Identification of *B. anthracis* N⁵-Carboxyaminoimidazole Ribonucleotide Mutase (PurE) Active Site Binding Compounds via Fragment Library Screening

Hao Lei^a, Christopher Jones^a, Tian Zhu^a, Kavankumar Patel^a, Nina M. Wolf^b, Leslie W.-M. Fung^b, Hyun Lee^{a*}, and Michael E. Johnson^{a*}

^a Center for Pharmaceutical Biotechnology and Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 900 S. Ashland Ave., IL 60607, USA

^b Department of Chemistry, University of Illinois at Chicago, 845 W. Taylor St., Chicago, IL 60607, USA

*To whom correspondence should be addressed.

Michael E. Johnson: (Phone) 312-996-9114, (Fax) 312-413-9303, (Email) mjohnson@uic.edu

Hyun Lee: (Phone) 312-413-9304, (Fax) 312-413-9303, (E-mail) danielhl@uic.edu

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Abbreviations: PurE, N⁵-carboxyaminoimidazole ribonucleotide mutase; FBDD, Fragment-Based Drug Design; NMR, Nuclear Magnetic Resonance; STD-NMR, Saturation Transfer Difference Nuclear Magnetic Resonance (NMR); WaterLOGSY NMR, Water ligand observed via gradient spectroscopy NMR; SPR, Surface Plasmon Resonance; TSA, Thermal shift assay; N⁵-CAIR, N⁵-carboxyaminoimidazole ribonucleotide; CAIR, 4-carboxyaminoimidazole ribonucleotide; AIR, aminoimidazole ribonucleotide.

Abstract (200 words)

The *de novo* purine biosynthesis pathway is an attractive target for antibacterial drug design, and PurE from this pathway has been identified to be crucial for *Bacillus anthracis* survival in serum. In this study we adopted a fragment-based hit discovery approach, using three screening methods – saturation transfer difference nucleus magnetic resonance (STD-NMR), water-ligand observed via gradient spectroscopy (WaterLOGSY) NMR, and surface plasmon resonance (SPR), against *B. anthracis* PurE (*BaPurE*) to identify active site binding fragments by initially testing 352 compounds in a Zenobia fragment library. Competition STD NMR with the *BaPurE* product effectively eliminated non-active site binding hits from the primary hits, selecting active site binders only. Binding affinities (dissociation constant, K_D) of these compounds varied between 234 and 301 μM . Based on test results from the Zenobia compounds, we subsequently developed and applied a streamlined fragment screening strategy to screen a much larger library consisting of 3,000 computationally pre-selected fragments. **Thirteen** final fragment hits were confirmed to exhibit binding affinities varying from 14 μM to 700 μM , which were categorized into five different basic scaffolds. All **thirteen** fragment hits have ligand efficiencies higher than 0.30. We demonstrated that at least two fragments from two different scaffolds exhibit inhibitory activity against the *BaPurE* enzyme.

Graphic abstract

1. Introduction

Bacillus anthracis is a highly infectious aerobic, spore-forming, gram-positive bacterium that causes anthrax, a life-threatening infectious disease.¹ It has been classified as a Category A pathogen due to its potential as a bioweapon. *B. anthracis* spores can be produced *in vitro*, aerosolized, and could be transmitted to large populations, potentially resulting in high mortality. Multiple drug resistant *B. anthracis* strains have been isolated from the natural environment, including strains resistant to penicillin G, amoxicillin, erythromycin, cefuroxime, sulfamethoxazole, trimethoprim, cefotaxime-sodium, aztreonam, and ceftazidime.²⁻⁵ The declining number of new antibiotics approved by the FDA in the past four decades, as well as the occurrence of the drug resistant strains, highlights the need for alternative therapies with novel drug targets.⁶

Bacterial purine *de novo* biosynthesis is a crucial pathway for bacterial growth and virulence. Genetic studies indicate that a *purE* knockout in strains of *Escherichia coli*, *Serovar typhimurium*, and *B. anthracis* leads to a bacterial growth defect in human blood serum.⁷ Additionally, the *purE* knockout *B. anthracis* strain exhibited a significant virulence attenuation in a murine model.⁷ In fungi, plants and most bacteria, PurK converts AIR to N^5 -CAIR, which PurE subsequently rearranges to CAIR (**Fig 1A**).^{8,9} The protein sequence identity between the human PurE domain and the *B. anthracis* PurE enzyme is ~34%, **potentially** providing favorable selectivity to target bacterial PurE.

B. anthracis PurE is an octamer that contains eight active site pockets that are shallow and partially open, with an open central solvent-accessible cavity (**Fig. 1B** and **Suppl. Fig. S1**).^{10, 11} The PurE active site features suggest that fragment-based screening may be a useful alternative to drug-like or lead-like compound library high throughput screening (HTS) for PurE inhibitor development. The typical fragment based drug discovery (FBDD) approach follows a pipeline of fragment library screening, fragment binding mode elucidation, and scaffold expansion.^{12, 13} Compared with traditional HTS of drug- or lead-like hits, fragment hits usually have lower binding affinities with very little to no effect on enzyme activity, but higher ligand efficiencies (LE).^{14, 15}

Figure 1. Reactions catalyzed by class I/class II PurE and the *B. anthracis* PurE structure.

(A) Most bacteria, fungi and plant PurE enzymes catalyze the reversible reaction of converting N⁵-CAIR to CAIR. (B) PurE (PDB: 1XMP¹¹) is an octamer and has eight active sites in total, four located on the front side and four on the back. A central, solvent-accessible cavity passes through the protein. Aliphatic protons from Leu, Ile and Val are extensively distributed in the central cavity (CC) while relatively few are located in the active sites (AS). The aliphatic protons will saturate first during the STD-NMR pulse sequence and aliphatic amino acid residues in the PurE central cavity provide hydrophobic areas for non-active site compound binding, which produces false-positive signals in STD-NMR screening.

