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Discovery and development of inhibitors of acetyltransferase Eis to combat *Mycobacterium tuberculosis*

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Abstract

Aminoglycosides are bactericidal antibiotics with a broad spectrum of activity, used to treat infections caused mostly by Gram-negative pathogens and, as a second-line therapy, against tuberculosis. A common resistance mechanism to aminoglycosides is bacterial aminoglycoside acetyltransferase enzymes (AACs), which render aminoglycosides inactive by acetylating their amino groups. In *Mycobacterium tuberculosis*, an AAC called Eis (enhanced intracellular survival) acetylates kanamycin and amikacin. When upregulated as a result of mutations, Eis causes clinically important aminoglycoside resistance; therefore, Eis inhibitors are attractive as potential aminoglycoside adjuvants for treatment of aminoglycoside-resistant tuberculosis. For over a decade, we have studied Eis and discovered several series of Eis inhibitors. Here, we provide a detailed protocol for a colorimetric assay used for high-throughput discovery of Eis inhibitors, their characterization, and testing their selectivity. We describe protocols for *in vitro* cell culture assays for testing aminoglycoside adjuvant properties of the inhibitors. A procedure for obtaining crystals of Eis-inhibitor complexes and determining their structures is also presented. Finally, we discuss applicability of these methods to discovery and testing of inhibitors of other AACs.

Keywords

Antibacterial agents; Crystal structure; Drug resistance; High-throughput assay; Infectious diseases

1. Introduction

Aminoglycosides are clinically important antibiotics with a broad spectrum of activity. Aminoglycosides are commonly used to treat infections with Gram-negative pathogens that are not susceptible to β-lactams. Aminoglycosides bind to the bacterial ribosome and inhibit its function, causing a bactericidal effect due to the inhibition of synthesis of essential proteins or accumulation of toxic peptide products generated by the aborted

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synthesis. An important mechanism of resistance to aminoglycosides besides ribosomal mutations is their enzymatic modification by aminoglycoside acetyltransferases (AACs). More than 50 different AAC enzymes have been identified [1]. AACs catalyze acetylation of amino groups of aminoglycosides by transferring the acetyl group of acetyl coenzyme A (AcCoA) onto an amino group of an aminoglycoside, thereby reducing its binding affinity towards the ribosome. Most of the AACs have a GCN5-related *N*-acetyltransferase (GNAT) fold. This basic fold and the enzymatic function of AACs are shared with histone acetyltransferases, which acetylate N_ε of lysine residues in histone proteins. Apart from Eis and its homologs [2–6], AAC enzymes reported to date are regioselective, modifying at one specific amino group position (1, 3, 2' or 6'). The position of the modification is included in the enzyme names. Clinically relevant AACs of this type include AAC(6')-Ie [7, 8] and AAC(6')-Ib [9, 10]. Recently, the Wright group discovered a non-GNAT AAC, called ApmA, which modified apramycin at the 2' position, a structurally unique aminoglycoside used to treat infection in animals [11]. ApmA was found to have a left-handed β-helix superfamily fold. In *Mycobacterium tuberculosis* (*Mtb*), the causative pathogen of tuberculosis (TB), upregulation of an AAC called Eis (enhanced intracellular survival) was shown to be responsible for clinically observed resistance of TB to kanamycin, a second-line anti-TB drug [12–15]. Subsequently, we showed that unlike other AACs, Eis was uniquely regioversatile, acetylating kanamycin, amikacin (another anti-TB drug), and other clinically used aminoglycosides at multiple amino group positions [16, 17]. We reasoned that Eis was an attractive candidate for inhibitor discovery and development, with the aim of using Eis inhibitors as aminoglycoside adjuvants against kanamycin- and amikacin-resistant TB. Over the past decade, we optimized and used a DTNB-based colorimetric assay to discover several structural families of Eis inhibitors *via* high-throughput screening (HTS) of chemical libraries [18–25], including those containing FDA-approved drugs [26] (Fig. 1A). In this chapter, we describe the details of this robust assay as well as its applications, from a high-throughput setup to downstream applications for inhibitor characterization. We include a cell-based assay used to validate the on-target activity of the inhibitors of the *Mtb* cell. We also describe a protocol for a crystallographic method of crystallizing Eis-inhibitor complexes. We expect that analogous approaches can be used for discovery and characterization of inhibitors of AACs and other acetyltransferases.

2. Method: Discovery and characterization of Eis inhibitors

2.1 Recombinant protein purification of *Mtb* Eis and other AACs

Eis has been expressed recombinantly in *Escherichia coli* BL21(DE3). The structural analysis of Eis from different species (*Bacillus anthracis* [2], *Anabaena variabilis* [5], *Mycobacterium smegmatis* [27]) was described by our group, as well as biochemical characterization of Eis from nine other species by our group [3]. Here, we will be focusing on *Mtb* Eis, which we have used for discovery and development of several inhibitors. The sequence of *Mtb* Eis (gene locus Rv2416c) was cloned into the pET28a plasmid, yielding an Eis protein with N-terminal His_6 tag. The placement of His_6 tag is crucial for biochemical and structural studies, as Eis with a C-terminal His_6 tag affects the acetylation activity and crystallization [17]. The other AACs utilized to determine the specificity of Eis inhibitors are AAC(6')-Ie/APH(2")-Ia, used solely for the AAC(6')-Ie activity (heretofore

called AAC(6')-Ie) from *Staphylococcus aureus*, AAC(3)-IV from *E. coli*, and AAC(2')-Ic from *Mtb*. These three enzymes were cloned and purified similarly to Eis. AAC(6')-Ie was cloned into the pET22b plasmid with a C-terminal His_6 tag [28], AAC(3)-IV was cloned into Int-pET19b-pps [29] with a N-terminal His_{10} tag [28], and AAC(2')-Ic was cloned into pET28a with a N-terminal His_6 tag [17].

2.1.1 Equipment and materials

1. Temperature controlled incubator shaker (with a range of 16 °C to 37 °C).
2. Cell lysis equipment: Sonicator, French press, or a cell homogenizer.
3. Centrifuge capable of reaching at least 30,000×g.
4. Ni-NTA agarose resin (Qiagen) or equivalent.
5. A fast protein liquid chromatography (FPLC) station with a Superdex S-200 size exclusion column (Cytiva).
6. Amicon Ultra-15 centrifugal filter (10-kDa MW cut-off, Millipore).
7. SDS-PAGE gel electrophoresis system.

