Unexpected N-acetylation of capreomycin by mycobacterial Eis enzymes

Jacob L. Houghton^{1,2†}, Keith D. Green^{2†}, Rachel E. Pricer^{2,3}, Abdelrahman S. Mayhoub^{2‡}
and Sylvie Garneau-Tsodikova^{1–3*}

¹Department of Medicinal Chemistry, University of Michigan, Ann Arbor, MI 48109, USA; ²Life Sciences Institute, University of Michigan, 210 Washtenaw Avenue, Room 4437, Ann Arbor, MI 48109, USA; ³Chemical Biology Doctoral Program, University of Michigan, Ann Arbor, MI 48109, USA

*Corresponding author. Tel: +1-734-615-2736; Fax: +1-734-615-5521; E-mail: sylviegt@umich.edu

†These authors contributed equally to this work.

‡On leave from Faculty of Pharmacy, Al-Azhar University, Cairo, 11884, Egypt.

Received 8 September 2012; returned 5 October 2012; revised 14 November 2012; accepted 16 November 2012

Objectives: The enhanced intracellular survival (Eis) protein from *Mycobacterium tuberculosis* (Eis_Mtb), a regio-versatile N-acetyltransferase active towards many aminoglycosides (AGs), confers resistance to kanamycin A in some cases of extensively drug-resistant tuberculosis (XDR-TB). We assessed the activity of Eis_Mtb and of its homologue from *Mycobacterium smegmatis* (Eis_Msm) against a panel of anti-tuberculosis (TB) drugs and lysine-containing compounds.

Methods and results: Both enzymes acetylated capreomycin and some lysine-containing compounds, but not other non-AG non-lysine-containing drugs tested. Modelling studies predicted the site of modification on capreomycin to be one of the two primary amines in its β -lysine side chain. Using Eis_Mtb, we established via nuclear magnetic resonance (NMR) spectroscopy that acetylation of capreomycin occurs on the ϵ -amine of the β -lysine side chain. Using Msm, we also demonstrated for the first time to our knowledge that acetylation of capreomycin results in deactivation of the drug.

Conclusions: Eis is a unique acetyltransferase capable of inactivating the anti-TB drug capreomycin, AGs and other lysine-containing compounds.

Keywords: antibiotic resistance, anti-tuberculosis drugs, mechanisms of resistance

Introduction

Causing 1.4 million deaths globally in 2010, tuberculosis (TB) is the second leading pathogen-related cause of death, surpassed only by HIV/AIDS. Although worldwide incidences of TB are beginning to modestly decline, occurrences of drug-resistant TB are increasing rapidly. Drug resistance to TB occurs when infected patients are treated with an improper regimen of antibiotics or when the patients fail to comply with the proper multi-drug regimen, which occurs most frequently in areas with sub-standard TB control programmes. Bacteria that are resistant to at least isoniazid and rifampicin, the two most powerful first-line treatments for TB, are designated as multidrug-resistant TB (MDR-TB). Strains of *Mycobacterium tuberculosis* (Mtb) are classified as extensively drug-resistant (XDR) if they are shown to be resistant to rifampicin and isoniazid, to one of the fluoroquinolones and to at least one of the second-line anti-TB drugs such as kanamycin A, amikacin or capreomycin. The cure rates for MDR- and XDR-TB are ~50% and ~30%, respectively.

In one-third of kanamycin A-resistant Mtb clinical isolates from a large and diverse set, the resistance to kanamycin A was shown to be a result of up-regulation of the enhanced intracellular survival (eis) gene due to mutations in its promoter.^{1,2} The Eis protein is a unique aminoglycoside (AG) acetyltransferase (AAC)² that inactivates AGs by unusual regio-versatile acetylation of AGs at multiple amine positions.³ Specifically, kanamycin A and amikacin, which are both multi-acetylated by Eis,^{3–6} are two important second-line anti-TB drugs. The structural and functional characterization of Eis from Mtb (Eis_Mtb) has been the focus of several recent studies.^{2,3,5,7–12} Eis homologues can be found in mycobacteria as well as in several non-mycobacterial prokaryotes. We have shown that Eis homologues from *Mycobacterium smegmatis* (Eis_Msm) and *Anabaena variabilis* (Eis_Ava) also act as a regio-versatile AG acetyltransferase (AAC) and that they can be inhibited by Eis_Mtb inhibitors.^{4,6} Additionally, it was recently reported that Eis_Msm and Eis_Mtb are also capable of acetylating

lysine residues of dual-protein phosphatase 16/mitogen-activated protein kinase phosphatase-7 (DUSP16/MPK-7).⁵

