Colistin requires de novo lipopolysaccharide biosynthesis for activity

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Colistin is a last resort antibiotic for infections caused by highly drug-resistant Gram-negative pathogens such as Pseudomonas aeruginosa [1,2]. Unfortunately, treatment failure is common despite low rates of resistance, and efforts to address this are compromised by our poor understanding of colistin’s mode of action [3-6]. Here, we show that colistin causes dose-dependent membrane disruption and killing of P. aeruginosa via a process that requires de novo lipopolysaccharide (LPS) biosynthesis. Colistin binds to LPS in the outer membrane (OM), leading to disruption of the cation-bridges that stabilise the lipopolysaccharide molecules. Due to the weakening of these bridges, de novo synthesis of LPS results in the release of lipopolysaccharide from the OM. The subsequent loss of membrane integrity enables colistin to access the cytoplasmic membrane (CM), leading to permeabilisation and bacterial lysis. Inhibition of LPS biosynthesis in Escherichia coli and Klebsiella pneumoniae also inhibited the bactericidal activity of colistin, suggesting a conserved mechanism across clinically-relevant pathogens. Together, these findings reveal the mechanism by which colistin kills bacteria and provide the foundations for the development of new approaches to enhance treatment outcomes.

The emergence of multi-drug resistant Gram-negative pathogens has led to a dramatic increase in the use of cyclic lipopeptide antibiotics such as colistin (polymyxin E) and polymyxin B as therapeutics of last resort [7,8]. Despite this, our understanding of the mechanism by which these drugs kill bacteria is poor. The currently proposed mode of action of colistin begins with binding of the antibiotic to LPS, which leads to disruption of the OM via the displacement of cations that form stabilising bridges between LPS molecules (Fig. 1a) [9-11]. It is hypothesised that colistin then migrates across the OM via a process termed ‘self-directed uptake’, although this has never been demonstrated experimentally [11-13]. This crucial step enables colistin to access the CM target, which it disrupts, leading to bacterial lysis and death [11-13]. Furthermore, the existence of colistin-tolerant persister cells suggests that bacterial processes contribute to the lethality of the antibiotic, although none have yet been identified [14-16]. Given these gaps in our understanding of this increasingly important class of antibiotics, the aim of this work was to determine the mechanism by which polymyxins kill bacteria.
To test the current model of colistin activity, we employed a panel of assays to investigate membrane damage, cell lysis and bacterial viability during exposure of *P. aeruginosa* PA14 to clinically-relevant concentrations of the antibiotic [17,18]. Colistin caused immediate and dose-dependent disruption of the OM, as determined by uptake of the normally membrane-impermeant dye N-phenyl-1-naphthylamine (NPN) (Fig. 1b, Supplementary Fig. 1). Within 30 min of colistin exposure, *P. aeruginosa* began to release periplasmic β-lactamase into the culture supernatant, indicative of severe OM damage (Fig. 1c). This was followed by disruption of the CM by 60 min, as detected by staining of cells with the membrane-impermeant DNA-reactive fluorophore propidium iodide (Fig. 1d, Supplementary Fig. 2). Subsequently, bacterial lysis induced by colistin was observed with a decrease in OD$_{595\text{nm}}$ from 90 min, and there was a corresponding drop in c.f.u. counts by 2 h (Fig. 1ef). These findings provide a timeline for the current model of polymyxin-mediated bacterial killing, whilst the survival of a sub-population of bacteria exposed to the antibiotic confirmed the existence of colistin-tolerant persister cells within *P. aeruginosa* populations (Fig. 1f, Supplementary Fig. 3).

To further investigate colistin-mediated membrane disruption, we measured the release of LPS and phospholipids into the extracellular space. *P. aeruginosa* released large quantities of phospholipids, most likely in the form of OMVs, in the absence of colistin, but this was almost completely blocked by colistin at bactericidal concentrations (Fig. 1g) [19]. By contrast, colistin exposure resulted in the time-dependent release of LPS into the culture supernatant (Fig. 1h, Supplementary Fig. 4).

As reported previously, released LPS completely inhibited the antibacterial activity of colistin at 2 µg ml$^{-1}$ (Supplementary Fig. 5) [20]. However, we also considered the possibility that colistin-induced LPS release contributed to the bactericidal activity of the antibiotic. This is because the loss of LPS from the OM results in lipid asymmetry and a loss of integrity of the OM, which if uncorrected culminates in cell death in most bacteria [21]. To counteract lipid asymmetry, bacteria increase the production and trafficking of LPS to the OM [22]. Therefore, we hypothesised that colistin activity would be enhanced by blocking LPS biosynthesis, thereby preventing *P. aeruginosa* from restoring the
composition of the outer leaflet of the OM. To test this, we reduced the ability of \textit{P. aeruginosa} to
restore LPS to the OM of colistin-treated cells by exposing bacteria to the polymyxin antibiotic in the
presence of sub-lethal concentrations of the LPS biosynthesis inhibitors CHIR-090 or cerulenin
(Supplementary Table 2, Supplementary Fig. 6) [23-25].

