AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

Synergistic Combinations and Repurposed Antibiotics Active against the Pandrug-Resistant *Klebsiella pneumoniae* Nevada Strain

Thea Brennan-Krohn,[a,b,c] James E. Kirby[a,c]

[a]Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA
[b]Division of Infectious Diseases, Boston Children's Hospital, Boston, Massachusetts, USA
[c]Harvard Medical School, Boston, Massachusetts, USA

KEYWORDS NDM-1, antimicrobial resistance, apramycin, carbapenem-resistant *Enterobacteriaceae*, colistin, multidrug resistance, repurposed, spectinomycin, synergy

In early 2017, the Centers for Disease Control and Prevention issued an alarming report describing a woman in Nevada who died in the setting of infection with a panresistant *Klebsiella pneumoniae* isolate that harbored an NDM-1 enzyme (AR-0636) and was colistin resistant as a result of inactivation of the *mgrB* regulator gene (1, 2). Our laboratory has previously identified colistin-containing combinations that demonstrated *in vitro* synergy against colistin-resistant, carbapenem-resistant *Enterobacteriaceae* (3). Here, we therefore tested the activity of 20 combinations, 18 of which contained colistin, against AR-0636 to assess whether they merit future investigation as treatment options for patients infected with otherwise-panresistant *Enterobacteriaceae*.

Synergy testing was performed using an inkjet printer-assisted checkerboard synergy assay developed in our laboratory (4, 5). The antimicrobials tested and suppliers were colistin and amikacin, Santa Cruz Biotechnology, Santa Cruz, CA; apramycin and spectinomycin, Alfa Aesar, Tewksbury, MA; ceftazidime, clindamycin, fusidic acid, linezolid, minocycline, and sulfamethoxazole, Chem-Impex; avibactam, MedChemExpress, Monmouth Junction, NJ; azithromycin, chloramphenicol, doxycycline, and levofloxacin, Sigma-Aldrich, St. Louis, MO; meropenem, Ark Pharm, Libertyville, IL; rifampin, Fisher Scientific, Pittsburgh, PA; tigecycline, Biotang, Inc., Lexington, MA; trimethoprim, Research Products International, Mt. Prospect, IL; vancomycin and aztreonam, MP Biomedicals, Santa Ana, CA; and eravacycline, Tetraphase Pharmaceuticals, Watertown, MA. Quality control testing was performed using bacterial strains recommended by the Clinical and Laboratory Standards Institute (6). The MIC for each antibiotic was determined from wells in the array containing only that drug. The modal colistin MIC was 16 μg/ml; the MICs for other drugs are shown in Table 1. For each well containing both antibiotics in which growth was inhibited, the fractional inhibitory concentration (FIC) for each antibiotic was calculated by dividing the concentration of the antibiotic in that well by the MIC of the antibiotic. The FIC index (FIC_I) was determined by summing the FICs of the two drugs. If the MIC of an antibiotic was off-scale, the highest concentration tested was assigned an FIC of 0.5 to permit FIC_I calculation. A minimum FIC_I ($FIC_{I\text{-}MIN}$) of $\leq$0.5 was considered synergistic, an $FIC_{I\text{-}MIN}$ of $>$4 was considered antagonistic, and intermediate values were considered indifferent. If the colistin MIC was $>$1 2-fold dilution above or below the modal colistin MIC, the results were not used, and the test was repeated with a new inoculum.

Synergy was seen when colistin was combined with all antibiotics assayed, with the exceptions of meropenem, vancomycin, amikacin, apramycin, and spectinomycin (Table 1). No combinations demonstrated antagonism. Although predictive correlations

Citation Brennan-Krohn T, Kirby JE. 2019. Synergistic combinations and repurposed antibiotics active against the pandrug-resistant *Klebsiella pneumoniae* Nevada strain. Antimicrob Agents Chemother 63:e01374-19. https://doi.org/10.1128/AAC.01374-19.

Address correspondence to James E. Kirby, jekirby@bidmc.harvard.edu.

