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Original Article

Screening of MMV pandemic response and pathogen box compounds against pan-drug-resistant *Klebsiella pneumoniae* to identify potent inhibitory compounds

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ABSTRACT

Background: The recent emergence of pan-drug-resistant (PDR) *K. pneumoniae* strains hinders the success rate of treatment procedures for patients. High mortality, extended duration of hospitalization with high costs is associated with such infections. Discovery and identification of new drugs are inevitable to combat PDR clinical pathogens. We aim to identify and evaluate new compounds *in vitro* against a PDR clinical *K. pneumoniae* isolate using compounds of Pathogen Box and Pandemic Response Box from Medicines for Malaria Venture (MMV).
Methods: The PDR strain was initially screened with the 601 compounds from both Boxes at 10 μM concentration. Formation of dormant cells against the drug activity was assessed using persister assay. MIC was determined for the drugs inhibiting PDR *K. pneumoniae* during initial screening.
Results: Five compounds were identified to inhibit the test strain. MMV1580854 (94.60 %), MMV1579788 (94.65 %), MMV1578574 (eravacycline; 93.13 %), MMV1578566 (epetraborole; 95.29 %) and MMV1578564 (96.32 %) were able to exhibit a higher percentage of growth inhibition. Persisters were found to be growing in a range from 10^4 to 10^7 CFU/ml. Minimum inhibitory concentrations (MIC) of all compounds were $\geq$ 2 μM except for MMV1579788, which had a MIC of $\geq$ 5 μM.
Conclusion: Five novel compounds were identified against the highly evolved pan-drug-resistant *K. pneumoniae*. Among the five, epetraborole andMMV1578564 were identified as highly potent based on the persister frequency and MICs. The pan-drug resistant clinical isolate used in this study was found to be acting differently from the reference or wild type strains against the test compounds in a previous study.

1. Introduction

Multidrug-resistant *Klebsiella pneumoniae* causing hospital acquired infections in immune-compromised patients leads to a significant burden to the healthcare systems. Patients with weakened immune systems have increased mortality and morbidity risks from secondary infections [1]. *K. pneumoniae* is a rod-shaped, non-motile, gram negative opportunistic pathogen belonging to the *Enterobacteriaceae* family. The pathogen has the potential to acquire multi drug resistance to different classes of drugs, and more often a causative agent of various infections, including pneumonia, urinary tract infections (UTIs), blood stream infections, meningitis and liver abscesses [2]. The World Health

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Organization (WHO) released a list of priority pathogens in 2017 for which novel antibiotics are urgently required. The top-tier pathogen in this belongs to Enterobacteriaceae producing ESBLs (extended spectrum beta lactamases) resistant to carbapenem [3]. *K. pneumoniae* is the second-most frequent etiologic agent responsible for hospital acquired infections and community-acquired infections [4]. Hospital acquired infections include ventilator associated pneumonia, catheter-associated urinary tract infections and wound or surgical site infections [5,6]. *K. pneumoniae* is well known for producing polysaccharide biofilms, adhesins and siderophores that help in survival through evading the host immune response during infection [7]. The majority of *K. pneumoniae* strains exhibit a mucoid phenotype during growth in agar media, due to the expression of a polysaccharide capsule on their surface [8]. Capsular polysaccharides are considered as an important virulence factor which can bind and neutralize the host antibodies that opsonize bacteria for phagocytosis [9,10]. *K. pneumoniae* has predominantly acquired resistance to various generations of antibiotics. It is resistant to ampicillin and other first generation β-lactam antibiotics due to the presence of *SHV* gene chromosomal DNA. Carbapenem was used as the gold standard treatment against ESBL producing strains [11]. Mutations responsible for changes in the function of efflux pumps and porin structure played a critical role in the development of carbapenem resistance [12, 13]. *K. pneumoniae* carbapenemase (KPC) producing *K. pneumoniae* strains emerged as a predominant clinical pathogen, causing a major public health burden associated with a significant high mortality rate of 40–50 % when compared to carbapenem susceptible *K. pneumoniae* with a death rate of 2.4–12.5 % [14,15]. The emergence of carbapenem resistance narrowed down the option to the last resorts colistin and tigecycline for treatment [16]. That notwithstanding, carbapenem resistant *K. pneumoniae* overcome colistin either via chromosomal mutations in the *mgr*B gene or by acquiring plasmid borne mobile colistin resistant genes [17–19]. Moreover, usage of both antibiotics comes with disadvantages such as neurotoxicity, nephrotoxicity and insufficient serum levels [20]. Pan-drug resistant *K. pneumoniae* is responsible for major burdens in clinical settings worldwide, which can lead to healthcare associated infections in patients with compromised immune systems, individuals undergoing organ transplantation and elderly patients [19,21–23]. On several occasions, the ceftazidime-avibactam (CAZ-AVI) complex regime was employed to treat bacteremia caused by Carbapenemase producing pan-drug resistant *K. pneumoniae pathogen strains* [24,25]. Existing drugs such as colistin and tigecycline are ineffective in treating infections caused by these extensively drug-resistant bacteria (XDR)/pan-drug resistant bacteria (PDR). Failure of antibiotics and the emergence of drug resistance in bacterial pathogens insist the serious demand to expand the antibiotic arsenal fo r tackling pan-drug resistant bacteria. MMV recently developed two drug libraries, namely the Pathogen Box and Pandemic Response Boxes, each containing 400 structurally diverse drugs [26].

