Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane

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Summary

Colistin is an antibiotic of last resort for infections caused by drug-resistant Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Escherichia coli*. For this reason, high rates of treatment failure and increasing resistance to this antibiotic are very concerning and attempts to resolve these issues are hampered by a poor understanding of colistin's mode of action. Whilst it is well established that colistin binds to lipopolysaccharide in the bacterial outer membrane, it was unclear how this led to bacterial killing. Here, we show that colistin also targets lipopolysaccharide in the cytoplasmic membrane and that this interaction is essential for cytoplasmic membrane permeabilisation, cell lysis and the bactericidal activity of the antibiotic. We also found that MCR-1-mediated colistin resistance confers protection against the antibiotic via the presence of modified lipopolysaccharide within the cytoplasmic membrane, rather than the outer membrane. These findings reveal key details about the mechanism by which colistin kills bacteria, providing the foundations for the development of new approaches to enhance therapeutic outcomes.

62 **Introduction**

63 The emergence of multi-drug-resistant Gram-negative pathogens such as *Pseudomonas aeruginosa*,
64 *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* has necessitated the use of polymyxin
65 antibiotics, which are often the only viable last-resort therapeutic option [1-4].

66 Two closely related polymyxin antibiotics are used clinically, colistin (polymyxin E) and polymyxin B, which
67 share a high degree of structural similarity, consisting of a cationic peptide ring of 7 amino acids connected
68 to a hydrophobic acyl tail by a linear chain of 3 amino acids [1,3]. Polymyxins target lipopolysaccharide (LPS)
69 in the outer membrane (OM) of Gram-negative bacteria via a two-step process (Supplementary Fig. 1). Firstly,
70 the positively-charged peptide ring binds to the anionic lipid A moiety of LPS, resulting in the disruption of
71 cation bridges that stabilise the outer leaflet of the OM. Secondly, the acyl tail of the antibiotic associates
72 with the fatty acid tails of lipid A, causing further OM disruption [1].

73 Polymyxins are rapidly bactericidal towards Gram-negative bacteria *in vitro* but are considerably less
74 efficacious *in vivo*, with up to 70% of patients infected by Gram-negative pathogens failing to respond to
75 colistin treatment [5-7]. This may be partly due to the nephrotoxicity associated with polymyxins, which
76 severely limits the dose that can be used [8]. Whilst polymyxins have a clinical breakpoint of 2 $\mu g\ ml^{-1}$, the
77 peak serum concentration of these drugs is typically only 1-4 $\mu g\ ml^{-1}$, which means that even small changes
78 in a pathogen's susceptibility can confer resistance [8].

79 Colistin resistance is a growing threat and typically arises via chromosomal mutations or the acquisition of
80 mobilized colistin resistance (*mcr*) genes [9]. Chromosomally mediated colistin resistance can occur via
81 several different pathways, most of which result in the addition of cationic moieties to the normally
82 negatively charged lipid A [9]. This is thought to result in electrostatic repulsion of the positively-charged
83 polymyxins away from the bacterial surface [10]. By contrast, MCR-mediated resistance is acquired via a
84 plasmid-borne copy of an *mcr* gene, of which 9 homologues have been described to date, following the first
85 report of *mcr*-1 in late 2015 [11,12]. The presence of *mcr* genes on plasmids has become a major cause for
86 concern, with reports of MCR-mediated resistance occurring in several important pathogens including *E. coli*,
87 *Salmonella enterica* and *K. pneumoniae* [13,14]. MCR-1 is thought to be the most prevalent MCR enzyme,
88 having been identified in bacteria isolated from humans, livestock and the environment in Asia, Africa,
89 Europe and North America [14]. Colistin resistance is conferred by the phosphoethanolamine transferase
90 activity of MCR-1, which adds a cationic phosphoethanolamine moiety to the anionic lipid A domain of LPS,
91 reducing its net negative charge and thus decreasing its affinity for the polymyxin antibiotic [11,14].

92 Whilst most colistin resistance occurs via LPS modifications, an alternative mechanism has been observed in
93 some strains of *A. baumannii*, which involves the total loss of LPS from the OM [15]. However, this
94 phenomenon appears to be limited to certain species since other major pathogens such as *P. aeruginosa*, *E.*
95 *coli* and *K. pneumoniae* cannot tolerate the absence of LPS from the OM [16].

96 Despite the growing importance of polymyxin antibiotics, there are significant gaps in our understanding of
97 their mode of action. Although it is established that the binding of polymyxins to LPS on the surface of Gram-
98 negative bacteria leads to disruption of the OM, it is not known how this results in cell lysis and bacterial
99 death (Supplementary Fig. 1) [3,17]. It is hypothesised that damage to the LPS monolayer enables polymyxins
100 to traverse the OM via a process of 'self-directed uptake', although this has not been demonstrated
101 experimentally [17,18]. Once they have crossed the OM, polymyxins permeabilise the cytoplasmic
102 membrane (CM), which is required for bacterial lysis and killing [1-3]. However, the mechanism by which
103 colistin damages the CM is unclear [17]. It has been proposed that the surfactant activity of polymyxins is
104 sufficient to compromise the phospholipid bilayer of the CM via a detergent-like effect [1,3]. In support of
105 this, polymyxins can interact with mammalian cell membranes, leading to changes in epithelial monolayer
106 permeability and also have some activity against the Gram-positive bacterium *Streptococcus pyogenes*,
107 where the CM is formed of a phospholipid bilayer [19,20].

108 However, several lines of evidence call into doubt the ability of physiologically relevant concentrations of
109 polymyxins to disrupt phospholipid bilayers. Firstly, the concentration of polymyxin B required to disrupt
110 mammalian epithelial cells is 30-fold greater than that needed to kill Gram-negative bacteria [19].
111 Additionally, the concentrations of colistin required to inhibit the growth of *S. pyogenes* (4-16 µg ml^{-1}) are
112 above typical serum concentrations of the antibiotic, and across a range of clinically-relevant concentrations
113 (0.25-4 µg ml^{-1}), colistin displayed no activity against a panel of Gram-positive organisms, including
114 *Staphylococcus aureus* and *Lactococcus lactis* (Supplementary Fig. 2) [8,20]. Furthermore, as described
115 above, polymyxins are 30-100-fold less active against colistin-resistant *A. baumanii* isolates that are LPS-
116 deficient, with an OM composed of a phospholipid bilayer [15,21,22]. Together, these observations led us to
117 question whether, at physiologically relevant concentrations, colistin disrupted the CM of Gram-negative
118 bacteria via the engagement of the polymyxin with membrane phospholipids.

119 Studies with model membranes have found that the presence of LPS in a phospholipid bilayer, even at very
120 low concentrations (3% of total membrane composition), significantly increased the susceptibility of
121 membranes to colistin-mediated permeabilisation [23]. In Gram-negative bacteria, LPS is synthesised in the
122 cytoplasm via the Raetz pathway, during which it is introduced into the inner leaflet of the CM [24,25]. It is
123 then flipped to the outer leaflet of the CM by MsbA before transportation to the OM via the LptABCDEFG
124 machinery [24-28]. Therefore, LPS is present in the CM in relatively large quantities, en route to the OM [24-
125 30]. Given the high affinity of polymyxin antibiotics for LPS, and that the presence of LPS in membranes
126 markedly sensitises them to polymyxins, we hypothesised that colistin targets LPS in the CM, enabling
127 efficient bacterial lysis and death at clinically relevant concentrations of the drug.

128

129

130 **Results**

131 **Colistin targets LPS in the cytoplasmic membrane**

132 LPS biosynthesis and transport are essential processes for most Gram-negative bacteria [16]. Therefore, to
133 investigate whether LPS present in the CM was a target for colistin, we attempted to reduce the amount of
134 LPS in the CM by partially inhibiting LPS biosynthesis in the cytoplasm of *P. aeruginosa* PA14 using sub-lethal
135 concentrations of the LpxC inhibitor CHIR090, or the fatty acid biosynthesis inhibitor cerulenin
136 (Supplementary Tables 1, 2) [16,31,32]. By generating spheroplasts of *P. aeruginosa* PA14, LPS levels in both
137 the CM and OM were quantified using the well-established and highly specific Limulus Amebocyte Lysate
138 (LAL) assay (Supplementary Fig. 3, 4, 5) [33]. Additionally, the amount of LPS in the CM and OM of *P.*
139 *aeruginosa* spheroplasts or whole cells was measured using fluorescent BoDipy-labelled polymyxin B
140 nonapeptide (PMBN) as a non-toxic probe for surface LPS (Supplementary Fig. 6) [34].

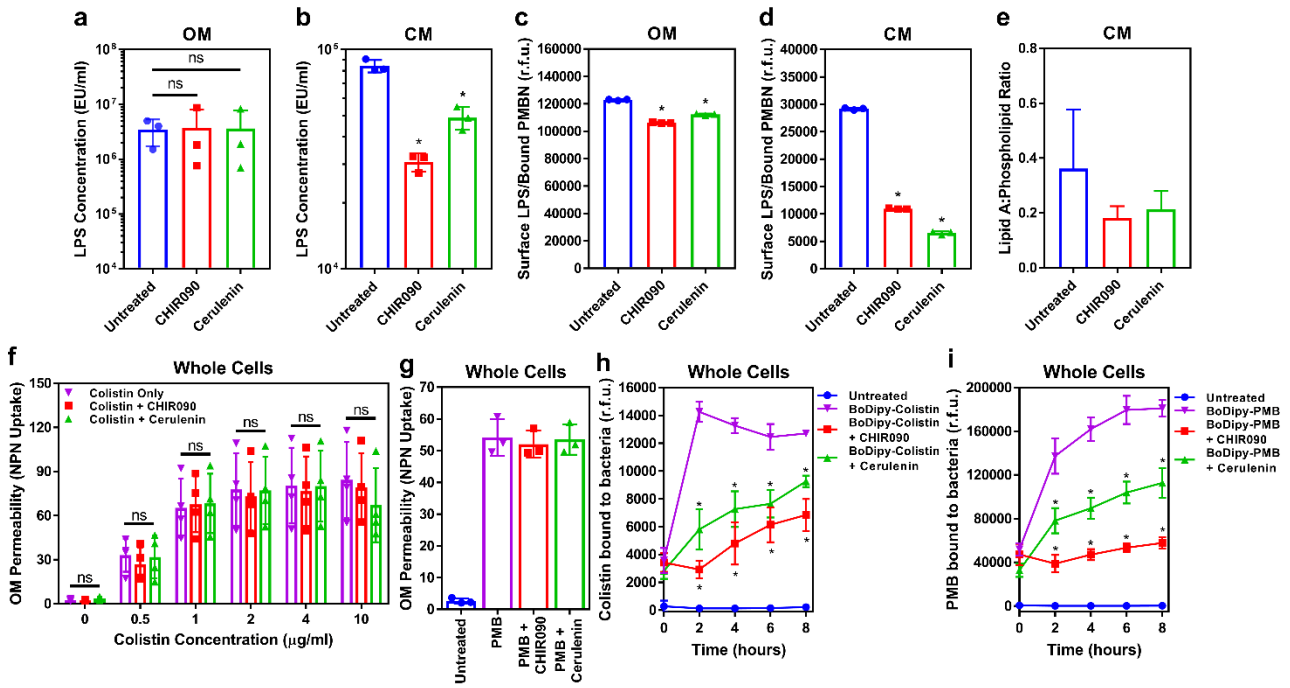
141 Using the LAL assay, we found that approximately 2.5% of total cellular LPS was present in the CM of
142 untreated *P. aeruginosa* cells, with the remaining 97.5% found in the OM (Fig. 1a,b). These findings are similar
143 to that reported for *E. coli,* where 4.3% of total cellular LPS was present in the CM, and slightly lower than
144 what was been found in *Salmonella typhimurium* (~10% total LPS present in the CM) [29,30]. When *P.*
145 *aeruginosa* was exposed to sub-lethal concentrations of CHIR090 or cerulenin, the levels of LPS in the CM
146 decreased ~2-fold, but there was no significant effect on LPS levels in the OM, and only a minimal effect on
147 the growth profile of bacteria (Fig. 1a,b, Supplementary Fig. 5). We found very similar results using BoDipy-
148 tagged PMBN, with a ~3-fold reduction in the LPS content of the CM in *P. aeruginosa* spheroplasts exposed
149 to CHIR090 or cerulenin relative to untreated cells, but only a very minor reduction in LPS levels in the OM
150 (Fig. 1c,d, Supplementary Fig. 6-9). To confirm these findings, we used MALDI-TOF-based lipidomics to
151 quantify the amount of LPS in the CM of *P. aeruginosa* spheroplasts [35]. This demonstrated identical results
152 to those obtained using the LAL assay, providing additional confidence that we were able to reduce LPS
153 abundance in the CM by ~2-fold with CHIR090 or cerulenin (Fig. 1e, Supplementary Fig. 10).

