Drug Repurposing for Antimicrobial Development: 
Teaching Old Drugs New Tricks

Learning Objectives
1. Describe major barriers of the traditional drug discovery/development process
2. Identify potential advantages that drug repurposing may have over traditional drug discovery
3. List examples of drugs that have been successfully repurposed
4. Examine the antimicrobial properties of sertraline

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Drug Discovery and Development

1. Review of Process
   a. Drug Discovery (3-6 years)
      i. Target identification
         • Finding a target that causes or leads to a condition/disease
         • Usually receptors or enzymes
      ii. Target validation
         • Validate to confirm functions and effects of the target
         • May involve knockout models, protein/gene expression profiles, etc.
      iii. Compound screening (~10,000) - screen target with thousands of compounds
      iv. Lead optimization
         • Chemically modify selected compound(s) to optimize pharmacokinetic/pharmacodynamic properties
         • Oral drug candidates are often gauged using Lipinski’s “rule of five” (Figure 1)
            a. A set of drug-design criteria
            b. Associated with lower attrition rate
   b. Preclinical (1-2 years)
      i. Animal modeling
      ii. Determine absorption, distribution, metabolism, excretion, toxicity (ADMET)
      iii. Investigational new drug application
   c. Clinical trials - Phase I through IV (5-6 years) (Table 1)
   d. Food and Drug Administration (FDA) review
      i. Generally 1-2 years, unless expedited FDA review (See Appendix A)

<table>
<thead>
<tr>
<th>Clinical Trial</th>
<th>Patients &amp; Duration</th>
<th>Purpose</th>
<th>Approximate % Success in Each Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I</td>
<td>20–100, several months</td>
<td>Test safety of a single dose (Phase Ia) and multiple doses (Phase Ib); includes maximum tolerated dose</td>
<td>70%</td>
</tr>
<tr>
<td>Phase II</td>
<td>100–300, months to 2 years</td>
<td>Assess efficacy and side effects</td>
<td>33%</td>
</tr>
<tr>
<td>Phase III</td>
<td>300–3,000, 1-4 years</td>
<td>Assess safety and monitoring of adverse reactions</td>
<td>25-30%</td>
</tr>
<tr>
<td>Phase IV</td>
<td>Varies</td>
<td>Post-marketing surveillance for continued monitoring of safety and efficacy</td>
<td>N/A</td>
</tr>
</tbody>
</table>

2. Current Barriers/Challenges in Drug Development
   a. Movement of a new compound from initial discovery to market launch is slow and costly:
      i. Average duration ≈ 10-15 years
      ii. Minimum cost of ~ $2 billion
   b. High attrition = low probability of success
      i. Only approximately one in 10,000 compounds screened will make it into the market
      ii. Only ~10% of drugs entering clinical trials will get FDA approval
c. Industry productivity gap
   i. Increased investment in research and development expenditures has *NOT* improved efficiency
   ii. Fewer drugs approved in 2016 than in recent years (Figure 2)

**Figure 2. Novel FDA Approvals since 1993**

![Graph showing novel FDA approvals from 1993 to 2016.](image)

