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Aspirin, sodium benzoate and sodium salicylate reverse resistance to colistin in Enterobacteriaceae and *Pseudomonas aeruginosa*

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Background: MDR bacterial infections are currently a serious problem for clinicians worldwide. *Klebsiella pneumoniae* and *Enterobacter* spp., among Enterobacteriaceae, and *Pseudomonas aeruginosa*, are part of the group of ESCAPE pathogens or bacteria that 'escape' from common antibacterial treatments. The lack of effectiveness of the first common line of antibiotics has led to the search for new therapies based on older antibiotics, such as colistin.

Objectives: We searched for new enhancers of the action of colistin against MDR Gram-negative bacteria that can be easily applicable to clinical treatments.

Methods: Colistin MICs were determined alone and with the protonophores CCCP, sodium benzoate, sodium salicylate and aspirin using the broth microdilution method and FIC indexes were calculated to assess synergy between colistin and each chemical. Time–kill assays of colistin with and without protonophores were performed to determine the bactericidal action of combinations of colistin with protonophores. Likewise, the effect of sucrose, L-arginine and L-glutamic acid on the MICs of colistin alone and combined with each protonophore was assessed.

Results: It was found that sodium benzoate, sodium salicylate and aspirin, at concentrations allowed for human and animal use, partially or totally reversed resistance to colistin in *P. aeruginosa* and highly resistant enterobacterial strains. The mechanism of action could be related to their negative charge at a physiological pH along with their lipid-soluble character.

Conclusions: Sodium benzoate, sodium salicylate and aspirin are good enhancers to use in antibiotic therapies that include colistin.

Introduction

The spread of MDR in Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp. and *Proteus* spp., among Enterobacteriaceae, and *Pseudomonas aeruginosa*, which are species included among the biggest threats of antibiotic resistance,[1] has led to the search for alternatives among older antibiotics, such as colistin.[2] However, the emergence of colistin resistance can seriously compromise this alternative against MDR Gram-negative bacteria.[2]

Colistin is a cationic cyclic polypeptide antibiotic that belongs to the family of polymyxins.[3,4] The main mode of action of colistin consists of the interaction between the positive charges of its amino groups and the negative charges of phosphate residues of lipid A in LPS, displacing the divalent metal cations Mg^{2+} and Ca^{2+}. This results in the destabilization of LPS,[5] weakening and disrupting the outer and inner membranes, which leads to bacterial lysis.[5–7]

Colistin, polymyxin E, is available as colistin sulphate and colistimethate sodium. They are clinically used according to the route of administration, although the active form of the drug is colistin sulphate.[8] Unfortunately, the clinical utility of colistin has been seriously threatened by the emergence of plasmid-mediated colistin resistance via *mcr* genes,[2] mainly the *mcr-1* gene.[9] The *mcr* genes encode phosphoethanolamine (pEtN) transferases that add pEtN to LPS, which confers low to moderate polymyxin resistance (MIC from 4 to 16 mg/L).[10] However, acquired chromosomal resistance to colistin still remains the most common in Enterobacteriaceae isolated from humans.[11] It consists of the binding of 4-amino-4-deoxy-L-arabinose (L-Ara4N), pEtN or galactosamine to phosphate groups of lipid A in LPS, neutralizing the negative charges, resulting in polymyxin repulsion.[5,10] Most mutations that result in acquired colistin resistance have been identified in two-component regulatory systems, Pho/Q and PmrA/B in *E. coli* and *K. pneumoniae*, and, in addition, ParR/S, CprR/S and ColR/S in *P. aeruginosa*.[5,10]

Furthermore, *Proteus*, *Morganella*, *Providencia* and *Serratia* are intrinsically resistant to polymyxins due to the natural modification of LPS phosphate groups by L-Ara4N.[12]

Recently, the reversal of colistin resistance induced by CCCP in *E. coli* and *K. pneumoniae* has been described;[13–15] however, the cause of this potentiating effect is unknown. CCCP is a lipid-soluble protonophore and a powerful uncoupler that has been extensively tested for the study of active efflux in Gram-negative bacteria.[16] The objective of this study was to search for chemicals capable of reversing colistin resistance in a similar way to that of CCCP, but which, unlike CCCP, can be used in clinical therapy.