As described above (Fig. 1f), colistin alone caused rapid killing of \textit{P. aeruginosa} (Fig. 2a).
However, in contradiction to our hypothesis, the presence of either inhibitor of LPS biosynthesis
almost completely blocked killing of \textit{P. aeruginosa} by colistin (Fig. 2ab). To ensure that the protective
effect of LPS biosynthesis inhibition wasn’t due to reduced LPS on the bacterial cell surface, we
exposed \textit{P. aeruginosa} to colistin for 2 h and then added CHIR-090 or cerulenin. The addition of either
LPS biosynthesis inhibitor paused or greatly slowed colistin-mediated killing of \textit{P. aeruginosa},
confirming that \textit{de novo} LPS biosynthesis is needed for the continuous bactericidal activity of the
polymyxin antibiotic (Fig. 2c).

Scanning electron microscopy (SEM) supported the data showing that colistin activity was
dependent on \textit{de novo} biosynthesis of LPS. Exposure to the polymyxin antibiotic alone caused
significant morphological changes to \textit{P. aeruginosa} consistent with a loss of membrane integrity (Fig.
2d). However, bacteria exposed to colistin in the presence of CHIR-090 or cerulenin had a similar
appearance to untreated cells (Fig. 2efghi). The protective effects of CHIR-090 and cerulenin against
colistin-mediated killing extended to clinical isolates of \textit{P. aeruginosa}, \textit{K. pneumoniae} and \textit{E. coli},
suggesting the polymyxin antibiotic has a broadly conserved mechanism against Gram-negative
pathogens (Supplementary Fig. 7). By contrast to the LPS biosynthesis inhibitors, neither sub-
inhibitory nor bacteriostatic concentrations of the protein synthesis inhibitor tetracycline prevented
colistin-mediated killing of \textit{P. aeruginosa} (Supplementary Fig. 8). Therefore, colistin requires \textit{de novo}
LPS but not protein biosynthesis for activity, and functions in the absence of bacterial replication.

However, \textit{de novo} LPS biosynthesis was not required for the bactericidal activity of EDTA, which also
disrupts cation bridges in the LPS monolayer, indicating that the requirement for \textit{de novo} LPS
biosynthesis is specific to polymyxin-mediated killing (Supplementary Fig. 9).
Next, we determined which of the steps in the current model of action of colistin were dependent on *de novo* LPS biosynthesis. Using NPN, we found that colistin rapidly permeabilised the OM, and this was unaffected by the presence of CHIR-090 or cerulenin (Fig. 3a). Therefore, *de novo* LPS biosynthesis is not required for the initial interactions of the polymyxin antibiotic with the bacterial surface. However, colistin-mediated release of β-lactamase from *P. aeruginosa* was blocked by cerulenin, indicating that *de novo* LPS biosynthesis is required for severe damage to the OM (Fig. 3b). This assay could not be repeated using CHIR-090 as it reacted with the chromogenic substrate used to detect and quantify β-lactamase. Nonetheless, the presence of cerulenin or CHIR-090 prevented colistin-mediated CM damage and lysis of *P. aeruginosa* (Fig. 3cd). This demonstrated that *de novo* LPS biosynthesis is required for colistin to cross the OM and provided a direct link between the degree of OM damage and the ability of colistin to access the CM. Colistin-mediated disruption of the CM and subsequent lysis of clinical *P. aeruginosa*, *K. pneumoniae* and *E. coli* isolates were also reduced or blocked when LPS biosynthesis was inhibited, showing that the dependency on LPS biosynthesis for colistin activity is conserved (Supplementary Fig. 10, 11).

To determine how *de novo* LPS biosynthesis modulated the degree of colistin-mediated damage to the OM, we tested whether CHIR-090 or cerulenin affected LPS release caused by the polymyxin antibiotic. Both inhibitors blocked colistin-induced LPS release, demonstrating that this process, which results in severe damage to the OM, was dependent upon *de novo* LPS biosynthesis (Fig. 3e, Supplementary Fig. 12-14). Therefore, *de novo* LPS biosynthesis triggers the loss of LPS from the cell surface, and thus enables colistin to cross the OM.

The data shown above confirmed that neither CHIR-090 nor cerulenin inhibited the initial interactions of colistin with the surface of *P. aeruginosa* (Fig. 2c, 3a). However, we hypothesised that the severe OM damage mediated by the combination of colistin and *de novo* LPS biosynthesis causing LPS release would increase binding of the antibiotic to *P. aeruginosa* by exposing the CM target. To test this, we measured the binding of colistin to bacterial cells in the absence or presence of CHIR-090 or cerulenin using colistin labelled with a BoDipy fluorophore (Supplementary Fig. 15, 16). Exposure
of *P. aeruginosa* to BoDipy-colistin resulted in strong binding of the antibiotic to the bacterial cells during the first two hours, which was maintained over the rest of the incubation period (Fig. 3f). By contrast, when the experiment was repeated in the presence of CHIR-090 or cerulenin, binding to bacteria was significantly reduced, with a slow accumulation of BoDipy-colistin over the course of the assay (Fig. 3f).

