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A novel colistin adjuvant identified by virtual screening for ArnT inhibitors

Francesca Ghirga[1,†], Roberta Stefanelli[2,3,†], Luca Cavinato[2], Alessandra Lo Sciuto[3], Silvia Corradi[1,4], Deborah Quaglio[4], Andrea Calcaterra[4], Bruno Casciaro[1], Maria Rosa Loffredo[5], Floriana Cappiello[5], Patrizia Morelli[6], Alberto Antonelli[7,8], Gian Maria Rossolini[7,8], Marialuisa Mangoni[5], Carmine Mancone[9], Bruno Botta[4], Mattia Mori[10,‡], Fiorentina Ascenzioni [2,*,‡] and Francesco Imperi[3]

[1]Center for Life Nano Science@Sapienza, Italian Institute of Technology, Rome, Italy; [2]Department of Biology and Biotechnology Charles Darwin, Sapienza University of Rome, Laboratory affiliated to Pasteur Italia-Fondazione Cenci Bolognetti, Rome, Italy; [3]Department of Sciences, 'Department of Excellence 2018–2022', Roma Tre University, Rome, Italy; [4]Department of Chemistry and Technology of Drugs, 'Department of Excellence 2018–2022', Sapienza University of Rome, Rome, Italy; [5]Department of Biochemical Sciences, Sapienza University of Rome, Laboratory affiliated to Pasteur Italia-Fondazione Cenci Bolognetti, Rome, Italy; [6]Microbiology Laboratory, Giannina Gaslini Institute, Genoa, Italy; [7]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy; [8]Clinical Microbiology and Virology Unit, Florence Careggi University Hospital, Florence, Italy; [9]Department of Molecular Medicine, 'Department of Excellence 2018–2022', Sapienza University of Rome, Rome, Italy; [10]Department of Biotechnology, Chemistry and Pharmacy, 'Department of Excellence 2018–2022', University of Siena, Siena, Italy

*Corresponding author. E-mail: fiorentina.ascenzioni@uniroma1.it
†These authors equally contributed to the work.
‡These authors contributed equally to the work.

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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 aminoarabinosylation, 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 aminoarabinosylation 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 aminoarabinosylation 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 aminoarabinosylation 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 aminoarabinosylation, 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 aminoarabinosylation 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 $\sim 5\times10^5$ 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 $\sim 5\times10^5$ 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 μM 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 μM), or equivalent concentrations of DMSO as control, was added to each well (200 μL 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 μL 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 aminoarabinosylation (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 μM) 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 μM, 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 μM and completely abrogated it at concentrations ≥31 μM (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 μM) (Figure S2). The potentiating effect of BBN149 on colistin activity was also corroborated by chequerboard 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 μM BBN149 and remained stable at higher BBN149 concentrations (up to 125 μM; 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

