Drug-Resistant and Persistent Tuberculosis: Mechanisms and Strategies for Improved Treatment

Ying Zhang, MD, PhD
Email: yzhang@jhsph.edu
Historical Perspective

- Old infectious disease, since antiquity: Egyptian mummies 4000 years ago had typical spine TB (Pott’s disease)
- Phthisis (“wasting”, Hippocrates), Consumption, White Plague
- Association with art, literature, unique in human civilization
- Robert Koch discovered M. tuberculosis in 1882
- Calmette and Guerin developed BCG vaccine in 1921
- 20% all deaths in 18th and 19th century due to TB (5% now)
- Improved sanitation and nutrition lowered TB incidence even before chemotherapy in 1950s
- Introduction of chemotherapy in 1950s made TB curable ->further decrease in TB cases
- TB declined in developed countries until 1970s
- In the late 1980s incidence in developed countries increased, with emergence of MDR-TB
TB: A Leading Infectious Killer
- Top 3 Infectious Killer

- One-third of world population is infected with TB bacillus – 2 billion people
- 9 million new TB cases and 2 million deaths each year
- TB is the leading killer of HIV/AIDS patients
- 50 million people infected with MDR-TB
- Each year 500,000 cases MDR-TB, and 50,000 cases XDR-TB
- Former Soviet Union countries (22.3%), India and China have highest incidence of MDR-TB
The New Tuberculosis

HIV and Drug-resistant TB – A lethal combination and a major threat to TB control

1990s: MDR-TB (resistant to RIF+INH)

WHO declared TB a global emergency in 1993

TB emergency declared in Africa - August 25, 2005

CDC announced emergence of XDR-TB – Mar 24, 2006

TDR: Totally Drug Resistant TB, 2009
TB Chemotherapy:
THE Effective TB Control

- **Pre-antibiotic era:** before 1940s (e.g., cod liver oils, bed rest, fresh air)

- **Drugs used to treat TB:** Streptomycin first TB drug (1944), followed by PAS (para-amino salicylate)(1946), isoniazid (1952), pyrazinamide (1952), rifampin (1963)
  
  - (a) Front-line Drugs: *isoniazid* (INH) rifampicin (RIF), *pyrazinamide* (PZA), streptomycin, *ethambutol*
  
  - (b) Second-line Drugs: *PAS*, *cycloserine*, *ethionamide*, *thiacetazone*, ofloxacin/levofloxacin, SLID (kanamycin, amikacin, capreomycin)

- (TB specific drugs are italicized and underlined, and the rest are broad spectrum antibiotics)
Role of British MRC in TB Chemotherapy

- Streptomycin clinical trial (Philip D’Arcy Hart): The first ever randomized clinical trial (RCT), basis for EBM (evidence based medicine) in 1946

- Principle of drug combination: streptomycin resistance developed easily, but addition of PAS led to prevention of drug resistance in 1948

- In 1950s, standard TB therapy contained INH, PAS and SM, and took 18-24 months

- Formulation of current 6 month TB therapy with INH, RIF, PZA, EMB in 1970-1980s
The Best TB Therapy: 6 months

- Initial phase (daily, 2 months) with 4 drugs: Isoniazid (INH), Rifampin (RIF), Pyrazinamide (PZA), Ethambutol (EMB)

- Continuation phase (4 months) with 2 drugs: INH and RIF
Treatment for MDR-TB

- PZA+EMB + second-line TB drugs (PAS, ethionamide, cycloserine, kanamycin, amikacin, capreomycin, quinolones etc.)
- Too expensive (TB case: $11 to $100, MDR-TB case: $150,000)
- MDR-TB requires extensive chemotherapy, for 18-24 months, more toxic, side effects, poor cure rates
TB Chemotherapy-cont

- **Treatment principle:** Drug combination
  for other infections, e.g. HIV-HAART therapy; Hp;
cancer MOPP therapy for lymphoma

- **Why drug combination?**
  (a) prevent drug resistance: Spontaneous mutations
      (calculations, e.g., resistance to INH 1 in a million bacilli,
      resistance to RIF at 1 in 100 million)
  (b) enhance efficacy of therapy (Mitchison hypothesis)
Special Bacterial Populations Theory (Mitchison Hypothesis)

