Antimicrobial Selection in Combating Resistance
(Dead Bugs Don’t Mutate!)

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The urgent need to curtail proliferation of antibacterial resistant bacteria has refocused attention on the proper use of antibacterial agents.

– HAS IT?

That the use of any antibacterial agent or class of agents over time will result either in the development of resistance to these agents or in the emergence of new pathogenic strains that are intrinsically resistant is now widely accepted.

– IS IT?
People in households who own pets were colonized with less similar (more diverse) microbiota compared to each other than to people in households without pets. This may indicate enhanced microbial sharing and perhaps more diverse microbial inputs related to pet exposure.

**Selection and use of antimicrobials**

- **BUG**
- **COST**
- **PD**
- **PK**
PK/PD Relationships

- Cmax = peak serum concentration
- AUC = area under the curve
- MIC = Min inhibitory concentration
- T > MIC should be > 40-50% of the dose

An MIC in the absence of PK/PD data is useless!

Joe Blondeau 2006

MPC

Mutant Prevention Concentration
(above the MIC)
Mutant Prevention Concentration

The MPC is the theoretical upper boundary of an antibiotic concentration window in which resistant mutants are selectively amplified.

Mutant Selection Window Hypothesis

- Resistant mutants are selected exclusively within a concentration range (mutant selection window) that extends from the point where growth inhibition begins, approximated by the MIC, up to the MPC.

- The ‘danger zone’ for the drug selective amplification of resistant subpopulations is postulated to occur in the mutant selection window (MSW). For drug concentrations falling below the MIC, neither mutant nor susceptible cells are inhibited.

- For drug concentrations falling within the MSW, susceptible cells are likely inhibited as the drug concentration is in excess of the MIC, however, mutant cells will not be inhibited as the drug concentration is below the MPC.
But what happens below the MIC?

Below the MPC...

- Fleming commented in 1945, ‘...But I would like to sound a note of warning...it is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to euthanize them and the same thing has occasionally happened in the body.’

- Undoubtedly, Fleming was warning against exposing bacteria to insufficient concentrations of drug and that doing so would ultimately encourage resistance selection.

Treatment of bacteria with low concentrations of bactericidal antibiotics can generate multidrug resistance through an increase in the mutation rate that is driven by the formation of reactive oxygen species (ROS).

Phenotypic, genetic and microarray analyses were performed with *Escherichia coli* treated with norfloxacin to identify additional contributors to cell death.

As expected the expression of DNA damage response and repair genes was markedly upregulated.

Surprisingly, significantly upregulated expression was also observed for genes related to superoxide stress, iron–sulfur (Fe–S) cluster synthesis and iron uptake and utilization.
The study showed that bacterial DNA gyrase inhibitors, including norfloxacin, induce a breakdown in iron regulatory dynamics which promotes the formation of reactive oxygen species (ROS) that contribute to cell death.

Phenotypic Response To Gyrase Inhibition.

An immediate 2 log reduction in CFU/ml was observed when 250ng/ml norfloxacin-treated cell growth (Gray triangles) was compared with untreated cell growth (Black triangles).

Conclusion

• Dwyer and colleagues proposed that a common mechanism of cell death underlies all bactericidal antibiotics, whereby hydroxyl radicals are formed as a function of leaching of iron from iron sulfur clusters, and stimulation of the Fenton reaction.
• BACTERICIDAL antibiotics (norfloxacin, ampicillin and kanamycin from 3 major drug classes) were shown to stimulate the production of hydroxyl radicals in both Gram negative and Gram positive bacteria, which ultimately contributed to cell death.

• In contrast, the bacteriostatic drugs erythromycin, spectinomycin, chloramphenicol, tetracycline, and rifamycin did not produce free radicals.

To test the hypothesis that ROS formation, due to treatment with low levels of bactericidal antibiotics, leads to an increase in mutation rates which can lead to drug resistance mutation rates were examined in *E. coli* following treatment with low levels of norfloxacin, ampicillin and kanamycin.
Low Levels of Bactericidal Antibiotics Increase Mutation Rate Due to Reactive Oxygen Species Formation

- Overnight treatment with low concentrations of antibiotics.
- All three treatment regimes led to a significant increase in the mutation rate (up to eightfold) compared with untreated.

E. coli - 5 days growth in 1 mg/ml ampicillin

Treatment of wild-type E. coli with 1 ug/ml ampicillin for 5 days led to an increase in the MIC for ampicillin AND to increased MICs for the unrelated drugs norfloxacin, kanamycin, tetracycline, and chloramphenicol.

