Collaborating with the Innate Immune System to Treat Multidrug-Resistant Superbugs

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Chief, Division of Host-Microbe Systems & Therapeutics
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Virulence Factor
Innate Immunity
Bacterial Pathogens
Research focus on common, invasive bacterial pathogens of humans ...

S. pyogenes (Group A Strep)  
S. agalactiae (Group B Strep)  
S. aureus (Golden Staph)  
S. pneumoniae (Pneumococcus) (e.g. Pseudomonas)  

... and their interaction with host innate immunity

Cathelicidin AMPs  
HIF and Immunity  
Neutrophil Traps  
Macrophage Signaling  
Host-Pathogen Glycobiology
Community-Acquired MRSA

Bryce, 14 months from Santee, CA
Human Neutrophil vs. Bacterial Pathogen
S. aureus Subversion of Host Phagocyte Defense

Staphylococcus aureus
Mechanisms of innate immune resistance

Cloaking of opsonins
Serotype 5, 8 capsules, PNAG
Clumping factor
Nonopsonic binding or degradation of immunoglobulins

Neutrophil lysis
γ-Hemolysin
Panton-Valentine leukocidin

Impairment of phagocyte recruitment
Formyl peptide receptor
C5a receptor

Interference with complement activation
C3b
C3 convertase complexes
SCIN

Resistance to oxidative burst killing
H₂O₂
tO₂
Superoxide
Singlet O₂
Catalase

Resistance to antimicrobial peptides
Aureolysin

Nizet, J Allergy Clin Immunol 2007
Seeking *alternatives* to classical antibiotics, especially very broad-spectrum agents, that kill bacteria or block their growth

* Drugs to block specific pathogen immune resistance factors
  Sensitize pathogens to clearance by normal host innate defenses
  More targeted therapy, avoid “collateral damage” to microbiome

* Modulation of innate immunity to treat bacterial infections
  Can we pharmacologically boost phagocyte function?

* Explore “repurposing” existing drugs for the above properties

* Synergy between pharmaceutical and endogenous antibiotics
Molecular Koch’s Postulates

Cloning
Gain of function analysis
What genes are sufficient?

Mutagenesis
Loss of function analysis
What genes are necessary?

Stanley Falkow
Idealized Approach to Analysis of New Bacterial Virulence Factors

- In vitro
- Ex vivo
- In vivo
- Target for therapy?
Aureus of Brutus
(Ancient Roman Coin)

Hapalemur aureus
(Golden Bamboo Lemur)

Amblyglyphidodon aureus
(Golden Damselfish)

aureus = “golden” (Latin)

Teinopalpus aureus
Golden swallowtail butterfly

Canis aureus
Golden jackal

Senecio aureus
Golden ragwort
Aureus = "golden" (Lat.)

Staphylococcus aureus

2 x Farnesyl-PP

Mutation → CrtM

Dehydrosqualene → CrtN

4,4'-Diaponeurosporene

Staphyloxyanthin

Fritz Götz Lab, Tuebingen
Beta-carotene

2x Farnesyl-PP
Mutation → CrtM
Dehydrosqualene
CrtN
4,4'-Diaponeurosporene
Staphyloxanthin

Glc - O - OC - C_{14}H_{29}
S. aureus Pigment Has Antioxidant Properties

Liu et al., J Exp Med 2005
First Steps of Staphyloxanthin Biosynthesis Resemble Those of Human Cholesterol Biosynthesis

**Diagram:**
- **Part a:** Staphyloxanthin Biosynthesis
  - Dehydrosqualene → CrtN → 4,4'-Diaponeurosporene → Staphyloxanthin
- **Part b:** Cholesterol, Ergosterol Biosynthesis
  - Squalene → Lanosterol → Cholesterol → Ergosterol

**Chemical Structures:**
- 2 Farnesyl diphosphate
- Presqualene diphosphate
- Inhibitor
- NADPH

**References:**
- Liu et al. Science 2008
X-Ray Crystal Structures of *S. aureus* CrtM Together With Bound Phosphonosulfonate Inhibitors