- High Speed of bacterial growth
  - A. Continuous growth
  - INH (RIF, SM, EMB)

- Low Speed of bacterial growth
  - D. Dormant
  - B. Spurts of metabolism
  - Semi-dormant
  - C. Acid inhibition
  - RIF
  - PZA
Yin and Yang Model: Effect of Drugs
(Y. Zhang, Clin Pharmacol Ther. 2007; 82:595-600)

Day and Night
Body and Mind
Consciousness and Subconsciousness
INH vs PZA

Reverters

RIF, TMC207, PA-824

PZA

INH, EMB, SM

Persisters

Bacterial populations
Genetic vs phenotypic resistance
LTBI vs active TB
Explains current TB therapy
Explains INH prophylaxis for LTBI
Drug Resistance in TB: Two Types

- Genetic drug resistance: due to chromosomal mutations – **Yang Resistance**

- Phenotypic drug resistance: due to changes in bacterial physiology as in stationary phase, persisters, dormant state – **Yin Resistance**

- Interconversion of Yang and Yin resistances
In vivo versus in vitro Drug Resistance

- Selection of pre-existing spontaneous mutants in vitro - simple
- Hetero-resistance in vivo: heterogeneous with different levels of resistance with different mechanisms – complex
- XDR-TB morphology change: multi-branching, coccoid/round

(P. Farnia et al., Int J Clin Exp Med 2010;3:308-314)
Drug resistance in M. tuberculosis is NOT mediated by plasmids or transposons as in many other bacteria, but due to mutations in chromosomal genes.

MDR/XDR-TB is caused by sequential accumulation of mutations in different genes due to poor compliance or inappropriate treatment.

Primary versus secondary resistance
Mechanisms of Drug Resistance

1. Reduced permeability/uptake
2. Enhanced efflux
3. Enzymatic inactivation (beta-lactamase)
4. Alteration of drug target
5. Loss of enzymes involved in prodrug activation
   - isoniazid resistance - KatG (1992)
   - pyrazinamide resistance - PncA (1996)
Timeline of TB Drug Resistance Genes

- 1993: *rpoB* RIF resistance (Telenti et al. NEJM)
- 1994: *gyrA* FQ resistance (Takiff et al., AAC)
- 1997: *rrs* KAN resistance (Taniguchi et al., 1997)
- 2000: *ethA/etaA* ETH resistance (Baulard et al.; DeBarber et al.)
## Mechanisms of Drug Resistance in *M. tuberculosis*

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Physiologic effect/inhibition</th>
<th>Molecular target</th>
<th>Genes associated with resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>Cell wall mycolic acid synthesis; Oxygen radical-associated damage</td>
<td>Enoyl acyl carrier protein reductase (InhA)</td>
<td>Catalase-peroxidase *(katG) (KatG315, 80-90%); *inhA (-15, -8, 10-20%)</td>
</tr>
<tr>
<td>RIF</td>
<td>RNA synthesis</td>
<td>RNA polymerase</td>
<td><em>rpoB</em> (95%)</td>
</tr>
<tr>
<td>PZA</td>
<td>Membrane function</td>
<td>Membrane energy</td>
<td><em>pncA</em> (85%); <em>rpsA</em></td>
</tr>
<tr>
<td>EMB</td>
<td>Cell wall arabinogalactan synthesis</td>
<td>Arabinosyl transferase</td>
<td><em>embB</em> (EmbB306 50%)</td>
</tr>
<tr>
<td>SM</td>
<td>Protein synthesis</td>
<td>Ribosome S12 protein; 16S rRNA; 16S rRNA methyltransferase</td>
<td><em>rpsL</em> (60%); <em>rrs</em> (20%); <em>gidB</em></td>
</tr>
<tr>
<td>SLID</td>
<td>Protein synthesis</td>
<td>16S rRNA</td>
<td><em>rrs</em> (1401A-&gt;G)</td>
</tr>
<tr>
<td>Quinolone</td>
<td>DNA synthesis</td>
<td>DNA gyrase</td>
<td><em>gyrA</em> (95%), <em>gyrB</em></td>
</tr>
</tbody>
</table>
Correlation between Mutations and Drug Resistance

