รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาระบบการศึกษาและค้นหายาปฏิชีวนะที่มีความจำเพาะต่อเชื้อ *Acinetobacter baumannii*

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มีนาคม 2562

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และ
มหาวิทยาลัยมหิดล

Abstract

An increasing number of multidrug-resistant *Acinetobacter baumannii* (MDR-*AB*) infections have been reported worldwide, posing a threat to public health. The establishment of methods to elucidate the mechanism of action (MOA) of *A. baumannii*-specific antibiotics is needed to develop novel antimicrobial therapeutics with activity against MDR-*AB*. We previously developed bacterial cytological profiling (BCP) to understand the MOA of compounds in *E. coli*, and *B. subtilis*. Given how distantly related *A. baumannii* is to these species, it was unclear to what extent it could be applied. Here we implemented bacterial cytological profiling (BCP) as an antibiotic MOA discovery platform for *A. baumannii*. We found that the BCP platform can distinguish between six major antibiotic classes and can also sub-classify antibiotics that inhibit the same cellular pathway but have different molecular targets. We used BCP to show that the compound NSC145612 inhibits the growth of *A. baumannii* via targeting RNA transcription. We confirmed this result by isolating and characterizing resistant mutants with mutations in the *rpoB* gene. We conclude that BCP provides a useful tool for MOA studies of antibacterial compounds that are active against *A. baumannii.*

บทคัดย่อ

ปัจจุบันสถานการณ์การเพิ่มขึ้นของการติดเชื้อแบคทีเรีย *Acinetobacter baumannii* ที่ดื้อยากำลังสร้างความวิตกกังวลแก่วงการสาธารณสุขทั่วโลกอย่างมาก ดังนั้นเพื่อเป็นการป้องกันและควบคุมเชื้อดื้อยาดังกล่าว การสร้างวิธีที่เหมาะสมในการศึกษากลไกการยับยั้งการเจริญเติบของสารต้านจุลชีพที่ออกฤทธิ์ต่อเชื้อแบคทีเรีย *A. baumannii* จึงมีความจำเป็นอย่างมาก การศึกษาก่อนหน้าบ่งบอกว่าเทคนิค bacterial cytological profiling (BCP) เป็นเทคนิคที่สามารถนำไปใช้ในการศึกษากลไกการทำงานของสารต้านจุลชีพได้เป็นอย่างดี และประสบความสำเร็จในแบคทีเรียชนิดอื่น เช่น *E. coli* และ *B. subtilis* แต่อย่างไรก็ตามเทคนิค BCP ยังไม่เคยนำมาประยุกต์ใช้กับเชื้อแบคทีเรีย *A. baumannii* ซึ่งมีความแตกต่างทางด้านวิวัฒนาการอย่างมากเมื่อเทียบกับ แบคทีเรีย *E. coli* และ *B. subtilis* ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์ที่จะทำการทดสอบว่าเทคนิค BCP สามารถนำมาประยุกต์ใช้กับแบคทีเรีย *A. baumannii* ได้หรือไม่ จากการทดลองพบว่าเทคนิค BCP สามารถแยกประเภทของสารต้านจุลชีพที่ยับยั้งการเจริญเติบโตของเชื้อ *A. baumannii* ได้มากถึง 6 ชนิดที่ยับยั้งกระบวนการหลักต่าง ๆ ที่แตกต่างกันของแบคทีเรีย อีกทั้งยังสามารถแยกกลไกย่อยของสารต้านจุลชีพที่แตกต่างกันได้อีกด้วย โดยนอกจากนี้งานวิจัยนี้ยังใช้เทคนิค BCP มาใช้ในการศึกษากลไกการยับยั้งการเจริญเติบโตของสาร NSC145612 ต่อ *A. baumannii* โดยพบว่า สาร NSC145612 ยับยั้งการบวนการสังเคราะห์ RNA ของ *A. baumannii* ซึ่งสอดคล้องกับผลการเหนี่ยวนำให้เกิดการกลายพันธุ์ดื้อยาของสารดังกล่าวที่ระบุว่า เชื้อที่สามารถต้านทานการออกฤทธิ์ของสาร NSC145612 เป็นเชื้อที่มีการกลายพันธุ์ในบริเวณยีน *rpoB* ซึ่งเป็นยีนที่ผลิตเอนไซม์ที่ทำหน้าที่หลักในกระบวนการสังเคราะห์ RNA ของแบคทีเรีย จากงานงานวิจัยนี้สามารถสรุปได้ว่าเทคนิค BCP สามารถนำมาประยุกต์ใช้ในการศึกษากลไกการทำงานของสารต้านจุลชีพที่สามารถยับยั้งการเจริญเติบโตของ *A. baumannii* ได้

Project Code : MRG6080081

Project Title : Developing an *Acinetobacter baumannii*-specific antibiotic discovery platform

Investigator : Poochit Nonejuie / Institute of Molecular Biosciences, Mahidol University

E-mail Address : pnonejuie@hotmail.com

Project Period : 2 years

Objectives

To investigate the utility of Bacterial Cytological Profiling (BCP) in identifying the MOA of antibacterial molecules that inhibit the growth of *A. baumannii*.

Materials and methods

Bacteria strain, growth and antibiotics. *Acinetobacter baumannii* strain ATCC 19606 and strain ATCC 17978 were used in this study. The bacteria were grown in LB medium or LB agar at 30°C. A total of twenty-two antibiotics were tested on *A. baumannii* from which fifteen antibiotics with minimal inhibitory concentration of less than 112 μg/ml were used in this study (Table 1). The compound NSC145612 was obtained from the National Cancer Institute's Developmental Therapeutics Program. Preparation of the antibiotics was according to the manufacture's recommendations.

Minimal Inhibitory Concentrations. Minimal inhibitory concentrations (MIC) of all antibiotics are shown in Table 1. MIC was obtained using microdilution method (1) . Overnight cultures of *A. baumannii* were diluted 1:100 in LB broth and allowed to grow at 30°C on a roller until exponential phase or until the OD_{600} of 0.2 was obtained. The bacteria culture was further diluted 1:100 into each well of 96 well plate containing antibiotics in LB media at appropriate concentrations. Cultures were allowed to grow at 30°C for 24 hours. MIC was determined by observing the concentration of the antibiotic in the well where the bacteria was unable to grow.

Fluorescence microscopy. Overnight cultures of *A. baumannii* were diluted 1:500 in LB broth and grown at 30°C on a roller until exponential phase. Antibiotics were added at various concentration. Cultures were then grown at 30°C on a roller for 2 hours. *A. baumannii* cultures were stained with FM 4-64 (2 μg/ml), DAPI (4 μg/ml) and SYTOX-green (0.5 μM). Stained bacterial cultures were harvested by centrifugation and resuspended in 1/10 volume of the same culture media. Three microliters of this was added to agarose pads on concave glass slides. Fluorescence microscopy was performed with consistent imaging parameters throughout all experiments.

Cytological profiling. Cytological profiles were determined by automated cell analysis using CellProfiller 3.0 (2). Briefly, images were pre-processed on Fiji software (3) and subsequently analyzed on CellProfiler 3.0 software. Cell morphological parameters such as length, width, and area were determined. To obtain the average intensity of SYTOX-green and DAPI, both the membrane and nucleoid outlines were used and subtracted by background intensity in corresponding images. Decondensation of the nucleoid was determined by the ratio of the area of the nucleoid to that of the cell membrane.

Statistical analysis. As described previously (1, 4, 5), the cytological parameters of each antibiotic were obtained from three independent experiments. Profiling data was from automated analysis of the cells in each imaging field. Only images containing more than 20 for long cells and for the rest, more than 30 cells per imaging field were selected into data points. Weighed principal component analysis (PCA) was performed using statistic tools on MATLAB 2017a. Euclidean cluster analysis was generated from Morpheus (https://software.broadinstitute.org/morpheus).

Isolation of NSC145612-resistant mutants. In *A. baumannii*, *A. baumannii* ATCC 19606 culture was diluted into the LB media containing NSC145612 starting at 0.5X MIC. This process was repeated with escalating concentration of NSC145612 until the NSC145612-resistant *A. baumannii* was obtained. The resistant strains were purified on LB agar plates and MIC for NSC145612 and rifampicin determined by broth dilution method, as mentioned above.

