

A novel colistin adjuvant identified by virtual screening for ArnT inhibitors

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Background: Colistin is a last-resort treatment option for many MDR Gram-negative bacteria. The covalent addition of L-aminoarabinose to the lipid A moiety of LPS is the main colistin resistance mechanism in the human pathogen *Pseudomonas aeruginosa*.

Objectives: Identification (by *in silico* screening of a chemical library) of potential inhibitors of ArnT, which catalyses the last committed step of lipid A aminoarabinylation, and their validation *in vitro* as colistin adjuvants.

Methods: The available ArnT crystal structure was used for a docking-based virtual screening of an in-house library of natural products. The resulting putative ArnT inhibitors were tested in growth inhibition assays using a reference colistin-resistant *P. aeruginosa* strain. The most promising compound was further characterized for its range of activity, specificity and cytotoxicity. Additionally, the effect of the compound on lipid A aminoarabinylation was verified by MS analyses of lipid A.

Results: A putative ArnT inhibitor (BBN149) was discovered by molecular docking and demonstrated to specifically potentiate colistin activity in colistin-resistant *P. aeruginosa* isolates, without relevant effect on colistin-susceptible strains. BBN149 also showed adjuvant activity against colistin-resistant *Klebsiella pneumoniae* and low toxicity to bronchial epithelial cells. Lipid A aminoarabinylation was reduced in BBN149-treated cells, although only partially.

Conclusions: This study demonstrates that *in silico* screening targeting ArnT can successfully identify inhibitors of colistin resistance and provides a promising lead compound for the development of colistin adjuvants for the treatment of MDR bacterial infections.

Introduction

The spread of drug resistance in Gram-negative bacterial pathogens and the paucity of new antimicrobials prompted the medical

community to re-use the old polymyxin antibiotic colistin when no other less-toxic or effective antibiotics are available. Colistin is a cationic polypeptide whose antibacterial activity relies on interaction with the anionic lipid A moiety of LPS, leading to

displacement of the LPS-stabilizing cations Ca^{2+} and Mg^{2+} , which in turn causes outer membrane derangement. This results in increased membrane permeability, leakage of cell contents and ultimately cell death.¹

Reintroduction of colistin in clinical practice has inevitably led to the emergence and spread of colistin-resistant isolates.² Colistin resistance rates in Gram-negative pathogens (e.g. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) are still relatively low (on average <10%), although some surveillance studies have reported resistance rates close to 50%.^{3–5}

P. aeruginosa is an opportunistic pathogen responsible for severe infections in immunocompromised patients and represents a major threat to patients suffering from cystic fibrosis (CF). Its high intrinsic antibiotic resistance, mostly due to low outer membrane permeability and active drug efflux, makes infections by this pathogen challenging to treat.^{6,7} The evolution of resistance in this pathogen is particularly problematic in CF patients, whose ability to resolve bacterial infection is impaired by defective host defences.^{8–10} Chronic lung infections in these patients require continuous therapeutic treatments that generally select for antibiotic-resistant clones.^{10–12} Although, in the past, colistin was mostly kept on the shelf, its use was never discontinued in CF, particularly for the treatment of MDR and XDR infections.¹³

Gram-negative bacteria acquire colistin resistance mostly through genomic mutations in regulatory genes causing transcriptional activation of genes responsible for remodelling of LPS, primarily by the covalent addition of 4-amino-4-deoxy-L-arabinose (Ara4N) or phosphoethanolamine (PEtN) groups to lipid A. The resulting positive charge reduces LPS affinity for colistin, leading to resistance.^{14,15} In *P. aeruginosa*, colistin resistance is always associated with the overexpression of the *arn* operon, encoding the enzymes for Ara4N modification of lipid A, whose expression is controlled by a complex regulatory network involving several two-component systems.² Accordingly, mutations within these regulatory systems that lead to constitutive activation of the *arn* operon are typically identified in colistin-resistant *P. aeruginosa* isolates.^{16–19} The crucial role of lipid A aminoarabinylation in the acquisition of colistin resistance in *P. aeruginosa* was proved definitively by the finding that Ara4N-defective mutants, in both reference and clinical isolates, are unable to develop colistin resistance in *in vitro* evolution experiments.^{19,20} This evidence, together with the observations that PEtN modification of lipid A, by either endogenous (*eptA*) or plasmid-harboured PetN transferase genes (*mcr-1*), has marginal effects on colistin resistance in *P. aeruginosa*,^{21,22} strongly supports the notion that the pharmacological inhibition of the Ara4N biosynthetic pathway could represent a suitable approach to extend the clinical lifetime of colistin for the treatment of *P. aeruginosa* infections.

ArnT is the integral cytoplasmic membrane enzyme responsible for the last committed step of lipid A aminoarabinylation, i.e. the attachment of Ara4N to the phosphate group(s) of lipid A.²³ Here, a docking-based virtual screening of an in-house library of natural products within the catalytic site of ArnT was carried out to identify potential inhibitors of Ara4N-dependent colistin resistance. This led to identification of the compound BBN149, a diterpene isolated from the leaves of *Fabiana densa* var. *ramulosa* (Solanaceae),^{24,25} able to potentiate colistin activity against colistin-resistant *P. aeruginosa* isolates, without affecting growth *per se* and with no activity on colistin-susceptible strains. This compound was also

effective against colistin-resistant clinical isolates of *K. pneumoniae*. MS analysis revealed only a partial reduction in the lipid A aminoarabinylation levels of treated cells, suggesting that BBN149 could also have additional effect(s) besides ArnT inhibition.

Materials and methods

Bacterial strains, culture media and chemicals

The bacterial strains used in this study are listed in Table S1 (available as [Supplementary data](#) at JAC Online). Mueller–Hinton broth (MH, Difco) was used for all bacterial assays. Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Milan, Italy). All the tested compounds are known structures belonging to our in-house library of natural products (Table S2).^{26,27} Chemical identity of the compounds was re-assessed by nuclear magnetic resonance (NMR) and proved to agree with literature data (see [Supplementary data](#) for details). Compound purity was checked by reversed-phase HPLC and was always >95%.

