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Identification and Characterization of Inhibitors of the Aminoglycoside Resistance Acetyltransferase Eis from *Mycobacterium tuberculosis*

Keith D. Green,[a] Wenjing Chen,[a, b] and Sylvie Garneau-Tsodikova*[a, b, c]

With an anticipated 9.8 million new cases this year,[1] the tuberculosis (TB) epidemic is one of the most serious health problems worldwide. The continuous emergence and global spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB, underscore the pressing clinical need for novel treatments of this deadly infectious disease and for new solutions to alleviate the resistance problem.[2,3]

Aminoglycoside (AG) antibiotics[4] such as kanamycin A (KAN) (**1**) and amikacin (AMK) (**2**) are currently used as a last resort for treatment of XDR-TB (Figure 1 A). However, resistance to KAN is constantly rising, and treatment options for patients affected with XDR-TB are becoming fewer.[5] In most bacterial strains, a major mechanism of resistance to AGs is the enzymatic modification of the drugs by AG-modifying enzymes such as AG acetyltransferases (AACs), AG phosphotransferases (APHs), and AG nucleotidyltransferases (ANTs).[6,7] In *Mtb*, resistance to AGs results either from mutations of the ribosome that prevent the drugs from binding to it,[8–10] or from up-regulation of the chromosomal *eis* (enhanced intracellular survival) gene caused by mutations in its promoter.[11,12] Other biological functions of the mycobacterial protein Eis have been the subject of numerous investigations.[13–20] We recently demonstrated that Eis is a unique AAC that inactivates a broad set of AGs via a multi-acetylation mechanism.[21]

Two main strategies to overcome the effect of Eis in *Mtb* can be envisioned: 1) the development of new AGs not susceptible to Eis and 2) the use of Eis inhibitors. We recently reported a chemoenzymatic methodology[22] and a complementary protecting-group-free chemical strategy[23] for the production of novel AG derivatives. However, as Eis is capable of multi-acetylation of a large variety of AG scaffolds, it is unlikely that novel AGs will provide a viable and/or sustainable solution to the resistance problem in *Mtb*. Blanchard and co-workers previously showed that, when used in conjunction, the β-lactamase inhibitor clavulanate and meropenem are effective against XDR-TB.[24] The AG tobramycin and the macrolide antibiotic clarithromycin have also showed promising synergistic effects in *Mtb* clinical isolates.[25] Wright and colleagues also demonstrated that, in general, combinations of antibiotics and non-antibiotic drugs could result in enhancement of antimicrobial efficacy.[26] Similarly, an inhibitor of the resistance acetyltransferase Eis in combination with the currently used second-line antituberculosis drugs KAN or AMK may provide a potential solution to overcome the problem of XDR-TB. Herein, by using in vitro high-throughput screening (HTS), we identified and characterized the first series of potent inhibitors of Eis (Figure 1 B).

To identify inhibitors of *Mtb* Eis, we used neomycin B (NEO) (**3**) owing to the robust activity of the enzyme with this AG. We screened a total of 23 000 compounds from three small-molecule libraries: ChemDiv, BioFocus NCC, and MicroSource MS2000 spectrum. From the 23 000 molecules tested, 300 (1.3 %) showed a reasonable degree of inhibition ($>3\sigma$ from the mean negative control) against Eis, out of which 56 showed dose-dependent inhibition. The 25 compounds discussed herein (Figure 1 B) were found to have IC_{50} values in the low-micromolar range (Table 1 and Figure 2, as well as figures S1 and S2 in the Supporting Information). While most of these have not yet been biologically characterized, compounds **7**, **14**, **27**, and **28** have found application as anti-HIV treatments (**27**[27,28] and **28**[27–29]), molecules to prolong eukaryote longevity (**7**),[30] antibacterials (**27** and **28**),[31] anticancer agents (**28**),[32] and hypoglycemia therapeutics (**14**).[33]

At first glance, the 25 identified compounds appear to have vastly different structures. However, upon closer inspection of their scaffolds, two structural features link these 25 Eis inhibitors: the presence of at least one aromatic ring and one amine functional group. In general, we observed that positively or potentially positively charged molecules, including chlorhexidine (**6**), displayed lower IC_{50} values than preferably negatively charged (**27** and **28**) or neutral compounds. The highly negatively charged AG-binding cavity of the Eis protein (PDB ID: 3R1K)[21] is consistent with this general trend.

