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In vitro synergism of colistin in combination with *N*-acetylcysteine against *Acinetobacter baumannii* grown in planktonic phase and in biofilms

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Objectives: To investigate the potential synergism of colistin in combination with *N*-acetylcysteine against *Acinetobacter baumannii* strains grown in planktonic phase or as biofilms.

Methods: Sixteen strains were investigated, including nine colistin-susceptible (MIC range 0.5–1 mg/L) and seven colistin-resistant (MIC range 16–256 mg/L) strains. Synergism of colistin in combination with *N*-acetylcysteine was investigated by chequerboard assays. The activity of colistin/*N*-acetylcysteine combinations was further evaluated by time–kill assays with planktonic cultures (three colistin-resistant strains and one colistin-susceptible strain) and by *in vitro* biofilm models (three colistin-resistant and three colistin-susceptible strains).

Results: Chequerboard assays revealed a relevant synergism of colistin/*N*-acetylcysteine combinations with all colistin-resistant strains, whereas no synergism was observed with colistin-susceptible strains. Time–kill assays showed a concentration-dependent potentiation of colistin activity by *N*-acetylcysteine against colistin-resistant strains, with eradication of the culture by combinations of *N*-acetylcysteine at 8000 mg/L plus colistin at 2 or 8 mg/L. A static effect during the first 8 h of incubation was demonstrated with the colistin-susceptible strain exposed to 0.25 × MIC colistin plus 8000 mg/L *N*-acetylcysteine. A remarkable antibiofilm synergistic activity of 8 mg/L colistin plus 8000 mg/L *N*-acetylcysteine was demonstrated with all colistin-resistant and colistin-susceptible strains. The effects were greater with colistin-resistant strains (marked reduction of viable biofilm cells was observed at sub-MIC colistin concentrations).

Conclusions: *N*-acetylcysteine, at concentrations achievable by topical administration, was shown to revert the colistin-resistant phenotype in *A. baumannii*, and to exert a relevant activity against biofilms of colistin-susceptible and colistin-resistant *A. baumannii* strains.

Introduction

Acinetobacter baumannii is a major respiratory pathogen, causing both acute infections, such as hospital-acquired and ventilator-associated pneumonia, and chronic infections in patients with cystic fibrosis (CF) and bronchiectasis.[1,2] Acute infections caused by *A. baumannii* are associated with higher morbidity and mortality rates, whereas chronic infections are associated with frequent exacerbations and hospitalizations.[1,2]

A. baumannii infections are often unresponsive to antibiotic treatment, as a consequence of the propensity of this pathogen to grow in biofilms and of the increasing dissemination of carbapenem-resistant *A. baumannii* strains (CRAb), which are usually resistant to most anti-*Acinetobacter* agents.[1,2] Colistin is among the last-resort agents for treatment of infections caused by CRAb, and nebulized colistin (alone or in combination with intravenous colistin) has been increasingly used for the treatment of

Table 1. Features of the *A. baumannii* strains tested in this work

Strain	Reference	Year of isolation	Origin[a]	Biological sample[b]	MLST[c] (IC)	Resistance profile[d]	MIC (mg/L)	
							colistin	NAC
ATCC 17978	ATCC	1951	France	CSF	ST437 (IC2)	WT	1	>32000
RUH134	6	1982	The Netherlands (NA)	urine	ST2 (IC2)	AG^R, SXT^R	0.5	>32000
Z44	this study	2012	S. Giovanni Rotondo, Italy (RM)	BAS	NA	CB^R, FQ^R, AG^R,	0.5	32000
Z45	this study	2012	Avellino, Italy (IM)	BAS	NA	CB^R, FQ^R, AG^R,	0.5	>32000
Z46	this study	2012	Avellino, Italy (GS)	BAS	NA	CB^R, FQ^R, AG^R,	1	32000
VA804/03	8	2003	Varese, Italy (NS)	sputum	NA	CB^R	1	16000
AB20C17	7	2011	Naples, Italy (RM)	BAS	ST78 (IC6)	CB^R, FQ^R, AG^R, SXT^R	1	>32000
AB19C05	7	2011	Lecce, Italy (TS)	BAS	ST2 (IC2)	CB^R, FQ^R, AG^R, SXT^R	0.5	>32000
AB11C29	7	2011	Modena, Italy (GS)	BAS	ST2 (IC2)	CB^R, FQ^R, AG^R, SXT^R	0.5	>32000
N50	8	2004	Rome, Italy (NA)	BAS	ST2 (IC2)[e]	CB^R, FQ^R, AG^R, CST^R	16	>32000
Z165	this study	2016	Rome, Italy (ICU)	BAL	ST2 (IC2)[e]	CB^R, FQ^R, AG^R, SXT^R, CST^R	32	32000
Z166	this study	2016	Rome, Italy (ICU)	RS	ST2 (IC2)	CB^R, FQ^R, AG^R, SXT^R, CST^R	128	>32000
Z167	this study	2016	Rome, Italy (IM)	blood	ST2 (IC2)[e]	CB^R, FQ^R, AG^R, SXT^R, CST^R	128	>32000
Z168	this study	2016	Rome, Italy (ICU)	blood	ST2 (IC2)	CB^R, FQ^R, AG^R, SXT^R, CST^R	256	>32000
Z169	this study	2016	Rome, Italy (ICU)	pus	ST2 (IC2)	CB^R, FQ^R, AG^R, SXT^R, CST^R	64	>32000
Z170	this study	2016	Rome, Italy (N)	urine	ST2 (IC2)	CB^R, FQ^R, AG^R, SXT^R, CST^R	64	32000