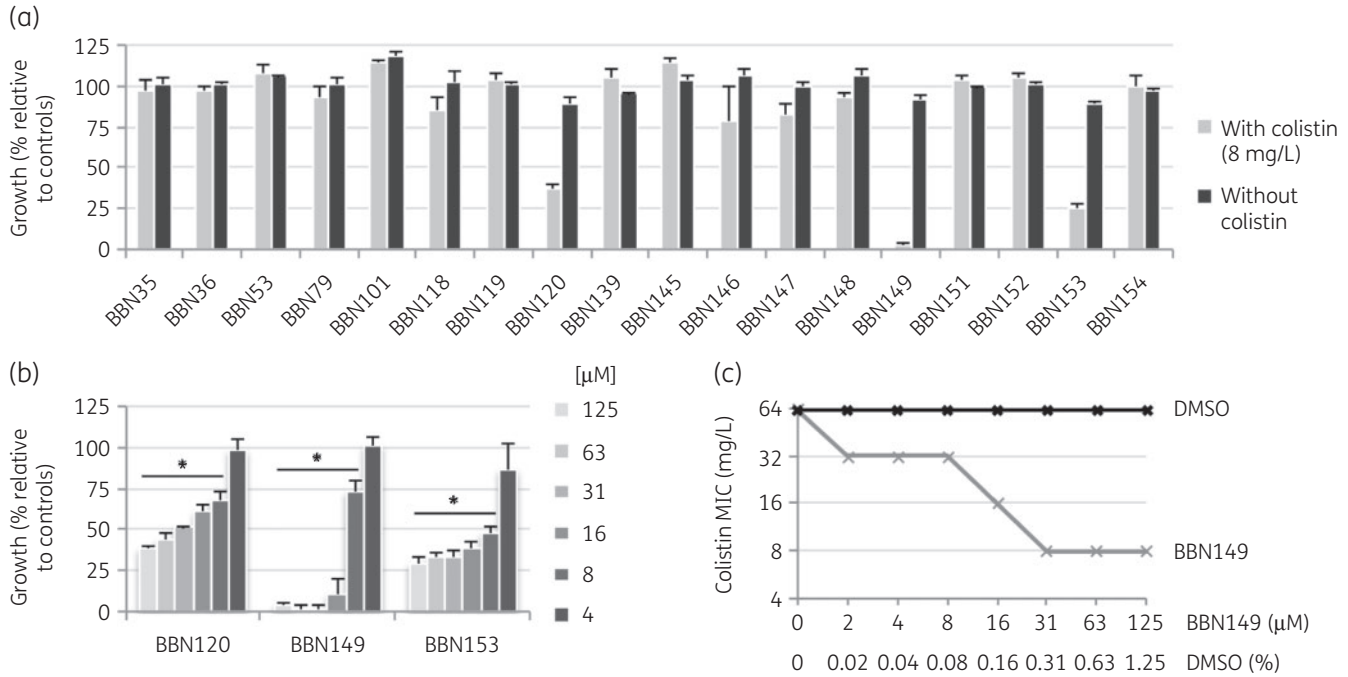

Figure 1. Validation of BBN149 as a colistin resistance inhibitor. (a) Effect of the 18 putative ArnT inhibitors identified by *in silico* docking at 50 μM on the growth of the colistin-resistant isolate *P. aeruginosa* PA14 col^R 5 after 24 h at 37°C in MH supplemented or not with a sub-MIC concentration 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 (±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).

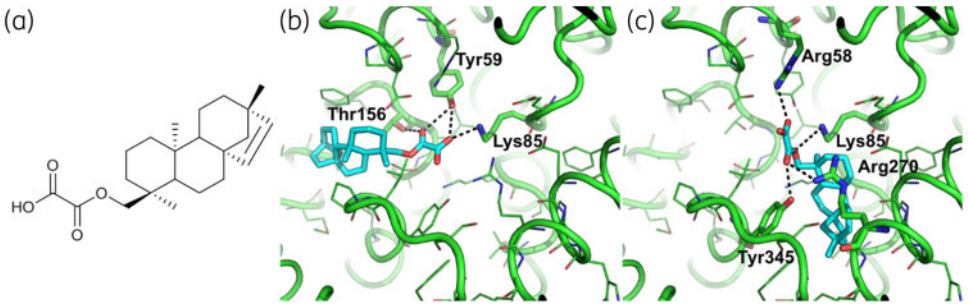

Figure 2. (a) Chemical structure of the diterpenoid *ent*-beyer-15-en-18-*O*-oxalate (BBN149). (b, c) Predicted binding mode of BBN149 to the catalytic site of the ArnT crystallographic structure. Two possible docking poses are shown in the two panels. The ligand is coloured cyan and shown as sticks, 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 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 μM BBN149 with colistin is mainly bacteriostatic at 1× MIC of colistin and bactericidal at 2× MIC (Figure 3).

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

		Colistin MIC (mg/L)	
Species	Strain	BBN149	DMSO
P. aeruginosa	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
K. pneumoniae	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
A. baumannii	5615	16	32
	12316	16	16
	12384	4	4
E. coli	4451 (*mcr-1*)	4	4
	4531 (*mcr-1*)	4	8
	4592 (*mcr-1*)	4	4

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 aminoarabinosylation.[39] BBN149 at 30 μM 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