Chia-I Liu
Wen-Ji Jeng
Andrew H.J. Wang
Academia Sinica
Taiwan

CrtM X-ray structure

Superposition of CrtM and hSQS structures, showing a rmsd of 5.5 Å

Liu et al. Science 2008

Farnesyl thiodiphosphate (FSPP-1 and FSPP-2)

BPH-698

BPH-652

BPH-700
A Cholesterol-Lowering Agent Prevents Staphylococcal Pigment Production

\[
\text{BPH-652}
\]

\[
\text{H}_2\text{O}_2 \text{ susceptibility}
\]

\[
\begin{align*}
\text{Percent of activity} & \quad \log_{10} \text{Surviving CFU} \\
-20 & \quad 4 \\
0 & \quad 5 \\
20 & \quad 6 \\
60 & \quad 7 \\
80 & \quad 6 \\
100 & \quad 5 \\
120 & \quad 4 \\
\end{align*}
\]

\[
\begin{align*}
\text{PBS} & \quad \text{BPH-652} \\
4 & \quad 6 \\
5 & \quad 5 \\
6 & \quad 4 \\
\end{align*}
\]

\[
\begin{align*}
\Delta \text{CrtM} & \quad 1000 \\
100 & \quad 10 \\
10 & \quad 1 \\
1 & \quad \text{WT} \\
\end{align*}
\]

Liu et al. Science 2008
A Cholesterol-Lowering Agent Blocks S. aureus Virulence In Vitro & In Vivo

![Graph showing whole blood survival and mouse intraperitoneal infection results.](Liu et al. Science 2008)
Neutrophil “NETs”: DNA-Based Extracellular Traps for Killing Pathogenic Bacteria
Invasive M1 Clone of GAS Has Potent Phage Encoded DNase (Sda1)

Ramy Aziz
Malak Kotb
Genetic Manipulation of GAS DNase Activity

M1 = invasive GAS isolate
M49 = noninvasive GAS isolate
L. lactis = nonpathogen

Buchanan et al., Current Biology 2006
DNAse Sda1 Promotes GAS Neutrophil Resistance

Targeted Mutagenesis of Sda1 DNAse

- Human Neutrophils
  - M1 WT
  - M1ΔSda1
  - MOI (bacteria:neutrophil): 0.01, 0.1, 1.0
  - % survival: 0-80
  - Decreases bacterial survival

Heterologous expression of Sda1 DNAse

- Human Neutrophils
  - + empty vector
  - + pSda1
  - MOI (bacteria:neutrophil): M49 GAS, L. lactis
  - % survival: 0-150
  - Increases bacterial survival

Buchanan et al., Current Biology 2006
The M1 GAS DNase Sda1 Degrades Neutrophil Extracellular Traps

**In vitro**

- **M1 WT**
- **M1ΔSda1**

**In vivo**

- **M1 WT**
- **M1ΔSda1**

*In vivo* staining of NETs from mouse skin biopsies (22 h)

*Buchanan et al., Current Biology 2006*
DNAse Inhibition Reduces GAS Virulence

G-Actin: Type I DNAse inhibitor

Extracellular Killing (+CytD)

M1 WT GAS

Lesion area (mm²)

- Actin + Actin

Days

- Actin + Actin

P = 0.0029

Buchanan et al., Current Biology 2006
Statins

30 million users in the US in 2005

3-Hydroxy 3-Methylglutaryl Coenzyme A (HMG-CoA) Reductase Inhibitors

Pharmacological Effects
- Treatment of Hyperlipidemia
- Lowers LDL
- Raises HDL
Clinical Data: Decreased Risk or Improved Outcomes of Infection in Patients Receiving Statins

<table>
<thead>
<tr>
<th>Disease</th>
<th>Effect of Statins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteremia</td>
<td>Reduced Mortality</td>
</tr>
<tr>
<td>Sepsis</td>
<td>Reduced Incidence, Reduced Mortality</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>Reduced Incidence, Reduced Mortality</td>
</tr>
</tbody>
</table>

Prevailing Hypothesis: Statin downregulates inflammatory mediatory release deleterious in sepsis (TNF, iNOS, IL-1, IL-6)

We sought to test an Alternative Hypothesis: Could statins improves the innate immune function of phagocytes?
Statin treated neutrophils and macrophages kill \textit{S. aureus} more efficiently

with C. Glass Lab

Effect is observed with multiple bacterial species

Chow et al. Cell Host Microbe (2010)
Statins actually REDUCED phagocytosis & oxidative burst; rather, they boosted NET production & killing.