IC, international clone; NAC, *N*-acetylcysteine; NA, not available.
[a]Wards: RM, respiratory medicine; IM, internal medicine; GS, general surgery; NS, neurosurgery; TS, thoracic surgery; N, neurology.
[b]BAS, bronchial aspirate; BAL, bronchoalveolar lavage; RS, rectal swab.
[c]According to MLST Pasteur scheme.
[d]WT, wild-type phenotype; CB^R, resistance to carbapenems (imipenem and meropenem); FQ^R, resistance to fluoroquinolones (ciprofloxacin); AG^R, resistance to aminoglycosides (amikacin, gentamicin); SXT^R, resistance to trimethoprim/sulfamethoxazole; CST^R, resistance to colistin.
[e]Strain was subjected to WGS and also analysed following the Oxford scheme (N50, ST350; Z165, ST281; Z167, ST281).

respiratory tract infections caused by these strains.[2,3] As a result there has been great concern regarding the increasing trends of resistance to colistin observed in this pathogen in recent years.[4]

N-acetylcysteine is a mucolytic agent commonly administered for the treatment of lower respiratory tract infections, especially in patients with chronic respiratory disorders such as COPD, CF and bronchiectasis.[5] In addition, an increasing amount of data points to an intrinsic antimicrobial and antibiofilm activity of *N*-acetylcysteine against some pathogens.[5]

Here, we investigated the potential synergism of colistin in combination with *N*-acetylcysteine against *A. baumannii* strains, including also CRAb and colistin-resistant CRAb, grown in planktonic phase or in biofilms.

Materials and methods

Bacterial strains

Sixteen strains were investigated, including nine colistin-susceptible and seven colistin-resistant strains (Table 1). The colistin-susceptible strains included two reference strains, namely *A. baumannii* ATCC 17978 and *A. baumannii* RUH134 [as the reference strain for International Clone 2 (IC2)],[6] and seven CRAb strains from previous surveillance studies[7,8] or referred to our laboratory for characterization. Of the seven colistin-resistant strains, six were from an outbreak that occurred between January and June 2016, and one was from a previous study. All colistin-resistant strains were of ST2, but exhibited different colistin MICs (Table 1). Identification of clinical strains was performed by MALDI–TOF MS and confirmed by PCR detection of $bla_{OXA\text{-}51}$-like alleles.[9] Antimicrobial susceptibility was determined using the reference broth microdilution method.[10]

MLST was performed as previously described,[7] according to the Pasteur scheme (http://pubmlst.org/abaumannii/). For the strains subjected to WGS (see below), *in silico* MLST according to the Oxford scheme (http://pubmlst.org/abaumannii/) was also applied.

Analysis of colistin resistance mechanisms was carried out in the three colistin-resistant strains used for time–kill assays with planktonic cultures and for testing of antibiofilm activity (i.e. *A. baumannii* N50, Z165 and Z167). Bacterial DNA was extracted using the phenol:chloroform method.[11] Genomic DNA was subjected to WGS with a MiSeq platform (Illumina, Inc., San Diego, CA, USA), using a 2×250 or 2×300 bp paired-end approach, and reads were assembled using SPAdes.[12] Draft genomes were used to investigate the presence of known mutations associated with colistin resistance in the *pmrA*, *pmrB*, *lpxA*, *lpxC* and *lpxD* genes.[13] The corresponding genes of the IC2 colistin-susceptible ACICU strain[14] were used as reference for sequence comparison. Strain N50 was found to have the *pmrB* gene disrupted by the insertion of a copy of the IS*Aba1* insertion sequence at nucleotide 531. In contrast, no known mutations accounting for colistin resistance were detected with respect to the reference sequence in strains Z165 and Z167, despite the high colistin MIC expressed by these strains, a matter that will require further investigation.