Virtual screening for ArnT inhibitors

The crystallographic structure of ArnT bound to undecaprenyl phosphate (Protein Data Bank ID: 5F15)²³ was used as a rigid receptor in molecular docking simulations. Docking was carried out with FRED 3.0.1 (OpenEye Scientific Software, <http://www.eyesopen.com>), using the default settings and the highest docking resolution.²⁸ The Chemgauss4 scoring function implemented in the FRED docking program was used to rank the compounds of the in-house library. The library was prepared for docking simulations with OMEGA 3.0.1.2 (OpenEye Scientific Software),²⁹ while the ligand ionization state was assigned by QUACPAC 1.6.3.1 (OpenEye Scientific Software). Chemical diversity of ligands was assessed as described previously.³⁰

In vitro screening assay for colistin adjuvants

P. aeruginosa PA14 col^R 5 was cultured in MH for 8 h and then diluted at a concentration of $\sim 5 \times 10^5$ cfu/mL in fresh MH with or without 8 mg/L colistin and a fixed concentration (50 μM), or increasing concentrations, of each putative ArnT inhibitor (or equivalent amounts of DMSO as control) in 96-well microtitre plates (200 μL volume per well). Growth (OD_{600}) was measured in a Wallac 1420 Victor3 V multilabel plate reader (Perkin Elmer) after 24 h at 37°C under static conditions and expressed as the percentage of growth with respect to the control wells containing the equivalent concentration of DMSO (corresponding to 100%).

MIC assays

Bacterial strains were cultured in MH for 8 h and then diluted to a concentration of $\sim 5 \times 10^5$ cfu/mL in fresh MH containing 30 μM BBN149 (or 0.3% DMSO as the control) and serial 2-fold dilutions of colistin (starting from 128 mg/L or 8 mg/L for colistin-resistant or colistin-susceptible isolates, respectively) in microtitre plates (200 μL volume per well). MIC was determined by naked eye as the lowest concentration of colistin able to inhibit bacterial growth after 24 h at 37°C under static conditions. Each strain was tested in at least three independent experiments. The same protocol was employed to assess the effect of BBN149 on the activity of ofloxacin, gentamicin or meropenem (antibiotic concentration range: 16–0.03 mg/L).

Chequerboard assay

Fifty microlitres each of 2-fold serial dilutions of BBN149 (0–500 μM) and colistin (0–512 mg/L) in MH were perpendicularly dispensed in 96-well microtitre plates and each well was inoculated with 100 μL of MH containing *P. aeruginosa* PA14 col^R 5 at $\sim 10^6$ cfu/mL, precultured in MH until

mid-exponential phase. Microtitre plates were incubated at 37°C under static conditions for 24 h and bacterial growth was visually assessed.

Time–kill assays

Exponential-phase bacterial cultures in MH were diluted in the same medium at 3×10^5 – 6×10^5 cfu/mL in the presence of 30 μ M BBN149, or 0.3% DMSO as control, and different colistin concentrations. Bacterial cultures were incubated at 37°C and, at different timepoints, serial dilutions were prepared in saline and plated on MH agar plates.

Cytotoxicity assay

Cytotoxicity was assessed as previously described,³¹ with a few modifications. Briefly, the bronchial epithelial cell line 16HBE was cultivated as previously reported^{32,33} and used at 3×10^5 cells/well in 96-well microtitre plates. Twenty hours after seeding, fresh medium containing serial 2-fold dilutions of BBN149 (starting from 125 to 4 μ M), or equivalent concentrations of DMSO as control, was added to each well (200 μ L volume per well). After 3 or 18 h at 37°C, MTT was added at the concentration of 0.5 g/L and the cells were incubated for 3 h at 37°C. Culture supernatant was then discarded, 100 μ L of DMSO was added to each well and absorbance at 570 nm (A_{570}) was read using a microtitre plate reader (Bio-Rad Novapath™ microplate reader). Cell viability was expressed as a percentage with respect to the control wells containing the equivalent concentration of DMSO (corresponding to 100%).

Lipid A extraction and analysis

Lipid A was extracted from bacterial cell pellets using the ammonium hydroxide-isobutyric acid-based procedure,²² with previously described modifications.³⁴ Samples were analysed in a MALDI-TOF plate (TOF/TOF 5800 System, Sciex, Ontario, Canada) in the negative-ion mode with reflectron mode. Calibration and spectral data analysis were performed as described.³⁴

Gene expression analysis

The expression level of the *arnB*, *arnT* and *arnF* genes was determined by quantitative RT–PCR as described,³⁵ using the primer pairs listed in Table S3. Relative gene expression with respect to the housekeeping gene *rpoD* was determined using the $2^{-\Delta\Delta Ct}$ method.³⁶

Statistical analysis

Statistical analysis was performed with the software GraphPad InStat, using one-way analysis of variance (ANOVA).

Results

Virtual screening-mediated identification of putative ArnT inhibitors

Recently, the crystal structure of ArnT, i.e. the integral membrane enzyme responsible for Ara4N attachment to lipid A, has been solved and its catalytic mechanism has been proposed based on functional mutagenesis experiments.²³ In an attempt to identify potential inhibitors of Ara4N-dependent colistin resistance, we carried out a docking-based virtual screening of an in-house chemical library with the catalytic site of ArnT. Briefly, the library contains about 1000 natural compounds from different classes, mostly isolated from plants used in the traditional medicine of South America. Besides natural products, the library has been recently enlarged with natural products from commercially available

sources and synthetic and semisynthetic compounds.^{26,27} By virtual screening, compounds were ranked based on their predicted binding mode and theoretical affinity within the ArnT catalytic site and further filtered by chemical diversity criteria, leading to the final selection of 18 candidate hits (Table S2) that were submitted to biological investigations.

In vitro validation of BBN149 as colistin adjuvant

Compounds identified by virtual screening were subjected to biological validation by using an *in vitro*-evolved colistin-resistant isolate of *P. aeruginosa* PA14 (PA14 col^R 5, colistin MIC = 64 mg/L), which overexpresses the *arn* operon responsible for lipid A aminoarabinylation (Figure S1).²⁰ First, in order to verify whether the compounds selected *in silico* were able to reduce or abrogate the growth of the reference strain PA14 col^R 5, they were tested at a fixed concentration (50 μ M) in the presence of a subinhibitory concentration of colistin (8 mg/L). Three compounds (BBN120, BBN149 and BBN153) showed significant inhibition (>60%) of PA14 col^R 5 growth in the presence of colistin, without notable effects on growth in the absence of the antibiotic (Figure 1a). None of the other compounds showed relevant inhibitory activity on either colistin-treated or untreated cells (Figure 1a).

