Clinical Use of Colistin Induces Cross-Resistance to Host Antimicrobials in *Acinetobacter baumannii*

Brooke A. Napier, Eileen M. Burd, Sarah W. Satola, Stephanie M. Cagle, Susan M. Ray, Patrick McGann, Jan Pohl, Emil P. Lesho, David S. Weiss

Department of Microbiology and Immunology, Emory Vaccine Center, Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, USA; Atlanta Veterans Affairs Medical Center, Georgia Emerging Infections Program, Atlanta, Georgia, USA; Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA; Multidrug-resistant Organism Repository and Surveillance Network, Walter Reed Army Institute of Research, Silver Spring, Maryland, USA; Division of Scientific Resources, Biotechnology Core Facility Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; Grady Memorial Hospital, Atlanta, Georgia, USA

ABSTRACT The alarming rise in antibiotic resistance has led to an increase in patient mortality and health care costs. This problem is compounded by the absence of new antibiotics close to regulatory approval. *Acinetobacter baumannii* is a human pathogen that causes infections primarily in patients in intensive care units (ICUs) and is highly antibiotic resistant. Colistin is one of the last-line antibiotics for treating *A. baumannii* infections; however, colistin-resistant strains are becoming increasingly common. This cationic antibiotic attacks negatively charged bacterial membranes in a manner similar to that seen with cationic antimicrobials of the innate immune system. We therefore set out to determine if the increasing use of colistin, and emergence of colistin-resistant strains, is concomitant with the generation of cross-resistance to host cationic antimicrobials. We found that there is indeed a positive correlation between resistance to colistin and resistance to the host antimicrobials LL-37 and lysozyme among clinical isolates. Importantly, isolates obtained before and after treatment of individual patients demonstrated that colistin use correlated with increased resistance to cationic host antimicrobials. These data reveal the overlooked risk of inducing cross-resistance to host antimicrobials when treating patients with colistin as a last-line antibiotic.

IMPORTANTENCE Increased use of the cationic antibiotic colistin to treat multidrug-resistant *Acinetobacter baumannii* has led to the development of colistin-resistant strains. Here we report that treatment of patients with colistin can induce not only increased resistance to colistin but also resistance to host cationic antimicrobials. This worrisome finding likely represents an example of a broader trend observed in other bacteria against which colistin is used therapeutically such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Furthermore, these data suggest that the possible future use of an array of cationic antimicrobial peptides in development as therapeutics may have unintended negative consequences, eventually leading to the generation of hyper-virulent strains that are resistant to innate host defenses. The potential for the induction of cross-resistance to innate immune antimicrobials should be considered during the development of new therapeutics.
Implimental material) and susceptibilities to multiple other antibi-
otics (Table S2). Bacterial suspensions were prepared using a
PROMPT (3M Company, St. Paul, MN) inoculation system, and
antibiotic susceptibilities were determined using Neg Breakpoint
Combo Panel type 41 on a Microscan WalkAway Plus automated
system (Siemens Healthcare Diagnostics Inc., West Sacramento,
CA). Additionally, the MICs for colistin were measured using
Etest strips (bioMérieux, Durham, NC), following the inoculation
and reading instructions of the manufacturer. The strains exhib-
ited a range of MIC values. Using Clinical and Laboratory Stan-
dards Institute (CLSI) interpretive criteria, we found that strains
CI-1, CI-2, CI-3, and 17978 were sensitive to colistin whereas
CI-4, ARLC, MU134, MU215, MU181, and MU52 were resistant
(Table S1).

We treated the colistin-sensitive and -resistant isolates with
LL-37 or lysozyme to determine their sensitivities, as previously
described (18). Briefly, overnight cultures were grown from fro-
zen stock in lysogeny broth (LB; also known as Luria broth) (BD
Biosciences, Sparks, MD) at 37°C with aeration and then diluted
to a final concentration of ~10^6 CFU/ml in 25% LB. Bacteria

