Identifying Mechanism of Action of Antibacterial Compounds Using Bacterial Cytological Profiling

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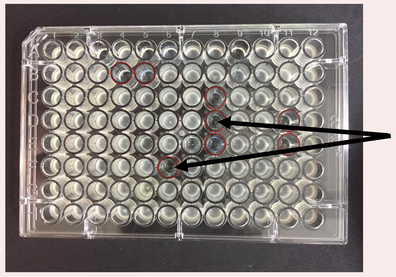
The extensive use of antibiotics has caused many pathogenic bacteria to develop resistance. This calls for not only new antibacterial compounds, but also compounds that have a unique mechanism of action (MOA). Unfortunately, while identifying antibacterial activity is fairly straightforward, determining the MOA of those compounds is not. In order to simplify and accelerate this process, we used bacterial cytological profiling (BCP). Through the use of fluorescence microscopy, BCP aids in the identification of MOA through analysis of cellular phenotypic changes upon treatment with compounds of interest. A series of experiments were performed to identify which compounds could kill or inhibit growth of a sensitized strain of *E. coli* with a deletion of the TolC efflux pump. We screened 576 synthetic compounds from a ChemBridge library to determine whether they would kill this strain as well as their minimal inhibitory concentration (MIC). After being treated with various concentrations of our identified compounds, the bacteria were examined using BCP to identify the MOA. Additionally, we isolated bacteria from soil samples collected around the UCSD campus and screened them for the production of antimicrobial compounds.

Kill Assays

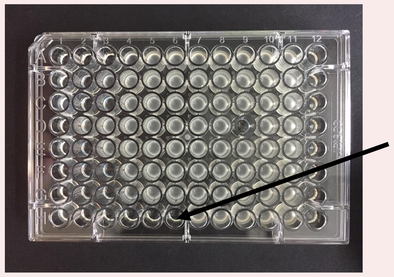
Kill assays were used to identify whether or not the antibiotic at the highest concentration provided by ChemBridge would kill a sensitized strain of *E. coli*, AD3644. One 96-well plate was prepared by filling each well with 100 µL of LB media. Using a multichannel pipette, 2 µL from each well in the compound plate was pipetted into its corresponding well in the media-filled 96-well plate. Then, 1 µL of a 0.05 OD bacterial culture was pipetted into each well. After incubating at 30°C with shaking for 24 hours, the wells with no bacterial growth were noted for further analysis.



We found a total of 52 compounds that killed AD3644 at their highest concentrations. The kill assay above shows several compounds that killed, based on the number of clear wells with no bacterial growth.

MIC lates

The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the compound that is still effective. We filled a 96-well plate with 100 µL of LB, except the first column, which was filled with 200 µL. Then we took 8 of the successful compounds at a time and pipetted 4 µL of the compound into a well in the first column of a 96-well plate. The compounds were then serially diluted at a 1:1 ratio, leaving columns for positive and negative controls. After adding 1µL of bacteria, we then incubated the plates overnight at 30°C with rolling.



Serial dilutions down the plate go from 100 µg/mL to 0.195 µg/mL. We found that the MICs of the compounds range from 50µg/mL to 3.125µg/mL.

Acknowledgements

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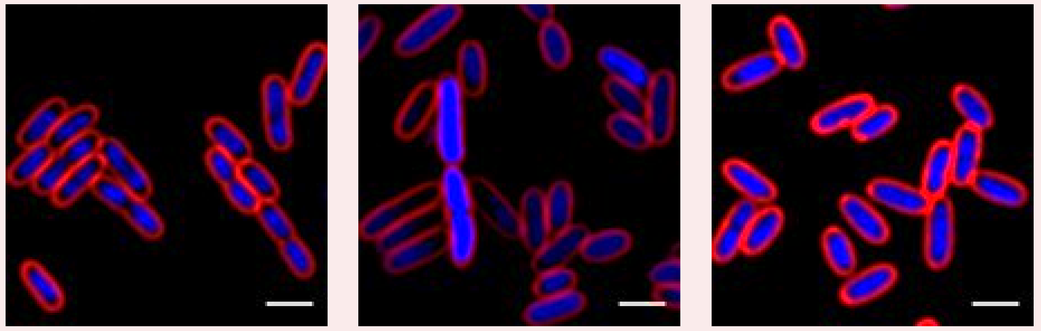
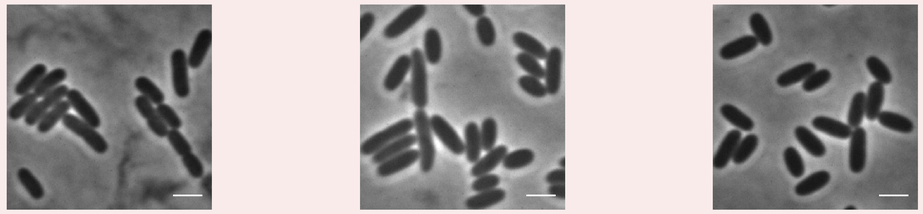
Microscopy

