are not present in the kinetic assay conditions. To compensate for the lower sensitivity very high inhibitor and enzyme concentrations are used in the structural study, much more protein that can be used in a kinetic study. Furthermore, attempted crystallization studies have confirmed the inability of growing crystals with the enzyme under any conditions with pH values greater than 7.0 (Potente, unpublished results). However, the kinetic studies show that the enzyme is significantly less active under low pH conditions and reproducible $K_i$ values cannot be obtained. To summarize, except for the factors that can not be changed such as pH value and the presence of substrates, several precipitants that are frequently used in structural studies were used in a kinetic study, as well as a few different buffer solutions, aiming to compensate for the different conditions.

As shown in Table 3.7, many of the precipitants caused the activity of the enzyme to decrease by at least 40%, suggesting that the protein is much less active under crystallization conditions. However, sodium citrate and sodium acetate show significant enhancement of the enzyme activity by at least 8-fold. At first there was a concern that either sodium citrate or sodium acetate could react with aspartate semialdehyde to produce aspartate. To test the hypothesis, aspartase was added to the assay and no detectable change at was observed 240 nm (from fumarate production).

To qualitatively examine the inhibitors under crystallization conditions, several $vc$-ASADH inhibitors were rescreened in the presence of 20% PEG 3350, because it has a less detrimental effect on the enzyme and the results are shown in Table 3.8.
Table 3.7. Effects of Precipitants in Kinetic Study

<table>
<thead>
<tr>
<th>Precipitant</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% PEG 3350</td>
<td>Decrease the enzyme activity by 40%</td>
</tr>
<tr>
<td>20% PEG 2000 (NaCl/MES)</td>
<td>Decrease the enzyme activity by 70% (60%)</td>
</tr>
<tr>
<td>20% PEG 400</td>
<td>Decrease the enzyme activity by 60%</td>
</tr>
<tr>
<td>0.8 M Na-Citrate</td>
<td>Increase the enzyme activity by 10-fold</td>
</tr>
<tr>
<td>0.8 M NaOAc</td>
<td>Increase the enzyme activity by 8-fold</td>
</tr>
<tr>
<td>1 M (NH₄)₂SO₄</td>
<td>Decrease the enzyme activity by 100%</td>
</tr>
</tbody>
</table>

Table 3.8. Altered Kᵢ values of νc-ASADH Inhibitors in the Presence of 20% PEG 3350

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Kᵢ w/o PEG 3350 vs. νc (mM)</th>
<th>Kᵢ w/ PEG 3350 vs. νc (mM)</th>
<th>Kᵢ change (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-2,3-diaminopropionate</td>
<td>0.46</td>
<td>&gt;100</td>
<td>- 200</td>
</tr>
<tr>
<td>DL-2-amino adipic acid</td>
<td>0.47</td>
<td>3.6</td>
<td>- 8</td>
</tr>
<tr>
<td>DL-5-aminocaproate</td>
<td>0.56</td>
<td>4.2</td>
<td>- 8</td>
</tr>
<tr>
<td>L-2,4-diaminobutyrate</td>
<td>0.14</td>
<td>1.7</td>
<td>- 12</td>
</tr>
<tr>
<td>L-2,5-diaminopentanoate</td>
<td>5.2</td>
<td>3.3</td>
<td>+ 1.6</td>
</tr>
<tr>
<td>3-nitropropanoate</td>
<td>0.24</td>
<td>1.8</td>
<td>- 9</td>
</tr>
</tbody>
</table>

As shown in table 3.8, the Kᵢ values of most of the inhibitors were increased by approximately 10-fold compared to the values in the absence of PEG 3350. These change suggest that the inhibitors appear to be only weakly bound to the enzyme under this crystallization condition and the amount of the inhibitors added may not sufficient to form a complex. This weaker binding of inhibitors in presence of PEG 3350 suggests the need for exploring new precipitants if the structures of enzyme-inhibitor complexes are to be determined.
Chapter 4: Conclusions