The Pathogen Box is an open source drug library containing 400 diverse drugs like compounds that are effective against infectious diseases such as tuberculosis, malaria, cryptosporidiosis and toxoplasmosis which are prevalent in tropical and sub-tropical regions. The compounds were developed to accelerate the search for novel activity against pathogens mediating neglected tropical diseases. They act as an important source for drug discovery since they were generated with a variety of chemical scaffolds and as they are very different from the antibiotics that are currently available in the market [27,28].

MMV and Drugs for Neglected Diseases initiative (DNDi) collaborated to create the Pandemic Response Box compounds as a joint effort in association with scientists from public and private sectors. These compounds help the researchers across the globe to explore the options against emerging pandemic diseases. This box contains four hundred structurally diverse compounds which can be tested against global pathogens causing infectious and neglected diseases. Researchers working in the drug discovery field have selected the compounds of their interest, with distinct mechanisms of action and unique qualities with potential antibacterial, antiviral or antifungal properties. Potential compounds with robust bio-activities are in different phases of clinical trials. These biologically active compounds have been selected by MMV and DNDi collaboration based on the literature information, albeit with limited knowledge against the pathogens responsible for the infectious diseases [26]. To this end, compounds available in Pathogen Box and Pandemic Response Boxes would serve as a screening library against emerging MDR and PDR pathogens of interest.

The study investigated the antibacterial activity of potential candidate against a pan drug-resistant *K. pneumoniae* clinical isolate. In this study, we used compounds from the Pathogen and Pandemic Response Boxes to evaluate their inhibitory activity against a PDR *K. pneumoniae* clinical isolate that was obtained from a patient undergoing treatment at atertiary hospital located in the Coimbatore District, state of Tamil Nadu, in the Southern part of India. Using the microbroth dilution method, we evaluated inhibitory activity by screening all the 400 compounds from the Pathogen Box and 201 compounds from the Pandemic Response Box predicted to have antibacterial activity against the PDR *K. pneumoniae.* Additionally, minimum inhibitory concentration of the compounds that exhibit activity against pan-drug resistant *K. pneumoniae* was also determined. Persister cells are the subpopulation of the bacteria that remain dormant even after the action by antibiotics. Later, they could restore their metabolic functions and cause recurrent infections in the host, also getting transmitted to other hosts [29]. Frequencies of persisters on antibiotic free solid media after exposure against the library compounds were recorded.