154 To test whether CHIR090 or cerulenin had any effect on the interaction of colistin or polymyxin B with LPS in
155 the OM of *P. aeruginosa* cells, we used the *N*-phenyl-1-napthylamine (NPN) fluorophore to measure OM
156 permeablisation caused by the polymyxin antibiotics [36]. This revealed that both colistin, across a range of
157 concentrations, and polymyxin B caused immediate and equal disruption of the OM of untreated bacteria
158 and those exposed to CHIR090 or cerulenin, further confirming that the LPS biosynthesis inhibitors did not
159 modulate the levels of LPS in the OM (Fig. 1f,g, Supplementary Fig. 11).

160 To further investigate the interaction of polymyxin antibiotics with *P. aeruginosa*, we measured the binding
161 of BoDipy-labelled colistin or BoDipy-polymyxin B to whole *P. aeruginosa* cells exposed, or not, to CHIR090
162 or cerulenin (Supplementary Fig. 6-9). In the absence of the inhibitors of LPS biosynthesis, there was a rapid
163 and high level of binding of the fluorescently-labelled polymyxin antibiotics to bacteria (Fig. 1h,i). By contrast,

164 when LPS was depleted from the CM using CHIR090 or cerulenin, the binding of BoDipy-colistin or BoDipy-
165 polymyxin B was significantly reduced (Fig. 1h,i).

166 Together, these findings demonstrated that we could reduce LPS abundance in the CM without affecting LPS
167 levels in the OM, and that the amount of LPS in the CM influenced the degree to which the polymyxin
168 antibiotics targeted bacterial cells.



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170 **Figure 1. Depletion of LPS from the cytoplasmic membrane of *P. aeruginosa* reduces colistin binding to cells. a, b,**
171 Quantification of LPS levels in the OM **(a)** and CM **(b)** of *P. aeruginosa* PA14 during conversion to spheroplasts from
172 whole cells pre-exposed, or not, to the LPS biosynthesis inhibitors CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for
173 2 h, as determined using the Limulus Amebocyte Lysate assay (n=3; ns: p>0.05, *p<0.001 compared to Untreated
174 conditions). **c, d,** Quantification of amount of LPS in the OM of *P. aeruginosa* PA14 whole cells **(c)** or in the CM of *P.*
175 *aeruginosa* PA14 spheroplasts **(d)** exposed, or not, to CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for 2 h, as
176 determined by fluorescence of BoDipy-PMBN (7 µg ml^{-1}) bound to the cell surface (n=3; *p<0.0001 compared to
177 Untreated conditions). **e,** Quantification of the ratio of lipid A to the membrane phospholipid phosphatidylglycerol (34:2)
178 in the CM of *P. aeruginosa* PA14 spheroplasts exposed, or not, to CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for 2
179 h, as determined by MALDI-TOF-based lipidomics (n=3 in duplicate). **f, g,** OM disruption of *P. aeruginosa* PA14 cells
180 during 10 minutes of exposure to the indicated range of colistin concentrations **(f)** or polymyxin B (PMB, 4 µg ml^{-1}) **(g)**,
181 in the absence or presence of CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), as assessed by uptake of the fluorescent
182 dye NPN (10 µM) (n=3, each data point represents the arithmetic mean of 20 replicate measurements; no significant
183 differences (p>0.05) between polymyxin treated bacteria). **h, i,** Binding of fluorescent BoDipy-labelled colistin (5 µg ml^{-}
184 ^{1}) **(h)** or BoDipy-PMB (10 µg ml^{-1}) **(i)** to *P. aeruginosa* PA14 cells in the absence or presence of CHIR090 (0.125 µg ml^{-1})
185 or cerulenin (32 µg ml^{-1}) (n=4; *p<0.0001 compared to BoDipy-labelled antibiotics alone). Data in **a-e** were analysed by
186 a one-way ANOVA with Dunnett's post-hoc test. Data in **f, h, i** were analysed by a two-way ANOVA with Dunnett's post-
187 hoc test. Data in **g** were analysed by a one-way ANOVA with Tukey's post-hoc test. Data are presented as the arithmetic

188 mean, and error bars represent the standard deviation of the mean. OM: outer membrane; CM: cytoplasmic membrane;
189 NPN: *N*-phenyl-1-napthylamine; r.f.u.: relative fluorescence units; PMB: polymyxin B; PMBN polymyxin B nonapeptide.

190

191

192 **The bactericidal activity of colistin is dependent upon the abundance of LPS in the cytoplasmic membrane**

193 Having demonstrated that colistin and polymyxin B target LPS in the CM, we wanted to understand whether
194 this interaction was needed for the bactericidal activity of the antibiotics. To do this, we reduced LPS levels
195 in the CM of *P. aeruginosa* PA14 using CHIR090 or cerulenin and measured bacterial survival over time during
196 exposure to the polymyxins. As expected, colistin alone caused a rapid and substantial drop in c.f.u. counts
197 of bacteria, with >10,000-fold reduction in survival within 4 h (Fig. 2a,b). By contrast, there was almost no
198 decrease in c.f.u. counts over 8 h when *P. aeruginosa* was exposed to colistin in the presence of CHIR090 or
199 cerulenin to reduce LPS abundance in the CM (Fig. 2a,b). These protective effects of CHIR090 and cerulenin
200 were apparent across multiple colistin concentrations and, whilst at the highest concentration tested (10 µg
201 ml^{-1}) a small decrease in cell viability was observed, this still represented a 1000-fold increased level of
202 survival (Supplementary Fig. 12). Furthermore, the ability to protect against killing by colistin was also
203 dependent on the dose of CHIR090 and cerulenin, with decreased survival of *P. aeruginosa* cells at lower
204 concentrations of the LPS biosynthesis inhibitors (Supplementary Fig. 13).

205 Reducing LPS in the CM with CHIR090 or cerulenin also prevented polymyxin B from exerting its bactericidal
206 effects, with >1000-fold increased survival of *P. aeruginosa* in the presence of either inhibitor compared to
207 bacteria exposed to polymyxin B alone (Fig. 2c). The protective effects of CHIR090 and cerulenin could also
208 be observed even after bacteria were first exposed to colistin. After 2 h incubation with colistin, c.f.u. counts
209 of *P. aeruginosa* dropped approximately 500-fold compared to the starting inoculum (Fig. 2d). In the absence
210 of CHIR090 or cerulenin, killing continued with an additional 1000-fold decrease in survival over the next 4 h
211 (Fig. 2d). However, the addition of cerulenin after 2 h completely halted bacterial killing, whilst the
212 introduction of CHIR090 reduced the rate of killing significantly (Fig. 2d). Therefore, reduction of LPS levels
213 in the CM significantly inhibited the bactericidal activity of colistin against *P. aeruginosa*, even after the
214 antibiotic had bound to cells.

215 Since perturbation of LPS biosynthesis by CHIR090 or cerulenin may have triggered stress responses
216 activating the PmrAB or PhoPQ two-component systems that modulate the susceptibility of *P. aeruginosa* to
217 colistin, we repeated the killing experiments using transposon mutants with disrupted copies of *pmrA*, *pmrB*,
218 *phoP* or *phoQ* [37,38]. As expected from previous work, the *phoQ*-disrupted mutant was less susceptible to
219 colistin than the WT strain (Supplementary Fig. 14) [39]. However, the presence of CHIR090 or cerulenin
220 significantly reduced the killing of all four mutants by colistin, indicating that reduction of LPS levels in the
221 CM, rather than the induction of stress responses and modification of LPS in the OM, was responsible for the

222 protection conferred by the LPS biosynthesis inhibitors against colistin (Supplementary Fig. 14). Moreover,
223 neither CHIR090 nor cerulenin had any effect on the ability of the bactericidal agents ciprofloxacin,
224 gentamicin, imipenem, chlorhexidine or ethylenediaminetetraacetic acid (EDTA) to kill *P. aeruginosa*,
225 providing further evidence that the effects of reducing LPS biosynthesis were specific to polymyxins, and not
226 a consequence of general stress responses reducing antibiotic susceptibility (Supplementary Fig. 15).

227 Since growth rate can affect the bactericidal activity of antibiotics, it was possible that the protection
228 provided by CHIR090 or cerulenin against colistin-mediated killing was due to their slight inhibitory effects
229 on cell replication (Supplementary Fig. 5) [40,41]. However, a range of completely or partially growth-
230 inhibitory concentrations of the bacteriostatic antibiotics tetracycline or chloramphenicol had almost no
231 effect on colistin-mediated killing, confirming that alterations in growth rate due to the presence of CHIR090
232 or cerulenin were not the basis of their protective effects against polymyxins (Supplementary Fig. 16).
233 Additionally, we confirmed using checkerboard broth microdilution assays that neither CHIR090 nor
234 cerulenin displayed synergy with colistin, suggesting that CHIR090 and cerulenin did not have enhanced
235 growth-inhibitory effects in combination with the polymyxin antibiotic (Supplementary Fig. 17). This also
236 revealed that the inhibitors of LPS biosynthesis did not directly interfere with colistin, for example by binding
237 to the polymyxin (Supplementary Fig. 17).

238 To confirm that the protective effects of CHIR090 were only due to its inhibition of LPS biosynthesis, we
239 isolated spontaneous mutants that were resistant to the LpxC inhibitor. These mutants were fully sensitive
240 to colistin but were not protected by CHIR090, demonstrating that the inhibitor was not blocking the
241 bactericidal effects of the polymyxin antibiotic via off-target effects (Supplementary Fig. 18). Next, we tested
242 whether either CHIR090 or cerulenin triggered *P. aeruginosa* to release outer membrane vesicles (OMVs)
243 that could inactivate colistin extracellularly by measuring the concentration of membrane phospholipids and
244 LPS in the culture supernatant [42]. Exposure of bacteria to the LPS biosynthesis inhibitors in combination
245 with colistin resulted in a decrease in OMVs shed from the cell surface, with higher levels of colistin activity
246 maintained in the supernatant in the presence of CHIR090 or cerulenin (Supplementary Fig. 19, 20). From
247 these experiments, we concluded that the protection conferred by CHIR090 and cerulenin against killing of
248 *P. aeruginosa* by polymyxins was specifically due to their effects on reducing LPS abundance in the CM.

