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Synergy between Colistin and the Signal Peptidase Inhibitor MD3 Is Dependent on the Mechanism of Colistin Resistance in *Acinetobacter baumannii*

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Synergy between colistin and the signal peptidase inhibitor MD3 was tested against isogenic mutants and clinical pairs of *Acinetobacter baumannii* isolates. Checkerboard assays and growth curves showed synergy against both colistin-susceptible strains (fractional inhibitory concentration index [FIC_{index}] = 0.13 to 0.24) and colistin-resistant strains with mutations in *pmrB* and phosphoethanolamine modification of lipid A (FIC_{index} = 0.14 to 0.25) but not against colistin-resistant Δ*lpx* strains with loss of lipopolysaccharide (FIC_{index} = 0.75 to 1). A colistin/MD3 combination would need to be targeted to strains with specific colistin resistance mechanisms.

Acinetobacter baumannii is an important nosocomial pathogen that is able to acquire or develop resistance to multiple antibiotics (1). The frequency of multidrug-resistant (MDR) clinical isolates has increased in recent years. One of "agents of last resort" with activity against *A. baumannii* MDR strains is colistin (COL) (polymyxin E) (2), a polycationic antimicrobial peptide which targets the polyanionic bacterial lipopolysaccharide (LPS) (3). Two mechanisms of resistance to colistin are the most studied in *A. baumannii* (4, 5). One involves the total loss of LPS by means of inactivation of the lipid A biosynthetic pathway. Mutations in any of the first three genes involved in lipid A biosynthesis (*lpxA*, *lpxC*, and *lpxD*) prevent interaction with colistin and result in high-level resistance (6). A second mechanism, previously studied by our group, involves mutations and increased expression of *pmrAB* genes. These result in the addition of phosphoethanolamine residues to lipid A, which decreases the negative charge displayed on the LPS (7, 8). Colistin resistance is concerning; with no new commercial antimicrobials available for use against MDR isolates, there is an urgent need to develop compounds to address this clinical issue (9, 10).

Recently, enhanced antimicrobial activity of colistin in combination with a synthetic β-aminoketone, MD3 [1-(2,5-dichlorophenyl)-3-(dimethylamino)propan-1-one], an inhibitor of bacterial type I signal peptidases (SPases), was described. It was demonstrated that SPase inhibition by MD3 in combination with outer membrane permeabilizing agents (colistin or sodium hexametaphosphate [NaHMP]) was increased in *A. baumannii* and other species (11). Bacterial SPases are involved in the maturation of proteins through cleavage of the amino-terminal signal peptides of translocated proteins (12). Permeabilization of the outer membrane should enable more-efficient access of MD3 to cytoplasm-located SPases. The aim of the present study was therefore to evaluate the synergistic effects of the combination of MD3 and colistin against strains of *A. baumannii* harboring well-characterized mechanisms of colistin resistance.

The combination of MD3 and colistin (COL) was tested against *A. baumannii* strains with deficits in LPS biosynthesis (inactivating mutations in *lpx* genes) and strains with lipid A modifications (mutations in *pmrB*). The bacterial strains used were the antibiotic-susceptible *A. baumannii* ATCC 19606 type strain and isogenically derived colistin-resistant (Col^r) mutants ATCC 19606Δ*lpxA*, ATCC 19606Δ*lpxC*, ATCC 19606Δ*lpxD* (6), and ATCC 19606*pmrB* (7). Also tested were the MDR but colistin-susceptible (Col^s) clinical isolate *A. baumannii* ABRIM (13) and its derived Col^r ABRIM*pmrB* mutant (7). Finally, two pairs of Col^s/Col^r *A. baumannii* isolates, AB248/AB249*pmrB* and AB299/AB347*pmrB*, recovered consecutively from two intensive-care units (ICU) patients before and after colistin therapy, were studied (14). Molecular mechanisms of colistin resistance in each of these strains have been extensively characterized previously (Table 1). The MICs of COL and MD3 were determined by broth microdilution following CLSI criteria (15). Colistin MICs were also confirmed by Etest (bioMérieux, France) in the presence of 1 to 4 mg/liter of MD3. The activity of COL in combination with MD3 was assessed in checkerboard assays (16, 17). After 20 h of incubation, 10-μl volumes of alamarBlue reagent (Thermo-Scientific, USA) were added to all wells to identify those containing viable bacteria. The fractional inhibitory concentration index (FIC_{index}) was calculated as follows: $FIC_{index} = FIC_{COL} + FIC_{MD3}$ = MIC [COL_{MD3}]/[COL] + MIC [$MD3_{COL}$]/[MD3]. The FIC data were interpreted as follows: $FIC_{index} = \leq 0.5$, synergy; $FIC_{index} = > 0.5$ to 4, no interaction (17). The MD3 compound was obtained from the Infectious Disease Research Institute of Seattle (USA).