2. Materials and methods

2.1. Isolation and identification of bacterial culture

The clinical isolate used in the study was isolated from a (uro) sepsis patient. A urine catheter swab was rubbed onto MacConkey and Blood agar (Himedia, India) media supplemented with sheep blood and then incubated at 37 °C for 16–18 h. A urine culture was also initiated. Resulting bacterial colonies from urine and catheter swab samples were further sub-cultured and subjected to bacterial identification. Bacterial isolate was streaked on to the MacConkey agar, incubated overnight at 37 °C and stored at 2 °C until use. Bacterial isolate was subjected to Vitek2® test (BioMérieux, Marcy-l'Étoile, France) with GN ID card and 16s rRNA sequencing by Sanger method. Bacterial suspension adjusted to 0.5 McFarland density was loaded into theVitek2®, BioMérieux GN ID card. QIAMP DNA mini kit (Qiagen, Germany) was used to extract DNA using manufacturer's instructions followed by 16s rRNA region amplification using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). Twenty-five μl of PCR reaction mixture consisted of 12.5 μl of Emerald Green 2× master mix (TakaraBio, Japan), 200 nM of forward and reverse primers (IDT Technologies, Singapore), 2μlof template and with nuclease-free water to make-up the volume. PCR conditions for 16s rRNA gene amplification are as follows: Initial denaturation for 94 °C for 5min, followed by 30 cycles of denaturation at 94 °C 60s, annealing at 55 °C for 1min and elongation at 72 °C for 90s and a final cycle of elongation at 72 °C for 10 min. PCR products were visualized with the 1.5 % agarose gel electrophoresis method with ethidium bromide staining. PCR product was purified by GENECLEAN® Turbo (MP Bio, France) and subjected to Sanger sequencing, using a Applied Biosystems 3500 Genetic Analyzer instrument (Hitachi Hi-tech, Tokyo, Japan). Sequenced raw reads and Chromatogram data were visualized and analyzed with FinchTV software. Trimmed reads were submitted in NCBI BLASTn against rRNA database to identify the hits.

2.2. Antimicrobial sensitivity and string test

The bacterial strain was subjected to antimicrobial sensitivity testingusingVitek2®, (BioMérieux, France) AST-N280 card, Kirby-Bauer

disk diffusion and micro-broth dilution methods, representing a standard antibiotics test panel (Himedia, Mumbai India; Table 1). Antibiotics including tobramycin, ceftazidime + avibactam, cefotaxime, ertapenem, cefoperazone + sulbactam, meropenem, ciprofloxacin, amikacin, cefuroxime-axetil, nitrofurantoin, nalidixic acid, doxycycline, trimethoprim + sulfamethoxazole, cefuroxime, ceftriaxone, cefepime, imipenem, colistin, piperacillin + tazobactam, ofloxacin, gentamycin, netilmicin, tigecycline, amoxcillin + clavulanic acid, norfloxacin, and ampicillin were used for antibiotic susceptibility profiling of the clinical isolate. Less than five disks were used per Mueller-Hinton agar (Himedia, India) plate with a test organism lawn for the agar disk diffusion method, or the Kirby Bauer method for antimicrobial susceptibility testing. The inhibition zone was measured as per the manufacturer's guidelines. Reports from the Vitek2®sytem were also used to identify the antibiotic susceptibility profile of the test isolate. In addition, hypermucoviscosity was measured by using the string test method to ascertain the virulence nature of the clinical isolate. The string test involves streaking the selected strain onto Muller Hinton agar and grown at 37 °C overnight. With the help of an inoculation loop, the colony was touched and pulled to produce a thin string between colony and loop. If a thin string up to 5 mm height is observed, it denotes hypermucoviscosity [30].

2.3. Colistin broth disk elution assay

Colistin broth disk elution method was applied to further confirm the colistin resistance status of the isolate. Four tubes containing cation-adjusted Mueller Hinton broth (10 ml) were added with 0, 1, 2, and 4 colistin disks (10 µg), representing 0, 1, 2 and 4 µg/ml concentrations, respectively. The tubes were left undisturbed for 1 h at room temperature to facilitate the release of colistin from the disks. In accordance with CLSI guidelines, 50 µl of 0.5 McFarland scale turbidity-adjusted culture was inoculated into each tube [31]. A tube containing cation–adjusted Mueller Hinton broth without colistin represented the growth control, whereas the negative control tube contained a colistin disk with *E. coli ATCC 25922* culture (Fig. 1). Following an 18–24 h incubation period at37 °C, turbidity resulting from bacterial growth in tubes with varying concentrations represents the resistance ability of the test organism to the given concentration.

2.4. Screening of PDR K. pneumoniae with pandemic response and pathogen boxes compounds

Upon arrival of the Pathogen and Pandemic Response Box compounds, each well contained 10 µl of drugs at a concentration of 10 mM in five 96 well microtiter plates. Di-methyl sulfoxide (DMSO; 90 µl) was added to each well to achieve a final concentration of 1 mM according to the manufacturer instructions. All the plates were stored at −20 °C and