2.1.2 Buffers and chemicals

1. 10× Eis purification buffer, 500 mM Tris-HCl pH 8.0, 1 L: Dissolve 60.55 g of Tris base (Trizma) in 800 mL of ddH_2O. Adjust the pH to 8.0 by addition of concentrated HCl before adding ddH_2O to a final volume of 1 L.
2. 10× AAC(6'/3) purification buffer, 500 mM sodium phosphate pH 8.0, 1 L: Dissolve 2.736 g of NaH_2PO_4 and 128.7 g of Na_2HPO_4 in 900 mL of ddH_2O. Adjust the pH to 8.0 by addition of 5 M NaOH before adding ddH_2O to a final volume of 1 L.
3. 10× AAC(2') purification buffer, 250 mM triethanolamine pH 7.8, 10 mM EDTA, 1 L: Dissolve 37.3 g of triethanolamine and 3.38 g of Na_2EDTA in 800 mL of ddH_2O. Adjust the pH to 7.8 by addition of concentrated HCl before adding ddH_2O to a final volume of 1 L.
4. 500 mM HEPES, pH 7.5, 1 L: Dissolve 119.15 g of HEPES in 800 mL of ddH_2O. Adjust the pH to 7.5 by addition of 5 M NaOH before adding ddH_2O to a final volume of 1 L.
5. 4 M NaCl, 1 L: Dissolve 234 g of NaCl in 1 L of ddH_2O.
6. 2 M imidazole, pH 8.0, 1 L: Dissolve 136.2 g of imidazole in 800 mL of ddH_2O. Adjust the pH to 8.0 by addition of concentrated HCl before adding ddH_2O to a final volume of 1 L.

   NOTE: Sterilize solutions 1–6 by autoclaving for 45 min at 121 °C, 15 psi, with subsequent slow exhaust. Do not tighten the lids or do use foil in place of lids when sterilizing in the autoclave.

7. 100 mg/mL AMP, 10 mL: Dissolve 1 g of AMP in 10 mL of ddH_2O. Store in 1 mL aliquots at −20 °C.

8. 50 mg/mL KAN, 10 mL: Dissolve 0.5 g of KAN in 10 mL of ddH_2O. Store in 1 mL aliquots at −20 °C.

9. 1 M IPTG, 10 mL: Dissolve 2.4 g of IPTG in 10 mL of ddH_2O. Store in 1 mL aliquots at −20 °C.

   NOTE: Sterilize solutions 7–9 by filtration through a 0.22 μm syringe filter.

10. Buffer A (1× purification buffer, 300 mM NaCl, 10% glycerol), 100 mL: Combine 10 mL of 10× purification buffer, 7.5 mL of 4 M NaCl, 10 mL of glycerol, then add ddH_2O to a final volume of 100 mL.

11. Buffer B (1× purification buffer, 300 mM NaCl, 5 mM imidazole, 10% glycerol), 50 mL: Combine 5 mL of 10× purification buffer, 3.75 mL of 4 M NaCl, 12.5 μL of 2 M imidazole, 5 mL of glycerol, then add ddH_2O to a final volume of 50 mL.

12. Buffer C (1× purification buffer, 300 mM NaCl, 20 mM imidazole, 10% glycerol), 50 mL: Combine 5 mL of 10× purification buffer (enzyme dependent), 3.75 mL of 4 M NaCl, 0.5 mL of 2 M imidazole, 5 mL of glycerol, then add ddH_2O to a final volume of 50 mL.

13. Buffer D (1× purification buffer, 300 mM NaCl, 40 mM imidazole, 10% glycerol), 50 mL: Combine 5 mL of 10× purification buffer, 3.75 mL of 4 M NaCl, 1 mL of 2 M imidazole, 5 mL of glycerol, then add ddH_2O to a final volume of 50 mL.

14. Buffer E (1× purification buffer, 300 mM NaCl, 250 mM imidazole, 10% glycerol), 50 mL: Combine 5 mL of 10× purification buffer, 3.75 mL of 4 M NaCl, 6.25 mL of 2 M imidazole, 5 mL of glycerol, then add ddH_2O to a final volume of 50 mL.

15. Buffer F (50 mM Tris-HCl pH 8.0, 10% glycerol), 2 L: Combine 200 mL of 500 mM Tris-HCl pH 8.0 and 200 mL of glycerol, then add ddH_2O to a final volume of 2 L.

16. Buffer G (50 mM HEPES pH 7.5, 10% glycerol), 2 L: Combine 200 mL of 500 mM HEPES pH 7.5 and 200 mL of glycerol, then add ddH_2O to a final volume of and dilute to 2 L.

17. Buffer H (25 mM triethanolamine pH 7.8, 1 mM EDTA, 10% glycerol), 2 L: Combine 200 mL 250 mM triethanolamine pH 7.8, 10 mM EDTA, and 200 mL of glycerol, then add ddH_2O to a final volume of 2 L.

2.1.3 Overexpression and purification protocol

1. Transform the vector containing the AAC gene of interest, *i.e.*, pET28a-Eis into *E. coli* BL21 (DE3) cells by the heat shock method. Add 1 μL of the plasmid (at least 50 ng/μL) into 50 μL of cells and gently mix. Keep on ice for 30 min before placing the tube in a 42 °C water bath for 45 s. Place the tube back on ice for 5

min before the addition of 250 μL of autoclaved (as described above) and cooled Luria-Bertani (LB) medium. Shake the cells for 45 min at 37 °C. Spread 150 μL of this cell culture on LB agar plates with KAN (50 μg/mL) or ampicillin (AMP; 100 μg/mL) for pET22b or Int-pET19b-pps, and incubate overnight at 37 °C.

2. Pick 5–10 colonies and inoculate in 5 mL of autoclaved LB medium. Shake the culture for 3–4 h before using it as a starter for 1 L of LB medium culture. All LB medium should be supplemented with KAN (50 μg/mL) or AMP (100 μg/mL). Shake the cell culture continuously at 200 rpm at 37 °C until, for Eis, it reaches an attenuance at 600 nm of 0.3–0.4. Move the culture to a 16 °C incubator shaker for 1 h. Add a final concentration of 500 μL of 1 M IPTG into the 1 L culture and keep shaking at the same speed overnight. Prepare at least 3–4 L of culture. For AAC(6')-Ie and AAC(3)-IV, incubate the cells until an attenuance at 600 nm of 0.6, at which point induce protein production by adding 1 mL of 1 M IPTG (1 mM final concentration). Keep growing the cells for an additional 4–5 h at 37 °C. Grow cells expressing AAC(2')-Ic to an attenuance at 600 nm of 1.0, induce by adding 200 μL of 1 M IPTG, and grow for 9 h at 20 °C.

3. Harvest cells by centrifugation for 10 min at 5000×g and resuspend the cell pellet in Buffer A. Spent LB medium should be treated with bleach (10% final concentration) and allowed to set for 10 min. Afterwards, all treated media can usually be poured down the drain. Disrupt cells on ice using a cell lysis apparatus and then centrifuge at 30,000×g for 45 min at 4 °C to clarify the lysate. Save a small amount of the insoluble cell debris for gel analysis to establish if the overexpressed protein is insoluble. The remaining debris can be scraped out of the tube into a paper towel using a spatula and placed in a biohazardous waste container for autoclaving.