NMEs = new molecular entity, BLAs = biologics license applications

**IMPACT ⇒ Lower productivity ⇒ Less treatments regimens available on the market**

**Antibiotic Development: Past and Present**
1. Antibiotic resistance has evolved into a global health crisis - new antibiotics are urgently needed.
   a. Increasing prevalence of multidrug resistant pathogens
   b. Without the development of more effective antibiotics, experts warn that a ‘post-antibiotic era’ is near\(^9\), \(^10\)
2. 40-year innovation gap (Figure 3)
   a. Despite rapid progression in science and technology (over the past several decades), efforts to bring new, novel antibiotics to market have been unsuccessful
   b. Not since the “golden age” of antibiotics, from 1940 to 1960, has the antibiotic pipeline thrived\(^11\)
   c. Expectations were high for more fruitful discoveries, given the advancements in medicinal chemistry, molecular biology, and arrival of genomics, but this led to disappointing results
   d. After discovery of nalidixic acid in 1962, no new structural classes of antibiotics were developed until linezolid in 2000.\(^12\), \(^13\) Antibiotic development has since been sparse
3. Major factors that led to the 40-year innovation gap\textsuperscript{11, 14, 15}
   a. Perception that the war on antibiotic resistance had been decisively “won.” The concept that bacterial resistance is inevitable under selective pressure was not appreciated at the time
   b. Focus shifted from novel discovery to improving pharmacological properties of existing compounds ⇒ the Golden Age of medicinal chemistry
   c. Adherence to “rule-based” drug design (Lipinski)
   d. Pharma retreat from antibiotic development programs due to low return on investment
   e. Screening approaches: molecular, target-based versus whole-cell phenotypic\textsuperscript{16-18}
      i. Much of the success during the ‘golden age’ was due to empiric whole-cell screening, where they progressed through clinical trials with little or no knowledge of mechanism of action or target.
      ii. Following arrival of genomics, bioinformatics, and high-throughput technologies, drug discovery efforts focused on molecular, target-based approaches, but this has not led to more drug approvals
      iii. Between 1999 and 2008, 28 of the first-in-class small molecule drugs approved by FDA were discovered by phenotypic screening, compared to 17 discovered by a molecular target-based approach\textsuperscript{19}

**Drug Repurposing: An Alternative Pathway**

1. Refers to the identification of new uses for existing drugs, candidates under development, or abandoned compounds/failed compounds\textsuperscript{20}
   a. Often used interchangeably with ‘drug repositioning’
   b. Other (similar) terms include:
      i. reprofiling
      ii. redirecting
      iii. redeployment
      iv. rediscovery
      v. drug rescue
   c. Some investigators have called for more standardized definitions and terminology in order to avoid confusion and misinterpretations of published works in the field\textsuperscript{21}
   d. Ideal candidate for repurposing = a drug that has passed safety and tolerability studies
e. Advantages of drug repurposing\(^3, 22-24\)
   i. Reduce development costs
   ii. Increase efficiency (faster time to approval)
   iii. Reduce investment risk (for compounds that have cleared Phase-I)
   iv. Other potential uses: diagnostic tool, biomarker, etc.

f. Repurposing commercially available drugs
   i. Example = Gabapentin. Originally indicated for seizure control, but was also found to provide relief for postherpetic neuralgia\(^25\)
   ii. Additional examples provided in Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Initial Approved Indication</th>
<th>Added Indication(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amantadine</td>
<td>Influenza</td>
<td>Parkinson’s</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Fungal infections</td>
<td>Leishmaniosis</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Inflammation, pain</td>
<td>Antiplatelet</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>Depression</td>
<td>Fibromyalgia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diabetic neuropathy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chronic musculoskeletal pain</td>
</tr>
<tr>
<td>Everolimus</td>
<td>Advanced kidney cancer</td>
<td>Prevention of organ rejection</td>
</tr>
<tr>
<td>Finasteride</td>
<td>Prostate hyperplasia</td>
<td>Hair loss</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>Epilepsy</td>
<td>Postherpetic Neuralgia</td>
</tr>
<tr>
<td>Infliximab</td>
<td>Crohn’s disease</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Severe plaque psoriasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>Minoxidil</td>
<td>Hypertension</td>
<td>Hair loss</td>
</tr>
</tbody>
</table>

a. ‘Rescued’ medications (repurposing drugs that had failed and/or been abandoned)\(^26\)
   iii. Thalidomide is a notable example of reviving a discontinued (and once disastrous) drug. Originally marketed in 1957 to pregnant women for morning sickness, it was found to have devastating teratogenic effects, causing more than 10,000 birth defects. \(^27, 28\)
   iv. Additional examples provided in Appendix B