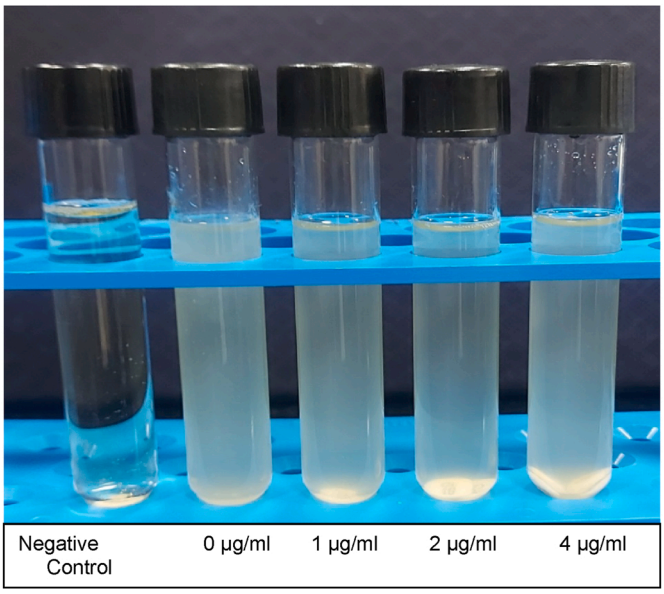


Fig. 1. Colistinbroth Disk Elution assay with *PDR K. pneumoniae*. Negative control denotes the tube containing one 10 µg colistin disk inoculated with *E. coli ATCC25922*. The second tube represents the positive control with no disk (0 µg/ml). Third, fourth and fifth tubes containing 1, 2 and 4 colistin disks.

thawed on ice before use. Four-hundred compounds from the Pathogen Box and 201 compounds from the Pandemic Box predicted for antibacterial activity (n = 601) were prepared by suspending 3 µl of stock solution (1 mM) in 147 µl of cation-adjusted Mueller Hinton broth (CaMHB) to achieve a final concentration of 20 µM in 150 µl suspension, and dispensed 50 µl in triplicate during screening in flat bottom 96-well microtiter plates. Prior to incubation, a fresh overnight culture from LB broth was inoculated into 5 ml of CaMHB, and the mixture was shaken at 150 rpm for 18 h at 37 °C. The CLSI-recommended micro-broth dilution method was used for screening 601 compounds against the PDR *K. pneumoniae* strain. The turbidity was adjusted to 0.5 McFarland units and then diluted (1:10) with CaMHB, resulting in a10 × 10^5 cells/ml inoculum. The prepared inoculum (50 µl) was transferred to the 96 well microtiter plates, which were already containing 50 µl of 20 µM compounds (n = 601) in triplicates. This resulted in a 100 µl suspension with test compounds at 10 µM concentration, cell density at 5 × 10^5 cells/ml and 1 % DMSO. The first column represented growth control having only the bacterial culture and DMSO (1 %), and the last column acted as a blank containing only media. The loaded microtiter plates were sealed using optically clear microplate sealer and incubated at 37 °C for 24 h. Varioskan Flash Microplate reader (Thermo Fisher Scientific, Waltham,

Table 1
Antimicrobial sensitivity test results with Vitek2® system.

Antibiotics	Susceptibility	MIC (mg/L)	Antibiotics	Susceptibility	MIC (mg/L)
Tobramycin	R	≥16	**Cefuroxime**	R	≥64
Ceftazidime + Avibactam	R	≥32	**Ceftriaxone**	R	≥64
Cefotaxime	R	≥64	**Cefepime**	R	≥64
Ertapenem	R	≥8	**Imipenem**	R	≥16
Cefoperazone + Sulbactam	R	≥64	**Colistin**	R	≥16
Meropenem	R	≥16	**Piperacillin + Tazobactam**	R	≥128
Ciprofloxacin	R	≥4	**Ofloxacin**	R	≥8
Amikacin	R	≥64	**Gentamycin**	R	≥16
Cefuroxime axetil	R	≥64	**Netilmicin**	R	≥32
Nitrofurantoin	R	≥256	**Tigecycline**	R	≥8
Nalidixic acid	R	≥32	**Amoxcillin + clavulanic acid**	R	≥32
Doxycycline	R	≥16	**Norfloxacin**	R	≥32
Trimethoprim + sulfamethoxazole	R	≥320	**Ampicillin**	R	≥32

Test strain *K. pneumoniae* was resistant to all classes of antibiotics and combinations available for treating Infections. *R- Resistant; S-Sensitive; MIC- Minimum inhibitory concentration (mg/l).