249 To determine whether the protective effects of CHIR090 or cerulenin also applied to other Gram-negative
250 pathogens, we exposed clinical isolates of *E. coli* and *K. pneumoniae*, as well as *P. aeruginosa*, to colistin in
251 the absence or presence of sub-lethal concentrations of the LPS biosynthesis inhibitors. As observed for *P.*
252 *aeruginosa* PA14, there was a large and rapid drop in c.f.u. counts of all strains exposed to colistin alone, but
253 the presence of either CHIR090 or cerulenin significantly reduced the rate of killing (Supplementary Fig. 21).
254 Similar to what was observed for cerulenin, we also found that a sub-lethal concentration of the FabI inhibitor
255 triclosan protected *K. pneumoniae* from the bactericidal effects of colistin (Supplementary Fig. 22).

Combined, these data suggested that colistin has a broadly conserved mechanism of action against Gram-negative bacteria.

Next, we used scanning electron microscopy (SEM) to examine the morphology of *P. aeruginosa* cells exposed to colistin in the absence or presence of either CHIR090 or cerulenin. As expected, in the absence of antibiotics, bacteria had a typical rod-like appearance, whilst cells exposed to colistin had a greatly altered morphology, with a distorted and deflated appearance (Fig. 2e,f). However, cells exposed to colistin in the presence of CHIR090 or cerulenin had an almost normal appearance, underlining the protective effects of reducing LPS levels in the CM (Fig. 2g,h, Supplementary Fig. 23, 24). In summary, these findings revealed that the bactericidal activity of polymyxin antibiotics was dependent upon the abundance of LPS in the CM of clinically important pathogens.

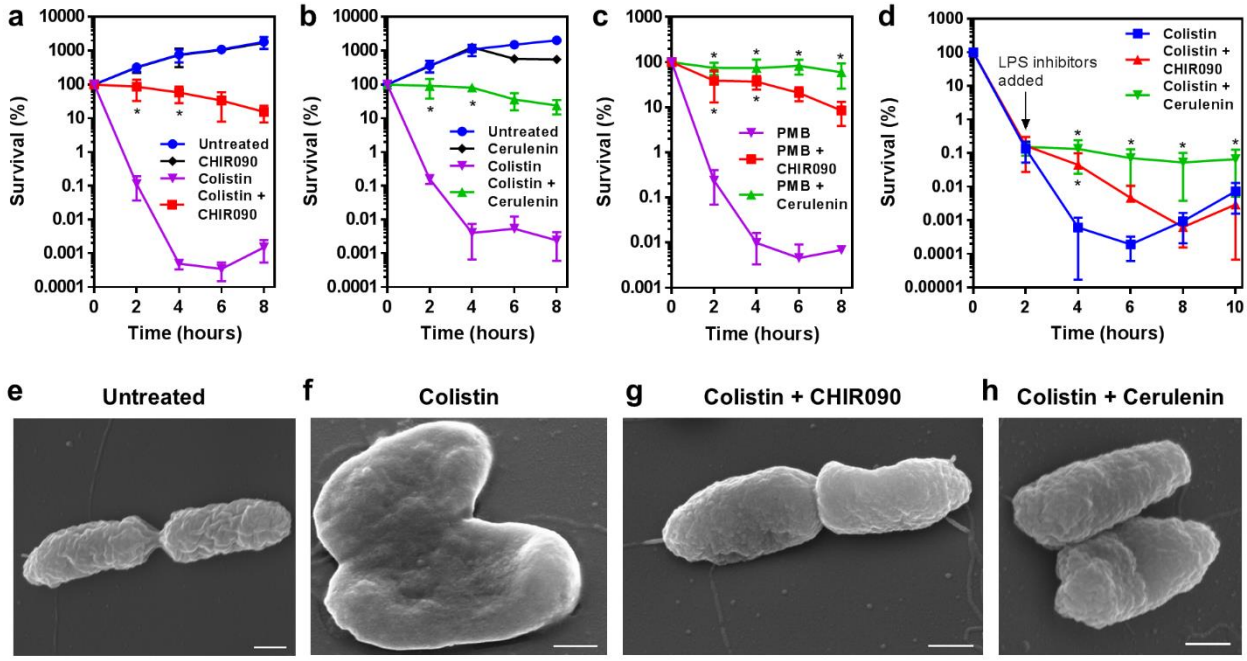


Figure 2. Depletion of LPS from the cytoplasmic membrane protects *P. aeruginosa* from colistin. a, Survival of *P. aeruginosa* PA14 cells exposed to colistin (4 µg ml^{-1}), the LPS biosynthesis inhibitor CHIR090 (0.125 µg ml^{-1}), both antibiotics, or neither (n=4; *p<0.01 compared to Colistin alone). Values for CHIR090 alone and Untreated overlap. **b,** Survival of *P. aeruginosa* PA14 cells 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 cells during exposure to polymyxin B (PMB, 4 µg ml^{-1}) alone, or in combination with CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) (n=4; *p<0.05 compared to PMB alone). **d,** Survival of *P. aeruginosa* PA14 cells exposed to colistin (4 µg ml^{-1}) alone for 2 h, followed by no further treatment, or the addition of either CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) (n=3; *p<0.05 compared to Colistin alone). **e-h,** Representative scanning electron microscopy (SEM) images of *P. aeruginosa* PA14 cells incubated in broth only **(e)**, broth containing colistin (4 µg ml^{-1}) alone **(f)**, colistin (4 µg ml^{-1}) and CHIR090 (0.125 µg ml^{-1}) **(g)**, or colistin (4 µg ml^{-1}) and cerulenin (32 µg ml^{-1}) **(h)**, for 2 h in each case (Scale bars: 200 nm). Data in **a-d** were analysed by a two-way ANOVA with Sidak's **(a, b)**, Dunnett's **(c)** or Holm-Sidak's **(d)** post-hoc tests. Data are presented as the arithmetic mean, and error bars represent the standard deviation of the mean.

280 **Colistin-mediated lysis is dependent on the presence of LPS in the cytoplasmic membrane**

281 Having established that the bactericidal activity of colistin was reduced when the abundance of LPS in the
282 CM was lowered, we wanted to determine whether this was due to colistin-mediated CM damage. To do
283 this, we first examined how variations in the amount of LPS in the CM of whole *P. aeruginosa* cells affected
284 permeabilisation of the CM by polymyxin antibiotics. As expected, exposure of bacteria to colistin or
285 polymyxin B alone caused disruption of the CM, resulting in a significant signal from the DNA-binding
286 fluorophore propidium iodide (PI) (Fig. 3a,b, Supplementary Fig. 25) [43]. By contrast, when LPS levels were
287 reduced in the CM using CHIR090 or cerulenin, colistin exposure triggered only a weak PI signal, indicating
288 that the CM remained intact (Fig. 3a,b). Similar results were seen with clinical isolates of *P. aeruginosa*, *K.*
289 *pneumoniae* and *E. coli,* with CHIR090 significantly reducing colistin-mediated permeabilisation of the CM
290 (Supplementary Fig. 26).

291 To confirm that these data arose from the reduced levels of LPS in the CM, we produced spheroplasts from
292 *P. aeruginosa* cells that had been exposed, or not, to CHIR090 or cerulenin. In the absence of any antibiotics,
293 there was a gradual loss of integrity of the spheroplast CM, as determined by the signal from PI staining (Fig.
294 3c). By contrast, challenging the spheroplasts with colistin resulted in a high level of fluorescence, indicative
295 of severe CM permeabilisation (Fig. 3c). However, when spheroplasts from bacteria pre-treated with CHIR090
296 or cerulenin to lower the abundance of LPS in the CM were exposed to colistin, the signal from PI was similar
297 to that seen in the absence of the polymyxin antibiotic (Fig. 3c). Therefore, the ability of colistin to disrupt
298 the CM of *P. aeruginosa* was directly related to the abundance of LPS in the CM.

299 To test whether the relationship between LPS levels in the CM and polymyxin-mediated CM disruption
300 extended to bacterial lysis, we measured the optical density (OD_{595nm}) of bacterial suspensions exposed to
301 colistin or polymyxin B in the absence or presence of CHIR090 or cerulenin. Whilst colistin alone caused lysis
302 of *P. aeruginosa*, with a steady decrease in OD_{595nm} readings observed, this was completely blocked in the
303 presence of CHIR090 or cerulenin (Fig. 3d). Almost identical data were produced when bacteria were
304 challenged with polymyxin B, or with *P. aeruginosa* spheroplasts exposed to colistin (Fig. 3e,f). In all cases,
305 when LPS levels in the CM were reduced using CHIR090 or cerulenin, there was no detectable cell lysis
306 triggered by the polymyxin antibiotics (Fig. 3e,f). These findings were then extended to clinical isolates of
307 Gram-negative pathogens, with CHIR090 significantly reducing lysis induced by colistin in *P. aeruginosa*, *E.*
308 *coli* and *K. pneumoniae* strains (Supplementary Fig. 27). Taken together, these data demonstrate that
309 polymyxin-mediated CM disruption and lysis were dependent on the abundance of LPS in the CM.