4. Load the clarified lysate onto a 2-mL Ni-NTA agarose resin column (see Note 1). To purify the protein of interest, wash the column with 5 mL of Buffer B followed by 15 mL of Buffer C (collected in 3 portions) followed by 15 mL of Buffer D (collected in 3 portions), and finally, elute the His-tagged protein with 15 mL of Buffer E (collected in 3 portions).

5. Dialyze the fractions containing the eluted protein, as determined by SDS-PAGE, in Buffer F (Eis), Buffer G (AAC(6')-Ie and AAC(3)-IV), or Buffer H (AAC(2')-Ie). Use 2 L of the dialysis buffer at a time for a total of 6 L, with at least 2–3 h between buffer changes. Buffered solutions can generally be poured down the sink without any treatment. Solutions containing a thiol-based reducing agent may have an odor.

6. For biochemical assays, concentrate the purified proteins to about 1 mg/mL by using an Amicon Ultra-15 centrifugal device. Store all proteins in 25-μL aliquots at −80 °C.

7. For crystallization, concentrate the eluted Eis protein to about 5 mL by using an Amicon Ultra-15 centrifugal device, then inject it onto a FPLC S-200 size exclusion column equilibrated in Buffer D. Run the column in the same buffer

at 2 mL/min and collect 5 mL fractions. Combine fractions containing Eis, as determined by SDS-PAGE (see Note 2). As a reference, Fig. 2 shows the S-200 gel filtration chromatogram as well as the SDS-PAGE gel profile. Concentrate the purified protein to 4–5 mg/mL by using an Amicon Ultra-15 centrifugal device. The protein should be stored on ice and used for crystallization within 1–2 weeks after purification. This is best accomplished by keeping an ice bucket in the refrigerator or cold room at 4 °C.

2.2 The acetylation activity assay

Eis protein and other AACs transfer the acetyl group of AcCoA to their aminoglycoside substrate, yielding an acetylated aminoglycoside and CoA. The released CoA can be detected by a colorimetric method, where the colorless Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid) or DTNB] reacts with the free thiol group of CoA yielding the yellow 2-nitro-5-thiobenzoate (TNB) and the disulfide bond linked product CoA-TNB. This color change is quantifiable by measuring light absorbance at 412 nm at room temperature [30, 31]. DTNB is readily accessible and inexpensive, but it is pH sensitive and reactive with thiol groups of solvent accessible cysteine residues. Because normally enzyme concentrations are much lower than the concentrations of the generated CoA product, the cysteine reactivity should not pose significant problems. We have successfully used this assay with Eis from *Mtb*, and several other species, as well as several other AACs without any complication, indicating that this assay is quantitative, replicable, and suitable for Eis and AACs in general.

2.2.1 Equipment and materials

1. Clear flat-bottom 96 or 384-well assay polystyrene plates.
2. A liquid handling multidrop dispenser instrument and/or multichannel pipette.
3. A plate reader such as PHERAstar plate reader or SpectraMax M5 plate reader.

2.2.2 Chemicals

1. *Mtb* Eis protein, prepared and purified following the protocol in Section 2.1.3, concentrated to 100 μM.
2. Other AAC proteins, purified following the protocol in Section 2.1.3, concentrated to 20–100 μM.
3. Aminoglycosides.
    a. Neomycin trisulfate salt hydrate (NEO), purchased from Sigma Aldrich, 100 mM: Dissolve 909 mg of NEO in ddH_2O to a final volume of 10 mL.
    b. Kanamycin sulfate (KAN), purchased from VWR, 100 mM: Dissolve 583 mg in ddH_2O to a final volume of 10 mL.
4. Acetyl coenzyme A (trilithium salt), purchased from Sigma Aldrich, 100 mM: Dissolve 827 mg of AcCoA in ddH_2O to a final volume of 10 mL.

5. 5,5’-Dithiosbis(2-nitrobenzoic acid) (DTNB), purchased from Sigma Aldrich, 1 M: Dissolve 4 g of DTNB in DMSO to a final volume of 10 mL. NOTE: Compounds dissolved in DMSO can be absorbed through the skin and caution should be taken by wearing appropriate gloves.

6. Screening plate. Obtain small molecule libraries from companies like ChemDiv, NIH Clinical Collection, and MicroSource Discovery Systems. Note that compounds containing free thiol groups may interfere with the DTNB reaction and produce false positive results. Prepare plates containing stock concentrations of 4 mM of these test compounds, except for the first and last columns (96-well) or the first two and last two columns (384-well), where on one side the empty columns contain no AcCoA and on the other 100% DMSO as positive and negative controls, respectively. Store the plates containing stock concentration of compounds at −20 °C.

7. Solutions for HTS:

    a. Eis/NEO mixture (50 mM Tris-HCl pH 8.0, 133.33 μM NEO, 0.33 μM Eis), 15 mL. This volume is sufficient for 500 reactions. Combine 750 μL of 1 M Tris-HCl pH 8.0, 20 μL of 100 mM NEO, and 49.5 μL of 100 μM Eis, then add ddH_2O to a final volume of 15 mL.

    b. AcCoA/DTNB mixture (50 mM Tris-HCl pH 8.0, 160 μM AcCoA, 2 mM DTNB), 5 mL. This volume is sufficient for 500 reactions. Combine 250 μL of 1 M Tris-HCl pH 8.0, 8 μL of 100 mM AcCoA, and 10 μL of 1 M DTNB, then add ddH_2O to a final volume of 5 mL.

8. Solutions for validation and dose-response assay in the HTS setup.

    a. Eis/NEO mixture (50 mM Tris-HCl pH 8.0, 400 μM KAN, 1 μM Eis), 15 mL. Combine 750 μL of 1 M Tris-HCl pH 8.0, 60 μL of 100 mM KAN, and 150 μL of 100 μM Eis, then add ddH_2O to a final volume of 15 mL.

    b. AcCoA/DTNB mixture (50 mM Tris-HCl pH 8.0, 2 mM AcCoA, 2 mM DTNB), 15 mL. Combine 750 μL of 1 M Tris-HCl pH 8.0, 300 μL of 100 mM AcCoA, and 150 μL of 1 M DTNB, then add ddH_2O to a final volume of 15 mL.

9. Solutions for validation and dose-response assay with fresh compounds.

    a. Eis/KAN mixture (50 mM Tris-HCl pH 8.0, 400 μM KAN, 1 μM Eis), 15 mL. Combine 750 μL of 1 M Tris-HCl pH 8.0, 60 μL of 100 mM KAN, and 150 μL of 100 μM Eis, then add ddH_2O to a final volume of 15 mL.

    b. AcCoA/DTNB mixture (50 mM Tris-HCl pH 8.0, 2 mM AcCoA, 2 mM DTNB), 15 mL. Combine 750 μL of 1 M Tris-HCl pH 8.0, 300 μL of 100 mM AcCoA, and 150 μL of 1 M DTNB, then add ddH_2O to a final volume of 15 mL.

NOTE: Used 96-well plates can be disposed of by emptying liquid contents into a sink followed by disposal in regular garbage.

