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Breaking the Phalanx: Overcoming Bacterial Drug Resistance with Quorum Sensing Inhibitors that Enhance Therapeutic Activity of Antibiotics

Jon-Michael Beasley[#1], Dorjbal Dorjsuren[#2], Sankalp Jain[#2], Marielle Rath[1], Ricardo Scheufen Tieghi[1], Alexander Tropsha[1], Anton Simeonov[2], Alexey V. Zakharov[2,*], Eugene Muratov[1,*].

[1]*UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA.*
[2] *Division of Preclinical Innovation, National Center for Advancing Translational Sciences, Rockville, MD, 20850*

[#] These authors contributed equally
* To whom correspondence should be addressed: *E-mail: alexey.zakharov@nih.gov, murik@email.unc.edu.*

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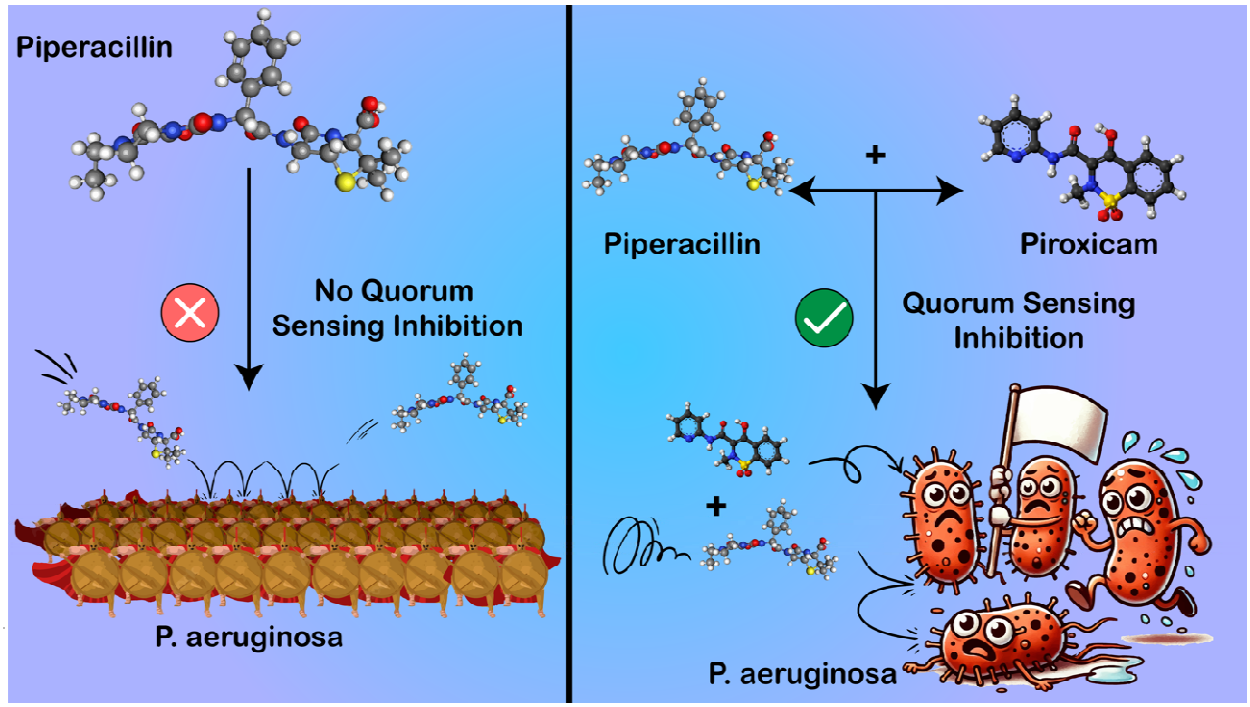
Table of Content Graphic



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Abstract

Antibiotic-resistant bacterial infections loom over humanity as an increasing deadly threat. There exists a dire need for new treatments, especially those that synergize with our existing arsenal of antibiotic drugs to help overcome the gap in antibiotic efficacy and attenuate the development of new antibiotic-resistance in the most dangerous pathogens. Quorum sensing systems in bacteria drive the formation of biofilms, increase surface motility, and enhance other virulence factors, making these systems attractive targets for the discovery of novel antibacterials. Quorum sensing inhibitors (QSIs) are hypothesized to synergize with existing antibiotics, making bacteria more sensitive to the effects of these drugs. In this study, we aimed to find the synergistic combinations between the QSIs and known antibiotics to combat the two deadliest hospital infections - *Pseudomonas aeruginosa* and *Acinetobacter baumannii.* We mined biochemical activity databases and literature to identify known, high efficacy QSIs against these bacteria. We used these data to develop and validate a Quantitative Structure-Activity Relationship (QSAR) model for predicting QSI activity and then employed this model to identify new potential QSIs from the Inxight database of approved and investigational drugs. We then tested binary mixtures of the identified QSIs with 11 existing antibiotics using a combinatorial matrix screening approach with ten (five of each) clinical isolates of *P. aeruginosa* and *A. baumannii*. Amongst explored drug combinations, 31 exhibited a synergistic effect, including mixtures involving naldemedine and telotristat, two drugs predicted by our model with previously undescribed QSI activity. Although no mixture inhibiting all the strains was found, piperacillin combined with curcumin, ketoprofen, indomethacin, and piroxicam demonstrated the broadest antimicrobial action. We anticipate that further preclinical investigation of these combinations of novel repurposed QSIs with a known antibiotic may lead to novel clinical candidates.

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Introduction

Antibiotic resistance, a global crisis of formidable proportions, has catapulted scientific communities into an urgent quest for innovative strategies to address the escalating threat to public health.[1] Most current antibiotics have been designed to directly kill pathogenic bacteria by destroying cell membranes or interfering with protein synthesis, triggering "Life or Death" selection pressure and promoting microbial resistance's evolution. Indeed, almost all pathogenic bacteria are resistant to commonly used antibiotics, which effectively renders frontline treatments ineffective.[2] The extensive use of antibiotics has led to the creation of "superbugs" that can resist a wide variety of common antibiotics.[3] A report by the British Government estimated that by 2050, antimicrobial resistance could cause 10 million deaths each year (**Figure 1**), rivalling cancer as a global cause of death, and cause a cumulative loss of US $100 trillion to world GDP.[4]

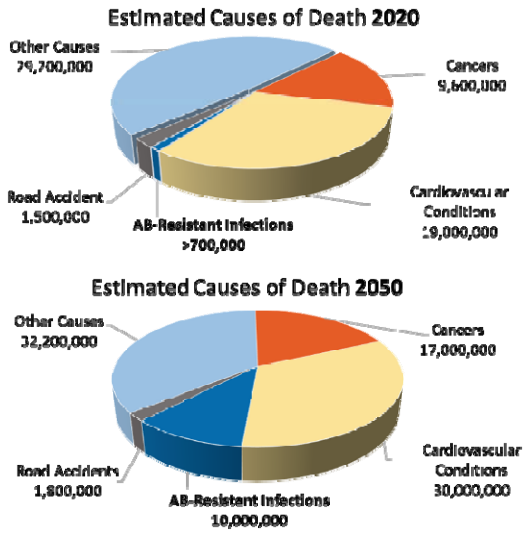

Figure 1. Antibacterial Resistance (ABRI) expected to become a major cause of death by 2050. ABRI-related deaths are expected to increase disproportionately, making up nearly 11% of total deaths in 2050 compared to an estimated 1% in 2020.[5,6]