Taken together, the data described in Figure 3 demonstrate that *de novo* LPS biosynthesis enables colistin to exert its bactericidal activity by causing LPS release and increasing the severity of the OM damage induced by the antibiotic. This provides colistin with access to the CM, which it disrupts, leading to lysis and bacterial killing.

Polymyxin antibiotics consist of a positively-charged peptide ring and a hydrophobic lipid tail [1,11,26]. Previous work demonstrated that the polymyxin peptide ring displaces cations which act as bridges between LPS molecules [27]. This leads to sufficient permeabilisation of the OM to allow the passage of small molecules such as NPN [11,13]. However, there is evidence that the lipid tail is required for CM damage and bacterial killing [28]. Therefore, we hypothesised that the lipid tail of colistin and *de novo* LPS biosynthesis act in concert to cause the severe OM damage required to enable the polymyxin antibiotic to access the CM.

To test this, we exposed *P. aeruginosa* to polymyxin B (PMB), which is structurally and functionally homologous to colistin, or a variant of PMB that consists of the cyclic peptide ring but not the lipid tail, known as polymyxin B nonapeptide (PMBN) (Supplementary Fig. 17). Both PMB and PMBN caused sufficient OM disruption to allow NPN to cross the LPS monolayer, albeit at a slower rate and to a lesser degree for PMBN than for PMB (Fig. 4ab). As with colistin, inhibition of LPS biosynthesis did not affect NPN staining in response to either PMB or PMBN (Fig. 4ab). This showed that *de novo* LPS biosynthesis was not required for minor OM disruption caused by the initial interaction of the polymyxin peptide ring with the bacterial surface. However, whilst PMB severely damaged the OM of *P. aeruginosa* cells, as determined by the release of periplasmic β-lactamase into the culture supernatant, this did not occur with PMBN (Fig. 4cd). Furthermore, in addition to the
requirement for the lipid tail of the polymyxin, the PMB-mediated release of β-lactamase was also 
inhibited by cerulenin, confirming the synchronous role of de novo LPS biosynthesis in this process 
(Fig. 4c). These findings are supported by previous work using scanning electron microscopy that 
demonstrated PMB caused much greater OM damage than PMBN [29].

The severe nature of the OM damage mediated by the polymyxin lipid tail appeared crucial 
for the bactericidal activity of the antibiotic, since PMB, but not PMBN caused permeabilisation of the 
CM, cell lysis and bacterial death, via a mechanism that was dependent upon de novo LPS biosynthesis 
(Fig. 4efghij). Therefore, whilst the peptide ring of the polymyxin antibiotics weakens the OM 
sufficiently to allow the uptake of small molecules, the lipid tail is required, in conjunction with de 
 novo LPS biosynthesis, for the severe damage to the OM that enables PMB to exert its bactericidal 
effects.

Combined, the findings described above, together with other studies, enable the construction 
of a novel model for the mechanism of action of colistin (Fig. 4k). Firstly, colistin interacts with LPS on 
the bacterial cell surface via the peptide ring of the antibiotic [11]. This displaces the cations that 
stabilise LPS molecules in the monolayer, and thus results in minor disruption of the OM [27]. As LPS 
is synthesised and transported to the cell surface, colistin-induced weakening of the lateral forces 
between LPS molecules causes LPS to be pushed out and released from the outer leaflet of the OM. 
The loss of LPS leads to severe OM damage, which is additionally dependent upon the lipid tail of the 
polymyxin antibiotic. This perturbation to the OM enables the release of periplasmic macromolecules 
and also facilitates access of the relatively large colistin molecules to the phospholipids in the CM, 
which the antibiotic disrupts via its detergent-like properties [28]. Once the OM and CM are 
compromised, the bacteria lyse and die.

This revised model addresses key gaps in our understanding of the mechanism of action of 
polymyxin antibiotics by revealing how these antibiotics cross the OM, explaining why the polymyxin 
acyl tail is required for bactericidal activity and providing a direct link between OM disruption and the 
bactericidal activity of the antibiotic. Such insight is needed because colistin is an increasingly
Important antibiotic for the treatment of infections caused by multi-drug resistant pathogens, but has a treatment failure rate of >70% [3-6]. This study provides two explanations for the extremely high rate of treatment failure. Firstly, bacteria release LPS in response to colistin, which sequesters and inactivates the antibiotic [20]. Secondly, we identify LPS biosynthesis as a bacterial process that could, when decreased, confer colistin tolerance to persister cells with reduced metabolic activity [14,15].