Currently there are three available assay methods for PurE, one to monitor PurE enzyme activity, and two to detect binding to PurE. In the inverse reaction, PurE converts CAIR to N⁵-CAIR, which is chemically labile and rapidly converts to AIR.¹⁶ This enzyme activity assay uses UV spectroscopy to continuously monitor the conversion of CAIR to N⁵-CAIR to AIR at 260 nm, ($\Delta\epsilon_{260}=8930 \text{ M}^{-1}\text{cm}^{-1}$, pH 8.0, where $\Delta\epsilon = \epsilon(\text{CAIR}) - \epsilon(\text{AIR})$).¹⁷ One of the two binding detection assays is an intrinsic tryptophan fluorescence (ITF) assay that monitors the intrinsic fluorescence signal of the tryptophan residue located near the active site of PurE using excitation/emission wavelengths of 295/350 nm.¹⁸ Since both of these methods use UV detection, they have limited application to compound screening because of potential signal interference from compounds at

these wavelengths. Another binding detection assay is the thermal shift assay (TSA).¹⁹ TSA has been adapted to a high throughput format for lead-like compound library screening, but has limited sensitivity for detecting low-affinity fragment compound binding. Therefore, there is a need to develop an alternative fragment screening method for this system.

In this study, we optimized three fragment screening methods – saturation transfer difference (STD) NMR, water-ligand observed via gradient spectroscopy (WaterLOGSY) NMR, and surface plasmon resonance (SPR) for the *B. anthracis* PurE. The Zenobia fragment library, a commercially available fragment library containing 352 compounds that follow the rule of three criteria²⁰ (**Suppl. Table S1**), was screened by these three methods as an initial test set for assay validation and comparison. Competition STD-NMR spectroscopy was introduced as a secondary confirmation method to further filter out non-active site binding hits followed by SPR titration or ITF titration to determine binding affinity of each fragment. Based on results from screening the relatively small Zenobia library, a streamlined screening strategy was designed and applied to the much larger 3,000-compound fragment library from ChemBridge (**Suppl. Table S2**).

2. Material and methods

2.1. Protein expression and purification

Protein expression and purification was carried out as previously described.²¹ 0.67 units/mg Thrombin (Sigma-Aldrich) was added to the purified his-tagged PurE protein for N-terminal His-tag cleavage. The mixture was incubated for 2 hours at 30 °C followed by another 30 minutes of incubation at room temperature (25 °C). Cleaved protein was loaded on a nickel immobilized metal-affinity column (HisTrap; HP, GE Healthcare) stacked with a Benzamidine column (GE Healthcare), both of which were equilibrated with buffer A (50 mM phosphate, pH 7.5, and 200 mM NaCl). The flow-through containing cleaved PurE was collected. The collected sample was then concentrated to a volume of less than 7 mL (~6 mg/mL) and loaded on a HiLoad 26/60 Superdex 200 size-exclusion column pre-equilibrated with buffer A. The purity of the protein was estimated to be ~95% by SDS-PAGE analysis. Purified PurE protein was flash frozen in liquid nitrogen and stored at -80 °C.

2.2. Enzyme activity test

The PurE enzyme activity was tested in various buffers, including 50 mM Tris, 50 mM phosphate, and 10 mM HEPES in the absence and presence of 75 mM NaCl at pH of 7.5. Assays were prepared in 96-well clear UV plates (Costar) with 200 μ L total assay volume. 100 μ L of 20 nM (2X) PurE enzyme in each buffer condition was distributed to wells along with corresponding buffer only as negative controls. The enzyme reaction was initiated by adding 100 μ L of 100 μ M (2X) substrate CAIR solution prepared in the corresponding buffer. Negative controls consisted of 50 μ M CAIR only. Plates were shaken for 30 seconds before reading. UV absorption at 260 nm was continuously monitored for 5 minutes at 25 °C by Envision™ 2104 Multilabel Reader (PerkinElmer). The slope of UV absorption vs. time plot for the first 30 sec, at each compound concentration, was calculated by linear regression.

2.3. Zenobia fragment library preparation

The Zenobia Fragment Library (Fragment Library 1) containing 352 fragment compounds was purchased in 96-well plates dissolved in 100% DMSO at 200 mM concentration. All fragments were clustered into a total number of 37 groups. The largest group has 53 fragment compounds, and 11 groups were singletons. The clustering was done by Schrödinger Canvas hierarchical clustering with the default parameter settings (Suppl. Table S3). For Zenobia library NMR screening, fragments were prepared as eight-compound mixtures in an “NMR cocktail”, with each compound per cocktail being from different clusters to maximize the diversity, which reduces the chance of spectral overlap, as well as signal intensity reduction due to potential competition for the same binding site by similar fragment compounds.

2.4. ChemBridge fragment library preparation

7,000 fragment compounds from the ChemBridge collection were computationally analyzed and filtered by the following criteria. The physicochemical characteristics of compounds were predicted by QikProp (Schrödinger). A slightly modified rule of three²⁰ for fragment library selection was applied. The criteria included molecular weight from 120 to 300, cLogP from 0 to 3, tPSA less than 70, and LogSw greater than -3. The molecules were further filtered to remove compounds that contained reactive groups (Supplementary Material). Cluster analysis was performed using the Leader-follower clustering method implemented in the Canvas software to

maximize diversity. The final fragment library containing 3,000 compounds was purchased from ChemBridge in 200 mM 100% deuterated DMSO stock.

2.5. Zenobia fragment library 1D reference NMR spectra

1D proton NMR reference spectra of the 352 Zenobia fragment compounds were collected individually. This step was essential for NMR spectral deconvolutions of binding fragments. Each reference NMR sample was prepared by adding 200 μ M of each fragment compound in 550 μ L PBS buffer (50 mM phosphate, 75 mM NaCl, and 10% D₂O, pH 7.5). Samples were equilibrated for 3 to 5 minutes until the lock signal stabilized. During data collection, a 1D double solvent suppression pulse sequence (lamellar pulses) was utilized to suppress both water and DMSO signals. Water and DMSO residual signal to noise (S/N) ratios were kept under 10 times the compound signal. Data acquisition was performed at a number of scan (NS) of 4 and dummy scan (DS) of 0. All NMR experiments were conducted at 10 °C on a Bruker 900 MHz NMR Spectrometer equipped with a cryoprobe. Data processing used Topspin 3.1.