Non-Antibiotic Drugs and Antibiotic Adjuvants
1. Non-antibiotics = drugs used (or under development) for non-infectious indications that may possess antimicrobial properties\(^29\)
2. Several drug classes have been known to demonstrate variable \textit{in vitro} antibiotic activity: \(^30-34\)
   a. Antipsychotic (i.e. selective serotonin reuptake inhibitors)
b. Cardiovascular (i.e. calcium channel blockers)
c. Antihistamines
d. Antihelmintics
e. Non-steroidal anti-inflammatory agents
f. Nucleoside and nucleobase analogues

3. Two general ways non-antibiotic drugs demonstrate *in vitro* activity:
   a. Directly as monotherapy
   b. Indirectly, in combination regimen, as 'helper' antibiotics or adjuvant antibiotics

   i. **Adjuvant antibiotics** – Examples of anti-virulence and anti-resistance mechanisms of select non-antibiotic drugsantibiotic potentiating activity are shown in Figure 4.

**Figure 4. Antibiotic Adjuvants Mechanisms**

Examining Sertraline as a Non-Antibiotic:
   a. Selective serotonin reuptake inhibitor, approved 1991
   b. Antifungal activity discovered serendipitously

   c. *Candida spp.*
      i. Sertraline was rapidly for all four *Candida spp*: *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis*
      ii. Sertraline minimal fungicidal concentrations (MFCs) (3 – 29 µg/ml) exceeded average steady-state plasma levels (55 – 250 ng/ml)

   d. *Aspergillus spp.*
      i. Sertraline *in vitro* activity was compared to fungicidal activity of other SSRIIs (paroxetine, fluoxetine, citalopram)
      ii. Sertraline and fluoxetine showed the greatest fungicidal activity
      iii. Minimum fungicidal concentrations (MFCs) exceeded average steady-state plasma levels
e. *Cryptococcus* spp.\(^{38}\)
   i. Sertraline is discovered to have potent activity, and was synergistic with fluconazole, against *Cryptococcus* spp.
   ii. Reduced the fungal burden alone or in combination with fluconazole in murine model

f. Adjunctive Sertraline for Treatment of Cryptococcal Meningitis in HIV patients\(^{39}\)
   i. Phase I/II
      - Open-label, dose-escalation trial of adjunctive sertraline (up to 400mg/day) for treatment of HIV-associated cryptococcal meningitis in Uganda
      - Primary outcome: clearance of *Cryptococcus* from cerebrospinal fluid (CSF) at two weeks, measured in colony forming units (CFU)/ml.
      - Secondary endpoints: incidence of paradoxical immune reconstitution inflammatory syndrome (IRIS), culture-positive relapse, and safety
      - Enrollment total = 172 participants
         - The first 60 patients received escalating sertraline doses of 100mg up to 400mg/day for 2 weeks
         - 112 participants were randomly assigned to three sertraline doses (200mg, 300mg, or 400mg) for the first 2 weeks, followed by a consolidation dose for 8 weeks.
      - Duration = 12 weeks
      - Number of patients per sertraline dose: 17 (100mg), 60 (200mg), 50 (300mg), 45 (400mg)
      - 2- and 12-week mortality was 22% (38/172) and 40% (69/172), respectively
      - Patients on any sertraline dose had a mean CSF clearance rate of -0.37 CFU/ml per day (95% CI -0.41 to -0.33), but there were no differences between doses (Figure 5)
      - No relapses occurred during the study period
      - No difference in grade 4 or 5 adverse event risk between lower doses of 100–200 mg/day and higher doses of 300–400 mg/day (hazard ratio 1.27, 95% CI 0.69–2.32; p=0.45).