The broad specificity of Eis towards a large number of AGs, its acetylation function towards some proteins and previous reports of acetylation by other AACs of drugs other than AGs, including the second-line anti-TB drug ciprofloxacin (Figure S1, available as Supplementary data at JAC Online),¹³ prompted us to ask whether Eis is capable of conferring resistance to other non-AG anti-TB drugs. As Eis was shown to acetylate protein lysine residues,⁵ in this study, we sought to explore the potential of Eis to acetylate capreomycin, a cyclic peptide antibiotic with a β -lysine side chain (commercially available as a mixture of capreomycin IA and capreomycin IB; Figure S1, available as Supplementary data at JAC Online), particularly effective against *Mtb* and used as a second-line anti-TB drug. We also investigated the potential of Eis to acetylate a variety of lysine-containing molecules (Figure S1). We demonstrated that Eis_{*Mtb*} and Eis_{*Msm*} are both capable of acetylating capreomycin IA and capreomycin IB, but neither shows activity against other non-AG anti-TB drugs that were investigated. The position of acetylation on capreomycin was confirmed as the ϵ -amine of the β -lysine side chain using Eis_{*Mtb*}. We also showed that, out of the 11 additional lysine-containing molecules tested, Eis_{*Mtb*} can efficiently acetylate tetra-lysine and fragment 28–43 of neurogranin, a calmodulin-binding protein.

Methods

Materials and instrumentation

Eis_{*Mtb*}³ and Eis_{*Msm*}⁴ were overexpressed and purified as previously described. Acetyl-coenzyme A (AcCoA), capreomycin (as a mixture of capreomycin IA and IB) (if IA or IB are not indicated, 'capreomycin' represents the mixture of both capreomycin IA and IB), isoniazid, pyrazinamide, ciprofloxacin, lysine, di-lysine, tri-lysine, tetra-lysine, lisinopril, tuftsin, thymulin, acetyl-amyloid β -protein fragment 15–20 amide, nisin, neurogranin fragment 28–43, thymopoietin II peptide fragment and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) were bought from Sigma-Aldrich (Milwaukee, WI, USA) (Figure S1). Thin layer chromatography (TLC) plates (Merck, Silica gel 60 F₂₅₄) were visualized using iodine. We recorded ¹H two-dimensional gradient correlation spectroscopy (gCOSY), gradient heteronuclear single-quantum correlation spectroscopy (gHSQC) and two-dimensional z-filter total correlation spectroscopy (zTOCSY) nuclear magnetic resonance (NMR) spectra on a Varian 500 MHz equipped with a 3 mm OneProbe. All NMR spectra were recorded in D₂O or 9:1/H₂O:D₂O at pH 8.0 (Figures S2–S9, available as Supplementary data at JAC Online). Liquid chromatography–mass spectrometry (LCMS) was performed on a Shimadzu LCMS-2019EV equipped with an SPD-20AV UV-Vis detector and an LC-20AD liquid chromatograph. UV-Vis assays were performed using a multimode SpectraMax M5 plate reader and 96-well plates (Fisher Scientific). *Msm* str. MC2 155 was a gift from Dr Sabine Ehrt (Weill Cornell Medical College, New York City, NY, USA).

Determination of Eis_{*Mtb*} and Eis_{*Msm*} acetylation activity with non-AG anti-TB drugs and lysine-containing molecules by UV-Vis assays

The acetylation of the non-AG anti-TB drugs isoniazid, pyrazinamide, ciprofloxacin and capreomycin and the lysine-containing molecules

lysine, di-lysine, tri-lysine, tetra-lysine, lisinopril, tuftsin, thymopoietin II peptide fragment, thymulin, acetyl-amyloid β -protein fragment 15–20 amide, nisin and neurogranin fragment 28–43 was explored using the previously described Ellman method,^{4,7} monitoring the reaction of coenzyme A (CoA) released by the Eis enzymes with Ellman's reagent (DTNB). Briefly, Eis (from *Mtb* or *Msm*, 0.5 μ M) was added to a mixture containing Tris (50 mM, pH 8.0, adjusted at room temperature), AcCoA (500 μ M), anti-TB drug or lysine-containing compound (100 μ M) and DTNB (2 mM) to initiate reactions (200 μ L total volume). The reaction progress at 25°C was monitored at 412 nm (ϵ_{412} = 14 150 M^{−1} cm^{−1}) by taking measurements every 30 s for 1 h (Figure 1c and Figure 2a and b).