To address the high rate of polymyxin treatment failure, there is increasing interest in the use of synergistic antibiotic combinations. For example, it has been shown that novobiocin enhances the activity of polymyxin B by promoting the transport of LPS to the OM and causing LPS release [30]. However, the reason why enhanced LPS transport conferred this increased susceptibility to the polymyxin antibiotic was unknown [30]. The data presented in this report provide a mechanistic explanation for this finding by showing that de novo LPS production and release from the OM is required for polymyxin activity. Thus, it is anticipated that a greater understanding of the mode of action of polymyxin antibiotics including colistin, combined with our growing appreciation of the processes of LPS biosynthesis and transport, will provide the necessary platform for the identification and development of therapeutic approaches to improve treatment efficacy.
Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Supplementary Table 1. For each experiment, bacteria were grown for 18 h to stationary-phase at 37°C with shaking (180 r.p.m.). All strains were grown in Luria broth (LB; Thermo Fisher Scientific, USA), with the exception of *P. aeruginosa* strain A23, which was cultured in Tryptic Soy Broth (TSB; BD Biosciences, USA), and *K. pneumoniae* strain KPC, which was grown in Brain Heart Infusion Broth (BHI; VWR International, USA). For routine culture of bacteria on solid media, strains were grown on the relevant media as described above supplemented with 1.5% technical agar (BD Biosciences). Growth media were supplemented with chloramphenicol (25 μg ml⁻¹) where required. Enumeration of bacterial c.f.u. was done by plating 10-fold serial dilutions of bacterial cultures on to Mueller-Hinton agar (MHA; Thermo Fisher Scientific) plates. Inoculated agar plates were incubated statically for 18 h in air at 37°C.

Determination of minimum inhibitory concentrations of antibiotics

The minimum inhibitory concentration (MIC) of colistin, polymyxin B, ciprofloxacin, tetracycline, cerulenin (all from Sigma-Aldrich, USA) and CHIR-090 (Axon Medchem, Netherlands) for bacterial strains was determined by the broth microdilution protocol [31]. A microtitre plate was used to prepare a range of antibiotic concentrations in 200 μl Mueller-Hinton broth (MHB) by two-fold serial dilutions. For certain experiments, the media were supplemented with purified LPS from *E. coli* (Sigma-Aldrich). Stationary-phase bacteria were diluted 1000-fold in fresh MHB, and seeded into each well of the microtitre plate to a final concentration of 5 x 10⁵ c.f.u. ml⁻¹. The microtitre plates were then incubated statically at 37°C for 18 h in air, after which point the MIC was defined as the lowest antibiotic concentration at which there was no visible growth of bacteria.
OM disruption assay

To detect damage to the OM of bacteria, the well-established NPN uptake assay was used [32]. Stationary-phase bacterial cells were washed in fresh MHB and diluted to an optical density (OD$_{600\text{nm}}$) of 0.5 in 5 mM pH 7.2 HEPES buffer (Sigma-Aldrich). This bacterial suspension was added to wells containing the relevant antibiotics in HEPES buffer, as well as the fluorescent probe N-phenyl-1-naphthylamine (NPN; Acros Organics, USA) at a final concentration of 10 μM. Samples were placed in a black microtitre plate with clear-bottomed wells and fluorescence measured immediately in a Tecan Infinite 200 Pro multiwell plate reader (Tecan Group Ltd., Switzerland) using an excitation wavelength of 355 nm and an emission wavelength of 405 nm. Fluorescence measurements were obtained every 30 seconds for a total period of 60 min.

β-lactamase release assay

*P. aeruginosa* cells were grown overnight with imipenem (Fresenius Kabi, Germany; 2 μg ml$^{-1}$) to induce expression of the endogenous β-lactamase AmpC [33]. Stationary-phase cells were washed twice by centrifugation (12,300 x g, 3 min) followed by resuspension in MHB, and added to 3 ml MHB containing the relevant antibiotics to a final inoculum of 10$^{8}$ c.f.u. ml$^{-1}$. Cultures were then incubated at 37°C with shaking (180 r.p.m.) for 60 min. Every 15 min, aliquots were obtained and bacteria removed by centrifugation (12,300 x g, 3 min). The resulting supernatant was then recovered and 200 μl added to the wells of a microtitre plate. Nitrocefin (Abcam, UK) was added to each well to a final concentration of 250 μM and the mixture incubated for 2.5 h at room temperature. Absorbance was measured at 490 nm in a Bio-Rad iMark microplate absorbance reader (Bio-Rad Laboratories, USA).

CM disruption assay

As described above for the β-lactamase release assay, stationary-phase bacterial cultures were washed and inoculated into 3 ml MHB containing relevant antibiotics. Cultures were incubated at 37°C with shaking (180 r.p.m.) for 4 h, and every 30 min, aliquots (200 μl) were taken and bacteria isolated
by centrifugation (12,300 x g, 3 min). Cells were then washed in sterile phosphate-buffered saline (PBS; VWR International), before being added to the wells of a black-walled microtitre plate, and propidium iodide (Sigma-Aldrich) was added to each well at a final concentration of 2.5 μM. Fluorescence was measured immediately in a Tecan Infinite 200 Pro multiwell plate reader (excitation at 535 nm and emission at 617 nm). To account for differences in fluorescence values arising from variations in cell number due to growth inhibition by antibiotics, relative fluorescence unit (r.f.u.) measurements were corrected for OD at 600 nm.

**Determination of bacterial lysis**

Washed stationary-phase bacteria were inoculated into 3 ml MHB containing the relevant antibiotics, as described above. Cultures were then placed in a shaking incubator (37°C, 180 r.p.m.) for 8 h, and every 30 min, samples (200 μl) were transferred to a microtitre plate, where OD_{595nm} measurements were obtained using a Bio-Rad iMark microplate absorbance reader.