Accepted manuscript posted online 15 July 2019
Published 23 August 2019

TABLE 1 Synergy of antimicrobials with colistin[a]

Drug	MIC (μg/ml)	$FIC_{I\text{-}MIN}$[b]	Drug concn at $FIC_{I\text{-}MIN}$ (drug/colistin)[c]
Doxycycline	>64	**0.094**	8/0.5, 4/1
Minocycline	64	**0.063**	2/0.5
Tigecycline	8	**0.125**	0.5/1
Eravacycline	4	**0.125**	0.25/1
Clindamycin	>32	**0.188**	8/0.5, 4/1
Fusidic acid	>32	**0.094**	2/1
Linezolid	>64	**0.313**	32/1
Chloramphenicol	64	**0.063**	2/0.5
Azithromycin	>64	**0.063**	4/0.5
Levofloxacin	4	**0.250**	0.5/1
Trimethoprim-sulfamethoxazole	4-76	**0.156**	0.5-9.5/0.5
Rifampin	32	**0.063**	1/0.25
Meropenem	32	0.501	NA
Ceftazidime-avibactam	>64-4	**0.125**	0.03-4/2
Amikacin	>128	0.504	NA
Apramycin	2	0.516	NA
Spectinomycin	8	0.531	NA
Vancomycin	>64	0.625	NA

[a]Colistin MIC, 16 μg/ml.
[b]$FIC_{I\text{-}MIN}$, minimum fractional inhibitory concentration index. Synergistic results are in bold.
[c]Values separated by a comma indicate two different concentration combinations that inhibited growth at the $FIC_{I\text{-}MIN}$. NA, combination not synergistic.

have not yet been established between concentrations that are active in *in vitro* synergy assays and clinical efficacy, we note that the concentration of colistin at the $FIC_{I\text{-}MIN}$ was $\leq$2 μg/ml for all synergistic combinations, which is within the susceptible range for colistin individually against *Enterobacteriaceae*, according to European Committee on Antimicrobial Susceptibility Testing breakpoints (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf). (CLSI does not have established breakpoints for colistin for *Enterobacteriaceae*.) Similarly, the concentration of each drug combined with colistin for which *Enterobacteriaceae* breakpoints have been promulgated by the CLSI (6) or the U.S. Food and Drug Administration (https://www.fda.gov/drugs/development-resources/antibacterial-susceptibility-test-interpretive-criteria) was within the susceptible range for those agents individually, except for doxycycline, which was in the intermediate range, suggesting the possibility that these combinations could be clinically useful at standard doses.

We hypothesize that the activity of colistin in combination with antibiotics that act intracellularly, including protein synthesis inhibitors that are inactive individually against Gram-negative bacteria (e.g., linezolid, clindamycin, and fusidic acid), against colistin-resistant Gram-negative organisms such as AR-0636 is the result of subinhibitory permeabilization of the outer membrane by colistin (3). Such permeabilization appears to be insufficient on its own to cause inhibition or killing but may still facilitate increased intracellular concentrations of drugs that normally either cannot pass through the outer membrane, like clindamycin, or are too efficiently expelled by efflux pumps (e.g., linezolid) to accumulate in the intracellular space.

This hypothesis is supported by the lack of synergistic activity observed when colistin was combined with meropenem and aminoglycosides, as the outer membrane does not constitute as significant a barrier for these drugs. The activities of apramycin and spectinomycin alone, however, were notable. AR-0636 is resistant to commonly used aminoglycosides, including amikacin (MIC, >128 μg/ml), but we found that the MICs of apramycin (2 μg/ml) and spectinomycin (8 μg/ml) for the strain were considerably lower. Apramycin is used in veterinary medicine but has low toxicity in animal models (7) and broad-spectrum activity against multidrug-resistant Gram-negative pathogens (8, 9). Spectinomycin, which is approved for the treatment of *Neisseria gonorrhoeae*, historically demonstrated efficacy in treating

TABLE 2 Synergy of antimicrobials with aztreonam[a]

Drug	MIC (μg/ml)	$FIC_{I\text{-}MIN}$[b]	Drug concn at $FIC_{I\text{-}MIN}$ (drug/aztreonam)
Ceftazidime-avibactam	>64-4	0.004	0.016-4/0.25
Avibactam	8	0.047	0.25/1, 0.125/2[c]

[a]Aztreonam MIC, 64 μg/ml.
[b]$FIC_{I\text{-}MIN}$, minimum fractional inhibitory concentration index. Both values represent synergistic results.
[c]Values separated by a comma indicate two different concentration combinations inhibited growth at the $FIC_{I\text{-}MIN}$.