Chow et al. Cell Host Microbe (2010)
Statin Boosting of Phagocyte Extracellular Traps is an On-Target Effect of HmgCoA Reductase Inhibition

Expression of Hmgcr in murine macrophages after siRNA transfection

Murine peritoneal macrophage ETs

Macrophage ETs RNA inhibition

Macrophage killing RNA inhibition

RAW 264.7 killing Mevalonate Rescue

Chow et al. Cell Host Microbe (2010)
Mice Treated With Statin Have Increased ET Production and Killing of S. aureus Ex Vivo and In vivo

Chow et al. Cell Host Microbe (2010)
Tamoxifen Induces NETs By Increasing Intracellular Ceramide Levels

Corriden et al. Nat Commun 2015
Tamoxifen Boosts Host Defense Against Staphylococcal Infection in vivo

Corriden et al. Nat Commun 2015
Now shifting gears ...

Cathelicidin antimicrobial peptides
“natural antibiotics” of innate defense

Hypoxia-induced factor (HIF)
as regulator of phagocyte function

HIF
(5′-TACGTGCT-3′)
HRE
Target gene
Cathelicidins

Produced on Epithelial Surfaces & By Granulocytes

- Skin
- Colon
- Salivary Gland
- Sweat Gland
- Neutrophil
- Mast Cell
Cathelicidins: Induced By Infection

Immunostain for murine CRAMP

Uninfected skin

GAS infection

CRAMP-KO Mouse Has Immune Defect

Wild-type Mice

Knockout Mice

Lesion size (mm²)

Days

+/-

+-

-/-


with R. Gallo Lab
HIF-1α expression is regulated by oxygen at the protein level (half life < 1 min)
Myeloid-Cell Specific Knockout of HIF-1α in Mice
cre/flox Deletion Driven by LysM promoter (Neutrophils and Macrophages)

Normal Phagocytosis

Defect in Bacterial Killing

Surviving Intracellular GBS

WT  HIF-1α -/-

Cramer et al., Cell 2003
How Does HIF-1\(\alpha\) Contribute to Phagocyte Killing?

Cathelicidin AMP

WT  HIF-/-  VHL-/-

- Anti-CRAMP
- Anti-\(\beta\)-actin

Granule Proteases

- Elastase activity

Units / min

Fibro  WT  HIF-/-  VHL-/-

TNF\(\alpha\) mRNA

Fold induction

- WT
- HIF-/-
- VHL-/-

iNOS mRNA

Fold induction

Peyssonnaux et al., J Clin Invest 2005
HIF-1α Activated in Response to Bacteria (Even at Normoxia) and Contributes to Innate Immunity

WT Mice

Wild-type mice

HIF-1α KO

GAS Skin Infection Model

Peyssonaux et al., J Clin Invest 2005
High $O_2$ (>10%)
- HIF absent and leukocytes quiescent

Low $O_2$ (2.5–5%)
- Initial HIF stabilization due to decreasing oxygen

Scarce $O_2$ (<1%)
- HIF transcripts induced by pathogen encounter through TLR–NF-$\kappa$B signalling

Endothelial cell
- Neutrophil

Blood vessel

Increased permeability
- Pro-inflammatory cytokines (TNF, IL-1 and IL-12)
- Apoptosis inhibited

VEGF
- Nitric oxide

Bacterial infection in tissue
- Phagocytosis of bacteria
- Antimicrobial peptides and proteases

Bactericidal and pro-inflammatory processes maximized
- TLR

Nizet & Johnson 2009
Nature Reviews | Immunology
Genetic Augmentation of HIF (vHL-/-) Boosts Bactericidal Capacity

![Graphs showing intracellular CFUs over time for GAS and PA under WT and vHL-/- conditions.](image-url)
New Generation HIF Agonists (PHD Inhibitors) Boost Phagocyte and Keratinocyte Killing of MRSA *in vitro* and *in vivo*

