Validation of a High-Throughput Screening Assay for Identification of Adjunctive and Directly Acting Antimicrobials Targeting Carbapenem-Resistant Enterobacteriaceae

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ABSTRACT

We describe development and validation of a high-throughput screen (HTS) for identifying small molecules that restore the efficacy of carbapenems (adjunctives) and/or directly inhibit growth of carbapenem-resistant Enterobacteriaceae (CRE). Our HTS assay is based on a screen-counterscreen approach using a representative multidrug-resistant CRE strain, *Klebsiella pneumoniae* BIDMC12A. Specifically, we tested the ability of small molecules to inhibit bacterial growth in the presence (screen) or absence (counterscreen) of meropenem, a representative carbapenem antibiotic. Primary screening of 11,698 known bioactive compounds identified 14 with adjunctive activity and 79 with direct antimicrobial effect. Secondary screening identified triclosan as a strongly synergistic meropenem adjunctive (fractional inhibitory concentration=0.48) and confirmed azidothymidine (AZT) (minimal inhibitory concentration [MIC]=4 $\mu\text{g mL}^{-1}$), NH125 (MIC=4 $\mu\text{g mL}^{-1}$), diphenyleiiodonium chloride (MIC=8 $\mu\text{g mL}^{-1}$), and spectinomycin (MIC=32 $\mu\text{g mL}^{-1}$) as potent direct antimicrobials. Spectrum of activity of AZT and spectinomycin was tested against a collection of 103 representative Enterobacteriaceae strains ($\approx$ 50% CRE). AZT, a nucleoside analog used to treat human immunodeficiency virus, demonstrated an MIC₅₀ of 2 $\mu\text{g mL}^{-1}$. Spectinomycin, an antibiotic used to treat gonorrhea, had an MIC₅₀ of 32 $\mu\text{g mL}^{-1}$. Therefore, a significant percentage of CRE strains appeared relatively susceptible to these antimicrobials. These data identified AZT and spectinomycin as available agents warranting further study for potential treatment of multidrug-resistant CRE infection. Our results provide proof of principle and impetus for performing a large-scale HTS for discovery of novel, small-molecule adjunctives and antibacterial agents directly targeting CRE.

INTRODUCTION

Gram-negative Enterobacteriaceae are the most frequent cause of urinary tract infections and the second most frequent cause of surgical site and bloodstream infections.¹ Unfortunately, these bacterial pathogens commonly express resistance to a wide variety of antibiotics. Such resistance has increased dramatically in recent decades.² Previously, carbapenem antibiotics remained a broadly effective, relatively nontoxic treatment even for these multidrug-resistant bacteria. However, of great concern, carbapenem resistance has now emerged in Enterobacteriaceae (most notably among *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter* spp.), in the United States and around the world, undermining carbapenem efficacy.³ Not surprisingly, carbapenem-resistant Enterobacteriaceae (CRE) infection is associated with a high mortality rate.^{4,5} Often, either toxic or no therapeutic options remain. Accordingly, CRE are now classified in the highest threat level by the Centers for Disease Control and Prevention.^{6–9}

Therefore, new effective treatments for CRE are desperately needed. These might include either new antimicrobials directly targeting CRE or adjunctive antimicrobials that restore susceptibility to existing antibiotics. Restoration of carbapenem activity may have particular merit based on the intrinsic broad-spectrum bactericidal activity and favorable safety profile of these β -lactam agents.¹⁰ Current understanding of carbapenem pharmacokinetics–pharmacodynamics (PKs–PDs) indicates that therapeutic success depends on the minimal inhibitory concentration (MIC) of the organism being treated.^{11,12} The MIC is defined as the lowest twofold dilution of antimicrobial that inhibits visible growth *in vitro* under standardized conditions. Specifically, Enterobacteriaceae with a meropenem (a carbapenem) MIC $\leq 1 \mu\text{g mL}^{-1}$ behave as clinically susceptible,^{11,12} without regard to the potential underlying genotypic resistance mechanisms. Therefore, the current recommendations in the United States are to treat solely based on the MIC.¹²

Carbapenem resistance in the United States is typically mediated by a β -lactamase called the *K. pneumoniae* carbapenemase (KPC). However, different strains possessing the same KPC gene show widely differing levels of inhibition by carbapenems, with MICs spanning the range of susceptibility to frank resistance.¹³ Furthermore, transfer of carbapenemase-containing plasmids into *E. coli* K-12 is associated with a substantially lower MIC in the recipient.¹⁴ These observations suggest that attributes of the host strain contribute substantially to the phenotypic expression of carbapenem resistance. We therefore hypothesized that CRE strains could be modulated using small molecules to lower the carbapenem MIC into the susceptible range and thereby restore carbapenem therapeutic efficacy.

Accordingly, we sought to develop and validate a high-throughput screen (HTS) to simultaneously detect antimicrobials that restore carbapenem susceptibility and/or directly target CRE. Identifying small molecules that successfully target Gram-negative organisms, especially those displaying a multidrug resistance phenotype, is notoriously difficult based on impermeability of the Gram-negative double membrane combined with extensive systems of efflux pumps.¹⁵ Of note, *K. pneumoniae* is the most broadly resistant, clinically aggressive, and widespread CRE in the United States. We therefore adopted a live cell screen-counterscreen approach using a representative *K. pneumoniae* CRE strain so that hits identified in our HTS will have already passed the bar for efficacy against a clinically relevant, multidrug-resistant bacterium. In this study, we describe HTS data and secondary analysis for >10,000 bioactive compounds. In addition to obtaining proof of principle for our approach, we identified several available agents that may prove useful in treating CRE infection.

MATERIALS AND METHODS

Bacterial Strains and Chemicals

BIDMC12A is a KPC-expressing *K. pneumoniae* clinical isolate. Its genome sequence (Carbapenem Resistance Initiative, Broad Institute, broadinstitute.org) is available online (GenBank accession number: JCNE000000000.1). This strain belongs to the most prevalent multilocus sequence type (MLST), a phylogenetic grouping based on the DNA sequence of several housekeeping genes, found among *K. pneumoniae* CRE isolates in the United States.¹⁶ Specifically, BIDMC12A sequence showed an exact match with the sequence type 258 (ST258) pattern defined in the Institut Pasteur *Klebsiella* MLST database (data not shown).¹⁷ Meropenem was from ArkPharm (Libertyville, IL). Azidothymidine (AZT, zidovudine) was from Synthonix (Wake Forest, NC). Spectinomycin was from MP Biomedicals (Santa Ana, CA). Triclosan and azithromycin

were from Sigma-Aldrich (St. Louis, MO). Colistin, diphenyleneiodonium chloride, calmidazolium chloride, and NH125 were from Santa Cruz Biotechnology (Dallas, TX). All compounds were dissolved in anhydrous dimethyl sulfoxide (DMSO; Sigma-Aldrich). Meropenem stocks were stored as aliquots at -20°C and used only once. New meropenem stocks were made biweekly from solid powder and quality controlled before HTS experiments using *E. coli* ATCC 25922 according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution.¹⁸

Primary Screening

Primary screening was performed in duplicate at the ICCB-Longwood Screening Facility (Harvard Medical School, Boston, MA) using known bioactive libraries. Bioactive libraries include FDA-approved drugs, compounds with a history of use in clinical trials, and specialized libraries containing compounds with at least partially known biological effects, such as kinase inhibitors, ion channel inhibitors, G-protein-coupled receptor inhibitors, and active lipolipids. See HTS Protocol (Table 1) for an overview of the HTS assay procedure.