The kinetic parameters (K_m and k_{cat}) for capreomycin and tetra-lysine were determined as above using varying concentrations of capreomycin or tetra-lysine [0, 20, 50, 100, 250, 500, 1000, 2000, 4000 (for tetra-lysine only) μ M] and AcCoA (500 μ M). The first 2–5 min of reactions was used to calculate the initial rates and the data were fitted to a Michaelis–Menten curve using SigmaPlot 11.0 to determine the K_m and k_{cat} values (Figure 1e for capreomycin and Figure 2c for tetra-lysine). All reactions were performed at least in triplicate.

TLC and LCMS assays showing the conversion of capreomycin into mono-acetyl-capreomycin products

An aliquot (5 μ L) of the capreomycin acetylation reaction used for NMR analysis [10 mM in Na₂HPO₄ buffer (25 mM, pH 8.0 adjusted at room temperature)] and an aliquot of the capreomycin standard used for NMR analysis (10 mM in D₂O) were loaded onto a silica gel TLC plate and eluted in a mixture of phenol:H₂O:NH₄OH/30:10:1. After drying for 2 h, the TLC plate was stained for visualization in a sealed chamber containing I₂/SiO₂ for 8 h (Figure 1b).

To confirm the single N-acetylation of both capreomycin IA and IB, the masses of the novel acetylated capreomycin were determined using the NMR sample (20 μ L) (see below for NMR sample preparation) by LCMS in positive mode using H₂O (0.1% formic acid). The mass spectrum obtained is provided in Figure 1(d).

Molecular modelling of capreomycin IB with Eis_{*Mtb*}

The capreomycin IB structure was built with Sybyl-X software and minimized to 0.01 kcal/mol by the Powell method, using Gasteiger–Hückel charges and the Tripos force fields. The protein coordinates (PDB code 3R1K)³ were downloaded from the Protein Data Bank web site. With the exception of CoA, the H₂O molecules, acetamide and all other substructures were removed from the structure. An acetyl group was added to CoA by modifying the natural ligand using Sybyl-X. The surrounding protein residues were then kept frozen while the AcCoA was subjected to energy minimization using the Steepest Descent method to get rid of steric clashes. Hydrogen atoms were added to the Eis_{*Mtb*}-AcCoA complex and its energy was minimized using the Amber force fields with Amber charges. The energy-optimized capreomycin IB was docked into the AG binding site in the Eis_{*Mtb*} protein using GOLD.¹⁴ The parameters were set as the default values for GOLD. The maximum distance between hydrogen bond donors and acceptors for hydrogen bonding was set to 3.5 Å. After docking, the top three capreomycin IB conformations were individually merged into the minimized Eis_{*Mtb*}-AcCoA complex. To relax the protein side chains, the three new Eis_{*Mtb*}-AcCoA–capreomycin IB complexes were subsequently subjected to energy minimization using the Amber force fields with Amber charges. During the energy minimization, the structures of capreomycin IB and residues within an 8 Å radius were allowed to move. The remaining residues were kept frozen in order to reduce calculation time. The energy minimization, in all three cases, was performed using the Powell method with a 0.05 kcal/mol energy gradient convergence

criterion and a distance-dependent dielectric function. The model of the *Eis_Mtb*-AcCoA-capreomycin IB complex is depicted in Figure S10 (available as Supplementary data at JAC Online).