Materials and methods

Strains

Twelve colistin-resistant clinical isolates of the species *E. coli*, *Enterobacter cloacae*, *K. pneumoniae*, *Serratia marcescens*, *P. aeruginosa*, *Proteus mirabilis* and *Morganella morganii* were collected. All the studied strains were selected based on their resistance to colistin. *E. coli*, *E. cloacae*, *K. pneumoniae*, *P. mirabilis* and *M. morganii* were ESBL-producing strains following EUCAST guidelines[17] and MDR according to Magiorakos *et al.*[18] Likewise, one *E. cloacae* and one *K. pneumoniae* clinical isolate that were susceptible to colistin were used as control strains for time–kill assays.

MIC determination

Colistin (colistin sulphate) and four lipid-soluble protonophores and uncouplers [CCCP, benzoic acid sodium salt or sodium benzoate (SB), salicylic acid sodium salt or sodium salicylate (SS) and acetylsalicylic acid or aspirin (AS)] were purchased from Sigma (Madrid). Colistin MICs were determined alone and with 10 and 25 μM CCCP, 14 and 28 mM SB and SS, and 0.8 and 1.6 mM AS using the broth microdilution method, by using 2-fold serial dilutions of colistin and fixed concentrations of CCCP, SB, SS and AS. MICs of each potential enhancer for the studied strains were initially determined (Table S1, available as Supplementary data at *JAC* Online). The MIC determinations were performed following CLSI guidelines and EUCAST recommendations.[17,19,20] The effects of CCCP, SB, SS and AS on colistin MICs were also determined for the WT strains *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853. The range of concentrations used for the determination of the MICs of colistin was from 0.063 to 4096 mg/L. Likewise, the concentrations of SB, SS and AS were within the range of concentrations allowed for use in humans and animals.[21–25] All the MICs were determined at least three times. Once MICs were determined, FIC indexes (FICIs) were calculated to assess synergy between colistin and each chemical. The FICI was calculated as follows: FICI = FICA + FICB, where FICA = MICA + B/MICA and FICB = MICA + B/MICB, where A is colistin and B is CCCP, SB, SS or AS. FICI $\leq$0.5 suggested synergy, FICI >0.5–4 suggested no interaction and FICI >4 suggested antagonism.[26]

Determination of plasmid-mediated resistance to colistin

The presence of *mcr* genes was determined according to their recently described prevalence in clinical isolates for *mcr-1*, *mcr-2*, *mcr-3* and *mcr-4* genes and by using previously described primers.[27,28] In addition, the transferability of colistin resistance was assessed by conjugation assays as previously described and by using the sodium azide-resistant *E. coli* J53 K12 strain as a recipient.[28,29] The transconjugants were selected on LB agar plates with sodium azide (100 mg/L) and colistin (1 mg/L).[30] Some transconjugants were also selected using either ceftazidime (2 mg/L) or cefotaxime (2 mg/L).

Assessment of the mode of action of CCCP, SB, SS and AS

MICs of sucrose, L-glutamic acid and L-arginine alone for the studied strains were determined using the broth microdilution method (Table S1). We next looked for subinhibitory concentrations of both amino acids that did not inhibit bacterial growth of the studied strains in the presence of both colistin and colistin + protonophore. Finally, the effects of 1 mM L-glutamic acid (Sigma), 1 mM L-arginine (Sigma) and 300 mM sucrose (Sigma) on the MICs of colistin alone and the MICs of combinations of colistin plus each of the four protonophores tested were assessed. To rule out synergy between each protonophore and sucrose, L-glutamic acid and L-arginine, FICIs of the combinations were also determined.

Time–kill assay

Time–kill assays of colistin with and without protonophores were performed for four highly colistin-resistant strains, two strains with acquired resistance (*E. cloacae* 19/3 and *K. pneumoniae* 19/5) and two strains with intrinsic resistance (*S. marcescens* 19/10 and *P. mirabilis* 19/11), following a previously described method with minor modifications.[13] Samples from overnight cultures of the studied strains were inoculated into fresh Mueller–Hinton broth (MHB) to yield a density of $\sim 5\times10^5$ cfu/mL. MHB contained colistin (4 mg/L), as previously described,[31] with or without 28 mM SB, 14 or 28 mM SS or 0.8 or 1.6 mM AS and 25 μM CCCP. The cultures were incubated at 37°C for 48 h and samples of each culture were obtained at 0, 2, 4, 8, 12, 24 and 48 h. The numbers of viable cfu in the samples were determined by serial dilutions, as previously described. The effects of protonophores alone and ethanol (the solvent for CCCP and AS) were also determined by time–kill assays. All time–kill assays were performed at least in triplicate and the SDs were also determined.