For the three hit compounds, we then determined the dose-dependent effect on PA14 col^R 5 growth in the presence of 8 mg/L colistin. BBN120 and BBN153 caused a significant growth reduction at concentrations ≥ 8 μ M, although they did not completely inhibit bacterial growth at any concentrations tested. In contrast, BBN149 significantly inhibited PA14 col^R 5 growth at 8–16 μ M and completely abrogated it at concentrations ≥ 31 μ M (Figure 1b). In agreement with the screening results, we confirmed that BBN149 has no inhibitory activity *per se*, as demonstrated by its marginal effect on PA14 col^R 5 cultured in the absence of colistin, even at the highest concentration tested (125 μ M) (Figure S2). The potentiating effect of BBN149 on colistin activity was also corroborated by checkerboard assays, which showed a dose-dependent reduction of colistin MIC for PA14 col^R 5 in the presence of increasing concentrations of BBN149 (Figure 1c). Colistin MIC decreased to the lowest level (8 mg/L, corresponding to an 8-fold reduction) with 31 μ M BBN149 and remained stable at higher BBN149 concentrations (up to 125 μ M; Figure 1c).

Overall, these results demonstrate that one of the compounds identified by *in silico* docking of ArnT, namely BBN149, actually potentiates colistin activity against a colistin-resistant *P. aeruginosa* strain.

Predicted binding mode of BBN149 to ArnT

BBN149 corresponds to *ent*-beyer-15-en-18-*O*-oxalate, a natural diterpenoid featuring an oxalate side chain (Figure 2a and Table S2).^{24,25} The binding mode of BBN149 within the catalytic site of ArnT was investigated by molecular docking simulations carried out with the FRED docking program (OpenEye). Two main binding poses were identified (Figure 2b and c), one extending towards the putative binding site of lipid A and the other occupying the undecaprenyl phosphate binding site with very good overlapping with the crystallographic ligand (Figure S3).²³ Notably, these poses are endowed with a comparable docking score as calculated by the Chemgauss4 function (–4.98 and –5.05, respectively) and might

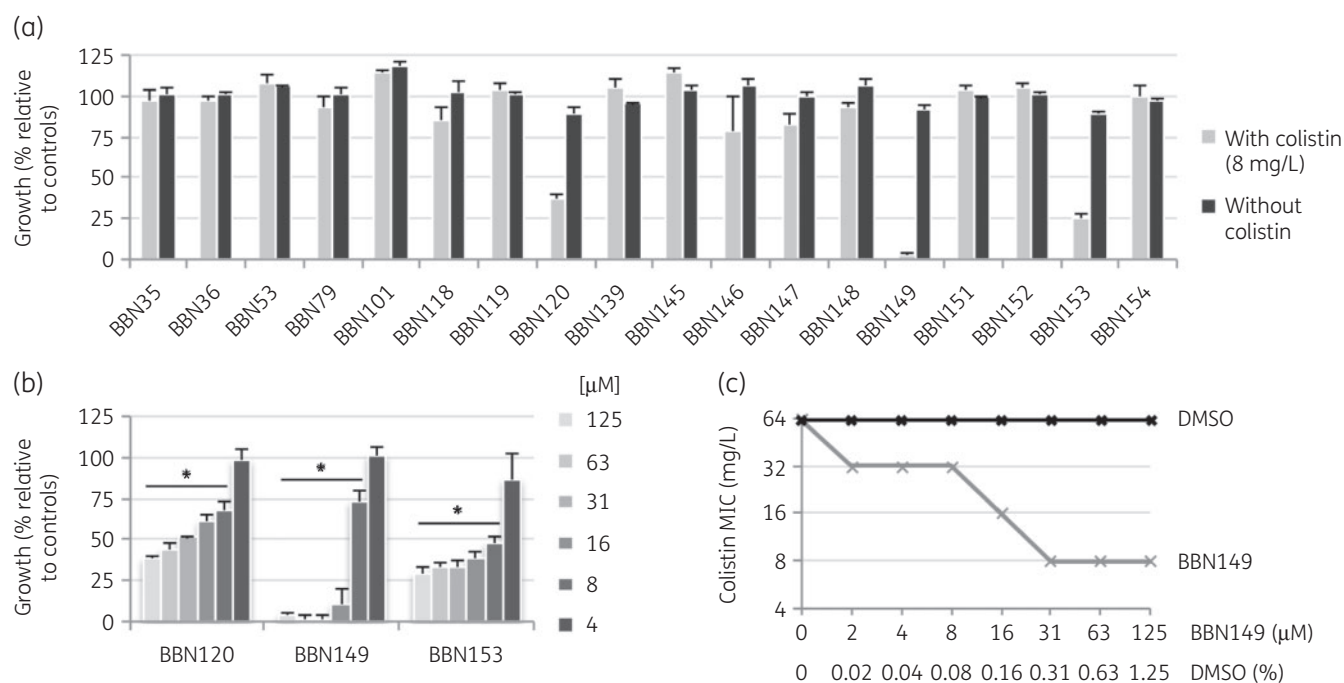


Figure 1. Validation of BBN149 as a colistin resistance inhibitor. (a) Effect of the 18 putative ArnT inhibitors identified by *in silico* docking at 50 μM on the growth of the colistin-resistant isolate *P. aeruginosa* PA14 col^R 5 after 24 h at 37°C in MH supplemented or not with a sub-MIC concentration of colistin (8 mg/L). (b) Dose-dependent effect of BBN120, BBN149 and BBN153 on PA14 col^R 5 growth after 24 h at 37°C in MH supplemented with 8 mg/L colistin. In panels (a) and (b), growth values are expressed as the percentage relative to the cultures treated with equivalent concentrations of DMSO and represent the mean (\pm SD) of at least three independent experiments. (c) Effect of different concentrations of BBN149, and DMSO as control, on the MIC of colistin for PA14 col^R 5 as determined by chequerboard assays. The graph is representative of four independent experiments. * $P < 0.05$ (ANOVA).