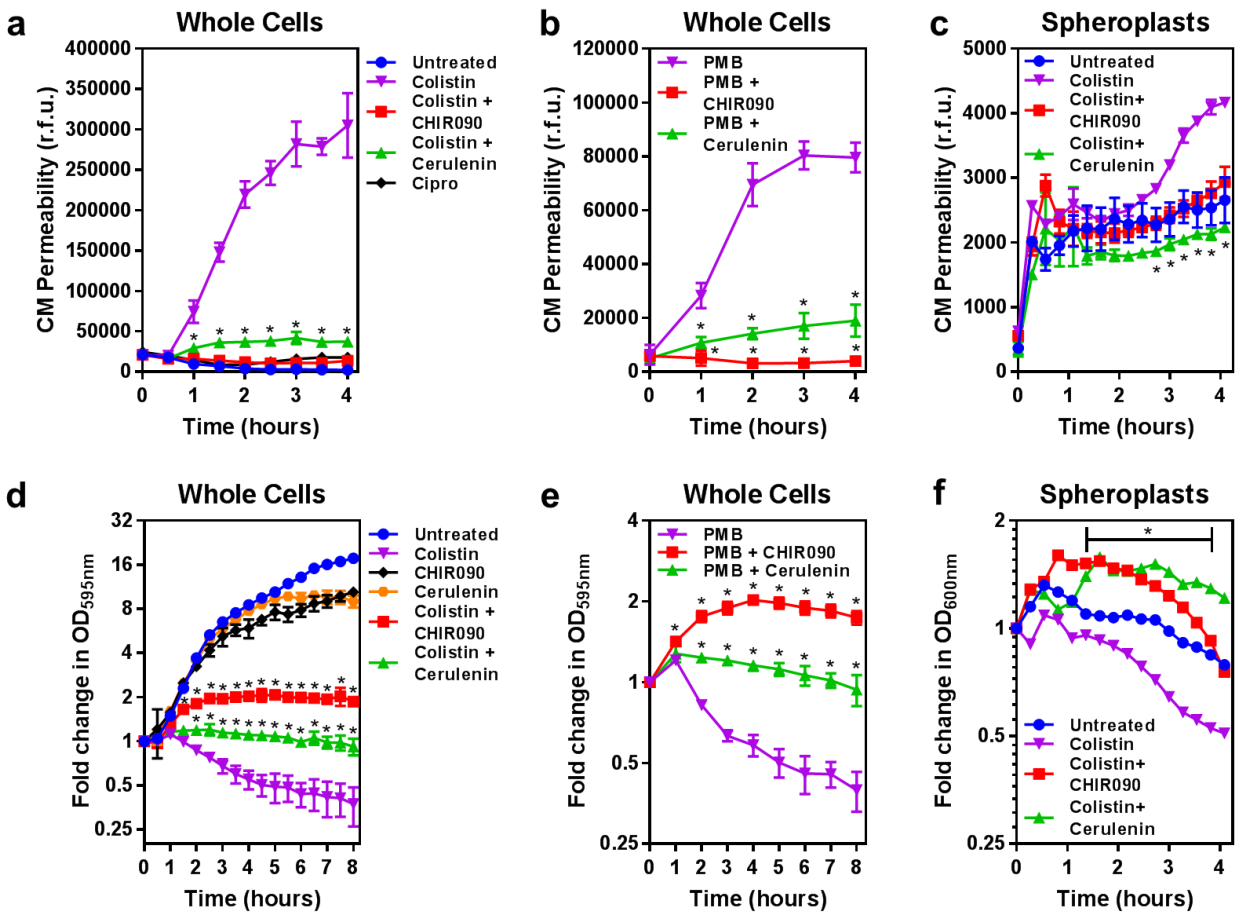


Figure 3. Depletion of LPS from the cytoplasmic membrane prevents colistin-mediated lysis of *P. aeruginosa*. a, Disruption of the CM of *P. aeruginosa* PA14 cells during incubation with colistin (4 µg ml^{-1}) in the absence or presence of CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), or with a bactericidal concentration of ciprofloxacin (4 µg ml^{-1}) as a negative control, as determined using 2.5 µM propidium iodide (PI) (n=3 in duplicate; *p<0.0001 compared to Colistin alone for all populations below the asterisks). **b,** CM disruption of *P. aeruginosa* PA14 cells exposed to polymyxin B (PMB, 4 µg ml^{-1}) in the absence or presence of CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), as determined using 2.5 µM PI (n=4; *p<0.0001 compared to PMB alone). **c,** Permeabilisation of the CM of *P. aeruginosa* PA14 spheroplasts by colistin (4 µg ml^{-1}) following exposure, or not, of bacteria to CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for 2 h prior to conversion to spheroplasts, as determined using 0.25 µM PI (n=3, experiment performed on 3 independent occasions; *p<0.05 for CHIR090 and cerulenin pre-treated spheroplasts compared to spheroplasts exposed only to colistin). **d, e,** Lysis of *P. aeruginosa* PA14 cells during exposure to colistin (4 µg ml^{-1}) **(d)** or PMB (4 µg ml^{-1}) **(e)** in the absence or presence of CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}), as measured using OD_{595nm} readings (n=4; *p<0.05 compared to Colistin/PMB alone). **f,** Lysis of *P. aeruginosa* PA14 spheroplasts in response to colistin (4 µg ml^{-1}) following exposure, or not, of bacteria to CHIR090 (0.125 µg ml^{-1}) or cerulenin (32 µg ml^{-1}) for 2 h prior to conversion to spheroplasts, measured by OD_{600nm} readings (n=3, experiment performed on 3 independent occasions; *p<0.05 for CHIR090 and cerulenin pre-treated spheroplasts compared to spheroplasts exposed only to colistin, error bars are omitted for clarity). Data in **a-f** were analysed by a two-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. CM: cytoplasmic membrane; r.f.u.: relative fluorescence units; OD: optical density; PMB: polymyxin B.

330 **LPS accumulation in the cytoplasmic membrane sensitises bacteria to colistin**

331 Having shown that reducing the LPS content of the CM reduced bacterial susceptibility to colistin, we
332 hypothesised that an increase in LPS levels in the CM would increase susceptibility to the polymyxin
333 antibiotic. Murepavadin is a peptide-based inhibitor of the LptD component of the LptABCDEFG complex in
334 *P. aeruginosa* that transports LPS from the CM to the OM [44]. Previous work has found that inhibition of any
335 one protein in the Lpt machinery leads to a total loss of function of the LPS transport bridge, preventing LPS
336 from being exported out of the CM [45]. Thus, as expected, partial inhibition of the Lpt system in *P.*
337 *aeruginosa* PA14 cells using a sub-lethal concentration of murepavadin resulted in an approximately 30%
338 increase in LPS levels in the CM, without significant changes to LPS abundance in the OM, as determined
339 using the LAL assay, the BoDipy-PMBN fluorescent probe and MALDI-TOF-based lipidomics (Fig. 4a,b,c,d,e,
340 Supplementary Fig. 28).

341 Next, we investigated how murepavadin exposure affected the ability of colistin to disrupt the OM and CM
342 of *P. aeruginosa*. We found that murepavadin had no impact on colistin-mediated disruption of the OM using
343 the NPN fluorophore (Fig. 4f). However, murepavadin significantly enhanced permeabilisation of the CM by
344 colistin in both spheroplasts and whole cells of *P. aeruginosa*, as determined via PI uptake assays (Fig. 4g,h).
345 Identical observations were made when measuring cell lysis with OD readings, with murepavadin exposure
346 causing a marked increase in colistin-induced lysis of *P. aeruginosa* whole cells and spheroplasts (Fig. 4i,j).

347 Subsequently, we demonstrated that a sub-lethal concentration of murepavadin sensitised *P. aeruginosa* to
348 a normally sub-lethal concentration of colistin (2 µg ml^{-1}), resulting in >10,000-fold reduction in c.f.u. counts
349 relative to bacteria incubated with murepavadin or colistin alone after 8 h (Fig. 4k). We also found that
350 murepavadin potentiated the activity of even lower concentrations of colistin (1 µg ml^{-1}), with exposure to
351 the LPS transport inhibitor increasing the ability of the polymyxin antibiotic to damage the CM, triggering
352 bacterial lysis and cell death (Supplementary Fig. 29). Synergy between murepavadin and colistin was
353 similarly apparent in a checkerboard MIC assay (Fig. 4l). We then confirmed that these findings applied to
354 clinical *P. aeruginosa* isolates, where in every case, sub-lethal murepavadin concentrations enhanced the
355 bactericidal effects of colistin, with significantly more CM disruption, cell lysis and killing by the polymyxin
356 (Supplementary Fig. 30).

357 Combined, these data provide additional evidence that LPS levels in the CM modulate the bactericidal and
358 lytic activity of colistin by showing that an increase in the abundance of LPS in the CM sensitised *P. aeruginosa*
359 PA14 to the polymyxin antibiotic. Our data also indicate that the simultaneous use of murepavadin and
360 polymyxin may provide an effective novel combination therapy that limits the toxic side effects associated
361 with both compounds [46].

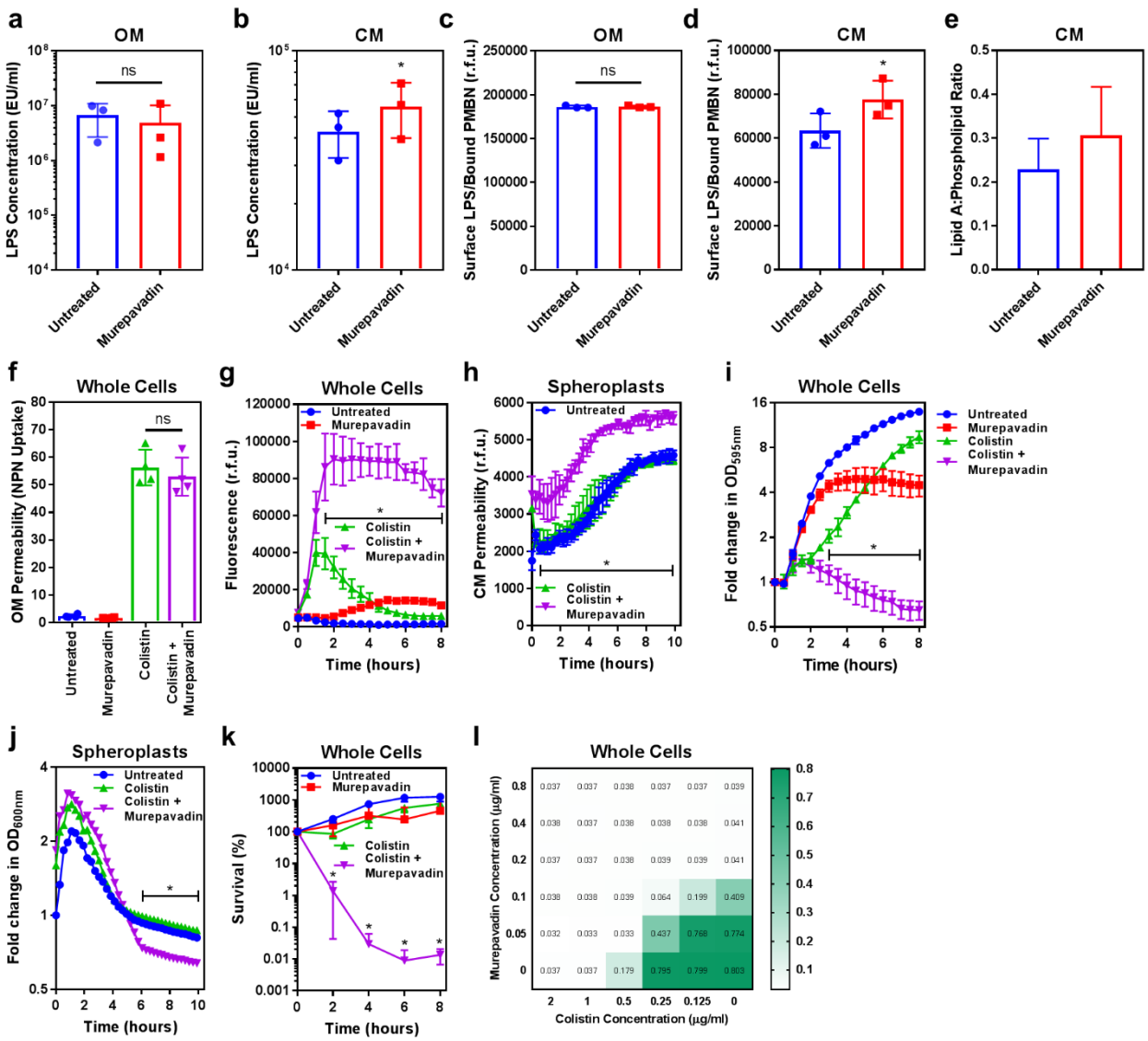


Figure 4. Murepavadin sensitises *P. aeruginosa* to colistin by increasing LPS abundance in the cytoplasmic membrane. **a, b,** Quantification of LPS levels in the OM **(a)** and CM **(b)** of *P. aeruginosa* PA14 during conversion to spheroplasts from whole cells pre-exposed, or not, to the LPS transport inhibitor murepavadin (0.05 µg ml^{-1}) for 2 h, as determined using the Limulus Amebocyte Lysate assay (n=3; ns: p>0.05, *p<0.05 compared to Untreated conditions). **c, d,** Quantification of amount of LPS in the OM of *P. aeruginosa* PA14 whole cells **(c)** or in the CM of *P. aeruginosa* PA14 spheroplasts **(d)** exposed, or not, to murepavadin (0.05 µg ml^{-1}) for 2 h, as determined by fluorescence of BoDipy-PMBN (7 µg ml^{-1}) bound to the cell surface (n=3; *p<0.001 compared to Untreated conditions). **e,** Quantification of the ratio of lipid A to the phospholipid phosphatidylglycerol (34:2) in the CM of *P. aeruginosa* PA14 spheroplasts exposed, or not, to murepavadin (0.05 µg ml^{-1}) for 2 h, as determined by MALDI-TOF-based lipidomics (n=3 in duplicate). **f,** OM disruption of *P. aeruginosa* PA14 cells during 10 minutes exposure to colistin (2 µg ml^{-1}) in the absence or presence of murepavadin (0.05 µg ml^{-1}), as assessed by uptake of the fluorescent dye NPN (10 µM) (n=4, each data point represents the arithmetic mean of 20 replicate measurements; ns: p>0.05 between colistin-treated bacteria). **g,** CM disruption of *P. aeruginosa* PA14 cells exposed to colistin (2 µg ml^{-1}) in the absence or presence of murepavadin (0.05 µg ml^{-1}), as determined using 2.5 µM PI (n=4; *p<0.0001 for Colistin and Murepavadin-exposed cells compared to Colistin alone). **h,** Permeabilisation of the CM of *P. aeruginosa* PA14 spheroplasts by colistin (2 µg ml^{-1}) following exposure, or not, of bacteria to murepavadin (0.05