As we face the antibiotic resistance crisis, where even routine infections pose a menacing risk, the development of novel solutions is imperative.[7] Despite the urgent need for new treatment options, the speed of antibiotic development lags far behind the rate at which bacteria are evolving to become multidrug resistant (MDR). The lack of innovation on this front can be attributed to the high cost of $1 billion[8] but low success rates of new drugs, as well as to the high likelihood that bacteria will eventually develop resistance to monotherapies. These expectations of low potential profitability effectively de-incentivize pharmaceutical companies to work on discovering and manufacturing such drugs.[9]

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Fortunately, existing antibiotics can still be leveraged against bacterial infections. Combination therapy with synergistic antibiotics having distinct mechanisms of action and different targets shows promise in reducing antibiotic resistance. In addition, dual therapies can more effectively and quickly treat bacterial infections.[10] Furthermore, lower doses of each antibiotic in the dual therapy can be used, which reduces the risk of toxicity and adverse effects.[11]

Several combination therapies are already on the market, such as amoxicillin/clavulanate (Augmentin), tazobactam/piperacillin (Zosyn), and trimethoprim/sulfamethoxazole (Bactrim). Other antibiotic combinations have shown success in experiments, such as vancomycin + trimethoprim and vancomycin + nitrofurantoin against *Escherichia coli*[12], polymixin B + meropenem + ampicillin/sulbactam against *Acinetobacter baumannii*[13], and polymixin B + aztreonam + amikacin against colistin-resistant *E. coli*[14]. Interestingly, several non-antibiotic compounds have synergistic effects with select antibiotics, including ursolic acid/oleanolic acid + ampicillin/oxacillin against MRSA[15] and plant-derived flavonoids catechol-type flavonoid-7,8-dihydroxyflavone, myricetin, and luteolin + colistin against MDR bacteria[16].

It is important to note that combination therapies do not necessarily need to be specially formulated, but rather each compound can be administered separately. Because combination therapies are less common, there are few dosing guidelines; therefore, physicians and pharmacists play a vital role when it comes to dosing in the clinic.[17]

Quorum Sensing Inhibitors and their Synergy with Antibiotics.

Among the diverse avenues of research aimed at circumventing antibiotic resistance, the inhibition of quorum sensing (QS) has emerged as a promising and relatively unexplored frontier.[4,18,19] Unlike traditional antibiotics that directly target essential cellular processes, QS inhibitors (QSIs) seek not to eliminate bacteria outright but disarm them, diminishing their ability to mount a coordinated defense.[20] At the heart of this exploration lies the recognition that as bacterial populations reach a critical density, they release signaling molecules into their environment.[20,21] These molecules serve as communal messages, allowing bacteria to gauge their numbers and orchestrate collective actions, such as forming biofilms, enhancing surface motility, or activating virulence factors, which have been associated with increased resistance to common antibiotics.[20,21] Disrupting this communication breaks their ability to act as a united front, potentially rendering them more susceptible to the host immune system and traditional antibiotics.[22,23]

The potential for synergistic interventions that simultaneously target both QS and traditional antibiotic pathways holds the promise of a two-pronged assault on bacterial resilience.[19] Synergistic combinations involving QS inhibitors may lower the required

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doses of each drug and help avoid adverse events while exploiting the greater therapeutic effect.[24,25] For example, low-dose gentamicin combined with amoxicillin improves treatment of bacterial endocarditis.[26,27] Furthermore, synergistic combinations have the potential to rescue the efficacy of antibiotics for which bacteria have previously developed resistance, enabling the practice of "antibiotic recycling" of the first line-of-defense drugs.[28]

Focusing on the Threat of Drug-Resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* Infections

Multidrug resistant ESKAPE pathogens, such as *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp.* have been recognized as emerging threats to public health. Among these 6 bacteria, the World Health Organization (WHO) prioritizes *Acinetobacter baumannii, Pseudomonas aeruginosa* as critical for R&D of new antibiotics. Antibiotic resistance in *P. aeruginosa* poses a challenge due to the variety of resistance mechanisms, complicating the selection of effective treatment. An analysis of bloodstream infections from a major US hospital revealed higher mortality rates associated with *P. aeruginosa* isolates exhibiting a 'difficult to treat' resistance phenotype (DTR), which is characterized by resistance to fluoroquinolones, cephalosporins and carbapenems. Patients infected with isolates resistant to all these antibiotics classes experienced a 40% increase in the adjusted mortality rates compared to those with susceptible strains.[29] Similarly, MDR of *Acinetobacter* infections further complicates treatment. Patients frequently have prolonged exposure to healthcare environments, increasing risk of exposure to antibiotics and colonization by resistant isolates.[30]

Study Goals and Outline

As stated above, WHO lists carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* as critical and high priority pathogens, respectively, as they are common, fatal nosocomial infectious agents.[3,31] This study aims to provide further evidence for the hypothesis that QSIs can synergize with existing antibiotic drugs, leading to novel combination therapies with the great potential to be effective against *A. baumannii* and/or *P. aeruginosa*. To achieve this goal, we focused on a knowledge-based discovery approach to identify QSIs reported in chemical bioactivity online databases and literature followed by Quantitative Structure Activity Relationship (QSAR) modeling to identify additional approved or investigational drugs predicted as QSIs. We endeavored to nominate and test the combinations of putative QSIs with known

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antibiotics against various strains of *A. baumannii* and *P. aeruginosa* to identify synergistic treatments.

Materials and Methods

ChEMBL Mining and Data Curation

We began the search for compounds that may inhibit QS by searching the ChEMBL database[32] for assays containing the phrase “quorum sensing”. Then, assays specific to the “Pseudomonas aeruginosa” and “Acinetobacter baumannii” species were selected for further curation. Data collected from ChEMBL were curated using KNIME (v4.5.2). These entries were separated by the assay “Standard Type” field annotated by ChEMBL. We discovered 1007 “Inhibition”, 141 “Activity”, 78 “Efficacy”, 71 “IC50”, 38 “Ratio”, 8 “CFU”, 4 “FC”, 3 “EC50”, 3 “Ratio IC50”, and 1 “IC85” entry Standard Types. The “Assay Description” field for each entry was reviewed to ensure that each assay was measuring the *inhibition* or *antagonism*, rather than *induction* or *agonism* of QS activity. Next, potential QS inhibitors were selected by choosing only entries with “Inhibition”, ”Activity”, or “Efficacy” Standard Values >= 50%, or those with an “Active” or “Dose-dependent effect” annotation in the “Comments” field. The Assay Descriptions for the included entries were again manually reviewed and only entries that describe assays using compound concentrations of 10 µM or less were kept. “IC50” and “IC85” Standard Type entries were included only if their Standard Values were <= 10 µM. The results of this data curation exercise included 49 entries describing 36 unique compounds with >= 50% inhibition of QS activity at concentrations of 10 µM or less. 838 entries describing 349 unique compounds were regarded as inactive with these criteria. Both chemical structures and biological activities were curated following the protocols developed by us earlier.[33,34] The resulting dataset is included in **Supplementary Data 1**.