Bactericidal activity of colistin + protonophore was defined as a decrease in cfu/mL $\geq$3 $\log_{10}$ in relation to the initial inoculum and synergy as a decrease in cfu/mL $\geq$2 $\log_{10}$ compared with cfu/mL in the presence of colistin alone.[32]

Results

Characterization of colistin resistance

The determination of colistin MICs revealed a range of 64 to >4096 mg/L for *E. cloacae*, *K. pneumoniae*, *S. marcescens*, *P. mirabilis* and *M. morganii* strains (Table 1). The PCR study of plasmid-mediated colistin resistance confirmed the absence of *mcr-1*, *mcr-2*, *mcr-3* and *mcr-4* genes in the 12 studied strains. Likewise, conjugation assays did not identify transferable resistance to colistin in any of the studied strains. Taken together, the results suggested that the 12 studied Gram-negative strains had chromosomally mediated colistin resistance.

Study of protonophores as enhancers of colistin activity

Our results showed that 25 μM (5 mg/L) CCCP decreased the MICs of colistin to values equal to or lower than the clinical breakpoint for colistin (MIC $\leq$2 mg/L) both for strains with acquired resistance (*E. coli*, *E. cloacae*, *K. pneumoniae* and *P. aeruginosa*) and for those with intrinsic resistance (*S. marcescens*, *P. mirabilis* and *M. morganii*) (Table 1). The effect of CCCP was concentration dependent, since 10 μM was 2- to 2048-fold less effective than 25 μM for *E. coli*, *E. cloacae*, *K. pneumoniae* and *S. marcescens* strains (Table 1). However, 25 and 10 μM CCCP did not induce any change in colistin MIC for any of the ATCC control strains (Table S2).

Table 1. Effects of CCCP and SB on colistin MICs (mg/L) and effects of sucrose, L-arginine and L-glutamic acid

Strain	CST	CST+ SC	CST+ Arg	CST+ Glu	CST+ CCCP-10	CST+ CCCP-25	CST+ CCCP-25+SC	CST+ CCCP-25+Arg	CST+ CCCP-25+Glu	CST+ SB-14	CST+ SB-28	CST+ SB-28+SC	CST+ SB-28+Arg	CST+ SB-28+Glu
E. coli 19/1	4	2	4	4	0.5	<0.063	<0.063	<0.063	0.5	2	2	0.5	<0.063	<0.25
E. coli 19/2	8	1	2	2	1	0.5	<0.063	<0.063	0.5	2	2	2	<0.063	1
E. cloacae 19/3	4096	512	512	4096	2	1	2	<0.063	0.5	4	1	2	<0.063	1
K. pneumoniae 19/4	64	8	64	64	4	1	1	<0.063	1	4	1	1	<0.063	1
K. pneumoniae 19/5	128	32	64	128	2	1	1	<0.063	1	8	2	2	<0.063	4
K. pneumoniae 19/6	64	32	64	64	4	2	1	<0.063	1	4	2	2	<0.063	4
K. pneumoniae 19/7	64	16	64	128	2	2	<0.063	<0.063	1	4	1	1	<0.063	2
K. pneumoniae 19/8	64	1	64	64	2	1	<0.063	<0.063	1	8	2	0.5	<0.063	2
P. aeruginosa 19/9	4	<0.063	4	2	2	2	2	<0.063	2	4	2	<0.063	<0.063	2
S. marcescens 19/10	4096	64	2048	4096	2	1	<0.063	<0.063	1	128	32	8	<0.063	2
P. mirabilis 19/11	>4096	>4096	>4096	>4096	1024	0.5	0.5	<0.063	1	>4096	>4096	0.5	>4096	>4096
M. morganii 19/12	>4096	>4096	>4096	>4096	1024	2	2	<0.063	64	>4096	>4096	>4096	>4096	>4096

CST, colistin; CCCP-10, 10 μM CCCP; CCCP-25, 25 μM CCCP; SB-14, 14 mM SB; SB-28, 28 mM SB; SC, 300 mM sucrose; Arg, 1 mM L-arginine; Glu, 1 mM L-glutamic acid.