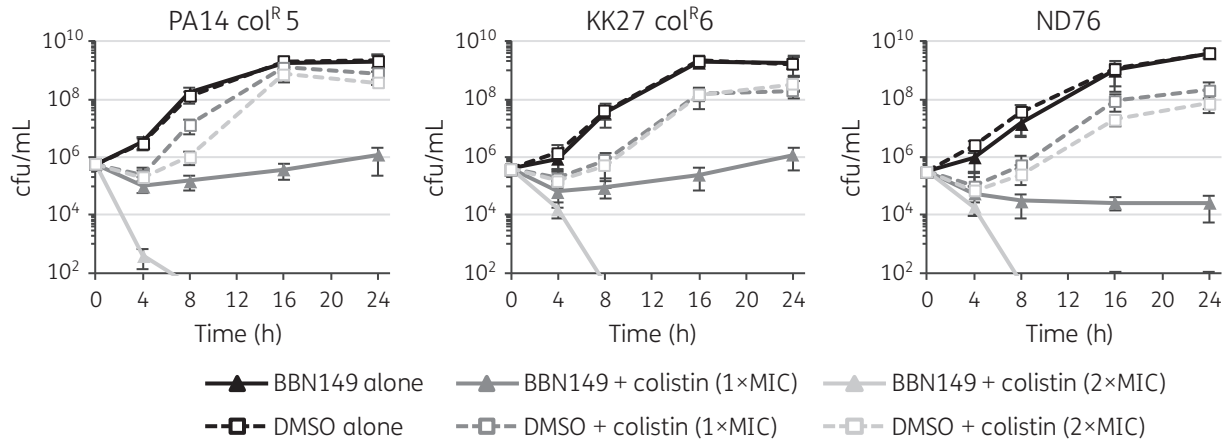

Figure 3. Time–kill curves of *P. aeruginosa* PA14 col^R 5, KK27 col^R 6 and ND76 exposed to 30 μM BBN149 in the presence or absence of colistin at 1× or 2× MIC (based on the values reported in Table 1). As a control, the strains were incubated in the presence of 0.3% DMSO and the same concentrations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

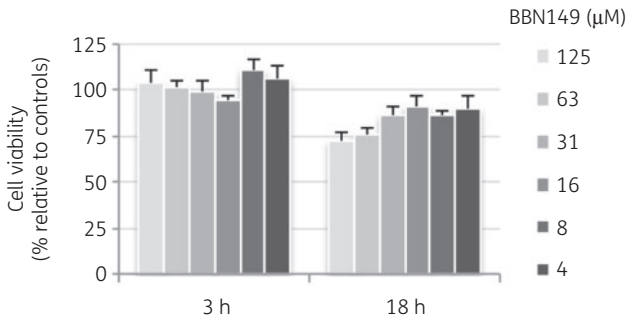

Figure 4. Viability of 16HBE epithelial cells exposed to BBN149 at the indicated concentrations for 3 or 18 h. Viability was assessed through the MTT assay and expressed as a percentage relative to vehicle-only (DMSO) controls. Data are the mean (±SD) of three independent experiments.

at 2× 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 μM) (Figure 4).

Effect of BBN149 on lipid A aminoarabinosylation

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 aminoarabinosylation 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 aminoarabinosylated lipid A species. In line with previous reports,[19,40,41] the level of lipid A aminoarabinosylation was only partial, with most lipid A still present as non-aminoarabinosylated forms. Overall, bacteria cultured with BBN149 showed slightly reduced levels of aminoarabinosylated 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 aminoarabinosylation 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 aminoarabinosylation 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 colistin[50] 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 aminoarabinosylation 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 aminoarabinosylated in colistin-resistant *P. aeruginosa* isolates (Figure 4).[19,40,41] This implies that minor variations in aminoarabinosylated lipid A levels could have a profound impact on colistin resistance and, on the other hand, that functionally relevant reductions in lipid A aminoarabinosylation may not always be easy to appraise. This would be in line with the finding that BBN149-mediated inhibition of lipid A aminoarabinosylation was much more evident in a strain with relatively high amounts of aminoarabinosylated lipid A compared with strains with lower lipid A aminoarabinosylation 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