Seven of the 25 Eis inhibitors identified were divided into three groups for a preliminary and limited structure–activity relationship (SAR) analysis: 1) compounds **4** and **5**, 2) **14**, **15**, and **16**, as well as 3) **27** and **28** (Figure 1 B). Compounds **14**, **15**, and **16** differ in their imidazolium versus benzoimidazolium substitution on one side of the ketone and in their *para* substituents at the phenyl ring on the opposite side of the carbonyl. These differences had no effect on the IC_{50} values, indicating the importance of the imidazolium, but a secondary role of the additional features to the core structure for biological activity. In contrary, the differences in benzyl ring substitutions in compounds **27** and **28** (alternative placement (*ortho* versus *meta*) of the carboxylic acid and replacement of the *para*-chloro group with a *para*-hydroxy group) resulted in a

[a] *Dr. K. D. Green, W. Chen, Dr. S. Garneau-Tsodikova*
Life Sciences Institute, University of Michigan
210 Washtenaw Ave., Ann Arbor, MI 48109 (USA)
Fax: (+1) 734-615-5521
E-mail: sylviegt@umich.edu

[b] *W. Chen, Dr. S. Garneau-Tsodikova*
Chemical Biology Doctoral Program, University of Michigan
210 Washtenaw Ave., Ann Arbor, MI 48109 (USA)

[c] *Dr. S. Garneau-Tsodikova*
Department of Medicinal Chemistry
College of Pharmacy, University of Michigan
210 Washtenaw Ave., Ann Arbor, MI 48109 (USA)

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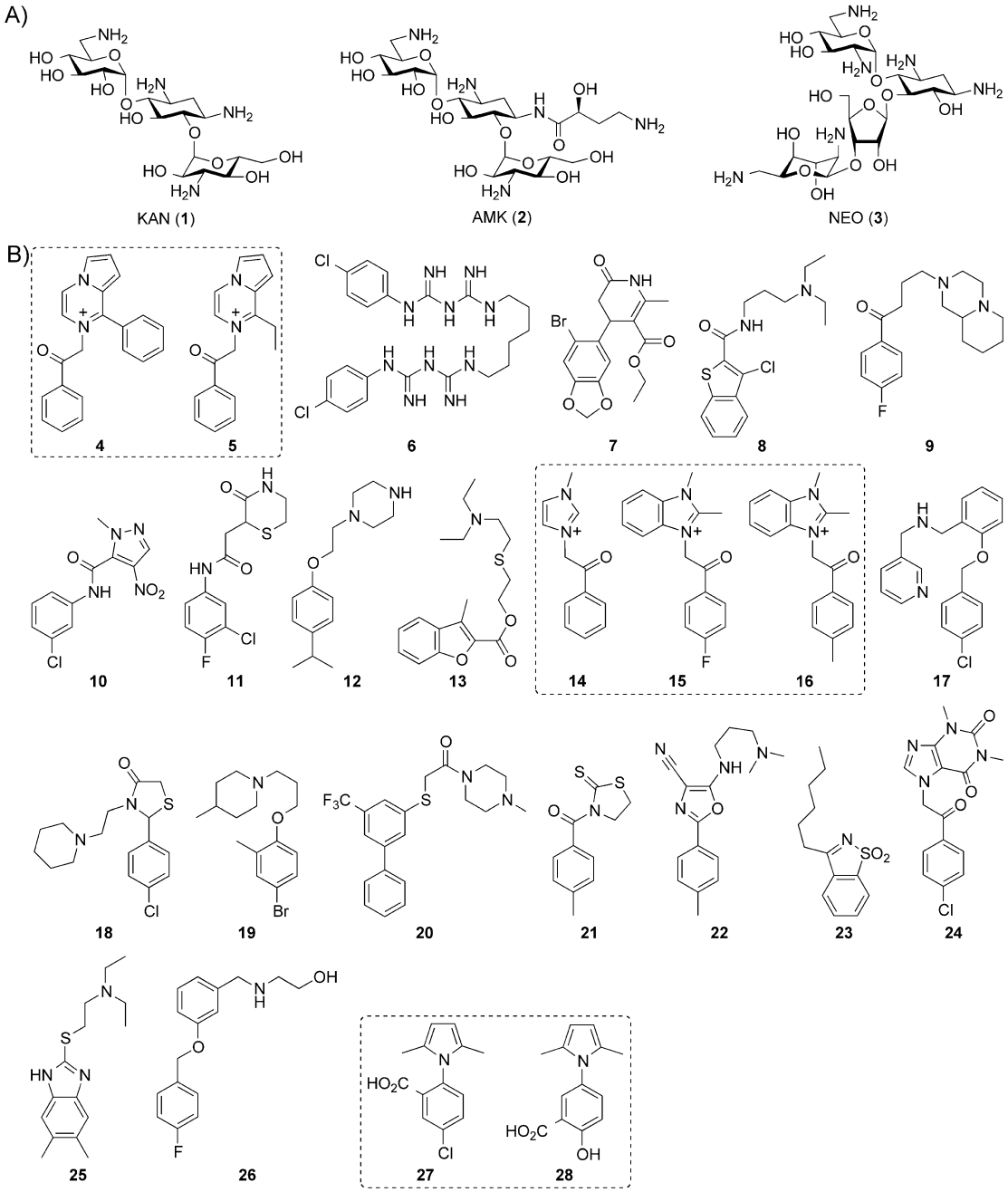

