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Novel small molecules that increase the susceptibility of *Neisseria gonorrhoeae* to cationic antimicrobial peptides by inhibiting lipid A phosphoethanolamine transferase

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Objectives: *Neisseria gonorrhoeae* is an exclusively human pathogen that commonly infects the urogenital tract resulting in gonorrhoea. Empirical treatment of gonorrhoea with antibiotics has led to multidrug resistance and the need for new therapeutics. Inactivation of lipooligosaccharide phosphoethanolamine transferase A (EptA), which attaches phosphoethanolamine to lipid A, results in attenuation of the pathogen in infection models. Small molecules that inhibit EptA are predicted to enhance natural clearance of gonococci via the human innate immune response.

Methods: A library of small-fragment compounds was tested for the ability to enhance susceptibility of the reference strain *N. gonorrhoeae* FA1090 to polymyxin B. The effect of these compounds on lipid A synthesis and viability in models of infection were tested.

Results: Three compounds, 135, 136 and 137, enhanced susceptibility of strain FA1090 to polymyxin B by 4-fold. Pre-treatment of bacterial cells with all three compounds resulted in enhanced killing by macrophages. Only lipid A from bacterial cells exposed to compound 137 showed a 17% reduction in the level of decoration of lipid A with phosphoethanolamine by MALDI-TOF MS analysis and reduced stimulation of cytokine responses in THP-1 cells. Binding of 137 occurred with higher affinity to purified EptA than the starting material, as determined by 1D saturation transfer difference NMR. Treatment of eight MDR strains with 137 increased susceptibility to polymyxin B in all cases.

Conclusions: Small molecules have been designed that bind to EptA, inhibit addition of phosphoethanolamine to lipid A and can sensitize *N. gonorrhoeae* to killing by macrophages.

Introduction

Neisseria gonorrhoeae causes the sexually transmitted infection gonorrhoea, with a worldwide yearly estimate of >106 million infections.[1] Empirical monotherapy has been used to treat gonorrhoea over the past 50 years, which has steadily resulted in the emergence of MDR strains that are resistant to all classes of antibiotics used in therapy.[2] The emergence of XDR isolates that are resistant to the standard dual antibiotic therapy (azithromycin and ceftriaxone) has led to the prospect of untreatable infections increasing morbidity, and the rapid escalation of economic health costs to society is a serious public health concern.[3,4]

The WHO has recommended research into novel therapies to stem the spread and improve treatment of MDR and XDR infections. One approach that has gained momentum in the last decade is the development of antivirulence compounds, which are

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designed to inhibit a key determinant in pathogenesis, thus enabling the natural immune responses of the host to clear the infection.[5] The virulence factor EptA (formerly termed lipid A phosphoethanolamine transferase, LptA), represents a promising target for antivirulence, as it is required for multiple aspects of gonococcal pathogenesis including colonization, inflammation and survival in neutrophils.[6] In a competitive infection with WT bacteria, EptA mutants are attenuated in female mouse models of colonization of the urogenital tract and the urethra of male humans, implying that this factor has an essential role in the pathogenesis of disease.[7,8] The loss of phosphoethanolamine (PEA) headgroups on lipid A increases susceptibility to complement-mediated killing mechanisms in serum[9] and reduces the cytokine response by the TLR-4/MD-2 pathways.[7,10,11] EptA mutants are unable to escape killing mechanisms in macrophages and neutrophils as they are unable to inhibit phagolysosome maturation[12–14] and are exquisitely susceptible to cationic antimicrobial peptides (CAMPs) such as cathelicidins and LL-37 in these cells.[13,15–17] Thus this enzyme represents an excellent target for the development of antivirulence compounds.[6,18]

EptA[19] catalyses the transfer of PEA from phosphatidylethanolamine (PtdE) to lipid A at the 1 and/or 4′ headgroup positions. The crystal structure of full-length EptA revealed that the N-terminal transmembrane (TM) domain is connected to the soluble domain (SD) by a bridging helix and an extended loop.[20] The SD of EptA adopts a hydrolase-type fold with a bound Zn^{2+} ion at the enzyme active site near to the catalytic nucleophile, Thr280.[21,22] Removal of the TM domain retains esterase activity required for cleavage of PtdE, but it is unable to function as a lipid A transferase in bacterial cells, suggesting that this domain is important for lipid A binding.