378 μg ml^{-1}) for 2 h prior to conversion to spheroplasts, as determined using 2.5 μM PI (n=3, experiment performed on 3
379 independent occasions; *p<0.0001 for murepavadin-exposed cells compared to Colistin alone except at 0 h). **i,** Lysis of
380 *P. aeruginosa* PA14 cells exposed to colistin (2 μg ml^{-1}) in the absence or presence of murepavadin (0.05 μg ml^{-1}), as
381 measured by OD_{595nm} readings (n=4; *p<0.01 for Colistin and Murepavadin-exposed cells compared to Colistin alone). **j,**
382 Lysis of *P. aeruginosa* PA14 spheroplasts by colistin (2 μg ml^{-1}) following exposure, or not, of bacteria to murepavadin
383 (0.05 μg ml^{-1}) for 2 h prior to conversion to spheroplasts, as determined with OD_{600nm} readings (n=3, experiment
384 performed on 3 independent occasions; *p<0.01 for murepavadin-exposed cells compared to Colistin alone, error bars
385 are omitted for clarity). **k,** Survival of *P. aeruginosa* PA14 cells exposed to colistin (2 μg ml^{-1}) in the absence or presence
386 of murepavadin (0.05 μg ml^{-1}), as determined by c.f.u. counts (n=4; *p<0.05 for Colistin and Murepavadin-exposed cells
387 compared to Colistin alone). **l,** Checkerboard broth microdilution assays showing the synergistic growth-inhibitory
388 interaction between colistin and the LPS transport inhibitor murepavadin against *P. aeruginosa* PA14 cells, as
389 determined by measuring OD_{595nm} after 18 h incubation (n=3; data are presented as the mean OD_{595nm} value, dark green
390 regions represent increased bacterial growth). Data in **a-e** were analysed by a two-tailed paired Student's *t*-test. Data
391 in **f** were analysed by a one-way ANOVA with Tukey's post-hoc test. Data in **g-k** were analysed by a two-way ANOVA
392 with Dunnett's post-hoc test. Data are presented as the arithmetic mean, and error bars, where shown, represent the
393 standard deviation of the mean. OM: outer membrane; CM: cytoplasmic membrane; NPN: *N*-phenyl-1-napthylamine;
394 r.f.u.: relative fluorescence units; OD: optical density. PMB: polymyxin B.

395

396 **MCR-1 confers colistin resistance via modified LPS in the cytoplasmic membrane**

397 The plasmid-borne mobilized colistin resistance (*mcr*) family of enzymes confer resistance to polymyxins by
398 adding a phosphoethanolamine group to the lipid A domain of LPS [10,11]. MCR-1 is located in the CM where
399 it modifies LPS before the molecules are transported to the OM [11]. As expected, *E. coli* expressing *mcr-1*
400 had a significantly greater colistin minimum inhibitory concentration (MIC, 2 μg ml^{-1}) compared to an
401 otherwise isogenic empty plasmid (pEmpty) control strain that did not express the resistance determinant
402 (0.25 μg ml^{-1}), confirming that the cells were producing functional MCR-1 (Supplementary Fig. 31). To
403 characterise the LPS-modifying activity of MCR-1 at the CM, we used MALDI-TOF-based lipidomics with whole
404 cells and spheroplasts of the *E. coli* strain expressing *mcr-1* [47]. This revealed that the ratio of LPS modified
405 with phosphoethanolamine to unmodified LPS was approximately 2:1 in whole cells producing MCR-1, but in
406 *mcr-1* expressing spheroplasts, the ratio of modified LPS to unmodified LPS was ~4:1 (Fig. 5a) (Supplementary
407 Fig. 32,33). Therefore, there was a significantly higher proportion of unmodified LPS in the OM compared to
408 the CM of MCR-1-producing *E. coli*.

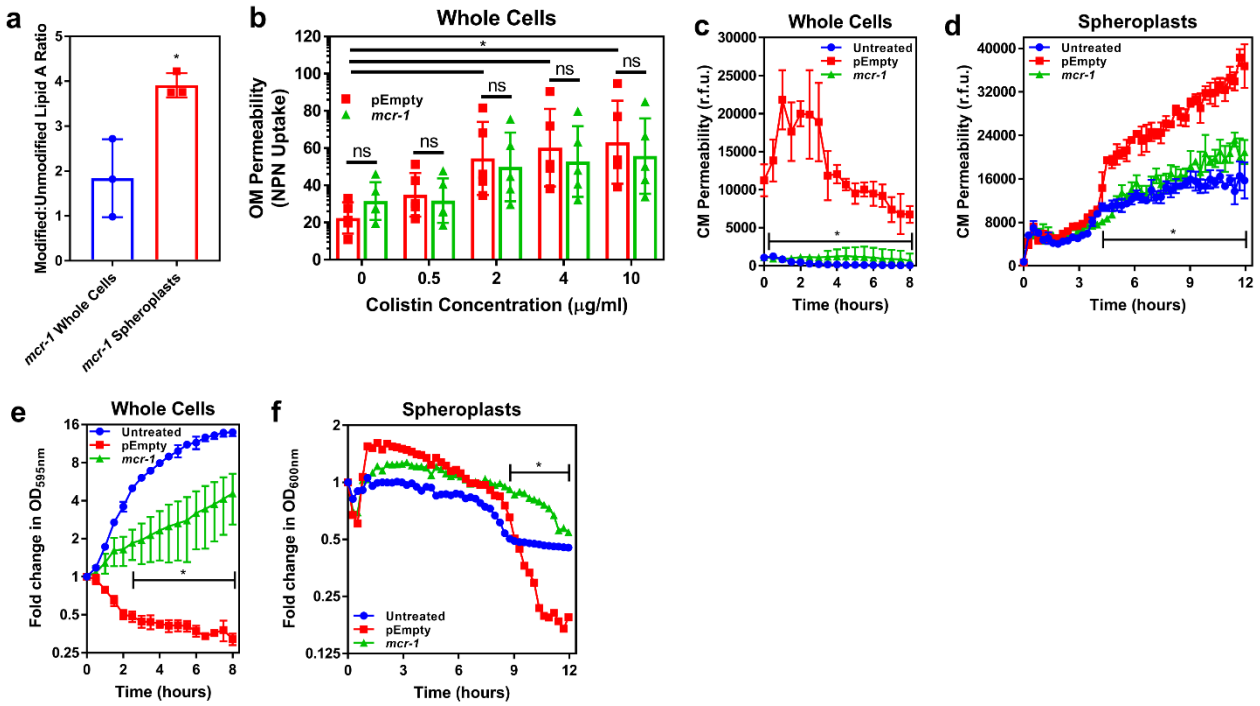
409 It has been proposed that the increased positive charge of LPS molecules conferred by the cationic
410 phosphoethanolamine moiety leads to electrostatic repulsion of colistin away from the surface of bacteria
411 [10,11]. However, previous work has shown that colistin permeabilises the OM of bacteria expressing *mcr*-1
412 almost as efficiently as strains that do not express *mcr*-1 [17]. This may reflect the presence of the relatively
413 large quantity of unmodified LPS in the OM as determined in our lipidomic analyses. Therefore, having

414 established that colistin kills Gram-negative pathogens by targeting LPS in the CM, we hypothesised that
415 MCR-1, which prevents polymyxin-mediated bacterial lysis, confers colistin resistance via the presence of
416 modified LPS in the CM rather than the OM.

417 We first verified previous findings by showing that colistin, across a range of antibiotic concentrations,
418 disrupted the OM of the *E. coli* strain expressing *mcr-1* as well as *E. coli* pEmpty (Fig. 5b) [17]. Thus, it
419 appeared that MCR-1 did not mediate colistin resistance at the level of the OM. By contrast, whilst colistin
420 compromised the CM of both whole cells and spheroplasts of *E. coli* pEmpty, it had no effect on the CM of
421 whole cells or spheroplasts expressing *mcr*-1, as determined by the entry of PI into the cytoplasm (Fig. 5c,d).
422 In keeping with this, colistin induced lysis of whole cells and spheroplasts of *E. coli* pEmpty, whereas
423 spheroplasts expressing *mcr-1* did not lyse in response to the polymyxin, and MCR-1 producing cells even
424 grew in the presence of the antibiotic, despite permeabilisation of the OM (Fig. 5e,f).

425 Taken together, these data support our hypothesis that MCR-1-mediated colistin resistance occurs due to
426 the presence of modified LPS in the CM, which prevented the antibiotic from permeabilising the CM and
427 causing bacterial lysis and death. Furthermore, these data support our hypothesis that colistin targets LPS in
428 the CM, because if phospholipids in the CM were the target of colistin then the modification of LPS would
429 not prevent the polymyxin from disrupting the CM.

430



431

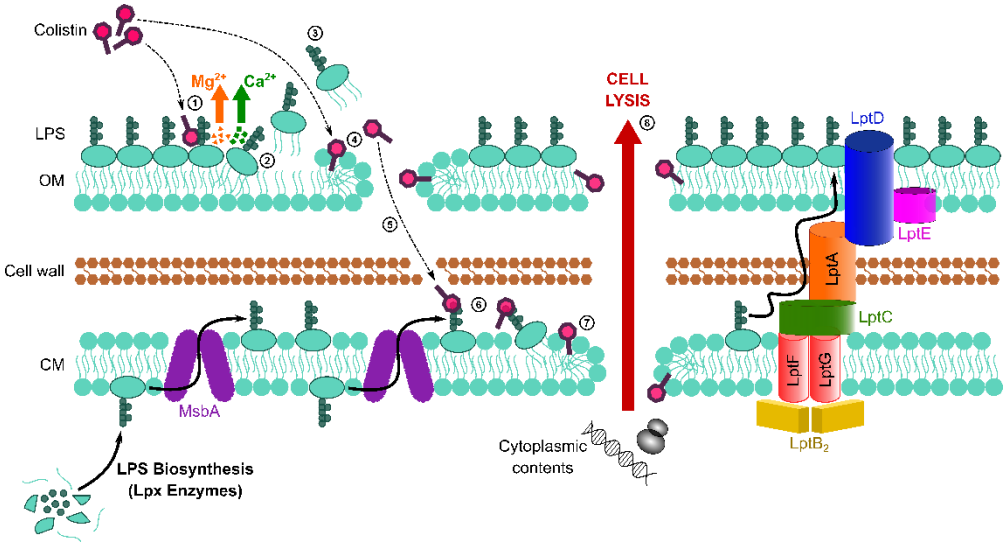
432 **Figure 5. Colistin disrupts the outer membrane but not the cytoplasmic membrane of *E. coli* expressing *mcr*-1. a,**
433 Quantification of LPS modified with phosphoethanolamine, expressed as the ratio of modified lipid A to unmodified
434 lipid A, in whole cells and spheroplasts of *E. coli* MC1000 expressing *mcr-1*, as determined by MALDI-TOF-based

435 lipidomics (n=3 in duplicate, *p<0.05 between Whole Cells and Spheroplasts). **b,** OM disruption of *E. coli* MC1000 cells
436 expressing *mcr-1* or an empty plasmid control strain (pEmpty) during 10 minutes of exposure to colistin at the indicated
437 antibiotic concentrations, as determined by uptake of the fluorescent dye NPN (10 µM) (n=5, each data point represents
438 the arithmetic mean of 20 replicate measurements; ns: p>0.05 between pEmpty and *mcr-1* strains, *p<0.05 between
439 the indicated concentrations of colistin). **c,** Permeabilisation of the CM of *E. coli* MC1000 cells expressing *mcr-1* or empty
440 plasmid-containing cells during incubation with colistin (4 µg ml^{-1}), as determined using 2.5 µM propidium iodide (PI)
441 (n=4, *p<0.0001 between pEmpty and *mcr-1* strains). **d,** Permeabilisation of the CM of *E. coli* MC1000 spheroplasts
442 generated from bacteria expressing *mcr-1* or empty plasmid control bacteria during incubation with colistin (4 µg ml^{-1}),
443 as determined using 0.25 µM PI (n=3, experiment performed on 4 independent occasions; *p<0.0001 compared to
444 pEmpty). **e,** Growth or lysis of *E. coli* MC1000 cells expressing *mcr-1* or empty plasmid control cells during exposure to
445 colistin (4 µg ml^{-1}), as measured using OD_{595nm} readings (n=4; *p<0.05 compared to pEmpty). **f,** Lysis of *E. coli* MC1000
446 spheroplasts generated from bacteria expressing *mcr-1* or empty plasmid-containing bacteria (pEmpty) during exposure
447 to colistin (4 µg ml^{-1}), as measured using OD_{595nm} readings (n=3, experiment performed on 4 independent occasions;
448 *p<0.01 compared to pEmpty, error bars are omitted for clarity). Data in **a** were analysed by a two-tailed paired
449 Student's *t*-test. Data in **c-f** were analysed by a two-way ANOVA with Sidak's **(c)** or Dunnett's **(d-f)** post-hoc tests. Data
450 are presented as the arithmetic mean, and error bars, where shown, represent the standard deviation of the mean.
451 OM: outer membrane; CM: cytoplasmic membrane; NPN: *N*-phenyl-1-napthylamine; r.f.u.: relative fluorescence units;
452 OD: optical density.