Likewise, the highest concentrations of the protonophores SB, SS and AS (28 mM SB, 28 mM SS and 1.6 mM AS) decreased colistin MICs 32- to >65 000-fold for *E. cloacae*, *K. pneumoniae* and *S. marcescens*, and 2- to 16-fold for the less resistant strains (*E. coli* 19/1, *E. coli* 19/2 and *P. aeruginosa* 19/9) (Tables 1 and 2). The effect of partial uncouplers was also concentration dependent since the reversal of colistin resistance was 2- to >4-fold less with 14 mM SB, 2- to 32-fold less with 14 mM SS and 2- to 256-fold less with 0.8 mM AS than the reversal of resistance induced by the highest concentrations of the same chemicals (Tables 1 and 2). Furthermore, the highest concentrations of SB, SS and AS decreased colistin MICs 2- to 4-fold for *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603, whereas colistin MIC for *P. aeruginosa* ATCC 27853 was only decreased by 28 mM SS, by 2-fold (Table S2). None of the concentrations assayed of the three partial uncouplers resulted in colistin MICs $\leq$2 mg/L for *P. mirabilis* and *M. morganii* strains, unlike 25 μM CCCP (Tables 1 and 2).

FICIs showed that all the combinations of colistin plus any of the assayed concentrations of SB, SS and AS were synergistic against *E. coli*, *E. cloacae*, *K. pneumoniae*, *P. aeruginosa* and *S. marcescens*, the same as using 25 μM CCCP, with FICIs $\leq$0.5, with the exception of the combinations of colistin + 14 mM SB and colistin + 0.8 mM AS (Table 3). No interaction was found for the combination colistin + 14 mM SB against *P. aeruginosa* (FICI of 1), the same as for the combination colistin + 0.8 mM AS against *E. coli* 19/1, *K. pneumoniae* 19/4, 19/7 and 19/8 and *P. aeruginosa* 19/9 strains with FICIs in the range 0.504–1.004. In addition, colistin + 0.8 mM AS was antagonistic against *S. marcescens* with an FICI of 4.141 (Table 3). Colistin + 25 μM CCCP was the only synergistic combination against *P. mirabilis* and *M. morganii*, although there was no interaction against *P. aeruginosa* (FICI of 0.643) (Table 3).

Time–kill assays

Colistin-resistant strains (*E. cloacae*, *K. pneumoniae*, *S. marcescens* and *P. mirabilis*) in the presence of 4 mg/L colistin showed growth similar to the same strains without the antibiotic; however, colistin + CCCP had a bactericidal effect on *E. cloacae*, *K. pneumoniae* and *S. marcescens* with decreases of growth in the period of measurement $\geq$3 $\log_{10}$ cfu/mL from 24 h (8 h in the case of *S. marcescens*) after exposure to colistin + 25 μM CCCP (Figure 1a, c and e). Colistin + 28 mM SS and colistin + 1.6 mM AS were bactericidal against *E. cloacae*, *K. pneumoniae* and *S. marcescens* from the first 4–8 h after exposure, with a slight regrowth for *E. cloacae* and *K. pneumoniae* strains after 24 h of colistin + 28 mM SS exposure (Figure 1a, c and e). Conversely, colistin + 0.8 mM AS was not bactericidal against any of the studied strains. On the other hand, the maximum tested concentration of SB + colistin was only bactericidal against *K. pneumoniae* and *E. cloacae*, after the first 4 and 12 h, respectively. This was similar to 14 mM SS, although 14 mM SS required 12 h of exposure to achieve a bactericidal effect and a slight regrowth was seen after 24 h (Figure 1a and c). None of the assayed combinations was bactericidal against *P. mirabilis* 19/11 (Figure 1g).

Likewise, time–kill assays confirmed the synergy of the combinations of colistin + 25 μM CCCP, colistin + 14 mM SS, colistin + 28 mM SS, colistin + 28 mM SB and colistin + 1.6 mM AS, from the first 2–8 h after exposure, for all the studied strains

Table 2. Effects of SS and AS on colistin MICs (mg/L) and effects of sucrose, L-arginine and L-glutamic acid