- INH resistance: KatG315 (80-95%), inhA (10-30%), KatG315 and inhA –15 C-to-T (95%)
- RIF resistance: *rpoB* (95%), 81 bp, 531, 526, 516
- PZA resistance: *pncA* (85%), scattered
- EMB resistance: EmbB306 (50%)
- Fluoroquinolone resistance: *gyrA* (95%)
- SM resistance: RpsL43/88(60%), *rrs* (20%), *gidB*
  amikacin, kanamycin, capreomycin: *rrs* 1400A->G
Molecular Methods for Detecting Mutations in Drug Resistance Genes

- PCR, followed by **DNA sequencing**, PCR-RFLP (HapII-KatG 315), SSCP, heteroduplex formation, WAVE-DHPLC, FRET (fluorescence resonance energy transfer) probes, molecular beacons, real-time PCR (TaqMan), hybridization (Line-Probe) tests in micro(macro)-array

- **Line-Probe assays** (Hain Lifescience GenoType MTBDRplus) currently being evaluated in the field with promising results

- **Xpert MTB/RIF TB test, Cepheid** (NEJM, 2010) 1,730 patients with drug-sensitive or MDR-TB, identified 98% TB cases and 98% patients with RIF-resistant TB in less than 2 hr

- **Limitations of Line-Probe, Xpert tests**: not for PZA resistance (pncA)

- **Next Generation DNA Sequencing:**
Bacterial Persisters

- The phenomenon first described by Gladys Hobby in 1942
- Joseph Bigger coined “persister” in 1944
- Penicillin killed 99%, residual 1% called “persisters”
- Genius: 1% inspiration and 99% perspiration; Less is more
Why Persisters and Cancer Stem Cells?
Dandelion Phenomenon
Features of Persisters

- Non-replicating, or slowly growing
- Phenotypic (Yin) antibiotic resistance, epigenetic changes, vs. stable genetic resistance (Yang).
- Not homogeneous! Heterogeneous (age, type of antibiotics, concentrations, exposure times, etc), different subpopulations (Yin-Yang model)
- Survival strategy
Persister Problem in TB

- Underlying lengthy TB therapy (6 month) -> increasing MDR/XDR-TB
- Post-treatment relapse
- Underlying latent TB infection (LTBI)
New TB cases are driven by the reservoir of latently infected people.

This “hidden epidemic” of people infected with latent TB is enormous - a time bomb.

To stop active TB cases, control LTBI by chemoprophylaxis or post-exposure vaccine.

Active TB
- 9 million new cases a year
- tip of the iceberg

Latent TB
- “hidden epidemic”
- 2 billion people infected
Yin and Yang of latent infection and active disease and their interconversions

LTBI
Reverters
Yin

Persisters
Yang
Active disease
Persister Models

- Based on definition of Bigger: Antibiotic (cidal) exposure of log phase cultures
- Antibiotic exposure of stationary phase cultures: Quinolones (ofloxacin in E. coli); 100 day old Mtb exposed to RIF and PZA
- Survivors after stresses (starvation, acid, oxidative, heavy metal, etc)
Mechanisms of Persisters

- Toxin-antitoxin module (TA) model: HipAB (Moyed in 1983); TisAB, RelBE

  - Overexpression of unrelated toxic proteins such as DnaJ, can cause higher persister formation (2006), raising question about specificity of TA model

- Other genes involved: relA, lexA, phoU, sucB, acnA, etc

- Stochastic expression of persister-related genes → persisters
Genes involved in persister formation or survival in *M. tuberculosis* (Zhang, Yew, Barer, 2012, AAC)