QSAR Modeling and Virtual Screening

We employed QSAR modeling, validation, and virtual screening protocols implemented in KNIME (v4.5.2). The 36 active and 349 inactive compounds were aggregated as a training set for QSAR modeling. Compound structure cleaning and curation was performed. This included removal of salts, mixtures, and duplicates, as well as standardizing structure representations (aromatic rings, nitro groups, etc.).[33] Following these procedures, 372 compounds (36 actives and 336 inactives) remained in the cleaned training set. We trained a random forest model and validated its performance using a five-fold external cross validation protocol. We then performed virtual screening

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of the Inxight pharmaceutical collection[35] to identify approved or investigational compounds predicted to inhibit quorum sensing in *P. aeruginosa*. From the 36 known active QSIs and QSAR predicted actives, those which are already U.S. FDA approved drugs were selected for combination screening.

Compound Selection Guided by Literature and Knowledge Graph Mining

Abstract Sifter (Version 5.5) is a publicly available Microsoft Excel workbook-based application that enhances PubMed's search capabilities.[36] The macro-enabled Microsoft Excel workbook, developed by the US EPA, was utilized to run a PubMed query to identify compounds that possess quorum-sensing inhibition against *A. baumannii*, since no relevant assay data was found in ChEMBL for this species. The keywords: “Acinetobacter baumannii” AND “quorum sensing” AND “inhibitors” were used. The search returned a total of 37 relevant articles. To further filter only the most relevant compounds demonstrating promising quorum sensing activity, all abstracts and papers were further investigated. If the compounds described in the research papers presented inhibition of >50% at 50 micromolar concentrations or less, the compounds were nominated for further testing, and the remaining compounds were removed.

In addition to Abstract Sifter, we used the ROBOKOP knowledge graph[37,38] and ChemoText[39] to find the additional evidence of nominated compounds being the QSI and/or their prior use as a constituent of antimicrobial mixture therapy. We also used these tools and QSAR models developed by us earlier[40] to exclude the combinations that may lead to undesired drug-drug interactions or side effects.

Bacterial Strains Tested

All strains of *P. aeruginosa*: MRSN 317 (NR-51516), MRSN 1344 (NR-51520), MRSN 1583 (NR-51524), PA14 (NR-50573), MRSN 315 (NR-51515) and *Acinetobacter Baumannii*: WC-136 (NR-19298), WC-487 (NR-19299), 137 (OIFC137) (NR-17777), BC-5 (NR-17783), and MRSN 1171 (NR-52153) were procured from ATCC.[41]
The selections of these resistant strains were based on their descriptions of the various resistant antibiotics.

Antibiotic Testing of *P. aeruginosa* and *A. baumannii* in 1536-well Plate Format

LB Liquid cultures of both *P. aeruginosa* and *Acinetobacter Baumannii* strains were used for AC_{50} determination for individual antibiotics used in 1536 well plate format. Briefly, 4 µL of LB medium was dispensed into multi-well plates by a Multidrop Combi Reagent Dispenser (Thermo Scientific, Pittsburgh PA). Test compounds were delivered as a DMSO solution via a Kalypsys pintool transfer (San Diego, CA) and arrayed as eleven-point titrations, with final drug concentrations ranging from 46 µM to 0.18 µM.

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Following compound transfers, 2 µL of diluted overnight culture of *P. aeruginosa* were for overnight growth assay. The bacterial growth was assessed using a BacTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) by measuring the ATP quantity, which was directly proportional to the number of viable cells in the well. The luminescent signal was read with a ViewLux reader (Perkin Elmer, Norwalk, CT).

Combinatorial Matrix Synergy Assay for *P. aeruginosa* and *A. baumannii* in 1536-well Plate Format

LB Liquid cultures of both *P. aeruginosa* and *Acinetobacter Baumannii* were utilized for predicted QSI and antibiotic mixtures in a matrix format in 1536-well plates. Briefly, 6 µL of LB medium was dispensed by a Multidrop Combi Reagent Dispenser (Thermo Scientific, Pittsburgh PA) into 1536-well solid bottom plates preplated with the selected compounds in combination using acoustic dispenser (Labcyte 650, Beckman Coulter, Indianapolis, IN) at six-point titrations. Final concentrations of the tested drugs ranged from 46 µM to 0.18 µM. Bacterial growth was monitored by assessing cell viability though OD at 600nm, which directly correlates with the number of viable cells in each well. Optical density readings were captured using Envision reader (Perkin Elmer, Norwalk, CT).

Selection of Antibiotics Tested

We selected 21 antibiotics commonly used in clinics for treatments of bacterial infections based on a variety of mechanisms, targeting different aspects of bacterial growth and replication: Chlorhexidine, Ciprofloxacin hydrochloride, Cefepime, Enrofloxacin, Piperacillin, Tazobactam, Difloxacin, Grepafloxacin, Pazufloxacin (mesylate), Tobramycin, Meropenem, Ceftazidime, Aztreonam, Garenoxacin, Clinafloxacin, Trovafloxacin, Delafloxacin (meglumine), Avibactam sodium, Gemifloxacin, Epetraborole and Cefiderocol. Descriptions of common uses of these drugs, their mechanisms of action, and drug resistance potential are provided in **Supplemental Data 2**.

We screened our in-house antibiotic library including these 21 drugs and selected those showing activity against most strains for matrix screening/analysis (**Supplemental Data 3**). This led to the selection of 11 antibiotics used in combination matrix screening: Avibactam sodium, Cefepime, Ceftazidime, Chlorhexidine, Ciprofloxacin Hydrochloride, Difloxacin, Meropenem, Piperacillin, Tazobactam, Tobramycin, and Trovafloxacin.