Table 1 Complete list of drugs tested in this study

Antibiotic Class	Antibiotic Name	MIC (μg/ml)	Target
	Drugs tested in *Acinetobacter baumannii* ATCC 19606		
Protein Synthesis Inhibitors			
Aminoglycoside	Amikacin	20	30s ribosome (promote mistranslation)
	Gentamicin	28	30s ribosome (promote mistranslation)
	Kanamycin	10	30s ribosome (promote mistranslation)
	Streptomycin	>112	30s ribosome (promote mistranslation)
	Tobramycin	7	30s ribosome (inhibit initiation complex formation)
Amphenicols	Chloramphenicol	50	50s ribosome (inhibit peptidyl transferase)
Macrolide	Azithromycin	16	50S ribosome (interfere aminoacyl translocation)
Tetracycline	Minocycline	0.75	30S ribosome (inhibit aminoacyl tRNA binding)
	Tetracycline	0.25	30S ribosome (inhibit aminoacyl tRNA binding)
	Tigecycline	2	30S ribosome (inhibit aminoacyl tRNA binding)
RNA Transcription Inhibitor			
Rifamycin	Rifampicin	1	DNA-dependent RNA polymerase
Cell Wall Synthesis Inhibitors			
Penicillin	Ampicillin	>112	Penicillin-binding proteins (PBPs)
	Amoxicillin	>112	Penicillin-binding proteins (PBPs)
	Mecillinam	>112	Penicillin-binding proteins (PBPs)
	Piperacillin	16	Penicillin-binding proteins (PBPs)
Carbapenem	Meropenem	1	Penicillin-binding proteins (PBPs)
Others	Fosfomycin	>112	UDP-N-acetylglucosamine enolpyruvyl transferase (MurA)
	$_{D}$-Cycloserine	>112	$_{D}$-Ala-$_{D}$-Ala terminal of peptidoglycan
Membrane Active Compounds			
Polymyxin	Colistin	1	Lipopolysaccharide (LPS)
Oxidative Phosphorylation uncoupling Agents	2,4-Dinitrophenol	>112	Energy poisoning agents
Lipid Synthesis Inhibitors			
Polychloro Phenoxy Phenols	Triclosan	0.2	Bacterial enoly-acyl carrier protein reductase enzyme (ENR: Fabl product)
Tested compound	NSC145612	25 μM	
	Drugs tested in *Acinetobacter baumannii* ATCC 17978		
DNA Synthesis Inhibitor			
Fluoroquinolone	Ciprofloxacin	1	DNA gyrase A

Results

BCP in *A. baumannii* can distinguish different classes of antibiotics

We first determined, based on cell morphological changes in *A. baumannii* ATCC 19606, if BCP can distinguish between antibiotics that interfere with six major cellular pathways: protein translation (chloramphenicol), RNA transcription (rifampicin), membrane integrity (colistin), lipid synthesis (triclosan), cell wall synthesis (piperacillin), and DNA replication (Ciprofloxacin). After incubation with antibiotics, we found that *A. baumannii* ATCC 19606 showed unique cell cytological profiles depending on the class of antibiotics used for treatment **(Fig. 1A-G).** Overall, *A. baumannii* cytological profiles of cells treated with different antibiotics were similar to those of *E. coli* shown in our previous study (1). Next, we quantitated 36 different cytological parameters of cells treated with each antibiotic and used principal component analysis (PCA) to determine if these cell profiles can be used to quantitatively classify the MOAs. The results showed that antibiotics with different MOAs were distinguishable from each other **(Fig. 1H**) and replicates of each antibiotic treatment were clustered together **(Fig. 1I).** These results suggest that BCP can be applied to *A. baumannii* in discriminating

antibiotics targeting six major cellular pathways including protein translation, RNA transcription, membrane integrity, lipid synthesis, cell wall synthesis, and DNA replication.

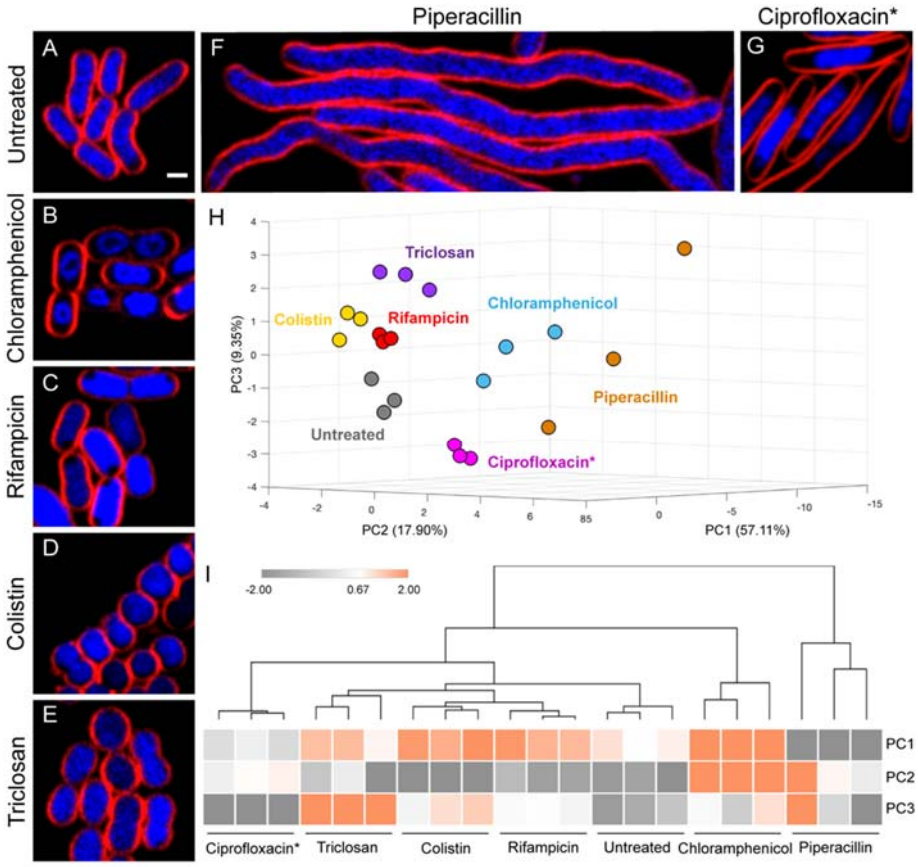


FIG 1 *A. baumannii* cells treated with antibiotics targeting different cellular pathways show distinct morphological changes. (*A*) Untreated bacterial cells. Bacterial cells were treated with (*B*, *D–F*) 5x MIC and (*C* and *G*) 2x MIC of each antibiotic for 2 hours and then stained with FM4-64 (red) and DAPI (blue). Scale bar represents 1 μm. (*H*) A 3D PCA graph constructed from PC1 (57.11%), PC2 (17.90%) and PC3 (9.35%) shows antibiotics that are distinguished into different subgroups as coded by colors. Three independent experiments were performed for each antibiotic treatment and cytological parameters measured as described in *Materials and Methods*. (*I*) Euclidean cluster map of antibiotics, using values from PC1, PC2 and PC3 of PCA. Ciprofloxacin* indicates that all data for treatment with ciprofloxacin was obtained in *A. baumannii* ATCC 17978 strain.

In *A. baumannii,* BCP can sub-classify different antibiotics that inhibit the same cellular pathway based on their mechanism of action

Our previous study in *E. coli* also showed that BCP can be used to classify sub-groups of antibiotics based on their MOA (1). To test if the ability of sub-classification by BCP is also observed in *A. baumannii*, we investigated whether BCP can differentiate various protein translation inhibitors and cell wall synthesis inhibitors. From all protein translation inhibitors tested **(Fig. 2A-I)**, we found that they were classified into 2 groups that correlated with their known MOA, similar to the previous study in *E. coli* (1): translation inhibition (P1) and aminoglycosides (P2) **(Fig. 2J and 2K)**. Protein translation inhibitors belonging to the P1 group bind directly to the ribosome to inhibit translation (6–9) resulting in the formation of toroidal-shaped DNA **(Fig. 1B and Fig. 2B-D)**. In addition to the

translation inhibition, aminoglycosides (10) displayed a significant effect on *A. baumannii* membrane permeability as indicated by the increase in SYTOX-Green uptake **(Fig. 2F-I, Right panel)** whereas SYTOX-Green signal was not detected in the untreated cells **(Fig. 2E, Right panel).** We also found that BCP could distinguish between two types of penicillin binding protein (PBPs) inhibitors in *A. baumannii* (**Fig. 3**), as expected (11, 12). Meropenem-treated *A. baumannii* cells were round and bloated compared to control cells in agreement with its affinity toward PBP2 (13) **(Fig. 3A-B).** Cells treated with piperacillin were elongated with an average length of 12 µm **(Fig. 3C)** likely due to the affinity of piperacillin toward PBP3 (14) which is required for cell septa formation in *A. baumannii*. Together, these results suggest that BCP in *A. baumannii* can also sub-classify antibiotics based on their MOA **(Fig. 2 and Fig. 3)** similar to what we previously reported in *E. coli* (1).