Due to the need for new therapies to treat gonococcal infections and the importance of EptA in the virulence of *N. gonorrhoeae*, we employed a pipeline-based methodology using saturation transfer difference (STD)-NMR to identify lead EptA-inhibiting compounds. Chemical modification of selected compounds improved their efficacy to reduce gonococcal resistance to the CAMP polymyxin B.

Materials and methods

Bacterial strains and growth conditions

FA1090 [Opa+, Pili+, PilP* (containing a stop codon in *pilP*)] was grown on gonococcal agar plates (GCA, Oxoid) supplemented with 1% (v/v) Deakin modified IsoVitaleX (DMIV) and 32 mg/L polymyxin B (Sigma–Aldrich). Opa (opacity) proteins are adhesins and PilP is a pilus biogenesis protein important for the multimerization of the PilQ porin.[23] Because of this, PilP* isolates have a higher intrinsic level of resistance to most compounds due to the reduction in PilQ porins.[24] *N. gonorrhoeae* FA1090Δ*eptA::aphA-3* (Opa+, Pili+, PilP+; see Supplementary materials and methods for construction, available as Supplementary data at *JAC* Online) was grown on GCA supplemented with 1% (v/v) DMIV and 50 mg/L kanamycin (Sigma–Aldrich). The 2008 WHO reference panel of MDR *N. gonorrhoeae* (MDR-NG) isolates was used to screen the compounds (*N. gonorrhoeae* 31536 to 31544).[25]

Compounds

The library of 1137 compounds was obtained from Maybridge Chemicals. The analogues encompassing 1–5, namely 16–55, were obtained from eMolecules. Synthetic derivatives of 48, namely 56–178, were synthesized by SYNthesis Pharm. Characteristics of these molecules [^{1}H NMR, HPLC (254 nm)] are detailed in the Supplementary materials and methods. All compounds were tested for identity, purity and solubility as we have described previously.[26] All compounds are covered under the Australian Provisional Patent Application No. 2021903584.

Conditions for STD-NMR and Carr-Purcell-Meiboom-Gill (CPMG)-NMR spectroscopy to identify compounds binding EptA

Compound binding to purified native EptA was first assessed by recording STD-NMR experiments[27] in cocktails containing up to six individual fragments at a concentration of ∼330 μM. Purified SD of EptA was prepared as described previously[22] and was used at a concentration of 5 μM in 50 mM sodium phosphate buffer, 100 mM NaCl, pH 7.4, 10% D_2O, 1% d_6-DMSO. The fragments were combined to minimize overlap in their 1D ^{1}H-NMR spectra and to allow facile identification of binders within each cocktail.[26] STD-NMR experiments[27] were conducted at 10°C and 600 MHz on a Varian Unity INOVA NMR spectrometer equipped with a cryogenically cooled probe. The magnitude of the signal in STD spectra was ranked by comparison with the most intense STD signal (I_{max}) identified across all the STD spectra for EptA as previously described.[28] The STD signal was categorized as strong where the intensity was ≥75% I_{max}, medium where the intensity was ≥50% I_{max} and <75% I_{max}, and weak where the intensity was ≥25% I_{max} and <50% I_{max}. Fragments showing the strongest STD signals from this analysis were validated by testing as singletons. Validation of these binders was conducted against recombinantly expressed and purified full-length EptA.[20] Single fragments were tested at a concentration of 400 μM in a buffer containing 50 mM phosphate, 100 mM NaCl, 0.024% (w/v) n-dodecyl-β-D-maltoside (DDM), pH 7.5, 0.2% d_6-DMSO. Fragments were tested for binding to full-length EptA, soluble EptA and buffer only (to exclude fragments that bound to the DDM micelles) by recording 1D STD and CPMG T2-weighted NMR experiments.

Microdilution assays to determine the effectiveness of small molecule inhibitors

Filter disc assays were used to identify the best hits that sensitized bacteria to subinhibitory concentrations of polymyxin B. The MICs of small molecule inhibitors of EptA were tested against strains FA1090 and FA1090Δ*eptA* using a 96-well plate-based microdilution format. For conditions, see Supplementary materials and methods.

