An Evaluation of Antibiotic Resistance:
Structure-Activity Relationship Studies of Tetracyclic Indolines as A Novel Class of
Resistance-Modifying Agents for MRSA
&
Analysis of Recent FDA Regulations on Antibiotic Use in Livestock

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Defended February 29, 2016

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Abstract

While the rate at which resistance develops against antimicrobials rises, research and development for new antimicrobials declines. By placing selective pressure on bacteria we are inadvertently forcing bacteria into expressing and propagating genes conferring high levels of resistance. Continued misuse and overuse of antibiotics, in light of the evident problem developing, must be resolved. To find a resolve, a multidisciplinary and multifaceted approach must be taken which involves 1) research and development of novel antimicrobial agents and 2) governmental regulation.

Strides in new antimicrobial drug development largely revolve around making old antibiotics usable again. Resistance-Modifying Agents (RMAs) act to re-sensitize resistant bacteria to antibiotics through a variety of mechanisms, although currently most target bacterial resistance mechanisms themselves, such as β-lactamases. Foreseeably, while these compounds have shown efficacy and certainly are of value in the present crisis, it is a short-term solution in light of the evidently rapid and dynamic capability of bacteria to respond evolutionarily. Nonetheless, a new class of RMAs, currently being researched and developed at Wang lab, hope to extend RMA lifespan through a model of synthetic compound development that targets gene expression.

Both clinically and community-acquired resistance contribute to the demolishment of a critical building block (antibiotics) of modern medicine. Arguably the most nonsensical piece of the puzzle is subtherapeutic antibiotic use in livestock, which accounts for 80% of all antibiotic use in the United States\textsuperscript{12}. FDA regulations
are seemingly the only feasible way to fix the problem, and yet their efforts in recent regulatory measures not only contain major loopholes, but seem altogether to be largely barren of any significant resolutions.
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Terms and Definitions

CFU: Colony Forming Unit

The FD&C Act: Federal Food, Drug, and Cosmetic Act, is a set of laws that allows the Food and Drug Administration (FDA) to regulate food and drugs in the United States. It was passed by congress in 1938 and is what gives FDA their authority.

GI50: GI50 is the concentration at which the compound must be at to inhibit 50% of cell proliferation. This is a measure of the compound’s toxicity to human cells. The higher the number, the less toxic the compound is.

Medically Important: FDA ranked each antibiotic as medically important (to human health) or not. This ranking was based upon the probability of transference of resistant bacteria from animal to human, and the subsequent consequences if a human were to be exposed to resistant bacteria (GIF #152 20).

MIC: MIC, or the Minimum Inhibitory Concentration, represents the current resistance level of the bacteria to a particular antibiotic.

MRC: MRC, or Minimum Resensitizing Concentration, is the concentration of the compound (with some antibiotics) at which no overnight growth is observed. The lower the number the better.

Multidrug-resistant: “Multidrug-resistant means that the associated bacterial illness can no longer be cured by at least three different classes of antibiotics.”

Nosocomial: A disease originating in a hospital or contracted from medical care. Also termed “hospital-acquired”.

RMA: Resistance-modifying agents (RMAs) specifically target non-essential bacterial genes that are responsible for the expression of bacterial resistance mechanisms (e.g. resistance-conferring genes) and their products. They act synergistically with antibiotics, but do not exhibit antibacterial activity on their own.

SAR: A SAR, or Structure-Activity Relationship study, is the process of making slight modifications to the compound to yield a variety of analogues. These analogues can lead to more potent compounds and reveal what parts of the structure are most important to its activity. In performing a structure-activity relationship study, the basic data to determine the compound’s activity include MIC, MRC, as well as GI50, a measure of mammalian cell toxicity.
CHAPTER 1: Clinically-Acquired Resistance and the Development of Novel Resistant-Modifying Agents

1.1 Introduction & Background

1.1.1 Introduction

While we have developed advanced surgery techniques to replace hips and create bionic legs, it may be forgotten that all of these advances would be rendered useless without the ability to fight infections with antibiotics. Antibiotics are a lynchpin in modern medicine, and now they are ceasing to work. Overuse and misuse of antibiotics in the community and clinic are leading us toward an era that those living today have only read of in history books (Photo 1.1), an era describing life when morbidity and mortality were much of the same.

Low-level resistance is rising across the population through community-acquired means, such as antibiotic use in agriculture. Imminently, the strength and frequency of clinically-acquired multi-drug resistant bacteria is escalating. Based of a 2013 report from the CDC, it is estimated that antibiotic resistance causes at a minimum over 2 million illnesses and 23 thousand deaths annually in the United States\(^1\). While the rate at which resistance develops against antimicrobials rises, research and development for new antimicrobials
declines. Continued misuse and overuse of antibiotics, in light of the evident problem developing, must be resolved.

To find a resolve, a multidisciplinary and multifaceted approach must be taken. Firstly, research and development of novel antimicrobial agents must flourish since resistance will continually develop\(^1\). Secondly, governmental regulation must step in to discontinue antibiotic misuse in agriculture and livestock. And finally, the conception of antibiotics bearing little consequence must shift in the minds of clinicians and the public, so as to treat antibiotics as serious drugs with global side-effects.

### 1.1.2 The History of Antibiotics in Clinical Applications

The first antibiotic, penicillin, was discovered by Alexander Fleming in 1928, and won him a noble prize in 1945. Here is an excerpt from his Nobel lecture on December 11, 1945: “But I would like to sound one note of warning...It is not difficult to make microbes resistance to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is a danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.”\(^2\) (Figure 1.1). Now, a century later, can we still claim ignorance?