Preparation of N-acetylcysteine-containing medium

N-acetylcysteine stock solutions (100 g/L) were prepared immediately before use, by dissolving *N*-acetylcysteine powder (Zambon, Bresso, Italy) in sterile double-distilled water, pH adjustment to 6.5–6.8 with NaOH, and filtering through a 0.22 μm membrane filter. All experiments were performed in CAMHB (Becton Dickinson, Milan, Italy), starting from an appropriately concentrated medium in order to avoid broth dilution when testing high *N*-acetylcysteine concentrations.

Table 2. FICI values for colistin/*N*-acetylcysteine (NAC) combinations against a collection of *A. baumannii* strains

	MIC (mg/L)		NAC concentration (mg/L)						
Strain	Colistin	NAC	500	1000	2000	4000	8000	16000	32000
ATCC 17978	1	>32000	1.01	1.02	1.03	1.06	1.13	1.25	0.75
RUH134	0.5	>32000	1.01	2.02	1.03	1.06	1.13	1.25	0.75
Z44	0.5	32000	1.02	1.03	1.06	1.13	1.25	1.00	1.01
Z45	0.5	>32000	1.01	1.02	1.03	1.06	0.63	0.75	0.56
Z46	1	32000	0.52	1.03	0.56	0.63	0.75	1.00	1.00
VA804/03	1	16000	1.03	1.06	1.13	2.25	1.50	1.00	2.00
AB20C17	1	>32000	1.01	1.02	0.53	0.56	0.63	0.75	0.56
AB19C05	0.5	>32000	1.01	1.02	1.03	1.06	1.13	0.75	0.63
AB11C29	0.5	>32000	1.01	1.02	1.03	1.06	1.13	0.75	0.56
N50	16	>32000	1.01	0.08	0.06	0.09	0.16	0.28	0.52
Z165	32	32000	0.52	0.28	0.13	0.14	0.26	0.51	1.01
Z166	128	>32000	0.26	0.27	0.06	0.07	0.13	0.25	0.50
Z167	128	>32000	0.51	0.52	0.06	0.07	0.13	0.25	0.50
Z168	256	>32000	0.51	0.27	0.09	0.06	0.13	0.25	0.50
Z169	64	>32000	0.51	0.14	0.06	0.08	0.14	0.27	0.50
Z170	64	32000	0.52	0.28	0.09	0.13	0.25	0.50	1.00

FICI values indicating synergy are shown with grey shading. FICI values were interpreted as follows: FICI ≤0.5, synergy; FICI >4.0, antagonism; FICI >0.5–4.0, no interaction.
Ranges of tested colistin concentrations were 0.003–4 and 0.25–256 mg/L for colistin-susceptible and colistin-resistant strains, respectively. When the MIC for a compound was higher than the tested range, FICI values were calculated using twice the maximum concentration tested. When the MIC for a compound was lower than the tested range , FICI values were calculated using the lowest concentration tested

Chequerboard assays

Chequerboard assays to investigate the potential synergism of colistin (Applichem, Darmstadt, Germany) with *N*-acetylcysteine were carried out as described previously.[15] The ranges of colistin concentrations tested were 0.003–4 and 0.25–256 mg/L for colistin-susceptible and colistin-resistant strains, respectively. The *N*-acetylcysteine range tested was 0.5–32 g/L for all strains, taking into account the high drug concentrations potentially achievable by topical administration.[5] Fractional inhibitory concentration index (FICI) values were interpreted as follows: ≤0.5, synergy; >4.0, antagonism; >0.5–4.0, no interaction. When the MIC of a compound was higher than the tested range, FICI values were calculated using double the maximum concentration tested. When the MIC of a compound was lower than the tested range, FICI values were calculated using the lowest concentration tested.

Time–kill assays with planktonic cultures

Time–kill assays were performed according to CLSI guidelines[16] with the colistin-susceptible reference strain for the IC2 (RUH134) and with three selected colistin-resistant strains (N50, Z165 and Z167) belonging to IC2. The latter strains were representative of two different STs according to the Oxford scheme, harboured at least two different colistin resistance mechanisms, and exhibited different colistin MICs (Table 1).

Two colistin concentrations (2 and 8 mg/L for colistin-resistant strains; 0.125 and 2 mg/L for colistin-susceptible strains) and three *N*-acetylcysteine concentrations (1600, 3200 and 8000 mg/L) were tested alone and in combination. Viable cell counts were performed at the beginning of the experiment and after 2, 4, 6, 8, 24 and 48 h of exposure (detection limit, 100 cfu/mL). Data were obtained from at least two independent experiments, with two replicates per condition per experiment.