Preparation of intact lipooligosaccharide (LOS) for analysis by MALDI-TOF MS

LOS was extracted and purified by a modification of the hot phenol–water method.[29] Conditions for MALDI-TOF MS and negative-ion MALDI-TOF MS of intact LOS are described in the Supplementary materials and methods.

Quantitative analysis of phosphorylated hexa-acylated lipid A ions in spectra of intact LOS

The quantitative analysis of phosphoethanolaminylation and phosphorylation of lipid A is enabled by observation of prompt Y-type reducing terminal fragment ions for the lipid A moieties of interest, including those that contained up to three phosphates and one PEA moiety in MALDI-TOF MS analysis. The MALDI-TOF spectra obtained on the SYNAPT G2 HDMS system were of high resolution, enabling detection of monoisotopic peaks for the lipid A species. Areas of the most abundant negative-ion peaks for hexa-acylated lipid A were determined for six to seven LOS spectra for each sample after using standard conditions for smoothing and correcting the baseline of the spectra using Mass Lynx

version 4.1 (Waters Corporation) software. Areas were included for the three most abundant isotopic peaks (M, M+1 and M+2) of the species listed in Table S1. Average ion abundances were calculated for peaks for hexa-acylated lipid A containing two or three phosphates (2P, 3P), with or without PEA, relative to the ion abundances for all quantified peaks for phosphorylated hexa-acylated lipid A. The ion abundance for peaks at *m/z* 1614.1 and 1737.1 were tabulated as fragment ions arising from facile loss of H_3PO_4 (98.0 Da) from 2P lipid A, and $H_4P_2O_7$ (177.9 Da) from 2P PEA lipid A, respectively, as previously described.[30] Differences in ion abundance for peaks of interest were analysed by ANOVA with a Bonferroni post-*t* test to determine the significance of levels of ions in LOS from each of the treated samples compared with the vehicle-only controls.

Cytokine expression from THP-1 cells and survival in RAW macrophage cell assays

The measurement of TNF-α from THP-1 cells stimulated by purified LOS is described in the Supplementary materials and methods. Compounds were tested for toxicity against RAW 264.7 murine macrophages (RAW) before use in bacterial survival assays (Supplementary materials and methods).

Results

Identification of hit small molecule inhibitors of EptA

The pipeline for the down-selection of anti-EptA compounds is described in Figure S1. Using STD-NMR, the compounds were ranked based on the intensity of the signals in the screening experiments in groups of six and validated by first recording STD-NMR experiments on single compounds against the soluble domain of EptA, and subsequently by recording both STD-NMR and CPMG-NMR experiments against the full-length EptA.[28] The top 15 ranked compounds by STD/CPMG-NMR, 1–15 (Figure S2), were then tested against nine MDR-NG isolates using a filter disc assay in the presence of the subinhibitory concentration of 48 mg/L polymyxin B. The results for 1–15 (data not shown) demonstrated that five compounds, 1–5, resulted in zones of inhibition of 1 mm or greater for two or more MDR-NG isolates.

In order to generate preliminary structure/activity relationships for the top ranked hits 1–5, a series of analogues spanning the chemical space of these molecules 16–55 (40 in total, Figure S3) were obtained and then tested using the broth microdilution assay against strain FA1090, using two original hits, 1 and 3, as controls. The bacteria were incubated in the presence of compound, with and without polymyxin B, to enable at least three cycles of bacterial replication to occur. This ensures inhibition of EptA over the course of bacterial replication, which leads to the sequential reduction of lipid A decorated with PEA and subsequently increases susceptibility to subinhibitory concentrations of polymyxin B. In this assay, the ability of the compounds to sensitize strain FA1090 to 48 mg/L polymyxin B, 1.3-fold lower than the WT polymyxin B MIC of 64 mg/L, was tested. A nominal selectivity index of >20-fold [fold change in maximum effective concentration (MEC) of compound, with and without polymyxin B] was used to stratify the 40 analogues into compounds that were more likely to be selective agents with a mode of action against EptA, while the remainder were more likely to be non-selectively toxic. From this approach, nine analogues (26, 27, 28, 29, 35, 38, 45, 48 and 50) and 3, but not 1, were shown to be effective at increasing the susceptibility of strain FA1090 to polymyxin B (Table S2). To determine whether these compounds were binding better to EptA than parental hit 3, STD/CPMG-NMR binding to full-length EptA was performed with analogues that were either above (29, 48, 50) or below (21, 23, 34, 36, 37, 53) the selectivity threshold of 20-fold in the bacterial polymyxin B susceptibility screen (Table S3). The results demonstrated that 21 and 50 were moderately better and 29, 34 and 48 were significantly better binders to EptA when compared with 3. Compounds 29 and 34 were discarded from further analysis as they belong to the promiscuous 2-aminothiazoles (PraATs), which have been identified as frequent hits in binding assays of this type.[31] In summary, 48 was chosen for further evaluation as the only hit that had a significantly higher affinity to EptA by STD/CPMG-NMR than 3 and also demonstrated a broad ability to sensitize MDR-NG to subinhibitory concentrations of polymyxin B (Table S3).