MA, USA) was used to obtain photometric readings at 600 nm with 300 ms delay. Optical density of the triplicates were averaged and used for further calculation. The percentage of growth inhibition was calculated using the formula: percentage of growth inhibition = [1- (OD_D - OD_B)/(OD_C - OD_B)] × 100; where OD_D represents the average absorbance in assay wells (drugs), OD_B is the optical density of the blank well and OD_C denotes the absorbance of growth control. Compounds that reduced the bacterial growth by more than 50 % were taken into consideration to further assess the MIC and persister subpopulations.

2.5. Minimum inhibitory concentration of hit compounds

Each hit compound that showed complete inhibition in initial screening was subjected to minimum inhibitory concentration (MIC) determination by microbroth dilution method in a 96 well plate using CLSI guidelines. The first and last columns remained as growth and negative control. The remaining columns contained 50 µl of MHB with each drug concentration ranging from 10 to 0.06 µM (MIC scale: 10, 8, 6, 5, 4, 2, 1, 0.5, 0.25, 0.125 and 0.06 µM) in triplicates. For an additional 50 µl of MHB inoculum, the compounds were diluted by twice the volume necessary to reach the desired concentration. The compounds were individually added in triplicates for concentrations 10 to 5 µM and for rest concentrations (4–0.06 µM) 4 µM was serially diluted in the Flat bottom 96 well microtiter plate. Fifty microliters of turbidity adjusted culture (10×10^5 cells/ml) were added to the wells already containing the drug at different concentrations. The final cell density was $2–5 \times 10^5$ cells/ml, with drug concentration ranging from 10 to 0.06 µM. After 24 h of incubation at 37 °C, the 96 well plates were measured for absorbance (optical density) at 600 nm with a 300 ms delay using a Varioskan Flash microplate reader.

2.6. Determination of persister population

One hundred microliters of the solution mixture from the wells with complete inhibition (optically clear) was serially diluted three times in a 2 ml centrifuge vial up to 10^{-3}dilution of the original cell density. One-hundred microliters from the final dilution were individually dispensed onto the Luria Bertani agar without antibiotics and spread evenly on the surface using L-rod, in triplicates. The spread plates were then allowed to dry and incubated for 18hrs at 37 °C. Following incubation, the colonies in the plates were counted manually and colony forming units (CFU) was calculated using the formula: CFU/ml = number of colonies × Dilution factor × volume of inoculum^{-1}. Average Colony Forming Units from the triplicate plates represent the persister frequency.

2.7. Prediction of applicability of potential compounds for human use

The selectivity index (SI) was calculated to determine the safety of the test compounds that were showing antibacterial activity for human use. SI was calculated using the formula: SI=CC_{50}(µM)/MIC(µM), where CC_{50} is the compound concentration at which 50 % of the mammalian cells get killed. Information regarding the cytotoxicity of all the compounds was provided by MMV. Compounds with a higher selectivity index (>1) indicate safety for human application.

3. Results

3.1. Identification and antimicrobial susceptibility of PDR K. pneumoniae

BLAST results showed that the 16s rRNA data from Sanger sequencing yielded a 100 % identity match with *K. pneumoniae*. Vitek2® system successfully identified the same with 98 % probability and high level of confidence. The clinical isolate was found to be resistant against all the test antibiotics (tobramycin, cefotaxime, ertapenem, meropenem, ciprofloxacin, amikacin, nitrofurantoin, nalidixic acid, doxycycline, cefuroxime, ceftriaxone, cefepime, imipenem, colistin, ofloxacin, gentamycin, netilmicin, tigecycline, norfloxacin, ampicillin) and combinations (ceftazidime + avibactam, cefoperazone + sulbactam, cefuroxime + axetil, trimethoprim + sulfamethoxazole, piperacillin + tazobactam, amoxcillin + clavulanic acid) used in the Kirby Bauer disk diffusion method and in Vitek2® system, (BioMérieux, Lyon, France) (Table 1). In colistin broth disk elution method growth was observed in all the tubes with different concentrations of colistin indicating the pathogen was resistant to colistin up to 4 µg/ml (Fig. 1). The string test results showed that the test organism was unable to produce hypermucoid capsule polysaccharide.