2.6. Saturation transfer difference (STD) NMR

The temperature for NMR experiments was optimized by comparing STD-NMR spectra at 10 °C and 25 °C. PurE and compound concentrations were optimized next by performing STD-NMR with various concentrations of PurE (20 – 50 μ M) and compounds (200 – 1000 μ M). In the Zenobia library study, each NMR sample contained eight fragment compounds, each at 400 μ M concentration with 20 μ M PurE (monomeric concentration), prepared in phosphate buffer (50 mM phosphate, 75 mM NaCl, and 10% D₂O, pH 7.5). Data were collected at 10 °C. A double solvent suppression (water and DMSO) STD-NMR pulse sequence was used for data collection. On and off-resonance spectra were collected separately in the STD-NMR pulse sequence as a pseudo 2D pulse program. Saturation was carried out at 0 ppm by 15 selective Gaussian pulses (50 milliseconds) in on-resonance pulse sequences, while saturation was carried out at 30 ppm during an off-resonance pulse sequence. Saturation difference spectra were acquired by subtracting on-resonance spectra from off-resonance spectra using Topspin. NMR spectra of binding fragment compounds were deconvoluted by matching the STD spectrum signals with individual compound 1D proton spectra. The same STD-NMR screening protocol was applied to the ChemBridge fragment library, except that each NMR sample contained only one fragment compound. (One sentence has been deleted here.) The on/off resonance spectra of each

compound were used as the references to deconvolute the compound-mixture spectra collected in the later competition STD-NMR experiments. Therefore, reference spectra collection and STD-NMR screening were achieved at the same time. 1D proton reference spectra collection of the entire fragment compound library was avoided.

2.7. Water ligand observed via gradient spectroscopy (WaterLOGSY) NMR

WaterLOGSY NMR screening samples of the Zenobia fragment library compounds were prepared as the same eight-fragment mixtures as those of the STD-NMR samples. Each screening sample contained eight fragment compounds at 400 μM concentration without protein, prepared in the same phosphate buffer as the STD-NMR. A double solvent suppression (water and DMSO) WaterLOGSY NMR pulse sequence was used, and data were collected with compound mixture first, and 50 μM PurE enzyme was subsequently added to collect another set of data. Spectra with/without PurE enzymes were aligned, and compounds with signal “flip-back” were identified as binders. Binding fragment compounds were also identified by the same deconvolution process as the STD-NMR.

2.8. Surface plasmon resonance (SPR) optimization and screening

Factors optimized for SPR screening were PurE immobilization level, buffer condition, assay flow rate, and association/dissociation time, using CAIR as a positive control. Immobilization of the PurE enzymes was done at three different levels, $\sim 7,000$, $\sim 10,000$, and $\sim 12,000$ response unit (RU) by a standard amine-coupling on a CM5 sensor chip (Biacore, GE Healthcare) using the Biacore T100 instrument. The PurE enzyme was diluted with 10 mM sodium acetate (pH 4.5) and immobilized on activated flow channels 2, 3, and 4 after sensor activation with a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC)/N-hydroxysuccinimide (NHS) mixture followed by blocking unoccupied surface areas with ethanolamine. Blank immobilization was done for flow channel 1 as a control. Two assay buffers, HBS-P (10 mM HEPES, 150 mM NaCl, and 0.05% v/v Surfactant P20, pH 7.4) and PBS-P (20 mM phosphate, 2.7 mM KCl, 137 mM NaCl and 0.05% v/v Surfactant P20, pH 7.4) and three assay flow rates (30, 50, and 100 $\mu\text{L}/\text{min}$) were tested and compared. In addition, association and dissociation times were tested at both 30 and 60 seconds.

For fragment library screening, PurE immobilization levels of flow channels 2 and 4 were achieved at $\sim 10,500$ RU and $\sim 11,500$ RU, respectively. Flow channels 1 and 3 were used as

controls immobilized with ethanolamine only. All Zenobia fragment library compounds were prepared as 10 mM stocks in 100% DMSO and were diluted in the PBS-P with 0.5 mM TCEP to 200 μ M final concentration in 384-well plates. A final DMSO concentration of 2% was used for both binding buffer and analyzing compound samples; solvent correction was performed using a series of eight solutions between 1.5% and 2.8% DMSO in PBS-P. Each compound was injected for 30 second association and 30 second dissociation times at a flow rate of 50 μ L/min into all four channels. All data were referenced for blank injections on flow channels 1 and 3. Sensorgrams were analyzed using Biaevaluation software 2.0.3, and the RU for each compound was measured during the binding equilibration phase. Fragment compounds that had abnormal binding sensorgrams were ruled out during analysis. All assays were done in duplicate. Hit compounds were selected with a cut-off of 8.0 RU. The same SPR screening conditions were used to screen the Chembridge fragment library.

2.9. Competition STD-NMR

Competition STD-NMR samples were prepared as four fragment combination cocktails for both Zenobia and ChemBridge fragment libraries, with each fragment concentration at 400 μ M in 550 μ L phosphate buffer. CAIR was synthesized following the previously described procedure.²² The ϵ_{260} of CAIR determined at pH 8.0 varied from 10,000~8,800 $M^{-1}cm^{-1}$ in different batches. CAIR was prepared as a 50 mM stock in the same buffer. Data were collected by the same STD-NMR pulse sequence in the absence and presence of 500 μ M CAIR. The signal intensity decrease was calculated by **Eq. 1** where y is % signal decrease, and I is the integrated value of the most intense peak of each compound signal.

$$y = \frac{I_{without\ CAIR} - I_{with\ CAIR}}{I_{without\ CAIR}} \quad (1)$$

Compounds with greater than 20% signal decrease were considered to be active site binding hits. The errors were calculated by the integrated value of 0.2 ppm noise area divided by $I_{without\ CAIR}$.