BCP helps identify the MOA, or how the antibiotic hinders the growth of the bacteria. Depending on the MIC of each tested compound, we took either 1x, 2x, or 5x times the MIC to ensure that we could witness the effects of the antibiotics on the cells during microscopy. We mixed three dyes into the bacteria: FM 4-64, a red dye that stains the cell membranes; DAPI, a blue dye that stains DNA; and SYTOX Green, which also stains DNA, but can only enter the cell when the membrane of the cells has been compromised. Therefore, SYTOX Green's presence in the cell is taken as an indicator of cell lysis.



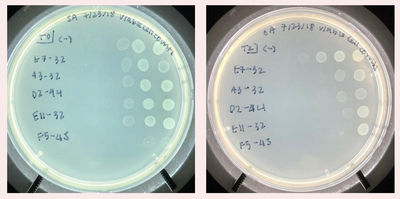
DMSO Control | Well E7 | Well F5



The different phenotypes of the treated bacteria give us clues as to what part of the cell the antibiotic targets. For example, well E7 shows bacteria with decondensed DNA that fills the entire cell. This implies that the antibiotic inhibits the bacteria's ability for RNA transcription. Small growths off of the cell's membrane could point to disruption of normal membrane development or energy production, as we can see in well F5.

Viable Cell Counts

Viable cell counts identify whether the tested compound kills all bacteria or simply slows their growth. We prepared 2 96-well plates for 4 timepoints, T0, T1, T2, and T4hr. In several test tubes, we added the cells into DMSO, as a control, or the compounds to be tested. In the first column, we pipetted 50 µL of the cell culture from the test tubes. The tubes were then placed back in the incubator at 30°C with rolling until the next timepoint. After adding T-base to the wells, we serially diluted them at a 1:10 ratio and spotted 5 uL on an LB agar plates. This was repeated for all four timepoints. After the bacteria colonies were grown, we counted visible single colonies per dilution and recorded them in a table.



Treatment	conc (µg/ml)	0	1	2	4
DMSO Control	--	0.0	0.8	1.7	1.9
Tetracycline 5X	1.823	0.5	No growth	No growth	No growth
E7-32 5x	31.25	-0.7	0.5	0.3	0.8
A3-32 2x	100	0.2	-1.3	-3.7	No growth
E11-32 5x	62.5	No growth	0.5	0.3	0.5
F5-45 5x	125	No growth	No growth	No growth	No growth

The table above shows the ratio of viable cells to the control for each compound at a certain hour . "No growth" indicates that the compound killed all cells.

Soil Samples

We collected soil from the UCSD campus and streaked it out on AIA plates supplemented with rifampicin and cycloheximide to prevent the growth of *Bacillus sp.*, bacteria commonly found in soil, and fungus. Once single colonies were grown, we streaked them again onto an LB plate and an AIA plate without antibiotics and incubated overnight at 30°C. 10µL of AD3644 and *B. subtilis* PY79 were spotted next to the streaked bacteria and incubated overnight at 30°C.

Zones of inhibition were measured if applicable.

In the image above, we can see that the soil bacteria grown on LB produced compounds that inhibited both PY79 and AD3644. On the AIA plate, PY79 was inhibited while AD3644 was not.

Conclusions

Bacteria are constantly developing resistance to antibiotics, and the need for uncommon MOAs is rising. Through kill assays, MICs, microscopy, and viable cell counts, we identified the compounds with antibiotic properties and the cellular pathways they target. 52 compounds out of total screened compounds managed to kill the sensitized *E. coli* strain, approximately a 10% success rate. We found several compounds that had relatively low MICs, ranging between 12.5 and 3.125 µg/mL. Additionally, we were able to identify the MOAs of several compounds that were visible using BCP, such as DNA and RNA synthesis inhibition. We were also able to identify whether the compounds of interest were bacteriostatic or bactericidal. Through soil samples, we were able to isolate a bacteria that produced compounds with antibiotic properties, and successfully test it against both *E. coli* and *B. subtilis*.

In the future, we would like to identify compound analogs for compounds H10 (not pictured) and E7 and screen them for their activity . For compounds that had low MICs, we would like to look into more into them by testing them on wild strains of *E. coli* or other pathogenic bacteria. As for the soil bacteria, we would like to extract their DNA for sequencing to identify what genus they belong to.

References

Nonejuie, P., Burkart, M., Pogliano, K. and Pogliano, J., 2013. Bacterial cytological profiling rapidly identifies the cellular pathways targeted by antibacterial molecules. ProcNatlAcadSciUSA **110:**16169-16174.