2.2.3 The enzyme activity assay—Before any inhibition assays, or inhibitor screening can be done, the enzyme needs to be checked for activity. In conjunction with Eis, test all AACs at the same substrate (aminoglycoside) and cosubstrate concentrations. Four different AACs use different buffers and enzyme concentrations depending on the activity and stability of the enzymes, as follows. Combine 100 μM of NEO, 500 μM of AcCoA, 2 mM of DTNB, and enzyme (0.5 μM of Eis, 0.5 μM of AAC(6')-Ie, 0.125 μM of AAC(3)-IV, 0.25 μM of AAC(2')-Ic) in buffer (50 mM MES pH 6.6 for AAC(6')-Ie and AAC(3)-IV, 50 mM Tris pH 8.0 for Eis, or 100 mM sodium phosphate for AAC(2')-Ic). Incubate the reactions at 25 °C (37 °C for AAC(6')-Ie) and monitor absorbance at 412 nm.

2.2.4 The high-throughput screening assay—Prior to the actual high-throughput screening assay, it is important to run a test control plate to identify the robustness of the assay. Use two controls in this assay: (1) a reaction without AcCoA (positive control), and (2) a reaction in the presence of 0.5% *v*/*v* DMSO without a compound (negative control). A Z' score can then be calculated to quantify the robustness of the assay [32, 33]:

$$Z' = 1 - 3\frac{\mathrm{SD(pos)} + \mathrm{SD(neg)}}{|\mathrm{Av(pos)} - \mathrm{Av(neg)}|}$$

where SD(pos) and SD(neg) are the standard deviations of the absorbance measurements for the wells without AcCoA and with DMSO, respectively; while the Av(pos) and Av(neg) are the average absorbance values for these respective wells. $Z' > 0.5$ indicates robustness of the assay. If the calculated Z' score, however, is too high (*i.e.*, between 0.9–1), the reaction may not be in a steady-state regime and certain conditions (such as reaction time) need to be adjusted accordingly. We previously reported a Z' score of 0.65 for this assay [34]. The above expression for Z' has been used in practice by many studies, including ours. A more statistically rigorous expression for Z' was recently suggested [33], which is predicted to serve as a better quality control parameter in the cases of marginal assay robustness.

The conditions reported herein were optimized and should be replicable.

1. Add 30 μL of Eis/NEO mixture to the assay plate using a multidrop dispenser.
2. Transfer 0.2 μL of solution from each well of the compound stock plate to the assay plate. Use a final concentration of 20 μM of compounds to test for inhibition.
3. After 10 min reaction at room temperature, add 10 μL of AcCoA/DTNB mixture to the assay plate.
4. After 5 min of incubation, measure the absorbance at 412 nm using a plate reader.

2.2.5 The dose response assay—A scatterplot can be created to identify hit compounds, which have a value of >3σ, where σ is the standard deviation to the negative

control (DMSO alone). To confirm that compounds are indeed hits that are worth pursuing, a single concentration (depending on the desired stringency) assay needs to be performed in triplicate using freshly prepared compounds for each initial hit. Compounds that result in a reduction of at least 30% in Eis activity in at least two of the independent assays are considered to be confirmed hits. Note that hit compounds containing a thiol group should be tested with 2 mM DTNB in 50 mM Tris pH 8.0 in the absence of the enzyme, as the compounds themselves may react with DTNB. Following this initial testing, perform a dose-response assay with varying concentration of compounds using a 2-fold dilution technique. Obtain IC_{50} value Hill coefficient values to assess the potency and specificity of the compounds.

1. Purchase hit compounds from the high-throughput assay in powder form and prepare fresh stock solution by dissolving the compound in 100% DMSO. Compound solubility vary and therefore stock concentration can differ from one another. If possible, aim to prepare 10 mM stock.
2. In a 96-well plate, add 100 μL of 50 mM Tris pH 8.0 and 5% DMSO in each well. To column 2, add an additional 20 μL of Tris/DMSO along with 5 μL of 10 mM compound. Perform a 5-fold dilution from column 2 to column 11 (200 μM to 0.1 nM) of a 96-well plate by taking 25 μL of the solution from column 2 and adding it to the next column. Keep columns 1 and 12 for positive and negative control.
3. Add 50 μL of an Eis/KAN mixture (see Note 3) into each well using a multichannel pipette.
4. After a 10 min of incubation at room temperature, add 50 μL of AcCoA/DTNB mixture to each well using a multichannel pipette.
5. After AcCoA addition, measure absorbance for 20–30 min in 30 s interval.
6. Subtract the signal from the positive control (no AcCoA) and convert the absorbance values to μM values. Calculate initial rates by using the first 2–5 min of the reaction. Normalize the data to the negative control (no compounds) to obtain % activity values.
7. Calculate IC_{50} and Hill coefficient values by using the equation below by nonlinear regression, by using software such as SigmaPlot or Origin. An example of an IC_{50} curve is shown in Fig. 3. For the dose-response curve to have a sigmoidal shape as in Fig. 3, the inhibitor concentration on the x-axis must be plotted on a log scale.

$$a = 100 - \frac{100 - r}{1 + \left(\frac{IC_{50}}{[I]}\right)^{h}}$$

2.3 Mode of inhibition analysis

Whether an inhibitor is reversible or irreversible can normally be tested by pre-incubating an enzyme with a slight molar excess of the inhibitor. If the inhibitor is irreversible, this

will result in a complete inactivation of the enzyme. To date, all Eis inhibitors have been reversible. To determine the mode of inhibition of such compounds, perform the reaction assays above by varying concentrations of aminoglycoside and inhibitor. Test at least four different concentrations, including 0 μM, of inhibitor and as many concentrations of aminoglycoside needed to generate an acceptable Michaelis-Menten curve. Present reaction rates as Lineweaver-Burk plots. The Lineweaver-Burk plots aids the identification if the mode of inhibition *via* the relationship between the slopes and intercepts. There is a possibility that Lineweaver-Burk analysis is insufficient. In this case, other modes of visualization can be used (*i.e.*, Eadie-Hofstee, Dixon, and/or rate/substrate *vs.* inhibitor concentration) to aid in visual interpretation of the mode of inhibition. It should be noted that data conversion to reciprocal coordinates leads to asymmetrical error bars. For this reason, when experimental uncertainty is significant, determination of the mode of inhibition by the visual inspection of the above plots can be misleading. Rigorous analysis of the mode of inhibition should be performed with the raw data (reaction rate *vs.* concentrations of substrate and inhibitor) using global nonlinear regression (using all the data in one regression run) with SigmaPlot (SYSTAT) assuming the most general, mixed mode of inhibition, as we reported recently for Eis inhibitors with different modes of action [26]. Here, use the software output (*p*-values) to establish the mode of inhibition. If a specific mode of inhibition is established that is simpler than mixed inhibition (*e.g.*, competitive, uncompetitive, or noncompetitive inhibition), obtain the V_{max}, K_m, and K_i values by nonlinear regression using the raw data for that mode of inhibition.