2.10. SPR Equilibrium dissociation constant (K_D) determination

For K_D determination of Zenobia fragment hits, PurE was immobilized to flow channels 2, 3, and 4, and immobilization levels were ~10,100 RU, ~10,000 RU, and ~9,500 RU, respectively. Immobilization of PurE for the ChemBridge fragment compounds was slightly lower than for the Zenobia compounds. All fragment hits showing competition with CAIR were prepared as 200

mM stocks in DMSO. Compound solutions with a series of increasing concentrations (0 – 1200 μ M) were prepared in the PBS-P with 0.5 mM TCEP, and applied to all four channels at a 50 μ L/min flow rate. Sensorgram analyses and RU value calculations were done in the same way as primary screening. The K_D values were determined by fitting the data to a single rectangular hyperbolic curve using **Eq. 2** where y is the RU, $y_{\max}$ is the maximum RU, and x is the compound concentration. All K_D values were determined in triplicate.

$$y = \frac{y_{\max} \cdot x}{K_D + x} \quad (2)$$

2.11. Intrinsic tryptophan fluorescence (ITF) assay K_D determination

Protein solutions were prepared in phosphate buffer (50 mM phosphate, 75 mM NaCl, pH 7.5) at a monomeric concentration of 8.5 μ M. Fragment compounds were prepared as 200 mM stock in DMSO. Samples were prepared by adding compound stock in 600 μ L protein solution at a series of concentrations (0 – 1000 μ M final concentrations) in a Varian fluorometer cell cuvette. Compounds at each concentration in the absence of PurE were also tested in 600 μ L phosphate buffer for potential background fluorescence signal as controls. Samples were scanned at 295/(310-500) nm (excitation/emission), and the fluorescence intensity at 350 nm was measured. $(F_0 - F)/F_0$ values were calculated and plotted with each compound concentration, where F_0 is the fluorescence intensity of the sample containing protein solution only, and F is the fluorescence intensity of samples at various compound concentrations with background interference subtracted. The binding affinity K_D was determined by fitting the data to a single rectangular hyperbolic equation, **Eq. 3**, using SigmaPlot 10.0, where $\Delta F_{\max}$ is the maximum value of $(F_0 - F)/F_0$, and $[L]$ is the compound concentration.

$$\frac{F_0 - F}{F_0} = \frac{\Delta F_{\max} \cdot [L]}{K_D + [L]} \quad (3)$$

2.12. Inhibitory activity by UV spectroscopy

Assays were prepared in 96-well clear UV plates (Costar) with 210 μ L total assay volumes. Compounds (10 mM stock in DMSO) were diluted in a series of decreasing concentration (0 – 200 μ M final concentration at 2-fold dilution) with DMSO. Compound, enzyme, and CAIR solutions were prepared at three times final assay concentration (3X) with assay buffer (50 mM Tris, 75 mM NaCl, pH 7.5). 70 μ L of 3X compound solution and 70 μ L of 3X enzyme solution were mixed first and incubated in the assay plates for 5 minutes before adding 70 μ L of 3X

CAIR solution to initiate the reaction. The final concentration of PurE enzyme, CAIR, and DMSO were 20 nM, 50 μ M, and 2%, respectively, and all assays were done at room temperature. The same plate reading program **was** used as described in **the** enzyme activity test section. The inhibition percentage was calculated by **Eq. 4**, where S_0 is the average slope calculated from the reaction without compound and S is the reaction **slope** at different compound concentrations.

$$Inhibition\% = \frac{S_0 - S}{S_0} \times 100\% \quad (4)$$

3. Results and discussion

3.1. Pre-screen optimization of three methods for PurE

STD-NMR and WaterLOGSY NMR are the most common ligand-based NMR techniques for fragment screening. Pre-screen optimization for NMR was done for buffers, salt, temperature, and protein and compound concentrations. PurE enzyme activity was similar in Phosphate and Tris buffers, while it was slightly lower in HEPES buffer. The addition of NaCl did not affect the activity (**Table 1**). Thus, phosphate buffer with 75 mM NaCl at pH 7.5 was selected as the buffer for the NMR sample since there is no NMR signal interference from the buffer itself. We selected 283 K, ~10 degrees lower than room temperature as the temperature for NMR screening. At this temperature, the 136 kDa octameric PurE will tumble slower (longer rotational correlation time, τ), adding line broadening, and reducing background signal interference from the protein. Although higher enzyme and ligand concentrations yield higher S/N ratios, reducing data collection time, reduced amounts of compound and enzyme are preferred to reduce the chance of compound aggregation as well as overall cost. Signal intensities were measured with various concentrations of PurE (20 – 50 μ M) and fragment compound (200 – 1000 μ M). The result is listed in **Supplementary Table S4**. The protein and compound concentrations were finalized at 20 μ M PurE with 400 μ M fragments for STD-NMR.

Table 1. Summary of PurE enzyme activity test

Buffer	NaCl	Slope ($\Delta A_{260}/\text{min}$)	Activity (unit/mg) ^a
Phosphate (50 mM)	None	-0.034 ± 0.002	20.03 ± 1.18
Phosphate (50 mM)	75 mM	-0.036 ± 0.004	21.22 ± 2.36
Tris (50 mM)	None	-0.033 ± 0.002	19.45 ± 1.18
Tris (50 mM)	75 mM	-0.035 ± 0.008	20.62 ± 4.72
HEPES (10 mM)	None	-0.026 ± 0.003	15.32 ± 1.77
HEPES (10 mM)	75 mM	-0.026 ± 0.003	15.32 ± 1.77

^a The activity is defined as the disappearance of **the number of** μmol CAIR in 1 min with 1 mg of PurE (unit/mg);²³ The CAIR concentration was calculated using $\Delta\epsilon_{260} = 8930 \text{ M}^{-1}\text{cm}^{-1}$.¹⁷

SPR pre-screen optimization was performed for assay buffer selection, immobilization level, assay flow rate, and association/dissociation time. First, obtaining reasonable response unit (RU) signals is crucial since our analytes are small fragment compounds (94 – 300 Da). Two assay buffers, PBS-P and HBS-P, were tested for binding signal, and no significant difference in RU changes was observed. Second, finding optimal immobilization level is another important factor for successful SPR results. Typically, high immobilization levels are not recommended for observing protein-protein or protein-ligand interactions due to mass transfer effect. However, low immobilization levels may not be ideal for screening fragment compounds. Our analytes are significantly smaller than immobilized protein (octamer, 136 kDa), resulting in a small RU change since the mass change upon compound binding is minimal in comparison to the large protein analytes. **We used CAIR as a positive control during our SPR optimization. In order to ensure that the immobilization level was sufficient to detect fragment binding,** a series of immobilization levels (7,000 – 12,000 RU) were tested, and ~10,000 RU showed adequate binding signal for fragment compound screening. Third, flow rate and association/dissociation time were optimized to reduce run time with detectable RU signals. A flow rate of 50 $\mu\text{L}/\text{min}$ and 30 seconds for both association and dissociation times resulted in optimal RU signals. All assay optimizations are summarized in **Supplementary Table S5**.