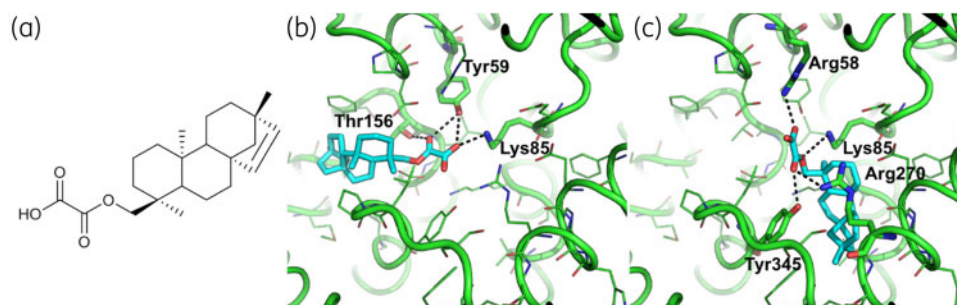


Figure 2. (a) Chemical structure of the diterpenoid *ent*-beyer-15-en-18-*O*-oxalate (BBN149). (b, c) Predicted binding mode of BBN149 to the catalytic site of the ArnT crystallographic structure. Two possible docking poses are shown in the two panels. The ligand is coloured cyan and shown as sticks, while the protein is coloured green. Residues within 5 Å from the ligand are shown as lines; those predicted to form H-bonds with BBN149 are shown as sticks and labelled. H-bond interactions are highlighted by black dashed lines. This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.

account for the inhibitory effect observed *in vitro*. In both poses, the oxalyl group nicely overlaps with the crystallographic phosphate moiety. In the case of BBN149 extending towards the lipid A binding site (Figure 2b), H-bond interactions are established with Tyr59, Lys85 and Thr156 while the lipophilic part of the molecule is well inserted in the hydrophobic cavity that projects towards the transmembrane region of the receptor. In the alternative pose, the oxalyl group establishes H-bond interactions with Arg58, Lys85, Arg270 and Tyr345, while the lipophilic part fills the undecaprenyl phosphate binding site (Figure 2c).

Notably, in both poses the H-bond interaction is predicted with the Lys85 residue, which was shown to be important for ArnT activity.^{23,37}

Range of activity, specificity and cytotoxicity of BBN149

To verify the specificity of BBN149 towards colistin resistance, the MIC of colistin for the colistin-susceptible parental strain PA14 was determined in the presence and absence of a BBN149 concentration active against PA14 col^R 5 (30 μM). Notably, BBN149 did not

decrease the colistin MIC for PA14 (Table 1), suggesting that the compound may exert adjuvant activity only on colistin-resistant isolates. This hypothesis was confirmed by testing three other *in vitro*-evolved colistin-resistant mutants of *P. aeruginosa* together with their parental CF isolates (Table S1).²⁰ While BBN149 efficiently reduced the MIC of colistin for all resistant strains, it did not potentiate colistin activity against their colistin-susceptible

Table 1. Colistin MIC for different bacterial strains in the presence of 30 μ M BBN149 or 0.3% DMSO as the control

Species	Strain	Colistin MIC (mg/L)	
		BBN149	DMSO
<i>P. aeruginosa</i>	PA14 col ^R 5	8	64
	PA14	1	0.5
	KK1 col ^R 1	8	128
	KK1	1	0.5
	KK27 col ^R 6	4	64
	KK27	1	0.5
	TR1 col ^R 6	4	16
	TR1	1	0.5
	ND76	4	32
	MG75	8	32
<i>K. pneumoniae</i>	KP-Mo-3	16	128
	KP-Mo-5	8	128
	KP-Mo-6	4	32
	KP-Mo-11	8	64
	KP-Mo-16	8	64
	KP-Mo-26	0.5	0.25
	KP-Mo-27	0.25	0.25
<i>A. baumannii</i>	5615	16	32
	12316	16	16
	12384	4	4
<i>E. coli</i>	4451 (<i>mcr-1</i>)	4	4
	4531 (<i>mcr-1</i>)	4	8
	4592 (<i>mcr-1</i>)	4	4

parental isolates (Table 1). Indeed, for all colistin-susceptible isolates (PA14 and the three CF isolates) we observed a slight (2-fold) increase in colistin MIC in the presence of BBN149 (Table 1); however, this remained less than or equal to the breakpoint for colistin-susceptible *P. aeruginosa* (2 mg/L).³⁸ Finally, the potentiating effect of BBN149 on colistin activity was also observed in two colistin-resistant clinical isolates, namely ND76 and MG75 (Table 1), isolated from the sputum of chronically infected CF patients (Table S1), indicating that BBN149 is also effective against colistin-resistant strains evolved *in vivo* during the infection. As previously observed for PA14 col^R 5, BBN149 alone had no inhibitory effect on the growth of any *P. aeruginosa* isolate (Figure S4). Finally, time-kill assays performed on a selection of *P. aeruginosa* colistin-resistant strains revealed that the combination of 30 μ M BBN149 with colistin is mainly bacteriostatic at 1 \times MIC of colistin and bactericidal at 2 \times MIC (Figure 3).

To rule out the adjuvant activity of BBN149 being due to a general destabilizing effect on the *P. aeruginosa* cell envelope, we assessed whether this compound also affects the MIC of other antibiotics with different mechanisms of action and intracellular targets, i.e. ofloxacin, gentamicin and meropenem. BBN149 did not cause any relevant variation in the susceptibility to these antibiotics, either in the parental strain PA14 or in its derivative PA14 col^R 5 (Table S4), indicating that BBN149 does not simply perturb the permeability barrier of the cell envelope, both in colistin-susceptible and colistin-resistant strains.