453

454 **Discussion**

455 Colistin is an increasingly important last-resort antibiotic used to combat infections caused by multi-drug-
456 resistant Gram-negative pathogens, including *P. aeruginosa*, *K. pneumoniae* and *E. coli* [1-3]. However,
457 treatment failure occurs frequently, and resistance is a growing concern [5-9]. Efforts to address these issues
458 are compromised by a poor understanding of colistin's bactericidal mode of action. Whilst the initial
459 interactions of colistin with LPS in the OM of Gram-negative bacteria were well-established, it was unclear
460 how the antibiotic traversed the OM and damaged the CM to cause cell lysis (Supplementary Fig. 1). In this
461 work, we demonstrate that colistin targets LPS in the CM, resulting in membrane permeabilisation, bacterial
462 lysis and killing (Fig. 6).



463

464 **Figure 6. Colistin kills bacteria by targeting LPS in both the outer and cytoplasmic membranes, leading to disruption**
465 **of the cell envelope and bacterial lysis.** Diagrammatic representation of the novel proposed mechanism of action of
466 colistin: Colistin binds to LPS in the OM (1), displacing cations that form bridges between LPS molecules, which leads to
467 destabilisation of the OM (2). As a consequence of the weakening of intermolecular bonds in the LPS monolayer, LPS is
468 released from the bacterial surface (3), allowing colistin to further damage the OM via the action of the polymyxin lipid
469 tail (4). This provides colistin with access to the periplasm, where colistin interacts with LPS in the CM (5) that is awaiting
470 transport to the OM by the LptABCDEFG machinery after being synthesized in the cytoplasm and flipped to the outer
471 leaflet of the CM by MsbA. As in the OM, colistin binding to LPS results in disruption of the CM (6), which it ultimately
472 permeabilises (7), culminating in the loss of cytoplasmic contents, cell lysis and bacterial death (8).

473

474 It has been hypothesised that polymyxins damage the phospholipid bilayer in the CM via the detergent-like
475 properties of the antibiotic [1,3]. However, our data showing that colistin requires a sufficient concentration
476 of LPS to be present in the CM in order to kill bacteria explains how an antibiotic with high affinity and
477 specificity for LPS causes disruption to both the OM and CM. Furthermore, these findings provide support
478 for the observations that colistin does not damage the OM of resistant *A. baumannii* isolates where LPS has

479 been replaced by a phospholipid bilayer, and that polymyxins cause only minimal disruption to model
480 phospholipid membranes unless LPS is present [15,23].

481 Whilst the interaction of colistin with LPS in the CM is likely to share similarities with the same process at the
482 OM, there are also likely to be differences owing to the differing concentrations of LPS between the two
483 membranes [23]. In the OM, LPS is a highly abundant component with molecules tightly packed and stabilised
484 with cation bridges. By contrast, LPS is a minority component in the CM, which may affect the rate and degree
485 to which the CM is disrupted by polymyxins. For example, previous work showed a direct correlation between
486 the concentration of LPS in a model membrane and the extent to which the membrane was permeabilised
487 by colistin [23]. Specifically, this study found that a model membrane was rapidly disrupted by colistin when
488 LPS was present at ~3% of the total composition of the membrane, but the permeabilising activity of the
489 polymyxin was greatly reduced when LPS was present at ~1% [23]. Whilst it is difficult to directly compare
490 data from synthetic membranes with live whole bacteria, this work explains how a relatively small drop in
491 LPS concentration in the CM caused by CHIR090 or cerulenin could make cells significantly less susceptible to
492 colistin.

493 Colistin induced OM damage occurred within minutes of bacterial exposure to the antibiotic, but disruption
494 of the CM took much longer. This delay could be due to the time taken for the polymyxin to traverse the OM,
495 since polymyxins accumulate on the bacterial surface before migrating to the CM [48]. Nonetheless, even
496 when spheroplasts, which lack an OM, were exposed to colistin, it still took more than 2 h for CM
497 permeabilisation to occur. Therefore, it appears that colistin-mediated disruption of the CM is considerably
498 less efficient than that of the OM, likely due to the much lower levels of LPS present in the CM.

499 Having shown that reducing the LPS content of the CM decreases susceptibility to colistin, we were then able
500 to use the LptD inhibitor murepavadin to accumulate LPS in the CM, and thereby increase the sensitivity of
501 bacteria to colistin. Combined, these complementary findings provide strong evidence that colistin targets
502 LPS in the CM, in addition to the OM, and that this is required for bactericidal and lytic activity at clinically
503 relevant concentrations of the antibiotic. At supra-physiological colistin concentrations, the protective
504 effects of reducing the LPS levels in the CM could be partially overcome, potentially explaining why at
505 concentrations above those achieved clinically, colistin displays partial activity against phospholipid bilayers
506 lacking LPS [19,20]. However, at concentrations of colistin achieved in serum, the lethal effects of the
507 antibiotic were directly proportional to the amount of LPS in the CM.

508 Our conclusion that colistin targets LPS in the CM is further supported by our studies with *E. coli* expressing
509 the *mcr-1* colistin resistance determinant. MCR-1 modifies lipid A with a phosphoethanolamine moiety as it
510 passes through the CM on its way to the OM [11]. However, recent work showed that MCR-1 did not prevent
511 colistin-mediated OM permeabilisation, suggesting that resistance is not conferred by modification of LPS
512 present in the OM [17]. Using lipidomics, we found that ~40% of the LPS in the OM of *E. coli* expressing *mcr-*

513 1 was not modified with phosphoethanolamine, which provides an explanation for the ability of colistin to
514 permeabilise this structure. By contrast, only ~20% of the LPS in the CM remained unmodified in the presence
515 of MCR-1. This observation, together with the fact that the abundance of LPS in the CM is very low, suggests
516 that very few targets (i.e. unmodified LPS molecules) exist for colistin in the CM of *mcr*-1 expressing *E. coli*.
517 This explains our data showing that the polymyxin antibiotic was unable to disrupt the CM of *E. coli* whole
518 cells and spheroplasts producing MCR-1, rendering the bacterium resistant to colistin.

519 Since all MCR homologues are present at the CM, it is likely that our findings are generalizable to this
520 mechanism of colistin resistance [11-14]. Polymyxin resistance can also occur via chromosomal mutations or
521 the induction of stress responses that lead to the addition of amino sugars and/or phosphoethanolamine to
522 the lipid A domain of LPS in the CM [37-39,49]. Therefore, our findings may also be applicable to non-MCR
523 mediated colistin resistance, although this remains to be tested.

524 In summary, this work contributes to our understanding of the mechanism of action of colistin by
525 demonstrating that polymyxin antibiotics target LPS in both the OM and the CM, and that this leads to the
526 disruption of both membranes, resulting in the bactericidal and lytic activities of the antibiotic. In addition,
527 our findings demonstrate that MCR-1 confers colistin resistance via modification of LPS in the CM, laying the
528 foundations for the development of new therapeutic strategies to overcome the growing threat posed by
529 globally disseminated colistin resistance.

530

531 ## Methods

532 ### Bacterial strains and growth conditions

533 The bacterial strains used in this study are listed in Supplementary Table 1. For each experiment, all strains
534 were grown for 18 h to stationary phase at 37°C with shaking (180 r.p.m.), except *S. pyogenes* GAS, which
535 was grown statically at 37°C in 5% CO_2, and *L. lactis* W2396231, which was grown statically at 30°C in air. All
536 strains were grown in Luria broth (LB; Thermo Fisher Scientific, USA), with the exception of *P. aeruginosa*
537 strain A23 and the Gram-positive organisms, which were cultured in Tryptic Soy Broth (TSB; BD Biosciences,
538 USA), and *K. pneumoniae* strain KPC, which was grown in Brain Heart Infusion Broth (BHI; VWR International,
539 USA). For routine culture of bacteria on solid media, strains were grown on the relevant media as described
540 above supplemented with 1.5% technical agar (BD Biosciences). Growth media were supplemented with
541 chloramphenicol (25 µg ml^{-1}), gentamicin (15 µg ml^{-1}), tetracycline (12.5 µg ml^{-1}) or 0.5 mM isopropyl-β-D-
542 thiogalactoside (IPTG, Melford Laboratories, UK) where required. Enumeration of bacterial c.f.u. was done
543 by plating 10-fold serial dilutions of bacterial cultures on to Mueller-Hinton agar (MHA; Thermo Fisher
544 Scientific) plates. Inoculated agar plates were incubated statically for 18 h in air at 37°C.

545 ### Determination of minimum inhibitory concentrations of antibiotics

546 The minimum inhibitory concentration (MIC) of colistin, polymyxin B, cerulenin, ciprofloxacin, EDTA,
547 tetracycline, chloramphenicol, triclosan (all from Sigma-Aldrich, USA), CHIR090 (Axon Medchem,
548 Netherlands), murepavadin (DC Chemicals, China), gentamicin (Thermo Fisher Scientific), imipenem
549 (Fresenius Kabi, Germany), and chlorhexidine (GlaxoSmithKline, UK) against bacterial strains was determined
550 by the broth microdilution protocol (Supplementary Table 2, 3) [50]. A microtitre plate was used to prepare
551 a range of antibiotic concentrations in 200 µl cation-adjusted Mueller-Hinton broth (CA-MHB) by two-fold
552 serial dilutions. For certain experiments, checkerboard analyses were performed by preparing two-fold serial
553 dilutions of two antibiotics in different directions, generating an 8 x 8 matrix to assess the MICs of the
554 antibiotics in combination [17]. Stationary-phase bacteria were diluted 1000-fold in fresh CA-MHB and
555 seeded into each well of the microtitre plate to a final concentration of 5 x 10^5 c.f.u. ml^{-1}. The microtitre
556 plates were then incubated statically at 37°C for 18 h in air, after which point the MIC was defined as the
557 lowest antibiotic concentration at which there was no visible growth of bacteria. In some cases, the extent
558 of bacterial growth after 18 h incubation was also determined by obtaining OD_{595nm} measurements using a
559 Bio-Rad iMark microplate absorbance reader (Bio-Rad Laboratories, USA).