3.2. Primary screening of the Zenobia library

Primary screening of the Zenobia fragment library containing 352 fragment compounds was performed by the three optimized methods, STD-NMR, WaterLOGSY NMR, and SPR. STD and WaterLOGSY NMR screening were each done once, and SPR was done in duplicate.

Primary screening results are shown in **Figures 2A – 2C**; SPR results were highly reproducible within replicates (**Fig. 2C**). In our ligand-based NMR screening study, samples were prepared as eight-compound mixtures. If compounds were mixed randomly, this would increase the chance of similar fragments competing for the active site, which could lead to decreased signal intensity and to difficulty in deconvoluting hit fragments.²⁴ To avoid this, Zenobia library compounds were clustered by their scaffolds, and compound mixtures added in NMR samples were combined from different clusters in order to maximize diversity. STD-NMR detected a total of 84 fragment hits ($S/N > 5.0$). NMR spectra from one of the NMR screening cocktail samples consisting of eight fragments are shown in **Figure 2D**. In the STD-NMR spectra, two double split peaks from ZT0430 and ZT0470 were identified while the remaining six fragment compounds showed no signal in the STD-NMR spectrum, indicating that only ZT0430 and ZT0470 are binders. WaterLOGSY NMR detected 48 hits ($S/N > 5.0$), and NMR spectra of the same set of eight fragments from STD-NMR are shown in **Figure 2E**. In WaterLOGSY NMR, the peak “flip-back” is the binding signature, which was the case for ZT0430 and ZT0470 after adding PurE. All the other six non-binding fragments’ signals remained inverted, indicating no binding. SPR screening produced 86 fragment hits within a cut-off of 8.0 RU. Example sensorgrams of a binder (ZT0022) and a non-binder (ZT0037) are shown in **Figure 2F**. The response unit of ZT0022 was ~22 RU, while that of ZT0037 was only ~2.2 RU.

Figure 2. Primary screening results by three methods for the Zenobia fragment library. The signal to noise ratio (S/N) values determined by STD-NMR (A) and WaterLOGSY NMR (B) for fragment compounds in the presence of PurE. 84 and 48 fragment hits were detected by STD-NMR and WaterLOGSY NMR, respectively, with a cut-off value of 5.0. Red lines represent cut-off values for each primary screening method. (C) Replicate plots of the screening of 352 fragment compounds by SPR. The red box indicates hit compounds from SPR with a cut-off 8.0 RU. (D) An example NMR spectrum of a cocktail consisting of eight fragment compounds. *S* is the STD-NMR spectrum of the eight fragment compound mixture aligned with the individual 1D proton NMR spectra of each of the eight fragment compounds. The double split peak noted by white open arrows corresponds to the binding signals from ZT0470, while the other double split peak noted by black filled arrows corresponds to the binding signal from ZT0430. (E) Spectrum W1 is the WaterLOGSY NMR spectrum with the same eight compound mixture in the absence of PurE. Spectrum W2 is the WaterLOGSY NMR spectrum of the same sample after the addition of PurE enzyme. The 1D proton NMR spectra of each eight compounds are similarly aligned. The white arrows indicate the binding of ZT0470, where a peak “flip-up” is observed after the addition of PurE in W2. The same peak “flip-up” is also observed from ZT0430 that is specified by black filled arrows. (F) SPR sensorgrams of a binding fragment ZT0022 (red) and a nonbinding fragment ZT0037 (black).

3.3. Selecting active site binders by secondary competition STD-NMR

Interaction between fragments and macromolecules at sites other than the target site is a common issue associated with fragment library screening. Neither ligand-based NMR techniques nor SPR can differentiate active site binding from binding at other locations on PurE. In our case, PurE exists as an octamer that contains eight active site pockets and one central cavity. There is a possibility that hit fragments from the primary screen may bind to the central cavity instead of the active sites, which would make them non-active site binding hits. Aliphatic protons on three amino acid residues (Leu, Ile, and Val) are saturated first during the STD-NMR on-resonance pulse sequence. Compounds bound to areas abundant with aliphatic protons build up irradiation saturation in a shorter period of time through cross relaxation. In the PurE octamer structure, the central cavity contains a crowded population of aliphatic protons (**Fig. 1B**), which quickly saturates the resonances of fragment compounds binding to this area. In addition, areas abundant with aliphatic amino acid residues provide a hydrophobic surface that could cause nonspecific interactions. Therefore we optimized a competition STD-NMR experiment as a secondary confirmation method to eliminate non-active site binding hits. Normally a known inhibitor is used as a competitor in a competition replacement experiment, but unfortunately there is no known inhibitor commercially available for *B. anthracis* PurE. 4-nitroaminoimidazole

ribonucleotide (NAIR), a known *E. coli* PurE inhibitor ($K_D = 86 \pm 31 \text{ nM}$)¹⁸, could be used as an active site competitor as an alternative to CAIR since NAIR is likely bind to *B. anthracis* PurE tighter than CAIR based on high active site homology between *E. coli* and *B. anthracis* PurE. However, NAIR was not commercially available. Therefore, we used the PurE enzyme product CAIR ($K_m = 9.7 \text{ }\mu\text{M}$ for *B. anthracis* PurE¹⁹) as a competitor instead to select compounds that bind specifically to the active sites. Although CAIR was used to set up the competition STD-NMR, it is the substrate, AIR, that served as a competitor due to the fast conversion from CAIR to AIR according to our NMR and PurE activity data ($20.03 \pm 1.18 \text{ unit/mg}$). 500 μM CAIR fully converts to AIR in 0.7 min by 20 μM PurE enzyme. The binding affinity of AIR was $283 \pm 94 \text{ }\mu\text{M}$, as determined by intrinsic tryptophan fluorescence assay. This method can identify active site binders by observing STD signal intensity decrease caused by competition replacement by AIR.^{25, 26} (Two sentences have been removed from here.) We thus used competitor concentrations higher than that of compounds during competition screening, to ensure that competitive replacement could be observed. The competition STD-NMR spectra of ZT0073 are shown in **Figure 3** as an example. The STD-NMR spectra of ZT0073 alone and compound ZT0073 with 500 μM competitor were aligned, and a 25% signal decrease was observed upon addition of competitor. The top 57 STD-NMR fragment hits (selected by cut-off S/N > 7) were cherry-picked and retested by competition STD-NMR. The top six fragments, which exhibited a signal intensity decrease of more than 20%, were retested by competition STD-NMR in deuterated HEPES buffer (10 mM, pH 7.5) and the results were reproducible. These six fragments were selected for further study.

