Bacterial Anti-Virulence Strategy as an Alternative To Antibiotics

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Antibiotic Resistance is One of Our Most Serious Health Treats

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**Europe**
- EU: ARB costs society $1.5 bn/yr & 600 million days of lost productivity.\(^5\)
- Russia: ARB a major concern with 83.6% of families imprudently use antibiotics at home.\(^6\)

**Asia**
- Thailand: >140,000 ARB infections/yr and >30,000/yr patients die; 2 bn in productivity losses/yr.\(^4\)
- Japan: Extensive levels of ARB found in Tokyo's urban watershed.\(^5\)
- China: Extreme over-prescription of antibiotics\(^5\) and rapid growth rate of ARB.\(^2\)
- India: Within 4 years (02-06) ARB went from being resistant to 7, to 21 drugs.\(^3\)
- Vietnam: Farming practices contributing to spread of ARB through environmental contamination.\(^4\)

**North America**
- USA: ARB causes majority of 99,000 deaths/yr from infections acquired in hospitals.\(^6\)
- USA: Health care costs of ARB are US$21-34 bn/yr.\(^6\)

**Middle East & North Africa**
- Egypt: 38% of blood infections contracted by young cancer patients are from ARB.\(^5\)
- Israel: ARB found fatal in ~50% cases when resistant to our strongest antibiotics.\(^3\)

**South America**
- Peru, Bolivia: >51% of hospital infections caused by ARB.\(^7\)
- Brazil: Rates of ARB are up >60%.\(^6\)

**Sub-Saharan Africa**
- Tanzania: Death rate of ARB infected children double that of malaria.\(^5\)
- Nigeria: Rapid spread of ARB that came to Africa from Asia.\(^8\)

**Antarctica**
- ARB found in Antarctic animals & water samples.\(^6\)

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70% of nosocomial infections in the US are now resistant to one or more antibiotics

Source: WHO
Excessive Antibiotic Use for Food Producing Animals

Animals in the USA consume more than twice as many medically important antibiotics as humans.

- 30% consumed by humans
- 70% are consumed by animals

Increase in antibiotic use from 2009 to 2014 in food producing animals

**Medically important**
- Aminoglycosides
- Cephalosporins
- Lincosamides
- Macrolides
- Penicillins
- Sulfas
- Tetracyclines
- NIR*

**Non-medically important**
- Ionophores
- NIR*
Just one gram of soil is estimated to contain about one billion bacterial cells.

Drop in New Antibiotics Approved by the FDA since 2000

Data Sources:
4. Drugs@FDA; www.accessdata.fda.gov/scripts/cder/drugsatfda

*Withdrawals took a mean of 15 years; safety-related withdrawals a mean of 33 months (Outterson et al [JLME]). Chart reflects original year of approval for subsequently withdrawn or discontinued drugs, not year withdrawn.

Adapted from Outterson et al, JLME 2013
Anti-Virulence as an Alternative Approach to Antibiotics

**Anti-Virulence approach:** Disarming bacterial pathogens from their ability to cause infection by targeting key mechanisms required for acute and persistent infections without affecting bacterial growth or viability.

**Advantages over antibiotics:**
- Less resistance to treatment
- Preservation of beneficial flora
- Active even on MDR bacteria
Main Conserved Targets for Anti-Virulence Strategies

- Biofilms
- Capsule
- Toxins
- c-di-GMP
- Quorum sensing
- Protein secretion
- Two component systems
Main Conserved Targets for Anti-Virulence Strategies

- **Biofilms**
- **Capsule**
- **Toxins**
- **Quorum sensing**
- **Two component systems**
- **c-di-GMP**
- **Protein secretion**

**References**
- Bretl et al. 2011 MMBR
- JM Ghigo, Institut Pasteur
Quorum Sensing is an Cell-to-Cell Density-Dependent Communication System that Synchronizes the Production of Multiple Virulence Factors
Phenotypes shown to be Regulated by Quorum Sensing In Gram Negative and Gram Positive Bacteria
**Model: Quorum Sensing in *Pseudomonas aeruginosa***

*Pseudomonas aeruginosa* infects multiple organisms

3 QS systems in *P. aeruginosa*:
- MvfR
- LasR
- RhlR

MvfR = Multiple virulence factor regulator

MvfR regulated genes

- Rest of the genome
- MvfR Regulated

MvfR regulated virulence factors

- Pyocyanin
- Cyanide
- Lectins
- Proteases
- Elastase
- LasR QS
- RhlR QS
- Rhamnolipids
- Peptides
- Chitinases

- Pyochelin
- Pyoverdin
- Type VI secretion
- Type II secretion
- HAQs, 2-AA
- Efflux pumps
- Fimbriae/pili
- Flagella
- Protein translation
- more...

