Combinations of Previously-Approved Drugs Targeting *Pseudomonas aeruginosa* Development

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Alexander Leung is a third year student in the Biomedical Sciences Specialization of the Bachelor of Health Sciences (Honours) Program. Under the supervision of Dr. Eric Brown, Alexander has been searching for novel therapies against the ubiquitous opportunistic pathogen, Pseudomonas aeruginosa. Herein, he introduces the bacterium and provides a primer on antibiotics with a particular focus on those used to combat P. aeruginosa infections. The focus of his research, conducted at the High Throughput Screening Laboratory in McMaster's Centre for Microbial Chemical Biology, is to utilize high throughput screening to identify compounds that synergize with known antibiotics.

Multidrug resistant bacteria remain an under-recognized epidemic.[1] Indeed, the top three infectious diseases (septicemia, influenza and pneumonia) together account for over 100,000 deaths per year in the United States.[2] In light of increasing antibiotic resistance in bacteria[3] and the emergence of multidrug resistant pathogens like *Pseudomonas aeruginosa*,[1,4] the need for new antibiotics is obvious. However, with reduced interest from pharmaceutical companies and the decade-long process of securing drug regulatory approval, the antibiotic pipeline has run dry.[1,3]

Antibiotics are toxic compounds which kill or perturb the growth of bacteria but not humans. This is achieved by targeting essential physiological and biochemical processes that are unique to bacteria. In general, the different classes of antibiotics affect five major targets: the bacterial cell wall, the cell membrane, protein synthesis, DNA and RNA synthesis, and folic acid metabolism. For example, penicillins, cephalosporins and carbapenems are classified as β-lactam antibiotics, which kill by disrupting synthesis of the bacterial cell wall.[5] Resistance to antibiotics is a natural bacterial phenomenon that results from the evolutionary selective pressure that comes hand in hand with continued exposure to such compounds. Bacteria achieve antibiotic resistance through four general mechanisms: target modification, efflux, immunity and bypass, and enzyme-catalyzed destruction.[5] Hence, the use of antibiotics paradoxically accelerates its disuse.[1,3] For instance, *P. aeruginosa* possess numerous families of efflux pumps with overlapping substrate ranges that effectively pump out antibiotics. In total, with insensitivity to almost all commercially available antibiotics, P. aeruginosa is a particularly worrisome opportunistic pathogen.[1,6,7] Indeed, *P. aeruginosa* lung infection is a common nosocomial infection and a major cause of morbidity and mortality in cystic fibrosis patients.[8] Pan-resistant strains of *P. aeruginosa* are becoming increasingly common in both the clinic and community, and the health hazard it presents is exacerbated by the lack of new antibiotics that would otherwise have the potential to treat such infections for the next decade.[1,6]

Treatment of *P. aeruginosa* infection in cystic fibrosis patients often consists of combinations of antibiotics, such as oral ciprofloxacin with inhaled tobramycin or colistin.[9] The rationale for the use of antibiotic combinations is based largely on empirical success in therapy, particularly in preventing the emergence of antibiotic resistance. With that, fresh approaches towards identifying antibacterial combinations beyond traditional antibiotic combinations are worth investigating. For example, it is possible that combining non-antibiotic drugs with traditional antibiotics may produce a synergistic effect that is greater than the antibiotic alone. Previously approved drugs are also have the benefit well characterized pharmacology and toxicology profiles, which greatly accelerates drug development timelines. Therefore, the strategy of discovering approved non-antibiotics which can augment the activity of conventional antibiotics against *P. aeruginosa* is a practical approach in addressing the shortage of new treatments for multidrug resistant infections.

HIGH THROUGHPUT SCREENING AND THE SEARCH FOR NOVEL THERAPIES AGAINST *P. AERUGINOSA*

To rapidly generate the desired antibiotic/non-antibiotic combinations, a process known as screening was employed. First, a diverse library of 2080 FDA-approved drugs (FAD) was assembled and 9 antibiotics (See Table 1), some of which are currently prescribed to treat P. aeruginosa infections in cystic fibrosis patients, were selected.[10] Using robots and liquid handling devices, aliquots of FAD compounds and antibiotic were dispensed into assay plates. This process was controlled by using custom protocols at the High Throughput Screening Laboratory in McMaster's Centre for Microbial Chemical Biology. A fixed amount of P. aeruginosa (strain PA01) was then added into the assay plates and allowed to incubate overnight. A FAD compound would be identified as a positive result or "hit" if in combination with an antibiotic there was significant planktonic growth inhibition of P. aeruginosa after overnight incubation.