Although the discovery of antibiotics was simultaneous with the discovery of antibiotic resistance, few heeded the latter. Indeed penicillin unveiled a golden age
in medicine, where there was no foreseen downside to prescribing what was seemingly a miracle drug. As Fleming put, “[antibiotics] to all intents and purposes [are] non-poisonous so there is no need to worry about giving an overdose and poising the patient”². Furthermore, the applications of antibiotic were truly expansive, curing the deadly and the dreadful: scarlet fever, Tuberculosis, and
syphilis (Photo 1.1) to name a few. Open-heart surgery and aggressive chemotherapy for cancers such as leukemia became possible as antibiotics greatly reduced the risk of infection.3

In lieu of such a ‘miraculous’ discovery, antibiotics were over-prescribed with a carefree zeal. For instance, dentists gave antibiotics to people with heart murmurs for the extremely unlikely case of a bacterial infection; and children with ear infections were given antibiotics without running tests to find out if it was bacterially caused, which was less likely than not (80% were virally caused).3 As one could imagine, a residual affect of such cavalier prescribing is that there is still a widely favored belief among patients worldwide that it’s reasonable to take antibiotics at any hint of illness; this is particularly problematic in many parts of the world where antibiotics require no prescription. Nonetheless, in countries that do require a prescription, patients often voluntarily request antibiotics from doctors, who often oblige given the demands of rapid patient-visit turnover, the precarious legal climate currently in healthcare, and the relatively time-consuming nature of bacterial laboratory testing.3

To note, on the other side of this same issue, there is some grounded reasoning as to why you would prescribe antibiotics “just in case”. For instance, in ICUs (Intensive Care Units) there is a positive relationship between mortality due to sepsis and every hour a bacterial infection is left untreated.4 As a result this evidence favors a heavy hand in precautionary antibiotic distribution before obtaining the results of patient cultures.
Today, in the United States 80% of all antibiotics are used for non-human purposes\(^5\) that foster the development of resistant bacteria in our food supply. Furthermore, the CDC estimates that up to 50% of antibiotics prescribed in the clinical setting are unnecessary or “not optimally effective as prescribed”\(^1\). The rate of resistance developing in bacteria is increasing and far surpasses humans’ capacity to develop new antibiotics; since 1996, resistance to new antibiotics approved by the U.S. Food and Drug Administration (FDA) usually appears within a year\(^1,6\) and the number of new antibacterials approved by the FDA dwindles (Figure 1.2).

There is little research into new antibiotics given the need; this is for several reasons. Foremost, large pharmaceutical companies see little profit in the development of antibiotics. As antibiotics have developed over time, resistance has emerged increasingly quickly to new antibiotics. Aside from foreseen resistance to new antibiotics forecasting a short market lifespan, likely the primary reason for the lack of antibiotic development in big pharmaceutical companies is in the curative

![Figure 1.2. Reverse development of new antibiotics versus resistant bacteria. The abscissa shows a time bar. The ordinate shows blue bars that indicate the number of antibiotics launched in the depicted period; the red line shows the percentage of bacteria resistant against the last resort antibiotic vancomycin in US hospital intensive care units; the black line shows a moving average trend line of antibiotics launched in the depicted period. The number of antibiotic-resistant bacteria infections is increasing whereas the development of new antibiotics is constantly decreasing. Schäberle, Till F., and Ingrid M. Hack. "Overcoming the current deadlock in antibiotic research." Trends in microbiology 22.4 (2014): 165-167.](image)
nature of antibiotics. Curative drugs are less profitable than drugs designed for chronic use (such as drugs to treat blood pressure, statins, and antacids). In addition, the merging of pharmaceutical companies has resulted in less diversified research teams.

1.2 Antibiotics

While hundreds of compounds exhibiting antibiotic activity have been isolated over the years, few of these may be used clinically because many compounds have unacceptable levels of toxicity to human cells (GI50) or lack physicochemical properties that allow them to function well in the human body (such as hydrophilicity). Antibiotics that may be used clinically share one of a few mechanisms of action, which largely correlate to differences between bacterial cells and human cells.

1.2.1 Cell Wall Synthesis and Penicillin-Binding Proteins

The first mechanism applies to bacterial cell-wall synthesis; bacteria have cell walls made of peptidoglycan, a sugary amino-acid mesh around the plasma membrane; human cells do not share this feature. Gram-negative bacteria have an additional “outer” plasma membrane exterior to the cell wall, generally making them more elusive targets amongst human cells (compared to gram-positive bacteria). Commonly proteins known as penicillin binding proteins (PBP)s are responsible for the final stages of assembling the cellular wall by cross-linking polymers (Figure 1.3, part 1-3); more simply, they may be imagined as a functioning
similar to zippers, acting to zip pieces of wall together. The name “Penicillin Binding Proteins” comes from enzymes in this class having a high affinity for penicillin. This feature has been heavily extorted with β-lactam antibiotics, which serve to effectively bind to the PBP’s active site(s), inhibiting the enzyme’s transpeptidase activity (its ability to cross-link the adjacent tetrapeptides chains); essentially the β-lactam antibiotic serves to jam the “zippers” (Figure 1.3, part 4,5). If enough of the zippers are jammed, the integrity of cell wall gives and the cell dies via lysis.