Growth curve analyses were performed using COL and MD3 at

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TABLE 1 Descriptions of bacterial strains and MICs of colistin and MD3 alone and in combination[a]

A. baumannii strain	MIC (mg/liter)				FIC_{index}	Description	Source or reference
	Colistin	MD3	COL_{MD3}	$MD3_{COL}$			
ATCC 19606	0.5	32	0.06	2	Synergy	*A. baumannii* type strain	ATCC
ATCC 19606*pmrB*	64	16	0.5	4	Synergy	Isogenic derivative mutant of ATCC 19606; single amino acid substitution (Ala227Val) in PmrB	7
ATCC 19606Δ*lpxA*	64	2	16	1	No interaction	Isogenic derivative mutant of ATCC 19606; 445-bp deletion at nucleotide 364 within the *lpxA* gene and frameshift after H121	6
ATCC 19606Δ*lpxC*	128	2	64	1	No interaction	Isogenic derivative mutant of ATCC 19606; 84-bp deletion within the *lpxC* gene	6
ATCC 19606Δ*lpxD*	2,056	2	1,028	1	No interaction	Isogenic derivative mutant of ATCC 19606; single-base deletion at nucleotide 364 of the *lpxD* gene and frameshift after K317	6
ABRIM	0.5	32	0.06	4	Synergy	*A. baumannii* clinical isolate	13
ABRIM*pmrB*	32	8	0.5	1	Synergy	Isogenic derivative mutant of ABRIM; single amino acid substitution (Asn353Tyr) in PmrB	7
AB248	0.25	64	0.03	1	Synergy	*A. baumannii* clinical isolate	14
AB249*pmrB*	256	32	0.25	8	Synergy	Isogenic clinical isolate derivative of AB248; single amino acid substitution (Pro233Ser) in PmrB	14
AB299	0.25	64	0.03	4	Synergy	*A. baumannii* clinical isolate	14
AB347*pmrB*	64	16	0.25	4	Synergy	Isogenic clinical isolate derivative of AB299; single amino acid substitution (Pro170Leu) in PmrB	14

[a] COL_{MD3}, colistin MICs in the presence of MD3; $MD3_{COL}$, MD3 MICs in the presence of colistin; ATCC, American Type Culture Collection.

the fixed concentrations that resulted in synergy when COL and MD3 were combined in checkerboards. *A. baumannii* cultures were grown overnight, and 5×10^5 CFU/ml was used to inoculate 100 μl of Mueller-Hinton II broth in 96-well plates. Optical density was monitored using an Epoch-2 Microplate Spectrophotometer for 18 h (BioTek, USA).

Synergy observed in Col^s and Col^r strains with amino acid modifications in PmrB. The MICs of colistin and MD3 for the studied strains are shown in Table 1. The MICs of colistin were concordant with those previously published (14, 18). MD3 alone had little activity against any of the *A. baumannii* strains tested. However, there was a clear inverse relationship between susceptibility to MD3 and the MIC of COL, which could have been due in part to easier transport of MD3 through a modified outer membrane. All the Col^r mutants showed a MD3 MIC 2-fold to 16-fold lower than that seen with the wild-type parents (Table 1).

Clear synergy was seen against the Col^s ATCC 19606 parental strain ($FIC_{index} = 0.18$), the Col^s clinical strains ($FIC_{index} = 0.13$ to 0.24), and all the Col^r strains with modifications in *pmrB* (including both the ATCC 19606*pmrB* strain [$FIC_{index} = 0.26$] and the clinical isolates [$FIC_{index} = 0.14$ to 0.25]). However, no synergy (interpreted as no interaction) was observed against the Δ*lpx* strains ($FIC_{index} = 0.75$ to 1) (Table 1 and Fig. 1A). In Col^r Δ*lpx* mutants, the use of MD3 resulted in a reduction in the MIC of COL of only 2-fold to 4-fold. This was in contrast to 128-fold and 1,028-fold reductions at 4 mg/liter and 8 mg/liter of MD3 for the ATCC 19606*pmrB* mutant (from 64 mg/liter to 0.5 and 0.06 mg/liter, respectively). Similarly, assays performed using the ABRIM*pmrB*, AB249*pmrB*, and AB347*pmrB* Col^r clinical strains showed reductions in the COL MIC from 32 to 0.5 mg/liter, 256 to 0.25 mg/liter, and 64 to 0.25 mg/liter, respectively (64-fold to 256-fold), with MD3 added at 1 mg/liter. A decrease in the COL MIC of 8-fold to 16-fold was also observed with the Col^s clinical strains. These results confirm a potent synergistic effect of MD3 in combination with COL against Col^r strains with *pmrB* gene mutations promoting phosphoethanolamine modifications but not against Col^r strains with complete loss of the LPS.