**Determination of bactericidal activity of antibiotics**

Stationary-phase bacteria were washed and added to a final inoculum of 10^8 c.f.u. ml^{-1} in 3 ml MHB containing the relevant antibiotics. Cultures were incubated with shaking (37°C, 180 r.p.m.) for 8 h. Bacterial survival was determined after 2, 4, 6 and 8 h through enumeration of c.f.u. counts by serial dilution in 10-fold steps of cultures in 200 μl sterile PBS.

**Emergence of colistin resistance assay**

Bacteria in stationary-phase were washed and added to 3 ml MHB containing colistin (2 μg ml^{-1}), and survival was determined every 2 h up to 8 h as described above. Following 8 h incubation, the MIC of colistin against the recovered bacterial population was determined by the broth microdilution method as stated above. The recovered bacteria were washed by centrifugation (12,300 x g, 3 min) and resuspension in fresh LB, before being inoculated into 3 ml LB and grown for 18 h to stationary-phase in a shaking incubator (37°C, 180 r.p.m.). This population was then exposed to colistin (2 μg ml^{-1}) for a
second time, with bacterial viability again measured over 8 h. After the second colistin exposure, the
MIC of colistin was again determined, before the bacteria were exposed to colistin for a third time,
with associated measurements of survival and the colistin MIC of the recovered population obtained.

Determination of membrane lipid release from bacteria

The supernatant from bacterial cultures exposed to colistin was recovered every 2 h by centrifugation
(12,300 x g, 3 min). Recovered supernatants were mixed with 5 μl of FM-4-64 styryl dye (Thermo Fisher
Scientific) at a final concentration of 5 μg ml\(^{-1}\) in the wells of a black-walled microtitre plate.
Fluorescence was measured using a Tecan Infinite 200 Pro multiwell plate reader (excitation at 565
nm, emission at 600 nm) to quantify the phospholipid released into the supernatant by bacteria
exposed, or not, to colistin.

Determination of LPS release from bacteria

The chromogenic Limulus Amebocyte Lysate (LAL) assay (all reagents from Thermo Fisher Scientific)
was used to detect and quantify the LPS released from bacteria into the culture supernatant as
described previously [34]. Samples of cell-free culture supernatant (50 μl) were equilibrated to 37°C
and loaded into the wells of a microtitre plate at the same temperature. Limulus amebocyte lysate
reagent (50 μl) was added to each well, and the mixture incubated at 37°C for 10 min. Chromogenic
substrate solution (100 μl, 2 mM) was subsequently added to each well and the microtitre plate was
incubated for a further 6 min at 37°C. The enzymatic reaction was stopped by adding 50 μl of 25% acetic acid to each well, and the presence of LPS was determined by measuring absorbance at 405 nm in a Tecan Infinite 200 Pro multiwell plate reader. A standard curve was generated using an *E. coli* endotoxin standard stock solution, which enabled the conversion of A\(_{405\text{nm}}\) values into concentrations of LPS in the supernatant.
Extraction of LPS and visualisation by silver staining

LPS was extracted from the supernatant of bacterial cultures as previously described, and the ultrafast silver staining method was used for visualising LPS on 12% SDS-PAGE gels [35,36]. Stationary-phase bacteria (1 ml) were washed and inoculated to a final concentration of $10^8$ c.f.u. ml$^{-1}$ into 9 ml MHB containing the relevant antibiotics. Bacterial cultures were incubated with shaking (37°C, 180 r.p.m.) for up to 8 h, and 9 ml of supernatant was recovered following centrifugation (3270 x g, 30 min) and filter sterilisation (0.2 µm filter) to remove bacterial cells. Absolute ethanol (30 ml) was added to the supernatant and the samples were stored at -20°C for 30 min. The precipitate was pelleted by centrifugation at 3270 x g for 30 min and resuspended in 500 µl Laemmli buffer. Precipitated protein was removed by digesting with proteinase K (50 µg) overnight, before 25 µl was run on an acrylamide mini-gel system. For certain experiments, the concentration of LPS in these samples was determined using the LAL assay described above. Electrophoresis was carried out at 12 mA in the stacking gel and 25 mA in the separating gel.

LPS preparations subjected to SDS-PAGE were visualised by first oxidising the gel with 200 ml 40% ethanol-5% acetic acid containing 0.7% sodium periodate for 20 min at room temperature. The gel was washed three times with distilled water for 5 min each time. Silver staining solution was freshly prepared by first adding concentrated ammonium hydroxide (4 ml) to 0.1 M sodium hydroxide (56 ml) before adding 200 ml of distilled water. Silver nitrate (20% w/v, 10 ml) was added in drops whilst stirring, before the addition of 30 ml of distilled water. This solution was used to stain the gel for 10 min, which was subsequently washed with distilled water for 5 min three times. The silver stain was developed with 200 ml of water containing formaldehyde (100 µl of a 37% w/v solution) and 10 mg citric acid. Development was stopped using 10% acetic acid before the gel was washed in distilled water and then imaged using a Bio-Rad Gel Doc EZ Imager (Bio-Rad Laboratories).
Determination of colistin activity