To verify whether BBN149 can also potentiate colistin activity against other Gram-negative pathogens, we assessed its ability to counteract colistin resistance in a panel of colistin-resistant *K. pneumoniae* clinical strains. These strains belonged to different STs and carried loss-of-function mutations in *mgrB*, encoding a repressor of the *arn* operon responsible for lipid A aminoarabinylation.³⁹ BBN149 at 30 μ M strongly reduced the colistin MIC for all the colistin-resistant *K. pneumoniae* strains, while it did not increase colistin activity against the colistin-susceptible KP-Mo-26 and KP-Mo-27 isolates (Table 1), implying that the inhibitory activity of this compound on colistin resistance is not restricted to *P. aeruginosa*. Time-kill and growth assays confirmed that in *K. pneumoniae* the BBN149/colistin combination is also bactericidal

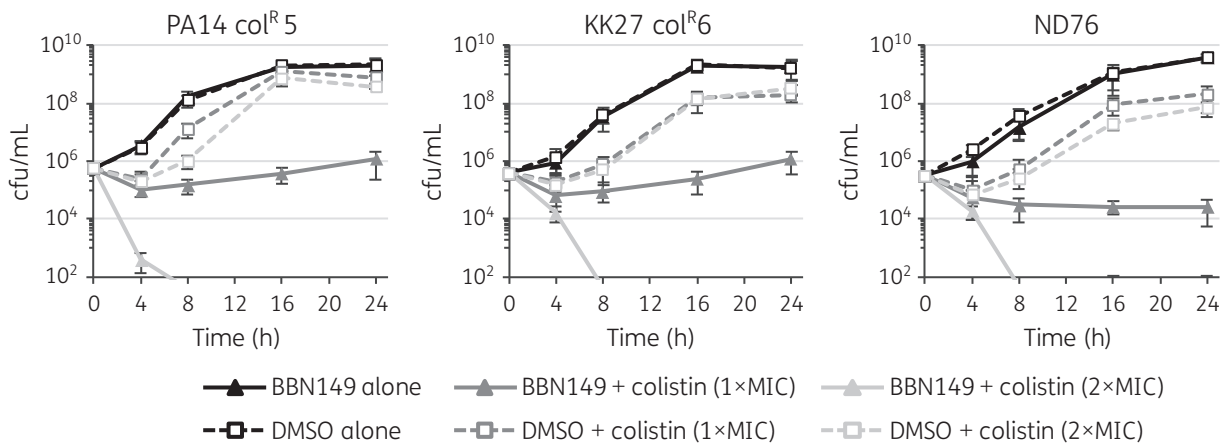


Figure 3. Time-kill curves of *P. aeruginosa* PA14 col^R 5, KK27 col^R 6 and ND76 exposed to 30 μ M BBN149 in the presence or absence of colistin at 1 \times or 2 \times MIC (based on the values reported in Table 1). As a control, the strains were incubated in the presence of 0.3% DMSO and the same concentrations of colistin. The results are the mean (\pm SD) of two independent assays.

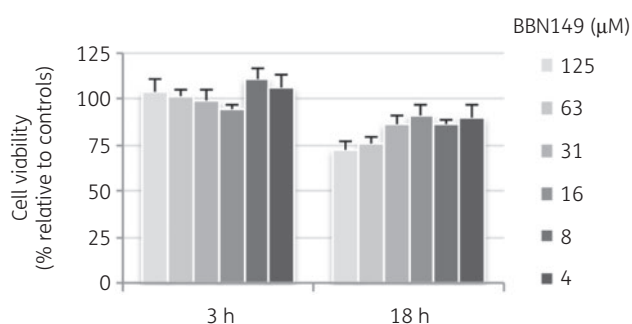


Figure 4. Viability of 16HBE epithelial cells exposed to BBN149 at the indicated concentrations for 3 or 18 h. Viability was assessed through the MTT assay and expressed as a percentage relative to vehicle-only (DMSO) controls. Data are the mean (\pm SD) of three independent experiments.

at $2 \times$ MIC of colistin (Figure S5) and that BBN149 has no inhibitory effect in the absence of colistin (Figure S4).

Interestingly, BBN149 had only minor or no adjuvant activity against *Escherichia coli* carrying the *mcr-1* gene or colistin-resistant *A. baumannii* (Table 1), in which colistin resistance is based on phosphoethanolamine modification of lipid A,² further supporting the hypothesis that the compound has specificity towards aminoarabinose-mediated colistin resistance.

To evaluate cytocompatibility of BBN149, with a particular focus on lung therapy, cytotoxicity was tested in the bronchial epithelial cells 16HBE at two timepoints (3 and 18 h) by the MTT assay. BBN149 did not affect cell viability at any concentration after 3 h while, after 18 h, a slight decrease in cell viability (about 25%) was observed for the highest concentrations tested (63–125 μ M) (Figure 4).

Effect of BBN149 on lipid A aminoarabinylation

BBN149 was identified by *in silico* screening as a potential inhibitor of the integral membrane enzyme ArnT, which catalyses the attachment of Ara4N to lipid A.²³ To evaluate whether BBN149 actually interferes with lipid A aminoarabinylation we compared, by MS, the lipid A profile of three different colistin-resistant *P. aeruginosa* strains cultured in the presence or absence of BBN149. As shown in Figure 5, lipid A spectra of colistin-resistant isolates were characterized by some peaks corresponding to aminoarabinylation of lipid A species. In line with previous reports,^{19,40,41} the level of lipid A aminoarabinylation was only partial, with most lipid A still present as non-aminoarabinylation forms. Overall, bacteria cultured with BBN149 showed slightly reduced levels of aminoarabinylation of lipid A, although the extent of such reduction appeared to be strain-dependent, being much more pronounced in the CF isolate TR1 col^R 6 compared with the reference strain PA14 col^R 5 or the other CF isolate KK27 col^R 6 (Figure 5).

Discussion

Given the growing importance of colistin as a last-resort option for the treatment of MDR Gram-negative bacterial infections, many groups have recently attempted to identify compounds capable of potentiating this antibiotic and/or restoring its activity against colistin-resistant isolates. In recent years, several colistin adjuvants have been identified, mainly by empirical screening of