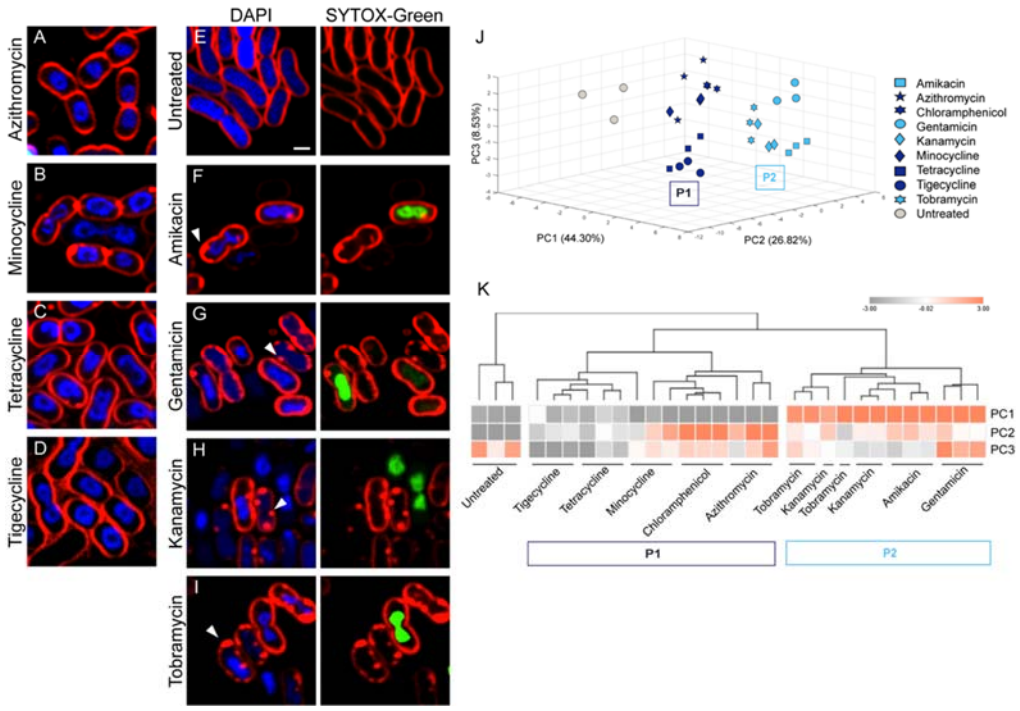


FIG 2 *A. baumannii* cytological profiling differentiating protein translation inhibitors into subgroups by their MOA.

Bacterial cells were treated with each antibiotic at 5x MIC for 2 hours and then stained with FM4-64 (red), DAPI (blue) and SYTOX green (green). Scale bar represents 1 µm. (*A–D*) Cells treated with protein translation inhibitors (P1 group) show distinct cell profiles. (*E*) Untreated cells. (*F–I*) Cells treated with aminoglycosides (P2 group) showing altered membrane permeability. Arrows indicate membrane pooling. SYTOX-green (*Right* panels) only stains nucleoids in the cells with permeabilized membranes. (*J*) PCA graph of protein translation inhibitors using PC1 (44.30%), PC2 (26.82%) and PC3 (8.53%) and (*K*) Euclidean cluster map, using PC1, PC2 and PC3 from PCA.

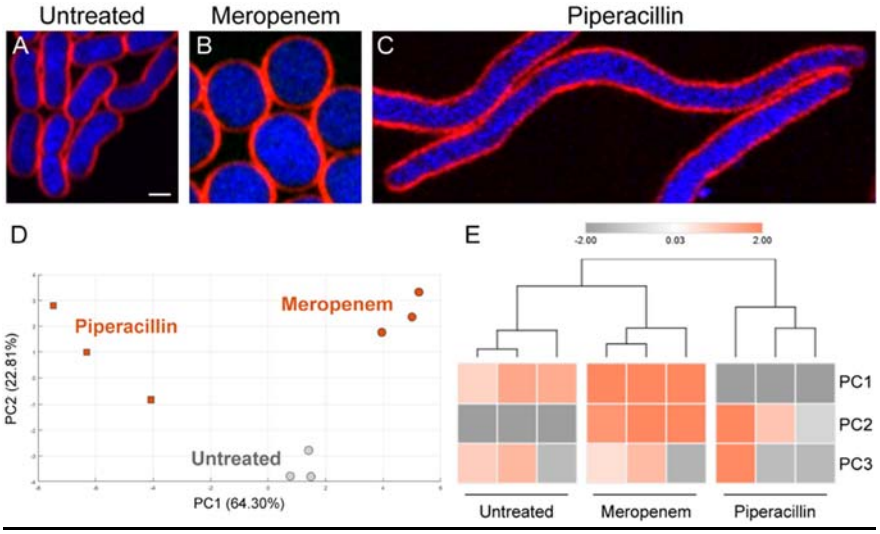


FIG 3 Cytological profiling of cell wall synthesis inhibitors; meropenem treated cells showing different profiles to the cells treated with piperacillin.

(*A–C*) Bacterial cells were treated with antibiotics at 5x MIC for 2 hours and then stained with FM4-64 (red) and DAPI (blue). Scale bar represents 1 μm. (*D*) PCA graph of cell wall synthesis inhibitors showing only PC1 (64.30%) and PC2 (22.81%) and (*E*) Euclidean cluster map using PC1, PC2 and PC3 from PCA showing distinct morphological clusters.

The compound NSC145612 inhibits the growth of *A. baumannii* via RNA transcription inhibition

In this study, we have tested 64 compounds from National Cancer Institute's Developmental Therapeutics Program library for their antibacterial activities against Gram-negative bacterium *E. coli* ATCC 25922 and found that 17 compounds were active. Among those Gram-negative active compounds, the compound NSC145612 (**Fig. 4A, right panel**) showed a promising MIC against *A. baumannii* ATCC 19606 at 25 μM (**Table 1**). Although the chemical scaffold of NSC145612 is closely related to rifampicin (15), which is an antibiotic inhibiting DNA-dependent RNA polymerase (16), the compound has never been tested for its mechanism of action. In order to investigate if the compound exhibits the same MOA as rifampicin, we performed BCP on the compound against *A. baumannii*. As expected, the result showed that NSC145612-treated *A. baumannii* cells exhibited a cytological profile identical to rifampicin-treated cells (**Fig. 4B-D**) and grouped together in PCA analysis (**Fig. 4E-F**), suggesting that NSC145612 inhibits RNA transcription of *A. baumannii* similar to rifampicin.

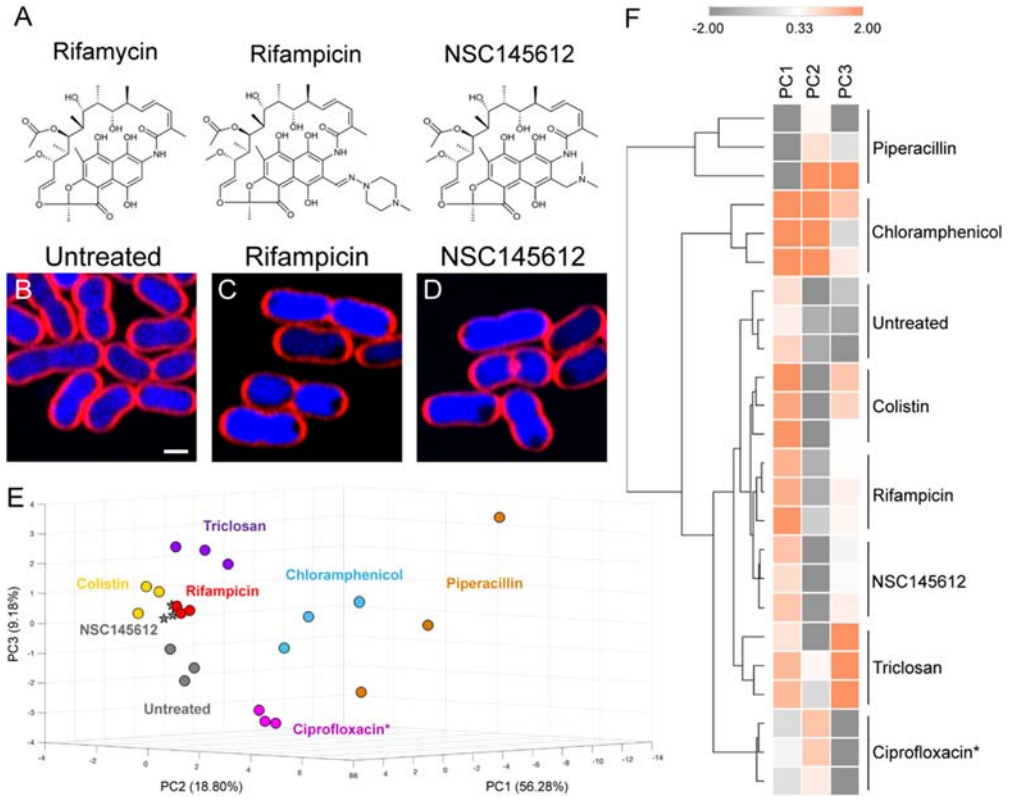


FIG 4 *A. baumannii* cell treated with NSC145612 show similar profiles to the RNA transcription inhibitor, rifampicin.

(*A*) Chemical structure of rifamycin, rifampicin and NSC145612. (*B*) Untreated cells. Bacterial cells were treated with 2x MIC of (C) Rifampicin or (*D*) NSC145612 for 2 hours and then stained with FM4-64 (red) and DAPI (blue). Scale bar represents 1 μm. (*E*) PCA graph of 6 major classes of representative antibiotics and NSC145612 using PC1 (56.28%), PC2 (18.80%) and PC3 (9.18%) and (*F*) Euclidean cluster map, using values from PC1, PC2 and PC3 from PCA, showing NSC145612 closely clustered to Rifampicin. Ciprofloxacin* indicates that all data for treatment with Ciprofloxacin was obtained in *A. baumannii* ATCC 17978 strain.