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Results

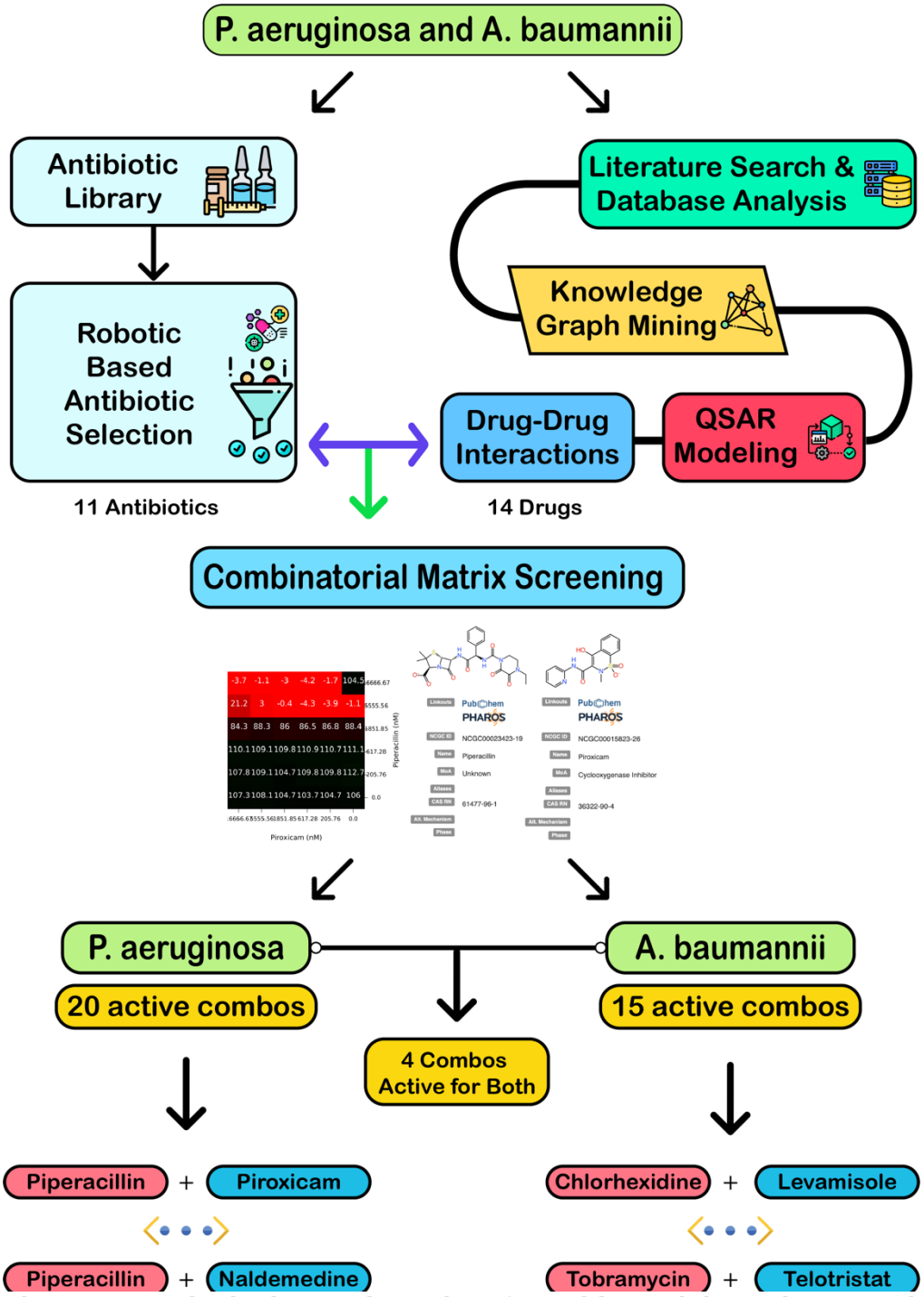

Figure 2. Study design and results of combinatorial matrix screening of antibiotics with QSIs. Antibiotics selected by robotic screening against drug-resistant bacteria and potential QSIs selected by knowledge mining and machine learning predictions were tested against *P. aeruginosa* and *A. baumannii* isolates, revealing synergistically active drug combinations.

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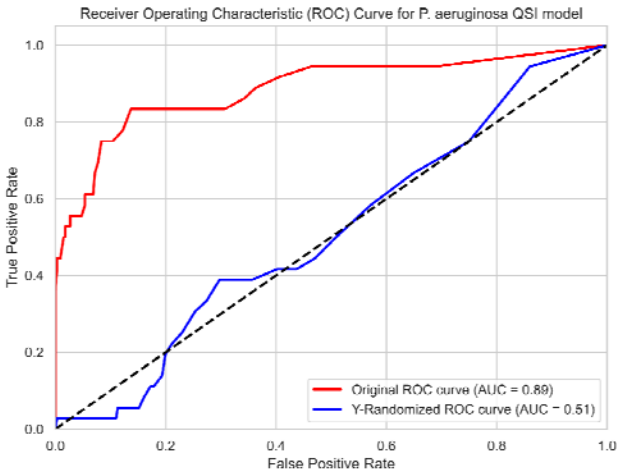
ChEMBL Quorum Sensing Inhibition Data

The result of the ChEMBL search yielded 1354 bioactivity entries for *P. aeruginosa,* but none were found for *A. baumannii*. The results of this data curation included 49 entries describing 36 unique compounds with >= 50% inhibition of QS activity at concentrations of 10 µM or less (**Supplemental Data 1**). 838 entries describing 349 unique compounds were regarded as inactive with these criteria. Of the 36 active compounds, 3 were chosen for synergy screening in this study due to their status as approved or investigational drugs in the Inxight pharmaceutical collection[35]: curcumin, azaguanine-8, and sulfathiazole.

QSAR Modeling and Virtual Screening

The RF model trained on compounds tested for *P. aeruginosa* QSI activity obtained an AUC-ROC of 0.89 and the same model trained on randomly labelled data obtained performance of only 0.51, indicating our model was not overfitted.



We further performed virtual screening of the Inxight pharmaceutical collection.[35] 49 of the 12,584 compounds were predicted to be active. Of these 49, seven compounds including abafungin, mycophenolic acid, telotristat, fenebrutinib, umbralisib (R enantiomer), relacorilant, and naldemedine, remained as these were predicted not to have undesired drug-drug interactions by our models and knowledge graph mining results. Due to significant deficit of reliable experimental data, we also utilized KGs for searching for any additional evidence of QSI or antibacterial activity of selected compounds, similarly to our previous antiviral studies.[42]

Figure 3. Receiver Operating Characteristic (ROC) curve of *P. aeruginosa* QSI classifier model demonstrating five-fold cross validation performance. This model achieved 0.89 AUC (red line), while cross validation on randomly labelled data achieved no better performance than random prediction (0.51 AUC, blue line).

Abstract Sifter for Literature Mining

Literature mining with Abstract Sifter resulted in 37 articles for investigation. From these 37 articles, 26 compounds of potential interest were identified (Compounds with inhibition greater than 50% for *A. baumannii* and clearly stated concentrations used).

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From these 26 compounds, known antibiotics were excluded, resulting in the nomination of 5 compounds for further investigation: ketoprofen, piroxicam, indomethacin, curcumin, and levamisole.

Table 1: Nominated Compounds from *A. baumannii* Literature Search.
Ketoprofen, piroxicam, indomethacin, curcumin, and levamisole were identified as QSIs in *A. baumannii*.[43–45]

Compound Name:	PUBCHEM ID	Inhibition	Concentration	DOI
Ketoprofen	PUBCHEM.COMPOUND:3825	72%	0.7-6.25 mg/mL	10.1111/jam.15609
Piroxicam	PUBCHEM.COMPOUND:54676228	91%	1.25-2.5 mg/mL	10.1111/jam.15609
Indomethacin	PUBCHEM.COMPOUND:3715	81%	3.12-12.5 mg/mL	10.1111/jam.15609
Curcumin	PUBCHEM.COMPOUND:969516	80%	0.01 and 0.05 mg/mL	10.3389/fmicb.2019.00990
Levamisole	PUBCHEM.COMPOUND:26879	72%	0.512 mg/mL	10.1007/s10096-020-03882-z

Synergy and Antagonism of Selected Combinations

Figure 4 summarizes the results of testing the combinations of Piperacillin with Piroxicam at different concentrations against *Pseudomonas aeruginosa* (MRSN 1344). The heat maps depict the response profiles when testing these combinations, indicating areas of synergy and antagonism. The response profiles in the heat maps are evaluated using the DBSumNeg value (**Figure 4** (**b**)), which quantifies the degree of synergy. In this context, DBSumNeg represents the sum of the differences between observed and expected growth inhibition values, with negative values indicating synergy. A DBSumNeg threshold of -3 is considered indicative of synergy because it reflects a significant deviation from additive effects, meaning the combined effect of the drugs is greater than the sum of their individual effects.[46]