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Physiological Function</th>
<th>Persister/Persistence/Nonreplication</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pcaA</em></td>
<td>Cording and mycolic acid cyclopropane ring synthesis</td>
<td>Required for persistent infection and lethality in mouse model</td>
</tr>
<tr>
<td><em>sucB</em> (DlaT)</td>
<td>TCA cycle/glycolysis</td>
<td>Identified as a drug target in nonreplicating (Acid and NO treated and NRP2) bacilli.</td>
</tr>
<tr>
<td><em>menA</em></td>
<td>Menaquinone synthesis</td>
<td>Inhibitors of MenA reduce recovery of bacilli from NRP2</td>
</tr>
<tr>
<td><em>tgs1</em></td>
<td>Triacylglycerol synthase 1 (member of DosR regulon)</td>
<td>Deletion reduces development of antibiotic tolerance (persisters) after multiple stresses</td>
</tr>
<tr>
<td><em>icl1</em></td>
<td>Isocitrate lyase</td>
<td><em>icl1</em> deletion ➞ loss of persistence in mice</td>
</tr>
<tr>
<td><em>cydC</em></td>
<td>Cytochrome <em>bd</em> assembly</td>
<td>Required for persistence in isoniazid treated mice</td>
</tr>
<tr>
<td><em>mce4</em></td>
<td>Cholesterol transport</td>
<td>Required for persistence in mice</td>
</tr>
<tr>
<td><em>relA</em></td>
<td>Stringent response, ppGpp synthesis</td>
<td>Deletion reduces persistence in mice</td>
</tr>
<tr>
<td><em>carD</em></td>
<td>Regulator of rRNA transcription</td>
<td>Knockdown reduces stress resistance and persistence in mouse infection</td>
</tr>
<tr>
<td><em>prcBA</em></td>
<td>Proteasome core subunits</td>
<td>Required for persistence in in mice and long term survival in vitro</td>
</tr>
<tr>
<td><em>phoY2</em></td>
<td>Global cellular metabolism regulator PhoU</td>
<td>Deletion reduces persister formation in vitro and persistence in mouse infection</td>
</tr>
<tr>
<td><em>rpsA</em> (S1)</td>
<td>Involved in trans-translation</td>
<td>Trans-translation required for survival under stress conditions</td>
</tr>
</tbody>
</table>
PhoU is a new persister switch in *E. coli*
(Li and Zhang, 2007, AAC, 51:2092-9)

- E. coli transposon mutant library screen with ampicillin and identified PhoU mutant that had defect to produce persisters
- Increased sensitivity to a diverse range of antibiotics (norfloxacin, gentamicin, tetracycline) in MIC/MBC tests (2 fold more susceptible)
- Increased sensitivity to various stresses (starvation, acid pH, weak acids, heat)
- The PhoU mutant phenotypes can be complemented with wild type *phoU* gene
Function of PhoU and its role in persister formation

- PhoU is a negative regulator for phosphate uptake
- PhoU is a global negative regulator involved in shut-down of cellular metabolism based on microarray data, a persister switch involved in persister formation
- PhoU is a ubiquitous protein (kinase-phosphatase) and drug target for persisters
*M. tuberculosis* has two PhoU homologs PhoY1 and PhoY2

- PhoY1 and PhoY2 has 63% amino acid identity with each other
- PhoY2 and PhoY1 mutants of MTB constructed
- PhoY2 is the equivalent of PhoU, since PhoY2 mutant showed increased sensitivity to TB drugs RIF and PZA and had reduced persistence in mice
## Drug exposure of *M. tuberculosis* H37Rv and *phoY1* and *phoY2* mutant strains