Figure 1. A) Structures of AGs used in this study. B) Structures of the 25 inhibitors of Eis identified by high-throughput screening.

greater than fivefold increase in the inhibition of Eis. Similarly, replacement of the ethyl group of **5** adjacent to the cationic nitrogen with a phenyl moiety in **4** resulted in a 25-fold increase in the inhibitory activity of compound **4**. Further kinetic analysis of compound **4** revealed a mixed mode of inhibition against NEO (Figure 2B). The observed mixed mode of inhibition could be explained by the three substrates (NEO, acetyl-NEO, and diacetyl-NEO) that are produced during the reaction of NEO with Eis. Here, compound **4**, may be competing differently with each possible substrate.

Interestingly, in contrast to compound **4**, the best inhibitor identified in this study with an IC_{50} value of 188 ± 30 nM, chlorhexidine (**6**), was found to behave as an AG-competitive inhibitor against NEO, KAN, and AMK (Figure 2A). Chlorhexidine is an antibiotic used mainly as a topical antibacterial, as a mouthwash, and as a sterilizing agent for surgical equipment.[34] Because of its toxic effects on pulmonary tissues,[35] chlorhexidine cannot be pursued as a potential TB treatment, but will continue to serve as a positive control for future HTS experiments for the identification of additional Eis inhibitor scaffolds.

With their structurally diverse scaffolds, the remaining compounds cannot be divided into distinct groups for SAR analyses. However, grouping the compounds by their IC_{50} values does reveal some trends. In comparison with compounds **4**

Table 1. Eis inhibition by hit compounds **4**–**28** for NEO acetylation.

Compd[a]	IC_{50} [μM][b]	Compd[a]	IC_{50} [μM][b]
4	0.364 ± 0.032	**15**	3.24 ± 0.32
4	0.331 ± 0.082 (AMK)[c]	**16**	3.84 ± 0.55
4	0.585 ± 0.113 (KAN)[c]	**17**	3.39 ± 0.61
5	9.25 ± 1.50	**18**	4.90 ± 0.75
6	0.188 ± 0.030	**19**	5.54 ± 0.63
6	0.321 ± 0.058 (AMK)[c]	**20**	5.68 ± 0.88
6	0.666 ± 0.193 (KAN)[c]	**21**	5.75 ± 0.66
7	1.09 ± 0.14	**22**	6.50 ± 1.32
8	1.24 ± 0.16	**23**	7.64 ± 0.60
9	2.01 ± 0.12	**24**	9.79 ± 1.97
10	2.29 ± 0.52	**25**	11.4 ± 1.6
11	2.37 ± 0.41	**26**	15.9 ± 2.6
12	2.63 ± 0.60	**27**	> 200
13	2.64 ± 0.36	**28**	41 ± 9
14	3.06 ± 0.56	–	–

[a] See Figure 1 B for structures. [b] Determined from at least three trials; best-fit values were obtained by using KaleidaGraph 4.1. [c] IC_{50} values were also determined for compounds **4** and **6** using AMK and KAN (figure S2, Supporting Information).

and **6**–**8**, the fewer hydrogen bonding sites of compounds **9**–**13** could explain the relatively higher IC_{50} values for these molecules. Likewise, the increased structural rigidity of compounds **17**–**26** could limit the ability of these molecules to adopt an ideal conformation for binding, potentially explaining the higher inhibitory constants observed for these molecules.