Antibiofilm assays

The potential synergism of colistin/*N*-acetylcysteine combinations against preformed biofilms was investigated using a standardized *in vitro* biofilm model.[17] The study was carried out with three colistin-susceptible strains (RUH134, ATCC 17978 and AB11C29, a recent CRAb clinical isolate) and the three colistin-resistant strains used in time–kill assays (see above). Biofilms were grown in the Nunc TSP Lid system (Thermo Fisher Scientific, Waltham, MA, USA), as previously described,[15] for 7 days in CAMHB that was replaced daily (static conditions, 35°C). Preformed biofilms were then exposed to different colistin concentrations (2 and 8 mg/L for colistin-resistant strains; 0.5, 2 and 8 mg/L for colistin-susceptible strains) and three *N*-acetylcysteine concentrations (1600, 3200 and 8000 mg/L), alone or in combination with colistin, for 24 h (static conditions, 35°C). At the end of the experiment, loosely adherent bacteria were removed by washing twice with 200 μL of PBS (Sigma–Aldrich, Milan, Italy), and sessile cells were removed from pegs by sonication for 30 min (Elma Transsonic T 460, Singen, Germany) in 200 μL of tryptic soy broth (Oxoid, Milan, Italy) supplemented with 0.1% Tween 20 (Sigma–Aldrich). Mean viable cell counts per peg (cfu/peg) were then determined by plating 10 μL of suitable dilutions of the recovery medium onto tryptic soy agar (TSA) (Oxoid) and incubating for 48 h at 35°C (detection limit, 20 cfu/peg). Data were obtained in at least two independent experiments, with at least six replicates per condition per experiment.

Statistical analysis

Statistical analysis of results from time–kill assays with planktonic cultures and from antibiofilm assays was performed using the unpaired *t*-test and the unpaired *t*-test with Welch's correction, respectively (GraphPad Prism version 6.0, San Diego, CA, USA).