**Figure 5. Rate of CSF Clearance by Sertraline Dose**

- The MIC for sertraline ranged from 1 to 8 \(\mu\)g/ml. Median sertraline plasma concentrations, quantified in certain participants, were 399 ng/ml (278 – 560; \(n = 30\)) for 400mg doses.
• Figure 6 shows the proportion of people achieving therapeutic brain sertraline concentrations based on susceptibility and dose. Sertraline levels have been shown to be up to 20 times higher in brain than in plasma.\textsuperscript{40}

**Figure 6. Probability of Achieving Therapeutic Sertraline Brain Tissue Levels**

![Probability of Achieving Therapeutic Sertraline Brain Tissue Levels](image)

• Conclusion: Given its fungicidal properties, safety profile, lack of relevant drug interactions, low cost, and brain penetration, sertraline appears to be a promising adjunctive agent for treatment of cryptococcal meningitis

ii. **Phase III**
- Randomized controlled trial to determine whether the addition of sertraline to standard amphotericin-based treatment improves survival for HIV-associated cryptococcal meningitis
- Outcomes will be evaluated for standard amphotericin-based treatment with and without sertraline 400mg (Table 4)
- Primary outcome: survival at 18 weeks
- Anticipated enrollment = 550 patients
- Currently recruiting participants
  - Updates available at [https://clinicaltrials.gov/ct2/home](https://clinicaltrials.gov/ct2/home)
  - Clinicaltrials.gov identifier: NCT01802385

**Table 3. Phase III – Use of Sertraline as Adjunct Therapy for Cryptococcal Meningitis**

<table>
<thead>
<tr>
<th>Placebo Comparator: Placebo</th>
<th>Experimental: Sertraline 400mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard cryptococcal meningitis therapy: amphotericin (0.7-1.0 mg/kg/day) + fluconazole (800-1200mg/day).</td>
<td>Standard cryptococcal meningitis therapy: amphotericin (0.7-1.0 mg/kg/day) + fluconazole (800-1200mg/day) PLUS <strong>sertraline 400</strong> mg/day x 2 weeks, then 200mg x 12 week, then tapered over 3 weeks.</td>
</tr>
</tbody>
</table>
Examination of Non-Antibiotic Drugs against *Staphylococcus aureus* Using Novel Screening Assays

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I. Study Objective: The objective of the proposed research is to utilize novel target-based whole cell screening tools to characterize antibiotic effects of select non-antibiotic drugs against *Staphylococcus aureus* clinical isolates.

II. Central hypothesis: One or more of the selected non-antibiotic drugs will demonstrate antibiotic effects against *S. aureus* either directly (as monotherapy) or indirectly (in combination with antibiotic drugs).

III. Specific Aims
   a. Specific Aim 1: Evaluate the *in vitro* antibiotic activity of non-antibiotic drugs against *S. aureus* clinical strains with known resistance genes.

      Objective: Determine MICs and MBCs for selected non-antibiotic drugs (amlodipine, azelastine, ebselen, and sertraline) against *S. aureus* using microplate alamar blue assays.

   b. Specific Aim 2: Evaluate the antibiotic-potentiating activity of non-antibiotic drugs against *S. aureus* clinical strains with known resistance genes.

      Objective 2.1: Determine if non-antibiotic drugs (amlodipine, azelastine, ebselen, and sertraline) potentiate ciprofloxacin or tetracycline antibiotic activity using checkerboard titration assays.

      Objective 2.2: Screen antibiotic-potentiating combinations using an efflux modulation assay and ethidium bromide cartwheel method.

IV. Aim 1 Methods & Rationale:
   a. Non-antibiotic drugs (Table 4)
      Amlodipine, azelastine, ebselen, and sertraline were selected as our test compounds based on several criteria.
      - Proven safety and tolerability in humans. Amlodipine, azelastine, and sertraline have been shown to be safe, tolerable, and each have FDA-approved indications. Ebselen is not currently FDA-approved, but has been shown to be safe in humans.¹¹
      - Drugs for which antibiotic activity has been demonstrated previously, but with limited data.
• Drugs that represented various therapeutic classes.
• Apart from ebselen, we also chose drugs that have been shown to inhibit P-glycoprotein efflux pumps, as this characteristic has been correlated with intrinsic antibiotic activity.42-44
• Note that our range of concentrations for susceptibility testing exceeds average plasma concentrations. These were chosen, in part, based on previous in vitro studies.38, 39, 45-49 Additional factors such as route of administration, site of infection, and drug concentration at tissue sites were also considered.