To confirm the molecular target of NSC145612 in *A. baumannii*, two NSC145612-resistant *A. baumannii* strains were also isolated with an MIC above 150 μM. As expected, NSC145612-resistant *A. baumannii* contained a mutation in the *rpoB* gene, *rpoB(G543S)*, which is located in the RRDR and is known to be responsible for rifampicin resistance in *A. baumannii* (17, 18). BCP results showed that neither NSC145612 nor rifampicin treatment resulted in cytological changes of NSC145612-resistant *A. baumannii* strains as compared to the controls (**Fig. 5**), confirming that NSC145612 and rifampicin are inactive against the resistant strains. Overall, these results suggest that NSC145612 inhibits RNA transcription of *A. baumannii* by targeting its RNA polymerase subunit B.

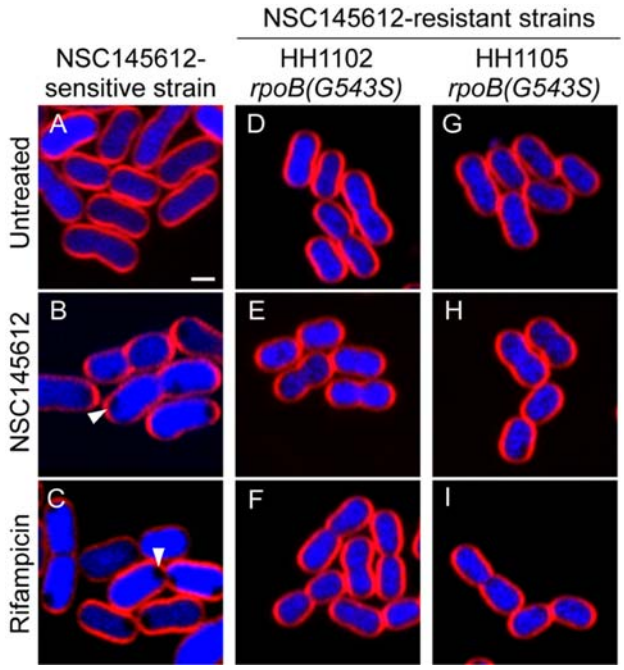


FIG 5 NSC145612-resistant *A. baumannii* cells show no morphological change upon NSC145612 and rifampicin treatment. (*A–C*) NSC145612-sensitive strain. Arrows indicate signature phenotype of RNA transcription inhibition. (*D–I*) NSC145612-resistant strains with *rpoB* mutations indicated. Bacterial cells were treated with 2x MIC of NSC145612 (*B, E*, and *H*) or Rifampicin (*C, F*, and *I*) for 2 hours and then stained with FM4-64 (red) and DAPI (blue). Scale bar represents 1 μm.

Conclusion and Discussion

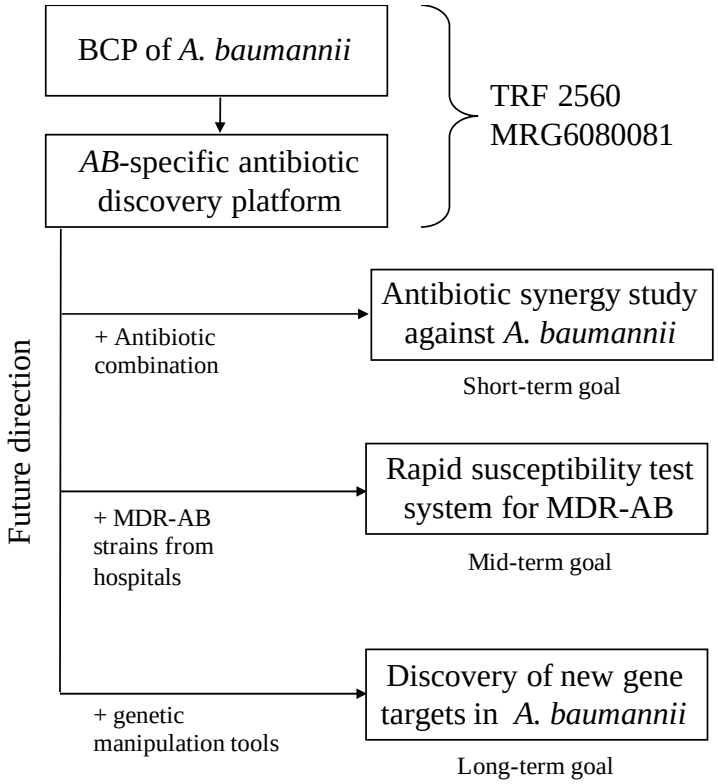
A recent report from the World Health Organization revealed that among the ESKAPE pathogens, *A. baumannii* poses a threat to public health and economies worldwide (19). *A. baumannii* is a successful pathogen due to its ability to survive in desiccated environments and its intrinsic antibiotic resistance (20). As a result, MDR-*AB* is spreading at an alarming rate (21–23). Multiple approaches exist in order to help mitigate the rise of MDR-*AB* including developing more stringent guidelines for antibiotic usage and establishing effective surveillance and containment programs (19). A direct approach to combat MDR-*AB* is to find new antibiotics that are effective against this pathogen.

BCP has been developed for several species of bacteria including *E.coli, S. aureus* and *B. subtilis*, but it has not been systematically applied to *A. baumannii* (4, 24, 25). Here we study the cytological profiles of antibiotics commonly used to treat *A. baumannii*. We show that BCP can be used to identify the MOA of newly discovered compounds to facilitate *A. baumannii*-specific antibiotic discovery. BCP successfully differentiates *A. baumannii* cells treated with different antibiotics targeting major cellular pathways: protein translation, RNA transcription, membrane integrity, lipid synthesis, cell wall synthesis, and DNA replication. Similar to *E. coli*, *A. baumannii* cytological changes can reveal subgroups of protein translation inhibitors and cell wall synthesis inhibitors suggesting similar cytological responses across Gram-negative bacteria species.

The compound NSC145612 from the National Cancer Institute's Developmental Therapeutics Program has previously been tested for anti-cancer and AIDS antiviral activity, all of which gave negative results (15). In this study, its antibacterial activity was tested by BCP and later confirmed by resistant mutant selection and genome sequencing. NSC145612 inhibits the growth of *A. baumannii* and *E. coli* via RNA transcription inhibition by targeting RpoB protein. Altogether, this study proves the

utility of BCP as a potential method to reveal the mechanism of action of compounds that are active against *A. baumannii*.

Future direction



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Keywords : *Acinetobacter baumannii*, antibiotic screening, mechanisms of action

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   ตีพิมพ์เผยแพร่ผลงานวิจัยระดับนานาชาติจำนวน 1 เรื่องในวารสาร Antimicrobial Agents and Chemotherapy(AAC) ซึ่งเป็นวารสารที่อยู่ใน Quartile in Category Q1 และมี Impact Factor 4.256

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2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
     ไม่มี
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
     ไม่มี
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

ความร่วมมือระหว่างการทำวิจัย ได้ร่วมมือกับ Professor Joe Pogliano จาก University of California, San Diego และผู้ก่อตั้งบริษัท Linnaeus Bioscience ในการทำวิจัยให้สำเร็จลุล่วงตามเวลาที่กำหนด

เครือข่ายความร่วมมือที่สร้างขึ้นใหม่ นอกจากความร่วมมือกับทาง Professor Joe Pogliano แล้ว ทางผู้วิจัยยังทำการสร้างเครือข่ายนักวิจัยที่สนใจใช้เทคนิค BCP ในการศึกษากลไกการทำงานของสารต้านจุลชีพ โดยในปัจจุบันมีการทำงานร่วมกับกลุ่มวิจัยในประเทศไทยต่าง ๆ ดังนี้

1. รองศาสตราจารย์ ดร. รศนา วงศ์รัตนชีวิน เทคโนโลยีชีวภาพ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น (Natural product)

2. ผู้ช่วยศาสตราจารย์ ดร. วรินทร ชวศิริ ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย (Natural product)

3. ผู้ช่วยศาสตราจารย์ ดร.กุลยา สมบูรณ์วิวัฒน์ ภาควิชาชีวเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย (Antimicrobial peptides)

4. ดร.สรชา ธรรมภิวัฒนา สถาบันวิศวกรรมชีวการแพทย์ คณะแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ (Nanoparticle)

- เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
  พัฒนาและถ่ายทอดความรู้เกี่ยวกับเทคนิค BCP ครบทั้งกระบวนการแก่นักวิจัยของทางสถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล 1 คน (Htut Htut Htoo) และเผยแพร่ความรู้เกี่ยวกับเทคนิค BCP แก่นักศึกษาที่สนใจ

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

   เสนอผลงาน Oral presentation ในงานประชุมวิชาการนานาชาติ The 6th International Conference on Biochemistry and Molecular Biology 2018 (BMB 2018) ที่จัดขึ้นระหว่างวันที่ 20-22 มิถุนายน พ.ศ. 2561