MvfR QS System is Critical for Infection in Multiple Hosts

- Lethality in flies
  - PA14: 100%
  - mvfR-: 0%

- Lethality in mice
  - PA14: 100%
  - mvfR-: 20%

- Multiplication in plants
  - Growth on Arabidopsis leaves
    - PA14: $10^8$
    - mvfR-: $10^6$

Adapted from Rahme et al. 1997 PNAS
Adapted from Lau et al. 2003 Infect. & Immun.
MvfR is Part of a Family of Transcriptional Regulators Widely Conserved in Bacterial Pathogens

LysR-type transcriptional regulators (LTTR)

Table 1. Examples of LTTRs

<table>
<thead>
<tr>
<th>LTTR</th>
<th>Regulation</th>
<th>Target gene function</th>
<th>Co-factor</th>
<th>Origin</th>
<th>Subclones</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Akr</td>
<td>Activator (local)</td>
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<td>Bacteria</td>
<td>ns</td>
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<td>AkrI</td>
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<td>Staphylococcus aureus</td>
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</tr>
</tbody>
</table>

*Subclones of proteobacteria indicated by y, ns = Gene positive; ns, not applicable.

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Maddocks et al. 2008 Microbiology

Ilangovan et al. 2013 PLoS Pathogens
MvfR QS System
and its Interconnection with the LasR & RhlR QS systems

virulence factors

LasR/lasI

pqsh

PqsH

pqABCDE

PqsABCD

PhnAB

virulence factors

MvfR

RhlR/rhlII

PqsE

PqsH

HHQ

Acute infection

Chronic infection

HHQ

PQS

2-AA

HQNO

Adapted from Maura et.al. 2016 Scientific Reports, 2016
HQNO and 2-AA Promote Chronic Infection Phenotypes

Bacterial translation → "dormancy" stage → antibiotic tolerance

2-AA

Host immune response → reduced bacterial clearance → chronic infection

HQNO

Hazan et al. 2016 Current Biology

High Levels of MvfR Controlled Small Molecules are Produced in Human Tissues

Objective of the project

???

inhibitor

MvfR

Acute infections

Chronic infections

Adapted from O’Connell 2007 Nat. Rev. Micro
High Throughput Screening for MvfR Inhibitors

~ 300,000 compounds
chemical HTS
whole cell assay (pqsa-sacB)
390 cherry picks (0.13%)

secondary screen
reporter gene assay (pqsa-GFP)
39 hits (10%)
(33 commercially available)

Active inhibitor → cells grow in sucrose
Inactive inhibitor → cells die in sucrose

vehicle
cpd#1
cpd#2
cpd#3
cpd#4
cpd#5

Starkey*, Lepine*, Maura* et al. 2014 PLoS Pathogens
High Throughput Screening for MvfR Inhibitors

Benzamide-Benzimidazole (BB) core structure

functional assays
HAQs & pyocyanin inhibition

Starkey*, Lepine*, Maura* et al. 2014 PLoS Pathogens
### BB Compounds Optimization

<table>
<thead>
<tr>
<th>ID</th>
<th>Structure</th>
<th>%HAQs (10μM)</th>
<th>ID</th>
<th>Structure</th>
<th>%HAQs (10μM)</th>
<th>%HAQs (1μM)</th>
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<td>M26</td>
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<td>M65</td>
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<td>M57</td>
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<td>M62</td>
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<td>M22</td>
<td></td>
<td></td>
<td>M50</td>
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</table>

**Synthesized**

H = HHQ
P = PQS
N = Pyocyanin

### Graphical Representation

- **M64**
  - IC₅₀ = 200-300nM

**% inhibition** vs **M64 Concentration (M)**

#### References

Starkey*, Lepine*, Maura* et al. 2014 PLoS Pathogens
### P. aeruginosa MDR Strains are Sensitive to M64

#### Antibiotic Resistance Profile

<table>
<thead>
<tr>
<th></th>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

- **R** = resistant
- **I** = intermediate
- **S** = sensitive

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**Starkey*, Lepine*, Maura* et al. 2014 PLoS Pathogens**
M64 Blocks Biofilm Formation
M64 Rescues Mice from *P. aeruginosa* Burn Infection