compound libraries.^{43–47} However, examples of rational design of potential colistin adjuvants also exist. For instance, Ara4N analogues have been tested for inhibition of lipid A aminoarabinylation on purified membranes, but not for antibacterial activity on whole cells,⁴⁸ and eukaryotic kinase inhibitors have been repurposed to inhibit the two-component systems that trigger the expression of the lipid A modification genes involved in colistin resistance.⁴⁹ Here, a unique in-house library of natural products was screened *in silico* against the catalytic site of the ArnT enzyme, leading to the identification of promising colistin adjuvant activity in the natural diterpenoid BBN149. Some evidence supports the notion that BBN149 could specifically act on colistin resistance mechanism(s). First, it significantly potentiates colistin activity against all colistin-resistant isolates tested, without exerting growth inhibitory activity *per se* and without relevant effects on colistin-susceptible strains (Figure 1 and Table 1). Moreover, BBN149 does not potentiate the activity of other antibiotics with different modes of action (Table S4), thus ruling out that it non-specifically affects the outer membrane permeability barrier. Regarding the mechanism of action, although molecular docking predicted that BBN149 might fit the catalytic cavity of ArnT with steric overlap with the binding mode of lipid A or the undecaprenyl phosphate (Figure 2), we observed only slight inhibition of lipid A aminoarabinylation in BBN149-treated *P. aeruginosa* cells (Figure 4). This could be explained by at least three non-mutually exclusive hypotheses. First, it is possible that BBN149 also has other targets and/or activities besides ArnT inhibition. Considering the observed specificity of BBN149 for colistin-resistant cells, these side activities should, however, affect mechanism(s) involved in colistin resistance rather than essential cellular functions. Since BBN149 has to reach the periplasmic space to exert its inhibitory activity on the inner membrane protein ArnT,²³ another possibility is that this compound requires the membrane-permeabilizing effect of colistin⁵⁰ to efficiently cross the outer membrane and bind to its cellular target. This would explain why BBN149 can synergize with colistin in growth inhibition assays but exerts a low inhibitory effect on lipid A aminoarabinylation when tested alone. An alternative hypothesis can be formulated if the lipid A profile of colistin-resistant isolates is considered. Indeed, our study and other studies have shown that the lipid A is only partially aminoarabinylation in colistin-resistant *P. aeruginosa* isolates (Figure 4).^{19,40,41} This implies that minor variations in aminoarabinylation of lipid A levels could have a profound impact on colistin resistance and, on the other hand, that functionally relevant reductions in lipid A aminoarabinylation may not always be easy to appraise. This would be in line with the finding that BBN149-mediated inhibition of lipid A aminoarabinylation was much more evident in a strain with relatively high amounts of aminoarabinylation of lipid A compared with strains with lower lipid A aminoarabinylation levels (Figure 4). Additional biochemical and/or genetic assays are required to verify these hypotheses and to further characterize the mechanism of action and ArnT-binding properties of BBN149.

Notably, BBN149 is not able to revert colistin-resistant isolates to susceptibility, as the colistin MIC for colistin-resistant *P. aeruginosa* and *K. pneumoniae* treated with BBN149 was ≥ 4 mg/L (Table 1), which is slightly higher than the colistin breakpoint for both species (2 mg/L).³⁸ However, clinical breakpoints are established on the basis of the plasma colistin concentrations that can be reached by IV administration with a low risk of side effects (mainly

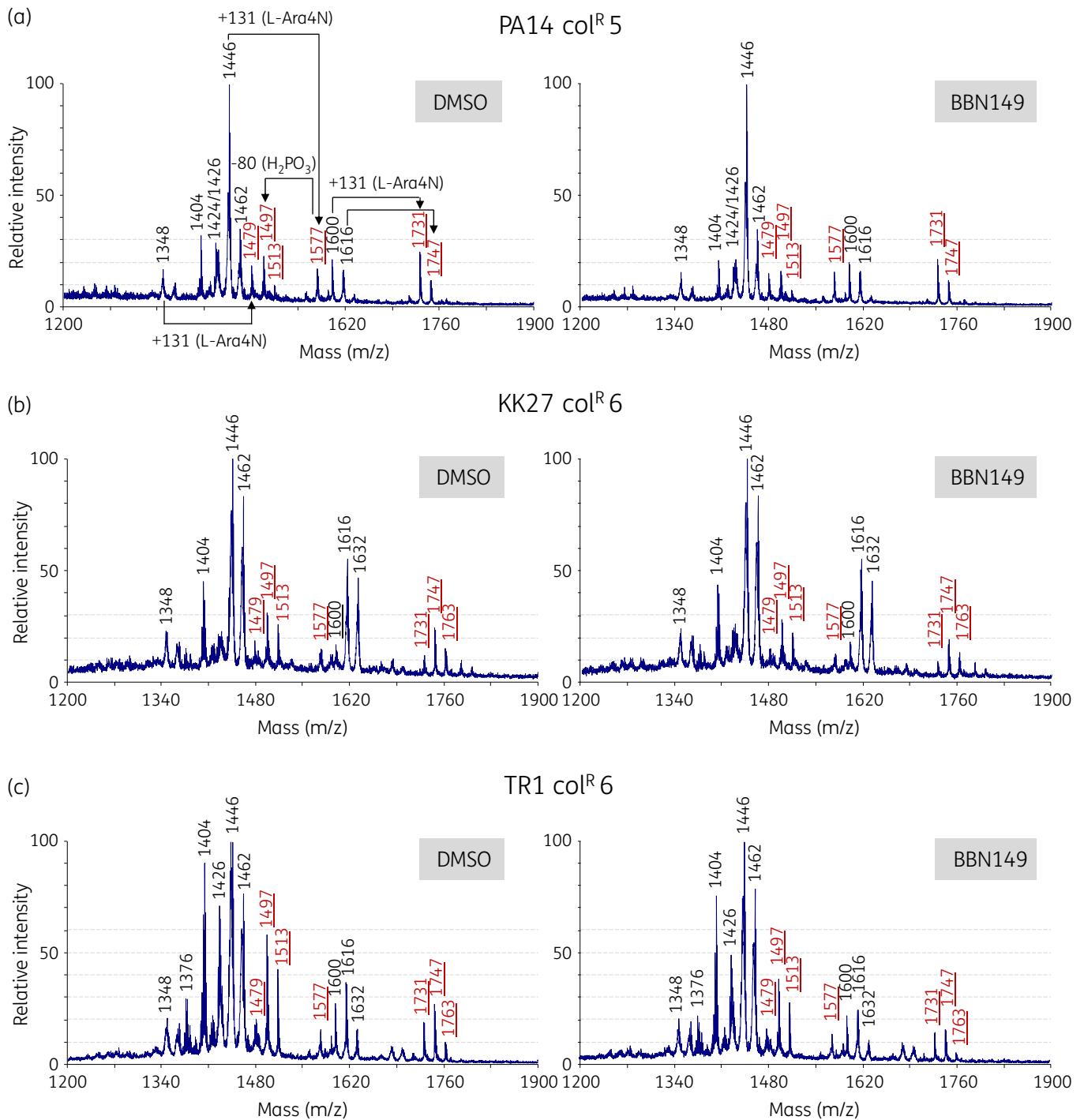


Figure 5. Effect of BBN149 on lipid A aminoarabinoacylation. MALDI-TOF analysis of lipid A extracted from *P. aeruginosa* PA14 col^R 5 (a), KK27 col^R 6 (b) or TR1 col^R 6 (c) cultured in MH supplemented with 30 μ M BBN149 or 0.3% DMSO as control. In (a) arrows indicate the addition of an aminoarabino molecule (L-Ara4N; $m/z + 131$) or the removal of a phosphate group ($m/z - 80$) from the penta-acylated lipid A ($m/z = 1446$), the hexa-acylated lipid A ($m/z = 1616$) or a still unidentified lipid A form at $m/z = 1348$. Lipid A peaks differing by $m/z \pm 16$ correspond to different hydroxylation states of the secondary C12 acyl chains.⁴² The m/z values of all peaks corresponding to aminoarabinoacylated lipid A forms are in red, bold and underlined. Spectra were obtained in the negative-ion mode, thus m/z values correspond to (molecular mass - 1)/1 and are representative of three biological replicates. This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.