(New Figure 3)

Figure 3. Secondary competition STD-NMR. Competition STD-NMR spectra. *S1* is the STD-NMR spectrum with ZT0073 only, and *S2* is the STD-NMR spectrum after adding the active site competitor. Black filled arrows indicate that one of the ZT0073 signals showed a 25% intensity drop after adding active site competitor (starred peaks). The bottom spectrum is the 1D proton spectrum of ZT0073.

3.4. Equilibrium dissociation constant (K_D) determination

In general, inhibitory activity of a small fragment compound is quite difficult to measure by enzymatic assay due to only partial occupancy of the active site. Instead, binding affinity (equilibrium dissociation constant, K_D) of fragment compounds has been commonly used as a good indicator for selecting fragments.¹² The binding affinity of fragment hits can be measured by isothermal titration calorimetry (ITC), SPR, or 2D HSQC NMR titration. 2D HSQC with ¹⁵N-labeled PurE was not successful due to the extreme line broadening effect caused by the large molecular size of PurE. Another method, an intrinsic tryptophan fluorescence (ITF) assay, was introduced to measure the ligand binding affinity to *E. coli* PurE.¹⁸ The single tryptophan (W20) residue of *E. coli* PurE is located near the PurE active site, the CAIR binding site, and hence W20 serves as a probe for ligand binding.¹⁸ This tryptophan residue is conserved in many PurE enzymes including *B. anthracis* PurE (W15). W15 is the only tryptophan in *B. anthracis* PurE, which eliminates the possibility of having unrelated fluorescence signals from other non-active site areas (Suppl. Fig. S1). Unfortunately, all six active site binding hits from the Zenobia library had strong fluorescence interference from the compounds themselves, which limited use of the ITF assay. Thus, we used SPR to determine the binding affinities of these hit fragments. Of the six hits, three fragments exhibited specific binding to PurE with K_D values in the range of 200–300 μ M, whereas the remaining three compounds did not show dose-response curves. The SPR sensorgrams and the fitting curves are shown in Figure 4. ZT0022 and ZT0073 exhibited solubility problems at higher concentrations (> 500 μ M) and displayed a two-phase binding interaction: specific binding at lower concentration and non-specific binding at higher concentrations. K_D values of the two fragment compounds were calculated from the lower concentration soluble portion of the binding curve (Fig. 4D and E). Compound aggregation could be the potential reason that causes nonspecific binding at higher concentration. It may result from the compounds' hydrophobic interaction, since ZT0022 and ZT0073 have fewer hydrophilic functional groups than ZT0580. Ligand efficiency (LE) is a parameter widely used

for fragment hit quality evaluation.¹⁴ A higher LE indicates more chemical space for affinity improvement as well as physicochemical property modification. The LE values of the three Zenobia final hits were all calculated to be above 0.3. Binding affinities of the three final Zenobia hits are listed in **Table 2**. Overall, ZT0580 can be considered to be a high-quality fragment with the LE above 0.4 and solubility above 1.2 mM in aqueous solution.

(New Figure 4)

Figure 4. Dissociation equilibrium constant (K_D) determination by SPR. (A), (B) and (C) are the SPR sensorgrams of ZT0022, ZT0073 and ZT0580 at various concentrations (0 – 1200 μM). (D), (E) and (F) are the SPR fitting curve of ZT0073, ZT0022 and ZT0580. The K_D values were determined with a single rectangular hyperbolic equation (See methods). The determined K_D values of ZT0022, ZT0073 and ZT0580 were 301 ± 36 , 234 ± 34 and $274 \pm 14 \mu\text{M}$, respectively.

Table 2. Final hits from the Zenobia fragment library

ID	Structure	MW (Da)	Δ STD (%) ^a	K _D (μM) ^b	LE ^c
ZT0022		143	24 ± 4	301 ± 36	0.44
ZT0073		179	25 ± 2	234 ± 34	0.38
ZT0580		154	57 ± 5	274 ± 14	0.44

^a Δ STD (%) is the percent of STD-NMR signal decreasing in the presence of CAIR. Experiments were repeated in deuterated HEPES buffer (10 mM, pH 7.5). The standard deviation are from two independent experiments.

^b K_D: Equilibrium dissociation constant determined by SPR; The standard deviation are from three independent experiments.

^c LE: Ligand efficiency ($LE = -RT \ln K_D / N$, where K_D is the binding affinity and N is the number of non-hydrogen atoms); LE was calculated from the K_D values determined by SPR.

3.5. Comparison of the three screening methods and the resulting screening strategy.

There were 84 STD-NMR hits and 48 WaterLOGSY-NMR hits with the same cut-off value applied ($S/N > 5$). STD-NMR is more sensitive in detecting PurE binding fragments. The sensitivity difference between these two NMR methods may result from the large molecular size of PurE, 136 kDa. PurE has an octameric structure that benefits STD-NMR detection because the spin diffusion process in STD-NMR is more efficient and requires less irradiation saturation time when dealing with larger target proteins. However, there are several reported cases where WaterLOGSY NMR displays significantly higher sensitivity than STD-NMR, which include ketoprofen–bovine serum albumin, *tert*-butyl hydroquinone–hemagglutinin, and chloramphenicol–ribosome (70S) interactions.²⁸ The sensitivity of ligand-based NMR screening methods may vary depending on target macromolecules. Hence, it is always necessary to compare both approaches when tackling a new target protein. (One sentence has been deleted.) 44 out of 48 WaterLOGSY-NMR hits overlap with STD-NMR hits. SPR detected 86 hits in total, 51 of which overlap with STD-NMR hits; and 33 overlap with WaterLOGSY-NMR hits. There are 32 hits selected by all three approaches (**Fig. 5A**). Hit fragment structures and properties from three methods were compared in different clusters. There was no significant difference of the hit distribution, suggesting that one technique might not bias properties of the hits over the other techniques for our target protein PurE.