FIG 1 Colistin resistance correlates with resistance to host cationic antimicrobials in A. baumannii clinical isolates. (A to J) Colistin-sensitive (A to D) and
colistin-resistant (E to J) A. baumannii clinical isolates were treated with 6.25 μg/ml of LL-37 (green) or 2.5 mg/ml of lysozyme (blue) for the indicated times and
plated for enumeration of CFU. Time zero CFU is indicated by dashed black lines. (K to L) Percent changes of CFU from time zero to 2 h after treatment with
LL-37 (K) or lysozyme (L) from 4 individual experiments with 3 to 4 samples of each isolate represented in panels A to J. Data were analyzed for significance using
the unpaired Student’s t test. **, P < 0.001; ***, P < 0.0001. Error bars represent the standard deviations of the results determined for triplicate samples.
were treated with host antimicrobials (LL-37, 6.25 μg/ml; lyso-
some, 2.5 mg/ml) and incubated with aeration at 37°C, and ali-
quots were plated at 0 h, 1 h, and 2 h for enumeration of CFU. The
colistin-sensitive clinical isolates CI-1 (Fig. 1A) and CI-2 (Fig. 1B)
were inhibited from replicating to wild-type levels in the presence
of LL-37, while CI-3 (Fig. 1C) and 17978 (Fig. 1D) were killed. In
contrast, the colistin-resistant clinical isolates CI-4 (Fig. 1E),
ARLC (Fig. 1F), MU134 (Fig. 1G), MU215 (Fig. 1H), MU181
(Fig. 1I), and MU52 (Fig. 1J) each replicated roughly 100- to 200-
fold after 2 h in the presence of LL-37. These data suggest that
colistin resistance correlates with increased resistance to the host
cationic antimicrobial peptide LL-37, which was further clearly
demonstrated when results from 4 experiments performed with
the aforementioned strains were pooled (Fig. 1K). We observed
similar phenotypes following lysozyme treatment, with the excep-
tion of the colistin-susceptible 17978 isolate (Fig. 1D), which was
able to persist in the presence of lysozyme instead of being killed,
and CI-2 (Fig. 1B), which exhibited very limited replication. These
results were again further illustrated when data from several ex-
periments were pooled (Fig. 1L) and suggest that colistin resis-
tance is highly correlated with resistance to lysozyme.

We next investigated the basis for the resistance phenotypes
observed. The A. baumannii PmrAB two-component regulatory
system induces phosphoethanolamine modifications to the lipid
A component of lipopolysaccharide (LPS) in response to the pres-
ence of polymyxins (19, 20). Arroyo et al. showed that point mu-
tations within the PmrB periplasmic domain, histidine kinase
(HisK) dimerization/phosphoacceptor domain, or C-terminal
ATP binding domain (ATPB) can confer constitutive polymyxin
resistance through these lipid A modifications (20). We sequenced
the pmrB genes in the colistin-resistant isolates used in this study
and found that each harbored nonsynonymous mutations in the
sequences encoding one or more of these three domains com-
pared to the pmrB sequence from the colistin-sensitive 17978
strain (see Table S1 in the supplemental material). In contrast,
only synonymous mutations were identified in the sequences of
pmrB from the colistin-sensitive strains. Nonsynonymous point
mutations conferring resistance can be selected rapidly and likely
explain the ability of some of the colistin-sensitive strains to even-
tually acquire resistance during extended exposure to LL-37 or
lysozyme (see Fig. S1A to D in the supplemental material) (20).
Taken together, these data suggest that mutations within pmrB
confer not only colistin resistance but also cross-resistance to host
cationic antimicrobial peptides.

Since the clinical isolates used here were very diverse (isolated
from different anatomical sites, from different areas of the United
States, and exhibiting differing antibiotic susceptibility patterns;
see Table S1 and S2 in the supplemental material), it is not sur-
prising that there was heterogeneity in their patterns of resistance
to host antimicrobials; specifically, the colistin-sensitive CI-2 iso-

**Fig 2** Clinical treatment with colistin can induce increased resistance to host cationic antimicrobials. Sequential A. baumannii isolates were collected from 2
patients (Ab6266 and Ab6267, patient 1; Ab3527 and Ab3941, patient 2) pre-colistin treatment (Ab6266 and Ab3527) and post-colistin treatment (Ab6267 and
Ab3941). Isolates were treated with 6.25 μg/ml of LL-37 (A and C) or 2.5 mg/ml of lysozyme (B and D). Percent changes from time zero to 2 h after treatment
with host antimicrobials from 3 experiments with 3 to 4 samples of each isolate are represented. Data were analyzed for significance using the unpaired Student’s
t test. *, P < 0.05; **, P < 0.001. Error bars represent the standard deviations of the results determined for triplicate samples.
late displayed low-level resistance to LL-37 and lysozyme (Fig. 1B) that was not observed with the other colistin-sensitive isolates (Fig. 1A and C to D). In order to overcome the complications of comparing diverse strains, and to directly determine whether colistin treatment can induce cross-resistance to host cationic antimicrobials, we obtained 2 pairs of A. baumannii clinical isolates from patients pre- and post-colistin treatment (21). Each pair of serial isolates (Ab6266 and Ab6267, and Ab3527 and Ab3941) was from a distinct patient, under 35 years of age, who had sustained severe trauma overseas (21). Ab6267 was collected after 3 weeks of patient treatment with colistin, and Ab3941 was collected after 6 weeks of patient treatment with colistin. Both pre-treatment isolates (Ab6266 and Ab3527) were colistin sensitive, while the post-treatment isolates (Ab6267 and Ab3941) displayed increased resistance to colistin (see Table S1 in the supplemental material). In addition, the transition to increased colistin resistance was not accompanied by changes in susceptibility to the other antibiotics tested (Table S2). While we cannot specify the event(s) (an induced mutational event, selection for a preexisting mutant, or recombination) that led to the selection for the post-treatment resistant isolates, the pre- and post-treatment isolates were shown to be highly similar by pulse-field gel electrophoresis (PFGE), using a 90% similarity cutoff, compared to 1,500 A. baumannii strains collected from 24 geographically separate hospitals (21). Additionally, optical genome mapping (OGM) analysis showed that the two sets of pre- and post-treatment isolates shared >99% homology (21).