Strain	CST	CST+ SS-14	CST+ SS-14+SC	CST+ SS-14+Arg	CST+ SS-14+Glu	CST+ SS-28	CST+ SS-28+SC	CST+ SS-28+Arg	CST+ SS-28+Glu	CST+ AS-0.8	CST+ AS-0.8+SC	CST+ AS-0.8+Arg	CST+ AS-0.8+Glu	CST+ AS-1.6	CST+ AS-1.6+SC	CST+ AS-1.6+Arg	CST+ AS-1.6+Glu
E. coli 19/1	4	<0.063	0.5	<0.063	0.5	<0.063	<0.063	<0.063	<0.063	4	1	2	2	0.5	0.5	1	4
E. coli 19/2	8	0.5	2	0.5	1	0.5	0.5	0.25	0.125	2	2	2	2	1	0.5	0.5	1
E. cloacae 19/3	4096	2	0.25	<0.063	8	<0.063	<0.063	<0.063	<0.063	4	1	2	4096	1	2	1	2048
K. pneumoniae 19/4	64	1	2	0.5	8	1	1	0.5	1	32	0.5	2	64	1	1	2	2
K. pneumoniae 19/5	128	2	2	0.5	16	1	1	<0.063	2	32	2	1	128	2	2	2	16
K. pneumoniae 19/6	64	2	2	0.5	8	1	1	<0.063	2	16	1	1	64	1	1	0.5	2
K. pneumoniae 19/7	64	1	1	0.5	4	1	1	<0.063	4	32	2	1	128	2	2	4	32
K. pneumoniae 19/8	64	2	1	1	8	1	1	<0.063	4	32	4	2	128	2	2	4	64
P. aeruginosa 19/9	4	2	2	<0.063	8	2	2	2	4	2	1	0.125	4	1	0.5	1	2
S. marcescens 19/10	4096	1	<0.063	<0.063	64	<0.063	1	<0.063	128	2048	1	1	2048	8	64	16	256
P. mirabilis 19/11	>4096	>4096	1	>4096	>4096	2048	>4096	4096	>4096	>4096	2	>4096	>4096	4096	>4096	>4096	>4096
M. morganii 19/12	>4096	>4096	0.063	>4096	>4096	2048	>4096	2048	>4096	>4096	>4096	>4096	>4096	2048	>4096	>4096	>4096

CST, colistin; SS-14, 14 mM SS; SS-28, 28 mM SS; AS-0.8, 0.8 mM AS; AS-1.6, 1.6 mM AS; SC, 300 mM sucrose; Arg, 1 mM L-arginine; Glu, 1 mM L-glutamic acid.

(Figure 1a, c, e and g). This finding was consistent with the analysis of FICIs, with the exception that FICIs showed a synergistic effect on Proteae isolates for the combination of colistin + 25 μM CCCP, but not for the other combinations with colistin (Table 3). On the other hand, the combination colistin + 0.8 mM AS did not show synergy against *S. marcescens* and *P. mirabilis* in time–kill assays (Figure 1e and g), which did correspond with FICI analysis (Table 3).

Effect of sucrose and L-arginine on MICs of colistin + uncouplers

The addition of 300 mM sucrose did not reverse the effect of CCCP on colistin MICs, but it enhanced it 2- to >32-fold for *K. pneumoniae* strains, >8-fold for *E. coli* strains and >16-fold for *S. marcescens* (Table 1).

The experiment with 300 mM sucrose was also performed with combinations of colistin + SB, colistin + SS and colistin + AS. Similar to when we assessed the combination of colistin + CCCP, the addition of 300 mM sucrose did not reverse the effects of SB, 14 mM SS and 0.8 mM AS on colistin MIC, but instead promoted their effects, decreasing the MICs of the above combinations 2- to 64-fold in the strains with acquired resistance (Tables 1 and 2). Sucrose at 300 mM increased the susceptibility of *S. marcescens*, *P. mirabilis* and *M. morganii* to colistin + SB, colistin + 14 mM SS and colistin + 0.8 mM AS by 4- to >65 000-fold (Tables 1 and 2). Like sucrose, the amino acid L-arginine (1 mM) also increased the susceptibility to colistin + CCCP, colistin + 28 mM SB, colistin + 14 mM SS and colistin + 0.8 mM AS by 2- to 2048-fold (Tables 1 and 2). However, the combinations of L-arginine and sucrose with any of the four uncouplers were not synergistic against any of the 12 studied strains. On the other hand, this amino acid alone, as with sucrose, decreased colistin MICs 2- to 8-fold for *E. coli*, *E. cloacae*, *K. pneumoniae* and *S. marcescens* strains (Table 1).

Effect of L-glutamic acid on MICs of colistin + uncouplers

L-Glutamic acid (1 mM) completely reversed the acquired susceptibility to colistin induced by 0.8 mM AS for *E. cloacae*, *K. pneumoniae*, *P. aeruginosa* and *S. marcescens* strains (Table 2). However, L-glutamic acid (1 mM) partially reversed the effect of CCCP (25 μM), SB and SS (14 and 28 mM concentrations) and AS (1.6 mM) on colistin MICs for the studied strains (Tables 1 and 2). Therefore, the highest concentration of CCCP, SB, AS and SS weakened the effect of L-glutamic acid.

FICIs for the combinations of any of the four uncouplers with L-glutamic acid did not show synergism against any of the 12 clinical isolates.