Two days before use in the primary or secondary screening assays, bacteria were recovered from a frozen stock and passaged on trypticase soy agar containing 5% sheep blood (Remel, Lenexa, KS). Single isolated colonies were inoculated into cation-adjusted Mueller-Hinton broth (BD Diagnostics, Franklin Lakes, NJ) and grown overnight before each experiment (~ 18 h at 37°C in a humidified 5% CO_2 atmosphere).

Experiments were performed in clear 384-well plates (Greiner Bio-One, Monroe, NC). Less than 1 h before compound transfer, test and negative control wells were filled with 30 μL of cation-adjusted Mueller-Hinton broth with or without 20 $\mu\text{g mL}^{-1}$ meropenem in the screen and counterscreen, respectively, using a Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific, Waltham, MA). Positive control wells were filled with 30 μL of cation-adjusted Mueller-Hinton broth with 10 $\mu\text{g mL}^{-1}$ colistin ± 20 $\mu\text{g mL}^{-1}$ meropenem.

Plates were then centrifuged at 150 g for 1 s. Three hundred nanoliters of each test compound was added to screening wells using a pin transfer robot. Bacterial inocula were prepared by dilution of an overnight culture of *K. pneumoniae* BIDMC12A to 1×10^6 CFU mL^{-1} in sterile, cation-adjusted Mueller-Hinton broth. Thirty microliters of this suspension was immediately added to all wells to achieve a final inoculum concentration of 5×10^5 CFU mL^{-1} , consistent with the CLSI guidelines for antimicrobial susceptibility testing.¹⁸ Note, the final meropenem and colistin concentrations cited in the previous paragraph were reduced twofold during addition of bacteria.

Table 1. HTS Protocol

Step	Parameter	Value	Description
1	Plate growth media	30 μ L	Sterile, cation-adjusted Mueller–Hinton broth with (screen) or without (counterscreen) 10 μ g mL ⁻¹ meropenem
2	Positive control	30 μ L	Mueller–Hinton broth containing 5 μ g mL ⁻¹ colistin with (screen) or without (counterscreen) 10 μ g mL ⁻¹ meropenem (final concentrations)
3	Library compounds	300 nL	Compounds are transferred using a custom pin-transfer robot
4	Negative control	—	No compounds transferred to negative control wells
5	Plate cells	30 μ L	<i>Klebsiella pneumoniae</i> BIDMC12A in sterile cation-adjusted Mueller–Hinton broth using the CLSI recommended inoculum
6	Incubation	48 h	37°C with 100% humidity
7	Assay readout	OD ₆₀₀	EnVision spectrophotometer
8	Data analysis	—	Data were analyzed using a custom Python script

Step Notes

- Growth medium was dispensed using a Multidrop Combi Automated Liquid Handler (Thermo Fisher Scientific, Waltham, MA).
- Positive controls were always located in column 24.
- Compounds are introduced into experimental wells at a 1:200 dilution. Final concentrations varied between libraries. Final compound concentrations used were 25 μ g/mL, 10 μ g/mL, 50 μ M, 16.7 μ M, or 5.6 μ M.
- Negative controls were always located in column 23.
- Final concentration of cells is $\sim 5 \times 10^5$ CFU/mL.
- Plates are stacked no more than four plates high with ample space between stacks for even heat distribution.
- OD₆₀₀ was read using an EnVision Plate Reader (PerkinElmer, Waltham, MA).
- Z-scores were calculated using the average and standard deviation of experimental wells. Hits were selected using the least significant z-score between replicates.

CLSI, Clinical and Laboratory Standards Institute; HTS, high-throughput screen.

Z-scores were calculated for each well using the formula $z = \frac{(x - \mu)}{\sigma}$, where x is the absorbance readout for an individual well, and μ and σ are the mean and standard deviation values calculated from all experimental wells in the same screening plate. Z-scores were used to designate hits as strong ($z < -6$), moderate ($-3 > z > -6$), and weak ($-1.5 > z > -3$) using the least significant z-score from the two replicate test wells. Compounds were considered a potential adjunctive if $|z|$ for the screen was at least threefold greater than that for the counterscreen ($|z_{\text{screen}}| \geq 3 \cdot |z_{\text{counterscreen}}|$). Compounds that were hits in both the screen and counterscreen were annotated as direct antimicrobials.

Hit Confirmation

Hits were cherry-picked from library plates using an EV075 Liquid Handling Platform (Tecan, Medford, MA) and analyzed in an abbreviated two-dimensional synergy/direct inhibition test to verify their activity. Specifically, fourfold serial dilutions of cherry-picked compounds (dissolved in DMSO) were dispensed into 384-well plates alone or in combination with 10 or 2.5 μ g mL⁻¹ meropenem using a D300 Digital Dispensing System (Hewlett-Packard, Palo Alto, CA). Total DMSO concentration in the wells was kept below 1%, as recommended by the CLSI guidelines for testing antibiotics dissolved in this solvent.¹² Wells were inoculated with *K. pneumoniae* BIDMC12A at a density of $\sim 5 \times 10^5$ CFU mL⁻¹ in cation-adjusted Mueller–Hinton broth in a 60 μ L assay volume. Plates were incubated at 37°C in ambient air at 100% humidity for 48 h.

Growth was quantified by reading OD₆₀₀. Percent growth inhibition was calculated compared with controls.

Plates were incubated for 48 h at 37°C in ambient air with 100% humidity in stacks no greater than four plates high with ample space for air circulation.

Data Collection and Analysis

Cell growth was quantified by optical density at 600 nm (OD₆₀₀) using an EnVision Plate Reader (PerkinElmer, Waltham, MA). Z'-scores were calculated for the screen and counterscreen on a plate-by-plate basis using data from 16 positive and 16 negative control wells using the following formula:

$$Z' = 1 - \frac{3 \cdot (\text{Positive control S.D.} + \text{Negative Control S.D.})}{|\text{Positive control mean} - \text{Negative control mean}|}$$

Two-Dimensional Synergy Testing

Two-dimensional synergy testing was conducted for select antimicrobials using the method of Chiaraviglio and Kirby with modifications. Briefly, $\sqrt{2}$ -fold dilutions of antimicrobials were made in 384-well microtiter plates using the D300 Digital Dispensing System. *K. pneumoniae* BIDMC12A cells were inoculated at a density of $\sim 5 \times 10^5$ CFU mL⁻¹ in a 50 μ L assay volume. Plates were incubated overnight at 37°C, and growth was quantified by OD₆₀₀ using an Epoch Microplate Reader (BioTek, Winooski, VT). An overnight (18–24 h), rather than the 48 h, HTS incubation period was used for two-

dimensional synergy, dose–response, and activity spectrum studies to conform with the CLSI consensus guidelines for clinical susceptibility testing of antimicrobials.^{18,19} Experiments were performed at least twice using three identical plates per experiment. Combinatorial MICs used for fractional inhibitory concentration (FIC) analysis and graphical representation in isobologram plots were defined as the concentration at which OD₆₀₀ was reduced by $\geq 95\%$ compared to a control containing media alone averaged across all experimental replicates. This cutoff is functionally equivalent to the complete growth inhibition after taking into account the $\approx 5\%$ variability in optical density of media alone. FIC values were calculated as previously described.²⁰ FIC values (Σ FIC) were calculated for each combination of test antimicrobial and meropenem that resulted in inhibition of *K. pneumoniae* BIDMC12A. FIC_A was defined as the concentration of test antimicrobial divided by the MIC of the test antimicrobial alone. FIC_B was defined as the concentration of meropenem divided by the MIC of meropenem alone. Σ FIC values were calculated by summing FIC_A+FIC_B. The lowest Σ FIC was used to test for synergy. Σ FIC ≤ 0.5 indicated synergy, whereas values >0.5 indicated lack of synergy.