3.2. Identification of effective antibacterial compounds against PDR K. pneumoniae

Five hit compounds from the Pandemic Response Box were found to be capable of inhibiting the growth of PDR *K. pneumoniae* at 10 µM concentration. In case of the Pathogen box containing 400 compounds, none were shown to be inhibiting the organism. The optical density (OD) of wells with the five compounds (n = 5) had similar absorbance values to that of negative control wells that represent no growth with visually clear wells. The average values of optical density OD_{600nm} from triplicate wells are represented in Table 2. These five compounds were found to be showing more than 93% of growth inhibition against the test isolate. At 10 µM drug concentration, five Pandemic Response Box compounds - MMV1580854, MMV1579788, eravacycline, epetraborole and MMV1578564 had a comparatively high inhibitory rate against PDR *K. pneumoniae*. The percentage of growth inhibition by the compounds ranged from the lowest of 93.13 % by eravacycline to the highest inhibition of 96.32 % by MMV1578564. Other compounds such as epetraborole, MMV1579788 and MMV1580854 inhibited 95.29 %, 94.65 % and 94.60 % of the bacterial growth, respectively. The remaining compounds were found to be yielding lower degrees of inhibition, or no inhibition at all.

3.3. Potential to inhibit persister subpopulation

Interestingly, our results showed a subset of dormant phenotypic variants evading the inhibitory activity of test compounds as shown in persister assays. The compounds with inhibitory activity were also able to reduce the viable persister population. The persister frequency ranged from 10^4 to 10^7 cells/ml for all five hit compounds. Eravacycline produced a high number of persisters (2.8×10^7 CFU/ml) on the antibiotic free LB agar plate; whereas MMV1580854, MMV1579788 and MMV1578564 exhibited a high anti-persister activity yielding1.3×10^6

Table 2
Screening of 601 compounds of MMV Pathogen Box and Pandemic Response Box against PDR *K. pneumoniae*.

Compounds	Triplicates			Avg OD	% IG	Pf (CFU/ml)	MIC (µM)
	OD1	OD2	OD3				
MMV1580854	0.082	0.081	0.067	0.076	94.60	1.3×10^6	≥2
MMV1579788	0.073	0.063	0.093	0.076	94.65	9.4×10^6	≥5
Eravacycline	0.095	0.081	0.084	0.086	93.13	2.8×10^7	≥2
Epetraborole	0.078	0.091	0.047	0.072	95.29	8.2×10^4	≥2
MMV1578564	0.09	0.057	0.048	0.065	96.32	9.0×10^5	≥2

Inhibition of the compounds is represented as %IG (Percentage of growth inhibition) based on the optical density (OD) at 600 nm. The persister subpopulation is denoted as PF(Persister frequency)in the table as CFU/ml units. Minimum Inhibitory Concentration (MIC) at which the compounds completely inhibit growth at a given concentration (µM) is represented.MMV ID for drugs: Eravacycline-MMV1578574, Epetraborole - MMV1578566.

CFU/ml, 9.4 × 10^6 CFU/ml and 9.0 × 10^5 CFU/ml, respectively. Epetraborole showed the lowest persister frequency with only 8.2 × 10^4 CFU/ml (Table 2), showing that the compound was highly effective and also reduced the surviving subpopulation of the bacterial cell.

3.4. Minimum concentration of antibacterial activity and selectivity index

MIC values of the compounds that showed inhibition during the initial screening were calculated at various concentrations. The PDR strain was inhibited by MMV1579788 at a concentration greater than, or equal to 5 μM. Four other compounds, such as MMV1580854, eravacycline, epetraborole and MMV1578564 inhibited the growth of the bacteria at comparatively lower concentrations of 2 μM and above. All the hit compounds were showing higher SI values (>20) (Table 3). The selectivity indices of MMV1580854, eravacycline and epetraborole were all 50, respectively, whereas MMV1578564's selectivity index was slightly lesser with a value of 45. With a selectivity index value of 20, MMV1579788 ranked lowest, but still above the predetermined threshold.