NMR analysis of the 6'-N-acetyl-capreomycin products from reaction of capreomycin with *Eis_Mtb*

To determine which position of capreomycin becomes acetylated by *Eis_Mtb*, the reaction was performed by monitoring the solution via NMR in a manner that did not require the need for purification of the acetylated products. A solution (200 μ L) containing capreomycin (10 mM, 1 eq), AcCoA (25 mM, 2.5 eq) and *Eis_Mtb* (5 μ M) in Na₂HPO₄ buffer (25 mM, pH 8.0 adjusted at room temperature) containing 10% D₂O was prepared and allowed to proceed to completion (overnight) at room temperature. ¹H gCOSY, zTOCSY and gHSQC NMR experiments were performed on the reaction solution, applying PURGE (presaturation utilizing relaxation gradients and echoes) solvent suppression.¹⁵ Proton connectivity was assigned using zTOCSY and gCOSY spectra. Representative spectra for 6'-N-acetyl-capreomycin products are provided in Figures S6–S9, available as Supplementary data at JAC Online. Note that according to standard nomenclature the ϵ -amine of the β -lysine is notated as the 6'-position, which is indicated on the spectra of the appropriate Figures S6–S9.

To unambiguously establish the position acetylated on capreomycin, the NMR spectra of the products were compared with those of a standard of pure capreomycin (as a mixture of capreomycin IA and IB) (10 mM in 10% D₂O) (Tables S1 and S2, available as Supplementary data at JAC Online). Representative spectra for the capreomycin standard are provided in Figures S2–S5, available as Supplementary data at JAC Online.

Activity of capreomycin and acetyl-capreomycin against *Msm* str. MC2 155 by disc diffusion assays

To establish if acetylation of capreomycin results in deactivation of the drug, reactions (250 μ L) were done with four concentrations of capreomycin (100 μ M, 500 μ M, 1.0 mM and 2.5 mM) with an ~5-fold excess of AcCoA (500 μ M, 2.4 mM, 5 mM and 12 mM, respectively) in Tris-HCl buffer (50 mM, pH 8.0 adjusted at room temperature) in the presence of *Eis_Mtb* (3.5 μ M) and incubated at room temperature overnight (~16 h). A second series of identical reactions were setup in the absence of the *Eis_Mtb* enzyme as controls. Prior to testing the activity of capreomycin and acetyl-capreomycin in disc diffusion assays, completion of the acetylation reactions was confirmed by taking an aliquot of each of the above reaction mixtures (10 μ L) to which a solution (100 μ L) containing AcCoA (500 μ M), *Eis_Mtb* (0.5 μ M), Tris-HCl buffer (50 mM, pH 8.0 adjusted at room temperature) and DTNB (2 mM) was added prior to monitoring potential additional acetylation by UV-Vis assays. The reaction progress was monitored at 412 nm taking measurements every 30 s for 1 h. As expected, acetylation was observed for the reactions lacking the *Eis_Mtb*, while no further acetylation was observed for the reactions containing already acetylated capreomycin, indicating that acetylation of capreomycin was complete. To test the antibacterial activity of capreomycin and acetyl-capreomycin, 10 μ L of the reaction mixture was spotted on sterile filter discs and allowed to air dry for 30 min. The discs were then placed on 7H9 plates and covered with soft 7H9 agar (0.75% agar) containing *Msm* str. MC2 155 (1 mL of a turbid culture of *Msm* str. MC2 155 per 25 mL of soft 7H9 agar). The bacteria were allowed to grow overnight (~16 h) at 37°C, and were then stained with 1.5 mL of an MTT solution (0.5 mg/mL of H₂O). Visualization was performed after 5 min of staining (Figure S11, available as Supplementary data at JAC Online).

Results and discussion

Exploration of non-AG anti-TB drugs and lysine-containing molecules as potential substrates of *Eis*

In addition to capreomycin, we chose other first- and second-line non-AG anti-TB drugs as candidate *Eis* acetylation substrates, as follows. Previous studies have shown that isoniazid (Figure S1) becomes acetylated by arylamine *N*-acetyltransferase 2 on the hydrazide functional group, demonstrating its potential for deactivation via acetylation.¹⁶ Pyrazinamide (Figure S1) was chosen for its structural similarity to isoniazid, allowing comparison of isoniazid with a similar drug that is not likely to be acetylated. Ciprofloxacin was selected because its acetylation by another resistance-conferring AAC was previously reported.¹³