Different bacteria express a variety of PBPs, most having somewhere between 4-20 types8. Co-expressed PBPs serve different functions, although not all are fully understood yet. Commonly there are two general classes of PBPs that are differentiated by molecular weight (high and low molecular mass). Of the PBPs that have been thoroughly investigated, such as those of E. Coli, it appears that inhibition of any single type of PBP does not disable cell-wall synthesis lethally8.
1.2.2 Membrane Permeability

A second mechanism of action involving the cell wall, although less common, is seen through a family of antibiotics known as lantibiotics. The most well-known member of this family is Nisin, which acts on gram-positive bacteria. Nisin’s function is two-fold; 1) It binds to an essential membrane-bound cell-wall precursor (lipid II); this inhibits transglycosylation, a step in cell wall synthesis; 2) then, it forms pores in the membrane below, increasing the permeability of the membrane 1000-fold, which ultimately leads to cell death.9 The outer membrane of gram-negative bacteria inhibits lantibiotic’s access to lipid II, and therefore lantibiotics cannot be used with gram-negative bacteria. Interestingly, while Nisin has been used for 50 years, little resistance has developed against it.10 Of the resistance that has been seen against Nisin, a correlation with increased expression of certain PBPs in combination with a histidine kinase is evident, but the mechanistic link is still unknown.

1.2.3 Protein Synthesis at Intracellular Ribosomes

A third mechanism of action targets protein synthesis at intercellular ribosomes. While both humans and bacteria have similarly functioning ribosomes, they are different sizes. This size difference is exploited by introducing antibiotics that prefer to bind to 50S or 30S ribosomal units (as oppose to the 40S or 60S subunits in human cells). Several classes of antibiotics target protein synthesis, however their mechanisms are slightly different. Although it is widely resisted among bacteria, Erythromycin is a macrolide that can grow to high concentrations
in gram-positive bacteria; it functions by reversibly binding to the 50S ribosome unit, inhibiting movement along mRNA. Similarly, Chloramphenicol and Clindamycin bind to the 50S subunit and inhibit peptide elongation; specifically through inhibition of the peptide bond formation between amino acids.

Alternatively, there are classes of antibiotics that target the 30S ribosomal subunit. Streptomycin, an aminoglycoside and effective antibiotic against gram-negative bacteria, allosterically irreversibly binds to the 30S subunit, causing a slight conformational change in the protein that causes the mRNA to be misread. Likewise, tetracyclines bind to the 30S subunit and interfere with anticodon-codon reading by blocking tRNA from binding to the ribosome\textsuperscript{11,12}.

\textbf{1. 3 The Emergence of Antibiotic Resistance}

Epidemiologically, the first documented nosocomial infections due to multi-drug resistant bacteria can be traced back to independent bacterial outbreaks in several areas of the world such as Turkey, England, and French Guiana. Interestingly, several of the outbreaks developed in neonatal units. That the outbreaks (and associated antibiotic resistance) were in neonatal units provides a model for how antibiotic resistance can develop from selective pressure. Due to undeveloped immunity in neonates, bacterial infections are particularly threatening. As a result, and likely in combination with high-levels of concern around the care of newborns, it has been commonplace to put neonates on broad-spectrum antibiotics for extended periods of time. Studies such as De Man, et al. have shown that broad-spectrum antibiotic therapy is much more likely (18-fold) to select for resistant
(especially multi-drug resistant) bacteria than narrow-spectrum antibiotic therapy\textsuperscript{13}. Furthermore, the use of antibiotics for extended periods of time contributes to the development of resistant bacteria due to killing off of nearly all variety of bacteria, aside from ones conferring resistance mechanisms. As a result, resistant bacteria proliferate and saturate the environment. As put by D. Isaacs in a review article, such treatment could be seen as “unnatural selection” for resistant bacteria\textsuperscript{14}.

Most bacteria already have resistance genes in their chromosomal make-up, as was discovered by a group of scientists studying bacteria from 30,000 years ago\textsuperscript{15}. With recent selective pressure, these genes have begun to be expressed more frequently. The study by D’Costa, et al. postulates that “new antibiotics will select for pre-existing resistant determinants that have been circulating the microbial pangenome for millennia,” and suggests that for each antibiotic existing in nature, discovered or not, exists simultaneously an antagonistic resistance determinate\textsuperscript{15}. In essence, when an antibiotic emerges in nature, so does its resistance determinant (or shortly thereafter).

\textbf{1.4 TYPES OF RESISTANCE MECHANISMS and RMA targets}

Given the lack of development of new antibiotics and the difficulty in doing so, one popular idea of combating antibiotic resistance is through making already-developed antibiotics useful again. Theoretically this could be accomplished by targeting the resistant-mechanisms that are rendering the antibiotics useless. There are several common techniques bacteria use to resist antibiotics; alteration of target site, enzymatic degradation, efflux pumps, and reduced permeability (Figure 1.4).

An important area of research is the development of compounds that specifically target these mechanisms. These compounds, termed Resistance Modifying Agents (RMAs), act synergistically with antibiotics, but do not exhibit antibacterial activity on their own (Figure 1.5).

The general idea is to create a compound to combine with current antibiotics (which are already streamlined for production) to create a new “cocktail” antibiotic. The concept of cocktail treatments is not new; it is common practice in the fight against cancer and viral infections such as HIV. For antibiotics, the cocktail-approach would increase the market life span of current antibiotics, which would appeal to pharmaceutical companies and people in the health sector.
1.4.1 Alteration of Target Site

New resistance mechanisms develop through a series of point mutations, in which those mutations that provide an advantage to the bacteria are propagated through the population over time. This basic schema of evolution and natural selection is not unlike human evolution; the key difference is the rate of bacterial evolution. For instance, in our time-kill experiments, we measured the growth of multi-drug resistant BAA44, a MRSA strain, and found that the bacterial population doubles every 8 minutes and 28 seconds.\(^1\) This rapid reproduction and population growth allows for natural selection to occur with dynamic responsiveness, being on a temporal scale much faster than our own. Favorable point mutations can quickly become common in a single population.