<table>
<thead>
<tr>
<th>Condition</th>
<th>Drug</th>
<th>Concentration ug/ml</th>
<th>Strain</th>
<th>Beginning CFU/ml</th>
<th>CFU/ml 3 day</th>
<th>CFU/ml 9 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH5.6</td>
<td>PZA</td>
<td>200</td>
<td>H37Rv</td>
<td>$3.1 \times 10^6$</td>
<td>$3.1 \times 10^5$</td>
<td>$5.3 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PhoY1</td>
<td>$3.2 \times 10^6$</td>
<td>$4.3 \times 10^5$</td>
<td>$3.3 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PhoY2</td>
<td>$2.4 \times 10^6$</td>
<td>$4.83 \times 10^5$</td>
<td>$&lt;10^2$</td>
</tr>
<tr>
<td>RIF 0</td>
<td></td>
<td></td>
<td>H37Rv</td>
<td>$3.1 \times 10^6$</td>
<td>$6.9 \times 10^5$</td>
<td>$1.33 \times 10^5$</td>
</tr>
<tr>
<td>RIF 0</td>
<td></td>
<td></td>
<td>PhoY1</td>
<td>$3.2 \times 10^6$</td>
<td>$2.07 \times 10^6$</td>
<td>$3.97 \times 10^5$</td>
</tr>
<tr>
<td>RIF 0</td>
<td></td>
<td></td>
<td>PhoY2</td>
<td>$2.4 \times 10^6$</td>
<td>$4.77 \times 10^5$</td>
<td>$2.77 \times 10^5$</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>RIF 8</td>
<td></td>
<td>H37Rv</td>
<td>$3.1 \times 10^8$</td>
<td>$3.37 \times 10^5$</td>
<td>$1.67 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PhoY1</td>
<td>$3.2 \times 10^8$</td>
<td>$4.97 \times 10^5$</td>
<td>$3.13 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PhoY2</td>
<td>$2.4 \times 10^8$</td>
<td>$2.27 \times 10^5$</td>
<td>$&lt;10^2$</td>
</tr>
<tr>
<td>RIF 0</td>
<td></td>
<td></td>
<td>H37Rv</td>
<td>$3.1 \times 10^8$</td>
<td>$1.33 \times 10^9$</td>
<td>$3.5 \times 10^8$</td>
</tr>
<tr>
<td>RIF 0</td>
<td></td>
<td></td>
<td>PhoY1</td>
<td>$3.2 \times 10^8$</td>
<td>$2.0 \times 10^9$</td>
<td>$3.57 \times 10^8$</td>
</tr>
<tr>
<td>RIF 0</td>
<td></td>
<td></td>
<td>PhoY2</td>
<td>$2.4 \times 10^8$</td>
<td>$1.37 \times 10^9$</td>
<td>$3.0 \times 10^8$</td>
</tr>
</tbody>
</table>
### Survival of *phoY1* and *phoY2* Mutants and their Complemented Strains in Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Beginning CFU</th>
<th>CFU/Spleen</th>
<th>CFU/Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>$0.84 \times 10^4$</td>
<td>$3.03 \times 10^3$</td>
<td>$1.20 \times 10^2$</td>
</tr>
<tr>
<td>H37Rv Δ<em>phoY1</em></td>
<td>$1.18 \times 10^4$</td>
<td>$7.39 \times 10^3$</td>
<td>$3.73 \times 10^2$</td>
</tr>
<tr>
<td>H37Rv Δ<em>phoY1</em> Complemented</td>
<td>$1.25 \times 10^4$</td>
<td>$4.38 \times 10^3$</td>
<td>$3.81 \times 10^2$</td>
</tr>
<tr>
<td>H37Rv Δ<em>phoY2</em></td>
<td>$1.13 \times 10^4$</td>
<td>$1.71 \times 10^2$</td>
<td>$1.6 \times 10^1$</td>
</tr>
<tr>
<td>H37Rv Δ<em>phoY2</em> Complemented</td>
<td>$1.35 \times 10^4$</td>
<td>$5.82 \times 10^3$</td>
<td>$4.89 \times 10^2$</td>
</tr>
</tbody>
</table>

PhoY2 mutant has defect in persister formation in vitro and in vivo. Shi and Zhang, 2010, JAC. 65(6):1237-42
Pyrazinamide (PZA):
A Remarkable Persister Drug

A most important front-line TB drug, plays a key role in shortening therapy, because PZA kills persister bacilli that are not killed by other TB drugs.