2.4 The inhibitor selectivity assay

Further assess hit compounds to verify if inhibition is selective to *Mtb* Eis. This can be done by following the protocol laid out in Section 3.5 but using other AACs. Generally, test three AACs with different regiospecificity: AAC(2')-Ic (0.125 μM), AAC(3)-IV (0.25 μM) and AAC(6')/APH(2")-Ia (0.25 μM). Protein production and purification for these AACs were previously described in detail [17, 28]. Some AACs may not accept and acetylate KAN and/or NEO; therefore, a different aminoglycoside substrate needs to be used.

2.5 Cell viability assays

The hit compounds discovered by HTS and validated by dose-response assays should then be tested for their activity in the bacterial cell. Because *Mtb* H37Rv is a virulent and pathogenic strain, it can only be handled in a biosafety level 3 BSL-3 facility. While this is the preferred strain to use, this may not be feasible for many researchers. Non-pathogenic BSL-1 and BSL-2 mycobacteria such as *M. smegmatis*, *Mtb* H37Ra, or one of the genetically modified BSL-2 strains of *Mtb* produced by the Jacobs laboratory (*i.e.*, *Mtb* mc^26230, *Mtb* mc^26206, etc.) [35] may be used in place of *Mtb* H37Rv. To test if compounds can inhibit Eis in mycobacterial cell, use *Mtb* mc^26230 [19] modified to mimic the BSL-3 *Mtb* K204 strain, which is a KAN-resistant overexpressing Eis strain to see if it can restore its KAN sensitivity. In addition, preliminary biosafety test can be carried out by assessing compound toxicity effect using different mammalian cell lines. Mycobacterial and mammalian cells should be handled with care and always in a sterile environment, specifically a biosafety cabinet suitable for handling cell cultures.

2.5.1 Materials and chemicals

1. Mycobacterial strains: *Mtb* $mc^2$6230, *Mtb* $mc^2$6230 K204. Store aliquots of mycobacterial cells at −80 °C.
2. Solid and liquid media for growth of *Mtb* $mc^2$6230. The solid medium contains Middlebrook 7H11 medium supplemented with 0.5% *v*/*v* glycerol, 10% *v*/*v* OADC, 0.2% *w*/*v* casaminoacids, and 24 μg/mL of pantothenate. Liquid medium is Middlebrook 7H9 supplemented with 0.05% *v*/*v* tyloxapol, 0.5% *v*/*v* glycerol, 10% *v*/*v* OADC, 0.2% *w*/*v* casaminoacids, and 24 μg/mL of pantothenate. NOTE: Sterilize OADC, casaminoacids, pantothenate, and tyloxapol by filtration through a 0.22 μm syringe filter, and add these to cooled autoclaved 7H11 or 7H9 medium.
3. 96-well plates with lids.
4. Plate reader.

2.5.2 General MIC value determination

1. Prepare mycobacterial strains by inoculating cells from frozen stock onto respective growth medium agar plates. Grow cells at 37 °C until colonies can be visualized.
2. Pick colonies of mycobacteria and inoculate them into 5 mL of appropriate growth medium, until the density reached 0.4–0.6 using a densitometer and a 0.5 McFarland standard, this serves as the stock culture. A working culture is prepared by taking 0.1 mL of stock culture and adding it into 10 mL of sterile growth medium.
3. Using a multichannel pipette, add 200 μL of sterile ddH_2O to the exterior wells of the 96-well plate (rows A and H, columns 1 and 12) to help prevent evaporation from the rest of the plate. Place 200 μL of growth medium in columns 2 rows B-G while 100 μL of growth medium in columns 4–11. In column 3, add 195 μL of growth medium, followed by 5 μL of a compound to be tested. Generally, initial testing is done using 10 mM (or 10 mg/mL) compound stock, which yields the highest concentration of compound to be tested of 125 μM (or 125 μg/mL). The inhibitor concentration can be adjusted accordingly by increasing or decreasing the concentration of compound stock. In each experiment, KAN and isoniazid are used as standard controls, following the same double dilution method.
4. Perform double dilution from columns 3 to 10 by taking 100 μL of growth medium with compounds from column 3 and adding to column 4, mixing the new solution by pipetting at least three times and repeating the procedure in the succeeding columns. Column 11 serves as growth control; hence no compound is added to this well.
5. Add 100 μL of working culture into columns 3–11 and rows B-G of the 96-well plate. No mycobacterium is added into column 2, which serves as sterile control.

6. Place both the 96-well plate and remaining working culture in a 37 °C incubator. Once turbidity can be seen in the control wells (column 11), the 96-well plate is ready for staining.

7. In each well, add 5 μL of 2.5 mg/mL resazurin. A color change from blue to pink indicates presence of metabolically active mycobacterial cells. The MIC value is taken on the blue well with the lowest concentration of compound present.

NOTE: All liquid waste should be treated with a final concentration of 10% bleach. Then plates can be put in the biohazardous waste and autoclaved.

2.5.3 The KAN adjuvant activity assay—To determine if the compound displays any KAN adjuvant activity, the procedure described in section 2.5.2 is slightly modified. This procedure is performed only with *Mtb* $mc^2$6230 and *Mtb* $mc^2$6230 K204.

1. Carry out steps 1 and 2 of section 2.5.2.

2. As in step 3 of section 5.2, place 200 μL of ddH_2O in the exterior border wells of the 96-well plate. To one row of the remaining wells, add 200 μL of growth medium, to a second row, add 196 μL of growth medium and 4 μL of a 1 mg/mL solution of KAN, and fill all remaining rows with 100 μL of the growth medium.

3. Using a multichannel pipette, double dilute KAN across rows, leaving the last row free of KAN for the growth control. This results in a concentration range of 10 μg/mL to 1.25 μg/mL KAN, which covers the resistant to susceptible range for *Mtb.*

4. To each well of a column, add 50 μL of the compound at the desired concentration taking into account the dilution with the other components of the assay. Use at least one column as a KAN control, where the column was free of compound.

5. Add 50 μL of the working solution of bacteria to each well, except for the well for sterile control.

6. Incubate the plate and stain following steps 6 and 7 of section 2.5.2. Examples of compounds that are KAN adjuvants are shown in Table 1.

NOTE: All liquid waste should be treated with a final concentration of 10% bleach. Then, plates can be put in the biohazardous waste and autoclaved.

2.6 The crystallographic approach

The crystal structures of Eis in complexes with inhibitors provide details of Eis-inhibitor interactions, informing on the mechanism of the inhibitors and aiding in their medicinal chemistry optimization. Here we provide a detailed procedure as to how to grow crystals of Eis and effectively soak in small molecule inhibitors. Our previous work established that one of the Eis cysteine residues, Cys204, located somewhat close to the substrate binding site, likely impeded substrate and inhibitor binding, because the CoA used for Eis crystallization formed a disulfide bond with this cysteine residue [16]. We showed that a point mutant Eis C204A retained the acetylation activity of the wild-type enzyme. This

mutant was successfully used in soaking inhibitors and tobramycin into Eis crystals in all of our crystallographic studies involving these ligands. This Eis mutant can be produced and purified following the protocol used the wild-type Eis (Section 2.1 [16]).