Dose–Response Curves

Inhibitory effects of AZT, diphenyleneiodonium chloride (DPI), NH125, and spectinomycin were tested using the CLSI guidelines for broth microdilution.¹⁸ Experiments were performed in triplicate with at least two replicates per experiment. Dose–response curves were generated by plotting log₁₀ concentration of compound versus percent growth normalized to an antibiotic-free control. As per convention in the antimicrobial resistance field, concentrations were plotted in micrograms per milliliter. IC₅₀ and IC₉₀ values were calculated using regression analysis. The MIC values in the same experiments were determined according to the CLSI guidelines for microbroth dilution, that is, the lowest concentration of compound resulting in complete visible inhibition of growth.¹⁸

Activity Spectrum Testing

A total of 103 clinical isolates (50 carbapenem resistant and 53 carbapenem susceptible) were used to investigate AZT and spectinomycin activity spectrum. Strains were stored at -80°C arrayed in 96-well plates (Corning, Corning, NY) in 50% glycerol. One day before experiments, 96-well plates containing 100 μL sterile, cation-adjusted Mueller–Hinton broth were inoculated using a 96-pin replicator (Boeckel, Feasterville, PA) and grown to stationary phase. On the day of the experiment, stationary phase cultures were diluted 1:400 into fresh media. A replicator tool was used to inoculate diluted

bacteria into 96-well plates, each containing a separate two-fold dilution of the appropriate drug. Plates were incubated overnight at 37°C in a humidified 5% CO₂ atmosphere. Cell growth was then quantified by measurement of OD₆₀₀ using an Epoch Microplate Reader (BioTek). MICs were defined as the concentration at which 95% of bacterial growth was inhibited compared to a control containing media alone. Experiments were performed in triplicate on separate days with independent inocula, and median MIC values were reported.

RESULTS

Primary Screening

Our HTS for adjunctives and direct antimicrobials was designed as a parallel screen–counterscreen assay, in which each test compound was simultaneously evaluated for its ability to potentiate a subinhibitory concentration of a widely used carbapenem, meropenem (screen), and for its direct antibacterial effect (counterscreen). Simply, a representative *K. pneumoniae* CRE strain, BIDMC12A, was inoculated into a 384-well plate in the presence (screen) or absence (counterscreen) of 10 $\mu\text{g mL}^{-1}$ meropenem. Inoculum concentrations and growth media corresponded to standards used for *in vitro* susceptibility testing.¹⁸ Output was optical density measurement of bacterial growth after 48 h. Compounds that inhibited growth solely in the screen were considered potential adjunctives, while those that inhibited growth in both the screen and counterscreen were considered direct antimicrobials. Selection of the 48-h incubation period was based on initial experiments showing superior Z' -scores in the screening assay at 48 (average $Z' = 0.77$) versus 24 h (average $Z' = 0.43$).

Importantly, our screening strain is a representative of *K. pneumoniae* ST258 (Carbapenem Resistance Initiative, Broad Institute, broadinstitute.org), the predominant, KPC-producing sequence type found clinically in the United States.¹⁶ This strain was further chosen based on its broad-spectrum resistance to clinical antimicrobials and a high-level resistance to meropenem. Notably, clinical microbiology laboratory testing found it sensitive to colistin, doxycycline, tigecycline, and gentamicin; intermediate to tetracycline and minocycline; and resistant to ampicillin, cephalosporins, carbapenems, amikacin, tobramycin, quinolones, nitrofurantoin, and trimethoprim/sulfamethoxazole.

The subinhibitory concentration of meropenem (10 $\mu\text{g mL}^{-1}$) used in the screening assay was fivefold below the highly reproducible MIC (50 $\mu\text{g mL}^{-1}$) of the screening strain determined by the CLSI broth microdilution reference method (data not shown). Therefore, in primary screening, we were asking for compounds that could lower the meropenem MIC significantly (≥ 5 -fold) but not necessarily into the susceptible range (MIC

≤ 1). Therefore, the meropenem screening concentration was chosen as a balance between sensitivity and stringency, with the knowledge that a subset of CRE might be shifted into the clinically susceptible range with a fivefold carbapenem MIC reduction, and the activity of potential hits might be improved upon during follow-up structure–activity relationship studies.

Initially, the assay was optimized for incubation time and well volume (data not shown) resulting in Z' that routinely exceeded 0.6 in the screen and counterscreen. Using the optimized assay, we screened 11,698 compounds from the known bioactive collection at the ICCB-Longwood Screening Facility (Harvard Medical School, Boston, MA). Both screen and counterscreen were performed in duplicate to reduce the false-positive rate with use of the least significant z -score from each pair for categorizing hits.²¹ The assay demonstrated good correlation between replicate wells (screen, $R^2 = 0.76$, and counterscreen, $R^2 = 0.74$; Fig. 1). Z' for all plates in the HTS effort averaged 0.69 in the screen and 0.62 in the counterscreen.

Screening hits are summarized in *Supplementary Tables S1 and S2* (Supplementary Data are available online at www.liebertpub.com/adt). The bioactive libraries contain >250 known antimicrobials, most of which are detailed in a prior article from our laboratory.¹⁹ Typical of CRE strains, *K. pneumoniae* BIDMC12A demonstrated an extensive *in vitro* antimicrobial resistance profile. As expected, most therapeutic antimicrobials, including the penicillins, cephalosporins,

carbapenems, sulfonamides, macrolides (with the exception of azithromycin and clarithromycin), and aminoglycosides (with the exception of apramycin and spectinomycin), were negative in our screen. Polymyxins (including colistin, our positive control compound), tetracyclines (minocycline, doxycycline, and demeclocycline), rifamycins, chloramphenicols, and fluoroquinolones (third and fourth generations only) showed variable antibacterial effect. Several other compounds with known antibacterial (auranofin,²² carbadox,²³ chloroxine, clioquinol,²⁴ and triclosan) or antifungal (zinc pyrithione) activity were also screening hits. Disinfectants (hexachlorophene,²⁵ quaternary ammonium compounds, and bisbiguanides²⁶), mercurics (thiomersal), DNA damage-inducing chemotherapeutics (mitomycin C and bleomycin), and, interestingly, several nucleoside analogs used as antivirals (zidovudine, didanosine, and PMEG) or chemotherapeutics (azacitidine, clofarabine, 5'-fluoroxuridine, and zebularine) demonstrated weak to strong antibacterial activity. In total, 14 distinct compounds were putative adjunctive hits, and 79 were putative direct antimicrobial hits.

Notably, z -scores correlated well with relative growth ($R^2 = 0.86$) and proved sensitive enough to identify hits with even modest inhibition (see Fig. 2 for representative data from

Fig. 1. Correlation between replicates. Our screen was performed as a screen–counterscreen experiment, each with two replicates (A and B). OD_{600} values are plotted for each replicate of the screen (x) and counterscreen (•), respectively. Screen, $R^2 = 0.74$, and counterscreen, $R^2 = 0.76$.