Screening the FAD library with 9 different antibiotics resulted in 607 unique hits for *P. aeruginosa* out of a possible 18720 (2080 FADs x 9 antibiotics) combinations. The list of hits was ranked

Antibiotic	# of hits (out of 2080 per screen)	# of uninteresting compounds	# of Hits - # of uninteresting molecules
Ceftazidime	47	47	0
Cefuroxime	66	61	5
Ciprofloxacin	63	54	9
Clarithryomycin	52	44	8
Erythromycin	63	58	5
Meropenem	105	95	10
Piperacillin	83	66	17
Tetracycline	79	75	4
Tobramycin	49	48	1
Total	**607**	**548**	**59** (48 unique compounds)

TABLE 1: Summary statistics of the 9 antibiotic screens performed. Compounds that were uninteresting (i.e. known antimicrobial compounds)were excluded from further analysis.

according to their magnitude of effect on the viability of *P. aeruginosa*, and filtered for known antibacterial agents and other uninteresting compounds. In total, 48 unique and unexpected FADs that potentiated growth inhibition of *P. aeruginosa* were identified, of which 7 were selected based on immediate availability and function for further experimentation (See Table 2).

The study then focused on 2 non-antibiotic FADs, 2-aminoheptane (2AH) and ticlopidine. Both compounds were observed to increase the antibacterial activities of certain known antibiotics, an effect known as synergy. This synergy was validated by growth recovery and other experiments which demonstrated growth inhibition of PA01 in combination with sub-lethal concentrations of antibiotics (See Figure 1). However, the synergy was limited to *P. aeruginosa*. Ticlopidine, an ADP receptor agonist used to decrease platelet aggregation and thrombotic events,[11] possessed anti-Pseudomonal activity in combination with cefuroxime, a 2nd generation cephalosporin which *P. aeruginosa* is clinically resistant.[12] Interestingly, 2AH, a nasal decongestant and vasoconstrictor[13] synergized with both ciprofloxacin and tetracycline, antibiotics which affect bacterial viability by inhibiting DNA and protein synthesis respectively.[5] Furthermore, *P. aeruginosa* is resistant to tetracycline.[14] The current results provide ample room for future exploration of the in vitro efficacy of the listed combinations. These include identifying the mechanism of action of the combination with a genome-scale collection of Escherichia coli deletion strains,[15] and further investigating the synergy of the 46 other FADs which were filtered out during analysis. From there, in vivo activity of drug combinations should also be tested in mouse models. The screening could also be expanded to include additional antibiotics and FADs which are not presently included.

Altogether, the systematic combination of FADs and antibiotics may prove to be useful in the search for new drug regimens against *P. aeruginosa*. By combining FADs and antibiotics in their screening process, researchers achieve a level of diversity that far exceeds the range of compounds explored by even the biggest

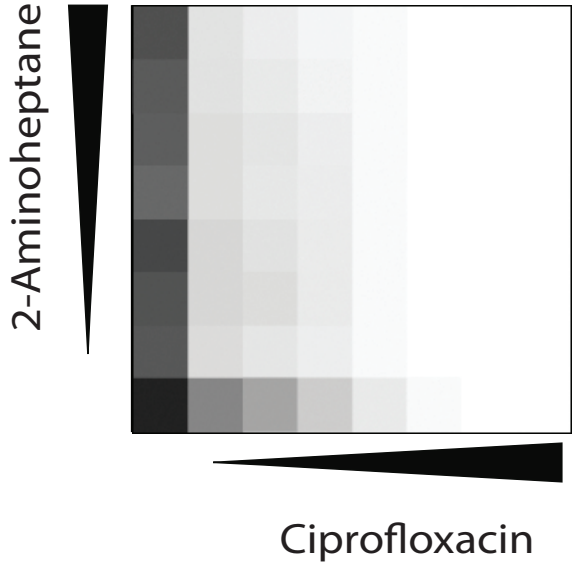

FIGURE 1: Representative checkerboard from a synergy experiment. The map shows the combined effect of 2-aminoheptane and ciprofloxacin in an 8x8 matrix. Each square representsadifferent concentration of 2-aminoheptane and ciprofloxacin(including a zero concentration point, bottom-left dark blue square) each. The absence of color in asquare indicates a higher level of growth inhibition.