2.6.1 Materials and chemicals

1. 24-well crystallization (VDX) plate for a hanging drop setup can be purchased with or without sealant from Hampton Research. Silicone grease sealant needed to be applied by the user for VDX plates without sealant.
2. 22 mm cover slips, purchased from Fisher Scientific.
3. Mounted litholoops.
4. KAN, 100 mM, 1 mL: Dissolve 58.3 mg in ddH_2O to a final volume of 1 mL.
5. Coenzyme A trilithium salt, 100 mM, 1 mL: Dissolve 78.5 mg in ddH_2O to a final volume of 1 mL.
6. 1 M Tris-HCl, pH 8.5, 50 mL: Dissolve 6.06 g of Tris base (Trizma) in 40 mL of ddH_2O. Adjust the pH to 8.5 by addition of concentrated HCl before adding ddH_2O to a final volume of 50 mL.
7. 50% *w*/*v* PEG 8000, 50 mL: Dissolve 25 g of PEG 8000 in ddH_2O to a final volume of 50 mL.
8. 4 M $(NH_4)_2SO_4$, 50 mL: Dissolve 26.4 g of ammonium sulfate in ddH_2O to a final volume of 50 mL.
9. Stabilization solution (0.1 M Tris-HCl pH 8.5, 10% PEG 8000), 1 mL: Combine 100 μL of 1 M Tris pH 8.5 and 200 μL of 50% PEG 8000 with 700 μL of ddH_2O.
10. Compound solution (0.1 M Tris-HCl pH 8.5, 10% PEG 8000, 1 mM compound, 20% glycerol), 0.1 mL: Prepare 10 mM compound stocks. Combine 10 μL of 1 M Tris pH 8.5 and 20 μL of 50% PEG 8000 with 20 μL of glycerol and 10 μL of 10 mM compound. Add 40 μL of ddH_2O to the mixture.

2.6.2 The crystallization protocol

1. Add 5 μL of 100 mM KAN and 4 μL of 100 mM CoA to 41 μL of EisC204A protein (~5 mg/mL). Note that even if the crystals can be obtained from frozen stocks of EisC204A, it is recommended to use freshly purified protein.
2. The general crystallization condition for *Mtb* Eis is 0.1 M Tris-HCl pH 8.5, 10% PEG 8000 and 0.5 M $(NH_4)_2SO_4$. Optimal crystallization condition, however, may differ somewhat due to minor variations in the preparation of protein sample and chemical stocks. As such, it is recommended to do a 2-dimensional grid screen around this condition by varying the concentration of PEG 8000 (6% to 15%) and $(NH_4)_2SO_4$ (0.3 to 0.6 M) in the first and second dimension, respectively. Different pH of Tris-HCl may also be tested, but from our experience, this is not necessary if the buffer used is within ~0.5 pH range.

3. Set up hanging drop crystal trays by mixing the Eis/KAN/CoA with the reservoir solution in 1:1 ratio. Set the trays in a temperature- and vibration-controlled incubator at 21 °C. Fully formed crystals can be observed after two weeks (Fig. 2C).

4. Gradually exchange drops containing crystals by slowly adding 1–2 μL of stabilization solution, followed by removal of 1–2 μL drop solution. Do this at least five times. Keep crystals in this condition for at least 10 min before gradually exchanging into compound solution. If multiple compounds are to be tested, crystals can be first moved to a new drop containing stabilization solution, before exchanging it to desired compound solution.

5. Incubate the crystals in compound solution for at least 30 min. Then pick up the crystals with mounted litholoops, and quickly plunge them in liquid nitrogen. The used crystal trays can be emptied into the laboratory sink and discarded into regular garbage.

2.6.3 Structure determination and analysis—Data collection can be carried out using an in-house X-ray diffractometer or synchrotron. Generally, synchrotron is preferred as it yields data of superior quality. Process collected data using HKL2000 [36]. Use our initial Eis crystal structure (PDB: 3R1K [17]) as the search model to obtain Eis-inhibitor crystal structures by molecular replacement with Phaser [37] or MOLREP [38]. Assess the presence of soaked compounds by examining the omit F_o-F_c electron density maps using Coot software [39]. PDB and CIF files for the soaked compounds need to be generated by JLigand software [40] in CCP4 suite. The JLigand software requires a SMILES String which can be obtain by drawing and selecting the chemical structure of the compound in ChemDraw and copying the structures into JLigand as SMILES strings. Both the PDB file and the CIF file for the compounds generated by JLigand must then be opened in COOT software to manually adjust the compound structure. Fig. 2D shows an example of small molecule. The superimposition of crystal structures of Eis bound to different ligands can be readily performed in Coot using the SSM superposition function.

Eis crystals can grow in different crystal forms with different space groups at the above conditions, but the most useful space group is R32, with one protomer of the hexameric Eis per asymmetric unit. The overall structure of an Eis protomer and its binding site for aminoglycosides, CoA and our previously identified small molecule inhibitors is shown in Fig. 2D. Two aminoglycosides tobramycin and paromomycin were previously observed in the aminoglycoside binding site in *Mtb* Eis [16] and its *M. smegmatis* [41] homolog by us and others, respectively.

2.7 Notes

1. Ni-NTA agarose resins supplied by a manufacturer contain ethanol. Make sure to wash the beads first with 1–2 column volume of ddH_2O before equilibrating with Buffer A.

2. Eis fractionates as hexamer in an S-200 column and elutes near the void volume.

3. NEO was used in HTS because it shows robust activity with *Mtb* Eis. However, subsequent assays and studies, KAN was used in place of NEO because it is more biologically and clinically relevant for *Mtb* Eis studies. Other aminoglycosides can be used with other AAC enzymes, as dictated by the enzymatic activity and clinical relevance.

3. Application of the method to other acetyltransferases

The described enzymatic assay monitors the formation of the co-product of the acetyl transfer reaction, CoA; therefore, this assay is applicable to discovery of inhibitors of other AAC enzymes. Indeed, a similar assay was used to discover and characterize low-μM inhibitors of AAC(6')-Ib that could be useful for their application as amikacin adjuvants against *Acinetobacter baumannii* infections [43]. Another example of the application of this assay is finding of Zn^{2+} as an inhibitor of AAC(6')-Ib with an IC_{50} value of 15 μM in a similar kanamycin acetylation assay [44]. This initial discovery was followed up on by our group, where we found by using an analogous assay that zinc pyrithione was a nonselective inhibitor of AAC enzymes [45]. The acetylation activity assay yields a robust and reproducible signal in a steady-state regime within a few minutes using sub-μM concentrations of the enzyme. This set up can be used to conduct initial high-throughput screening to identify low-μM hits. Lower concentrations of the enzymes and longer reaction times can be used to identify inhibitors with nM potency, owing to generally high stability of AAC enzymes. We expect that this assay will be used more broadly in high throughput to discover inhibitors of AAC and other acetyltransferases of biomedical and biological significance.