We first tested acetylation of capreomycin, ciprofloxacin, isoniazid and pyrazinamide by *Eis* proteins from *Mtb* and *Msm* by using a UV-Vis spectrophotometric assay. We found that *Eis_Mtb* and *Eis_Msm* did not acetylate ciprofloxacin, isoniazid and pyrazinamide to any observable levels. For pyrazinamide, this result is not surprising, given the lack of nucleophilic amines likely to undergo acetylation. In contrast, we observed efficient acetylation of capreomycin by both *Eis_Mtb* and *Eis_Msm* (Figure 1a and c). Even though *Eis* can acetylate AGs at multiple positions, we confirmed, by TLC (Figure 1b) and LCMS (Figure 1d), that both capreomycin IA and capreomycin IB were converted to mono-acetylated products. Steady-state kinetic experiments (Figure 1e) yielded the following values of Michaelis-Menten parameters for capreomycin acetylation by *Eis_Mtb*: $K_m = 654 \pm 45 \mu\text{M}$ and $k_{\text{cat}} = 1.25 \pm 0.03 \text{ s}^{-1}$. Both K_m and k_{cat} values are slightly greater than the respective values for AG acetylation by *Eis_Mtb* or *Eis_Msm*.^{3–5} The catalytic efficiency (k_{cat}/K_m) of $1911 \pm 139 \text{ M}^{-1} \text{ s}^{-1}$ is equivalent to or better than that for many of the AGs previously tested,^{3,4} suggesting that a somewhat lower affinity of *Eis* for capreomycin than for AGs is over-compensated by the more efficient acetyl transfer onto capreomycin or a quicker product release.

The findings that the lysine-containing anti-TB drug capreomycin can be acetylated by *Eis* proteins and the previously reported ability of *Eis* to acetylate lysine residues on the DUSP16/MPK-7 proteins⁵ raised the question of whether *Eis* proteins could accommodate a large number of substrates that feature a lysine side chain. To address this question, we tested 11 lysine-containing molecules. We first explored if lysine, di-lysine, tri-lysine and tetra-lysine could act as *Eis* substrates (Figure 2a). It appeared that a minimum of four amino acid residues are required for lysine *N*-acetylation by *Eis* as only tetra-lysine was found to be a good substrate of *Eis_Mtb*. Steady-state kinetic experiments (Figure 2c) yielded the following values of Michaelis-Menten parameters for tetra-lysine acetylation by *Eis_Mtb*: $K_m = 1130 \pm 168 \mu\text{M}$ and $k_{\text{cat}} = 0.208 \pm 0.010 \text{ s}^{-1}$. The catalytic efficiency (k_{cat}/K_m) of $184 \pm 29 \text{ M}^{-1} \text{ s}^{-1}$ is 10-fold worse than that for capreomycin, indicating a higher specificity for capreomycin. We next investigated the *N*-acetylation of a variety of molecules containing at least one lysine residue: (i) the angiotensin-converting enzyme inhibitor lisinopril; (ii) the tetra-peptide tuftsin, a product of the enzymatic cleavage of the Fc-domain of the heavy chain for immunoglobulin G; (iii) the thymopoietin II peptide fragment; (iv) the nona-peptide

Figure 1. 6'-N-acetylation of capreomycin (CAP) by Eis. (a) Scheme showing the conversion of CAP IA and CAP IB into 6'-N-acetyl-CAP IA (Ac-CAP IA) and 6'-N-acetyl-CAP IB (Ac-CAP IB), respectively, by Eis_Mtb. (b) TLC showing the Ac-CAP IA and Ac-CAP IB products (left-hand lane) generated by Eis_Mtb using CAP (mixture of CAP IA and CAP IB) (right-hand lane) with 2.5 eq of AcCoA. (c) Spectrophotometric assay plot monitoring the conversion of CAP into Ac-CAP by Eis_Mtb (black circles) and Eis_Msm (grey circles). (d) Mass spectrum of Ac-CAP (a mixture of Ac-CAP IA and Ac-CAP IB). (e) Michaelis-Menten analysis of the Eis_Mtb-catalysed N-acetylation of CAP.

thymulin, believed to be involved in T cell differentiation; and (v) the Aβ aggregation inhibitor acetyl-amyloid β-protein fragment 15–20 amide. We found that Eis_Mtb did not acetylate any of these molecules. We next explored the N-acetylation of the food preservative nisin and of the fragment 28–43 of the calmodulin-binding protein neurogranin, each containing three lysines. We found neurogranin fragment 28–43 to be a good substrate of Eis_Mtb (Figure 2b). Collectively, these results demonstrate the specificity of Eis proteins towards a selected group of lysine-containing compounds, including capreomycin, tetra-lysine and neurogranin fragment 28–43.