Gram-negative urinary tract infections caused by susceptible isolates (10). Apramycin and spectinomycin remain active against strains expressing circulating ribosomal methyltransferases (e.g., *rmtC* in AR-0636 [1]), in contrast to 4,6-disubstituted 2-deoxystreptamine aminoglycosides, such as plazomicin (11). Therefore, it is possible that apramycin and spectinomycin could have clinically meaningful activity against strains like AR-0636.

Synergy was also observed when aztreonam was combined with ceftazidime-avibactam and with avibactam alone (Table 2). It has previously been observed that the combination of aztreonam, which is stable to hydrolysis by metallo-β-lactamases (MBLs) but susceptible to many of the other β-lactamases that MBL-containing bacteria usually also possess, with avibactam, which inhibits these other enzymes but not MBLs, results in activity against MBL-containing bacteria (12). The synergistic activity of this combination against AR-0636 further underscores the potential of aztreonam-avibactam as a therapeutic option for multidrug-resistant MBL-producing *Enterobacteriaceae*. We also noted that avibactam alone had activity against AR-0636, with an MIC of 8 μg/ml. Although avibactam, a non-β-lactam–β-lactamase inhibitor, has generally been described as lacking significant intrinsic antibiotic activity, *in vitro* efficacy against extended-spectrum β-lactamase (ESBL)-containing *Enterobacteriaceae* at concentrations in the range of as low to 4 to 16 μg/ml has previously been noted (13), and *in vivo* activity of ceftazidime-avibactam against MBL-producing *Enterobacteriaceae* has been demonstrated in a mouse model (14). Our results suggest that avibactam alone may have potential activity even against multidrug-resistant *Enterobacteriaceae*.

AR-0636 provides a vivid demonstration of how limited the options for standard, single-agent antimicrobial therapy have become for multidrug-resistant *Enterobacteriaceae*. Our findings suggest that existing antibiotics, some of which have been in use for decades, may have activity against such strains when used in combination or individually. Further evaluation by means of *in vitro* pharmacokinetic and pharmacodynamic assays, animal models, and, ultimately, studies in human patients, will be needed to further elucidate their potential role in clinical practice.

ACKNOWLEDGMENTS

This work was supported by a Boston Children's Hospital Office of Faculty Development Faculty Career Development fellowship, National Institute of Allergy and Infectious Diseases Career Development award K08AI132716 to T.B.-K., and National Institute of Allergy and Infectious Diseases awards R21AI146485 and R21AI142040 to J.E.K.

The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The HP D300 digital dispenser used in synergy analysis was provided for our use by Tecan (Morrisville, NC). Tecan had no role in the study design, data collection/interpretation, manuscript preparation, or decision to publish.

REFERENCES

1. de Man TJB, Lutgring JD, Lonsway DR, Anderson KF, Kiehlbauch JA, Chen L, Walters MS, Sjolund-Karlsson M, Rasheed JK, Kallen A, Halpin AL. 2018. Genomic analysis of a pan-resistant isolate of *Klebsiella pneumoniae*, United States 2016. mBio 9:e00440-18. https://doi.org/10.1128/mBio.00440-18.
2. Chen L, Todd R, Kiehlbauch J, Walters M, Kallen A. 2017. Notes from the