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Abbreviations

AAC	aminoglycoside *N*-acetyltransferase
AcCoA	acetyl coenzyme A
AMP	ampicillin
CoA	coenzyme A
CoA-TNB	coenzyme A-thionitrobenzoic acid adduct
DMSO	dimethylsulfoxide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid
EDTA	ethylenediamine tetraacetic acid
Eis	enhanced intracellular survival

FDA	food and drug administration
FPLC	fast protein liquid chromatography
GCN5	general control non-depressible 5
GNAT	GCN5 *N*-acetyltransferase
HEPES	4-(2-hydroxyethyl)-1-piperazinetheanesulfonic acid
HTS	high-throughput screen(ing)
IPTG	isopropyl-β-D-1-thiogalactopyranoside
KAN	kanamycin A
LB	Luria-Bertani
Mtb	Mycobacterium tuberculosis
NEO	neomycin B
NTA	nitrilotriacetic acid
OADC	oleic acid bovine albumin dextrose catalase
PEG	polyethyleneglycol
SD	standard deviation
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
TB	tuberculosis
TNB	5-thio-2-nitrobenzoic acid

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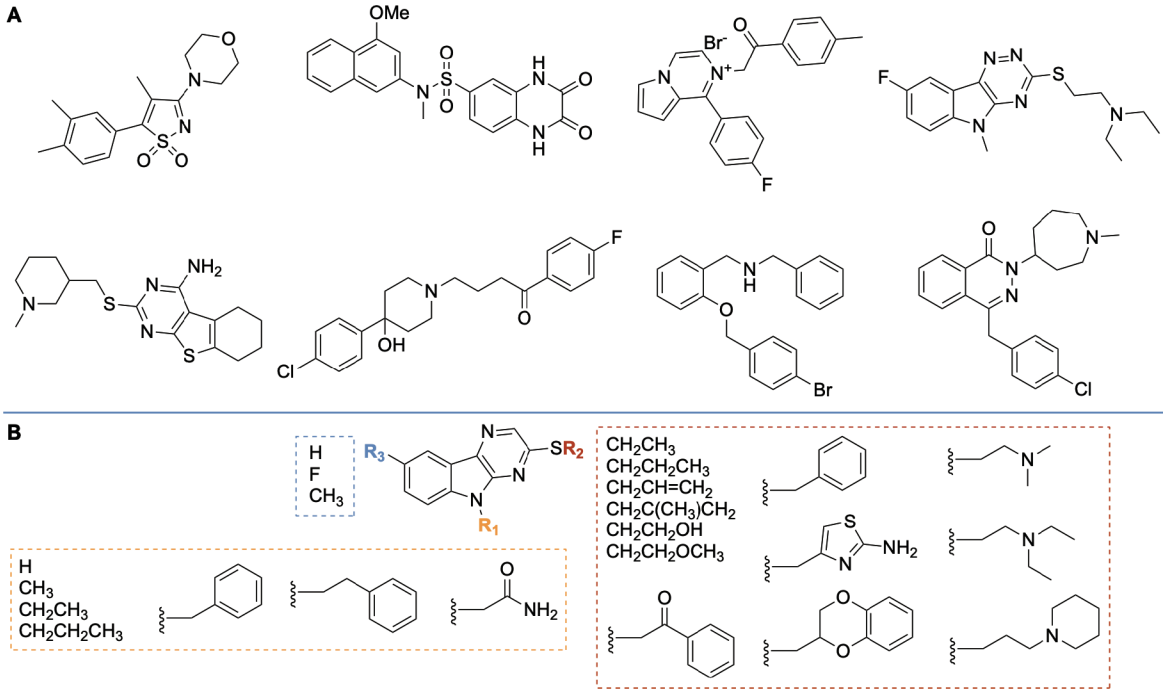

Fig. 1.

Small molecules identified as Eis inhibitors. **A.** Selected examples of Eis inhibitors our laboratory discovered and studied over the past decade [18–26]. **B.** An example of SAR study for the 1,2,4-triazino[5,6*b*]indole-3-thioether scaffold of Eis inhibitor where inhibition potency was investigated for compounds with varying R_1, R_2, and R_3 groups [21].

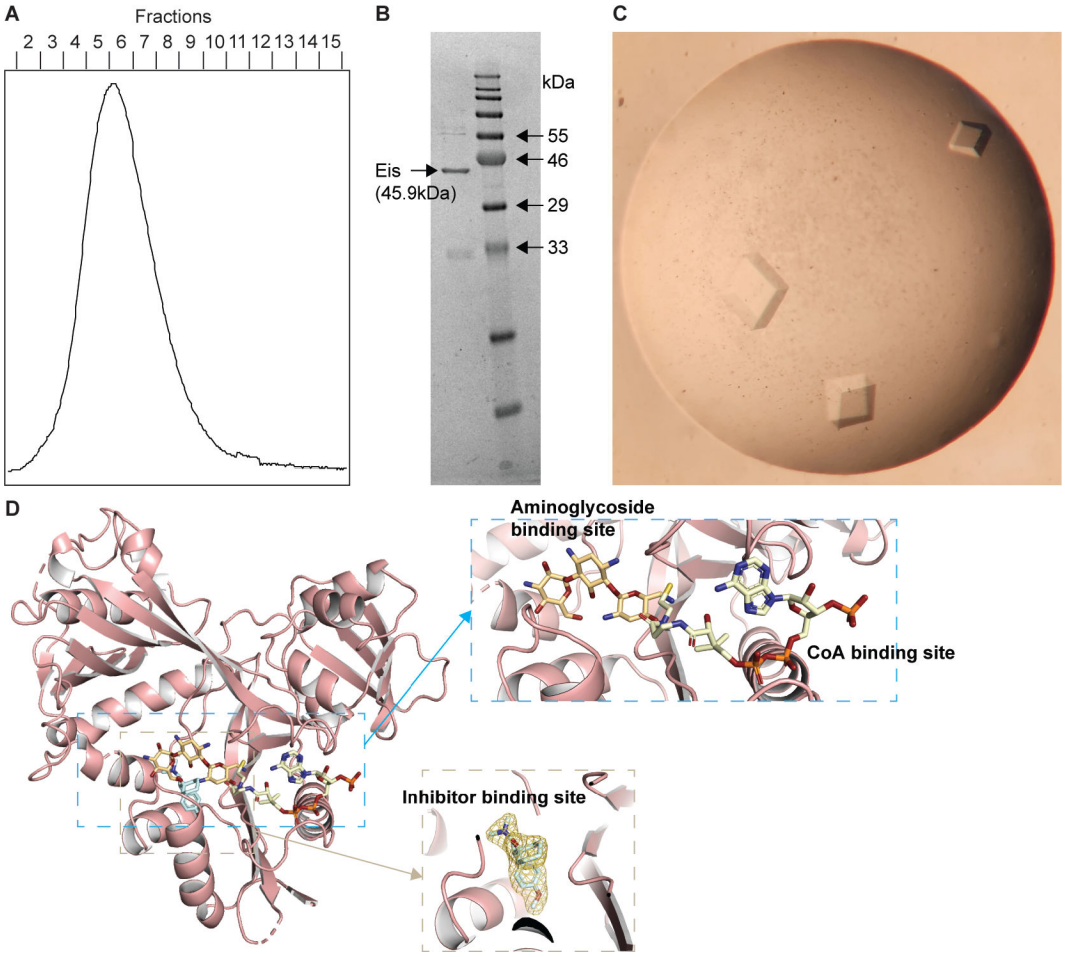

Fig. 2.