Regio-specificity of capreomycin acetylation by Eis_Mtb

In order to analyse which amine of capreomycin could be acetylated by Eis_Mtb, we performed computational docking of capreomycin IB onto Eis_Mtb in complex with AcCoA (PDB code 3R1K)³ (Figure S10, available as Supplementary data at JAC Online). The docking simulation indicated that the large binding pocket of Eis_Mtb could accommodate the scaffold of capreomycin. A number of hydrogen bonding interactions between capreomycin and the side chains of amino acid residues Arg192, Glu203, Asp211, Ser239, Arg242 and Glu401 of Eis_Mtb are predicted to play an important role in binding of capreomycin in the active site of the enzyme (Figure S10C and D). More specifically, capreomycin is predicted to bind in a manner that places both amines of the β-lysine side chain in the proximity of the acetyl thioester of AcCoA (Figure S10B). This suggests the potential for two acetylations, yet capreomycin is only mono-acetylated (Figure 1a and b). It has been reported that the lysine 55

residue in DUSP16/MPK-7 is acetylated at the α- and ε-amine by Eis_Msm and Eis_Mtb, respectively.⁵ Given these observations, acetylation of the ε-amine of the β-lysine side chain of capreomycin by Eis_Mtb appeared more likely.

In order to unambiguously establish the site of acetylation by Eis_Mtb on capreomycin, 1D- and 2D-NMR experiments were performed with the Eis_Mtb reaction products (Figures S6–S9) and with non-acetylated capreomycin as a standard (Figures S2–S5). Tables were constructed for a side-by-side comparison of the chemical shifts of the protons of interest (Tables S1 and S2), to facilitate the interpretation of the data. The analysis of the Eis_Mtb reaction with capreomycin indicated that the ε-amine of the β-lysine side chain was acetylated (Figure 1a), as manifested by a change in the chemical shift of the protons directly adjacent to the ε-amine of the β-lysine side chain.

Effect of capreomycin acetylation on its activity

Can this acetylation mechanism of capreomycin by Eis play a role *in vivo*? A recent systematic review of Mtb mutations that are associated with resistance to capreomycin, as well as kanamycin A and amikacin, found that Eis promoter mutations have been reported in as many as 9% of capreomycin-resistant strains.¹⁷ In this review,¹⁷ a weak correlation between a C12T mutation in the eis promoter strongly associated with amikacin resistance and resistance to capreomycin has been observed,¹⁷ suggesting that Eis may play a role in acetylating capreomycin *in vivo*. The authors of this review¹⁷ suggested that capreomycin-resistance of Mtb may be predicted by concurrent mutations in the rrs gene and eis promoter region, suggesting that Eis may inactivate

Figure 2. Spectrophotometric assay plots monitoring the acetylation of (a) lysine (circles), di-lysine (triangles), tri-lysine (squares) and tetra-lysine (diamonds) by *Eis_Mtb*, as well as of (b) neurogranin (triangles). (c) Michaelis-Menten analysis of the *Eis_Mtb* catalysed N-acetylation of tetra-lysine.

capreomycin *in vivo*, although evidence suggests that mutations in the *eis* promoter region alone are insufficient to cause significant levels of resistance to capreomycin in *Mtb*. Furthermore, capreomycin acetylation by an N-acetyltransferase (albeit at a yet

unknown position) was shown to be a resistance mechanism ensuring protection from capreomycin of the capreomycin-producing organism, *Streptomyces capreolus*.¹⁸ The X-ray crystal structure of capreomycin IA in complex with the 70S ribosome (PDB code 3KNN) shows that the ϵ -amine of the β -lysine residue of capreomycin makes electrostatic interactions with the backbone phosphates of the tRNA at the A-site while the capreomycin core is bound to the ribosome.¹⁹ As capreomycin may act by blocking translocation of the ribosome,^{20–23} the acetylation of the ϵ -amine observed here is predicted to abolish the electrostatic interaction of capreomycin with tRNA, possibly inactivating capreomycin. Replacing the β -lysine with ornithine was shown to increase the MIC of capreomycin against *Mtb* and other pathogens,²⁴ and ϵ -amine acetylation of the β -lysine of viomycin, a related cyclic peptide antibiotic produced by *Streptomyces vinaceus*, was shown to abolish viomycin activity. These observations, although all indirect, suggest that ϵ -amine acetylation may significantly decrease or abolish the activity of capreomycin variants containing the β -lysine residue.