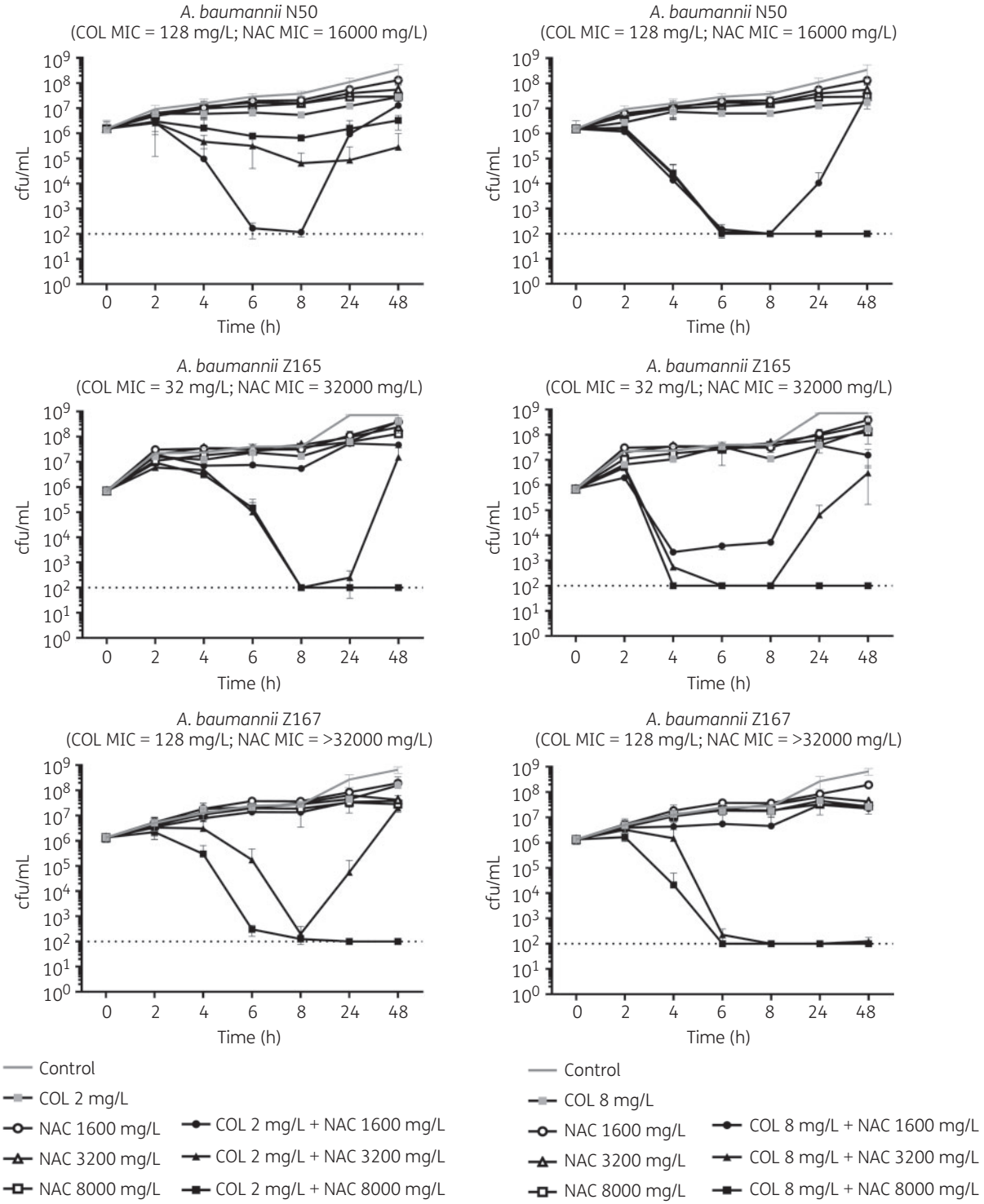

Figure 1. Time–kill assays performed with exponentially growing planktonic cultures of the colistin (COL)-resistant strains N50, Z165 and Z167 exposed to different colistin/*N*-acetylcysteine (NAC) combinations. Dotted lines indicate the detection limit (100 cfu/mL).

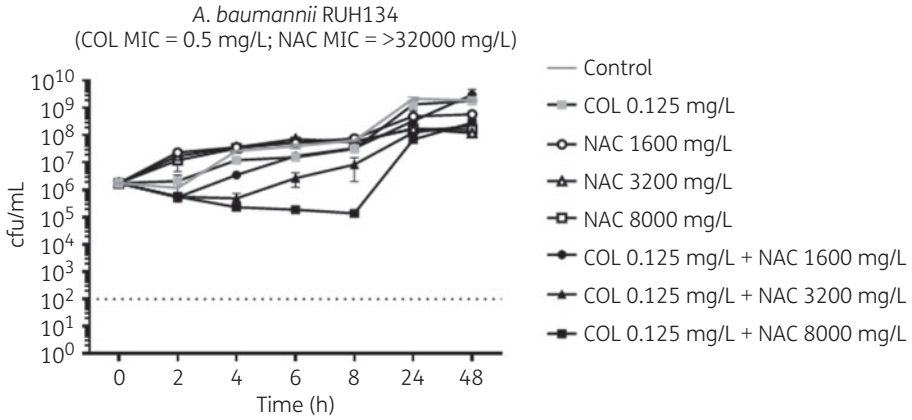

Figure 2. Time–kill assay performed with exponentially growing planktonic cultures of the colistin (COL)-susceptible strain RUH134, reference strain for IC2, exposed to different COL/*N*-acetylcysteine (NAC) combinations. Dotted lines indicate the detection limit (100 cfu/mL).

Results and discussion

Synergism of colistin/N-acetylcysteine combinations versus CRAb in chequerboard assays

Results of chequerboard assays showed a remarkable synergism of colistin/*N*-acetylcysteine combinations against the seven colistin-resistant *A. baumannii* strains (Table 2). In particular, in the presence of 4000 mg/L *N*-acetylcysteine (i.e. a concentration likely achievable by topical administration),[5] colistin MICs for all the seven colistin-resistant strains was lowered to <2 mg/L (i.e. the susceptibility breakpoint) (MIC range 0.5–1 mg/L). The correct interpretation of the chequerboard results was confirmed by plating 5 μL of each well of the chequerboard microtitre plate onto TSA and evaluating bacterial growth after 48 h of incubation (an example of such data is provided in Figure S1 available as Supplementary data at *JAC* Online).

In contrast, no synergism of colistin/*N*-acetylcysteine combinations was observed with the nine colistin-susceptible strains (Table 2).

Together, these results suggested that *N*-acetylcysteine could affect the mechanisms of acquired colistin resistance, potentially restoring a susceptible phenotype. Interestingly, this effect was observed with IC2 strains of different ST and harbouring different colistin resistance mechanisms (e.g. N50 versus Z165 and Z167), regardless of the colistin MIC. Further studies including a wider collection of colistin-resistant *A. baumannii* strains with characterized mechanisms of colistin resistance should be carried out to further investigate this phenomenon.