nephrotoxicity).⁵¹ It can therefore be speculated that BBN149 could be effectively used as a colistin adjuvant *in vivo* for the treatment of localized infections, such as wound or pulmonary infections, when higher colistin concentrations can be safely reached. For instance, it has been demonstrated that colistin can accumulate in the lung at high concentrations upon inhalation without relevant toxicity.^{52,53}

In conclusion, this work demonstrates that *in silico* molecular docking for the identification of ArnT inhibitors represents a suitable strategy to identify new colistin adjuvants, complementing a recent study in which the same approach was successfully applied to identify inhibitors of the lipid A phosphoethanolamine transferase MCR-1.⁵⁴ Moreover, the compound identified here, BBN149, represents a promising candidate for lead optimization in order to develop colistin resistance inhibitors with improved activity and/or pharmacological properties.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S5 and Tables S1 to S4 are available as [Supplementary data](#) at JAC Online.

References

- Storm DR, Rosenthal KS, Swanson PE. Polymyxin and related peptide antibiotics. *Annu Rev Biochem* 1977; **46**: 723–63.
- Jeannot K, Bolard A, Plésiat P. Resistance to polymyxins in Gram-negative organisms. *Int J Antimicrob Agents* 2017; **49**: 526–35.
- Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resist Updat* 2010; **13**: 132–8.
- Pedersen MG, Olesen HV, Jensen-Fangel S *et al.* Colistin resistance in *Pseudomonas aeruginosa* and *Achromobacter* spp. cultured from Danish cystic fibrosis patients is not related to plasmid-mediated expression of *mcr-1*. *J Cyst Fibros* 2018; **17**: e22–3.
- Schülin T. *In vitro* activity of the aerosolized agents colistin and tobramycin and five intravenous agents against *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in southwestern Germany. *J Antimicrob Chemother* 2002; **49**: 403–6.
- Poole K. *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol* 2011; **2**: 65.
- Karakonstantis S, Kritsotakis EI, Gikas A. Pandrug-resistant Gram-negative bacteria: a systematic review of current epidemiology, prognosis and treatment options. *J Antimicrob Chemother* 2020; **75**: 271–82.
- Del Porto P, Cifani N, Guarnieri S *et al.* Dysfunctional CFTR alters the bactericidal activity of human macrophages against *Pseudomonas aeruginosa*. *PLoS One* 2011; **6**: e19970.
- Cifani N, Pompili B, Anile M *et al.* Reactive-oxygen-species-mediated *P. aeruginosa* killing is functional in human cystic fibrosis macrophages. *PLoS One* 2013; **8**: e71717.
- Malhotra S, Hayes D Jr, Wozniak DJ. Cystic fibrosis and *Pseudomonas aeruginosa*: the host-microbe interface. *Clin Microbiol Rev* 2019; **32**: e00138–18.
- Folkesson A, Jelsbak L, Yang L *et al.* Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. *Nat Rev Microbiol* 2012; **10**: 841–51.
- Elborn JS. Cystic fibrosis. *Lancet* 2016; **388**: 2519–31.
- Tümmler B. Emerging therapies against infections with *Pseudomonas aeruginosa*. *F1000Res* 2019; **8**: 1371.
- Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 2014; **5**: 643.
- Baron S, Hadjadj L, Rolain JM *et al.* Molecular mechanisms of polymyxin resistance: knowns and unknowns. *Int J Antimicrob Agents* 2016; **48**: 583–91.
- Schurek KN, Sampaio JL, Kiffer CR *et al.* Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2009; **53**: 4345–51.
- Barrow K, Kwon DH. Alterations in two-component regulatory systems of *phoPQ* and *pmrAB* are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2009; **53**: 5150–4.
- Moskowitz SM, Brannon MK, Dasgupta N *et al.* *PmrB* mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother* 2012; **56**: 1019–30.
- Jochumsen N, Marvig RL, Damkjaer S *et al.* The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat Commun* 2016; **7**: 13002.
- Lo Sciuto A, Imperi F. Aminoarabinylation of lipid A is critical for the development of colistin resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2018; **62**: e01820–17.
- Nowicki EM, O'Brien JP, Brodbelt JS *et al.* Extracellular zinc induces phosphoethanolamine addition to *Pseudomonas aeruginosa* lipid A via the ColRS two-component system. *Mol Microbiol* 2015; **97**: 166–78.
- Liu YY, Chandler CE, Leung LM *et al.* Structural modification of lipopolysaccharide conferred by *mcr-1* in Gram-negative ESKAPE pathogens. *Antimicrob Agents Chemother* 2017; **61**: e00580–17.
- Petrou VI, Herrera CM, Schultz KM *et al.* Structures of aminoarabinose transferase ArnT suggest a molecular basis for lipid A glycosylation. *Science* 2016; **351**: 608–12.
- Erazo S, Zaldivar M, Delporte C *et al.* Antibacterial diterpenoids from *Fabiana densa* var. *ramulosa*. *Planta Med* 2002; **68**: 361–3.
- Quaglio D, Corradi S, Erazo S *et al.* Structural elucidation and antimicrobial characterization of novel diterpenoids from *Fabiana densa* var. *ramulosa*. *ACS Med Chem Lett* 2020; **10**: 1021/acsmedchemlett.9b00605.