Elaboration of lead molecule 48

Lead molecule 48 was modified to gain insight into which groups were required for potency (Tables S4–S11). A library of variants was prepared where the amide moiety was modified (56–96). Next the carbonyl group was removed to understand the role of this moiety (97–106) and finally the effect of the N-linked cyclopentyl group was investigated, with this coupled to the effects of having a sulphone (107–122), sulphoxide (123–126) and thioether moiety (127–178).

To identify active compounds, compound at the MEC (a 2-fold dilution below the MIC of the compound) was incubated with strain FA1090 and a subinhibitory concentration of polymyxin B that did not affect growth (32 mg/L polymyxin B, a 2-fold dilution below the MIC of the WT) in the broth microdilution assay. A supplementary screen to test ‘off target’ cytotoxicity examined the effect of the compound on the mutant strain FA1090Δ*eptA* (which is resistant to 1 mg/L polymyxin B). Compounds that increased susceptibility of FA1090Δ*eptA* in the presence of 1 mg/L polymyxin B were considered to be ‘off target’ as these compounds had a mode of action independent of EptA expression. From these screens, a total of 44 compounds were considered to be ‘on target’ for EptA inhibition (Figure S4). In comparison to 48, which reduced the MIC of strain FA1090 2-fold from 64 to 32 mg/L (at a concentration of 200 μM), 26 compounds reduced the polymyxin B MIC of strain FA1090 2-fold, 14 compounds reduced the MIC 4-fold, and 4 compounds reduced the MIC 8-fold. Only four compounds, 56, 135, 136 and 137, exhibited the least bactericidal activity at 100 μM after 3 h incubation, and of these, 135, 136 and 137 reduced the polymyxin B resistance by 4-fold from WT levels. Therefore, 135, 136 and 137 (Figure 1) were considered to be improved hits derived from the parent scaffold 48 and were further investigated to determine whether they represented novel EptA inhibitors.

Pre-treatment of bacterial cells with compounds increases killing of N. gonorrhoeae by RAW macrophage cell lines

EptA mutants of *N. gonorrhoeae* are known to be highly susceptible to killing by macrophages due to the presence of defensins and cathelicidins in these cells.[13] The improved compounds 135,

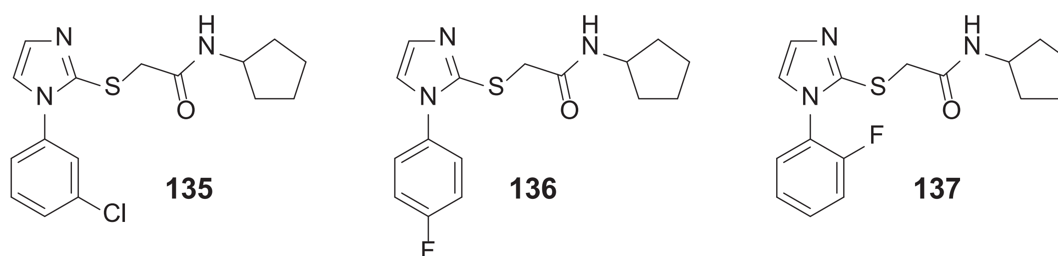

Figure 1. Structures of lead compounds that were investigated further for EptA inhibition.