Synergism of colistin/N-acetylcysteine combinations versus CRAb in time–kill assays with planktonic cultures

Results of time–kill assays performed with three colistin-resistant strains (N50, Z165 and Z167), representative of IC2 strains of different ST and harbouring different colistin resistance mechanisms showed a notable concentration-dependent potentiation by *N*-acetylcysteine of the activity of colistin at 8 mg/L (i.e. a concentration easily achievable with an inhaled formulation,[3] and lower than the MIC of colistin for these strains) (Figure 1). In particular, eradication of the starting inoculum (i.e. absence of regrowth after 48 h of incubation) was achieved for the three tested strains with combinations of 8000 mg/L *N*-acetylcysteine and 8 mg/L colistin, and for two of them (N50 and Z167) also with combinations of 3200 mg/L *N*-acetylcysteine and 8 mg/L colistin (Figure 1).

Similar results were obtained in time–kill assays performed with 2 mg/L colistin with strains Z165 and Z167 (i.e. *N*-acetylcysteine concentration-dependent potentiation of colistin activity). Nonetheless, a paradoxical effect was observed with strain N50. For this strain, combinations including 2 mg/L colistin and 3200 or 8000 mg/L *N*-acetylcysteine showed a substantially bacteriostatic effect across the entire assay, whereas 1600 mg/L *N*-acetylcysteine was responsible for a remarkable decrease in viable cells after 6–8 h of incubation, followed by regrowth. The mechanisms accounting for this phenomenon remain unclear, and deserve further investigation. However, because colistin concentrations achievable by inhalation are far above 2 mg/L,[3] it might not have a relevant clinical impact.

Time–kill assays performed with the colistin-susceptible strain RUH134 revealed a bacteriostatic effect of 0.125 mg/L colistin (i.e. 0.25 × MIC) and 8000 mg/L *N*-acetylcysteine during the first 8 h of incubation ($P < 0.001$ compared with controls), followed by regrowth (Figure 2). Experiments performed with 2 mg/L colistin (i.e. 4 × MIC) showed complete eradication of the starting inoculum from the first timepoint evaluated (2 h) (data not shown).

Overall, data obtained with time–kill assays were in accordance with chequerboard results, supporting the notion that *N*-acetylcysteine could restore the activity of colistin against colistin-resistant strains at concentrations achievable following nebulized administration of the two drugs.

Activity of colistin/N-acetylcysteine combinations against A. baumannii biofilms

The activity of colistin/*N*-acetylcysteine combinations was further investigated using a standardized *in vitro* biofilm model.[17] For this purpose, 7-day-old biofilms of three colistin-resistant (N50, Z165