Discussion

Previous studies described that CCCP reversed resistance to colistin for *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Proteus* spp., *S. marcescens*, *M. morganii* and *Providencia* sp.[13–15] and, more recently, this has also been demonstrated for anthelmintic salicylanilides against Gram-negative bacilli and partially for SS for *E. coli*.[31,33,34] CCCP and SS are uncouplers, protonophores and lipid soluble,[16,35,36] although the mechanism of action is unknown.

Table 3. FICIs of the combinations of colistin with protonophores

	CST+CCCP-10		CST+CCCP-25		CST+SB-14		CST+SB-28		CST+SS-14		CST+SS-28		CST+AS-0.8		CST+AS-1.6	
Strain	FICI	DI[a]	FICI	DI[a]	FICI	DI[a]	FICI	DI[a]	FICI	DI[a]	FICI	DI[a]	FICI	DI[a]	FICI	DI[a]
E. coli 19/1	0.143	S	0.018	S	0.500	S	0.500	S	0.016	S	0.016	S	1.004	NI	0.125	S
E. coli 19/2	0.161	S	0.080	S	0.250	S	0.250	S	0.063	S	0.063	S	0.252	S	0.126	S
E. cloacae 19/3	0.143	S	0.072	S	0.001	S	4×10^{-4}	S	0.001	S	2×10^{-5}	S	0.008	S	0.002	S
K. pneumoniae 19/4	0.205	S	0.051	S	0.063	S	0.016	S	0.016	S	0.016	S	0.557	NI	0.017	S
K. pneumoniae 19/5	0.087	S	0.044	S	0.063	S	0.016	S	0.016	S	0.008	S	0.307	S	0.019	S
K. pneumoniae 19/6	0.205	S	0.103	S	0.063	S	0.031	S	0.032	S	0.016	S	0.278	S	0.017	S
K. pneumoniae 19/7	0.103	S	0.103	S	0.063	S	0.016	S	0.016	S	0.016	S	0.557	NI	0.035	S
K. pneumoniae 19/8	0.103	S	0.051	S	0.126	S	0.031	S	0.032	S	0.016	S	0.557	NI	0.035	S
P. aeruginosa 19/9	0.643	NI	0.643	NI	1.001	NI	0.500	S	0.500	S	0.500	S	0.504	NI	0.252	S
S. marcescens 19/10	0.143	S	0.072	S	0.044	S	0.011	S	4×10^{-4}	S	2×10^{-5}	S	4.141	A	0.016	S
P. mirabilis 19/11	73.393	A	0.036	S	1.410	NI	1.410	NI	1.410	NI	0.705	NI	4.640	A	4.641	A
M. morganii 19/12	73.393	A	0.143	S	1.410	NI	1.410	NI	1.410	NI	0.705	NI	4.641	A	2.320	NI

CST, colistin; CCCP-10, 10 μM CCCP; CCCP-25, 25 μM CCCP; SB-14, 14 mM SB; SB-28, 28 mM SB; SS-14, 14 mM SS; SS-28, 28 mM SS; AS-0.8, 0.8 mM AS; AS-1.6, 1.6 mM AS.
[a]Drug interaction: S, synergy; NI, no interaction; A, antagonism.

Therefore, we decided to test SB and AS, which are partial uncouplers and lipid soluble,[35–37] and to compare their effects on colistin MICs with those of CCCP and SS. The effect of CCCP on the nine highly resistant strains resulted in decreases in colistin MICs similar to those previously described.[13–15] Likewise, SB, SS and AS induced reversals of colistin resistance similar to those of CCCP, except for Proteae strains. The ranges of decrease in colistin MICs induced by the four protonophores are not totally explained by efflux-pump inhibition, contrary to what Baron and Rolain[15] proposed, since this last mechanism results in 2-to 10-fold decreases in antibiotic MICs.[29,38] Furthermore, we generally found the strongest synergism for all combinations against non-Proteae highly colistin-resistant strains, as previously described for anthelmintic salicylanilides.[31] Interestingly, the efflux pump inhibitor CCCP did not induce any change in the colistin MIC for the ATCC susceptible strains.

On the other hand, the highest concentrations of CCCP, SS and AS were the most effective in enhancing the bactericidal action of colistin; thus, our results suggest a concentration-dependent potentiating effect on colistin bactericidal activity by the assayed protonophores. In fact, we found a higher effective bactericidal action of the combination of colistin + CCCP when using 25 μM CCCP for the studied strains than was found by Sundaramoorthy *et al.*[34] by using 10 μM CCCP for their studied strain. Furthermore, we have shown the bactericidal action of 14 and 28 mM SS against highly colistin-resistant Enterobacteriaceae, whereas the use of 5 mM salicylate by Sundaramoorthy *et al.*[34] was not bactericidal against a highly colistin-resistant *E. coli* strain and only decreased the colistin MIC by 4-fold. Unlike Sundaramoorthy *et al.*,[34] our experiment with SS was not based on it being an inhibitor of MarR.