560 ### Production of spheroplasts

561 Spheroplasts of *P. aeruginosa* and *E. coli* strains lacking an OM and cell wall were generated as previously
562 described [51]. Briefly, stationary-phase bacteria grown overnight were washed twice by centrifugation
563 (12,300 x *g*, 3 min) followed by resuspension in CA-MHB, and added at a final inoculum of 10^8 c.f.u. ml^{-1} to 9

564 ml CA-MHB, containing for some experiments the LPS biosynthesis inhibitors CHIR090 or cerulenin, or the
565 LPS transport inhibitor murepavadin. Cultures were then incubated at 37°C with shaking (180 r.p.m.) for 2 h.
566 After the incubation, bacteria were washed twice by centrifuging (3,273 x *g*, 20 min, 4°C) and resuspending
567 first in 10 ml Tris buffer (0.03 M, pH 8.0, Sigma-Aldrich), and subsequently in Tris buffer (0.03 M, pH 8.0)
568 containing 20% sucrose. EDTA (250 µl, 10 mg ml^{-1}) and lysozyme (1 ml, 10 mg ml^{-1}, Roche, Switzerland) were
569 added to remove the OM and cell wall respectively, and the cell suspension was incubated for 1 h in a water
570 bath shaker at 30°C. Trypsin (500 µl, 10 mg ml^{-1}, Sigma-Aldrich) was then added, and the culture again
571 incubated at 30°C in a water bath shaker for 15 min. The resulting spheroplasts produced were harvested by
572 mild centrifugation (2,000 x *g*, 20 min, 4°C), with the supernatant containing the removed OM extracted for
573 further analysis. Successful conversion of bacterial whole cells into spheroplasts was confirmed using phase-
574 contrast microscopy, as detailed below.

575 **Confirmation of successful spheroplast formation**

576 Whole cells of *P. aeruginosa* grown overnight were washed twice by centrifugation (12,300 x *g*, 3 min) and
577 resuspension in CA-MHB, added at a final inoculum of 10^8 c.f.u. ml^{-1} to 9 ml CA-MHB, and incubated for 2 h
578 at 37°C with shaking (180 r.p.m.). Outer membrane proteins of these bacteria were subsequently labelled
579 with fluorescein isothiocyanate (FITC, Sigma-Aldrich) as previously described [52]. Bacterial cells were
580 washed twice by centrifugation (3,273 x *g*, 20 min, 4°C) and resuspension in 10 ml Labelling Buffer (50mM
581 Na_2CO_3, 100 mM NaCl, pH 8.0), to which FITC was added at a final concentration of 0.5 mg ml^{-1}. Bacteria were
582 incubated for 30 mins at room temperature, before labelled cells were harvested by centrifuging (3,273 x *g*,
583 20 min, 4°C) and washed thrice by resuspending in 10 ml Tris buffer (0.03 M, pH 8.0) containing 20% sucrose.
584 1 ml of FITC-labelled bacteria was extracted and centrifuged (12,300 x *g*, 3 min), and the cells were fixed in
585 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The remaining 9 ml of FITC-labelled cells
586 were converted into spheroplasts, as described above. The spheroplasts produced were recovered by mild
587 centrifugation (2,000 x *g*, 20 min, 4°C) and resuspension in 9 ml Tris buffer (0.03 M, pH 8.0) containing 20%
588 sucrose, before 1 ml of spheroplasts were fixed in the same way as with whole cells. The amount of FITC
589 fluorescence in the OM of whole cells and CM of spheroplasts was observed using fluorescence microscopy,
590 as described below. For quantification of FITC fluorescence, 200 µl samples of the fixed bacterial suspensions
591 were seeded into the wells of a black-walled microtitre plate, and fluorescence measured with a Tecan
592 Infinite 200 Pro multiwell plate reader (Tecan Group Ltd., Switzerland), using an excitation wavelength of 490
593 nm and an emission wavelength of 525 nm.

594 **Microscopic analysis**

595 For phase-contrast and fluorescence microscopy, a 5 µl sample of fixed bacterial whole cells or spheroplasts
596 was spotted onto a thin 1.2% agarose gel patch prepared in distilled water on a microscope slide. Bacteria
597 were imaged using an Axio Imager.A2 Zeiss microscope (Carl Zeiss Microscopy GmbH, Germany) at 1000x

598 magnification with an oil immersion objective lens. The ZEN 2012 software was used for image acquisition,
599 whilst analysis of cell length:width ratios was done using the FIJI/ImageJ software by measuring two
600 perpendicular lines drawn through the centre of bacteria. For each experiment, all microscopy images were
601 acquired and processed using identical settings throughout.

602 **Determination of LPS concentration by Limulus Amebocyte Lysate assay**

603 Stationary-phase bacteria grown overnight were washed and exposed to CHIR090, cerulenin or murepavadin
604 for 2 h, before conversion to spheroplasts as described above. During formation of spheroplasts, the OM
605 extracted from the bacterial cells was recovered, and the concentration of LPS in the OM, as well as the
606 concentration of LPS in the CM of spheroplasts, was determined using the chromogenic Limulus Amebocyte
607 Lysate (LAL) assay (all reagents from Thermo Fisher Scientific) as described previously [33]. OM samples and
608 spheroplasts lysed by freeze-thaw were diluted in 10-fold steps, and 50 µl of each sample was equilibrated
609 to 37°C and loaded into the wells of a microtitre plate at the same temperature. LAL reagent (50 µl) was
610 added to each well, and the mixture incubated at 37°C for 10 min. Chromogenic substrate solution (100 µl, 2
611 mM) was subsequently added to each well and the microtitre plate was incubated for a further 6 min at 37°C.
612 The enzymatic reaction was stopped by adding 50 µl of 25% acetic acid to each well, and the presence of LPS
613 was determined by measuring absorbance at 405 nm in a Tecan Infinite 200 Pro multiwell plate reader. A
614 standard curve was generated using an *E. coli* endotoxin standard stock solution, which enabled the
615 conversion of A_{405nm} values into concentrations of LPS. The LAL assay was also used to quantify the amount
616 of LPS released into the culture supernatants from bacteria exposed to the relevant antibiotics.

617 **Bacterial growth assay**

618 Stationary-phase bacteria were diluted 1000-fold in fresh CA-MHB, and 4 µl was seeded into the wells of a
619 microtitre plate containing 200 µl CA-MHB, and for some experiments the inhibitors CHIR090, cerulenin or
620 murepavadin, to give a final inoculum of 5×10^5 c.f.u. ml^{-1}. The microtitre plate was incubated with shaking
621 (180 r.p.m.) at 37°C for up to 24 h in a Tecan Infinite 200 Pro multiwell plate reader and optical density
622 measurements were taken at 600 nm every 15 min.

623 **Fluorescent labelling of antibiotics**

624 Colistin, polymyxin B and polymyxin B nonapeptide (PMBN) were labelled with the fluorophore BoDipy FL SE
625 D2184 (Thermo Fisher Scientific) by incubating 100 µl of the BoDipy NHS ester compound (10 mg ml^{-1} in
626 dimethyl sulfoxide, DMSO) with 250 µl of the antibiotics (10 mg ml^{-1}) and 650 µl sodium bicarbonate (0.2 M,
627 pH 8.5) for 2 h at 37°C. BoDipy molecules that had not bound to the antibiotics were removed by dialysis
628 using a Float-A-Lyser G2 dialysis device (Spectrum Laboratories, USA) that had a molecular weight cut-off of
629 0.5 kDa. Dialysis was carried out at 4°C against sterile distilled water, which was changed four times during
630 the course of the 24 h dialysis period. Time of flight mass spectrometry analysis confirmed successful labelling

631 of the polymyxin compounds with the fluorophore, and the antibiotic activity of BoDipy-labelled colistin and
632 BoDipy-polymyxin B was assessed using MIC and bacterial survival assays, as described in the relevant
633 sections.

634 **Determination of LPS levels on cell surface using labelled polymyxin B nonapeptide**

635 To detect and quantify the amount of LPS present in the OM of whole cells and CM of spheroplasts, a
636 fluorescently-labelled form of PMBN (BoDipy-PMBN) was used as a probe, as described previously [34]. For
637 whole cells, stationary-phase bacterial cultures grown overnight were washed twice by centrifugation
638 (12,300 x *g*, 3 min) and resuspension in CA-MHB, before being added at a final inoculum of 10^8 c.f.u. ml^{-1} to
639 9 ml CA-MHB containing CHIR090, cerulenin or murepavadin where required. Bacteria were then incubated
640 at 37°C with shaking (180 r.p.m.) for 2 h, after which point cells were washed by centrifuging (3,273 x *g*, 20
641 min, 4°C) and resuspending in 9 ml Tris buffer (0.03 M, pH 8.0). Washed bacteria were recovered by
642 centrifugation (3,273 x *g*, 20 min, 4°C) and fixed by resuspending in 10 ml of 4% PFA in phosphate-buffered
643 saline (PBS). Similarly, spheroplasts generated as detailed above were harvested by mild centrifugation
644 (2,000 x *g*, 20 min, 4°C), and fixed in 10 ml of 4% PFA in phosphate-buffered saline (PBS) containing 20%
645 sucrose. Following fixation for 1 h at room temperature, both whole cells and spheroplasts were washed
646 twice by centrifuging (3,273 x *g*, 20 min, 4°C) and resuspending in 10 ml Tris buffer (0.03 M, pH 8.0) containing
647 20% sucrose. Subsequently, BoDipy-PMBN (7 µg ml^{-1}) was added, and the fixed bacterial suspensions were
648 incubated at 37°C for 30 min with shaking (180 r.p.m.). After the incubation, to remove any BoDipy-PMBN
649 that was not bound to the cell surface, the whole cells and spheroplasts were again washed twice as stated
650 above. For quantification of BoDipy-PMBN bound to the cell surface, 200 µl samples of the bacterial
651 suspensions were seeded into the wells of a black-walled microtitre plate, and fluorescence measured with
652 a Tecan Infinite 200 Pro multiwell plate reader, using an excitation wavelength of 490 nm and an emission
653 wavelength of 525 nm. To account for differences in fluorescence values arising from variations in cell
654 number, relative fluorescence unit (r.f.u.) measurements were corrected for OD at 600 nm.

655 **Determination of antibiotic binding to bacterial cells**

656 Stationary-phase bacterial cultures grown overnight were washed twice in CA-MHB by centrifugation (12,300
657 x *g*, 3 min) and resuspension, then added at an inoculum of 10^8 c.f.u. ml^{-1} to 3 ml CA-MHB containing
658 fluorescently-labelled BoDipy-Colistin, BoDipy-Polymyxin B or BoDipy-PMBN, either alone or in combination
659 with CHIR090 or cerulenin. The cultures were incubated for 8 h at 37°C with shaking (180 r.p.m.), and every
660 2 h bacteria were recovered by centrifugation (12,300 x *g*, 3 min). The pelleted cells were washed thoroughly
661 by four rounds of centrifugation (12,300 x *g*, 3 min) and resuspension in fresh CA-MHB in order to remove
662 any BoDipy-labelled antibiotic that was not attached to the bacteria. The amount of BoDipy-Colistin, BoDipy-
663 Polymyxin B or BoDipy-PMBN bound to the bacterial cells was quantified by measuring the fluorescence of
664 200 µl samples in a black-walled clear-bottomed microtitre plate, using a Tecan Infinite 200 Pro multiwell

665 plate reader (excitation at 490 nm, emission at 525 nm). The relative fluorescence values of samples were
666 corrected using OD_{600nm} measurements. Binding of BoDipy-Colistin, BoDipy-Polymyxin B or BoDipy-PMBN to
667 bacterial cells was also observed by fluorescence microscopy, as described above.