A frequent resistant mechanism of bacteria, albeit somewhat unintentional, is alteration of the binding site where the antibiotic attaches (such as on a PBP) so that it no longer binds to, or binds with less affinity to, the antibiotic. This often occurs through the propagation of favorable point mutations that cause conformational changes to the active site, or result in an amino acid substitution in the active site that is less favorable for binding. For instance, the portion of DNA coding for a certain PBP may have a point mutation that results in the active site having a lower affinity for β-lactam antibiotics; as is the case for the well-known PBP, PBP2a. Such mutations could be propagated, mutate again, and eventually

\(^1\) Based off the rate equation \( P(t) = P_i e^{kt} \) where \( P_i \) is the initial population, \( k \) is the growth coefficient, and \( t \) is the time in hours. OD was measured at \( t=0 \) and \( t=24 \) hours; OD was converted to CFUs using a standard curve we empirically derived. Conditions of growth were 37°C in MHB solution.
result in the active site losing all binding affinity for the antibiotic. The mutation of antibiotic target sites is a leading problem for resistance against antibiotics targeting intracellular ribosomes\textsuperscript{16}.

\textbf{1.4.2 Enzymatic Degradation: \(\beta\)-lactamases}

Enzymatic degradation of the antibiotic is most widely known through \(\beta\)-lactamases, which are the first line of resistance against \(\beta\)-lactam antibiotics. \(\beta\)-lactamases are enzymes that hydrolyze the defining and functionally essential \(\beta\)-lactam ring structure shared by all \(\beta\)-lactam antibiotics. As \(\beta\)-lactam antibiotics are one of the largest and most valuable classes of antibiotics, much research has gone into the re-sensitization of bacteria to \(\beta\)-lactam antibiotics.

While \(\beta\)-lactamases can be sorted into several broad categories, there are hundreds of variants \(\beta\)-lactamases due to point mutations resulting in slightly altered amino acid residues in the active sites. The frequency and culmination of these point mutations makes identifying and combating \(\beta\)-lactamases particularly challenging. Additionally, with horizontal gene transfer, the issue becomes even more convoluted.

A previous (and notably successful) development for re-sensitizing bacteria to \(\beta\)-lactam antibiotics has been \(\beta\)-lactamase inhibitors. Essentially an inhibitor shares structural features with the antibiotic, such as the \(\beta\)-lactam ring, and fits into the active site of the resistance-conferring (hydrolyzing) enzyme (\(\beta\)-lactamase). The inhibitor's irreversible binding and occupation of the enzyme's active site permanently disables it. While this system has shown some efficacy, resistance has
developed. Since one inhibitor binds irreversibly to one enzyme, it would make sense that this system of inhibition functions in a one-to-one inhibitor-to-enzyme ratio. Resistance develops from up-regulation of enzymatic gene expression; ultimately the resistance-conferring enzymes will be left to hydrolyze the antibiotic if the number of enzymes outweighs the number of inhibitors. The development of resistance to β-lactamase inhibitors, as seen with Clavulonic Acid, speaks to the inevitability of bacteria developing resistance mechanisms to antibiotics and inhibitors.

1.4.3 Efflux Pumps

Bacteria can also up-regulate expression of efflux pumps; this essentially causes antibiotics that enter the cell to be pumped back out before they exert their effects. This mechanism is particularly effective for bacteria and is a major contributor to multi-drug resistance. Often the efflux pumps lack specificity; this plays to the bacteria’s advantage because a pump can expel a variety of products (e.g. antibiotics) ranging in class and structure\(^\text{16}\). RMAs targeting efflux pumps could greatly aid in increasing antibiotic sensitivity to a number of clinically relevant multidrug resistant bacteria\(^\text{16}\).

1.4.4 Membrane Permeability

A common point of entrance (porins) for antibiotics aiming to act intracellularly may be altered or expression down-regulated so that the antibiotics cannot enter into the bacterial cell. This is largely important in gram-negative
bacteria, which have an additional outer plasma membrane (exterior to the cell wall), generally making them more elusive targets amongst human cells and more difficult to penetrate with antibiotics. Overall gram-negative bacteria are considered particularly dangerous in infectious disease and reduction in permeability can result in reduced uptake of antibiotics.

1.4.5 Escalating Resistance Levels

Once a bacterial resistance mechanism is expressed, the bacteria may up-regulate or down-regulate the expression of these genes under selective pressure, such as antibiotics. For instance bacteria may up-regulate the production of β-lactamases in the presence β-lactam antibiotics; this can make it more challenging to overcome resistance in cases such as β-lactamases and β-lactamase inhibitors.

Ultimately however, the primary mechanism of widespread genetic antibiotic resistance is through horizontal gene transfer including conjugation, transduction and transformation (Figure 1.6). Essentially, once a resistance mechanism is developed in bacteria, this resistance can be shared, most commonly though plasmids, to other bacteria. This is particularly problematic because bacteria can become multi-drug resistant very quickly as they share and pick-up resistance mechanisms from neighboring bacteria. Many plasmids evolve to carry DNA for multiple resistance mechanisms (often all of which are passed during conjugation)\(^17\); this is one factor suggesting that DNA resistance-conferring genes are quite close (physically) on the bacterial chromosome.
As an example, CTX-M-15, a β-lactamase that originated from a strain of E. Coli and has extended spectrum β-lactamase (ESBL) activity (meaning it has activity against most β-lactam antibiotics) has spread worldwide to other members of Enterobacteriaceae, a large family of gram-negative bacteria. This is particularly concerning as pathogenic Enterobacteriaceae are often associated with high levels of resistance, even to Carbapenems, which are considered “the last resort for treatment of serious gram-negative infections”\(^1\). The wide-scale presence of CTX-M-15 is likely to have been passed through conjugation, as it is often found on IncFII plasmids, which are highly active and associated with the genetic material of E. Coli.