Fig. 2. Representative screening data. OD_{600} values from a representative 384-well plate (screened in duplicate) were averaged between the screen and counterscreen and plotted on the x and y axes, respectively. Experimental compounds (○) that result in low growth in the screen but high growth in the counterscreen (upper left-hand corner of the graph) were considered putative adjunctives. Those that show inhibition in both the screen and counterscreen were considered direct antimicrobials. Positive (▲) and negative (◆) controls are located in the lower left and upper right, respectively. Color images available online at www.liebertpub.com/adt

a single screening plate). Due to this sensitivity, 25.7% of hits had less than 50% growth reduction. To limit our secondary analysis to compounds likely to be highly active, we excluded those that consistently displayed less than 50% growth reduction in primary screening.

Secondary Analyses and Two-Dimensional Synergy Testing

Based on this criterion, secondary analysis was selectively performed on 56 potential direct antimicrobials and 13 potential adjunctives (see *Supplementary Table S2* for a list of included and excluded compounds and *Supplementary Table S3* for results of secondary analysis) using small volume cherry picks from screening libraries. Fourteen (25%) suspected direct antimicrobials showed at least 50% growth inhibition in secondary analysis and were considered confirmed. Seven (12.5%) compounds initially classified as direct antibacterials demonstrated growth inhibition ranging from 25% to 49% and were classified as weakly active. In contrast, adjunctive activity detected in the primary screen was not confirmed during low-resolution checkerboard analysis using a fourfold dilution series. However, 14 (25%) compounds initially identified as direct antimicrobials displayed lower MIC or IC₅₀ values in the presence of meropenem and were therefore reclassified as adjunctive. Compounds for which an MIC or IC₅₀ could not be observed and showing less than 25% inhibition at concentrations equal to those tested in the HTS (50.7% of all compounds tested) were classified as negative.

Three putative adjunctives (azithromycin, calmidazolium, and triclosan) based on low-resolution testing were selected for follow-up high-resolution ($\sqrt{2}$ -fold combinatorial dilution series) checkerboard synergy assays in combination with meropenem. Neither azithromycin (FIC=0.75) nor calmidazolium (FIC=1) showed synergistic activity. However, triclosan, identified in primary screening as a direct antimicrobial, demonstrated synergy (FIC=0.48, graphically demonstrated by a markedly concave isobologram plot in *Fig. 3*).

Generation of Dose-Response Curves

Several compounds not typically considered antibacterial agents were found to have a strong antimicrobial activity in primary and secondary analyses. Among these compounds, DPI, NH125, and AZT were selected for further examination by dose-response curve analysis (*Fig. 4*). Although the MICs of these compounds were similar (*Table 2*), the dose-response curves differed (*Fig. 4*). NH125 showed a sharp decline in growth inhibition at dilutions below its MIC. In contrast, DPI exhibited a more gradual inhibition. AZT, a nucleoside analog used to treat human immunodeficiency virus infection,²⁷

Fig. 3. Isobologram suggested synergy between triclosan and meropenem. The isobologram was generated using $\sqrt{2}$ -fold dilutions of compounds in combination. Plotted are the lowest combinatorial concentrations of triclosan and meropenem that led to complete growth inhibition (*i.e.*, the combinatorial MICs). Markedly concave isobologram plots, such as this one, suggest synergy,³⁶ more formally defined by fractional inhibitory concentration analysis described in the text. Data used to generate the isobologram plot were from two experiments, each performed in triplicate. MIC, minimal inhibitory concentration.

showed a sigmoidal inhibition curve, with appreciable inhibition at concentrations well below its MIC (4 $\mu\text{g mL}^{-1}$), manifesting in an IC₈₀ (see Discussion) of 0.53 $\mu\text{g mL}^{-1}$ and an IC₅₀ 20-fold below the MIC. Spectinomycin, an aminoglycoside antibiotic used to treat *Neisseria gonorrhoeae* infections,²⁸ was also selected for further analysis (*Fig. 4*) based on growth inhibition in primary screening at concentrations significantly below those achievable in human serum²⁹ (*Supplementary Table S1*). In dose-response testing, spectinomycin

Fig. 4. Dose-response curves for selected compounds. Data points represent mean $\pm$ one standard deviation (error bars) of at least three experiments; AZT ($\blacklozenge$), NH125 ($\blacktriangle$), DPI ($\bullet$), and spectinomycin ($\blacksquare$). AZT, azidothymidine; DPI, diphenyleneiodonium chloride.

Table 2. Inhibitory Activity ($\mu\text{g mL}^{-1}$) of Selected Compounds

Compound	MIC	IC ₅₀	IC ₉₀
AZT	4 (0.53 ^a)	0.2	1.54
NH125	4	— ^b	—
DPI	8	1.49	6.37
Spectinomycin	32	9.6	27.62

^aTrailing endpoint MIC for AZT (listed in parentheses) was calculated using guidelines for thymidine salvage pathway inhibitors, trimethoprim and sulfamethoxazole.

^bIC₅₀ and IC₉₀ values were calculated by regression analysis, except for NH125, due to the abrupt change near the MIC.

DPI, diphenyleneiodonium chloride; MIC, minimal inhibitory concentration.

inhibited growth at concentrations as low as $8 \mu\text{g mL}^{-1}$, with an MIC of $32 \mu\text{g mL}^{-1}$ (Table 2).

Spectrum of Activity

Based on the relatively low MICs, AZT and spectinomycin were tested for activity spectrum against 103 carbapenem-resistant and carbapenem-susceptible clinical isolates collected at our institution over the past 7 years. Assays were performed in triplicate on consecutive days. The median MIC calculated from all three experiments is shown in Tables 3 and 4. For all strains tested, the MIC₅₀ for AZT was $2 \mu\text{g mL}^{-1}$. Importantly, the MIC value was $\leq 4 \mu\text{g mL}^{-1}$ for 56% of CRE strains. The MIC₅₀ for spectinomycin was $32 \mu\text{g mL}^{-1}$. Forty-one percentage of the CRE strains were susceptible to

Table 3. AZT Susceptibility Data for Clinical *Enterobacteriaceae* Strains

Organism, n (%)	Number (Cumulative%) of Strains with an AZT MIC ($\mu\text{g mL}^{-1}$)						MIC ($\mu\text{g mL}^{-1}$)	
	≤ 1	2	4	8	16	>16	50%	90%
<i>Klebsiella pneumoniae</i>								
Total—44 (100)	5 (11)	14 (43)	6 (56.5)	6 (70)	10 (93)	3 (100)	4	16
Meropenem susceptible—20 (45)	4 (20)	7 (55)	1 (60)	2 (70)	4 (90)	2 (100)	2	16
Meropenem resistant ^a —24 (55)	1 (4)	7 (33)	5 (54)	4 (71)	6 (96)	1 (100)	4	16
<i>Escherichia coli</i>								
Total—41 (100)	10 (24)	22 (78)	0	4 (87.7)	3 (95)	2 (100)	2	16
Meropenem susceptible—23 (56)	5 (22)	13 (78)	0	2 (87)	2 (96)	1 (100)	2	16
Meropenem resistant—18 (44)	5 (28)	9 (78)	0	2 (89)	1 (94.5)	1 (100)	2	16
<i>Enterobacter cloacae</i>								
Total—13 (100)	0	4 (31)	0	1 (39)	3 (62)	5 (100)	16	>16
Meropenem susceptible—8 (61.5)	0	3 (37.5)	0	1 (50)	3 (87.5)	1 (100)	ND	ND
Meropenem resistant—5 (38.5)	0	1 (20)	0	0	0	4 (100)	ND	ND
Others ^b								
Total—5 (100)	0	0	0	0	0	5 (100)	ND	ND
Meropenem susceptible—2 (40)	0	0	0	0	0	2 (100)	ND	ND
Meropenem resistant—3 (60)	0	0	0	0	0	3 (100)	ND	ND
All organisms—103 (100)	15 (14.6)	40 (53.6)	6 (59.4)	11 (70)	16 (85.5)	15 (100)	2	>16
All meropenem susceptible—53 (51.5)	9 (17)	23 (60.4)	1 (62.4)	5 (71.4)	9 (88.4)	6 (100)	2	>16
All meropenem resistant—50 (48.5)	6 (12)	17 (46)	5 (56)	6 (68)	7 (82)	9 (100)	4	>16