![Chemical structures of AKB-4924 and Mimosine](image1)

![Bar graphs showing % survival vs. untreated cells](image2)

![Graph showing lesion size (% vehicle)](image3)

![Graph showing chug tissue (% vehicle)](image4)

HIF Boosting Protects Bladder Epithelium From *E. coli* Urinary Tract Infection

Anthrax Lethal Factor Suppresses HIF, but Pharmacological Boosting Saves the Host

Macrophage Killing of *B. anthracis* (Sterne)

Systemic Mouse Infection *B. anthracis* (Sterne)

Raza Ali, unpublished
John Howard Mueller  
PhD (1891-1954)

William Augustus Hinton  
MD, PhD (1891-1954)

.... the father of

Jane Hinton  
DVM (1919-2003)
A Protein-Free Medium for Primary Isolation of the Gonococcus and Meningococcus.

J. Howard Mueller and Jane Hinton.

From the Department of Bacteriology* and Immunology, Harvard Medical School, and School of Public Health, and the Boston Dispensary, Boston, Mass.

30.0%  Beef infusion
1.75%  Casein hydrolysate
0.15%  Starch
1.70%  Agar
pH to neutral at 25°C

Later – cation-adjusted
(for *Pseudomonas*)
Calcium 20-25 mg/L
Magnesium 10-12.5 mg/L
Broth Dilution MIC Testing
(CLSI standard)
(cation-adjusted) Mueller-Hinton Broth

Disc Diffusion or E-testing
(CLSI Standard)
(cation-adjusted) Mueller-Hinton Agar
A SINGLE TEST, NAMELY MIC/MBC DETERMINATION IN STANDARDIZED BACTERIOLOGIC MEDIA (i.e. CA-MHB), DEFINES AN ENTIRE FIELD OF PHARMACOTHERAPY IN HUMAN MEDICINE – STARTING FROM HOW ANTIBIOTICS ARE DISCOVERED AND DEVELOPED, TO WHICH DRUGS ARE CHOSEN FOR THE HOSPITAL FORMULARY, TO WHICH INFORMATION IS PROVIDED TO DOCTORS WHEN THE PATHOGENIC MICROBE IS CULTURED FROM THE PATIENT’S BLOOD OR TISSUES
Before a patient has even seen the doctor ...

... their infection is already being treated by dozens of antibiotics
Reintroduction of β-Lactam Antibiotics in Refractory M.R.S.A. Bacteremia – With Surprising Results

Day 1
Vancomycin dosed for serum trough 15-20 mg/L
VAN MIC 1 DAP MIC 0.5 (isolate D592)

Day 12
Daptomycin 6 mg/kg
Persistent (+) blood cultures for MRSA

Day 17
Daptomycin 8 mg/kg + Gentamicin

Day 21
VAN MIC 4.0 DAP MIC 2.0 (isolate D712)
Daptomycin 10 mg/kg + Nafcillin 2 g IV q 4hr

Day 22
Bacteremia Resolved

Day 23-76
Cure

George Sakoulas, MD
Rapid MRSA Bacteremia Clearance with High-Dose Daptomycin plus a β-lactam

UCSD RPDP Project 2 – Publications


Exposure to Sub-MIC Nafcillin Increases Daptomycin Binding to *S. aureus* Cell Wall

Daptomycin-resistant VISA Clinical Isolate

Bodipy-Dapto (Green/yellow)

Daptomycin binds Ca^{2+} in vivo as an integral part of its mechanism of action – i.e. it becomes a de facto cationic peptide.

Cationic antimicrobial peptides such as cathelicidin are a critical component of mammalian innate immunity ……
Sublethal Nafcillin Dramatically Sensitizes MRSA/VISA Strains to Human Cathelicidin AMP LL-37 Killing

**Similar results with human alpha-defensin, platelet-derived AMP, and murine cathelicidin**

Nafcillin Increases Binding to MRSA by Rhodamine-Labeled Cathelicidin LL-37

MRSA + LL-37

MRSA + LL-37 + Naf 10

Sublethal Nafcillin Sensitizes MRSA/VISA Strains to Whole Blood, Neutrophil & Keratinocyte Killing