Because many AACs have a negatively charged AG-binding site that could be accessible for ligand binding,[36–38] in order to confirm the specificity of the identified inhibitors for Eis, we tested whether the four best compounds (**4**, **6**, **7**, and **8**) inhibit other AAC enzymes with negatively charged AG-binding sites from three different classes: AAC(2′)-Ic from *Mtb*, AAC(3)-IV from *Escherichia coli*, and AAC(6′)/APH(2″) from *Staphylococcus aureus*. With the exception of compound **4** against AAC(2′)-Ic, which displayed an IC_{50} value of 367 ± 129 μM (1000-fold worse than with Eis), no significant inhibition was observed for the combinations tested. This lack of cross-inhibition indicates that the inhibitors identified display high selectivity toward the Eis AG-binding site. Eis has been shown to multi-acetylate a large number of AGs[21] and is therefore potentially able to accommodate various conformations of structurally diverse and/or similar molecules in contrast to the mono-acetylating AACs [AAC(2′), AAC(3), and AAC(6′)] for which substrates can only bind in a single conformation. The unique flexibility of the AG-binding site of Eis could therefore explain the intriguing selectivity of the inhibitors identified for this enzyme. For example, the selectivity of chlorhexidine (**6**) for Eis, normally non-selectively binding to negatively charged sites and therefore expected to inhibit AAC(2′), AAC(3), and AAC(6′), could be justified by the uniqueness of the Eis AG-binding site that could accommodate compound **6** in conformation(s) that the other AACs cannot.

In summary, by using an in vitro HTS UV/Vis assay, we have identified 25 inhibitors of Eis from *Mtb* with 21 distinct scaffolds. The compounds display selective and potent inhibitory activity in vitro against the purified *Mtb* Eis and different modes of inhibition, with the known antibacterial chlorhexidine (**6**) competing with the AG for binding Eis. These findings provide the foundation for testing whether the Eis inhibitors will overcome KAN resistance in *Mtb* strains in which Eis is upregulated. This work also lays the groundwork for exploration of scaffold diversification and SAR studies of the identified biologically active compounds to be used in combination therapies with KAN or AMK against TB.

Experimental Section

Reagents and small-molecule libraries: All reagents including DTNB, NEO, KAN, AMK, and acetyl-CoA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Eis was screened against 23 000 compounds from three diverse libraries of small molecules: 1) the BioFocus NCC library, 2) the ChemDiv library (20 000 compounds), and 3) the MicroSource MS2000 library, composed of ~2000 bioactive compounds (343 molecules with reported biological activities, 629 natural products, 958 known therapeutics, and 70 compounds approved for agricultural use). The activity of promising compounds was confirmed by using repurchased samples from Sigma–Aldrich (compound **6**) and ChemDiv (San Diego, CA, USA) (compounds **4**, **5**, and **7**–**28**).

Expression and purification of Eis and other AAC proteins: The Eis and AAC(2′)-Ic from *Mtb*,[21] as well as the AAC(3)-IV from

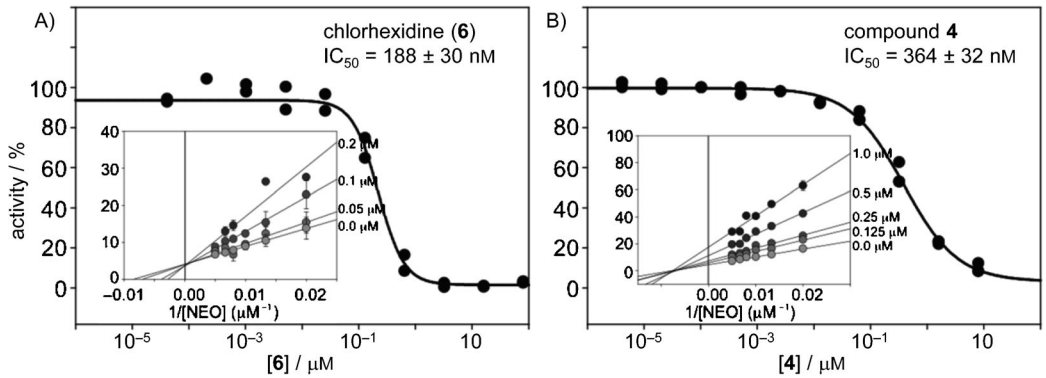

Figure 2. Representative examples of IC_{50} curves for A) chlorhexidine (**6**) and B) compound **4**. Plots showing the competitive and mixed inhibition with respect to NEO for compounds **6** and **4**, respectively, can be viewed as the inset in each panel.