เสนอผลงาน Poster presentation ในงานประชุมวิชาการ “นักวิจัยรุ่นใหม่...พบ...เมธีวิจัยอาวุโส สกว.” ครั้งที่ 18 ที่จัดขึ้นระหว่างวันที่ 9-11 มกราคม พ.ศ. 2562

Bacterial Cytological Profiling (BCP) as a tool to study mechanism of action of antibiotics that are active against *Acinetobacter baumannii*

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Running title: BCP for *A. baumannii* antibiotic research

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Abstract

An increasing number of multidrug-resistant *Acinetobacter baumannii* (MDR-*AB*) infections have been reported worldwide, posing a threat to public health. The establishment of methods to elucidate the mechanism of action (MOA) of *A. baumannii*-specific antibiotics is needed to develop novel antimicrobial therapeutics with activity against MDR-*AB*. We previously developed bacterial cytological profiling (BCP) to understand the MOA of compounds in *E. coli* and *B. subtilis*. Given how distantly related *A. baumannii* is to these species, it was unclear to what extent it could be applied. Here we implemented bacterial cytological profiling (BCP) as an antibiotic MOA discovery platform for *A. baumannii*. We found that the BCP platform can distinguish among six major antibiotic classes and can also sub-classify antibiotics that inhibit the same cellular pathway but have different molecular targets. We used BCP to show that the compound NSC145612 inhibits the growth of *A. baumannii* via targeting RNA transcription. We confirmed this result by isolating and characterizing resistant mutants with mutations in the *rpoB* gene. Altogether, we conclude that BCP provides a useful tool for MOA studies of antibacterial compounds that are active against *A. baumannii*.

Introduction

The discovery of penicillin led to the "golden era" of antibiotic research which lasted for many decades before fading away in the 1970s. Since then, the rate of discovery of novel antibacterial molecules has decreased dramatically, and most of the newly commercialized antibiotics are analogues of existing ones (1–3). Although five new classes of Gram-positive acting antibiotics were recently discovered, fewer novel Gram-negative antibiotics have been developed (4). The incidence of Gram-negative pathogens that are resistant to almost all existing antibiotics is growing rapidly (5, 6). As a result, the options for treating drug-

resistant Gram-negative infections are limited; thus, new antibiotics that act against Gram-negative bacteria are urgently needed (7). Among the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (5), *A. baumannii* is of particular concern as it is (8, 9) responsible for a wide range of hospital-acquired infections including meningitis, bacteremia, and skin infections (10). Apart from their intrinsic resistance, some clinically isolated *A. baumannii* strains have developed resistance to antibiotics commonly used for treatment such as β-lactams, aminoglycosides and tetracyclines (11). Also, a number of cases have been reported of strains that are resistant to colistin (11–13) and tigecycline (11, 14, 15), antibiotics considered to be the last line of defense (16), emphasizing the need of novel antibiotics that are active against the pathogen.

In order to minimize the harmful effects of antibiotics on the microbiome and prevent the spread of antibiotic resistance across various pathogens, narrow spectrum-antibiotics may be preferable over broad spectrum ones in some cases (2). Species-specific antibiotic screening platforms have been proposed as a potential approach to discover narrow spectrum-antibiotics (17). A Mycobacteria-specific screening platform is an example of a successful case of such screening approaches (4, 18). These screens resulted in the discovery of many antibiotics exhibiting both broad spectrum, such as streptomycin (19), and mycobacteria-specific activity including isoniazid, pyrazinamide, ethionamide, ethambutol (4), and bedaquiline (20). Recently, Gram-specific (21–24) and pathogen-specific (25, 26) antibiotic discovery were also proven to be successful, leading to the identification of narrow spectrum compounds including some that are active only against *A. baumannii* (27–29). As more candidate compounds are revealed through screening, there will be a need for better methods to elucidate their MOA in *A. baumannii*.

In recent years, we have developed a method for antibiotic mechanism of action (MOA) study called bacterial cytological profiling (BCP) that can be applied to various bacterial species (30–32). BCP generates reference cytological profiles of bacterial cells upon treatment with different classes of antibiotics. BCP has been proven to be beneficial in MOA studies of antibiotics (30, 31, 33–37) and in a rapid antibiotic susceptibility test (32). Although a BCP-derived method was successfully used in a synergy study between azithromycin and human antimicrobial peptide LL-37 against multidrug resistant *A. baumannii* (MDR-*AB*) (38), reference BCP profiles of *A. baumannii* treated with various types of antibiotics have not been reported. *A. baumannii* is very distantly related to *E. coli* and it was therefore unclear to what extent BCP could be applied. Here we investigated the utility of BCP for *A. baumannii*. We showed that BCP is a useful tool for identifying the MOA of antibacterial molecules that inhibit the growth of *A. baumannii* (**Table S1**) and used this platform to determine that the compound NSC145612 inhibits transcription in *A. baumannii*.

Results

BCP in *A. baumannii* can distinguish different classes of antibiotics

We first determined, based on cell morphological changes in *A. baumannii* ATCC 19606, if BCP can distinguish between antibiotics that interfere with six major cellular pathways: protein translation (chloramphenicol), RNA transcription (rifampicin), membrane integrity (colistin), lipid synthesis (triclosan), cell wall synthesis (piperacillin), and DNA replication (Ciprofloxacin). After incubation with antibiotics, we found that *A. baumannii* ATCC 19606 showed unique cell cytological profiles depending on the class of antibiotics used for treatment **(Fig. 1A-F).** Chloramphenicol-treated cells had a signature toroidal-shaped chromosome (**Fig. 1B**) while treatment with the transcription inhibitor, rifampicin,

resulted in diffuse DAPI staining throughout the cell except for a small rounded region near the cell membrane (**Fig. 1C**) similar to the BCP profile of actinomycin D-treated *E. coli* from the previous study (30). Colistin-treated cells were attached together creating a long chain of small and round cells (**Fig. 1D**) similar to what we previously reported (38). Triclosan-treated cells were shorter and slightly rounder than untreated cells (**Fig. 1E**). The Cell wall synthesis inhibitor piperacillin resulted in cell elongation without visible cell septation (**Fig. 1F**). In the case of DNA replication inhibitors, we found that even though DNA replication inhibitors effectively inhibited growth of *A. baumannii* ATCC 19606 as measured by MIC, only less than 10% of the cells treated with these inhibitors showed a possible DNA replication inhibition phenotype in this strain (**FIG S1**). Thus, another well-studied *A. baumannii* strain ATCC 17978 (39) was used in DNA replication experiment **(FIG S2 and Table S1)**. We found that the cell morphology of *A. baumannii* ATCC 17978 changed upon ciprofloxacin treatment. The treated cells were elongated and their chromosomes formed a single large nucleoid in the cell center (**Fig. 1G**). Overall, *A. baumannii* cytological profiles of cells treated with different antibiotics were similar to those of *E. coli* shown in our previous study (30). Next, we quantitated 36 different cytological parameters of cells treated with each antibiotic **(Table S2)** and used principal component analysis (PCA) to determine if these cell profiles can be used to quantitatively classify the MOAs. The results showed that antibiotics with different MOAs were distinguishable from each other **(Fig. 1H**) and replicates of each antibiotic treatment were clustered together **(Fig. 1I).** These results suggest that BCP can be applied to *A. baumannii* in discriminating antibiotics targeting six major cellular pathways including protein translation, RNA transcription, membrane integrity, lipid synthesis, cell wall synthesis, and DNA replication.

In *A. baumannii*, BCP can sub-classify different antibiotics that inhibit the same cellular pathway based on their mechanism of action

Our previous study in *E. coli* also showed that BCP can be used to classify sub-groups of antibiotics based on their MOA (30). To test if the ability of sub-classification by BCP is also observed in *A. baumannii*, we investigated whether BCP can differentiate various protein translation inhibitors and cell wall synthesis inhibitors. From all protein translation inhibitors tested **(Fig. 2A-I)**, we found that they were classified into 2 groups that correlated with their known MOA, similar to the previous study in *E. coli* (30): translation inhibition (P1) and aminoglycosides (P2) **(Fig. 2J and 2K)**. Tetracycline, tigecycline and minocycline, which are structurally related, were closely clustered in the analysis **(Fig. 2K)**. Protein translation inhibitors belonging to the P1 group bind directly to the ribosome to inhibit translation (40–43) resulting in the formation of toroidal-shaped DNA **(Fig. 1B and Fig. 2B-D)**. In addition to the translation inhibition, aminoglycosides (44) displayed a significant effect on *A. baumannii* membrane permeability as indicated by the increase in SYTOX-Green uptake **(Fig. 2F-I, Right panel)** whereas SYTOX-Green signal was not detected in the untreated cells **(Fig. 2E, Right panel).** The increase in SYTOX-Green intensity found in aminoglycoside-treated cells is more than 20 times higher than those of untreated cells (**Table S2**). This permeability effect of aminoglycosides separated them from the untreated and the others in the P1 group **(Fig. 2J and 2K).** We also found that BCP could distinguish between two types of penicillin binding protein (PBPs) inhibitors in *A. baumannii* (**Fig. 3**), as expected (45, 46). Among all cell wall synthesis inhibitors tested in this study (**Table S1**), only meropenem, a preferred choice for treating *A. baumannii* infections (10, 47–49), and piperacillin are active against the strain according to an MIC assay. Meropenem-treated *A. baumannii* cells were round and bloated compared to control cells in agreement with its affinity toward PBP2 (50) **(Fig. 3A-B).** Cells treated with piperacillin were elongated with

an average length of 12 µm **(Fig. 3C and Table S2)** likely due to the affinity of piperacillin toward PBP3 (49) which is required for cell septa formation in *A. baumannii*. Together, these results suggest that BCP in *A. baumannii* can also sub-classify antibiotics based on their MOA **(Fig. 2 and Fig. 3)** similar to what we previously reported in *E. coli* (30).