136 and 137 were shown to have no cytotoxicity towards macrophages at 300 μM using a lactate dehydrogenase activity assay (data not shown). Bacteria were pre-incubated with each compound at 50 μM and then exposed to the RAW macrophage cell line and total association was measured after 15 min. The viability of FA1090Δ*eptA* in the presence of RAW macrophages was significantly reduced by 2.4-fold compared with the WT control (Dunn's multiple comparisons test; $P=0.0025$) (Figure 2). Bacteria treated with either 135, 136 or 137 also showed reduced viability compared with WT of 1.9-fold, 2.6-fold and 2.2-fold, respectively (Dunn's multiple comparison test; $P=0.0260$, $P=0.0018$, $P=0.0078$).

Reduction of PEA decoration of lipid A from N. gonorrhoeae treated with 137

To confirm that the compounds were directly inhibiting EptA in bacteria and resulting in the polymyxin B-susceptible phenotype, strain FA1090 was grown in the presence of either 135, 136 or 137 and the abundance of lipid A phosphorylation assessed by MS of LOS extracted from broth-grown bacteria incubated at mid-log phase with 50 μM compound for 3 h (Figure 3 and Figure S5). Compound 137 significantly reduced the amount of PEA-decorated lipid A in the LOS relative to the LOS in control untreated bacterial cells and those bacterial cells treated with 135 and 136 (Figure 3a). As the level of phosphoethanolaminylation was reduced in 137-treated bacteria, the relative abundance of 2P phosphoforms increased (Figure 3b and c).

To further verify the reduction of PEA decoration of lipid A, the same purified lipid A extractions were used to induce cytokine responses from THP-1 cells (Figure 4). The LOS from FA1090Δ*eptA* and 137-treated bacteria induced significantly less TNFα expression by the THP-1 cells than untreated LOS ($P<0.05$ and $P<0.001$, respectively) while LOS from 135- and 136-treated bacteria were not significantly different from LOS from untreated bacteria.

The lead compound 137 has a higher binding affinity for EptA than the original hit compound 48

The binding of 137 to EptA was investigated by recording STD-NMR and CPMG experiments using a similar approach that was used to elucidate the original hit 48 and to gain insight into any improvement in binding (Figure 5). Compound 137 showed a significant increase in the intensity of the peaks in the STD difference spectrum, having an average STD intensity

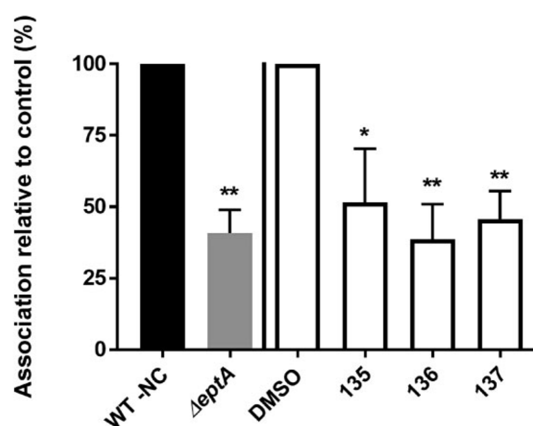

Figure 2. Total association of *N. gonorrhoeae* strain FA1090 in the presence and absence of compounds with RAW murine macrophages expressed as percentage survival relative to the appropriate control. Percentage survival of strain FA0190Δ*eptA* was calculated relative to WT. Percentage survival of WT with compounds 135, 136 and 137 was calculated relative to the WT in the presence of 1% DMSO, which is the carrier for the compounds. Statistical analysis was performed using Dunn's multiple comparisons test against appropriate controls. (Error bars = standard error of the mean; $^{*}P<0.05$, $^{**}P<0.01$).

of 4.6% in comparison with the original hit 48, which showed an average STD intensity of 0.4% using the same experimental parameters.