Eis purification, crystallization, and the crystal structure. **A.** Eis protein exists as a hexamer and elutes close to the void volume in a size-exclusion S-200 column. **B.** Purified Eis on a 15% denaturing SDS-PAGE gel. **C.** Eis forms rhombohedral crystals that appear after 2 weeks of incubation. **D.** The crystal structure of a protomer of EisC204A. Tobramycin and CoA bound to Eis (PDB ID: 4JD6) [16]. An inhibitor (venlafaxine, PDB ID: 8F4W [26]) is bound in a hydrophobic pocket near the aminoglycoside binding site.

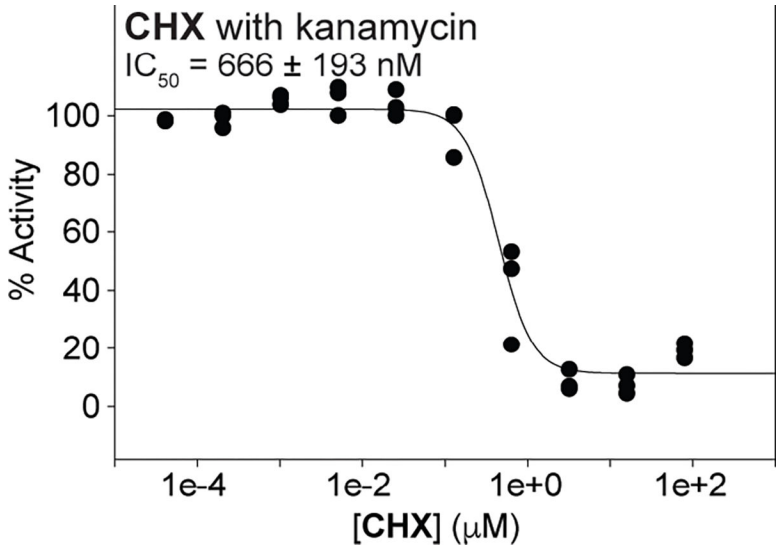

Fig. 3.

Example of an IC_{50} curve for chlorhexidine (CHX), one of the earliest identified *Mtb* Eis inhibitor [34].

Table 1.

Example IC_{50}, MIC, and KAN adjuvancy data for *Mtb* $mc^2$6230.

Compound	**IC_{50} against purified Eis (μM)**	**MIC *Mtb* $mc^2$6230 (μM)**	**MIC_{KAN} *Mtb* $mc^2$6230 (μg/mL)**	**MIC_{KAN} *Mtb* $mc^2$6230 K204 (μg/mL)**	**References**
SGT512	1.4 ± 0.5	50	≤1.25	>10	[18]
SGT773	5.4 ± 1.2	12.5	≤1.25	>10	[18]
SGT522	>200	12.5	≤1.25	5	[18]
SGT523	12 ± 3	50	≤1.25	≤1.25	[18]
SGT731	2.4 ± 0.7	50	≤1.25	≤1.25	[18]
Kanamycin (KAN)	--	--	≤1.25	320	[18]

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
E. coli BL21 (DE3)	Invitrogen	EC0114
Chemicals, Peptides, and Recombinant Proteins		
7H11	Millipore-Sigma	M0428
7H9	HIMEDIA	M198
AAC(2')-Ic	Generated in our lab	Ref [17]
AAC(3)-IV	Generated in our lab	Ref [28]
AAC(6')-Ie	Generated in our lab	Ref [28]
AcCoA trilithium salt	CoALA Biosciences	AC02
Ammonium sulfate	Fisher Scientific	BP212-212
Ampicillin, sodium salt	Millipore-Sigma	A9518
Casaminoacids	VWR	J851
CoA, trilithium salt	Millipore-Sigma	C3019
DMSO	TCI	D0798
D-pantothenic acid hemicalcium salt	Millipore-Sigma	P2250
DTNB	Millipore-Sigma	D8130
EDTA dibasic	Millipore-Sigma	E5134
Eis	Generated in our lab	Ref [17]
Eis C204A	Generated in our lab	Ref [17]
Glycerol	Millipore-Sigma	G7893
HCl	Millipore-Sigma	258148
HEPES free acid	Millipore-Sigma	H3375
Imidazole	Millipore-Sigma	I202
IPTG	GoldBio	I2481C
Isoniazid	Millipore-Sigma	I3377
Kanamycin sulfate	VWR	O408
LB broth	VWR	J106
McFarland standard	Pro-Lab Diagnostics	SD2300
Neomycin trisulfate	Millipore-Sigma	N1876
OADC	Prepared in our lab	Ref [42]
PEG 8000	Millipore-Sigma	89510
Resazurin, sodium salt	Millipore-Sigma	199303
Screening plate/compounds		
Sodium chloride	Millipore-Sigma	S9888
Sodium hydroxide	Millipore-Sigma	221465
Sodium phosphate dibasic	Millipore-Sigma	S9763
Sodium phosphate monobasic	Millipore-Sigma	331988

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Triethanolamine hydrochloride	Millipore-Sigma	T1502
Tris	Millipore-Sigma	T1503
Tyloxapol	Millipore-Sigma	T0307
Deposited Data		
Eis crystal structure	Protein Data Bank	3R1K
Experimental Models: Organisms/Strains		
Mtb mc^{2}6230	Provided by William Jacobs (Albert Eistein College of Medicine, USA)	
Mtb mc^{2}6230 K204	Generated in our lab	Ref [19]
Oligonucleotides		
Int-pET19b-pps	Generated in our lab	Ref [29]
pET22b	Millipore-Sigma	69744
pET28a	Millipore-Sigma	69864
Software and Algorithms		
CCP4 suite	https://www.ccp4.ac.uk/	
COOT	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/	
HKL2000	https://hkl-xray.com/hkl-2000	
JLigand	Included in CCP4 suite	
MOLREP	Included in CCP4 suite	
Phaser	Included in CCP4 suite	
SigmaPlot 15.0	SYSTAT	
Other		
Ni-NTA resin	Qiagen	30250