SPR is medium-throughput and can be optimized in 384-well plates with automated instruments. For large fragment compound library screening, SPR is a good option as a primary screening technique, and is cost efficient due to its higher degree of throughput and automation capability. In our opinion, NMR is lower-throughput and is less readily automated, although it is very sensitive. Hence NMR was chosen as a secondary screening method for binding confirmation in our study. Competition STD-NMR, as a tertiary screen, can identify fragments that bind to the active site. Finally, either the SPR or ITF assay can be used for binding affinity determination. Our screening strategy is shown in **Figure 5B** as applied to screening the 3,000-compound ChemBridge fragment library.

(New Figure 5)

Figure 5. Summary diagram of Zenobia library fragment hits and the screening pipeline for the ChemBridge fragment library. (A) Number of hits summary diagram from the three screening methods. Numbers located at each area are exclusive to each other, and the overlapping areas indicate hits jointly detected by corresponding methods. (B) Fragment screening strategy and the screening results for the ChemBridge fragment library, with sequential reduction of hits at each stage. The compound hit percentage of the full library and cutoff values applied in each step are displayed in parentheses.

3.6. ChemBridge 3,000 fragment screening and hit validation

Primary SPR screening of 3,000 fragments detected 750 fragment hits binding to PurE ($RU > 12.0$); the replicate plot showed very consistent RU values (**Fig. 6A**). Primary hits were rescreened by STD-NMR, with the binding of 194 out of 750 confirmed with S/N ratios above

10. These secondary hits were selected for a further competitive binding assay. ChemBridge fragments exhibited higher average signal intensity than those from the Zenobia library, as would be expected since the ChemBridge fragment library compounds have a larger average molecular size (225 Da) than those of the Zenobia library (154 Da). Competition-STD-NMR effectively eliminated non-active site binders, selecting only **thirteen** active site binding fragments with more than a 20% decrease in signal intensity after adding CAIR as a competing molecule. Alternatively, competition SPR could also be used to eliminate non-active site binding fragments. **The average cLogP values of thirteen active site binders is 1.70, while the average cLogP values of the remaining 178 nonspecific binders is 1.82. The slightly higher hydrophobicity of non-active site binders might be diagnostic of binding to the hydrophobic PurE central cavity area although their structures are diverse.”**

Both ITF and SPR were applied to determine the binding affinities of active site binders, and results are summarized in **Table 3**. Overall, the K_D values of each hit compound determined by the two methods are comparable within experimental error range. Of 15 hit fragments, CB-1715 exhibited the strongest binding affinity by both ITF and SPR. CB-0611 showed very weak binding with a K_D value of $707 \pm 101 \mu\text{M}$ by SPR, while ITF binding was not detected for this compound. The ITF titrations for both CB-1715 and CB-0611 are shown in **Figure 6B** and **C**. This observation indicates that, although ITF and SPR results are consistent for most of the tested compounds, each detection method still has its own limitations. Sometimes an additional detection method may be necessary to salvage some true low affinity fragment hits.

Table 3. Final hits from the ChemBridge fragment library

Scaffold	ID	MW (Da)	Δ STD (%) ^a	K_D (μ M) _{ITF} ^b	K_D (μ M) _{SPR} ^b	Inhibition (%) ^c	LE ^h
Scaffold 1	CB-1675	222	25 $\pm$ 1	39.6 $\pm$ 4.3	20.3 $\pm$ 4.1	ND ^d	0.43
	CB-1715	239	23 $\pm$ 4	19.8 $\pm$ 1.2	13.5 $\pm$ 1.5	49 $\pm$ 2 ^f	0.44
	CB-1744	220	30 $\pm$ 3	95.4 $\pm$ 7.0	79.8 $\pm$ 5.2	ND	0.35
Scaffold 2	CB-0611	198	32 $\pm$ 5	NB ^c	707 $\pm$ 100	ND	0.31
	CB-2217	251	22 $\pm$ 2	61.6 $\pm$ 1.1	73.0 $\pm$ 12.6	ND	0.33
	CB-2150	192	30 $\pm$ 2	81.3 $\pm$ 8.0	60.8 $\pm$ 26.1	36 $\pm$ 3 ^g	0.44
Scaffold 3	CB-0581	174	21 $\pm$ 3	73.8 $\pm$ 8.1	261 $\pm$ 28	ND	0.38
	CB-0164	178	25 $\pm$ 4	35.7 $\pm$ 3.6	35.3 $\pm$ 20.4	ND	0.51
	CB-2959	217	30 $\pm$ 4	604 $\pm$ 88	121 $\pm$ 14	ND	0.36
Scaffold 4	CB-0134	189	32 $\pm$ 7	214 $\pm$ 12	149 $\pm$ 11	ND	0.37
	CB-0467	208	28 $\pm$ 7	402 $\pm$ 53	444 $\pm$ 28	ND	0.33
Scaffold 5	CB-1150	179	23 $\pm$ 4	182 $\pm$ 12	103 $\pm$ 6	ND	0.54
	CB-1598	261	35 $\pm$ 7	367 $\pm$ 48	157 $\pm$ 13	ND	0.37

^a Δ STD (%) is the percent of STD-NMR signal decreasing in the presence of CAIR. Experiments were repeated in deuterated HEPES buffer (10 mM, pH 7.5). The standard deviations are from two independent experiments.

^b K_D (μ M) ITF: Equilibrium dissociation constant determined by intrinsic tryptophan fluorescence assay; K_D (μ M) SPR: Equilibrium dissociation constant determined by SPR; The standard deviations are from three independent experiments.

^c NB: No binding.

^d ND: not determined.

^e The enzyme inhibitory activities of only CB-1715 and CB-2150 were measurable; the remaining thirteen hits were not determined due to strong compound UV signal interference.