Effectiveness of 137 in reducing the polymyxin B MIC for MDR-NG

Since the original screen to detect compound 137 was performed using the antibiotic-susceptible strain FA1090, the effectiveness of 137 to reduce the polymyxin B MIC of MDR-NG isolates was tested. In all instances, 50 μM 137 was able to reduce the MIC of polymyxin B for MDR isolates by 2-fold (Table S12). A kill curve in the presence of LL-37 defensin revealed a qualitative increase in the rate of killing for WHO-F, -K, -G and -L, but resulted in a 2-fold increase to defensin activity for WHO-L only (Figure S6). To determine whether the reduction of polymyxin B MIC by 137 had a synergistic or antagonistic effect on killing with other antibiotics, the assays were repeated with subinhibitory concentrations of penicillin, azithromycin, gentamicin and ceftriaxone,

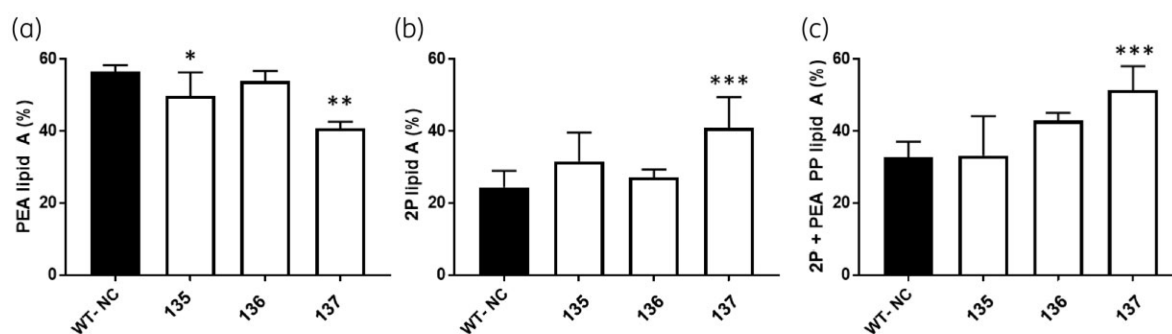

Figure 3. Graphs of average percentages of the areas of the negative-ion prompt fragment ion peaks in MALDI-TOF MS for: lipid A with PEA (a); 2P lipid A (b); and 2P lipid A and 2P PEA lipid A (c). In (a) the bars represent 2P PEA lipid A and 3P PEA lipid A ions, prominent fragment ion peaks at *m/z* 1737.1 for 3P PEA lipid A–$H_4P_2O_7$, fragment ion peaks at *m/z* 1614.1 for 2P lipid A–H_3PO_4, and peaks for these fragment ions but also with sodium adducts. The data represent six spectra for each LOS sample, FA1090 with no compound (NC), and FA1090 incubated with compound 135, 136, or 137. A total of nine peaks per LOS (each including areas for 'M, M+1 and M+2' resolved isotope peaks) were analysed. Error bars = SD. *$P \leq 0.05$, ***$P < 0.001$.

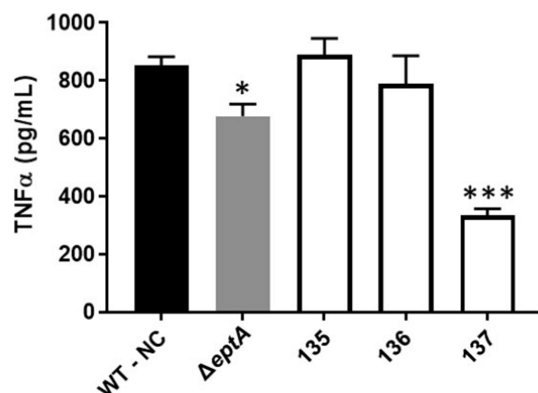

Figure 4. ELISA was used to quantify TNFα levels in supernatants from human THP-1 cells that were incubated for 18 h with LOS (100 ng/mL) purified from compound-treated and untreated FA1090 (WT) and the Δ*eptA* mutant. Treatment with compound 137 significantly reduced the induction of TNFα by FA1090 LOS compared with the untreated LOS ($P < 0.001$), whereas bacteria treated with either 135 or 136 had no effect on the induction of TNFα by LOS. The lipid A from FA1090Δ*eptA* induced significantly less TNFα than WT ($P < 0.05$). Results are expressed as the mean ± SD of six biological replicates with statistical analysis performed using one-way analysis of variance with Bonferroni's *post hoc* tests. *$P < 0.05$, ***$P < 0.001$.

which are used as first-line therapies. Of the seven WHO strains tested, weak synergism was noted for WHO-G and WHO-K in the presence of penicillin-G. WHO-G and WHO-K do not share any common resistance determinants, suggesting there is an unknown commonality that makes these isolates more susceptible to co-therapy. No antagonism was observed in any other combination of drugs with the seven isolates (Table S13).