The compound NSC145612 inhibits the growth of *A. baumannii* via RNA transcription inhibition

In this study, we have tested 64 compounds from National Cancer Institute's Developmental Therapeutics Program library for their antibacterial activities against Gram-negative bacterium *E. coli* ATCC 25922 and found that 17 compounds were active. Among those Gram-negative active compounds, the compound NSC145612 (**Fig. 4A, right panel**) showed a promising MIC against *A. baumannii* ATCC 19606 at 25 µM (**Table S1**). Although the chemical scaffold of NSC145612 is closely related to rifampicin (51), which is an antibiotic inhibiting DNA-dependent RNA polymerase (52), the compound has never been tested for its mechanism of action. In order to investigate if the compound exhibits the same MOA as rifampicin, we performed BCP on the compound against *A. baumannii*. As expected, the result showed that NSC145612-treated *A. baumannii* cells exhibited a cytological profile identical to rifampicin-treated cells (**Fig. 4B-D**) and grouped together in PCA analysis (**Fig. 4E-F**), suggesting that NSC145612 inhibits RNA transcription of *A. baumannii* similar to rifampicin. This conclusion was supported by examining NSC145612 in *E. coli* Δ*tolC*, whose growth was inhibited at 30 µM (**Table S3**). BCP of NSC145612-treated *E. coli* Δ*tolC* cells showed decondensed DNA (**Fig. S3**), which is a hallmark of transcription inhibition in *E. coli* (30).

In order to gain more information regarding molecular target of the compound NSC145612, we isolated and characterized resistant mutations in both *A. baumannii and E.*

coli Δ*tolC*. A total of four NSC145612-resistant mutants of *E. coli* were isolated (**Table S3**). Whole genome sequencing of the resistant mutants revealed various mutations in DNA-dependent RNA polymerase subunit B (*rpoB* gene) (**Table S3**), a well-known gene responsible for rifampicin-resistance in *E. coli* and *Mycobacterium tuberculosis* (52, 53). Notably, three of our four resistant mutants contain mutations (**Table S3**) which are located in the Rifampicin Resistance Determining Region (RRDR) of the *rpoB* gene spanning from codon 507 to 533 (54). The rare mutation *rpoB(V146F)*, which is located near the rifampicin-binding pocket of the enzyme (55), was also found in one of the NSC145612-resistant mutants (LB143 strain). In accordance with the genetic profiles, the BCP profile of resistant mutants treated with NSC145612 and rifampicin showed no cytological changes as compared to the untreated controls (**Fig. S3**), confirming that NSC145612 and rifampicin are inactive against the strains containing the *rpoB* mutation.

To confirm the molecular target of NSC145612 in *A. baumannii*, two NSC145612-resistant *A. baumannii* strains were also isolated with an MIC above 200 µM (**Table 1**). As expected, NSC145612-resistant *A. baumannii* contained a mutation in the *rpoB* gene, *rpoB(G543S)* (**Table 1**), which is located in the RRDR and is known to be responsible for rifampicin resistance in *A. baumannii* (56, 57). BCP results showed that neither NSC145612 nor rifampicin treatment resulted in cytological changes of NSC145612-resistant *A. baumannii* strains as compared to the controls (**Fig. 5**), confirming that NSC145612 and rifampicin are inactive against the resistant strains. Overall, these results suggest that NSC145612 inhibits RNA transcription of *A. baumannii* by targeting its RNA polymerase subunit B.

Discussion

A recent report from the World Health Organization revealed that among the ESKAPE pathogens, *A. baumannii* poses a threat to public health and economies worldwide (9). *A. baumannii* is a successful pathogen due to its ability to survive in desiccated environments and its intrinsic antibiotic resistance (8). As a result, MDR-*AB* is spreading at an alarming rate (58–60). Multiple approaches exist in order to help mitigate the rise of MDR-*AB* including developing more stringent guidelines for antibiotic usage and establishing effective surveillance and containment programs (9). A direct approach to combat MDR-*AB* is to find new antibiotics that are effective against this pathogen.

BCP has been developed for several species of bacteria including *E. coli*, *S. aureus* and *B. subtilis*, but it has not been systematically applied to *A. baumannii* (31, 32, 61). Here we study the cytological profiles of antibiotics commonly used to treat *A. baumannii*. We show that BCP can be used to identify the MOA of newly discovered compounds to facilitate *A. baumannii*-specific antibiotic discovery. BCP successfully differentiates *A. baumannii* cells treated with different antibiotics targeting major cellular pathways: protein translation, RNA transcription, membrane integrity, lipid synthesis, cell wall synthesis, and DNA replication. Similar to *E. coli*, *A. baumannii* cytological changes can reveal subgroups of protein translation inhibitors and cell wall synthesis inhibitors suggesting similar cytological responses across Gram-negative bacteria species.

While *A. baumannii* ATCC 17978 treated with ciprofloxacin showed a clear cytological profile consistent with inhibiting DNA replication (**Fig. S2**), treatment of *A. baumannii* ATCC 19606 with ciprofloxacin did not induce similar cytological changes (**Fig. S1**). The fact that *A. baumannii* ATCC 19606 did not respond to DNA replication inhibitors made it unpractical for data from this strain to be used in the analysis. DNA damage and replication inhibition caused by quinolone antibiotics induce SOS responses in *E. coli* (62–64) and other bacteria (65, 66). In previous studies of *E. coli* (30, 67), filamentous *E. coli*

observed after quinolone antibiotic treatment was a result of replication halt-induced SOS response (65). Upon SOS response induction, *sulA* is derepressed due to the decrease in LexA protein, a master regulator of SOS response genes. SulA then inhibits FtsZ polymerization which leads to cell division inhibition and filamentation (68, 69). However, the SOS response of *Acinetobactor* spp. is not well-understood due to the lack of similar SOS response genes including *lexA* and *sulA* (68, 70–72). Since distinct responses to DNA damage have been observed in different species of *Acinetobacter* (73, 74), it is possible that *A. baumannii* ATCC 19606 and ATCC 17978 respond differently to the DNA replication inhibitors. Based on these results, multiple *A. baumannii* strains should be used to establish a comprehensive database of cytological profiles.

The compound NSC145612 from the National Cancer Institute's Developmental Therapeutics Program has previously been tested for anti-cancer and AIDS antiviral activity, all of which gave negative results (51). In this study, its antibacterial activity was tested by BCP and later confirmed by resistant mutant selection and genome sequencing. NSC145612 inhibits the growth of *A. baumannii* and *E. coli* via RNA transcription inhibition by targeting RpoB protein. Altogether, this study proves the utility of BCP as a potential method to reveal the mechanism of action of compounds that are active against *A. baumannii*.

Materials and Methods

Bacteria strain, growth and antibiotics. *Acinetobacter baumannii* strain ATCC 19606, strain ATCC 17978 and *Escherichia coli* strain AD3644 (Δ*tolC*) were used in this study. The bacteria were grown in LB medium or LB agar at 30°C. A total of twenty-two antibiotics were tested on *A. baumannii* from which fifteen antibiotics with minimal inhibitory concentration of less than 112 μg/ml were used in this study (**Table S1**). The compound NSC145612 was obtained from the National Cancer Institute's Developmental Therapeutics

Program. Preparation of the antibiotics was according to the manufacture's recommendations.

Minimal Inhibitory Concentrations. Minimal inhibitory concentrations (MIC) of all antibiotics are shown in **Table S1**. MIC was obtained using microdilution method (30) . Overnight cultures of *A. baumannii* were diluted 1:100 in LB broth and allowed to grow at 30°C on a roller until exponential phase or until the OD_{600} of 0.2 was obtained. The bacteria culture was further diluted 1:100 into each well of 96 well plate containing antibiotics in LB media at appropriate concentrations. Cultures were allowed to grow at 30°C for 24 hours. MIC was determined by observing the concentration of the antibiotic in the well where the bacteria was unable to grow.