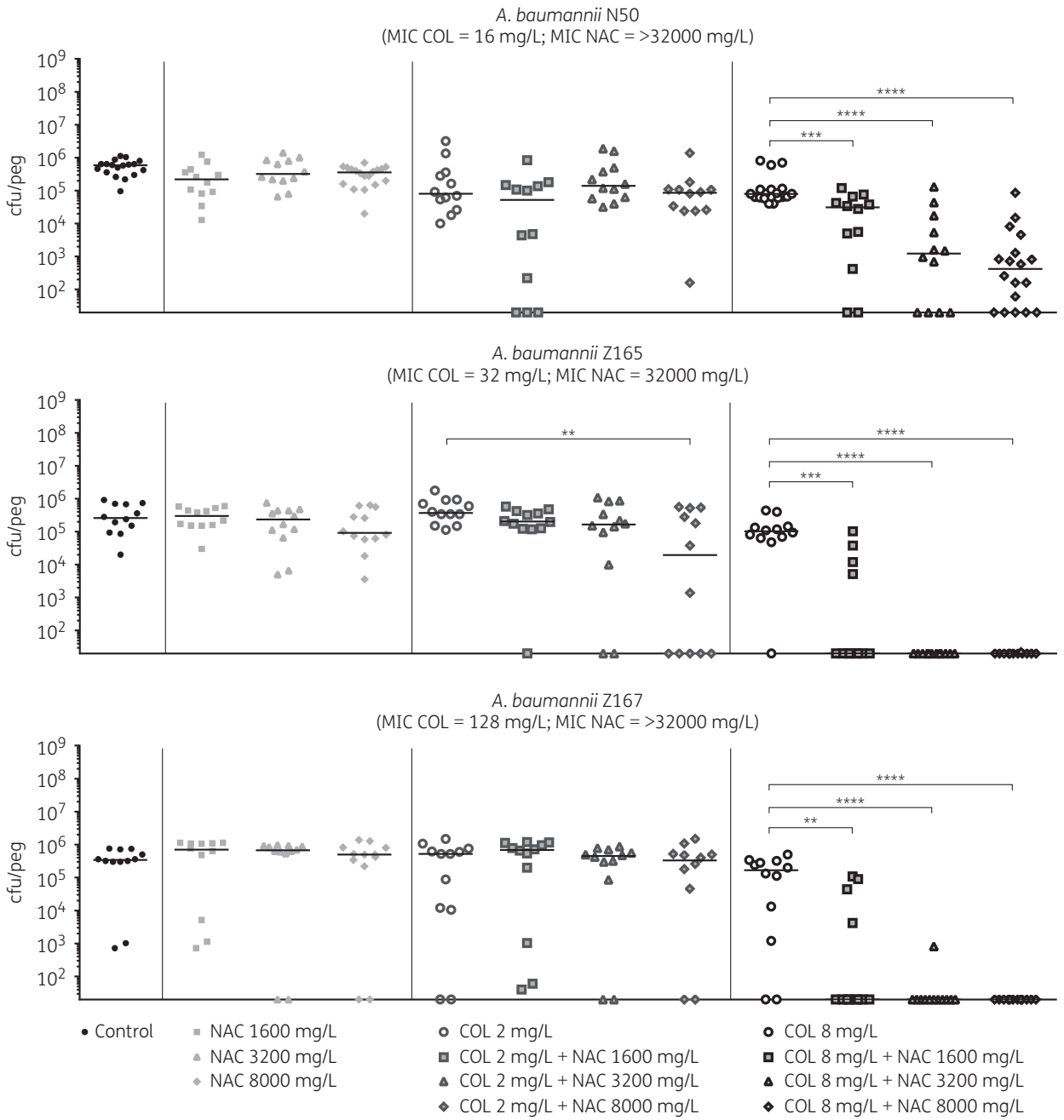

Figure 3. Antibiofilm activity of colistin (COL)/*N*-acetylcysteine (NAC) combinations against colistin-resistant *A. baumannii* strains N50, Z165 and Z167. The *x*-axis is set at the limit of detection (20 cfu/peg).

and Z167) and three colistin-susceptible (ATCC 17878, RUH134 and AB11C29) strains were exposed for 24 h to different colistin/*N*-acetylcysteine combinations and to each drug alone.

Colistin and *N*-acetylcysteine alone, even at the highest concentrations tested, had no or little effect compared with controls against the colistin-susceptible strains as well as the colistin-resistant strains (Figures 3 and 4).

Results of biofilm susceptibility testing performed with the three colistin-resistant strains showed a remarkable antibiofilm synergistic activity of combinations including colistin 8 mg/L (a concentration lower than the MICs of colistin for these strains), with a marked reduction in viable biofilm cells also observed in the presence of the lowest *N*-acetylcysteine concentration tested (i.e. 1600 mg/L) (Figure 3). These data partially differed from those observed in time–kill assays performed with planktonic cells, probably due to the markedly different physiological state of planktonic and biofilm-associated cells. Indeed, combinations of 8 mg/L colistin with 1600 mg/L *N*-acetylcysteine had no or little effect on planktonic