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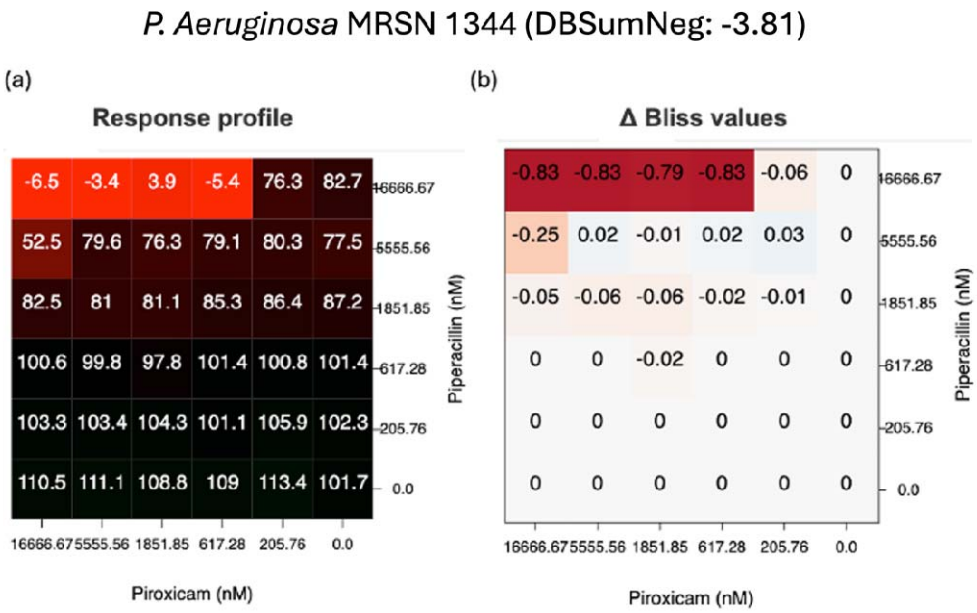

Figure 4. Representative Dose-Response Profiles for Piperacillin + Piroxicam against *P. aeruginosa* (MRSN 1344). DBSumNeg values are calculated from combination response profiles. Lower DBSumNeg Values (highlighted in red) represent stronger synergy.

Review of all response profiles for the drug combinations (**Supplemental Data 4-5**) resulted in selection of 26 combinations with DBSumNeg less than -3 in any tested strain (**Table 2**). Of these combinations, we sought to select the combinations, for each bacterial species, which achieved high synergy in multiple strains or across multiple species. We found that all 26 of these combinations achieved synergy in only one strain per species, but the combination of curcumin and piperacillin had strong synergy in both *A. baumannii* WC-487 and *P. aeruginosa* MRSN 1344, the combination of piroxicam and piperacillin had strong synergy in both *A. baumannii* WC-136 and *P. aeruginosa* MRSN 1344, the combination of indomethacin and piperacillin had strong synergy in both *A. baumannii* WC-136 and *P. aeruginosa* MRSN 1344, and the combination of ketoprofen and piperacillin had strong synergy in both *A. baumannii* WC-136 and *P. aeruginosa* MRSN 1344 (**Figure 5**).

We also found the combinations of naldemedine with avibactam, tazobactam, tobramycin, ceftazidime, chlorhexidine, and piperacillin in *P. aeruginosa* MRSN 315, the combination of telotristat and chlorhexidine in *P. aeruginosa* MRSN 315, and the combination of telotristat with tobramycin and avibactam in *A. baumannii* MRSN 1171 noteworthy as all of these combinations achieved exceptionally high DBSumNeg scores < -5 not achieved by any other combinations in the study (**Table 2**).

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We found that 43 drug combinations displayed anti-synergistic, antagonistic behavior in at least one strain. All but 4 of these antagonistic combinations were observed in only one bacterial strain. The combinations of curcumin and meropenem, curcumin and ciprofloxacin HCl, azaguanine-8 and ciprofloxacin HCl, and abafungin and clinafloxacin showed antagonism in two *A. baumannii* strains. **Figure 6** shows all synergistic and antagonistic combinations side-by-side in a circle plot for each bacterial species. A tabular form of these synergistic and antagonist interactions is available in **Supplemental Data 6.**

Table 2: Combinations of known antibiotics with nominated drugs showing highest synergistic activity against *P. aeruginosa* and *A. baumannii* strains. Combinations with DBSumNeg less than -3.0 were selected for further review (drug names in bold font), but combinations with -2.5 or less are still shown.

Antibiotic	Combination Drug	DBSumNeg	Strain
Levamisole	**Meropenem**	**-4.87**	**Pseudomonas aeruginosa, MRSN 317**
Levamisole	**Trovafloxacin mesylate**	**-4.14**	
Sulfathiazole	**Trovafloxacin mesylate**	**-3.9**	
Sulfathiazole	**Meropenem**	**-3.7**	
Sulfathiazole	**Chlorhexidine**	**-3.19**	
Levamisole	Cefepime HCl	-2.92	
Mycophenolic acid	Meropenem	-2.54	
Curcumin	**Piperacillin**	**-4.36**	**Pseudomonas aeruginosa, MRSN 1344**
Piroxicam	**Piperacillin**	**-3.81**	
Indomethacin	**Piperacillin**	**-3.8**	
Azaguanine-8	**Piperacillin**	**-3.7**	
Ketoprofen	**Piperacillin**	**-3.65**	
Levamisole	Piperacillin	-2.75	
Naldemedine	**Avibactam**	**-11.93**	**Pseudomonas aeruginosa, MRSN 315**
Naldemedine	**Tazobactam**	**-9.49**	
Telotristat	**Chlorhexidine**	**-8.76**	
Naldemedine	**Tobramycin**	**-7.71**	
Naldemedine	**Ceftazidime**	**-7.59**	
Naldemedine	**Chlorhexidine**	**-7.25**	
Naldemedine	**Piperacillin**	**-7.07**	
Telotristat	**Difloxacin HCl**	**-3.11**	
Piroxicam	**Piperacillin**	**-4.98**	**Acinetobacter baumannii, WC-136**
Indomethacin	**Piperacillin**	**-3.94**	
Ketoprofen	**Piperacillin**	**-3.76**	
Ketoprofen	**Ciprofloxacin HCl**	**-4.2**	**Acinetobacter baumannii, WC-487**
Piroxicam	**Ciprofloxacin HCl**	**-4.17**	

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Indomethacin	**Ciprofloxacin HCl**	**-4.13**	
Curcumin	**Piperacillin**	**-3.73**	
Sulfathiazole	Ciprofloxacin HCl	-2.64	
Telotristat	**Tobramycin**	**-6.73**	**Acinetobacter baumannii, MRSN 1171**
Telotristat	**Avibactam**	**-5.92**	
Telotristat	**Cefepime HCl**	**-4.97**	
Telotristat	**Piperacillin**	**-4.34**	
Levamisole	**Chlorhexidine**	**-3.02**	
Sulfathiazole	Tobramycin	-2.88	
Telotristat	Ceftazidime	-2.83	
Piroxicam	Chlorhexidine	-2.78	
Telotristat	Tazobactam	-2.63	