4.1 Summary

The long term goal of this project is to identify ASADH inhibitors that can be further developed as new species-selective antibiotics. The screening of two different fragment libraries has identified several good inhibitors against three different enzyme forms purified from a representative Gram-positive, Gram-negative and fungal species. Overall, 20 compounds from the two libraries were identified as ASADH inhibitors with 7 of them inhibiting Gram-positive ASADH, 14 inhibiting Gram-negative ASADH and only 5 inhibiting yeast ASADH. None of these compounds showed good inhibition against all three forms of this enzyme, while 7 out of these 20 compounds had inhibition against both of the bacterial forms of the enzyme. Several cysteine containing compounds were eliminated from future consideration because structural studies (Pavlovsky, unpublished results) show that they covalently modify the cysteine at the active site and would also likely react with mammalian proteins. Compounds that showed good selectivity among the enzyme forms, with low millimolar inhibition constants and high L. E. values were selected for further core structural development.

The analogue development of the initial amino acid inhibitors showed that the D-isomer of 4-6 carbon dicarboxylates with 1 or 2 amino groups tend to show inhibition against either Gram-positive or Gram- negative enzyme forms with good ligand efficiency and selectivity. Several inhibitors were identified from the benzophenone
analogues that showed enhanced inhibition against vc-ASADH. The core structural development studies showed that derivatives on the benzene ring are likely to bind at the ASADH active site. For the fungal form of the enzyme, small carboxylic acids with a halo-derivative showed very good inhibition constants and ligand efficiencies. The development of a series of phenyl acids yielded several inhibitors with comparable inhibition constants and acceptable ligand efficiencies.

Kinetic studies have successfully guided structural studies of these inhibitors bound to a target enzyme but difficulties still remained. There were a few compounds that were identified kinetically as good inhibitors, but did not show binding in the structural studies, and some inhibitors were found to bind to other enzyme forms instead of their original targets that were identified by kinetic studies. Studies have been carried out to examine and minimize the differences between kinetic and structural experimental conditions, with changes in pH values and the presence of precipitants identified as the major interferences. The activity of the enzymes decrease significantly in any conditions that has a pH value less than 7. Many of the precipitants also decreased the activity of the enzyme, requiring and the $K_i$ values to be re-determined in presence of one of the major precipitants, PEG 3350. Overall, the project has developed a systematic protocol for fragment library screening and inhibitor development. The differences between kinetic and structural studies have also been refined to produce kinetic results that will more accurately guide the structural studies.

4.2 Future studies

With the selectivity that has now been obtained between the three enzyme forms, it
is necessary to screen additional enzyme forms for each class of organism to verify and extend the species selectivity. Based on the low success rate of the initial structural studies, current inhibitors need to be re-screened kinetically in the presence of precipitants and the $K_i$ values should be re-determined under conditions that more closely match the crystallization conditions. It would also be beneficial if additional enantiomers of the amino acids inhibitors can be tested to obtain more results so that can be applied to confirm the chiral selectivity between enzyme forms. To avoid the strong absorbance of DMSO-soluble inhibitors at the monitoring wavelength, some alternative experiments such as fluorescence spectroscopy or other biophysical methods such as calorimetry or surface Plasmon resonance can be applied for a more accurate and complete screening of the DMSO-soluble library. Cell-based assays will be used to measure the antibiotic properties of both these initial fragment library compounds and the more selective inhibitors that will be produced against several microbial cell lines. Toxicity assays will also be conducted against cardiac, kidney, neural and hepatic cell lines. Those inhibitors that were confirmed both kinetically and structurally will be moved into further fragment elaboration based on their binding properties and modeling studies. Alternatively, more potent inhibitors can be developed synthetically with the coupling of strong or moderate inhibition fragments that bind at different sites on these target enzymes.
References