When treated with LL-37 or lysozyme, the post-colistin treatment Ab6267 isolate displayed a significant increase in resistance compared to the corresponding pre-colistin treatment isolate (Ab6266) (Fig. 2A and B). Ab3941 displayed a significant increase in resistance to killing by lysozyme compared to Ab3527 (Fig. 2D), although there was no change in its level of resistance to LL-37 (Fig. 2C). Neither pre-colistin treatment isolate demonstrated resistance to LL-37 or lysozyme over 22 h of treatment (see Fig. S1E and F in the supplemental material). As with the colistin-resistant isolates tested above, the post-colistin treatment strains both harbored nonsynonymous mutations in critical PmrB domains (Table S1). These data from sequentially isolated strains pre- and post-colistin treatment strongly support the conclusion that clinical use of colistin induces cross-resistance to host cationic antimicrobial peptides.

Implications. The rising prevalence of antibiotic resistance is linked to increases in mortality, morbidity, length of hospitalization, and health care costs (1–5). Understanding the mechanisms and consequences of antimicrobial resistance is critical to our ability to combat this problem. Here we have determined that there is a strong correlation between colistin resistance and cross-resistance to the host antimicrobials LL-37 and lysozyme (Fig. 1 and 2). This is likely a phenomenon that occurs broadly and is relevant to a range of nosocomial pathogens, including *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, against which colistin is used as a therapy. This is also critical information in light of the fact that numerous cationic antimicrobial peptides are currently in development as novel therapeutics (22–24). Our data caution that the use of such agents in the clinic may have the unintended negative consequence of inducing resistance to host antimicrobials, which could lead to the selection of hypervirulent strains in the long term.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00021-13/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Brandi Limbag (CDC) for generously providing clinical isolates CI-1, CI-2, CI-3, CI-4, and ARLC. The MU134, MU215, MU181, and MU52 isolates were collected by the Georgia Emerging Infections Program (GaEIP) as part of the active population-based surveillance for multidrug-resistant Gram-negative bacteria. Additionally, we would like to thank Chui-Yoke Chin, Phil Rather, Tim Sampson, and William Shafer for critical review of the manuscript.

The findings and conclusions in this report are ours and do not necessarily represent the official position of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry.

This work was supported by NIH grant R21-AI098800. Its contents are solely our responsibility and do not necessarily represent the official views of the NIH.

REFERENCES

1. Brusselaers N, Vogelaers D, Blot S. 2011. The rising problem of antimicrobial resistance in the intensive care unit. Ann. Intensive Care 1:47.

2. de Kraker ME, Wolkewitz M, Davey PG, Koller W, Berger J, Nagler J, Icket C, Kalenic S, Horvatic J, Seifert H, Kaasch AJ, Paniara O, Argyropoulou A, Bompola M, Smyth E, Skally M, Ragilo A, Dumpis U, Kelmere AM, Borg M, Xuereb D, Ghita MG, Noble M, Kolman J, Grabbevec S, Turner D, Lansbury L, Grundmann H, BRDFEN Study Group. 2011. Clinical impact of antimicrobial resistance in European hospitals: excess mortality and length of hospital stay related to methicillin-resistant Staphylococcus aureus bloodstream infections. Antimicrob. Agents Chemotherapy. 55:1598–1605.

3. Maragakis LL, Perencevich EN, Cosgrove SE. 2008. Clinical and economic burden of antimicrobial resistance. Expert Rev. Anti Infect. Ther. 6:751–763.

4. Salgado CD, O’Grady N, Farr BM. 2005. Prevention and control of antimicrobial-resistant infections in intensive care patients. Crit. Care Med. 33:2373–2382.