^aMeropenem resistance defined as an MIC of $\geq 2 \mu\text{g/mL}^{-1}$.

^bIncludes four strains of *Serratia marcescens* and one strain of *Citrobacter freundii*.

ND, too few strains represented to make an accurate determination.

Table 4. Spectinomycin Susceptibility Data for Clinical *Enterobacteriaceae* Strains

Organism, <i>n</i> (%)	Number (Cumulative%) of Strains with a Spectinomycin MIC ($\mu\text{g mL}^{-1}$)					MIC ($\mu\text{g mL}^{-1}$)	
	16	32	64	128	>128	50%	90%
<i>Klebsiella pneumoniae</i>							
Total—44 (100)	1 (2)	20 (48)	0	0	23 (100)	>128	>128
Meropenem susceptible—20 (45)	1 (5)	13 (70)	0	0	6 (100)	32	>128
Meropenem resistant ^a —24 (55)	0	7 (29)	0	0	17 (100)	>128	>128
<i>Escherichia coli</i>							
Total—41 (100)	1 (2)	22 (56)	1 (58)	10 (83)	7 (100)	32	>128
Meropenem susceptible—23 (56)	1 (4)	11 (52)	1 (57)	4 (74)	6 (100)	32	>128
Meropenem resistant—18 (44)	0	11 (61)	0	6 (94)	1 (100)	32	128
<i>Enterobacter cloacae</i>							
Total—13 (100)	0	7 (54)	3 (77)	0	3 (100)	32	>128
Meropenem susceptible—8 (61.5)	0	5 (62.5)	3 (100)	0	0	ND	ND
Meropenem resistant—5 (38.5)	0	2 (40)	0	0	3 (60)	ND	ND
Others ^b							
Total—5 (100)	0	0	2 (40)	0	3 (60)	ND	ND
Meropenem susceptible—2 (40)	0	0	0	0	2 (100)	ND	ND
Meropenem resistant—3 (60)	0	0	2 (67)	0	1 (33)	ND	ND
All organisms—103 (100)	2 (2)	49 (50)	6 (55)	10 (65)	36 (100)	32	>128
All meropenem susceptible—53 (51.5)	2 (4)	29 (59)	4 (66)	4 (74)	14 (100)	32	>128
All meropenem resistant—50 (48.5)	0	20 (40)	2 (44)	6 (56)	22 (100)	128	>128

^aMeropenem resistance defined as an MIC of $\geq 2 \mu\text{g/mL}$.^bIncludes four strains of *S. marcescens* and one strain of *C. freundii*.
ND, too few strains represented to make an accurate determination.

spectinomycin ($\text{MIC} \leq 32 \mu\text{g mL}^{-1}$) based on interpretive criteria for *N. gonorrhoeae*.¹²

DISCUSSION

In this work, we describe an HTS-counterscreen assay to simultaneously detect compounds that act either directly or indirectly, through potentiation of carbapenem activity, to inhibit growth of CRE. The whole-cell bioassay demonstrated robust statistical performance and allowed us to identify potentially clinically useful hits from a large known bioactive collection.

Options for treatment of CRE are extremely limited. Therefore, there is a critical need for new antimicrobials that

overcome the plethora of resistance mechanisms associated with CRE. This motivated us to design an HTS assay capable of identifying two types of agents that inhibit multidrug-resistant, carbapenemase-producing *K. pneumoniae*. The nature and rationale for the first—direct antimicrobial compounds—are obvious. However, the rationale for the second—compounds adjunctive with a carbapenem—deserves further comment. Carbapenems are broadly acting, bactericidal, β -lactam agents with a very favorable safety profile. Carbapenemase enzymes aside, carbapenems are effectively immune to hydrolysis by the multiplicity of additional β -lactamase enzymes carried by CRE strains (noted in a homology search of genomes available through the Carbapenem Resistance

Initiative, Broad Institute, broadinstitute.org, using the Comprehensive Antibiotic Resistance Database).³⁰ Therefore, identification of adjunctives, which would restore activity of a class of antimicrobials that was, before the emergence of CRE, the best and last line of defense against multidrug-resistant Enterobacteriaceae, seems highly desirable.

Importantly, variable phenotypic expression of the same KPC gene in different bacterial host backgrounds suggests that certain attributes of the host strain could be modulated to restore carbapenem susceptibility. Indeed, there is some precedent for use of adjunctives to restore carbapenem efficacy. The conceptually simplest are small-molecule adjunctives that directly inhibit carbapenemase activity. Two examples are avibactam,³¹ recently FDA approved, and RPX7009, currently in stage III clinical trials.³² Modulation of carbapenem susceptibility in CRE strains through use of double carbapenem therapy or tigecycline–carbapenem combination has also been suggested in human case reports,^{33,34} animal models, and/or *in vitro* studies.³⁵ However, it could be imagined that there are many other classes of compounds that might restore carbapenem efficacy through effects on antimicrobial transport, cell wall dynamics, or currently unknown mechanisms.

Our screen–counterscreen is capable of identifying three broad groups of adjunctives: (1) *pure adjunctives* that have no direct antimicrobial action in the absence of meropenem; (2) *mixed adjunctives* that display enhanced antimicrobial activity in the presence of meropenem, however, without reaching the formal definition of synergy in high-resolution checkerboard testing; specifically, their Σ FIC values were between 0.5 and 1; and (3) *synergistic adjunctives* that display enhanced antimicrobial activity in the presence of meropenem, with an Σ FIC value of <0.5. Hits from known antimicrobials often showed mixed adjunctive activity.

Interestingly, in replicate testing either in different primary screening libraries and/or in secondary screening, some compounds tested alternatively either as direct antimicrobials or pure, mixed, or synergistic adjunctives (*Supplementary Tables S1–S3*). These results are not surprising. Concentrations of the same compound in different libraries may differ either by design or through differential degradation during storage or freeze–thaw cycles. Our primary screening test for adjunctive activity was also necessarily limited to one concentration of meropenem, which may not have been optimal or discriminatory for detecting adjunctive activity.