Table 4. Selected Non-Antibiotic Drugs

<table>
<thead>
<tr>
<th>Generic drug name</th>
<th>Drug class or primary indication</th>
<th>FDA approved</th>
<th>Mean peak plasma concentrations</th>
<th>Concentrations for MIC determination (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amlodipine</td>
<td>Ca+ channel blocker</td>
<td>Yes</td>
<td>6-14 ng/ml</td>
<td>12.5 - 256</td>
</tr>
<tr>
<td>Azelastine</td>
<td>Antihistamine</td>
<td>Yes</td>
<td>200 pg/ml</td>
<td>12.5 - 400</td>
</tr>
<tr>
<td>Ebselen</td>
<td>Antioxidant</td>
<td>No</td>
<td>N/A</td>
<td>0.25 - 8</td>
</tr>
<tr>
<td>Sertraline</td>
<td>SSRI</td>
<td>Yes</td>
<td>100 - 500 ng/ml</td>
<td>2.5 - 160</td>
</tr>
</tbody>
</table>

SSRI = selective serotonin reuptake inhibitor

b. Bacterial isolates
Non-antibiotic MICs and MBCs were determined for five clinical isolates (Table 5) and one quality control strain (S. aureus 29213). Each isolate is genetically and phenotypically distinct from one another.
• Clinical isolates were selected from previous clinical and epidemiological studies by our research group.50-52
• Isolates were obtained from wound swab cultures of patients with skin and soft tissue infections (SSTIs) who were seen at primary care clinics in the South Texas Ambulatory Research Network (STARNet) from 2010 to 2013.
• Our group has already identified resistance genes and determined susceptibilities in these isolates using whole genome sequencing and Vitek 2 automated testing system (bioMerieux, Durham, NC), respectively.52

Table 5. Whole Genomic Sequencing and Antibiotic Susceptibilities† for Clinical Isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Resistance Genes</th>
<th>OX</th>
<th>CLIN</th>
<th>ERY</th>
<th>GENT</th>
<th>TMP-SMX</th>
<th>DOX</th>
<th>TET</th>
<th>VAN</th>
<th>CIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1019</td>
<td>meca</td>
<td>≥ 4, R</td>
<td>≤ 0.25</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
<td>≤ 10</td>
<td>≤ 2</td>
<td>≤ 4</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>A32</td>
<td>meca, ermC, tetK, gyrA(S84L)</td>
<td>≥ 4, R</td>
<td>≥ 4, R</td>
<td>≥ 8, R</td>
<td>≤ 0.5</td>
<td>20</td>
<td>≤ 2</td>
<td>≥ 16, R</td>
<td>1</td>
<td>≥ 8, R</td>
</tr>
<tr>
<td>B60</td>
<td>gyrA(S84L), norA</td>
<td>≤ 0.5</td>
<td>≤ 0.25</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
<td>≤ 10</td>
<td>≤ 2</td>
<td>≤ 4</td>
<td>≤ 0.5</td>
<td>≥ 8, R</td>
</tr>
<tr>
<td>B72</td>
<td>ermC, tetK</td>
<td>≤ 0.5</td>
<td>≥ 4, R</td>
<td>≥ 8, R</td>
<td>≤ 0.5</td>
<td>&gt; 320, R</td>
<td>4</td>
<td>≥ 16, R</td>
<td>1</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>J1019</td>
<td>norA</td>
<td>≤ 0.5</td>
<td>≤ 0.25</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
<td>≤ 10</td>
<td>≤ 2</td>
<td>≤ 4</td>
<td>≤ 0.5</td>
<td>≤ 0.5</td>
</tr>
</tbody>
</table>

†From VITEK® 2 automated system; shaded areas indicate resistance; OX = oxacillin, CLIN = clindamycin, ERY = erythromycin, GENT = gentamicin, TMP-SMX = trimethoprim-sulfamethoxazole, DOX = doxycycline, TET = tetracycline, VAN = vancomycin, CIP = ciprofloxacin
c. Susceptibility testing

- **MICs.** Standard broth microdilution procedures were followed according to Clinical Laboratory and Standards Institute (CLSI) methods, but with the addition of alamar blue dye. This method follows standard broth microdilution procedures, but with the addition of alamar blue dye, which indicates bacterial growth by change in color (blue to pink) and fluorescence intensity.