5. Vandijck DM, Depaelemiere M, Labeau SO, Depuydt PO, Annemans L, Buyse FM, Oeyen S, Colpaert KE, Pelleman RP, Blot SI, Decruyenaere JM. 2008. Daily cost of antimicrobial therapy in patients with intensive care unit-acquired, laboratory-confirmed bloodstream infection. Int. J. Antimicrob. Agents 31:161–165.

6. Dijskstraan L, Nemec A, Seifert H. 2007. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. Nat. Rev. Microbiol. 5:939–951.

7. Peleg AY, Seifert H, Paterson DL. 2008. Acinetobacter baumannii: emergence of a successful pathogen. Clin. Microbiol. Rev. 21:538–582.

8. Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA. 2007. Global challenge of multidrug-resistant Acinetobacter baumannii. Antimicrob. Agents Chemotherapy. 51:3471–3484.

9. Cai Y, Chai D, Wang R, Liang B, Bai N. 2012. Colistin resistance of Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. J. Antimicrob. Chemother. 67:1607–1615.

10. Hancock RE. 1997. Peptide antibiotics. Lancet 349:418–422.

11. Bucki R, Leszczynska K, Namiot A, Sokołowski W. 2010. Cathelicidin LL-37: a multitask antimicrobial peptide. Arch. Immunol. Ther. Exp. (Warsz) 58:15–25.

12. Düring K, Porsch P, Mahn A, Brinkmann O, Gieffers W. 1999. The non-enzymatic microbicidal activity of lysozymes. FEBS Lett. 449:93–100.

13. Ibrahim HR, Thomas U, Pellegrini A. 2001. A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. J. Biol. Chem. 276:43767–43774.

14. Laible NJ, Germaine GR. 1985. Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against Streptococcus sanguis and Streptococcus faecalis: inhibition by chitin oligosaccharides. Infect. Immun. 48:720–728.
15. Thennarasu S, Tan A, Penumatchu R, Shelburne CE, Heyl DL, Ramamoorthy A. 2010. Antimicrobial and membrane disrupting activities of a peptide derived from the human cathelicidin antimicrobial peptide LL37. Biophys. J. 98:248–257.
16. Vaara M, Vaara T. 1981. Outer membrane permeability barrier disruption by polymyxin in polymyxin-susceptible and -resistant Salmonella typhimurium. Antimicrob. Agents Chemother. 19:578–583.
17. Vandamme D, Landuyt B, Luyten W, Schoofs L. 2012. A comprehensive summary of LL-37, the factoculum human cathelicidin peptide. Cell. Immunol. 280:22–35.
18. Mai J, Tian XL, Gallant JW, Merkley N, Biswas Z, Svyitski R, Douglas SE, Ling J, Li YH. 2011. A novel target-specific, salt-resistant antimicrobial peptide against the cariogenic pathogen Streptococcus mutans. Antimicrob. Agents Chemother. 55:5205–5213.
19. Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA. 2009. Resistance to colistin in Acinetobacter baumannii associated with mutations in the PmrAB two-component system. Antimicrob. Agents Chemother. 53:3628–3634.
20. Arroyo LA, Herrera CM, Fernandez L, Hankins JV, Trent MS, Hancock RE. 2011. The pmrCAB operon mediates polymyxin resistance in Acinetobacter baumannii ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. Antimicrob. Agents Chemother. 55:3743–3751.
21. Lesho E, Yoon E, McGann P, Snrerd E, Kwak Y, Milillo M, Onnus-Leone F, Preston L, St. Clair K, Nikolich M, Viscount H, Wortmann G, Zapor M, Grillot-Courvalin C, Courvalin P, Clifford R, Waterman P. Emergence of colistin-resistance in Acinetobacter baumannii containing a novel pmrCAB operon during colistin therapy of extremely-drug-resistant wound infections. J. Infect. Dis, in press.
22. Chen Z, Wang D, Cong Y, Wang J, Zhu J, Yang J, Hu Z, Hu X, Tan Y, Hu F, Rao X. 2011. Recombinant antimicrobial peptide hPAB-β expressed in pichia pastoris, a potential agent active against methicillin-resistant Staphylococcus aureus. Appl. Microbiol. Biotechnol. 89:281–291.
23. Lamb HM, Wiseman LR. 1998. Pexiganan acetate. Drugs 56:1047–1052; discussion 1053–1054.
24. Peters BM, Shirtliff ME, Jabra-Rizk MA. 2010. Antimicrobial peptides: primeval molecules or future drugs? PLoS Pathog. 6:e1001067.