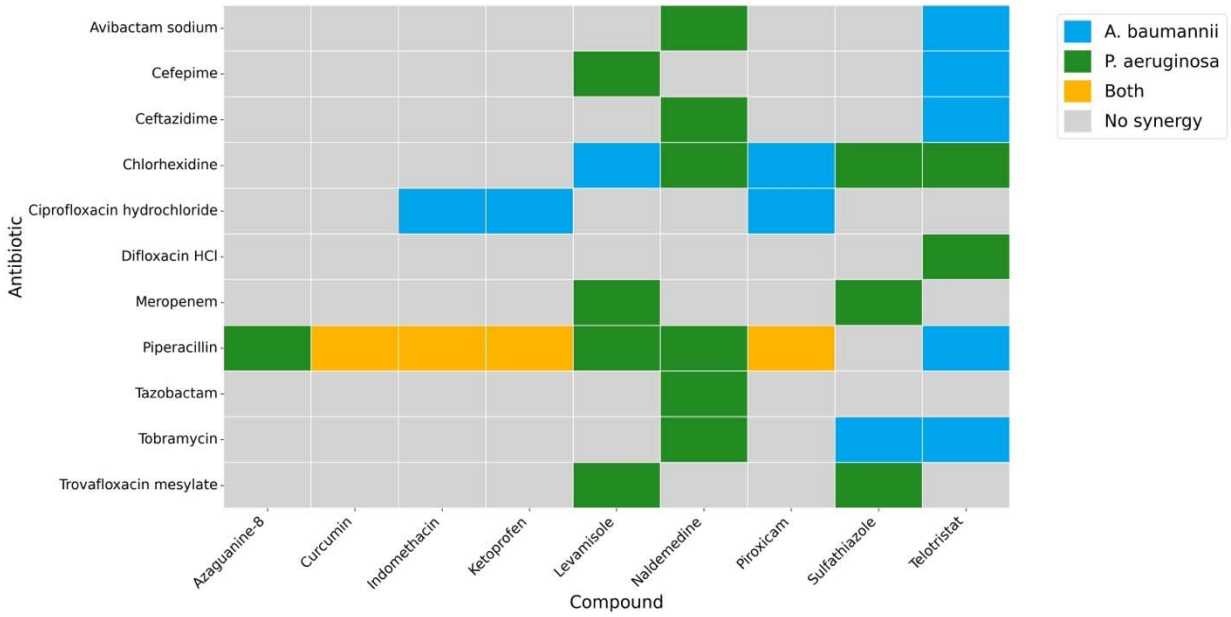

Figure 5: Synergistic Interaction Heatmap for Antibiotics and Compounds Against *A. baumannii* and *P. aeruginosa*. The figure displays a heatmap representing the number of strains showing synergy between various antibiotics and compounds, as evaluated against A. baumannii and P. aeruginosa. Number of strains showing synergy was quantified for combinations that showed a DBSumNeg value of less than –3.

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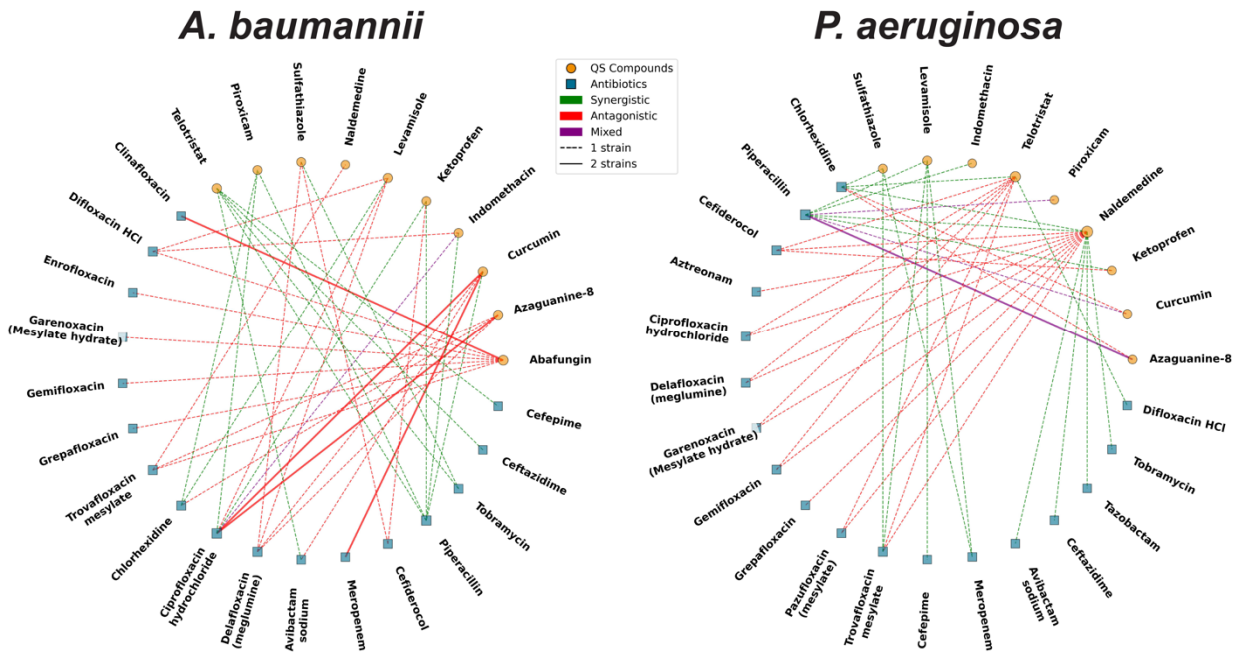

Figure 6: Interaction Network of *A. baumannii and P. aeruginosa* with Antibiotics and Compounds. Network represents the interactions between antibiotics and QSIs against *A. baumannii* (left) and *P. aeruginosa* (right), classified by their synergistic (green), and antagonistic (red).

Drug Approval Status, Pharmacokinetics, and Dosing Considerations

We must also consider pharmacokinetic properties which might affect drug concentration at the site of infection. An important consideration is that the non-antibiotic quorum sensing inhibitors are not indicated for bacterial infections to date, so dosing regimens may need to be created to ensure the concentration reaches the IC50 in the site of infection, while mitigating potential toxicity. We have provided reported and predicted pharmacokinetic data for these drugs in **Supplemental Data 7.** We have not included CMax, as this is dependent on dosing and only directly applicable for bloodstream infections. Clinical use of these drug combinations is also contingent upon approval status of the drugs. Some of these drugs have either not yet been approved for clinical use in the US or may have been withdrawn due to safety concerns. Approval status of these drugs is compiled in **Table 3.**

Table 3. Approval status of tested drugs (found on DrugBank[47])

Drug name	Approval status	Comments
Ketoprofen	Approved	
Meropenem	Approved	
Aztreonam	Approved	

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Cefepime hydrochloride	Approved	
Cefiderocol	Approved	
Piperacillin	Approved	
Clinafloxacin	Investigationall	Investigational new drug application was withdrawn due to safety concerns[48]
Ciprofloxacin hydrochloride	Approved	
Delafloxacin (meglumine)	Approved	
Difloxacin HCl	Vet approved, not approved for human use	
Enrofloxacin	Vet approved, not approved for human use	
Garenoxacin (Mesylate hydrate)	Investigational in the US, approved in Japan	
Gemifloxacin	Approved	
Grepafloxacin	Withdrawn	Due to deaths from Torsade de Pointes
Pazufloxacin (mesylate)	Investigational in the US, approved in Japan	
Trovafloxacin mesylate	Withdrawn	Due to risks of fatal liver failure
Sulfathiazole	Withdrawn	All forms except for those indicated for vaginal use have been withdrawn
Epetraborole (hydrochloride)	Investigational	Risk of nephrotoxicity, safer sulfonamide antibiotics are available
Tobramycin	Approved	
Abafungin	Investigational	Development discontinued in 2009 in Europe and Japan
Ketoprofen	Approved	
Piroxicam	Approved	
Indomethacin	Approved	
Azaguanine-8	Experimental	
Chlorhexidine	Approved	
Curcumin	Approved	
Levamisole	Withdrawn; vet approved	Withdrawn from market for human use due to risk of

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		agranulocytosis
Mycophenolic acid	Approved	
Naldemedine	Approved	
Relacorilant	Investigational	
Telotristat	Experimental	
Umbralisib (R-enantiomer)	Withdrawn	Withdrawn due to risk of death

Tables 4a and 4b. Potential interactions between selected synergistic combinations.