The effects of MD3 and COL on the growth and fitness of *A. baumannii* ATCC 19606, ATCC 19606*pmrB*, and ATCC 19606Δ*lpxD* are shown in Fig. 1B. When *A. baumannii* ATCC 19606 was grown in the presence of concentrations of COL and MD3 that were below the MICs of the compounds (0.06 mg/liter and 2 mg/liter, respectively), the strain was able to grow, although its fitness was affected. However, when the two compounds were combined at those concentrations, synergy and complete inhibition of growth were observed. Similarly, the ATCC 19606*pmrB* mutant was able to grow in the presence of 1 mg/liter of COL or 4 mg/liter of MD3 (64-fold below the MIC for COL and 4-fold below the MIC for MD3) but growth was totally inhibited in the presence of COL and MD3 in combination. In contrast, in the case of ATCC 19606Δ*lpxD* (COL and MD3 MICs of 2,056 mg/liter and 2 mg/liter, respectively), no synergistic inhibition of growth was seen even with COL at 512 mg/liter. Against the clinical pairs ABRIM/ABRIM*pmrB*, AB248/AB249*pmrB*, and AB299/AB347*pmrB*, the combination of the two compounds at subinhibitory concentrations inhibited growth, while with the Δ*lpxA* and Δ*lpxC* Col^r mutants, the growth was not affected, as seen with the Δ*lpxD* mutant (data not shown). These assays confirmed the results obtained using the checkerboard method.

This study confirmed that combining MD3 and COL increases the susceptibility of Col^s *A. baumannii* strains, as previously reported (11). Moreover, we have demonstrated that there is also a significant synergistic effect against Col^r isolates that is dependent on the mechanism of colistin resistance. In Col^r *A. baumannii* strains harboring phosphoethanolamine modifications in lipid A, potent synergy between MD3 and COL was observed, but no interaction was seen when colistin resistance was mediated by lipid A deficiency. We also observed that the acquisition of colistin