668 **Determination of LPS concentration and modification by mass spectroscopy**

669 Spheroplasts from bacterial cells exposed to CHIR090, cerulenin or murepavadin were prepared as described
670 above and then resuspended in ddH_2O (200 µl), before mild acid hydrolysis was performed via the addition
671 of 2% (vol/vol) acetic acid in ddH_2O (200 µl) and incubation at 100°C for 30 min. For experiments with whole
672 cells, bacteria grown overnight to stationary-phase were washed three times by centrifuging and
673 resuspending in ddH_2O, and a mild acid hydrolysis was performed on these whole cells as described for
674 spheroplasts. Acid-treated whole cells or spheroplasts were recovered by centrifugation (17,000 × *g*, 2 min),
675 and the resulting pellet was washed before being resuspended in 50 µl ultrapure water. The whole cell or
676 spheroplast suspension (0.5 µl) was then loaded immediately onto the target and overlaid with 1.2 µl of a
677 matrix consisting of 9H-Pyrido[3,4-B]indole (Norharmane) (Sigma-Aldrich) dissolved in 90:10 (vol/vol)
678 chloroform/methanol to a final concentration of 10 mg ml^{-1}. The bacterial suspension and matrix were then
679 mixed on the target before gentle drying under air at room temperature. MALDI-TOF mass spectroscopy
680 analysis was undertaken with a MALDI Biotyper Sirius system (Bruker Daltonics, USA), using the linear
681 negative-ion mode as described previously [35]. Manual peak picking at masses relevant to phospholipids or
682 lipid A was performed on the mass spectra obtained, and the corresponding signal intensities at the defined
683 masses were determined. Peaks were considered only if their signal/noise ratio was at least 5. To determine
684 the relative abundance of LPS, the sum of the area under the lipid A peaks (*m/z* 1447-1700) was divided by
685 the sum of the area under a representative phosphatidylglycerol phospholipid peak (34:2, *m/z* 717-747). To
686 determine the ratio of modified lipid A to unmodified lipid A, the area under the pETN-modified lipid A peak
687 (*m/z* 1,919.2) was divided by the area under the peak corresponding to native lipid A (*m/z* 1,796.2). All mass
688 spectra were generated and analysed with three biological replicates and two technical replicates.

689 **Outer membrane disruption assay**

690 To detect damage to the OM of bacteria, the well-established NPN uptake assay was used [36]. Stationary-
691 phase bacterial cells were washed in fresh CA-MHB and diluted to an optical density (OD_{600nm}) of 0.5 in 5 mM
692 pH 7.2 HEPES buffer (Sigma-Aldrich). This bacterial suspension was added to wells containing the relevant
693 antibiotics in HEPES buffer, as well as the fluorescent probe *N*-phenyl-1-naphthylamine (NPN; Acros Organics,
694 USA) at a final concentration of 10 µM. Samples were placed in a black microtitre plate with clear-bottomed
695 wells and fluorescence measured immediately in a Tecan Infinite 200 Pro multiwell plate using an excitation
696 wavelength of 355 nm and an emission wavelength of 405 nm. Fluorescence measurements were obtained
697 every 30 seconds for 10 min, and the degree of OM permeabilisation, referred to as the NPN Uptake Factor,
698 was calculated using the following formula:

699 $$\frac{Fluorescence\ of\ sample\ with\ NPN - Fluorescence\ of\ sample\ without\ NPN}{Fluorescence\ of\ HEPES\ buffer\ with\ NPN - Fluorescence\ of\ HEPES\ buffer\ without\ NPN}$$

700 **Determination of bactericidal activity of antibiotics**

701 As described above, stationary-phase bacteria grown overnight were washed twice and added at a final
702 inoculum of 10^8 c.f.u. ml^{-1} to 3 ml CA-MHB containing the relevant antibiotics. Cultures were incubated with
703 shaking (37°C, 180 r.p.m.) for up to 10 h. Bacterial survival was determined after 2, 4, 6, 8 and 10 h by serially
704 diluting cultures in 10-fold steps in 200 µl sterile PBS (VWR International), before enumeration of c.f.u. counts
705 on MHA plates.

706 **Isolation of CHIR090-resistant mutants**

707 Cultures of *P. aeruginosa* PA14 grown overnight to stationary-phase were washed twice in CA-MHB as
708 described above and diluted 10-fold to an inoculum of 10^8 c.f.u. ml^{-1} in CA-MHB, before 100 µl of this
709 inoculum was spread across the surface of an MHA plate. A well measuring 10 mm in diameter was made in
710 the agar plate and filled with 130 µl of the LPS biosynthesis inhibitor CHIR090 (8 µg ml^{-1}). After static
711 incubation of the agar plate at 37°C for 18 h in air, mutants with spontaneous resistance to CHIR090 were
712 isolated by picking colonies that had grown within the zone of inhibition around the well. These resistant
713 mutants were grown overnight to stationary-phase, and again exposed to CHIR090 in a well as described
714 above to confirm that the strains had a reduced susceptibility to the antibiotic. Bacterial growth, survival and
715 MIC assays were performed as detailed in the relevant sections to characterise the properties of the resistant
716 *P. aeruginosa* isolates.

717 **Determination of membrane lipid release from bacteria**

718 Stationary-phase bacteria grown overnight were washed twice in CA-MHB and exposed to the relevant
719 antibiotics in 3 ml CA-MHB at a final inoculum of 10^8 c.f.u. ml^{-1} as described above. Cultures were incubated
720 for 8 h at 37°C with shaking (180 r.p.m.), and every 2 h the supernatants were recovered by centrifugation
721 (12,300 x *g*, 3 min). Recovered supernatants (200 µl) were mixed with 5 µl of FM-4-64 styryl dye (Thermo
722 Fisher Scientific) at a final concentration of 5 µg ml^{-1} in the wells of a black-walled microtitre plate.
723 Fluorescence was measured using a Tecan Infinite 200 Pro multiwell plate reader (excitation at 565 nm,
724 emission at 600 nm) to quantify the phospholipid released into the supernatant by bacteria [53].

725 **Determination of colistin activity**

726 As described above, spent culture supernatants from bacteria exposed to the relevant antibiotics were
727 recovered and the activity of colistin in the supernatant was determined using an established zone of
728 inhibition assay [53]. Stationary-phase *P. aeruginosa* was diluted in CA-MHB to a concentration of 10^6 c.f.u.
729 ml^{-1} before 60 µl of this bacterial culture was spread across the surface of an MHA plate and allowed to air-
730 dry. Wells measuring 10 mm in diameter were made in the agar plate and filled with 130 µl of the culture

731 supernatant recovered from colistin-treated bacterial populations. Agar plates were incubated in air statically
732 at 37°C for 18 h and the zone of growth inhibition around each well was measured at four perpendicular
733 points. A standard plot was generated showing a linear relationship between the size of the zone of inhibition
734 and colistin concentration, which enabled inhibitory zone size to be converted to percentage colistin activity.

735 **Scanning electron microscopy (SEM) imaging**

736 Stationary-phase bacterial cells grown overnight were washed and inoculated into 3 ml CA-MHB containing
737 the relevant antibiotics as described above. After 2 h incubation (37°C, 180 r.p.m.), 25 µl of the cultures was
738 spotted onto a silicon chip (University Wafer, USA) and allowed to air-dry. The sample was fixed using
739 glutaraldehyde (2.5% in 0.01 M PBS) for 30 min at room temperature, before the chip was washed three
740 times in PBS (0.01 M). The sample was dehydrated using increasing concentrations of ethanol (10%, 30%,
741 50%, 70%, 90% and 100%) for 5 min each time, before a coating of 15 nm chromium (Quorum Technologies,
742 UK) was applied. Representative images of bacterial cells were obtained using a LEO Gemini 1525 field
743 emission gun scanning electron microscope (FEG SEM; Carl Zeiss Microscopy GmbH).

744 **Cytoplasmic membrane disruption assay**

745 To measure CM disruption of whole cells, bacteria grown to stationary-phase overnight were washed and
746 inoculated into 3 ml MHB containing the relevant antibiotics. Cultures were incubated at 37°C with shaking
747 (180 r.p.m.) for up to 8 h, and every 30 min, aliquots (200 µl) were taken and bacteria isolated by
748 centrifugation (12,300 x *g*, 3 min). Cells were then washed in sterile PBS before being added to the wells of a
749 black-walled microtitre plate, and propidium iodide (PI, Sigma-Aldrich) was added to each well at a final
750 concentration of 2.5 µM. Fluorescence was measured immediately in a Tecan Infinite 200 Pro multiwell plate
751 reader (excitation at 535 nm, emission at 617 nm). To measure disruption of the CM in spheroplasts,
752 spheroplasts of *P. aeruginosa* and *E. coli* generated as detailed above were washed by centrifugation (4,000
753 x *g*, 5 min) and resuspension in Tris buffer (0.03 M, pH 8.0) containing 20% sucrose. Spheroplast samples (20
754 µl) were then added in the wells of a black-walled microtitre plate to 180 µl of Tris buffer (0.03 M, pH 8.0)
755 containing 20% sucrose, the relevant antibiotics, and PI at a final concentration of 0.25 µM. The microtitre
756 plate was incubated with shaking (180 r.p.m.) at 37°C for up to 12 h in a Tecan Infinite 200 Pro multiwell plate
757 reader and fluorescence (excitation at 535 nm, emission at 617 nm) measured every 15 min. For both whole
758 bacterial cells and spheroplasts, to account for differences in fluorescence values arising from variations in
759 cell number, relative fluorescence unit (r.f.u.) measurements were corrected for OD at 600 nm.

760

761

762 **Determination of bacterial lysis**

763 In the case of whole bacterial cells, washed stationary-phase bacteria were inoculated into 3 ml CA-MHB
764 containing the relevant antibiotics, as described above. Cultures were then placed in a shaking incubator
765 (37°C, 180 r.p.m.) for 8 h, and every 30 min, samples (200 µl) were transferred to a microtitre plate, where
766 OD_{595nm} measurements were obtained using a Bio-Rad iMark microplate absorbance reader. For
767 spheroplasts, washed spheroplasts (20 µl) were added to 180 µl of Tris buffer (0.03 M, pH 8.0) containing
768 20% sucrose and the relevant antibiotics in a microtitre plate as detailed above. The microtitre plate was
769 incubated for up to 12 h at 37°C with shaking (180 r.p.m.) in a Tecan Infinite 200 Pro multiwell plate reader,
770 and readings of OD_{600nm} were made every 15 min.

771 **Statistical analyses**

772 Experiments were performed on at least three independent occasions, and the resulting data are presented
773 as the arithmetic mean of these biological repeats, unless stated otherwise. Error bars represent the standard
774 deviation of the mean. For single comparisons, a two-tailed Student's *t*-test was used to analyse the data.
775 For multiple comparisons at a single time point or concentration, data were analysed using a one-way
776 analysis of variance (ANOVA). Where data were obtained at several different time points or concentrations,
777 a two-way ANOVA was used for statistical analyses. Appropriate post-hoc tests (Dunnett's, Tukey's, Welch's,
778 Sidak's, Holm-Sidak's) were carried out to correct for multiple comparisons, with details provided in the figure
779 legends. Asterisks on graphs indicate significant differences between data, and the corresponding p-values
780 are reported in the figure legend. All statistical analyses were performed using GraphPad Prism 7 software
781 (GraphPad Software Inc., USA).

782 **Data availability**

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

785

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Author Contributions

A.S. and A.M.E. designed the experiments. A.S., A.K., M.B., K.L.H.H., L.E.E. and R.C.D.F. performed experiments or generated bacterial strains or reagents. All authors analysed data and contributed to writing of the manuscript.

Competing interests

The authors declare no competing interests.

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