Interactions were checked using Facts and Comparisons[49] and DrugBank[47]. If a potential interaction was found, we performed a further literature and database search for the nature of the interaction.

Table 4a. Drug combinations that are predicted to have drug-drug interactions.

Agent 1	**Agent 2**	**Outcome of the potential interaction**	**Potential mechanism of interaction**
Meropenem	Ketoprofen	Increased meropenem concentration	Ketoprofen can decrease renal excretion of meropenem, leading to a higher concentration of meropenem in serum.[50] Ketoprofen inhibits the following transporters: SLC1A2, SLC22A6, SLC22A8, SLC22A11, SLC22A7, several of which play a role in renal excretion. Meropenem is renally excreted.
Piperacillin	Piroxicam	Increased piperacillin concentration	Reduced excretion of piperacillin.[51] Piroxicam is an inhibitor of SLC22A6 and SLC22A8.[52] Piperacillin is an inhibitor of SLC22A6 and substrate of SLC22A8.[51]
Tobramycin	Naldemedine	Increased tobramycin concentration	Naldemedine could reduce the excretion of tobramycin, resulting in a higher serum level of tobramycin.[53]

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			Both drugs are renally excreted (tobramycin 90-95% and naldemedine 57%).[49]
Piperacillin	Naldemedine	Increased naldemedine concentration	Piperacillin could reduce the excretion of naldemedine, resulting in a higher serum concentration of naldemedine.[53] Piperacillin is primarily renally excreted (68% as unchanged drug in the urine).[49]
Piperacillin	Indomethacin	Increased piperacillin and indomethacin concentrations	Reduced renal clearance of both drugs. Piperacillin is an inhibitor of SLC22A6, and indomethacin is a substrate and inhibitor of SLC22A6. Piperacillin is a substrate of SLC22A8, and indomethacin is an inhibitor.
Ceftazidime	Naldemedine	Increased naldemedine concentrations	Ceftazidime could reduce the excretion of naldemedine, resulting in a higher serum concentration of naldemedine.[53] Both drugs are renally excreted (ceftazidime IV or injection 80-90% and naldemedine 57% in urine).[49,5449]
Difloxacin HCL	Indomethacin	Neuroexcitatory activity	NSAIDs can increase risk of fluoroquinolone-induced neuroexcitatory activity. Difloxacin and indomethacin specific combination has not been evaluated.[55]
Enrofloxacin	Indomethacin	Neuroexcitatory activity	Indomethacin may increase risk of fluoroquinolone-induced neuroexcitatory activity.[56] The specific combination of enrofloxacin and indomethacin has not been evaluated.
Pazufloxacin mesylate	Indomethacin	Neuroexcitatory activity	Indomethacin may increase risk of fluoroquinolone-induced neuroexcitatory activity, although there are no case reports of this but rather a class effect.[57,58]

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Cefepime HCL	Ketoprofen	Nephrotoxicity	Increased risk of nephrotoxicity. Renal function should be monitored. One animal study showed that cefepime and diclofenac (a different NSAID) increased risk of tissue damage.[59]

Table 4b. Drug combinations that have **no known** drug-drug interactions.

Agent 1	**Agent 2**
Piperacillin	Azaguanine-8
Piperacillin	Curcumin
Piperacillin	Levamisole
Piperacillin	Ketoprofen
Avibactam	Naldemedine
Tazobactam	Naldemedine
Chlorhexidine	Naldemedine
Chlorhexidine	Telotristat
Tobramycin	Telotristat
Avibactam	Telotristat
Piperacillin	Sulfathiazole
Ciprofloxacin HCL	Sulfathiazole
Meropenem	Curcumin
Cefiderocol	Curcumin

Of the 24 hit combinations, 10 are expected to have potential drug-drug interactions. Of note, many of these interactions pertain to one drug reducing the clearance of another, through inhibiting transporters (as in the case of ketoprofen + meropenem, piperacillin + piroxicam, and others). If these treatments are implemented in the hospital/clinic, dose adjustments may be necessary. However, there are several interactions more directly related to toxicity, as is the case with indomethacin + fluoroquinolones (risk of neuroexcitatory activity) and cefepime + ketoprofen (risk of acute kidney injury).

Discussion

In this study, we substantiated the hypothesis that treating drug-resistant *P. aeruginosa* and *A. baumannii* isolates with QSIs in combination with existing antibiotic drugs could confer a synergistic bactericidal effect. To prioritize clinical translational potential, we chose to focus on screening combinations involving known QSIs which are also approved drugs with well-known safety and pharmacokinetic profiles in humans. To

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select additional test compounds with unknown QSI activity, we developed a QSAR model to predict QSI activity from among approved compounds in the Inxight pharmaceuticals collection. We identified several combinations involving piroxicam, curcumin, indomethacin, ketoprofen, naldemedine, and telotristat which synergize with existing antibiotics. However, the exact mechanism of this synergistic activity has yet to be fully understood. Here, we discuss the putative mechanisms of action of these compounds based on prior literature.

Putative Mechanisms of Action of the Synergistic QSIs

Piroxicam

While piroxicam has been shown to inhibit various QS virulence factors in several *A. baumannii* strains[43], no studies, to the best of our knowledge, demonstrated its ability to inhibit QS, specifically, in *P. aeruginosa.* However, piroxicam was predicted to inhibit QS proteins LasR and PqsE by molecular docking and structure analysis.[60] Piroxicam has been shown to protect mice from a lethal challenge of *P. aeruginosa* and diminish inflammatory response to *P. aeruginosa* pneumonia, although this protection was not due to direct bactericidal effects of the drug.[61] Interestingly, other oxicam NSAID drugs, meloxicam and tenoxicam, have demonstrated QSI activity in *P. aeruginosa*.[62,63]

It is known that the combination of piroxicam with piperacillin can reduce the renal clearance of piperacillin. This is likely because piroxicam is an inhibitor of SLC22A6 and SLC22A8[52], while piperacillin is an inhibitor of SLC22A6 and substrate of SLC22A8[51]. It is plausible that this drug interaction may improve treatment outcomes by extending the half-life of piperacillin, although additional *in vivo* studies are required to support this hypothesis.