Whole Blood Killing

<table>
<thead>
<tr>
<th>Strain</th>
<th>Survival (%)</th>
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</thead>
<tbody>
<tr>
<td>D592 VISA</td>
<td>40</td>
</tr>
<tr>
<td>D712 VISA</td>
<td>20</td>
</tr>
<tr>
<td>Sanger 252 MRSA</td>
<td>0</td>
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Neutrophil Killing

<table>
<thead>
<tr>
<th>Strain</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D592 VISA</td>
<td>60</td>
</tr>
<tr>
<td>D712 VISA</td>
<td>40</td>
</tr>
</tbody>
</table>

Human Keratinocyte (HaCat) Survival at 2h

<table>
<thead>
<tr>
<th>Condition</th>
<th>Survival %</th>
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</thead>
<tbody>
<tr>
<td>No Abx</td>
<td>100</td>
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<tr>
<td>NAF 5</td>
<td>200</td>
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<tr>
<td>NAF 20</td>
<td>150</td>
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</tbody>
</table>

P < 0.05

Sublethal Nafcillin (Monotherapy) Influences MRSA Lesion Development in Mouse Skin Infection Models

Antibiotic pretreatment of Sanger 252 MRSA followed by mouse subcutaneous challenge

Representative gross appearance of skin lesions at 48 h time point

Mouse s.c. challenge with Sanger 252 MRSA +/- antibiotic treatment

Mouse s.c. challenge with Sanger 252 MRSA +/- antibiotic treatment

Loss of susceptibility to daptomycin is an increasing concern.

Available antibiotics have side effects with long-term use:
- linezolid-induced thrombocytopenia
- quinupristin-dalfopristin (QD)-associated myalgias, P450 effects
- serotonin syndrome concerns with linezolid and concomitant serotonin reuptake inhibitors.

Great clinical need for more potent and/or more tolerable therapies.
Adding Ampicillin to Daptomycin for Treatment of Refractory Amp-R, Vanco-R *Enterococcus faecium*

Aortic Valve Endocarditis in a hemodialysis patient with refractory bacteremia

Failed to clear after 7 d Daptomycin (6 mg/kg q 48 h) + linezolid (600 mg IV q 12 h)

D/C linezolid, start Ampicillin (1 gm q 6 h) in combo with Daptomycin as above → bacteremia cleared < 24 hours, then cured

Sakoulas, Bayer et al. 2011
Daptomycin plus Ceftaroline Synergy Against Daptomycin-NS VRE

Figure 1. In vitro antibiotic killing assays performed in calcium-supplemented LB (50 mg/L) for daptomycin susceptible VRE 8019 (left panel) and daptomycin nonsusceptible VRE 5938 (right panel) demonstrating log$_{10}$ CFU/mL at 6 hours (top) and 24 hours (bottom). Vertical bar denotes the starting inoculum. Note only daptomycin plus ceftaroline resulted in net killing of 2 log$_{10}$ CFU/mL at 24 hrs against the daptomycin nonsusceptible strain.

Sakoulas et al. AAC 2014
**Carbapenem-Resistant Enterobacteriaceae**

- Threat level: Urgent
- This bacteria is an immediate public health threat that requires urgent and aggressive action.
- 9,000 drug-resistant infections per year
- 600 deaths
- Carbapenem-resistant Klebsiella spp.
  - 7,900
  - 1,400
- CRE have become resistant to all or nearly all available antibiotics

**Multidrug-Resistant Pseudomonas Aeruginosa**

- Threat level: Serious
- This bacteria is a serious concern and requires prompt and sustained action to ensure the problem does not grow.
- 6,700 multidrug-resistant Pseudomonas infections
- 440 deaths
- 51,000 Pseudomonas infections per year