field: pan-resistant New Delhi metallo-beta-lactamase-producing *Klebsiella pneumoniae*–Washoe County, Nevada, 2016. MMWR Morb Mortal Wkly Rep 66:33. https://doi.org/10.15585/mmwr.mm6601a7.
3. Brennan-Krohn T, Pironti A, Kirby JE. 2018. Synergistic activity of colistin-containing combinations against colistin-resistant Enterobacteriaceae. Antimicrob Agents Chemother 62:e00873-18. https://doi.org/10.1128/AAC.00873-18.
4. Brennan-Krohn T, Truelson KA, Smith KP, Kirby JE. 2017. Screening for synergistic activity of antimicrobial combinations against carbapenem-resistant Enterobacteriaceae using inkjet printer-based technology. J Antimicrob Chemother 72:2775–2781. https://doi.org/10.1093/jac/dkx241.
5. Brennan-Krohn T, Kirby JE. 2019. Antimicrobial synergy testing by the inkjet printer-assisted automated checkerboard array and the manual time-kill method. J Vis Exp 146:e58636. https://doi.org/10.3791/58636.
6. Clinical and Laboratory Standards Institute. 2019. Performance standards for antimicrobial suceptibility testing: 29th informational supplement. CLSI document M100S-29. Clinical and Laboratory Standards Institute, Wayne, PA.
7. Matt T, Ng CL, Lang K, Sha S-H, Akbergenov R, Shcherbakov D, Meyer M, Duscha S, Xie J, Dubbaka SR, Perez-Fernandez D, Vasella A, Ramakrishnan V, Schacht J, Böttger EC. 2012. Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin. Proc Natl Acad Sci U S A 109:10984–10989. https://doi.org/10.1073/pnas.1204073109.
8. Kang AD, Smith KP, Eliopoulos GM, Berg AH, McCoy C, Kirby JE. 2017. In vitro apramycin activity against multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Diagn Microbiol Infect Dis 88:188–191. https://doi.org/10.1016/j.diagmicrobio.2017.03.006.
9. Juhas M, Widlake E, Teo J, Huseby DL, Tyrrell JM, Polikanov YS, Ercan O, Petersson A, Cao S, Aboklaish AF, Rominski A, Crich D, Bottger EC, Walsh TR, Hughes D, Hobbie SN. 2019. In vitro activity of apramycin against multidrug-, carbapenem- and aminoglycoside-resistant Enterobacteriaceae and *Acinetobacter baumannii*. J Antimicrob Chemother 74:944–952. https://doi.org/10.1093/jac/dky546.
10. Pickering LK, DuPont HL, Satterwhite TK. 1977. Evaluation of spectinomycin and gentamicin in the treatment of hospitalized patients with resistant urinary tract infections. Am J Med Sci 274:291–296. https://doi.org/10.1097/00000441-197711000-00007.
11. Garneau-Tsodikova S, Labby KJ. 2016. Mechanisms of resistance to aminoglycoside antibiotics: overview and perspectives. Medchemcomm 7:11–27. https://doi.org/10.1039/C5MD00344J.
12. Marshall S, Hujer AM, Rojas LJ, Papp-Wallace KM, Humphries RM, Spellberg B, Hujer KM, Marshall EK, Rudin SD, Perez F, Wilson BM, Wasserman RB, Chikowski L, Paterson DL, Vila AJ, van Duin D, Kreiswirth BN, Chambers HF, Fowler VG, Jr, Jacobs MR, Pulse ME, Weiss WJ, Bonomo RA. 2017. Can ceftazidime-avibactam and aztreonam overcome beta-lactam resistance conferred by metallo-beta-lactamases in Enterobacteriaceae? Antimicrob Agents Chemother 61:e02243-16. https://doi.org/10.1128/AAC.02243-16.
13. Bonnefoy A, Dupuis-Hamelin C, Steier V, Delachaume C, Seys C, Stachyra T, Fairley M, Guitton M, Lampilas M. 2004. In vitro activity of AVE1330A, an innovative broad-spectrum non-beta-lactam beta-lactamase inhibitor. J Antimicrob Chemother 54:410–417. https://doi.org/10.1093/jac/dkh358.
14. MacVane SH, Crandon JL, Nichols WW, Nicolau DP. 2014. Unexpected in vivo activity of ceftazidime alone and in combination with avibactam against New Delhi metallo-beta-lactamase-producing Enterobacteriaceae in a murine thigh infection model. Antimicrob Agents Chemother 58:7007–7009. https://doi.org/10.1128/AAC.02662-14.