Discussion

We describe a successful pipeline for the detection of anti-EptA compounds using *N. gonorrhoeae* as the screening platform. *N. gonorrhoeae* has a high intrinsic resistance to over 125 mg/L polymyxin B during laboratory culture, which is predominantly due to the expression of EptA.[32] This phenotype provides a stable dynamic range in which to detect low affinity and low potency inhibitors targeting EptA. A bacterial screening platform is ideally suited to analyse the ability of small molecules to enter bacterial cells and, in this case, inhibit EptA activity in the periplasm. The iterative approach of assessing the binding of the small molecules to purified EptA by STD/CPMG-NMR, followed by a selection pipeline for the detection of compounds that increased the susceptibility of the bacteria to subinhibitory concentrations of polymyxin B by 2-fold (48 mg/L) below the MIC of the WT bacteria, proved successful in identifying an early hit, 48. The addition of a specificity screen to test for non-selective toxicity against FA1090Δ*eptA* also ensured that compounds that were non-selectively toxic were removed from further development. The threshold for non-selectivity, as determined by a nominal 2-fold improvement in killing the bacteria by compound and polymyxin B together and then testing the binding of the compounds to purified EptA, showed the validity of this approach, which identified 48 as a specific binder to EptA. The same approach was used successfully to select elaborated variants of 48, namely 135, 136 and 137, which were more potent, being capable of sensitizing the bacteria to 16 mg/L polymyxin B, which was 4-fold lower than the WT MIC. Pre-incubation of the bacteria with these compounds also resulted in increased susceptibility to macrophage killing to the same extent as the FA1090Δ*eptA* mutant.

To verify whether 135, 136 and 137 specifically targeted EptA as inhibitors or had a different mode of action, lipid A was extracted from bacteria exposed to the compounds and the amount of PEA decoration was quantified using negative-ion MALDI-TOF MS. Previous studies of phosphorylation and phosphoethanolaminylation in *Neisseria* spp.[11,32] have indicated that approximately 60% of lipid A headgroups are decorated with PEA in broth-grown bacteria. In this study, it was shown that *N. gonorrhoeae* strain FA1090 also displayed a similar proportion of phosphoethanolaminylation and that upon exposure to 137, but not 135 or 136, this proportion was reduced by 17% (Figure 3). This result also correlated with STD-NMR studies, which showed that 137 binds with higher affinity than 48 to EptA

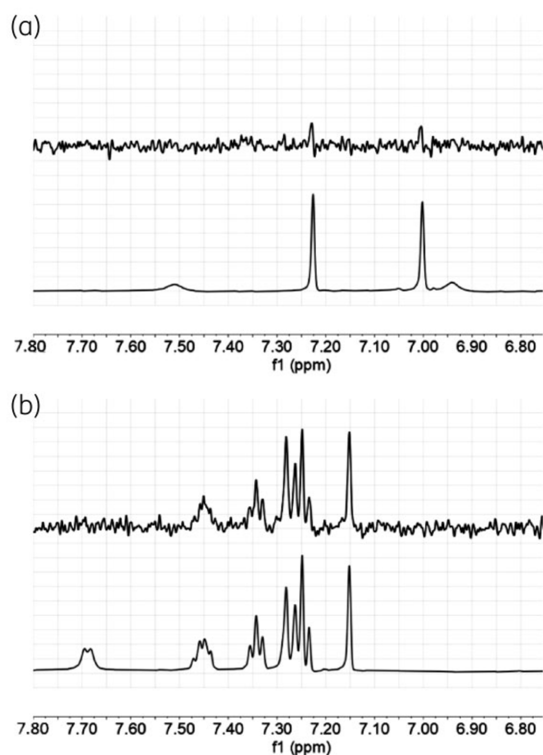

Figure 5. Analysis of binding to purified EptA by STD-NMR spectroscopy.[27] (a) Data generated for 48, where the bottom plot shows the aromatic region off-resonance spectrum from the STD-NMR experiment and the top plot shows the same region of the STD difference spectrum. (b) The same data as for (a) but for 137. In each case, the off-resonance experiment contains resonances that are consistent with the structure of the compound, which verifies the structure and confirms that it is present and soluble under the experimental conditions.