Based on our findings, SB reversed colistin resistance for *E. cloacae*, *K. pneumoniae* and *P. aeruginosa* strains at a concentration of 28 mM, 261-fold lower than its AUC_{0-t} of 7.3 M/h that was previously described.[21] Likewise, we found that SS and AS were as good enhancers of colistin action as 25 μM CCCP at 14 and 1.6 mM concentrations, respectively, which are lower than their AUC_{0-t}s of 16 and 5 mM/h, respectively.[23,25] Therefore, the above three chemicals, unlike CCCP, could be good adjuvants to colistin in antibiotic therapies, since they had a greater bactericidal effect than CCCP on both strains with acquired resistance and strains with intrinsic resistance and at concentrations that were within their AUC_{0-t}s. Furthermore, SB, SS and AS, unlike CCCP, are approved for use in humans. SB is frequently used in syrups and in the food industry[39] and SS and AS are frequently used in humans and animals for the treatment and prevention of a large number of symptoms and diseases.[40,41]

Ni *et al.*[13] and Sekyere and Amoako[14] suggested that the ability of CCCP to restore susceptibility to colistin was due to its activity as an uncoupler and depolarizing of the cell wall. To assess this hypothesis, we created a hyperosmolar microenvironment by adding 300 mM sucrose to the culture medium for MIC determination, since it has been described that 300 mM sucrose reverses the effect of CCCP on the transmembrane electrical potential of *E. coli* by reactivating the exchange of H^+/K^+.[42] In our experiment, the addition of 300 mM sucrose did not reverse the effects of CCCP, SB, SS and AS on colistin MICs but rather enhanced them. Similar results were found with L-arginine, which, like sucrose, has been shown to restore the proton gradient in bacterial cells.[42,43] Therefore, the results of our experiments with 300 mM sucrose and L-arginine do not support depolarization as the mechanism of action of CCCP, SB, SS and AS in the reversal of colistin resistance. Sucrose and L-arginine have a strong ability to bind water molecules,[44,45] which could increase the hydrophobicity of the bacterial outer membrane, improving its interaction with colistin. In this regard, Hart and Vreeland[46] noted that low salinity in culture medium increases the hydrophobicity of the cell surface.

Domalaon *et al.*[31] suggested the reversal of colistin resistance by disruption of the outer membrane by anthelmintic salicylanilides; however, this explanation is inconsistent with the restoration of colistin resistance that we have shown with L-glutamic acid.