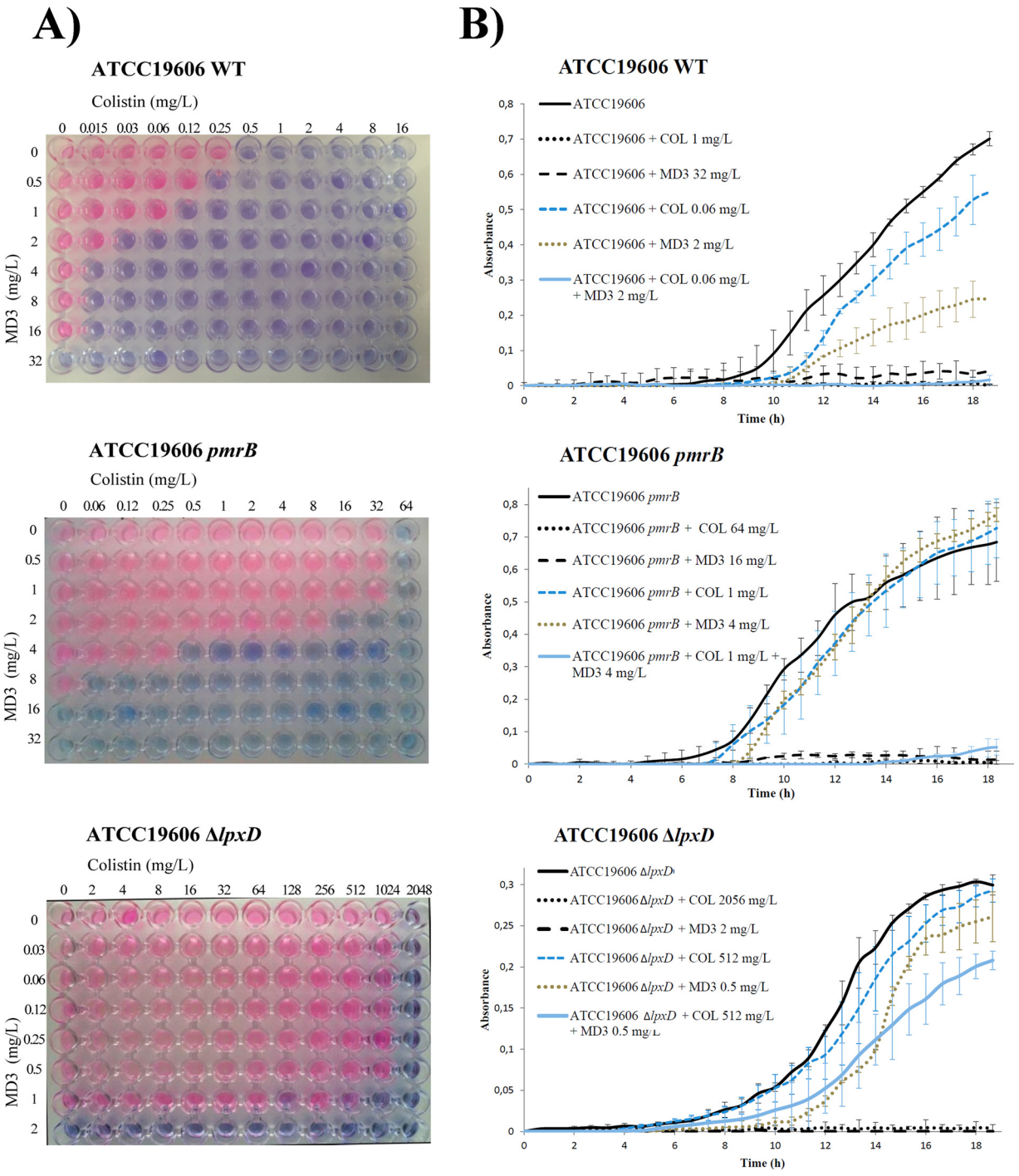

FIG 1 Assessment of synergy. (A) Checkerboard assays using colistin and MD3 performed on cultures of ATCC 19606, ATCC 19606*pmrB*, and ATCC 19606Δ*lpxD A. baumannii* strains. Pink wells indicate growth. Blue wells indicate growth inhibition. (B) Growth curves of the same strains in the absence of antimicrobials (black line) and in the presence of colistin at their MIC (black dotted line), of MD3 at their MIC (black dashed line), of colistin at their COL_{MD3} MIC (blue dashed line), of MD3 at their $MD3_{COL}$ MIC (green dotted line), and of colistin and MD3 at the COL_{MD3} and$MD3_{COL}$ MICs (blue line). COL_{MD3}, MICs of colistin in the presence of MD3; $MD3_{COL}$, MICs of MD3 in the presence of colistin. Because of the different methodologies used in the two assays, for the growth curve assays, strains ATCC 19606 and ATCC 19606*pmrB* were grown in 1 mg/liter of colistin instead 0.5 mg/liter as MIC controls. Independent assays were performed at least three times.

resistance in *A. baumannii* by means of a loss of LPS paradoxically increases the susceptibility to MD3.

In most published reports from studies of Col^r *A. baumannii* clinical isolates, resistance has been found to be mediated by modifications of the *pmrAB* genes (14, 19–22), as in those included in this study. Although clinical isolates with loss of LPS have been described (6, 23), complete loss of LPS promotes drastic changes in bacterial cellular architecture and significant loss of fitness (24),

whereas colistin resistance due to lipid A modifications leads a low or null fitness cost in the presence of antimicrobial selective pressure (18, 25). The relevant fitness burdens may affect the ability of Col^r *A. baumannii* to persist and spread in the clinical environment; therefore, it is most likely that Col^r clinical isolates of *A. baumannii* carry mutations in *pmrAB* genes rather than in *lpx* genes.

Colistin resistance due to phosphoethanolamine modification mediated by a plasmid-encoded enzyme, MCR-1, has recently been described in *Enterobacteriaceae* (26). This highlights the need for compounds, alone or in combination, able to target Col^r bacteria. We conclude that the MD3 compound could be used in the future for development of therapy against infections caused by Col^r *A. baumannii* and other MDR Gram-negative pathogens.

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