**Multidrug-Resistant Acinetobacter**

- Threat level: Serious
- This bacteria is a serious concern and requires prompt and sustained action to ensure the problem does not grow.
- 7,300 multidrug-resistant Acinetobacter infections
- 500 deaths from multidrug-resistant infections
- 12,000 Acinetobacter infections per year
- At least three different classes of antibiotics no longer core resistant Acinetobacter infections
<table>
<thead>
<tr>
<th>ANTIBIOTIC</th>
<th><em>Pseudomonas aeruginosa</em>, P4 (MDR)</th>
<th><em>Klebsiella pneumoniae</em>, K1100 (MDR, KPC)</th>
<th><em>Acinetobacter baumannii</em>, AB5075 (MDR)</th>
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<tr>
<td></td>
<td>MIC</td>
<td>Interpretation</td>
<td>MIC</td>
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<td>Nitrofurantoin</td>
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<tr>
<td>TMP/SFX</td>
<td>&gt; 320</td>
<td>R</td>
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</table>
Colistin (Polymyxin E2) from *Paenibacillus polymyxa*
“Drug of Last Resort” for MDR Gram-Pathogens

Pentacationic polypeptide consisting of a cyclic heptapeptide, a linear tripeptide and a fatty acid tail linked to the N-terminal of the tripeptide.

The five L-diaminobutyric acid (L-Dab) molecules are positively charged.
Colistin Nephrotoxicity: Dose-Limiting

Dai et al. AAC 2014

Eadon et al. Physiol Genom 2013
What is the most commonly prescribed antibiotic in the United States? (53 million/yr in 2010)
AZITHROMYCIN
Dramatic Differences in Azithromycin Activity vs. Multidrug-Resistant Gram-Negative Rods in Tissue Culture Media vs. Bacteriologic Media

Lin et al. eBiomedicine 2015

Leo Lin
UCSD MSTP
Azithromycin is Cidal for MDR Gram-Negative Rods at low Concentrations in RPMI + 5% LB

Lin et al. eBiomedicine 2015
MDR *A. baumannii*

**Ca-MHB**
- No abx
- AZM 2

**RPMI (5% LB)**
- No abx
- AZM 2

RPMI (5% LB), no Abx

RPMI (5% LB) + AZM 2

Lin et al. eBiomedicine 2015
Subinhibitory Azithromycin Induces Marked Ultrastructural Changes in Pseudomonas

MDR *P. aeruginosa* in RPMI (5% LB)
Synergy Between Azithromycin and Colistin in Killing MDR Gram-Negative Rods (done in MHB)

Lin et al. eBiomedicine 2015
Synergy Between Azithromycin and Colistin in Killing MDR *Acinetobacter baumannii* (done in MHB)

![Image of bacterial stains showing untreated and treated conditions with DAPI and SYTOX Green stains.](Lin et al. eBiomedicine 2015)
Synergy Between Azithromycin and LL-37 in Killing MDR Gram-Negative Rods

Lin et al. eBiomedicine 2015
Azithromycin Synergy with LL-37: Increased Cell Wall Permeability and Azithromycin Entry

MDR *Acinetobacter baumannii*

**f**
- Untreated
- NBD-AZM 5
- LL-37 (5 μM)
- NBD-AZM + LL-37

**g**
- Relative intensity
- Toroid nuclei
  - Untreated
  - NBD-AZM 5
  - LL-37 (5 μM)
  - NBD-AZM + LL-37

Lin et al. eBiomedicine 2015
Azithromycin Monotherapy Reduces CFU, Lung Inflammation and Mortality in Mouse Model of A. baumannii Pneumonia

Lin et al. eBiomedicine 2015
Azithromycin Activity vs. Carbapenem-Resistant *P. aeruginosa* & *K. pneumoniae*  
(Lin et al. eBiomedicine 2015)

Unrecognized Azithromycin Activity vs. MDR *Stenotrophomonas maltophilia*  
(Kumaraswamy et al. J Antimicrob Chemother 2016)
CURRENT “OLD” ANTIBIOTIC DISCOVERY PARADIGM

SCREEN IN BACTERIOLOGIC MEDIA

DISCOVERY ONLY “CLASSICAL” ANTIBIOTIC ACTIVITIES

BETTER FUTURE ANTIBIOTIC DISCOVERY PARADIGM

SCREEN IN BLOOD OR IMMUNE CELL CULTURE SYSTEM

DISCOVER SIMULTANEOUSLY:

CLASSICAL ANTIBIOTICS

VIRULENCE FACTOR INHIBITORS

INNATE IMMUNE SENSITIZERS

PHAGOCYTIC CELL BOOSTERS
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