E. coli[22,39] and AAC(6′)/APH(2″)-Ia from *S. aureus*[22,40] were overexpressed and purified as previously described.

Eis chemical library screening: The inhibition of Eis activity was determined by a UV/Vis assay monitoring the increase in absorbance at 412 nm ($\varepsilon_{412}=13600\,M^{-1}cm^{-1}$) resulting from the reaction of DTNB with the CoA–SH released upon acetylation of NEO. The final reaction mixtures (40 μL) contained Eis (0.25 μM), NEO (100 μM), Tris-HCl (50 mM, pH 8.0 adjusted at RT), AcCoA (40 μM), DTNB (0.5 mM), and the potential inhibitors (20 μM). Positive and negative control experiments were performed using chlorhexidine (**6**) (5 μM) and DMSO (0.5% *v/v*), respectively, instead of the potential inhibitors. Briefly, a mixture (30 μL) containing Eis (0.33 μM) and NEO (133.33 μM) in Tris-HCl (50 mM, pH 8.0 adjusted at RT) was added to 384-well non-binding-surface plates (Thermo Fisher Scientific, Waltham, MA, USA) using a Multidrop dispenser (Thermo Fisher Scientific). The potential inhibitors (0.2 μL of a 4 mM stock), chlorhexidine (**6**) (0.2 μL of a 1 mM stock), or DMSO (0.2 μL) were then added to each well by Biomek HDR (Beckman, Fullerton, CA, USA). After 10 min at RT, reactions were initiated by the addition of a mixture (10 μL) containing AcCoA (160 μM), DTNB (2 mM), and Tris-HCl (50 mM, pH 8.0 adjusted at RT). After an additional 5 min of incubation at RT, the absorbance was measured at 412 nm using a PHERAstar plate reader (BMG Labtech, Cary, NC, USA). The average *Z′* score for the entire HTS assay was 0.65.

Hit validation: Using the above conditions, all compounds deemed a hit ($>3\sigma$ as a statistical hit threshold from the mean negative control) were tested in triplicate. Compounds that displayed inhibition at least in two of the three independent assays were then tested for a dose–response using twofold dilutions from 20 μM to 78 nM. IC_{50} values were determined for all compounds displaying dose-dependent activity.

Inhibition kinetics: IC_{50} values were determined on a multimode SpectraMax M5 plate reader using 96-well plates (Thermo Fisher Scientific) by monitoring absorbance at 412 nm taking measurements every 30 s for 20 min. Eis inhibitors were dissolved in Tris-HCl (50 mM, pH 8.0 adjusted at RT containing 10% *v/v* DMSO) (100 μL) and a two- or fivefold dilution was performed. To the solution of inhibitors, a mixture (50 μL) containing Eis (1 μM), NEO (400 μM), and Tris-HCl (50 mM, pH 8.0 adjusted at RT) was added. After 10 min, the reactions were initiated by addition of a mixture (50 μL) containing AcCoA (2 mM), DTNB (2 mM), and Tris-HCl (50 mM, pH 8.0 adjusted at RT). Overall, inhibitor concentrations ranged from 200 μM to 4 pM. Initial rates (first 2–5 min of reaction) were calculated and normalized to reactions containing DMSO only. All assays were performed at least in triplicate. IC_{50} values were calculated by using a Hill plot fit with KaleidaGraph 4.1 software. Two representative examples of IC_{50} curves are provided in Figure 2, while the other 23 IC_{50} curves are presented in figure S1 (Supporting Information). Determination of IC_{50} values of compounds **4** and **6** against AMK and KAN were also performed as described above (figure S2, Supporting Information). All IC_{50} values are listed in Table 1.

Mode of inhibition: By using the conditions described for inhibition kinetics with varying concentrations of NEO (50, 75, 100, 125, 150, and 200 μM) and compounds **4** (1, 0.5, 0.25, and 0.125 μM) or **6** (5, 10, 20, and 40 nM), mixed inhibition was determined for compound **4**, and compound **6** was found to be a competitive inhibitor of NEO. Resulting reaction rates were plotted as Lineweaver–Burk plots (Figure 2 inserts of panels A and B). Using the same assay conditions, chlorhexidine (**6**) was also found to be a competitive inhibitor of KAN and AMK.

Inhibitor selectivity for Eis: To determine if the identified inhibitors are selective for Eis, we tested the four best Eis inhibitors (**4**, **6**, **7**, and **8**) with three other AACs: AAC(2′)-Ic, AAC(3)-IV, and AAC(6′)/APH(2″)-Ia. The conditions described for inhibition kinetics were used with varying concentrations of compounds **4**, **6**, **7**, or **8** (200–0.2 μM, 10-fold serial dilution) and AAC(2′)-Ic (0.125 μM), AAC(3)-IV (0.25 μM), or AAC(6′)/APH(2″)-Ia (0.25 μM) instead of Eis. For AAC(2′)-Ic with compound **4**, the concentration of inhibitor ranged from 1 μM to 500 pM and a fivefold serial dilution was used.

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