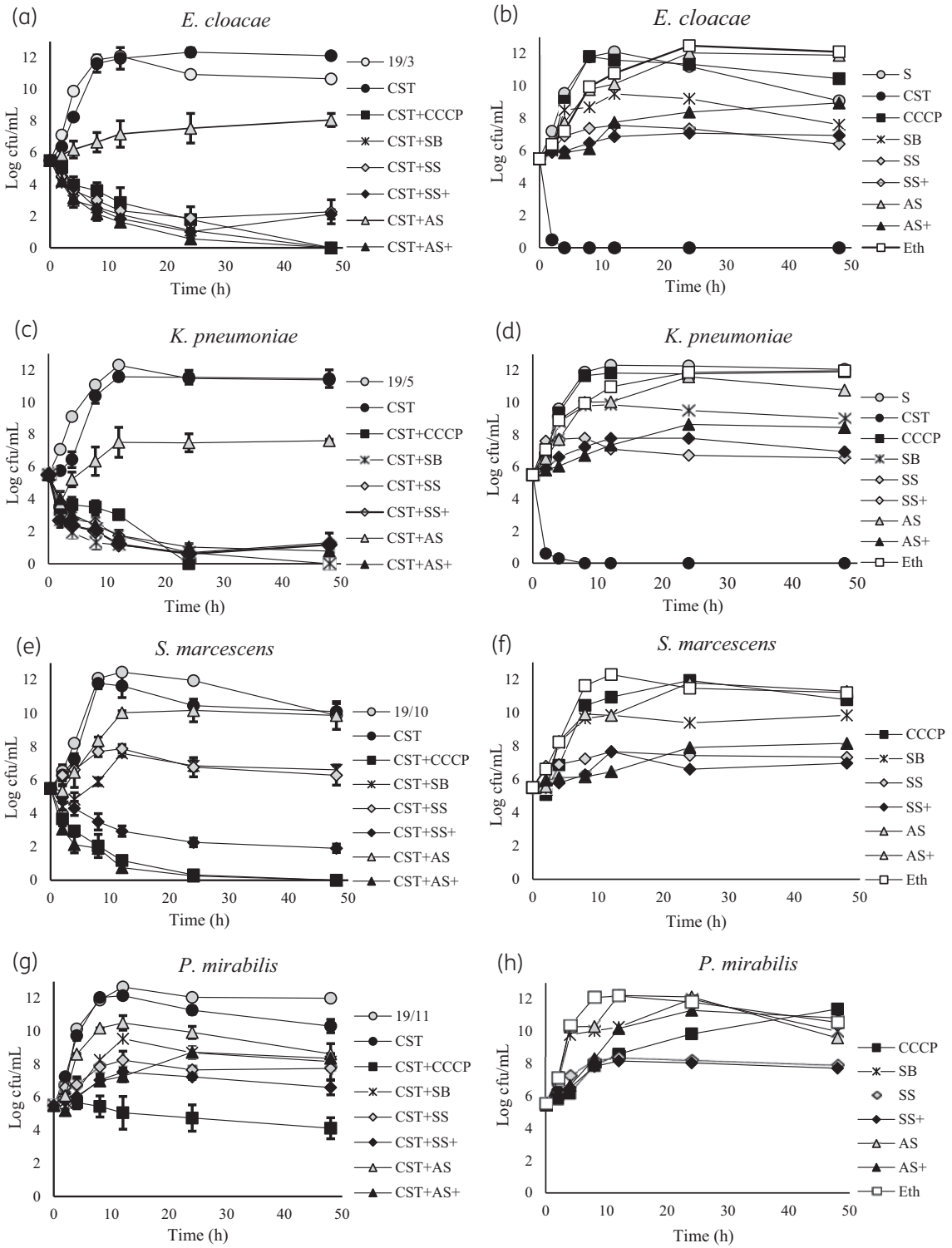

Figure 1. Time–kill assays with colistin. Growth (log cfu/mL) of colistin-resistant strains (19/3, 19/5, 19/10 and 19/11) and colistin-susceptible control strains (S) and growth (log cfu/mL) of strains with 4 mg/L colistin (CST), with 1.4% (v/v) ethanol (Eth) and with the following combinations: CST + 25 μM CCCP (CST+CCCP), CST + 28 mM SB (CST+SB), CST + 14 mM SS (CST+SS), CST + 28 mM SS (CST+SS+), CST + 0.8 mM AS (CST+AS) and CST + 1.6 mM AS (CST+AS+). The experiments were performed in triplicate and the error bars represent the SDs.

Unlike previous authors,[13–15,31] we propose that reversal of colistin resistance by CCCP, SB, SS and AS is based on their lipid-soluble properties and negative charge,[36,37,47–49] whereas colistin is positively charged.[5,7] In fact, the assays with L-glutamic acid support our hypothesis, since L-glutamic acid fully or partially restored colistin resistance in the presence of CCCP, SB, SS and AS. Therefore, though this amino acid is highly interactive with metal cations, such as Ca^{2+}, at a physiological pH,[50] it did not potentiate the action of colistin against enterobacterial strains except against the 19/2 strain. L-Glutamic acid is negatively charged at pH 7.0 and water soluble,[50–52] unlike CCCP, SB, SS and AS, which behave as lipid-soluble anions at pH 7.0.[35–37] Sundaramoorthy *et al.*[34] found that salicylate increased the cell-surface negative charge of colistin-resistant *E. coli*; thus, we suggest that the negative charge of the outer membrane could be restored by CCCP, SB, SS and AS based on their anionic charge at pH 7.0 along with their lipid solubility, which could allow an effective interaction of colistin with LPS. LPS of WT Gram-negative bacteria is a strongly negatively charged molecule[53] and resistance to colistin is mainly due to net change of charge of lipid A from −1.5 to 0.[10] Conversely, L-glutamic acid, which is not lipid soluble, would not restore the negative charge of LPS; however, it could bind to the colistin molecule, preventing the antibiotic from interacting with the outer membrane in the presence of protonophores.

Further studies are in progress to assess SB, SS and AS as adjuvants to polybasic/cationic antimicrobial peptides and antibiotics that have a self-promoted uptake mechanism, such as colistin.[54,55]

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Transparency declarations

None to declare.

Supplementary data

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

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