In order to investigate directly the effect of acetylation of capreomycin on activity of the drug, we performed disc diffusion assays of capreomycin and acetyl-capreomycin at 0.8 $\times$, 4 $\times$, 8 $\times$ and 18 $\times$ MIC of capreomycin against *Msm* str. MC2 155 (Figure S11). *Msm* str. MC2 155 was selected because it is a faster-dividing and non-pathogenic mycobacterial strain that is most commonly used as a model to gain insight into *Mtb* mechanisms.^{25–27} Doing so, we demonstrated that acetylation of capreomycin abolishes its activity against the bacteria.

Conclusions

We have assessed the potential of *Eis* proteins from *Mtb* and *Msm* to acetylate various first- and second-line non-AG anti-TB drugs, finding that capreomycin is the only non-AG anti-drug tested to date that can be modified by either protein. We have also established that tetra-lysine and the lysine-containing neurogranin fragment 28–43 can be efficiently acetylated by *Eis_Mtb*. These observations expand upon our understanding of *Eis* proteins as acetyltransferases, specifically in terms of their ability to modify a broad range of small molecules as well as proteins; all known acetylations occur at a primary amine. We conclusively confirmed via NMR spectroscopy that capreomycin is modified at the ϵ -amine of its β -lysine residue. *In silico* assessment of the binding mode of a novel peptide scaffold such as capreomycin with *Eis* will aid the discovery of novel inhibitors of *Eis*, an emerging strategy for overcoming the resistance conferred by *Eis*.^{4,7} Our finding that acetylation of capreomycin inactivates the drug against *Msm* supports the potential role of acetylation in resistance to capreomycin proposed in the MIC studies of *Mtb* that have shown that A1401G *rrs* mutations combined with mutations in the *eis* promoter may confer some low-level resistance to capreomycin.

Supplementary data

Figures S1–S11 and Tables S1 and S2 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

Acknowledgements

We thank Dr Sabine Ehrt (Weill Cornell Medical College, New York City, NY, USA) for providing Msm str. MC2 155. We thank Dr Oleg V. Tsodikov for critical reading of this manuscript and helpful suggestions (prior to submission).

Funding

This work was supported by the National Institutes of Health (NIH) grant AI090048 (S. G.-T.). J. H. L. was supported by the Cellular Biotechnology Training Program (CBTP) and an American Foundation of Pharmaceutical Education (AFPE) Fellowship. R. E. P. and J. H. L. were supported by Rackham Merit Fellowships at the University of Michigan. R. E. P. was supported by the Chemistry Biology Interface (CBI) Training Program at the University of Michigan.

Transparency declarations

No financial conflicts of interest to declare.

