Synergistic combinations and repurposed antibiotics active against the pandrug-resistant *Klebsiella pneumoniae* Nevada strain

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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 pan-resistant *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 CRE (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 pan-resistant *Enterobacteriaceae*.

Synergy testing was performed using an inkjet printer-assisted checkerboard synergy assay developed in our laboratory (4, 5). 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 minimal inhibitory concentration (MIC) for each antibiotic was determined from wells in the array containing only that drug. The modal colistin MIC was 16 μg/mL; 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 ≤0.5 was considered synergistic; an $FIC_{I\text{-}MIN}$ of >4, antagonistic; and intermediate values, indifference. If the colistin MIC were >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 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 ≤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/clinical_breakpoints/). (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 CLSI (6) or the US 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, 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 activity of apramycin and spectinomycin alone, however, was 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, approved for treatment of *N. gonorrhoeae*, historically demonstrated efficacy in treating 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 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 ESBL-containing *Enterobacteriaceae* at concentrations in the range of as low to 4-16 μg/mL has previously been noted (13), and *in vivo* activity of ceftazidime-avibactam against MBL-producing *Enterobacteriaceae* has been demonstrated in 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 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.

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TABLE 1 Synergy of antimicrobials with colistin[a]

Drug	MIC (ug/mL)	$FIC_{I\text{-}MIN}$[b]	Drug concentrations at $FIC_{I\text{-}MIN}$ (Drug/Colistin)
Doxycycline	>64	**0.094**	8/0.5 and 4/1[c]
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 and 4/1[c]
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	N/A
Ceftazidime-avibactam	>64-4	**0.125**	0.03-4/2
Amikacin	>128	0.504	N/A
Apramycin	2	0.516	N/A
Spectinomycin	8	0.531	N/A
Vancomycin	>64	0.625	N/A

[a]Colistin MIC = 16 ug/mL.

[b]$FIC_{I\text{-}MIN}$, Minimum fractional inhibitory concentration index. Synergistic results are bolded.

[c]Two different concentration combinations inhibited growth at the $FIC_{I\text{-}MIN}$.

N/A: Combination not synergistic

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TABLE 2 Synergy of antimicrobials with aztreonam[a]

Drug	MIC (ug/mL)	$FIC_{I\text{-}MIN}$[b]	Drug concentrations 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 and 0.125/2[c]

[a]Aztreonam MIC = 64 ug/mL.

[b]$FIC_{I\text{-}MIN}$, Minimum fractional inhibitory concentration index. Synergistic results are bolded.

[c]Two different concentration combinations inhibited growth at the $FIC_{I\text{-}MIN}$.

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