4. Discussion

The emergence of multidrug-resistant bacteria needs to be tackled through the identification of new antimicrobial agents, combinatorial therapy and drug repurposing. The emergence of carbapenemase-producing *K. pneumoniae* prompted the usage of advanced drugs such as aminoglycosides and colistin. Eventually, *K. pneumoniae* evolved into pan-drug-resistant strains through increased usage of colistin and aminoglycosides. Pan drug resistant pathogens have posed a global threat because there are not many alternative treatment options are available with only few empirical combinatorial approaches. The ceftazidime-avibactam complex can be used for treating infections caused by of pan-drug resistant *K. pneumoniae* and proven to be effective, but, the pan drug resistant *K. pneumoniae* used in our study was already resistant to CAZ-AVI combination. Furthermore, test strain in this study was resistant to one or more number of antibiotics of different antibiotic classes and antibiotic combinations (Table .1)compared to previously reported PDR *K. pneumoniae* observed worldwide [32]. Thus, we screened the MMV open source Pathogen Box and Pandemic Response Box compounds against our clinical PDR *K. pneumoniae* isolate. Previously, compounds from pathogen box and pandemic response box were reported to be effective against various clinically relevant bacterial pathogens such as *Acinetobacter baumanii* and *Pseudomonas aeruginosa* [28,33,34], exhibited fungicidal or anti-biofilm activity against fungal pathogens causing chromoblastomycosis and candidiasis [35,36]. Eravacycline is a broad-spectrum antibiotic that works against both Gram-positive and Gram-negative bacteria. The mode of action of eravacycline is similar to that of tetracycline. These cyclic compounds bind irreversibly to the 30s subunit of bacteria, thereby blocking the incorporation of amino acids to the elongating chain and impairing the bacterial protein synthesis, which ultimately results in bacterial cell death. Compared to tetracycline, eravacycline has a ten-fold higher affinity to ribosomal subunits and inhibits protein synthesis at four times lower concentrations [37]. This compound was also found to be unaffected by the efflux systems and ribosomal protection protein systems and it is currently under phase III clinical trial (NCT01844856) evaluation [38]. MMV1580854 andMMV1579788 showed a similar degree of growth inhibition (94.6 %), but with 10^1less CFUs than eravacyclineof1.3 × 10^6 CFU/ml and 9.4 × 10^6 CFU/ml, respectively. Both compounds differed by MIC values, with MMV1580854inhibiting the study isolate at a concentration ≥2 μM and MMV1579788at ≥ 5 μM. Although both compounds had similar inhibition rates, the persister frequency and MIC of MMV1579788 was found to be higher than that of MMV1580854. MMV1580854, also known as 2-(2-aminopyridin-3-yl)oxy-5-ethyl-4-fluorophenol, was found to target the oxidoreductase group of enzymes and shown to inhibit methicillin-resistant *Staphylococcus aureus* (MRSA), *E. coli* and *Pseudomonas aeruginosa* at lower concentrations [39]. MMV1579788 or (E)-3-(3, 3-dimethyl-2-oxo-1H-pyrrolo [2,3-b]pyridin-5-yl)-N-methyl-N-[(3-methyl-1-benzofuran-2-yl)methyl]prop-2-enamide acts as an inhibitor of fatty acid biosynthesis in bacteria [40].

Four-(4-ethyl-5-fluoro-2-hydroxyphenoxy)-3-fluorobenzamide or MMV1578564, was shown to modulate short chain-dehydrogenases reductase (SDR) of bacteria [41]. SDRs play a significant role in carbohydrate, lipid, amino acid and xenobiotic metabolisms [42]. The compound MMV1578564 showed a highest rate of growth inhibition at 96.32 % with MIC values at 2 μM. It produced a persister population of about 9.0 × 10^5 CFU/ml that was less than previously explored compounds.

Epetraborole (MMV1578566) showed to be effective, inhibiting the test pathogen and also able to reduce the surviving persister population to only 8.2 × 10^4 CFU/ml in the subsequent plating onto LB agar. Another supporting factor of epetraborole being the most effective was the MIC value of more than, or equal to, 2 μM.Epetraboroleis a novel boron-containing antibiotic belonging to the class of non-halogenated 3-aminomethyl benzoxaboroles. It targets a novel site in leucyl t-ribose nucleic acid (RNA) synthetase or leucyl t-RNA synthetase (LeuRS), surpassing the resistance mechanisms of most Gram-negative bacteria. Inhibiting LeuRS, which is an essential component in protein synthesis, will render the bacteria incapable of protein synthesis [43,44]. Epetraborole was also reported to be effective against *Mycobacterium avium, Mycobacterium abscessus* and *Mycobacterium intracellulare* [45,46]. In Phase II clinical trials (NCT01381549; NCT01381562) safety and efficacy of epetraborole compared to imipenem-cilstatin for the adults with febrile complicated UTIs and pyelonenephritis was evaluated [47,48]. Most of the five hit compounds in the study had trace cytotoxicity of CC_{50}at>100 μM of compound concentration, whereas MMV1578564 exhibited CC_{50} dosage of approximately 90 μM (Table 4). Notably, MMV1579788 showed no cytotoxicity towards mammalian cells at 30 μM as per the data provided by the MMV(Table 4) [26]. Excellent selectivity index values (Table 3) were observed with the various compounds, essentially due to less or no cytotoxicity to the mammalian cell lines and also achieving inhibition at lower concentration against the pan drug resistant pathogen. Selectivity index was considerably reasonable for all five compounds that could be suitable for human application. Limitations of the study include that a) screening against several field isolates with mono-resistant, multi-drug resistant and pan-drug resistant groups would add more valuable information; b) an *in vivo* study was not included in this study which may have yielded interesting results potentially leading to some pre-clinical studies.