  **Rationale for alamar blue.** The MIC measure itself, as a single inhibitory concentration, provides little insight about the antibiotic properties of a drug. Both rate and extent of bacterial killing (or inhibition) are defining characteristics of an antibiotic and are therefore critical to understand during early drug discovery. These additional endpoints can be captured by simply adding a growth indicator, such as alamar blue. Resazurin, the active ingredient in alamar blue, is a cell permeable non-toxic blue dye that undergoes an oxidation-reduction reaction after entering the cell, changing from non-fluorescent blue (oxidized form) to highly fluorescent, pink-colored resorufin (reduced form). The extent of this color conversion represents cell viability and can be assessed qualitatively, by visual inspection of color change, and quantitatively, with fluorometric readings.

- **MBCs.** Minimum bactericidal concentrations were determined according to CLSI procedures. After MICs were recorded, contents from wells for which there is no growth will be plated onto Mueller-Hinton agar plates and incubated 18-24 hours at 35 ± 2°C. MBCs were defined as the minimum drug concentration that results in a ≥ 99.9% reduction (≥ 3-log10) in CFU/ml from initial inoculum.

d. Fluorometric analysis.

- Fluorescence monitoring was included as an additional measure of antibiotic activity and was measured with Synergy HT Microplate Reader (excitation, 530 nm; emission 590 nm).
- Growth control wells represent 100% fluorescence, while Mueller-Hinton broth with alamar blue represents negative controls. The percent of bacterial growth inhibited by non-antibiotic drugs will be calculated with respect to the growth control. Percent (%) inhibition for a given drug concentration will be calculated as: \(1 - \left(\frac{\text{Fluorescence}_{\text{test drug}}}{\text{Fluorescence}_{\text{growth control}}}\right) \times 100\).

V. **Aim 2 Methods & Rationale (for antibiotic-potentiating activity):**

a. Antibiotics and reference efflux pump Inhibitor. Two antibiotics, ciprofloxacin and tetracycline, and a reference efflux pump inhibitor, reserpine, will be included in this study.

  **Rationale.** Ciprofloxacin and tetracycline were chosen for the purposes of determining antibiotic-potentiating activity of non-antibiotics and to screen for efflux-inhibition activity (to identify efflux-related mechanism).

  Ciprofloxacin and tetracycline are known substrates for several multidrug resistant efflux pumps (Appendix C).

  NorA, one of the most widely studied efflux pumps in *S. aureus*, is frequently evaluated with ciprofloxacin as the antibiotic substrate.
• TetK is another important efflux pump in *S. aureus*, conferring high-level resistance to tetracycline. Reserpine has been well-described in its ability to reduce or reverse efflux pump activity on antibiotic and/or biocide substrates. \(^{57-59}\)

• As a known efflux pump inhibitor, reserpine is used as an indicator of NorA and TetK efflux pump activity, but is not used clinically due to toxic concentrations required for efflux inhibition.

b. Susceptibility testing. MICs, MBCs, and fluorometric analysis for ciprofloxacin, tetracycline, and reserpine were performed using Aim 1 methods (See IV).

c. Antibiotic-potentiating activity

• **Checkerboard Assay.**
  - We determined if non-antibiotic drugs potentiate ciprofloxacin or tetracycline antibiotic activity using a checkerboard titration assay with alamar blue dye.
  - Serial dilutions of a non-antibiotic drug (amlodipine, azelastine, ebselen, or sertraline) and ciprofloxacin or tetracycline were added at concentrations below the MIC. An example of checkerboard design is shown in Figure 7, where tetracycline was added vertically in each column and non-antibiotics were added across rows.