Fluorescence microscopy. Overnight cultures of *A. baumannii* were diluted 1:500 and those of *E. coli* at 1:100 in LB broth and grown at 30°C on a roller until exponential phase. Antibiotics were added at concentrations of 0.75 times MIC for colistin, 2 times the MIC for rifampicin, NSC145612, and ciprofloxacin and, 5 times the MIC for the rest of the tested antibiotics. Cultures were then grown at 30°C on a roller for 2 hours. *A. baumannii* cultures were stained with FM 4-64 (2 µg/ml), DAPI (4 µg/ml) and SYTOX-green (0.5 µM). *E. coli* the cells were stained with FM 4-64 (1 µg/mL), DAPI (2 µg/mL), and SYTOX-Green (0.5 µM). Stained bacterial cultures were harvested by centrifugation at 6,000 g for 30 seconds and resuspended in 1/10 volume of the same culture media. Three microliters of this was added to agarose pads (1.2% agarose in 20% LB broth) on concave glass slides. Fluorescence microscopy was performed with consistent imaging parameters throughout all experiments.

Cytological profiling. Cytological profiles were determined by automated cell analysis using CellProfiller 3.0 (75). Briefly, images were pre-processed on Fiji software (76) and subsequently analyzed on CellProfiler 3.0 software. Cell morphological parameters such as length, width, area, perimeter, form factor, ferret diameter, radius, compactness, solidity and eccentricity of both cell membrane and nucleoid were determined. To obtain the average intensity of SYTOX-green and DAPI, both the membrane and nucleoid outlines were used and subtracted by background intensity in corresponding images. The fold-increase in permeability of aminoglycosides (P2 group) was determined by dividing the SYTOX-Green intensity of aminoglycoside-treated cells with that of the untreated cells (**Table S2**). Decondensation of the nucleoid was determined by the ratio of the area of the nucleoid to that of the cell membrane.

Statistical analysis. As described previously (30, 31, 38), the cytological parameters of each antibiotic were obtained from three independent experiments. Profiling data was from automated analysis of the cells in each imaging field. Only images containing more than 20 for long cells and for the rest, more than 30 cells per imaging field were selected into data points. Weighed principal component analysis (PCA) was performed using statistic tools on MATLAB 2017a. Euclidean cluster analysis was generated from Morpheus (https://software.broadinstitute.org/morpheus).

Isolation of NSC145612-resistant mutants. In *E. coli*, resistant mutants were obtained by plating *E. coli* AD3644 (Δ*tolC*) on LB agar plates containing 2X MIC of NSC145612. The plates were incubated at 30°C and resistant mutants were purified and stabilized on additional 2X MIC of NSC145612 selection plates. In *A. baumannii*, *A. baumannii* ATCC 19606 culture was diluted into the LB media containing NSC145612 starting at 0.5X MIC. This process

was repeated with escalating concentration of NSC145612 until the NSC145612-resistant *A. baumannii* was obtained. The resistant strains were purified on LB agar plates and MIC for NSC145612 and rifampicin determined by broth dilution method, as mentioned above.

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Figure legends and Tables

FIG 1 *A. baumannii* cells treated with antibiotics targeting different cellular pathways show distinct morphological changes.

(*A*) Untreated bacterial cells. Bacterial cells were treated with (*B*, *D–F*) 5x MIC and (*C* and *G*) 2x MIC of each antibiotic for 2 hours and then stained with FM4-64 (red) and DAPI (blue). Scale bar represents 1 μm. (*H*) A 3D PCA graph constructed from PC1 (57.11%), PC2 (17.90%) and PC3 (9.35%) shows antibiotics that are distinguished into different subgroups as coded by colors. Three independent experiments were performed for each antibiotic treatment and cytological parameters (Table S2) measured as described in *Materials and Methods*. (*I*) Euclidean cluster map of antibiotics, using values from PC1, PC2 and PC3 of PCA. Ciprofloxacin* indicates that all data for treatment with ciprofloxacin was obtained in *A. baumannii* ATCC 17978 strain.

FIG 2 *A. baumannii* cytological profiling differentiating protein translation inhibitors into subgroups by their MOA.

Bacterial cells were treated with each antibiotic at 5x MIC for 2 hours and then stained with FM4-64 (red), DAPI (blue) and SYTOX green (green). Scale bar represents 1 μm. (*A–D*) Cells treated with protein translation inhibitors (P1 group) show distinct cell profiles. (*E*) Untreated cells. (*F–I*) Cells treated with aminoglycosides (P2 group) showing altered membrane permeability. Arrows indicate membrane pooling. SYTOX-green (*Right* panels) only stains nucleoids in the cells with permeabilized membranes. (*J*) PCA graph of protein translation inhibitors using PC1 (44.30%), PC2 (26.82%) and PC3 (8.53%) and (*K*) Euclidean cluster map, using PC1, PC2 and PC3 from PCA.

FIG 3 Cytological profiling of cell wall synthesis inhibitors; meropenem treated cells showing different profiles to the cells treated with piperacillin.

(*A–C*) Bacterial cells were treated with antibiotics at 5x MIC for 2 hours and then stained with FM4-64 (red) and DAPI (blue). Scale bar represents 1 μm. (*D*) PCA graph of cell wall synthesis inhibitors showing only PC1 (64.30%) and PC2 (22.81%) and (*E*) Euclidean cluster map using PC1, PC2 and PC3 from PCA showing distinct morphological clusters.

FIG 4 *A. baumannii* cell treated with NSC145612 show similar profiles to the RNA transcription inhibitor, rifampicin.

(*A*) Chemical structure of rifamycin, rifampicin and NSC145612. (*B*) Untreated cells. Bacterial cells were treated with 2x MIC of (C) Rifampicin or (*D*) NSC145612 for 2 hours and then stained with FM4-64 (red) and DAPI (blue). Scale bar represents 1 μm. (*E*) PCA graph of 6 major classes of representative antibiotics and NSC145612 using PC1 (56.28%), PC2 (18.80%) and PC3 (9.18%) and (*F*) Euclidean cluster map, using values from PC1, PC2 and PC3 from PCA, showing NSC145612 closely clustered to Rifampicin. Ciprofloxacin* indicates that all data for treatment with Ciprofloxacin was obtained in *A. baumannii* ATCC 17978 strain.

FIG 5 NSC145612-resistant *A. baumannii* cells show no morphological change upon NSC145612 and rifampicin treatment.

(*A–C*) NSC145612-sensitive strain. Arrows indicate signature phenotype of RNA transcription inhibition. (*D–I*) NSC145612-resistant strains with *rpoB* mutations indicated. Bacterial cells were treated with 2x MIC of NSC145612 (*B*, *E*, and *H*) or Rifampicin (*C*, *F*, and *I*) for 2 hours and then stained with FM4-64 (red) and DAPI (blue). Scale bar represents 1 μm.

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362 **Table 1. MIC of NSC145612 and rifampicin against *A. baumannii* strains**

Strains	*rpoB* mutations	MIC	
		NSC145612 (μM)	Rifampicin (μM)
A. baumannii	-	25	1.2
A. baumannii HH1102	*rpoB(G543S)*	>200	24
A. baumannii HH1105	*rpoB(G543S)*	>200	24

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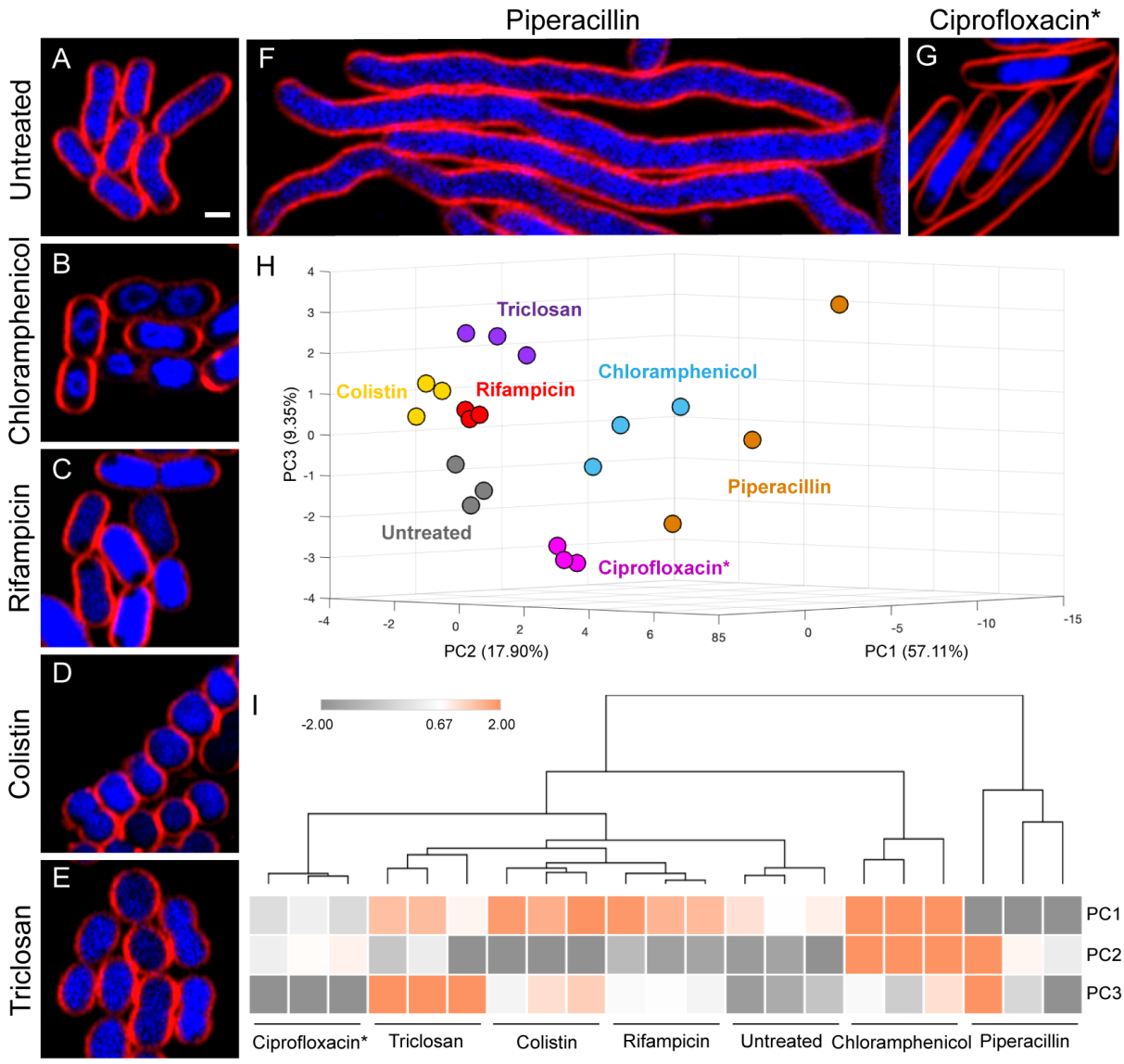