The activity of colistin during incubation with *P. aeruginosa* was determined using an established zone of inhibition assay [37]. Bacterial cultures exposed to colistin were centrifuged (12,300 x g, 3 min), and the supernatant recovered. Stationary-phase *P. aeruginosa* was diluted in MHB to a concentration of 10⁶ c.f.u. ml⁻¹, before 60 μl of this bacterial culture was spread across the surface of an MHA plate and allowed to air dry. Wells measuring 10 mm in diameter were made in the agar plate and filled with 130 μl of the culture supernatant recovered from colistin-treated bacterial populations. Agar plates were incubated in air statically at 37°C for 16 h and the zone of growth inhibition around each well was measured at four perpendicular points. A standard plot was generated showing a linear relationship between the size of the zone of inhibition and colistin concentration, which enabled inhibitory zone size to be converted to percentage colistin activity. Colistin activity was also measured after 4 h incubation (37°C, end-over-end rotation) in the presence of purified LPS, or in MHB alone over the course of 8 h in a shaking incubator (37°C, 180 r.p.m.).

Determination of colistin binding to bacterial cells

Colistin was labelled with the fluorophore BoDipy FL SE D2184 (Thermo Fisher Scientific) by incubating 100 μl of the BoDipy NHS ester compound (10 mg ml⁻¹ in dimethyl sulfoxide, DMSO) with 250 μl colistin (10 mg ml⁻¹ in water) and 650 μl sodium bicarbonate (0.2 M, pH 8.5) for 2 h at 37°C. BoDipy molecules that had not bound to colistin were removed by dialysis using a Float-A-Lyser G2 dialysis device (Spectrum Laboratories, USA) that had a molecular weight cut-off of 0.5 kDa. Dialysis was carried out at 4°C against sterile distilled water, which was changed four times during the course of the 24 h dialysis period. Time of flight mass spectrometry analysis confirmed successful labelling with the fluorophore, and the antibiotic activity of BoDipy-labelled colistin was assessed using MIC and bacterial survival assays, as described above.

Stationary-phase bacterial cultures were washed and added to 3 ml MHB containing the relevant antibiotics at an inoculum of 10⁸ c.f.u. ml⁻¹, as stated above. Cultures were incubated for 8 h (37°C,
180 r.p.m.), and every 2 h bacteria were recovered by centrifugation (12,300 x g, 3 min). The pelleted cells were washed thoroughly by four rounds of centrifugation (12,300 x g, 3 min) and resuspension in fresh MHB in order to remove any BoDipy-colistin that was not attached to the bacteria. BoDipy-colistin bound to the bacterial cells was quantified by measuring fluorescence in a Tecan Infinite 200 Pro multiwell plate reader (excitation at 490 nm, emission at 525 nm) using a black-walled clear-bottomed microtitre plate. The relative fluorescence values of samples containing cells were corrected using OD_{600nm} measurements.

**Bacterial growth assay**

Stationary-phase bacteria were diluted 1000-fold in fresh MHB, and 4 μl was seeded into the wells of a microtitre plate containing 200 μl MHB and the relevant antibiotics at a final density of 5 x 10^5 c.f.u. ml^{-1}. The microtitre plate was incubated with shaking (180 r.p.m.) at 37°C in a Tecan Infinite 200 Pro multiwell plate reader and optical density measurements were taken at 600 nm every 15 min over 14 h.

**Scanning electron microscope (SEM) imaging**

Stationary-phase bacterial cells were washed and inoculated into 3 ml MHB containing the relevant antibiotics as described above. After 2 h incubation (37°C, 180 r.p.m.), 25 μl of the cultures was spotted onto a silicon chip (University Wafer, USA) and allowed to air-dry. The sample was fixed using glutaraldehyde (2.5% in 0.01 M PBS) for 30 min at room temperature, before the chip was washed three times in PBS (0.01 M). The sample was dehydrated using increasing concentrations of ethanol (10%, 30%, 50%, 70%, 90% and 100%) for 5 min each time, before a coating of 15 nm chromium (Quorum Technologies, UK) was applied. Representative images of bacterial cells were obtained using a LEO Gemini 1525 field emission gun scanning electron microscope (FEG SEM; Carl Zeiss Microscopy GmbH, Germany).
Experiments were performed on at least three independent occasions, and resulting data are presented as the arithmetic mean of these biological repeats, unless stated otherwise. Error bars represent the standard deviation of the mean. For single comparisons, a two-tailed unpaired Student’s t-test was used to analyse the data. For multiple comparisons at a single time point or concentration, data were analysed using either a one-way analysis of variance (ANOVA) for parametric data, or a Kruskal-Wallis test for non-parametric data. Where data were obtained at several different time points or concentrations, a two-way ANOVA was used for statistical analysis. Appropriate post-hoc tests (Dunnett’s, Welch’s, Sidak’s, Holm-Sidak) were carried out to correct for multiple comparisons, with details provided in the figure legends. Asterisks on graphs indicate significant differences between data, and the corresponding p-values are reported in the figure legend. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., USA).

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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Author contributions

A.S. and A.M.E. designed the experiments. A.S., A.K. and M.B. performed experiments. All authors analysed data and contributed to writing of the manuscript.