1.4.6 A New Class of Resistance-Modifying Agents

Resistance-modifying agents (RMAs) specifically target bacterial non-essential genes that are responsible for the expression of bacterial resistance mechanisms (e.g. resistance-conferring genes) and their products. As of yet, the RMAs used clinically target a specific bacterial resistant mechanism (e.g. degrading enzymes, efflux pumps, or membrane permeability). While this has shown some efficacy, there is little doubt that resistance to these RMAs will emerge (if it hasn’t already, as seen the case of Clavulanic Acid). Furthermore, bacteria can express multiple resistance mechanisms, so even if one mechanism is inhibited, another mechanism could just be up-regulated or expressed. For instance, it is estimated that at least 10% of resistance in MRSA strains is due to efflux pumps alone\textsuperscript{16}. Therefore, while such RMAs offer short-term solutions, not to undermine their value clinically, but they will be transient in the fight against resistance.

A new class of RMAs may show a renewed promise. Instead of targeting the resistant mechanisms specifically, or the product of gene expression, what if we could target the very expression of resistant mechanisms (e.g. resistance-conferring genes) (Figure 1.7)?

If the bacteria cannot express the resistance mechanisms or up-regulate production of such mechanisms, theoretically we could cut off the very development of the resistance problem to begin with. Under the theory that resistance-conferring genes are (physically) close to one another on the bacterial chromosome (based upon plasmid transference of multiple resistant genes for one); it is reasonable to postulate that any compound affecting this region of the chromosome could regulate expression of several resistance mechanisms (Figure 1.7).

As an example, while the normal response of bacteria under antibiotic pressure may be to up-regulate expression of β-lactamase, the RMA would antagonistically prevent this increase. If expression were unable to increase, then our ability to target resistance mechanisms would become more manageable. For instance, in the case of the β-lactamase inhibitor clavulanic acid, bacterial up-regulation of β-lactamases can overcome inhibition (due to the 1:1 irreversible binding ratio). If β-lactamase expression could not up-regulated, the combination of β-lactams and β-lactamase inhibitors, such as clavulanic acid, could remain effective.

1.5 Working in Biochemical Space

While this all may sound sensible and clever, by no means is it easy. There are several components of working in biochemical space that are worth highlighting. Firstly, the significance of mutation and specificity should not be underestimated. To illustrate this point the physiological example of two human hormones, vasopressin and oxytoxin may be used. These two nonapeptides differ only by two amino acids, which is the result of only three different nucleotides.\textsuperscript{22,23}
and yet they have completely different effects in the body. Vasopressin primarily helps regulate water balance and blood pressure, while oxytocin stimulates uterine contractions. Clearly their structures are very deliberate and any change in either hormones’ amino acid sequence (which could result from a single point mutation) could result in dysfunction. To extend this example to the development of RMAs, only the slightest alteration in RMA structure could make it both incredibly effective or completely futile; thus the task of discovering novel, effective RMAs in a vast chemical space is comparable to (strategically) finding a needle in a haystack. Likewise, based upon the significance of only three nucleotides making the difference between these two human hormones, clearly it would take very little mutation to make significant alterations (or lead to resistance). With the right point mutation(s), theoretically the active site of a β-lactamase (or any other protein) could change dramatically.

Also it is worth highlighting some points on protein function in biochemical space. Biochemically, protein folding into tertiary structures is incredibly complicated to model due to the variety of interactions that occur in the folding process as a result of different charges, structures, and electronegativity. Even if we know the amino acid sequence for a given protein (which is possible with DNA sequencing) the three dimensional nature the protein takes is harder to fully model. The active sites of such proteins are also complicated three-dimensional pockets, and while we may know some features within them, only slight changes to the RMA chemical structures can greatly increase binding affinity because it may induce a better fit into the pocket (as would apply to β-lactamase inhibitors for instance).
Furthermore, when a protein binds to a compound or, for instance DNA, the protein often undergoes a conformational change. This is even harder to model. Protein binding systems, which includes the system of DNA expression, therefore are highly complicated however also open to manipulation in a variety of ways. For instance, theoretically a compound (e.g. RMA) could alter a regulatory protein by allosteric binding (binding to a site on the protein other than the active site) which could induce a conformational change resulting in an unfuctional active site, or stabilize the repressor protein’s binding to DNA (as is theorized with Of₁20), resulting in reduced expression. In essence, the systems in which we are working are highly varible, sensitive, and complicated to model; yet, due to those same features, open to manipulation, so RMA development is a perpetual task but highly hopeful.

1.6 Wang Lab
1.6.1 Background

A key hypothesis in development of new antimicrobials in Wang lab is that synthetic structures, as compared to natural compounds, will have a longer effective lifespan before resistance develops against them. The theory behind this lies in that most bacteria already have resistance genes in their chromosomal make-up15. Under this theory, if bacteria have ancient encoded DNA for resistance genes, it would be a precondition that it was through exposure to some natural compound(s) (e.g. antibiotics). Therefore, as bacteria have not yet come across the synthetic compounds, they will not already have coordinated resistance genes buried
somewhere in their microbial genome. While it is inevitable that resistance genes, even against synthetic compounds, will develop in bacteria\textsuperscript{1}, it will be more time consuming to develop new resistance genes than express genes already in their genetic make up.