**Abdominal burn + PA14 infection**

<table>
<thead>
<tr>
<th>Day</th>
<th>CFUs</th>
<th>Days</th>
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<tr>
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<tr>
<td>2</td>
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<td>5</td>
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<td>8</td>
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<td>11</td>
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<td></td>
</tr>
<tr>
<td>14</td>
<td>//</td>
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</tbody>
</table>

Assess survival

**Survival**

- **PA14**
- **PA14 + M64**

**Bacterial load in underlying muscle**

- **PA14**
- **PA14 + M64**

Starkey*, Lepine*, Maura* et al. 2014 PLoS Pathogens
M64 Rescues Mice from *P. aeruginosa* Lung Infection

Starkey*, Lepine*, Maura* et al. 2014 PLoS Pathogens
BB Compounds Block the Formation of Antibiotic Tolerance Cells \textit{in vitro}

% of 2-AA production

\begin{tabular}{c c c c c c}
    & PA14 + DMSO & PA14 + M64 & PA14 + M59 & PA14 + M50 & PA14 + M51 \\
\hline
0% & \\
20% & \\
40% & \\
60% & \\
80% & \\
100% & \\
\end{tabular}

% of Meropenem tolerant cells

\begin{tabular}{c c c c c c}
    & PA14 + DMSO & PA14 + M64 & PA14 + M59 & PA14 + M50 & PA14 + M51 \\
\hline
0% & \\
20% & *** \\
40% & *** \\
60% & *** \\
80% & *** \\
100% & *** \\
\end{tabular}

Starkey*, Lepine*, Maura* et al. 2014 PLoS Pathogens
M64 Prevents Relapsing *P. aeruginosa* Infection in Mice

**M64 Prevents Relapsing P. aeruginosa Infection in Mice**

Burn + PA14 infection

Ciprofloxacin IV 4mg/kg 1 2 3 4 5 6 days

CFUs

Ciprofloxacin IV 10mg/kg

M64 Prevents Relapsing *P. aeruginosa* Infection in Mice

Starkey*, Lepine*, Maura* et al. 2014 PLoS Pathogens
**MvfR = LTTR family**

Ilangovan et al. 2013 PLoS Pathogens

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**LysR-type transcriptional regulators (LTTR)**

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<th>Subclade(s)*</th>
<th>References</th>
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<tbody>
<tr>
<td>ABR</td>
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<td>Sterigmatosporidium sp.</td>
<td>Gly+</td>
<td>Yang et al. (2005)</td>
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<td>Gly+</td>
<td>Yang et al. (2005)</td>
</tr>
</tbody>
</table>

*Subclades of proteobacteria indicated by s, e, p. Gly+ = Gly+; Gly– = Gly−; ns = not applicable.

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**Table 1. Examples of LTTRs**

**Maddocks et al. 2008 Microbiology**
M64 Blocks *Escherichia coli* Invasion of Intestinal Epithelial Cells

**Invasion of Caco2 cells**

![Graph showing invasion of Caco2 cells](image)

**Bacterial viability**

![Graph showing bacterial viability](image)

**Caco2 cells viability**

![Graph showing Caco2 cells viability](image)

**Bacterial gene expression**

![Graph showing bacterial gene expression](image)

<table>
<thead>
<tr>
<th></th>
<th>fimD</th>
<th>fimH</th>
<th>flhB</th>
<th>flhD</th>
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<tr>
<td>DMSO</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
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<td>M64 10μM</td>
<td>80%</td>
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<td>M64 15μM</td>
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<tr>
<td>M64 25μM</td>
<td>40%</td>
<td>40%</td>
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</tbody>
</table>
M64 also Blocks the Virulence of Many Bacterial Pathogens

- **Bacteria**
  - Fly food
  - M64
  - 24h
  - Quantify gut inflammatory cells (immuno-histo-chemistry)

![Bar chart showing the number of inflammatory cells with and without M64 treatment for different bacterial species.](chart.png)

- **vehicle**
- **P. aeruginosa**
- **E. coli**
- **V. cholerae**
- **E. faecalis**
- **S. agalactiae**
- **Serratia**
- **Citrobacter**
- **P. multocida**
- **B. thetaiotaomicron**

*Significance levels: **p < 0.01, ***p < 0.001*
Summary & Perspectives

Acute virulence
- pyocyanin
- acute infection

Chronic virulence
- antibiotic tolerance
- biofilms
- relapsing infection

BB compounds = potential to be the next generation anti-bacterial drugs for *Pseudomonas* infections
BB compounds have an anti-virulence efficacy on a broad range of bacterial pathogens.

Potential applications?
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