(Figure 5). Interestingly, as the level of phosphoethanolaminylation reduced, the level of 2P lipid A and 3P lipid A increased, suggesting that there is an equilibrium maintained between these glycolipid decorations. The production of 2P lipid A and 3P lipid A in *Escherichia coli* is the result of the lipid A 1-diphosphate synthase, LpxT.[33] This study suggests that LpxT activity is present in *Neisseria* spp. and further work is required to identify the genetic locus.

To examine whether the reduction of phosphoethanolaminylation by 137 treatment was biologically relevant, the ability of the purified lipid A from 137-treated WT bacteria to elicit a cytokine response from THP-1 cells was measured. Consistent with prior observations,[10,11] the LOS from FA1090Δ*eptA* was less stimulatory for THP-1 cells than WT LOS. Even though the amount of PEA decoration was inhibited by 17%, the LOS of 137-treated WT bacteria demonstrated an almost 2-fold reduction in induction of cytokines compared with the untreated WT. However, concomitantly 2P lipid A increased in 137-treated samples due to decreased levels of both 3P lipid A and 3P PEA lipid A, whereas the EptA mutant displayed decreased levels of 3P PEA lipid A only. These differences in the microheterogeneity of lipid A phosphorylation may affect the inflammatory potential of the sample, as discussed in a recent study by John *et al.*[34] Substantiation of this hypothesis would require top-down MS/MS on the 137-treated and EptA mutant samples to examine the phosphate distribution at the 4′ and 1 positions of lipid A as it is the predominance of phosphorylation at the 4′ position that is associated with inflammatory potential. Pre-incubation of the bacteria with the compound was sufficient to phenocopy the FA1090Δ*eptA* mutant survival in macrophages. MDR-NG have mutations that lead to the derepressed overexpression of the MtrCDE efflux pump, which contributes to resistance to many antibiotics. All of the nine WHO isolates, including those with derepressed expression of MtrCDE efflux, were sensitized to polymyxin B by 2-fold. However, there was no substantial synergistic killing with the antibiotics penicillin, gentamicin, ceftriaxone or azithromycin, suggesting that the levels of phosphoethanolaminylation and pyrophosphorylation of lipid A did not result in exclusion of these antibiotics from the bacterial cell.

Overall, this work demonstrates that targeting EptA with small molecule inhibitors can sensitize *N. gonorrhoeae* to killing by CAMPs inside macrophages. Compound 137 is the first lead compound in the development of this class of novel therapeutic agents. Importantly, gonococcal EptA has a similar architectural structure to that of the mobile colistin resistance protein, Mcr-1, of Enterobacteriaceae. Future work will determine how these novel small molecules interact with the active site of this family of enzymes.

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Transparency declarations

The authors declare the following competing financial interest(s): Australian Provisional Patent Application No. 2021903584 has been filed, which covers the compounds described in this manuscript and their methods of use. The contents of this article are solely the responsibility of the authors and do not necessarily reflect the official views of the National Institutes of Health, the U.S. Department of Veterans Affairs, the United States government or the National Health and Medical Research Council of Australia.

Author contributions

C.M.K., A.V. and K.A.S. co-jointly designed the study. S.B. and C.M. performed the microbiology screening of the compounds. K.A.S. designed

the compounds. K.A.S. and A.V. managed the synthesis of the compounds. V.C.T. performed the defensin and antimicrobial susceptibility assays. A.A. prepared the enzyme for screening. M.J.S. performed the STD-NMR and CPMG-NMR spectroscopy. G.A.J. and C.M.J. performed the MALDI-TOF MS on the endotoxin and the cytokine assays. K.Y.L.L., C.M.S. and M.S.T. designed and performed the screening of the compounds in the macrophage assays. C.M. and C.M.K. co-wrote the draft of the manuscript and all participants edited the content for accuracy.

Supplementary data

Supplementary materials and methods, Figures S1 to S6 and Tables S1 to S13 are available as Supplementary data at *JAC* Online.

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