A catch of course to the development of synthetic compounds that will be effective against antimicrobials is that the vast majority of compounds existing in chemical space have no antimicrobial activity; as was realized in the late 1990s when pharmaceutical giant GlaxoSmithKline spent $200 million on putting a library of 500,000 synthetic compounds through 70 biochemical screens, only to yield no clinically exploitable compounds\textsuperscript{18}. Thus, approaching synthetic drug development can be a challenge.

Several years ago a polycyclic indoline library was synthesized and screened by Wang lab. This library was bio-inspired based upon the principle that naturally occurring indole alkaloids have a wide array of bioactivity, the majority of which were plant secondary metabolites. For instance, melatonin and serotonin, well known human hormones with psychoactive qualities, have indole-based structures. Indole-derivatives have a major role in many areas of medical pharmaceuticals including applications as anticancers, vasodialators, antihypertensives, anti-HIVs, antivirals, β-blockers, and opioid agonists – many of these are derivatives of naturally occurring parent compounds\textsuperscript{19}. To make a synthetic library similar to highly-active natural indoles alkaloids, Wang lab synthesized a library of polycyclic indolines (Figure 1.9); the basic structural difference is the absence of a double bond in the five-carbon ring of the latter (Figure 1.8).
The library consisted of approximately 170 different synthetic indoline compounds and 26 unique skeletal structures, a comparatively small compound library compared to GlaxoSmithKline’s. To test for bioactivity, the library was screened for anticancer activity (since many indoles have anticancer properties) and antimicrobial activity.

Several compounds were hits, showing bioactivity in the screens. That there were several hits supported the concept that a successful library should be synthesized with some strategic methodology in terms of the chemical space it is derived from.

The indoline library was further screened specifically for RMA activity with MRSA ATCC BAA-44, a strain resistant to a variety of classes of antibiotics including β-lactams and tetracyclines, and S. aureus (VRSA) NR-46421, a bacterial strain.
specifically resistant to vancomycin. There were 10 hits; the most promising of these hits was a compound known as Of1 (Figure 1.10).

Of1 was further analyzed to understand its mechanistic function in re-sensitizing resistant *S. aureus* to β-lactam antibiotics. It was shown that Of1 targeted non-essential, resistance-conferring bacterial genes. This was shown through gene expression studies performed by Wang lab and described more thoroughly in Dr. Podoll’s PhD thesis, but is worth highlighting. Essentially, a RT-qPCR gene expression assay was performed on two strains of bacteria, one which expresses a common β-lactamase (blaZ), and the other both β-lactamase (BlaZ) and PBP2a (MecA), a common ‘resistant’ variant of a penicillin binding protein that weakly binds β-lactam antibiotics. Each was grown with varying amounts of amoxicillin and Of1 for an hour, the cells were lysed, and RNA collected; primers for BlaZ and mecA were then used to find the transcript levels with RT-qPCR and normalized against 16S rRNA levels. In both strains, BlaZ transcription was reduced in a dose-dependent manner correlated to increasing concentrations of Of1 (in combination with a constant concentration of amoxicillin). This supported a previous hypothesis on Of1’s action of reducing β-lactamase expression, however, the real excitement came with the empirical data showing that PBP2a (mecA) transcription was also reduced in a similar dose-dependent manner. While the exact mechanism of action is still unknown, the results of this study indicate, “...that Of1 targets some aspect of the
bla/mec induction pathway and accordingly reduces transcription of the resistance determinants”\textsuperscript{20}.

This promising discovery suggests that it is an entirely new class of resistance-modifying agents. While there have been discoveries of mechanism-targeting resistance-modifying agents, such as β-lactamase inhibitors, the targeting of and foreseen regulatory ability of gene expression is exciting because it could inhibit a variety of resistance mechanisms, as oppose to just one.

1.6.2 YXL-166

Although Of1 was the most promising of the compounds screened, there were 9 other “hits”. The second most promising compound from this screen was a compound called “YXL-166”, which is now undergoing further analysis and is the compound featured in this study. In the initial screens, YXL-166 showed an ability to potentiate methicillin, a narrow spectrum β-lactam, in MRSA BAA-44. Tests were then run with a panel of antibiotics and YXL-166 showed to potentiate a variety of β-lactam antibiotics but did not show significant potentiation with other antibiotic classes (Table 1.1).
### Table 1.1

<table>
<thead>
<tr>
<th>Class</th>
<th>MRSA (BAA-44)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
</tr>
<tr>
<td><strong>β-lactam</strong></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td>256</td>
</tr>
<tr>
<td>Amoxicillin/Amoxicillin Acid Mix</td>
<td>32/16</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>256</td>
</tr>
<tr>
<td>Cefazolin (1g cephalosporin)</td>
<td>256</td>
</tr>
<tr>
<td>Meropenem</td>
<td>32</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>64</td>
</tr>
<tr>
<td>Lipopeptide</td>
<td></td>
</tr>
<tr>
<td>Daptomycin</td>
<td>256</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>16</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&gt;256/128</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>256</td>
</tr>
<tr>
<td>Linezolid</td>
<td>2</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>2</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>32</td>
</tr>
<tr>
<td><strong>Vancomycin</strong></td>
<td>1</td>
</tr>
</tbody>
</table>

NR = non-resistant, so no MIC can be taken

### 1.6.3 SAR Analysis

A structure-activity relationship study is performed to fully understand which parts of the compound are most crucial to the compound's activity and if any modifications can be made to discover a more potent lead compound. To be clear, there are several factors that must be accounted for in the potency of a compound. An ideal compound has the qualities of being effective at low concentrations, minimally toxic to mammalian cells, and versatile in the strains of resistant bacteria and a specific class of antibiotics it can work with. Furthermore, there are considerations that must be made in terms of the end goal of its use in humans, or in other words the transition from in vitro to in vivo testing and efficacy. For instance,
in vivo, high water solubility and hydrophilicity (and by effect polarity) are necessary physiochemical properties for the RMA to be effective in the human body.