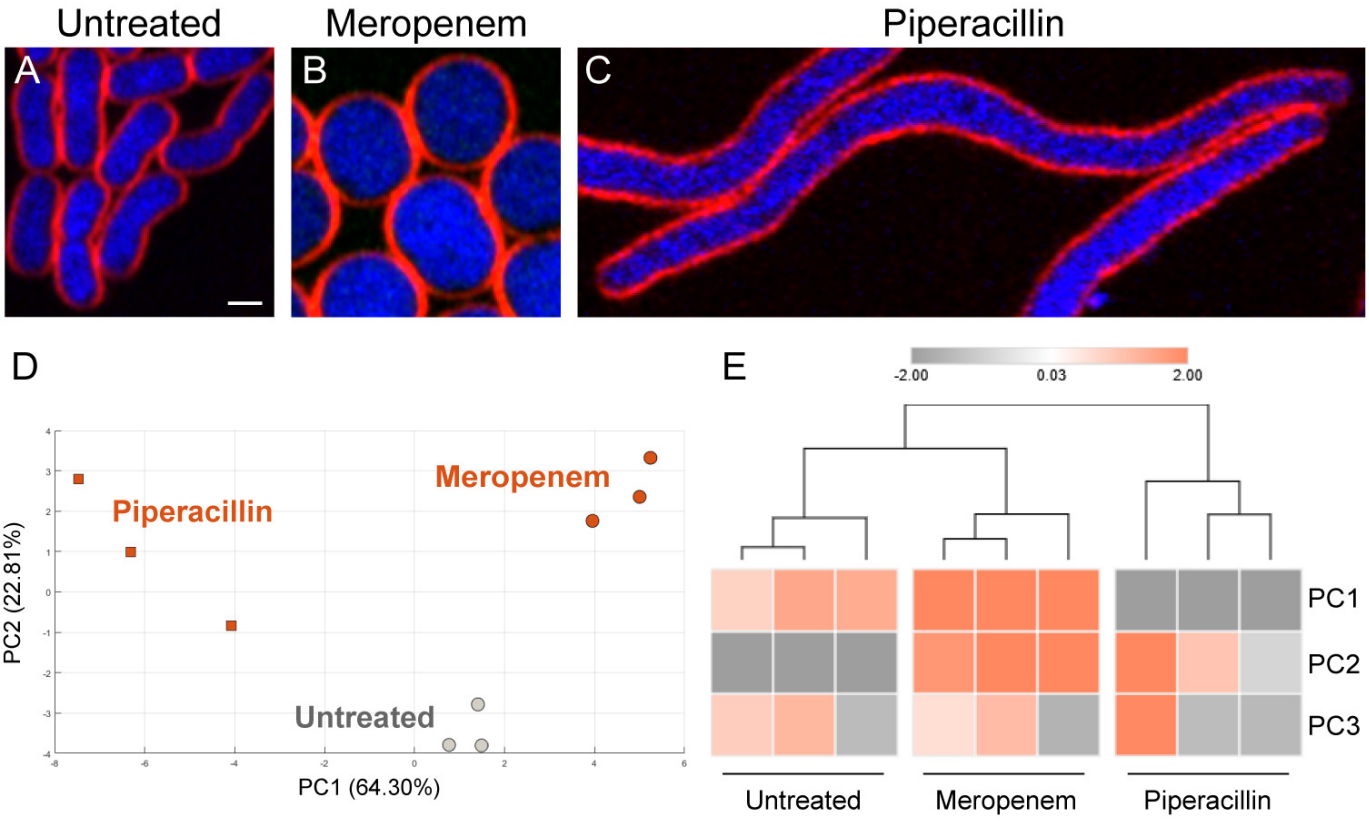

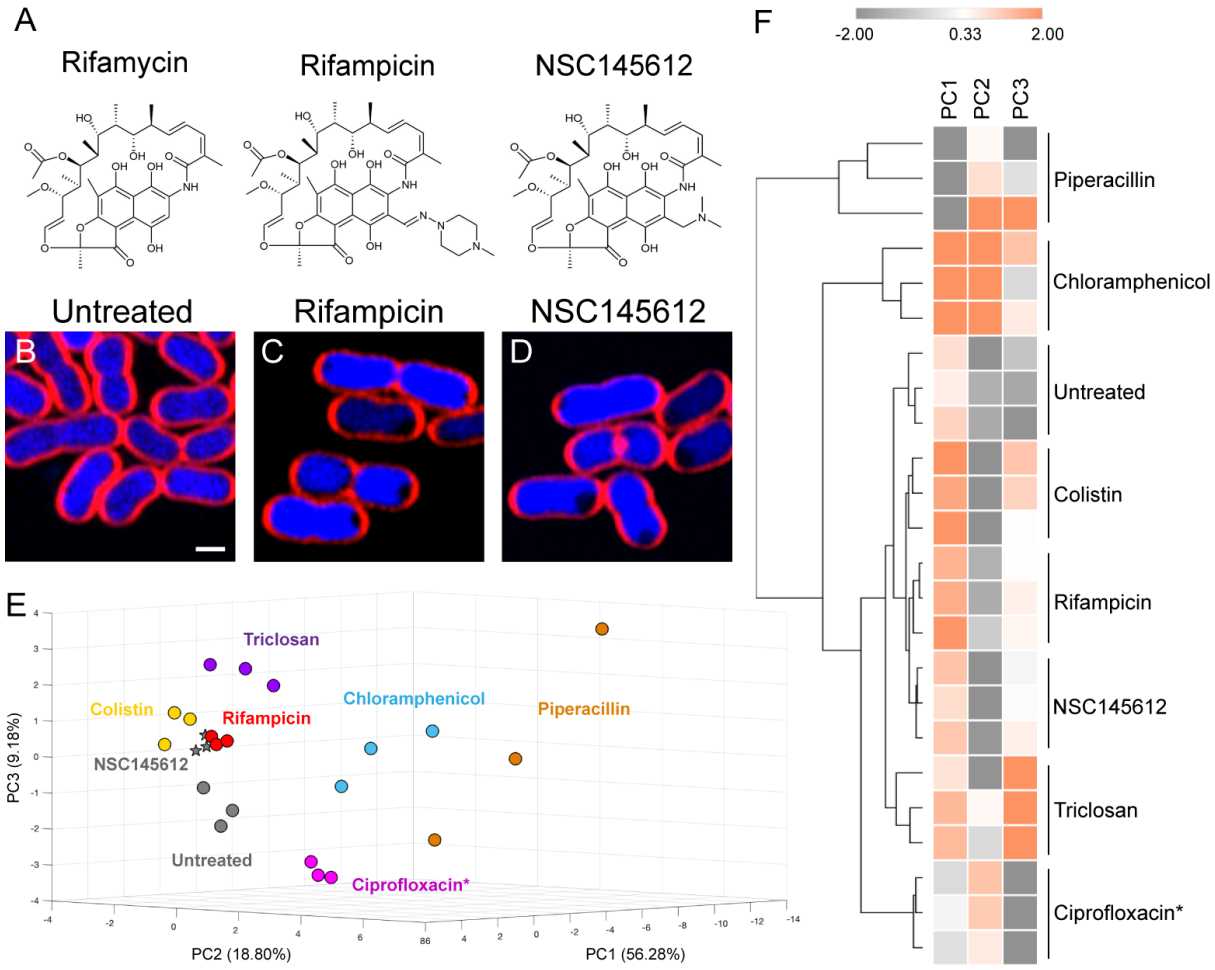

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