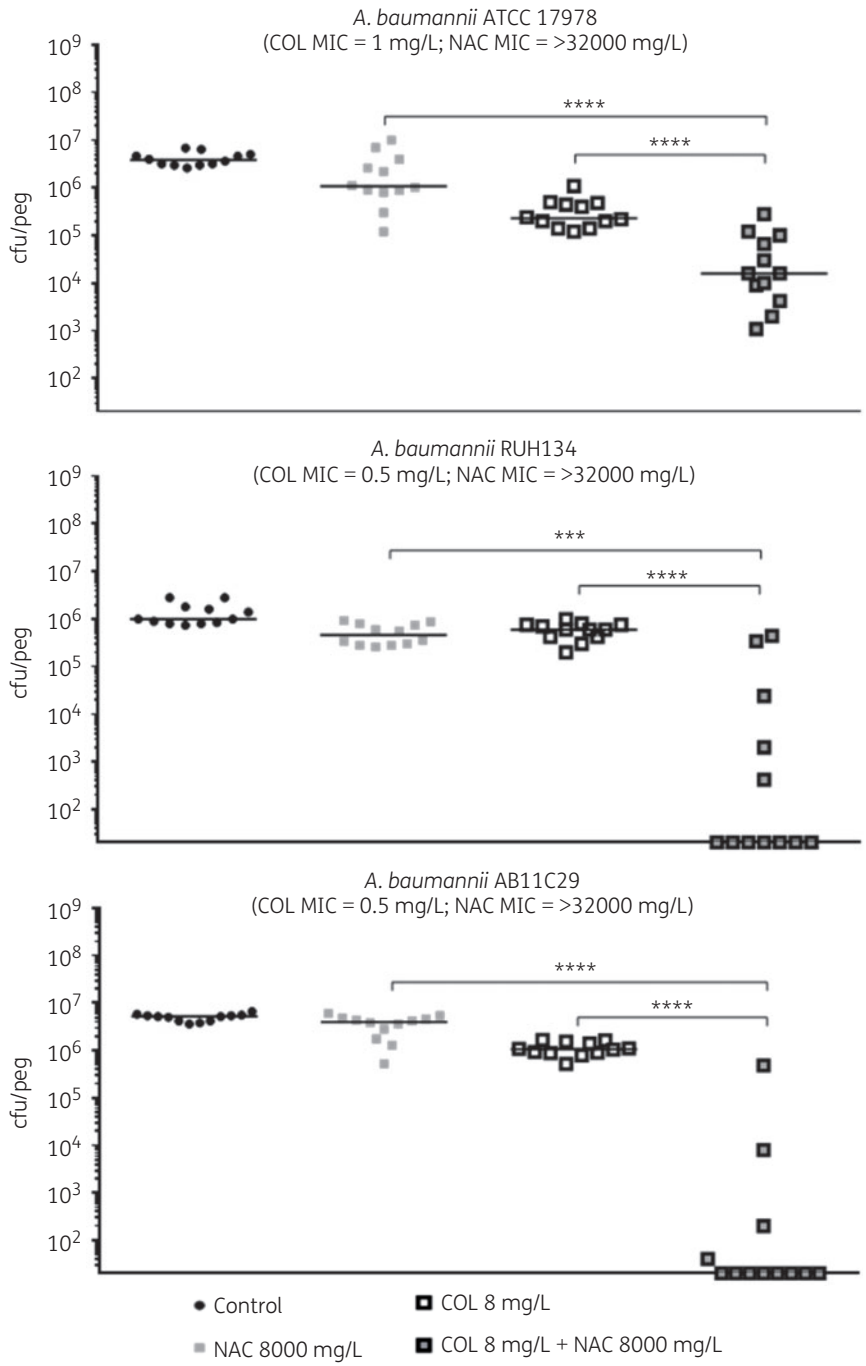

Figure 4. Antibiofilm activity of 8 mg/L colistin (COL) in combination with 8000 mg/L *N*-acetylcysteine (NAC) against the colistin-susceptible *A. baumannii* strains ATCC 17978, RUH134 and AB11C29. The *x*-axis is set at the limit of detection (20 cfu/peg).

cultures, while achieving a remarkable reduction in viable biofilm cells. On the other hand, 2 mg/L colistin in combination with 8000 mg/L *N*-acetylcysteine led to eradication of the starting inocula in planktonic cultures of Z165 and Z167 strains, while having no or little effect on biofilms (Figures 1 and 3).

As shown in Figure 4, a relevant antibiofilm synergistic activity of 8 mg/L colistin in combination with 8000 mg/L *N*-acetylcysteine was also observed with all the three colistin-susceptible strains (Figure 4), while combinations including lower colistin concentrations (i.e. 0.5 or 2 mg/L, representing from 1× to 4 × MIC of colistin for the tested strains) had shown no interaction (Figure S2).

These data strongly supported the hypothesis that *N*-acetylcysteine, at concentrations likely to be achievable by inhaled formulations, could exert two important and independent biological effects

against *A. baumannii*, namely reversal of colistin resistance phenotype and antibiofilm activity. Nonetheless, further studies aimed at elucidating the molecular mechanisms underlying the synergism and investigating the potential clinical relevance of inhaled colistin/*N*-acetylcysteine combinations in animal models are needed.

Conclusions

The results of this study demonstrated a relevant antibiofilm synergism of colistin/*N*-acetylcysteine combinations against *A. baumannii*, and the ability of *N*-acetylcysteine to reverse the colistin resistance phenotype in this pathogen. These interesting findings deserve further investigation for potential clinical applications of inhaled colistin/*N*-acetylcysteine formulations. To the best of our knowledge, this is the first report of a synergistic activity of colistin in combination with *N*-acetylcysteine.

Inhaled colistin/*N*-acetylcysteine combinations could represent an effective treatment against infections caused by biofilm-associated *A. baumannii* strains, while potentially reducing the risk of *in vivo* selection of colistin resistance (i.e. the expansion of a resistant mutant that may be selected during colistin treatment would likely be prevented by the capacity of *N*-acetylcysteine to reverse the colistin resistance phenotype).

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Transparency declarations

F. S. is an employee in Corporate Respiratory Medical Affairs at Zambon S.p.A.

G. M. R., F. B., S. A. and L. P. are Advisory Board members for Zambon S.p.A. The remaining authors have none to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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