![Figure 7. Checkerboard Template.](image)

A reduction of MIC ≥ 4-fold (or more than one dilution) was considered significant for antibiotic-potentiation.

• **Efflux Modulation & Ethidium Bromide Agar Cartwheel Assay.** \(^{57-59}\)
  This methodology assesses the presence of efflux pump activity in clinical bacterial isolates.
  - The isolates are streaked in solid media containing increasing concentrations of EtBr and the fluorescence emitted—which is inversely proportional to their capacity to extrude the compound—is compared to the fluorescence of control strains (i.e. isolates with known efflux activity). An example is described in Appendix D.

VI. **Summary of Results**
(Only tetracycline, and not ciprofloxacin, combinations are described here)
a. Amlodipine, azelastine, and sertraline MICs were 128 µg/ml, 200 µg/ml, and 20 µg/ml, respectively, for all *S. aureus* isolates.
b. Ebselen MICs ranged from 0.25 µg/ml (SA 29213) to 1 µg/ml (isolate B72).
c. Baseline tetracycline (TET) MICs were 32 µg/ml for resistant isolates A32 and B72
Combining TET with sub-MIC concentrations of non-antibiotics led to a four- to 16-fold decrease in TET MICs (Table 6).

Eight- to 16-fold reductions in TET MICs occurred for seven of the eight TET/non-antibiotic combinations, which effectively restored TET susceptibility to S. aureus (≤ 4 µg/ml).

**Table 6. TET MICs (µg/ml), Alone and in Combination with Non-Antibiotics**

<table>
<thead>
<tr>
<th>Clinical Isolate</th>
<th>TET Baseline MICs</th>
<th>TET MICs (In Combination with Non-Antibiotic Drugs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AML 64 µg/ml</td>
</tr>
<tr>
<td>A32 (MRSA)</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>B72 (MSSA)</td>
<td>32</td>
<td>2</td>
</tr>
</tbody>
</table>

AML = amlodipine, AZE = azelastine, EBS = ebselen, SERT = sertraline

**VII. Conclusions**

a. Complete reversal of tetracycline resistance occurred in seven of the eight TET/non-antibiotic combinations against two S. aureus clinical isolates.

b. Reversal of tetracycline resistance in isolates with tetK gene is suggestive of efflux pump inhibition. However, additional studies are needed for confirmation.

c. Overall, further studies exploring the antibiotic-potentiating effects of non-antibiotics tested herein.

**VIII. Strengths**

a. This study is novel in that it combines two strategic approaches: drug repurposing and target-based whole cell screens.

b. Another unique aspect of this study is the use of the microplate alamar blue assays. The extent of this color conversion represents cell viability and can be assessed qualitatively, by visual inspection of color change, and quantitatively, with fluorometric readings.

**IX. Limitations**

a. Static drug concentrations: A disadvantage of MIC and MBC testing is that each drug is tested at a fixed concentration. Although a range of drug concentrations are evaluated, each drug concentration remains constant throughout the incubation period. This differs from drug concentrations in vivo, which fluctuate with dose administration and elimination.

b. Variability in MBC determinations: We anticipate that some variability may be observed with MBC results. What defines bacteriostatic vs. bactericidal has been controversial, as no standardized definition exists. Instead these values (i.e. MBC/MIC ratios) have been arbitrarily assigned based on concept. Furthermore, MBC determinations have been shown to be less reproducible as compared to the MIC by broth microdilution, which maintains a precision of ± 1 dilution.
References