Heterogeneous Increase in MIC

Drug resistance in a population of cells may not always be uniform, a phenomenon known as heteroresistance.

To test this, following ampicillin treatment individual colonies were isolated and the MIC was measured.

A range of MICs were observed for ampicillin (<0.25 ug/ml to >10-12.5ug/ml). Norfloxacin MICs ranged from <100 ng/ml to > 1000 ng/ml.
Below the MIC – the fast track to MDR?

• While the majority of the multidrug cross-resistant strains exhibited resistance against the treatment drug, ampicillin, the results demonstrate that treatment with ampicillin can also generate mutants that are not resistant to ampicillin yet are resistant to other classes of antibiotics.

• Prolonged exposure to weakly inhibitory drug concentrations can springboard *E. coli* and *S. aureus* from drug-sensitive to MDR (Kohanski et al 2010)

The Fast Track to Multidrug Resistance

In this issue of Molecular Cell, Kohanski et al. demonstrate that even subinhibitory concentrations of bactericidal antibiotics result in the generation of reactive-oxygen species, leading to an increase in mutation rate and the emergence of multidrug-resistant bacterial strains. Many classes of bactericidal antibiotics have been shown to provoke bacteria to generate ROS.

Consequently these results carry a startling corollary: that any bactericidal drug in a therapeutic cocktail may assist bacteria in attaining resistance to the entire combination.

The ramifications of these results are broad because high concentrations are difficult to achieve and maintain and antibiotics routinely fall to subinhibitory levels in patients between doses or in drug-inaccessible tissues.
• Reexamined the role of ROS in cell death and consequently found that killing by antibiotics is unrelated to ROS production.

• Found no correlation between an individual cell’s probability of survival in the presence of antibiotic and its level of ROS. There was essentially no difference in survival of bacteria treated with various antibiotics under aerobic or anaerobic conditions.

• Concluded that "this suggests that ROS do not play a role in killing of bacterial pathogens by antibiotics."

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**Persisters**

- Persisters represent a small subpopulation of cells that spontaneously enter a dormant, non-dividing state.
- In contrast to the well-understood mechanisms of bacterial resistance to antibiotics, molecular mechanism(s) of persistence have so far remained elusive.

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**Model of a Relapsing Biofilm Infection**

- Regular cells and persister cells form in the biofilm and are shed into surrounding tissue and the bloodstream. Antibiotics kill regular cells, and the immune system eliminates escaping persister cells. The matrix protects persister cells from the immune system, and when the concentration of antibiotic drops, they repopulate the biofilm, causing a relapse.
exposure to 0.1 mg/ml DNA damage induces SOS response which activates the RecA protein, which in turn activates the LexA repressor, causing it to cleave.

repair enzymes that contain lex boxes in their promoter regions are transcribed. TisB is transcribed

TisB-dependent Persister Formation in E. coli

TisB is transcribed in tisAB mutant strains showed a 10- to 100-fold drop in the level of persisters – strengthens the case for TisB as a specialized persister protein

To clarify issues surrounding a debate that may have clinical importance, the hypothesis that ROS contribute to antimicrobial lethality was reviewed.

Collectively the data support the idea that primary damage caused by antibiotics can trigger ROS-mediated effects.

Summary

• Low doses of bactericidal antibiotics cause mild and transient stress in bacteria and that allows ROS accumulate to a level that is sufficient for ROS to be beneficial mutagens and inducers of protective functions.

• From an evolutionary perspective – is the destructive role of ROS is merely collateral damage arising from protective activities or does self-destruction confer a selective advantage to bacterial populations?
2010 - 2017

- In the past few years new research has shown some interesting new twists.
- Factors that interfere with antibiotic lethality can compromise efficacy and contribute to emergence of resistance. One of these is the consumption of antioxidant dietary supplements, since they interfere with antimicrobial lethality.

- ROS may also be clinically significant if ways are found to boost intracellular ROS production.
- Such work could lead to novel strategies to increase the lethal action of many antibiotics (at low doses?)

Exogenous Alanine and/or Glucose plus Kanamycin Kills Antibiotic-Resistant Bacteria

A strategy to overcome bacterial resistance to antibiotics is described that bypasses the need for discovery or design of novel drugs or reagents. This strategy uses non-toxic compounds to modulate the metabolome of antibiotic-resistant bacteria, promote the TCA cycle, increase PMF, and stimulate transport of extracellular antibiotics through the bacterial cell wall/membrane into the intracellular environment.
Thank you!

Questions?

"This is one sick dog!"