In secondary analysis, digital dispensing technology allowed us to maximally use volume-limited cherry picks to test for basic adjunctive activity with two subinhibitory dilutions of

meropenem and several fourfold dilutions of test compound. In this study, adjuvancy was defined as increased antimicrobial activity in the presence of meropenem as this analysis was not powered to detect or refute synergism by commonly used standards (*i.e.*, FIC <0.5). For select compounds, we therefore tested formally for synergism by checkerboard titrations. Triclosan is an example of an antimicrobial compound that was not picked up as adjunctive in primary screening likely because concentrations tested (10 and 14.5 $\mu\text{g mL}^{-1}$) were 10-fold greater than its MIC (0.88 $\mu\text{g mL}^{-1}$). However, in secondary cherry-pick screening, we were able to test concentrations as low as 0.04 $\mu\text{g mL}^{-1}$, more than 20-fold below the MIC. This allowed us to detect previously unidentified adjunctive activity. Triclosan was therefore selected for checkerboard testing, where it was found to be synergistic with meropenem. Interestingly, there are previous reports of synergy between triclosan and amoxicillin, another β -lactam antibiotic, reminiscent of our findings.³⁶

Notably, in checkerboard synergy testing, the synergistic demarcation of FIC <0.5 is a conservative cutoff based on the inherent error in twofold dilution checkerboard testing. It is possible to have true but less dramatic adjuvancy/synergism with a higher FIC index. However, reliable detection requires more robust methods, for example, through use of more discriminatory $\sqrt{2}$ -fold combinatorial dilution series³⁷ as we performed rather than the more standard, lower resolution, twofold dilution series. Therefore, the FIC index of 0.75 determined for the combination of azithromycin and meropenem likely represents true but weak synergy.

Most direct antimicrobials identified in primary screening were neither adjunctive nor synergistic with meropenem. The most potent of these direct antimicrobials were NH125, DPI, spectinomycin, and AZT. Only the last two are approved for human therapy. NH125 is reported to have bactericidal and/or growth inhibitory activity against Gram-negative³⁸ and Gram-positive^{38,39} organisms. Our calculated MIC of 4 $\mu\text{g mL}^{-1}$ (*Fig. 4*) is similar to that found for *E. coli* K-12 (3.1 $\mu\text{g mL}^{-1}$).³⁸ DPI is a nonspecific flavoenzyme inhibitor^{40,41} with, to our knowledge, no previously reported antimicrobial activity. However, in a previous screen conducted by our laboratory, both compounds showed toxicity to the J774A.1 eukaryotic cell line at concentrations relevant to bacterial killing,¹⁹ suggesting unacceptable selectivity. As such, they were not examined further.

Interestingly, several nucleoside analogs showed direct activity in primary and secondary screening assays. These results highlight nucleoside analogs as a promising class for future exploration to combat CRE as they appear to have some

ability to avoid entrenched resistance mechanisms in these strains. AZT stood out as particularly potent.

AZT is best known as an inhibitor of HIV reverse transcriptase.^{42,43} However, AZT is also a substrate for bacterial thymidine kinase (TK).⁴⁴ TK, a critical part of the thymidine salvage pathway, catalyzes the first of three phosphorylation events that make thymidine or AZT competent for incorporation into DNA.⁴⁴ Incorporation of AZT into DNA results in chain termination and ultimately cell death.⁴⁴ Therefore, AZT also acts as an antibacterial agent, and previous studies have reported activity against *Escherichia*, *Enterobacter*, *Klebsiella*, *Salmonella*, and *Shigella*.^{44–47} Our findings are therefore consistent with prior studies performed with a small number of carbapenem-susceptible Enterobacteriaceae strains.

Notably, the antibiotics, trimethoprim and sulfamethoxazole, also interfere with DNA synthesis through modulation of the thymidine salvage pathway. These drugs require special MIC interpretive criteria¹⁸ as they commonly exhibit a phenomenon known as a “trailing endpoint.” Whereas the MIC value is standardly defined as the lowest concentration in a doubling dilution series with no visible microbial growth, the MIC values for trimethoprim and sulfamethoxazole are defined as the lowest concentration with 80% growth inhibition.¹⁸ Therefore, if we apply the same 80% growth threshold for interpretation of the AZT data, the trailing endpoint MIC for the screening strain is 0.53 $\mu\text{g mL}^{-1}$, nearly eightfold lower than the complete growth inhibition MIC (Fig. 4). Relevance of AZT trailing endpoints to *in vivo* efficacy will have to be determined through further study.

In vitro resistance to AZT has been reported to arise spontaneously in *E. coli*,⁴⁵ a phenomenon we observed with *K. pneumoniae* BIDMC12A at a nearly identical frequency ($\sim 10^{-7}$, data not shown). Interestingly, this spontaneous mutation rate did not undermine successful AZT monotherapy in high-inoculum, *E. coli*, systemic calf or mouse pyelonephritis infection models.⁴⁸ Additionally, AZT treatment is correlated with a significant reduction in *Salmonella* relapses among HIV-infected patients,⁴⁹ further supporting the hypothesis that development of resistance *in vitro* does not necessarily preclude AZT efficacy *in vivo* and the potential for a significant antibacterial effect in humans.

PK parameters previously observed for AZT relative to the MIC values (calculated using complete inhibition rather than trailing endpoint inhibition) determined in our survey of carbapenem-resistant strains ($\text{MIC}_{50} = 4$) suggest the potential for treatment efficacy. Specifically, in phase I dose escalation trials, intravenous administration of 7.5 mg kg^{-1} every 4 h was

associated with a 4.7 $\mu\text{g mL}^{-1}$ peak serum concentration.⁵⁰ Overall, these observations suggest that AZT may be a therapeutic adjunct for CRE worthy of further experimental investigation. Of note, AZT did not appear to act synergistically with meropenem ($\text{FIC} > 0.5$, data not shown).

Spectinomycin and apramycin, aminoglycoside/aminocyclitol antibiotics, were also identified as potent directly acting antimicrobials, with MIC values for the former frequently below cutoffs used to predict susceptibility in human infection (i.e., for *N. gonorrhoeae*).¹² Both differ structurally from other aminoglycosides (gentamicin, tobramycin, and amikacin). These structural differences presumably account for the absence or reduction in potentially limiting toxicities to hearing and kidney function generally associated with aminoglycosides. Specifically, spectinomycin appears potentially to be neither ototoxic⁵¹ nor nephrotoxic.⁵² Similarly, it was previously reported in animal and cochlear explant models that apramycin does not cause ototoxic side effects.⁵³ Renal toxicity may also be reduced.⁵³

Therefore, it was of interest to test spectrum of activity performed specifically for spectinomycin because of its availability as a human therapeutic. Spectinomycin proved broadly active against clinical Enterobacteriaceae isolates. More than 59% of carbapenem-susceptible strains and 40% of CRE have an $\text{MIC} \leq 32 \mu\text{g mL}^{-1}$, considered susceptible by the CLSI guidelines for *N. gonorrhoeae*.¹² Importantly, serum levels for spectinomycin reach 200 $\mu\text{g mL}^{-1}$ after a 4-g dose delivered by intramuscular injection, the typical route of administration for treatment of *N. gonorrhoeae*.⁴⁹ Furthermore, this dosing regimen is not associated with significant side effects.⁴⁹ Achievable serum concentrations are well above the MIC_{50} for our strain collection and presumably at levels favorable to concentration-dependent killing⁵⁴ associated with aminoglycoside antibiotics. Therefore, based on *in vitro* activity and promising side effect profiles, spectinomycin and potentially apramycin as well might provide benefit in treatment of human CRE infection and deserve further investigation.