^f Determined at 25 μ M CB-1715 concentration

^g Determined at 100 μ M CB-2150 concentration

^h Ligand efficiency (LE) was calculated by using the K_D values determined by SPR

These thirteen final hits were grouped into five classes according to their chemical scaffolds (**Fig. 6D**), four of which possess two ring systems, either joined by an intermediate linker, or fused together. Fragments in scaffold 1 contain an amide linker between two rings, a five-membered hetero-aromatic ring with another five or six-membered aromatic ring, while scaffold 2 compounds have a single atom carbon/nitrogen linker between two rings. Fragments in scaffold 3 have a double fused ring core with single or double substitutes, and scaffold 4 compounds have a pyrazole or isoxazole directly linked to a phenyl group. Scaffold 5 possesses

the simplest structure, a multi-substituted benzene. Interestingly, hits from scaffold 1 have far tighter average binding affinities than those of any other scaffolds. Scaffold 1 compounds have slightly larger molecular size and more expanded chemical structures that might allow more chemical interactions with active site residues.

Figure 6. Thirteen competition STD-NMR hits from the ChemBridge fragment library and K_D determination by intrinsic tryptophan fluorescence assay. (A) Replicate plots of the primary screening of 3,000 fragment compounds by SPR. The red box indicates hit compounds from SPR with cut-off > 12 RU. (B) The fitting curve of CB-1715 generated from intrinsic tryptophan fluorescence (ITF) titration. The K_D was determined to be $19.8 \pm 1.2 \mu\text{M}$. (C) The low ITF signal intensities of CB-0611 at a series of concentrations (0 – 1200 μM) indicates no binding. (D) The final 13 hits were classified to 5 groups according to their chemical scaffolds. Single five and six membered rings are highlighted in cyan and green, respectively. Amide linkers are highlighted in pink. Single carbon/nitrogen linkers are highlighted in orange. The two fragments with detectable enzyme inhibition activity are shown in red boxes.

Two of our final fragment hits showed quantifiable PurE inhibition – CB-1715 and CB-2150 showed enzyme inhibitions of $49 \pm 2\%$ (at 25 μM) and $36 \pm 3\%$ (at 100 μM), respectively. The UV inhibitory activity test result is shown in Figure 7. The enzyme inhibitory activities of the remaining eleven hits were not determined either due to strong compound UV signal

interference or inhibition not observed. All **thirteen** final hits have ligand efficiencies above 0.30.

(New Figure 7)

Figure 7. The inhibitory activity of CB-2150 and CB1715 tested by UV spectroscopy. The negative controls have 50 μ M CAIR without PurE, while the positive controls have 50 μ M CAIR and 20 nM PurE. CB-1715 was tested at 25 μ M and showed $49 \pm 2\%$ inhibition. CB-2150 was tested at 100 μ M and it showed $36 \pm 3\%$ inhibition. The UV readings of CB-1715 and CB-2150 were normalized to the same level as that of the controls for easier visual comparison.

4. Conclusion

We designed a large-scale fragment compound library screening strategy based on the results of screening a small-scale Zenobia fragment library against *B. anthracis* PurE. The funnel-type procedure enriches the fragment hits with high quality while filtering out ones of either weak or nonspecific binding at each step. From the beginning to the end of the process, the degree of throughput of each screening technique decreases. This arrangement balances the screening efficiency with detection sensitivity. By applying the proposed screening strategy to the ChemBridge fragment library consisting of 3,000 fragment compounds, we identified **thirteen** active site binders. These **thirteen** final hits display binding affinities ranging from ~ 14 μ M to 900 μ M, and were categorized into five scaffolds based on their chemical structures. Our best lead fragment, CB-1715, exhibits $\sim 49\%$ inhibition at 25 μ M concentration, which is very

encouraging considering that CB-1715 is still a small fragment compound. Based on the ChemBridge fragment library screening results, the proposed screening strategy proved to be effective and efficient in identifying high quality fragment hits against *B. anthracis* PurE.

Acknowledgements

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Figure Legends

Figure 1. Reactions catalyzed by class I/class II PurE and the *B. anthracis* PurE structure.

(A) Most bacteria, fungi and plant PurE enzyme catalyze the reversible reaction of converting N⁵-CAIR to CAIR. (B) PurE (PDB: 1XMP¹¹) is an octamer and has eight active sites in total, four located on the front side and four on the back. A central, solvent-accessible cavity passes through the protein. Aliphatic protons from Leu, Ile and Val are extensively distributed in the central cavity (CC) while relatively few are located in the active sites (AS). The aliphatic protons will saturate first during the STD-NMR pulse sequence and aliphatic amino acid residues in the PurE central cavity provide hydrophobic areas for non-active site compound binding, which produces false-positive signals in STD-NMR screening.

Figure 2. Primary screening results by three methods for the Zenobia fragment library.

The signal to noise ratio (S/N) values determined by STD-NMR (A) and WaterLOGSY NMR (B) for fragment compounds in the presence of PurE. 84 and 48 fragment hits were detected by STD-NMR and WaterLOGSY NMR, respectively, with a cut-off value of 5.0. Red lines represent cut-off values for each primary screening method. (C) Replicate plots of the screening of 352 fragment compounds by SPR. The red box indicates hit compounds from SPR with cut-off 8.0 RU. (D) An example NMR spectrum of a cocktail consisting of eight fragment compounds. *S* is the STD-NMR spectrum of the eight fragment compound mixture aligned with the individual 1D proton NMR spectra of each of the eight fragment compounds. The double split peak noted by white open arrows corresponds to the binding signals from ZT0470, while the other double split peak noted by black filled arrows corresponds to the binding signal from ZT0430. (E) Spectrum *W1* is the WaterLOGSY NMR spectrum with the same eight compound mixture in the absence of PurE. Spectrum *W2* is the WaterLOGSY NMR spectrum of the same sample after the addition of PurE enzyme. The 1D proton NMR spectra of each eight compounds are similarly aligned. The white arrows indicate the binding of ZT0470, where a peak “flip-up” is observed after the addition of PurE in *W2*. The same peak “flip-up” is also observed from ZT0430 that is specified by black filled arrows. (F) SPR sensorgrams of a binding fragment ZT0022 (red) and a nonbinding fragment ZT0037 (black).

Figure 3. Secondary competition STD-NMR. Competition STD-NMR spectra. *S1* is the STD-NMR spectrum with ZT0073 only, and *S2* is the STD-NMR spectrum after adding the active site competitor. Black filled arrows indicate that one of the ZT0073 signals showed a 25% intensity drop after adding active site competitor (starred peaks). The bottom spectrum is the 1D proton spectrum of ZT0073.

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