pharmceutical companies in their search for single agents.[1] Since the pharmacology and toxicology profiles of FADs have been well characterized, the unfavourable economics associated with antibiotic development can be ameliorated.[1,3,5] The surprising combinations of drugs that were identified can potentially give rise to novel multi-component therapies in the treatment of diseases like cystic fibrosis. ▪

Compound	Antibiotic	Function of Compound
2-Aminoheptane	Ciprofloxacin	Nasal decongestant
2-Aminoheptane	Tetracycline	Nasal decongestant
Biperiden	Clarithromycin	Antiparkinson
Fipexide	Clarithromycin	Psychoactive drug
Lidoflazine	Clarithromycin	Calcium channel blocker
Simvastatin	Cefuroxime	Statin
Ticlopidine	Piperacillin	Antiplatelet drug, ADP receptor inhibitor
Ticlopidine	Cefuroxime	Antiplatelet drug, ADP receptor inhibitor
Ursolic Acid	Cefuroxime	STAT3 inactivation, anti-cancer

TABLE 2: Summary of compounds identified as hits after screening and selected for further investigation to identify potential antimicrobial activity.

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Reviewed by Maya Farha

Maya Farha is a Ph.D. candidate under the supervision of Dr. Eric Brown at McMaster University in the Department of Biochemistry and Biomedical Sciences. She is currently studying chemical-chemical combinations as tools to gain biological insight and as routes to drug discovery.

REFERENCES

[1]Spellberg, B. Rising Plague. Amherst, New York: Prometheus Books, 2009.

[2]Xu J, Kockanek KD, Murphy SL, Tejada-Vera BS. National Vital Statistics Reports: Deaths –Final Data for 2007, National Center for Health Statistics 2010. http://www.cdc.gov/NCHS/data/nvsr/nvsr58/nvsr58_19.pdf (accessed December 31, 2010)

[3]Wright GD. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biol. 2010 Sep 20;8:123.

[4]Arias CA, Murray BE. Antibiotic-resistant bugs in the 21st century--a clinical super-challenge. N Engl J Med 2009 Jan 29;360(5):439-43.

[5]Walsh C. Antibiotics. Washington, D.C.: American Society for Microbiology Press: 2003.

[6]Paterson DL. The epidemiological profile of infections with multidrug-resistant Pseudomonas aeruginosa and Acinetobacter species. Clin Infect Dis. 2006 Sep 1;43 Suppl 2:S43-8.

[7]Spellberg B. Antibiotic resistance and antibiotic development. Lancet Infect Dis. 2008 Apr;8(4):211-2;2-4.

[8]Salyers AA, Whitt DD. Bacterial pathogenesis: a molecular approach. Washington, D.C.: American Society for Microbiology Press: 1994.

[9]Bilton D, Henig N, Morrissey B, Gotfried M. Addition of inhaled tobramycin to ciprofloxacin for acute exacerbations of Pseudomonas aeruginosa infection in adult bronchiectasis. Chest. 2006 Nov;130(5):1503-10.

[10]Doring G, Conway SP, Heijerman HG, Hodson ME, Hoiby N, Smyth A, et al. Antibiotic therapy against Pseudomonas aeruginosa in cystic fibrosis: a European consensus. Eur Respir J. 2000 Oct;16(4):749-67.

[11]Hankey GJ, Sudlow CL, Dunbabin DW. Thienopyridine derivatives (ticlopidine, clopidogrel) versus aspirin for preventing stroke and other serious vascular events in high vascular risk patients. Cochrane Database Syst Rev. 2000(2):CD001246.

[12]Fischbach MA, Walsh CT. Antibiotics for emerging pathogens. Science. 2009 Aug 28;325(5944):1089-93.

[13]Proetz AW. Certain aliphatic compounds as nasal vasoconstrictors. Arch. Otolaryngol. 1943 Jan;37(1):15-22.

[14]Tseng JT, Bryan LE. Mechanisms of R factor R931 and chromosomal tetracycline resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 1973 May;3(5):638-41.

[15]Liu A, Tran L, Becket E, Lee K, Chinn L, Park E, et al. Antibiotic sensitivity profiles determined with an Escherichia coli gene knockout collection: generating an antibiotic bar code. Antimicrob Agents Chemother. 2010 Apr;54(4):1393-403.