We point out several potential limitations in our study. Specifically, false negatives may occur in primary screening through degradation of labile compounds during either storage or assay incubation period. We chose to use a 48 rather than a 24-h assay incubation period based on an increased assay specificity (higher Z') with the former; however, this choice was potentially at the expense of missing hits with labile activity. Of note, BIDMC12A is known to be susceptible to tigecycline based on clinical testing. Nevertheless, tigecycline was not detected as a direct antimicrobial hit in the HTS despite theoretical inclusion in libraries at concentrations

above its MIC. Oxidation of tigecycline during long-term storage in screening libraries^{12,55} presumably explains its lack of activity, consistent with evidence from a prior HTS effort.¹⁹ It is well known that meropenem is labile, and we paid particular attention to handling of this reagent to ensure consistency of data throughout the HTS, specifically through single use of stock solutions, avoidance of freeze-thaw cycles, and meticulous quality control as described in the “Materials and Methods” section. False negatives may also arise through inclusion of too low a screening concentration, for example, through limitations in dissolving polar compounds in screening libraries. Lastly, the potential for clinical efficacy of known bioactive hits (AZT and spectinomycin) against CRE will need to be explored in PK-PD studies, experiments that we plan to pursue in the future. We note that the MIC₉₀ values for the agents (Tables 3 and 4) likely exceed achievable serum concentrations (i.e., some CRE demonstrate frank resistance). If proven clinically efficacious in follow-up studies, these antimicrobials presumably could not be reliably used for empiric CRE therapy and would require susceptibility testing of clinical isolates to direct appropriate therapeutic use. Nevertheless, with few therapeutic options available for CRE, the potential to treat even a subset of strains may provide substantial clinical benefit.

We believe that the ability to detect direct antimicrobial activity and/or adjunctive activity in the same assay is a particular strength of our approach. Specifically, antimicrobials with the potential to synergize with meropenem, a therapeutically desirable property, might only be picked up as direct antimicrobials in our HTS. However, using our screening strategy, synergy will be uncovered in our more granular secondary assays, as observed for triclosan. Conversely, compounds detected initially as a pure adjunctive may have direct antimicrobial properties uncovered during either synergy or dose-response testing with fresh compound. Therefore, we believe our screening-counterscreening strategy enhances our ability to detect relevant activity against highly drug-resistant CRE.

Taken together, our data provide proof of principle for a screen-counterscreen approach for detecting direct and adjunctive inhibitors of CRE. Intriguingly, data generated with known bioactive libraries highlighted classes of antimicrobials, such as nucleoside analogs and aminoglycosides, that maintain activity despite many potential barriers associated with multidrug resistance. Presumably, their properties and those of small molecules identified in future large-scale screening efforts may be exploited to develop more effective therapeutics against these largely intractable pathogens.

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DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB: Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* 2004;39:309-317.
2. Sanchez GV, Master RN, Karlowsky JA, Bordon JM: In vitro antimicrobial resistance of urinary *Escherichia coli* isolates among U.S. outpatients from 2000 to 2010. *Antimicrob Agents Chemother* 2012;56:2181-2183.
3. Boucher HW, Talbot GH, Bradley JS, et al.: Bad bugs, no drugs: no ESCAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis* 2009;48:1-12.
4. Morrill HJ, Pogue JM, Kaye KS, LaPlante KL: Treatment options for carbapenem-resistant enterobacteriaceae infections. *Open Forum Infect Dis* 2015;2:ofv050.
5. Falagas ME, Tansarli GS, Karageorgopoulos DE, Vardakas KZ: Deaths attributable to carbapenem-resistant Enterobacteriaceae infections. *Emerg Infect Dis* 2014;20:1170-1175.
6. Thaden JT, Lewis SS, Hazen KC, et al.: Rising rates of carbapenem-resistant enterobacteriaceae in community hospitals: a mixed-methods review of epidemiology and microbiology practices in a network of community hospitals in the southeastern United States. *Infect Control Hosp Epidemiol* 2014;35:978-983.
7. Bratu S, Landman D, Haag R, et al.: Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City: A new threat to our antibiotic armamentarium. *Arch Intern Med* 2005;165:1430-1435.
8. Centers for Disease Control and Prevention: Vital signs: Carbapenem-resistant Enterobacteriaceae. *MMWR Morb Mortal Wkly Rep* 2013;62:165-170.
9. Centers for Disease Control and Prevention: Antibiotic/antimicrobial resistance biggest threats. www.cdc.gov/drugresistance/biggest_threats.html (accessed December 31, 2015).
10. Linden P: Safety profile of meropenem: An updated review of over 6,000 patients treated with meropenem. *Drug Saf* 2007;30:657-668.
11. Esterly JS, Wagner J, McLaughlin MM, Postelnick MJ, Qi C, Scheetz MH: Evaluation of clinical outcomes in patients with bloodstream infections due to Gram-negative bacteria according to carbapenem MIC stratification. *Antimicrob Agents Chemother* 2012;56:4885-4890.
12. Clinical and Laboratory Standards Institute: *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CLSI Document M100-S25*. Clinical and Laboratory Standards Institute, Wayne, PA, 2015.
13. Davies TA, Marie Queenan A, Morrow BJ, et al.: Longitudinal survey of carbapenem resistance and resistance mechanisms in Enterobacteriaceae and