- 26 Mascarello A, Mori M, Chiaradia-Delatorre LD et al. Discovery of *Mycobacterium tuberculosis* protein tyrosine phosphatase B (PtpB) inhibitors from natural products. *PLoS One* 2013; **8**: e77081.
- 27 Infante P, Alfonsi R, Ingallina C et al. Inhibition of Hedgehog-dependent tumors and cancer stem cells by a newly identified naturally occurring chemotype. *Cell Death Dis* 2016; **7**: e2376.
- 28 McGann M. FRED pose prediction and virtual screening accuracy. *J Chem Inf Model* 2011; **51**: 578–96.
- 29 Hawkins PC, Skillman AG, Warren GL et al. Conformer generation with OMEGA: algorithm and validation using high quality structures from the Protein Databank and Cambridge Structural Database. *J Chem Inf Model* 2010; **50**: 572–84.
- 30 Mori M, Tottone L, Quaglio D et al. Identification of a novel chalcone derivative that inhibits Notch signaling in T-cell acute lymphoblastic leukemia. *Sci Rep* 2017; **7**: 2213.
- 31 Costabile G, d'Angelo I, Rampioni G et al. Toward repositioning niclosamide for antivirulence therapy of *Pseudomonas aeruginosa* lung infections: development of inhalable formulations through nanosuspension technology. *Mol Pharm* 2015; **12**: 2604–17.
- 32 Gruenert DC, Willems M, Cassiman JJ et al. Established cell lines used in cystic fibrosis research. *J Cyst Fibros* 2004; **3** Suppl 2: 191–6.
- 33 De Rocco D, Pompili B, Castellani S et al. Assembly and functional analysis of an S/MAR based episome with the cystic fibrosis transmembrane conductance regulator gene. *Int J Mol Sci* 2018; **19**: E1220.
- 34 Lo Sciuto A, Martorana AM, Fernández-Piñar R et al. *Pseudomonas aeruginosa* LptE is crucial for LptD assembly, cell envelope integrity, antibiotic resistance and virulence. *Virulence* 2018; **9**: 1718–33.
- 35 Visaggio D, Pasqua M, Bonchi C et al. Cell aggregation promotes pyoverdine-dependent iron uptake and virulence in *Pseudomonas aeruginosa*. *Front Microbiol* 2015; **6**: 902.
- 36 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001; **25**: 402–8.
- 37 Tavares-Carreón F, Patel KB, Valvano MA. *Burkholderia cenocepacia* and *Salmonella enterica* ArnT proteins that transfer 4-amino-4-deoxy-L-arabinose to lipopolysaccharide share membrane topology and functional amino acids. *Sci Rep* 2015; **5**: 10773.
- 38 EUCAST. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0. 2019. <http://www.eucast.org>.
- 39 Esposito EP, Cervoni M, Bernardo M et al. Molecular epidemiology and virulence profiles of colistin-resistant *Klebsiella pneumoniae* blood isolates from the hospital agency “Ospedale dei Colli,” Naples, Italy. *Front Microbiol* 2018; **9**: 1463.
- 40 Lo Sciuto A, Cervoni M, Stefanelli R et al. Effect of lipid A aminoarabinylation on *Pseudomonas aeruginosa* colistin resistance and fitness. *Int J Antimicrob Agent* 2020; 105957: doi:10.1016/j.ijantimicag.2020.105957.
- 41 Lee JY, Park YK, Chung ES et al. Evolved resistance to colistin and its loss due to genetic reversion in *Pseudomonas aeruginosa*. *Sci Rep* 2016; **6**: 25543.
- 42 Lo Sciuto A, Cervoni M, Stefanelli R et al. Genetic basis and physiological effects of lipid A hydroxylation in *Pseudomonas aeruginosa* PAO1. *Pathogens* 2019; **8**: E291.
- 43 Barker WT, Martin SE, Chandler CE et al. Small molecule adjuvants that suppress both chromosomal and *mcr-1* encoded colistin-resistance and amplify colistin efficacy in polymyxin-susceptible bacteria. *Bioorg Med Chem* 2017; **25**: 5749–53.
- 44 Minrovic BM, Jung D, Melander RJ et al. New class of adjuvants enables lower dosing of colistin against *Acinetobacter baumannii*. *ACS Infect Dis* 2018; **4**: 1368–76.
- 45 Barker WT, Chandler CE, Melander RJ et al. Tryptamine derivatives disarm colistin resistance in polymyxin-resistant gram-negative bacteria. *Bioorg Med Chem* 2019; **27**: 1776–88.
- 46 Domalaon R, De Silva PM, Kumar A et al. The anthelmintic drug niclosamide synergizes with colistin and reverses colistin resistance in gram-negative bacilli. *Antimicrob Agents Chemother* 2019; **63**: e02574–18.
- 47 Zhou Y, Wang J, Guo Y et al. Discovery of a potential MCR-1 inhibitor that reverses polymyxin activity against clinical *mcr-1*-positive Enterobacteriaceae. *J Infect* 2019; **78**: 364–72.
- 48 Kline T, Trent MS, Stead CM et al. Synthesis of and evaluation of lipid A modification by 4-substituted 4-deoxy arabinose analogs as potential inhibitors of bacterial polymyxin resistance. *Bioorg Med Chem Lett* 2008; **18**: 1507–10.
- 49 Barker WT, Nemeth AM, Brackett SM et al. Repurposing eukaryotic kinase inhibitors as colistin adjuvants in Gram-negative bacteria. *ACS Infect Dis* 2019; **5**: 1764–71.
- 50 Brennan-Krohn T, Pironti A, Kirby JE. Synergistic activity of colistin-containing combinations against colistin-resistant Enterobacteriaceae. *Antimicrob Agents Chemother* 2018; **62**: e00873–18.
- 51 Karaiskos I, Souli M, Galani I et al. Colistin: still a lifesaver for the 21st century? *Expert Opin Drug Metab Toxicol* 2017; **13**: 59–71.
- 52 Antoniu SA, Cojocar I. Inhaled colistin for lower respiratory tract infections. *Expert Opin Drug Deliv* 2012; **9**: 333–42.
- 53 Gurjar M. Colistin for lung infection: an update. *J Intensive Care* 2015; **3**: 3.
- 54 Lan XJ, Yan HT, Lin F et al. Design, synthesis and biological evaluation of 1-phenyl-2-(phenylamino) ethanone derivatives as novel MCR-1 inhibitors. *Molecules* 2019; **24**: E2719.