References

- Campbell PJ, Morlock GP, Sikes RD *et al.* Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2011; **55**: 2032–41.
- Zaunbrecher MA, Sikes RD Jr, Metchock B *et al.* Overexpression of the chromosomally encoded aminoglycoside acetyltransferase *eis* confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 2009; **106**: 20004–9.
- Chen W, Biswas T, Porter VR *et al.* Unusual regioversatility of acetyltransferase Eis, a cause of drug resistance in XDR-TB. *Proc Natl Acad Sci USA* 2011; **108**: 9804–8.
- Chen W, Green KD, Tsodikov OV *et al.* Aminoglycoside multiacetylating activity of the enhanced intracellular survival protein from *Mycobacterium smegmatis* and its inhibition. *Biochemistry* 2012; **51**: 4959–67.
- Kim KH, An DR, Song J *et al.* *Mycobacterium tuberculosis* Eis protein initiates suppression of host immune responses by acetylation of DUSP16/MKP-7. *Proc Natl Acad Sci USA* 2012; **109**: 7729–34.
- Pricer RE, Houghton JL, Green KD *et al.* Biochemical and structural analysis of aminoglycoside acetyltransferase Eis from *Anabaena variabilis*. *Mol Biosyst* 2012; **8**: 3305–13.
- Green KD, Chen W, Garneau-Tsodikova S. Identification and characterization of inhibitors of the aminoglycoside resistance acetyltransferase Eis from *Mycobacterium tuberculosis*. *ChemMedChem* 2012; **7**: 73–7.
- Lella RK, Sharma C. Eis (enhanced intracellular survival) protein of *Mycobacterium tuberculosis* disturbs the cross regulation of T-cells. *J Biol Chem* 2007; **282**: 18671–5.
- Roberts EA, Clark A, McBeth S *et al.* Molecular characterization of the *eis* promoter of *Mycobacterium tuberculosis*. *J Bacteriol* 2004; **186**: 5410–7.
- Samuel LP, Song CH, Wei J *et al.* Expression, production and release of the Eis protein by *Mycobacterium tuberculosis* during infection of macrophages and its effect on cytokine secretion. *Microbiology* 2007; **153**: 529–40.
- Shin DM, Jeon BY, Lee HM *et al.* *Mycobacterium tuberculosis* Eis regulates autophagy, inflammation, and cell death through redox-dependent signaling. *PLoS Pathog* 2010; **6**: e1001230.
- Wei J, Dahl JL, Moulder JW *et al.* Identification of a *Mycobacterium tuberculosis* gene that enhances mycobacterial survival in macrophages. *J Bacteriol* 2000; **182**: 377–84.
- Vetting MW, Park CH, Hegde SS *et al.* Mechanistic and structural analysis of aminoglycoside N-acetyltransferase AAC(6′)-Ib and its bifunctional, fluoroquinolone-active AAC(6′)-Ib-cr variant. *Biochemistry* 2008; **47**: 9825–35.
- Verdonk ML, Cole JC, Hartshorn MJ *et al.* Improved protein-ligand docking using GOLD. *Proteins* 2003; **52**: 609–23.
- Simpson AJ, Brown SA. Purge NMR: effective and easy solvent suppression. *J Magn Reson* 2005; **175**: 340–6.
- Sim E, Walters K, Boukouvala S. Arylamine N-acetyltransferases: from structure to function. *Drug Metab Rev* 2008; **40**: 479–510.
- Georgiou SB, Magana M, Garfein RS *et al.* Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review. *PLoS One* 2012; **7**: e33275.
- Skinner RH, Cundliffe E. Resistance to the antibiotics viomycin and capreomycin in the *Streptomyces* species which produce them. *J Gen Microbiol* 1980; **120**: 95–104.
- Stanley RE, Blaha G, Grodzicki RL *et al.* The structures of the anti-tuberculosis antibiotics viomycin and capreomycin bound to the 70S ribosome. *Nat Struct Mol Biol* 2010; **17**: 289–93.
- Ermolenko DN, Spiegel PC, Majumdar ZK *et al.* The antibiotic viomycin traps the ribosome in an intermediate state of translocation. *Nat Struct Mol Biol* 2007; **14**: 493–7.
- Marrero P, Cabanas MJ, Modolell J. Induction of translational errors (misreading) by tuberactinomycins and capreomycins. *Biochem Biophys Res Commun* 1980; **97**: 1047–42.
- Modolell J, Vazquez D. The inhibition of ribosomal translocation by viomycin. *Eur J Biochem* 1977; **81**: 491–7.
- Peske F, Savelsbergh A, Katunin VI *et al.* Conformational changes of the small ribosomal subunit during elongation factor G-dependent tRNA-mRNA translocation. *J Mol Biol* 2004; **343**: 1183–94.
- Nomoto S, Shiba T. Syntheses of capreomycin analogs in relation to their antibacterial activities. *Bull Chem Soc Jpn* 1979; **52**: 1709–15.
- Shiloh MU, DiGiuseppe Champion PA. To catch a killer. What can mycobacterial models teach us about *Mycobacterium tuberculosis* pathogenesis? *Curr Opin Microbiol* 2010; **13**: 86–92.
- Tyagi JS, Sharma D. *Mycobacterium smegmatis* and tuberculosis. *Trends Microbiol* 2002; **10**: 68–9.
- Reyrat JM, Kahn D. *Mycobacterium smegmatis*: an absurd model for tuberculosis? *Trends Microbiol* 2001; **9**: 472–4.