Modifications were first made on the indole nitrogen. The initial compound (entry 1, Table 1.2) had a methyl group and the MRCs for cefazolin and amox/clav (a combination of amoxicillin and the β-lactamase inhibitor clavulanic acid), were 8 µg/mL and 16 µg/mL, respectively. By changing the group to an ethyl (entry 3, Table 1.2), the MRC decreased four-fold for both cefazolin and amox/clav and the GI\textsubscript{50} also increased slightly. Exchanging the methyl with hydrogen (entry 2, Table 1.2) kept the MRCs the same and adding a propargyl (entry 4, Table 1.2) dramatically increased the MRCs.
Next, modifications were made on the phenyl ring. The initial compound (entry 1, Table 1.3) had a methyl on C4 (R2) and C6 (R4) of indoline, with a hydrogen in between at the C5 (R3). The analogues all had hydrogens at R2 and R4, and then had either methyl (entry 12, Table 1.3), hydrogen (entry 13, Table 1.3), or chlorine (entry 14, Table 1.3) at R3. The hydrogen at R3 dramatically reduced RMA activity, while the methyl and chlorine both increased RMA activity equally, two-fold for cefazolin and four-fold for amox/clav. However, chlorine was better overall because it increased the GI$_{50}$ by nearly 200 percent.

<table>
<thead>
<tr>
<th>Table 1.3</th>
<th>BAA-44</th>
</tr>
</thead>
<tbody>
<tr>
<td>entry</td>
<td>compd</td>
</tr>
<tr>
<td>1</td>
<td>166</td>
</tr>
<tr>
<td>12</td>
<td>YG-44-1</td>
</tr>
<tr>
<td>13</td>
<td>YG-46-1</td>
</tr>
<tr>
<td>14</td>
<td>YG-55-1</td>
</tr>
</tbody>
</table>

$^a$All GI$_{50}$ and MRC values are in µg/mL.
Next, a variety of modifications were made on the second nitrogen, most of which did not improve RMA activity. The general trend pointed to the importance of the carboxyl group skeleton remaining on the second nitrogen, although it could withstand slight modifications on the distal end, which improved RMA activity. The initial compound (entry 1, Table 1.4) had a methyl group coming off the carboxyl group. Enlarging the methyl to an ethyl (entry 5, Table 1.4) or tert-butyl (entry 18, Table 1.4) group improved RMA activity two-fold for cefazolin and four-fold for amox/clav. Also both dramatically improved mammalian cell toxicity, the ethyl

![Chemical Structure](attachment:image.png)

**Table 1.4**

<table>
<thead>
<tr>
<th>entry</th>
<th>compd</th>
<th>R₅</th>
<th>GI₅₀ (HeLa)</th>
<th>Amox.Clav</th>
<th>Cefazolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>166</td>
<td>NCOOMe</td>
<td>20</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>YG-I-12-1</td>
<td>NCOOEt</td>
<td>&gt;200</td>
<td>4/8</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>YG-I-14-1</td>
<td>NCOOBn</td>
<td>&gt;200</td>
<td>64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>7</td>
<td>YG-59-1</td>
<td>NTfa</td>
<td>&gt;200</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>8</td>
<td>YG-58-2-1</td>
<td>NAc</td>
<td>15</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>9</td>
<td>YG-I-13-1</td>
<td>NSO2PhCl</td>
<td>179</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>10</td>
<td>YG-I-09-1</td>
<td>N-Me</td>
<td>25</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>11</td>
<td>YG-I-16-1</td>
<td>N-Et</td>
<td>23</td>
<td>32/&gt;64</td>
<td>16</td>
</tr>
<tr>
<td>17</td>
<td>YG-I-90-1</td>
<td>NCONHBn</td>
<td>&gt;200</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>18</td>
<td>YG-I-95-1</td>
<td>NCOOEtBu</td>
<td>159</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>YG-I-109-1</td>
<td>NSuc</td>
<td>&gt;200</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>22</td>
<td>YG-98-1</td>
<td>NCON6NMe</td>
<td>&gt;200</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>23</td>
<td>YG-I-115-1</td>
<td>NCONCOCCl3</td>
<td>19</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>24</td>
<td>YG-I-99-1</td>
<td>NGuaBoc2</td>
<td>11</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>25</td>
<td>YG-I-116-1</td>
<td>NGua</td>
<td>19</td>
<td>32</td>
<td>64</td>
</tr>
</tbody>
</table>

*aAll GI₅₀ and MRC values are in µg/mL.*
(entry 5, Table 1.4) to levels above the testing range (>200), suggesting nominal toxicity to human cells.

![YG-I-101-1](image)

![YG-I-108-2](image)

**Table 1.5**

<table>
<thead>
<tr>
<th>entry</th>
<th>compd</th>
<th>R₆</th>
<th>R₇</th>
<th>GI₅₀ (HeLa)</th>
<th>Amox.Clav a</th>
<th>Cefazolin a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>166</td>
<td>CH₂CH₂</td>
<td>Closed Ring</td>
<td>20</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>19</td>
<td>YG-I-101-1</td>
<td>CH₂</td>
<td>Closed Ring</td>
<td>40</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>YG-I-108-2</td>
<td>CH₂CH₂</td>
<td>Open Ring</td>
<td>&gt;200</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

aAll GI₅₀ and MRC values are in µg/mL.