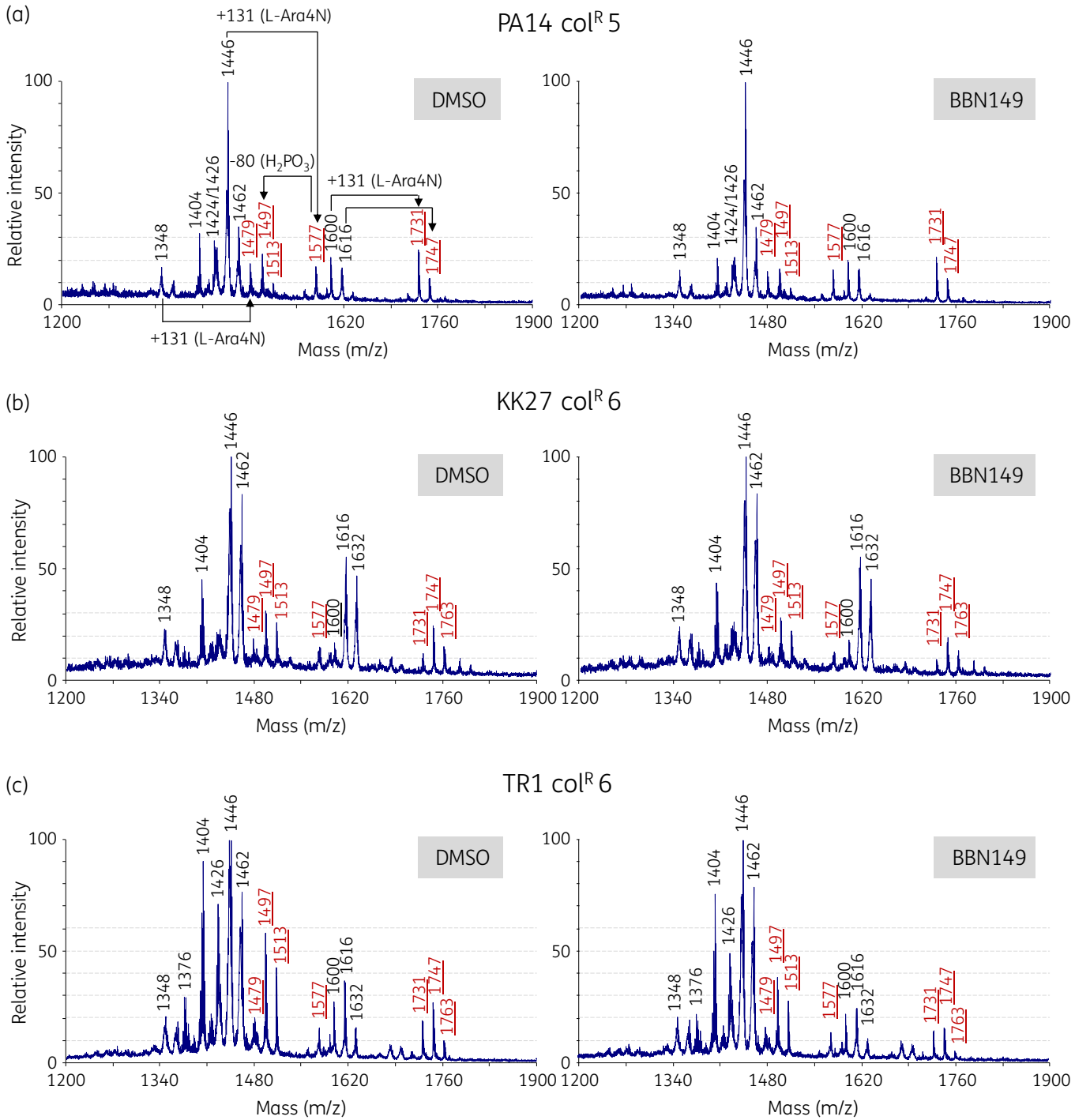

Figure 5. Effect of BBN149 on lipid A aminoarabinosylation. MALDI-TOF analysis of lipid A extracted from *P. aeruginosa* PA14 col^R 5 (a), KK27 col^R 6 (b) or TR1 col^R 6 (c) cultured in MH supplemented with 30 μM BBN149 or 0.3% DMSO as control. In (a) arrows indicate the addition of an aminoarabinose 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 aminoarabinosylated 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.

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