APPENDICES

A. FDA Expedited Review Programs

<table>
<thead>
<tr>
<th>Program name</th>
<th>Year instituted</th>
<th>Criteria for qualifying products</th>
<th>Key characteristics</th>
</tr>
</thead>
</table>
| Accelerated approval     | 1992            | - Treatment of serious condition  
- Evidence suggests reasonable advantage over existing therapies                                                                                                                                         | - Can be approved on basis of surrogate endpoint  
- Approval granted on a conditional basis (Phase IV confirmatory trials are required)                      |
| Priority review          | 1992            | - Treatment of serious condition or a drug designed as a Qualified Infectious Disease Product (QIDP)  
- Improvement in safety or effectiveness over existing therapies                                                                                           | Shorter FDA review timeline (ten vs six months)                                                        |
| Fast track               | 1997; 2012*     | - Treatment of serious condition or a drug designed as a QIDP  
- Preclinical or clinical evidence demonstrating potential to address unmet medical need.                                                                                               | Can be approved after one phase-II study                                                              |
| Breakthrough therapy     | 2012            | - Treatment of serious condition  
- Demonstrates substantial improvement over existing therapies on one or more clinically important endpoints                                                                 | Intensive FDA guidance throughout development to generate additional safety and efficacy data           |

* Fast track designation was amended by the FDA Safety and Innovation Act (FDASIA) in 2012 to include the Generating Antibiotics Incentives Now Act (GAIN Act)

Appendix B. Examples of ‘Rescued’ Drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Intended Indication</th>
<th>&quot;Rescue&quot; Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allopurinol</td>
<td>Antineoplastic</td>
<td>Gout</td>
</tr>
<tr>
<td>Atomoxetine</td>
<td>Antidepressant</td>
<td>Attention-deficit/hyperactivity disorder</td>
</tr>
<tr>
<td>Azidothymidine (AZT)</td>
<td>Cancer</td>
<td>HIV/AIDS</td>
</tr>
<tr>
<td>Eflornithine</td>
<td>Cancer</td>
<td>Hirsutism</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>Antiviral</td>
<td>Anticancer agent</td>
</tr>
<tr>
<td>Lomitapide</td>
<td>Common lipidemia</td>
<td>Familial hypercholesterolemia</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>Cutaneous metastases of breast cancer</td>
<td>Visceral leishmaniasis</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>Nausea, morning sickness</td>
<td>Leprosy, multiple myeloma</td>
</tr>
</tbody>
</table>
## Appendix C. Important Multidrug Resistant Efflux Pumps in *S. aureus*61-63

<table>
<thead>
<tr>
<th>Transport Protein Family</th>
<th>Efflux Transporter</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major Facilitator Superfamily (MFS)</strong></td>
<td>NorA</td>
<td>ciprofloxacin, norfloxacin, chloramphenicol, pentamidine, biocides (i.e. cetrimide), dyes (i.e. ethidium bromide)</td>
</tr>
<tr>
<td></td>
<td>NorB</td>
<td>ciprofloxacin, norfloxacin, moxifloxacin, tetracycline, cetrimide, ethidium bromide (EtBr)</td>
</tr>
<tr>
<td></td>
<td>NorC</td>
<td>ciprofloxacin, norfloxacin, moxifloxacin</td>
</tr>
<tr>
<td></td>
<td>MdeA</td>
<td>macrolides, mupirocin, fusidic acid, EtBr</td>
</tr>
<tr>
<td></td>
<td>TetK, TetL, Tet38</td>
<td>tetracycline</td>
</tr>
<tr>
<td></td>
<td>LmrS</td>
<td>linezolid, erythromycin, chloramphenicol, trimethoprim, lincomycin, dyes, detergents, EtBr</td>
</tr>
<tr>
<td></td>
<td>QacA</td>
<td>cetrimide, chlorhexidine, pentamidine, EtBr</td>
</tr>
<tr>
<td><strong>Multidrug and Toxic Compound Extrusion (MATE)</strong></td>
<td>MepA</td>
<td>ciprofloxacin, norfloxacin, moxifloxacin, tigecycline, cetrimide, dyes</td>
</tr>
<tr>
<td><strong>ATP-Binding Cassette (ABC)</strong></td>
<td>Msr(A)</td>
<td>erythromycin, macrolides, type B streptogrammins</td>
</tr>
</tbody>
</table>

## Appendix D.

[Diagram of efflux pumps and substrates]