Curcumin

Curcumin has been shown to disrupt QS in *A. baumannii* strain ATCC 17978. Specifically, curcumin reduced biofilm formation by ~45% at 10 µg/ml and ~86% at 100 µg/ml, significantly reduced pellicle formation at 50 µg/ml, reduced surface motility by >75% at 10 µg/ml, and reduced *C. elegans* killing by infection from 80% to 35% at 50 µg/ml.[44] Furthermore, molecular docking studies predicted interactions with curcumin and the active site of the biofilm response regulator BfmR as putative mechanism of action for regulation of *A. baumannii* QS virulence factors.[44] In *P. aeruginosa* PA14, inhibited the LasI/LasR QS system, as evidenced by 21% reduction in 3-oxo-C12-HSL production (via LasI) and 7% reduction in 3-oxo-C12-HSL detection (via LasR) at 200 µg/ml.[64] Curcumin has been shown to attenuate virulence of *P. aeruginosa* in whole plant and animal models. It is also thought to inhibit *P. aeruginosa* efflux pumps[65], presenting another possible mechanism for its observed synergy with beta-lactam antibiotics, such as piperacillin. Furthermore, the combination of curcumin and

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meropenem has previously been shown to have synergistic activity against several meropenem-resistant *Klebsiella pneumoniae* isolates, suggesting broad spectrum potential for this treatment combination.[66]

Indomethacin

Like piroxicam, indomethacin has been shown to inhibit *A. baumannii* QS virulence factors, including biofilm formation, and surface motility, and bacterial tolerance to oxidative stress.[43] In another study, indomethacin was tested for inhibition of QS in *Chromobacterium violaceum* CV026 and effects on virulence production in *Pseudomonas aeruginosa* PAO1, but was found to lack anti-QS activity as measured by the inhibition of production of violacein pigment.[67] While we show the synergistic effects of indomethacin with piperacillin in our current study, these prior results suggest that indomethacin may be acting through mechanisms unrelated to QS. Further experiments beyond violacein pigment inhibition in *P. aeruginosa* are warranted to investigate the activity reported in our current study.

Ketoprofen

Ketoprofen, like piroxicam and indomethacin, was shown to inhibit QS virulence factors with MICs 0.7-6.25 mg/mL in various *A. baumannii* strains.[43] The QSI activity of ketoprofen has also previously been confirmed in *P. aeruginosa* via assays showing attenuation of virulence factors and biofilm formation, as well as reduction in the expression of lasI, lasR, rhlI, and rhlR genes, by 35-47, 22-48, 34-67, and 43-56%, respectively.[68] Furthermore, *in silico* studies and comparison of chemical structures to natural QS activator ligands suggest that ketoprofen and its analogues inhibit QS in *P. aeruginosa* by acting directly on the *PqsR* protein target.[69]

Naldemedine

Naldemedine, a peripherally acting mu-opioid receptor (OPRM1) antagonist used in the treatment of opioid induced constipation, was a particularly surprising hit which showed exceptionally strong synergy with multiple antibiotics when tested against *P. aeruginosa* MRSN 315. We selected this drug for combinations screening as it was a predicted by our QSAR model as a QSI in *P. aeruginosa*, but we could find very little literature linking the drug to use in infectious diseases. However, one study used molecular docking to identify naldemedine, along with telmisartan and azilsartan, as potential inhibitors of the sortase A (SrtA) virulence factor in *Staphylococcus aureus.*[70] SrtA in Gram-positive infectious bacteria, like *S. aureus*, recruits other virulence proteins to the bacterial cell wall to aid in cell adhesion, host cell invasion, immune system evasion, biofilm formation, and nutrient acquisition.[71,72] SrtA represents an attractive drug target as it is localized to the exterior of the cell wall, has no known homologs in humans, and is not essential to cell viability, thus targeting this protein is unlikely to apply an evolutionary

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selective pressure towards resistance mechanisms.[73–75] However, most Gram-negative bacteria, including *P. aeruginos*a, lack sortase enzymes, so the exact mechanism of naldemedine's strong synergy with antibiotics in *P. aeruginosa* is unclear. We believe the evidence in the current study motivates further investigation into the mechanisms of its antimicrobial effect and its clinical use as a therapeutic agent.

Telotristat

Telotristat ethyl, the prodrug of telotristat, is a tryptophan hydroxylase inhibitor used to treat carcinoid syndrome diarrhea from neuroendocrine tumors. To the best of our knowledge, there have been no prior studies evaluating the direct effects of telotristat on bacterial QS, however we hypothesize that telotristat may inhibit QS by disrupting serotonin signaling. Tryptophan hydroxylases (TPH1/2) are critical to the production of serotonin, and serotonin is known to be a key modulator of bacteria in the host gut microbiome.[76] Serotonin upregulates QS signaling in *P. aeruginosa* and these bacteria are known to synthesize their own serotonin from available tryptophan in the environment.[77] Therefore, we hypothesize that the observed synergistic effects of telotristat in our current study could be due to inhibition of bacterial tryptophan hydroxylase, which could deplete serotonin and reduce QS activity, even in an *in vitro* environment.

Considerations for Clinical Use

Clinical use of these drug combinations will require consideration of drug distribution throughout the body and study of appropriate dosing strategies. Furthermore, clinicians will need to consider half-lives of each combination therapy and devise dosing regimens to ensure sufficient therapeutic concentrations of both drugs concomitantly. Thus, additional monitoring may be required if these combinations are implemented in practice. As usual, clinical judgment will be necessary for implementing these treatments in patients.

Conclusions

In this study, we substantiated the hypothesis that treating drug-resistant *P. aeruginosa* and *A. baumannii* isolates with QSIs in combination with existing antibiotic drugs could confer a synergistic bactericidal effect. To prioritize clinical translational potential, we chose to focus on screening combinations involving known QSIs which are also approved drugs with well-known safety and pharmacokinetic profiles in humans. To select additional test compounds with unknown QSI activity, we developed a QSAR model to predict QSI activity from among approved compounds in the Inxight pharmaceuticals collection.

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We used a combinatorial matrix screening approach to test these 14 identified compounds in combination with 11 antibiotics with antimicrobial activity against our *P. aeruginosa* and *A. baumannii* isolates at varied concentrations. We discovered several pairs of compounds with a strong antimicrobial synergy effect, including combinations of curcumin, piroxicam, indomethacin, and ketoprofen with piperacillin, which showed synergy in both *P. aeruginosa* and *A. baumannii* strains, and combinations involving naldemedine and telotristat, drugs with previously unreported QSI or antimicrobial activity.

We also investigated potential drug-drug interactions between the agents in the synergistic combination hits. Of the 24 combinations, 10 have potential drug-drug interactions. Of these, 6 pertain to the alteration in excretion. If implemented in the clinic/hospital, this could require dose adjustment, but it is not a reason to discount the combination. There are four combinations for which toxicity is a concern (NSAIDs + fluroquinolones). These combinations could require further monitoring in clinic.

In summary, our results and previous evidence for the hypothesis that QSIs could enhance the antimicrobial activity of the existing antibiotic arsenal. We anticipate that these results will motivate further preclinical investigations to confirm antibacterial synergy and combination safety *in vivo* before translational use in the clinic.

Conflict of Interest

A.T. and E.N.M. are co-founders of Predictive, LLC, which develops novel alternative methodologies and software for toxicity prediction. All the other authors declare no conflicts.

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