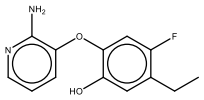
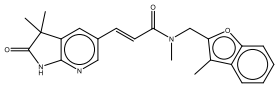
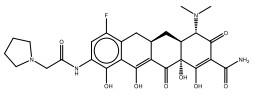
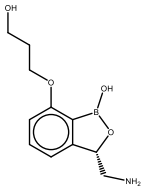
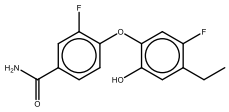
Overall, we identified five promising agents against the PDR *K. pneumoniae* on the basis of MIC, rate of inhibition, and persister subpopulation assessment. Among the five compounds, epetraborole and MMV1578564 were the most-highly potent molecules. Other compounds also exhibited significant effects with respect to above mentioned evaluations; nevertheless *in vitro* characteristics may differ. Notably, clinical isolates could be used in inhibitory screening assays, serving as the representative sample of field strains infecting human population and spreading between the patients and individuals. These field isolates could behave in a different manner when compared to standard reference strains due to their exposure to the drug compounds in the host, different genetic background, acquired extensive mutations

Table 3
Selectivity Index of the effective compounds against *PDR K. pneumoniae.*

Compounds	CC_{50}(μM)	MIC (μM)	SI
MMV1580854	>100	≥2	50
MMV1579788	>100	≥5	20
Eravacycline	>100	≥2	50
Epetraborole	>100	≥2	50
MMV1578564	~90	≥2	45

CC_{50} represent the drug concentration that kills 50 % of the cells. *MIC- Minimum inhibitory concentration; SI- Selectivity Index.

Table 4
Chemical structures and alternative names of the hit compounds that inhibited pan-drug-resistant *K. pneumoniae* in the study.

Compounds	Trivial or common name	Chemical name	Structure	M.W	Cell viability at 30 μM
MMV1580854	–	2-(2-aminopyridin-3-yl)oxy-5-ethyl-4-fluorophenol		248.3	98 %
MMV1579788	–	(E)-3-(3,3-dimethyl-2-oxo-1H-pyrrolo [2,3-b]pyridin-5-yl)-N-methyl-N-[(3-methyl-1-benzofuran-2-yl)methyl]prop-2-enamide		389.4	100 %
MMV1578574	Eravacycline	(4S,4aS,5aR,12aR)-4-(dimethylamino)-7-fluoro-1,10,11,12a-tetrahydroxy-3,12-dioxo-9-[(2-pyrrolidin-1-ylacetyl)amino]-4a,5,5a,6-tetrahydro-4H-tetracene-2-carBoxamide		631.5	91 %
MMV1578566	Epetraborole	3-[[(3S)-3-(aminomethyl)-1-hydroxy-3H-2,1-benzoxaborol-7-yl]oxy] propan-1-ol		273.5	93 %
MMV1578564	MUT056399	4-(4-ethyl-5-fluoro-2-hydroxyphenoxy)-3-fluorobenzamide		293.3	93 %

The cytotoxicity of the compounds at 30 μM concentration was represented as a percentage of cell survival (Viability) [26].*MW- molecular weight.

through exposure to the drugs in the environment, and immune pressure from both the infected and healthy individuals of the community. Epetraborole (NCT01381549; NCT01381562) and eravacycline (NCT01844856) are well known to be effective against various pathogens, including drug-resistant Gram-negative bacteria, *Mycobacterium* spp. and advanced into phase II and III clinical trials. MMV1580854, MMV1579788 and MMV1578564 could be further explored in *in-vitro* and *in-vivo* to advance knowledge about drug characteristics.

Author contributions

S.J conceptualized the study. Methodology: S.S and M.C.P. Supervision: S.J. Original draft was prepared by S.S. Reviewing and editing: S. J, S.S, M.C.P, A.B, M.P.G. All the authors reviewed and approved the final version of the manuscript.

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Declaration of competing interest

The authors declare no competing interests.

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