Despite its powerful in vivo activity in shortening the therapy, PZA has no activity against TB bacilli in vitro in culture condition at neutral pH.
Paradoxical Features of PZA

- PZA is not active at normal culture conditions (neutral pH), but is active at acidic pH (pH 5.5)
- In vitro, MIC is high = 50-100 μg/ml at acid pH 5.5-6.0, and kills MTB slowly (50-70% kill in two weeks)
- PZA kills old, dormant bacilli more effectively than the actively growing bacilli
- In vivo (mice or humans), it has impressive sterilizing activity against persister bacilli and shortens therapy
PZA achieves high sterilizing activity with INH-basis for its use in DOT

Mode of Action of PZA

Model can explain unusual properties of PZA: Acid pH, preferential activity for non-replicating persisters, hypoxic conditions
Enhanced PZA Activity by Energy Inhibitors

PZA=100 \mu g/ml; 5 day incubation at pH5.5
Synergy Between Diarylquinoline (J) and PZA (Andries et al., 2005, Science, 307: 223-7)

Like DCCD, J compound Inhibits F1F0 H-ATPase
What is the Target of PZA?

- Fas-I was proposed as a target of PZA (Nature Med, 2000, 6: 1043-7)
- Boshoff et al. showed that Fas-I is the target of 5-Cl-PZA, but not the target of PZA (J Bacteriol 2002, 184: 2167-72)
A New Target of PZA: RpsA
(Shi et al. Science, 2011, 333: 1630-2)

A new target of PZA: RpsA binds to POA

Overexpression of RpsA conferred 5-fold PZA resistance from 100 to 500 μg/ml

A low level PZA-resistant *M. tuberculosis* DHM444 (MIC 200-300 μg/ml PZA) without *pncA* mutation (Scorpio 1997), contained 3-bp deletion (ΔGCC) Alanine missing in C-terminus of RpsA
RpsA and Trans-translation

Under stress, trans-translation rescues ribosomes stalled on defective mRNAs and adds a peptide tag sequence encoded by tmRNA to aberrant polypeptides for degradation by proteases.

Trans-translation is dispensable during active growth but becomes critical under stress conditions in managing stalled ribosomes or damaged mRNA and proteins, involved in stress survival (L-form) and virulence (Y. pestis, H. pylori, S. typhi).

RpsA binds tmRNA and facilitates trans-translation mediated by a multimeric complex of tmRNA, SmpB, Ef-Tu, RpsA.
POA Inhibits Trans-translation in MTB in Concentration-dependent Manner

Implication for developing new drugs that target trans-translation, to avoid acid pH so it can be used throughout therapy.
A Revised Model of PZA Action

(Shi et al. Science, 2011, 333: 1630-2)
Mechanisms of Action: Five Major Classes of Antibiotics

- Inhibition of cell wall synthesis (beta-lactams, glycopeptides)
- Disruption of membrane permeability (polymyxin B, daptomycin)
- Inhibition of protein synthesis (aminoglycoside, tetracycline, macrolides)
- Inhibition of nucleic acid synthesis (quinolones, rifampin)
- Anti-metabolite (sulfa drugs)
- Inhibition of persister targets, e.g. trans-translation (PZA), a new generation of antibiotics for persisters
PZA and New TB Drug Candidates – Indispensable, Synergy

FDA approved drugs:
- Rifapentine:
- Linezolid: Phase I and II trials

Drug candidates under clinical development:
- Moxifloxacin/gatifloxacin, Phase II, III
- Diarylquinoline (TMC207): Phase II trial (MDR-TB, DS-TB)
- Nitroimidazoles: PA-824 and OPC-67683, Phase II trials
- Ethambutol analog, SQ-109, Phase II trial

Limitation of current drug discovery efforts: None can replace PZA

CPTR: Build new regimens: PZA + TMC207 or PA-824 +…
Control of Persisters

- Develop persister antibiotics
  - Whole cell based
  - Target/mechanism (PhoU, SucB, RpsA etc)
- Novel drug combo
- Wake up (revert) persisters
- Host immune control
Future Challenges

- Improved detection of MDR/XDR TB
- More effective vaccines: therapeutic and latency vaccines
- New drug development: Beat two types of resistance - not only active against M(X)DR-TB (genetic resistance), but can kill persisters and shorten TB therapy (phenotypic resistance)
Thank You!