Competing interests

The authors declare no competing financial interests.
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Figure 1. Colistin-mediated membrane damage causes the release of LPS but not membrane lipid from the surface of *P. aeruginosa*.

a, Diagrammatic representation of the current hypothesised mechanism of action of colistin: colistin binds to LPS on the OM, causing displacement of cations that form bridges between LPS molecules and leading to membrane disruption (1). The antibiotic then crosses the OM via ‘self-directed uptake’ (2). Colistin subsequently disrupts the CM (3), resulting in cell lysis (4) and bacterial death (5). 

b, Relative fluorescence of *P. aeruginosa* PA14 cells incubated with the indicated concentrations of colistin and the phospholipid-reactive membrane impermeant dye NPN (10 µM). Disruption of the LPS monolayer enables NPN to access phospholipids in the inner leaflet of the OM, resulting in fluorescence (n=4; *p*<0.05 compared to untreated bacteria, error bars are omitted for clarity). 

c, Release of periplasmic β-lactamase into the culture supernatant in response to colistin as determined using the chromogenic substrate nitrocefin (250 µM), which produces a product detectable at A$_{490}$nm.
d, Colistin-mediated permeabilisation of the CM of *P. aeruginosa* PA14 as determined by the binding of the membrane impermeant dye propidium iodide (2.5 μM) to DNA, which results in fluorescence (n=3 in duplicate; *p<0.01 compared to untreated cells for all colistin-exposed populations). e, Lysis or growth of *P. aeruginosa* PA14 exposed, or not, to colistin over time as determined by OD$_{595\text{nm}}$ measurements (n=4; *p<0.05 compared to untreated bacteria for all colistin-exposed populations). f, Killing or replication of *P. aeruginosa* PA14 exposed, or not, to colistin over time, as determined by c.f.u. counts (n=4; *p<0.01 compared to untreated cells). g, The concentration of phospholipids released from the surface of *P. aeruginosa* PA14 over time in the absence or presence of colistin, as determined by reactivity with the fluorescent probe FM-4-64 (n=4; *p<0.05 compared to values at 0 hours). h, The presence of LPS in the culture supernatant following exposure of *P. aeruginosa* PA14 to colistin (2 μg ml$^{-1}$), as determined using the Limulus Amebocyte Lysate assay (n=3; *p<0.05 compared to 0 hours). Data in a-g were analysed by a two-way ANOVA with Dunnett’s post-hoc test. Data in h were analysed by a Kruskal-Wallis test with Dunn’s post-hoc test. Data are presented as the arithmetic mean, and error bars, where shown, represent the standard deviation of the mean. r.f.u.: relative fluorescence units; OD: optical density; EU: endotoxin units; OM: outer membrane; CM: cytoplasmic membrane; NPN: *N*-phenyl-1-naphthylamine.
Figure 2. The bactericidal activity of colistin requires *de novo* LPS biosynthesis.

**a,** Survival over time of *P. aeruginosa* PA14 exposed to colistin (4 μg ml⁻¹), the LPS biosynthesis inhibitor CHIR-090 (0.125 μg ml⁻¹), both antibiotics or neither (n=4; *p*<0.01 compared to colistin alone). Values for CHIR-090 alone and untreated overlap. **b,** Survival of *P. aeruginosa* PA14 during exposure to colistin (4 μg ml⁻¹), the LPS biosynthesis inhibitor cerulenin (32 μg ml⁻¹), both antibiotics or neither (n=4; *p*<0.0001 compared to colistin alone). **c,** Survival of *P. aeruginosa* PA14 exposed to colistin (4 μg ml⁻¹) alone for 2 hours followed by no further treatment, or the addition of either CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹) (n=3 in duplicate; *p*<0.05 compared to colistin alone). **d-i,** Representative scanning electron microscopy (SEM) images of *P. aeruginosa* PA14 exposed to colistin (4 μg ml⁻¹) only (**d**), colistin (4 μg ml⁻¹) and CHIR-090 (0.125 μg ml⁻¹) (**e**), colistin (4 μg ml⁻¹) and
cerulenin (32 μg ml⁻¹) (f), in the absence of antibiotics (g), exposed to CHIR-090 (0.125 μg ml⁻¹) only (h), or cerulenin (32 μg ml⁻¹) only (i), for 2 hours in each case (Scale bars: 200 nm). Data in a-c were analysed by a two-way ANOVA with Sidak’s (a, b) or Holm-Sidak’s (c) post-hoc tests. For panels a, b, and c, data are presented as the arithmetic mean, and error bars represent the standard deviation of the mean.
Figure 3. Colistin-mediated LPS release requires *de novo* LPS biosynthesis and enables the antibiotic to access the cytoplasmic membrane.

**a**, OM disruption of *P. aeruginosa* PA14 cells during exposure to colistin (4 μg ml⁻¹) in the absence or presence of CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹) as assessed by fluorescence generated with NPN dye (10 μM) (n=4; no significant differences (p>0.05) between datasets, error bars are omitted for clarity).

**b**, Release of β-lactamase from the periplasm of *P. aeruginosa* PA14 cells exposed to colistin (4 μg ml⁻¹) in the absence or presence of cerulenin (32 μg ml⁻¹) as detected using 250 μM nitrocefin (n=4; *p<0.01 compared to colistin alone).