- non-fermenters from the USA in 2007–09. *J Antimicrob Chemother* 2011;66: 2298–2307.
14. Navon-Venezia S, Chmelnitsky I, Leavitt A, Schwaber MJ, Schwartz D, Carmeli Y: Plasmid-mediated imipenem-hydrolyzing enzyme KPC-2 among multiple carbapenem-resistant *Escherichia coli* clones in Israel. *Antimicrob Agents Chemother* 2006;50:3098–3101.
 15. Lewis K: Platforms for antibiotic discovery. *Nat Rev Drug Discov* 2013;12:371–387.
 16. Kitchel B, Rasheed JK, Patel JB, et al.: Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* isolates in the United States: Clonal expansion of multilocus sequence type 258. *Antimicrob Agents Chemother* 2009;53:3365–3370.
 17. INSTITUT Pasteur MLST and whole genome MLST databases: *Klebsiella* sequence typing home page. <http://bigsd.bweb.pasteur.fr/klebsiella/klebsiella.html> (accessed February 25, 2016).
 18. Clinical and Laboratory Standards Institute: *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Tenth Edition. CLSI Document M07-A10*. Clinical and Laboratory Standards Institute, Wayne, PA, 2015.
 19. Chiaraviglio L, Kirby JE: High-throughput intracellular antimicrobial susceptibility testing of *Legionella pneumophila*. *Antimicrob Agents Chemother* 2015;59:7517–7529.
 20. Hitchings GH, Falco EA, Vanderwerff H, Russell PB, Elion GB: Antagonists of nucleic acid derivatives. VII. 2, 4-Diaminopyrimidines. *J Biol Chem* 1952;199: 43–56.
 21. Malo N, Hanley JA, Cerquozzi S, Pelletier J, Nadon R: Statistical practice in high-throughput screening data analysis. *Nat Biotechnol* 2006;24: 167–175.
 22. Harbut MB, Vilcheze C, Luo X, et al.: Auranofin exerts broad-spectrum bactericidal activities by targeting thiol-redox homeostasis. *Proc Natl Acad Sci U S A* 2015;112:4453–4458.
 23. Davis JW, Libke KG, Kornegay ET: Carbadox in the prevention of experimentally induced swine dysentery. *J Am Vet Med Assoc* 1968;153:1181–1184.
 24. Finkelstein RA, Raungthum J: A laboratory evaluation of clioquinol as a prophylactic against cholera. *Bull World Health Organ* 1968;38:803–807.
 25. Silvernale JN, Joswick HL, Corner TR, Gerhardt P: Antimicrobial actions of hexachlorophene: Cytological manifestations. *J Bacteriol* 1971;108:482–491.
 26. Weinberg ED: Antimicrobial activities of biguanides. *Ann N Y Acad Sci* 1968;148:587–600.
 27. Tan X, Chu CK, Boudinot FD: Development and optimization of anti-HIV nucleoside analogs and prodrugs: A review of their cellular pharmacology, structure-activity relationships and pharmacokinetics. *Adv Drug Deliv Rev* 1999;39:117–151.
 28. Unemo M: Current and future antimicrobial treatment of gonorrhoea—The rapidly evolving *Neisseria gonorrhoeae* continues to challenge. *BMC Infect Dis* 2015;15:364.
 29. Kusumi R, Metzler C, Fass R: Pharmacokinetics of spectinomycin in volunteers with renal insufficiency. *Chemotherapy* 1981;27:95–98.
 30. McArthur AG, Waglechner N, Nizam F, et al.: The comprehensive antibiotic resistance database. *Antimicrob Agents Chemother* 2013;57: 3348–3357.
 31. Watkins RR, Papp-Wallace KM, Drawz SM, Bonomo RA: Novel beta-lactamase inhibitors: A therapeutic hope against the scourge of multidrug resistance. *Front Microbiol* 2013;4:392.
 32. Efficacy, Safety, Tolerability of Carbavance Compared to Best Available Therapy in Serious Infections Due to Carbapenem Resistant Enterobacteriaceae, in Adults. <https://clinicaltrials.gov/ct2/show/NCT02168946> (accessed January 11, 2016).
 33. Bulik CC, Nicolau DP: Double-carbapenem therapy for carbapenemase-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2011;55: 3002–3004.
 34. Ceccarelli G, Falcone M, Giordano A, et al.: Successful ertapenem-doripenem combination treatment of bacteremic ventilator-associated pneumonia due to colistin-resistant KPC-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2013;57:2900–2901.
 35. Lim TP, Cai Y, Hong Y, et al.: In vitro pharmacodynamics of various antibiotics in combination against extensively drug-resistant *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2015;59:2515–2524.
 36. Wignall GR, Goneau LW, Chew BH, Denstedt JD, Cadieux PA: The effects of triclosan on uropathogen susceptibility to clinically relevant antibiotics. *J Endourol* 2008;22:2349–2356.
 37. Pillai K, Eliopoulos G: Antimicrobial combinations. In: *Antibiotics in Laboratory Medicine*. Lorian V, (ed.), pp. 365–440. Lippincott, Williams and Wilkins, Philadelphia, PA, 2005.
 38. Yamamoto K, Kitayama T, Ishida N, et al.: Identification and characterization of a potent antibacterial agent, NH125 against drug-resistant bacteria. *Biosci Biotechnol Biochem* 2000;64:919–923.
 39. Kim W, Conery AL, Rajamuthiah R, Fuchs BB, Ausubel FM, Mylonakis E: Identification of an antimicrobial agent effective against methicillin-resistant *Staphylococcus aureus* persists using a fluorescence-based screening strategy. *PLoS One* 2015;10:e0127640.
 40. Riganti C, Gazzano E, Polimeni M, Costamagna C, Bosia A, Ghigo D: Diphenyleneiodonium inhibits the cell redox metabolism and induces oxidative stress. *J Biol Chem* 2004;279:47726–47731.
 41. Stuehr DJ, Fasehun OA, Kwon NS, et al.: Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. *FASEB J* 1991;5:98–103.
 42. Mitsuya H, Broder S: Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proc Natl Acad Sci U S A* 1986; 83:1911–1915.
 43. Furman PA, Fyfe JA, St Clair MH, et al.: Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci U S A* 1986;83:8333–8337.
 44. Elwell LP, Ferone R, Freeman GA, et al.: Antibacterial activity and mechanism of action of 3'-azido-3'-deoxythymidine (BW A509U). *Antimicrob Agents Chemother* 1987;31:274–280.
 45. Lewin CS, Allen R, Amyes SG: Zidovudine-resistance in *Salmonella typhimurium* and *Escherichia coli*. *J Antimicrob Chemother* 1990;25:706–708.
 46. Lewin CS, Watt B, Paton R, Amyes SG: Isolation of zidovudine resistant *Escherichia coli* from AIDS patients. *FEMS Microbiol Lett* 1990;58:141–143.
 47. Sandrini MP, Clausen AR, On SL, Aarestrup FM, Munch-Petersen B, Piskur J: Nucleoside analogues are activated by bacterial deoxyribonucleoside kinases in a species-specific manner. *J Antimicrob Chemother* 2007;60:510–520.
 48. Keith BR, White G, Wilson HR: In vivo efficacy of zidovudine (3'-azido-3'-deoxythymidine) in experimental gram-negative-bacterial infections. *Antimicrob Agents Chemother* 1989;33:479–483.
 49. Schroeter AL, Reynolds GH, Holmes KK, Pyke T, Wiesner PJ: Spectinomycin in the treatment of gonorrhea. *J Am Venereal Dis Assoc* 1975;1:139–145.
 50. Cload PA: A review of the pharmacokinetics of zidovudine in man. *J Infect* 1989;18 Suppl 1:15–21.
 51. Akiyoshi M, Yano S, Ikeda T: Ototoxicity of spectinomycin (author's transl). *Jpn J Antibiot* 1976;29:771–782.
 52. Trobicin™ spectinomycin for injectable suspension, USP. www.accessdata.fda.gov/drugsatfda_docs/label/2012/050347s060lbl.pdf (accessed January 11, 2016).
 53. Matt T, Ng CL, Lang K, et al.: Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin. *Proc Natl Acad Sci U S A* 2012;109:10984–10989.
 54. Gonzalez LS, 3rd, Spencer JP: Aminoglycosides: A practical review. *Am Fam Physician* 1998;58:1811–1820.

55. Bradford PA, Petersen PJ, Young M, Jones CH, Tischler M, O'Connell J: Tigecycline MIC testing by broth dilution requires use of fresh medium or addition of the biocatalytic oxygen-reducing reagent oxyrase to standardize the test method. *Antimicrob Agents Chemother* 2005;49:3903–3909.

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Abbreviations Used

ATCC = American Type Culture Collection
AZT = azidothymidine (zidovudine)
CFU = colony-forming units
CLSI = Clinical and Laboratory Standards Institute
CRE = carbapenem-resistant Enterobacteriaceae
DMSO = dimethyl sulfoxide
DPI = diphenyleneiodonium chloride
FIC = fractional inhibitory concentration
HTS = high-throughput screen
IC₅₀/IC₉₀ = concentration at which 50/90% of growth is inhibited
KPC = *Klebsiella pneumoniae* carbapenemase
MIC = minimal inhibitory concentration
MIC_{50/90} = minimal inhibitory concentration for 50/90% of strains
MLST = multilocus sequence type
OD₆₀₀ = optical density at 600 nm
PK = pharmacokinetic
PD = pharmacodynamic
TK = thymidine kinase