**c**, Permeabilisation of the CM of *P. aeruginosa* PA14 cells during incubation with colistin (4 μg ml⁻¹) in the absence or presence of CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹), or with a bactericidal concentration of ciprofloxacin (4 μg ml⁻¹), as determined using 2.5 μM propidium iodide (n=3 in duplicate; *p<0.0001 compared to colistin alone for all populations below the asterisks).

**d**, Lysis of *P. aeruginosa* PA14 cells during exposure to colistin (4 μg ml⁻¹) in the absence or presence of CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹) as measured using OD₅₉₅nm readings (n=4; *p<0.05 compared to colistin alone).

**e**, Binding of BoDipy-labelled colistin (5 μg ml⁻¹) to *P. aeruginosa* PA14 cells in the absence or presence of CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹).
cerulenin (32 μg ml\(^{-1}\)) (n=4; *p<0.0001 compared to BoDipy-colistin alone). f, Concentration of LPS in the supernatant of cultures of \textit{P. aeruginosa} PA14 exposed for 4 hours to colistin (4 μg ml\(^{-1}\)) in the absence or presence of CHIR-090 (0.125 μg ml\(^{-1}\)) or cerulenin (32 μg ml\(^{-1}\)) as determined with the Limulus Amebocyte Lysate assay (n=3; *p<0.0001 compared to colistin alone). Data in a-d, f were analysed by a two-way ANOVA with Dunnett’s post-hoc test. Data in e were analysed using a one-way ANOVA with Dunnett’s post-hoc test. Data are presented as the arithmetic mean, and error bars, where shown, represent the standard deviation of the mean. r.f.u.: relative fluorescence units; OD: optical density; EU: endotoxin units; OM: outer membrane; NPN: \(N\)-phenyl-1-naphthylamine; CM: cytoplasmic membrane.
Figure 4. The polymyxin acyl tail is required for damage of both the outer and cytoplasmic membranes.

**a, b,** Permeability of the OM of *P. aeruginosa* PA14 during exposure to 4 μg ml⁻¹ PMB (**a**) or an equivalent molar concentration (2.8 μg ml⁻¹) of PMBN (**b**), in the absence or presence of CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹), as detected using 10 μM NPN dye (n=4; all data with LPS biosynthesis inhibitors not significantly different (p>0.05) compared to PMB or PMBN alone, error bars are omitted for clarity). **c, d,** Release of periplasmic β-lactamase from *P. aeruginosa* PA14 incubated with 4 μg ml⁻¹ PMB (**c**) or 2.8 μg ml⁻¹ PMBN, in the absence or presence of cerulenin (32 μg ml⁻¹), as determined with 250 μM nitrocefin (n=4; *p<0.0001 compared to PMB or PMBN alone). **e, f,** Disruption to the CM of *P. aeruginosa* PA14 caused by 4 μg ml⁻¹ PMB (**e**) or 2.8 μg ml⁻¹ of PMBN (**f**), in
the absence or presence of CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹), quantified using 2.5 μM propidium iodide (n=4; *p<0.0001 compared to PMB or PMBN alone). g, h, Lysis or growth of P. aeruginosa PA14 exposed to 4 μg ml⁻¹ PMB (g) or 2.8 μg ml⁻¹ PMBN (h), in the absence or presence of CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹), as assessed by OD₅₉₅ nm readings (n=4; *p<0.001 compared to PMB or PMBN alone). i, j, Survival of P. aeruginosa PA14 exposed to 4 μg ml⁻¹ PMB (i) or 2.8 μg ml⁻¹ PMBN (j), in the absence or presence of CHIR-090 (0.125 μg ml⁻¹) or cerulenin (32 μg ml⁻¹) (n=4; *p<0.05 compared to PMB or PMBN alone). k, Diagrammatic representation of the proposed mechanism of action of colistin based on the findings described here: colistin binds to LPS on the OM, leading to the displacement of cations that form bridges between LPS molecules and causing minor membrane disruption (1). The weakening of the intermolecular bridges in the LPS monolayer means that as de novo LPS is synthesised and transported to the cell surface, LPS molecules are forced out and released by the bacteria (2). This released LPS sequesters and inactivates free colistin extracellularly (3). Loss of LPS from the cell surface also results in significant damage to the OM via the action of the polymyxin lipid tail (4), and thus facilitates the access of colistin to the periplasm. This enables colistin to bind to the CM (5), which it disrupts, culminating in the loss of cytoplasmic contents, cell lysis and bacterial death (6). Data in a, b, e-j were analysed by a two-way ANOVA with Dunnett’s post-hoc test. Data in c, d were analysed by a two-way ANOVA with Sidak’s post-hoc test. Data are presented as the arithmetic mean, and error bars, where shown, represent the standard deviation of the mean. r.f.u.: relative fluorescence units; OD: optical density; OM: outer membrane; PMB: polymyxin B; PMBN: polymyxin B nonapeptide; NPN: N-phenyl-1-naphthylamine; CM: cytoplasmic membrane.