Analogs with ring modifications including ring openings and decreasing ring size were made. Opening the ring (entry 20, Table 1.5), although improving the GI₅₀ to nominal toxicity levels, dramatically increased the MRCs (>64 µg/mL), so the ring structure was deemed an important part of the compound. The initial ring was seven-membered (entry 1, Table 1.5); a change to a six-member ring (entry 19, Table 1.5) improved RMA activity two-fold and slightly increased GI₅₀.
After each part of the structure was analyzed for activity the most promising qualities were combine to create a “super” analogue. R1 remained the mostly the same as in the initial compound (entry 1, Table 1.6) as a methyl. R2, R3, and R4 which was initially methyl, hydrogen, methyl (entry 1, Table 1.6) was exchanged for a hydrogen, methyl, hydrogen (entry 12, Table 1.6) or hydrogen, chlorine, hydrogen (entry 14, Table 1.6) based off of both (entry 12, Table 1.3) and (entry 14, Table 1.3) equally reducing the MRCs two-fold for both amox/clav and cefazolin. R5 was initially NOOMe (entry 1, Table 1.6), and was exchanged for several substitute varieties, including NCOOEt (entry 5, Table 1.2) which had reduced the MRC by at least two-fold for both amox/clav and cefazolin. Although R5 also had good results with NOOtBu (entry 18, Table 1.4), this large group is very hydrophobic so would likely not work well in vivo, thus was not continued into the final analogue.
structures. R6 which initially was a 7-member ring (entry 1, Table 1.5) was replaced mostly with a 6-member ring (entry 19, Table 1.5) as it had reduced the MRC two-fold for both amox/clav and cefazolin.

Interestingly, none of these analogues had superior activity to some of the earlier analogues in the study. Ultimately the best analogue to move forward with was determined to be (entry 5, Table 1.4), or YG-I-12-1 (Figure 1.11). While other analogues also had MRCs for both amox/clav and cefazolin of 4 μg/mL, (entry 5, Table 1.4) also had a GI50 of >200, indicating that it was nominally toxic to human cells.

1.6.4 Future Directions

This process of compound development is ongoing, and a new set of 166 analogues are currently undergoing testing. It is important to recognize that in this methodological, yet exploratory form of research it is common to continue to develop the project over extended period of time. For instance, even Of1, the landmark RMA compound developed by Wang lab in 2013, is still undergoing further experimental modifications. This is because slight alterations can make a world of a difference due to the complexity of biochemical space and the interactions within it.

Nonetheless, we do plan on significantly moving forward with several compounds in the coming year. Specifically, there will be further testing of several
more (new) YXL-166 analoges with several strains of multi-drug resistant MRSA in the coming month. We anticipate discovering a more potent lead compound based upon subsequent modifications following the SAR analysis (above). Time-kill trials will be run with Of1 and the lead compound(s) derived from YXL-166. And excitingly, we are in the process of writing a proposal for mice trials, which we anticipate will commence this year.

1.6.6. Materials and Methods

BAA-44 was scratched on triptcase soy broth (TSB) agar plates and incubated at 37°C overnight. Individual colonies were then selected and grown in Mueller Hinton Broth (MHB) for approximately six hours at 37°C in a shaker at 220 RPM. Using a spectrophotometer, the optical density was then measured at a wavelength of 600 nm to determine the absorbance of the sample (OD<sub>600</sub>), which correlates to cell density; increased incubation time exponentially increases the number of cells in the sample until saturation. For our purposes, an OD of 0.200-0.400 was ideal because it suggests the bacteria are in a healthy prolific state (as oppose to saturation where they would be in a starving state). The OD does not provide an absolute value for cells/mL, so a standard curve must be created to appropriately quantify cell density.

A standard curve was created by using 10x serial dilutions of a sample starting at an OD of 0.100. 5 μl of each sample was plated on 33 cm<sup>2</sup> agar plates, spread with 5 glass beads each. Each diluted sample’s OD was also measured and recorded. After overnight incubation at 37°C, individual colonies, or Colony-
Forming Units (CFU) were counted. It is assumed that each cell will give rise to a single colony, thus cell count can be determined as equivalent to CFUs. When a preliminary dilution range was determined as diluted enough to count individual cells (some dilutions resulted in too high of a cell concentration to count, appearing as “lawns” on the plates), this range was repeated in triplicate to determine a standard error. Using Microsoft Excel, a standard curve was calculated (Figure 1.12).

![Graph](image)

**Standard Curve for BAA-44 OD<sub>600</sub> vs. CFU**

The MIC, or minimum inhibitory concentration, of BAA-44 with cefazolin and amox/clav, respectively, was measured using a standard microdilution method. Each antibiotic was serial diluted two fold from 256 - 2 µg/mL for Cefazolin and 64 - 0.5 µg/mL for Amox/clav, with 40 µl in each well. Bacteria was diluted in MHB to an OD of 0.005 and 10 µl of was added to each well (calculated for a final bacteria OD of 0.001 based on a 5:1 dilution); the plates were then incubated in a shaker at 37°C
for 18 hours. Following the 18-hour growth period, the wells were examined by holding them up to a light; clear wells indicated inhibited bacterial growth and cloudy were not inhibited. This was repeated in triplicate. The lowest concentration of antibiotic where growth was inhibited, as indicated by a clear well, was the MIC. The BAA-44 MICs for cefazolin and amox/clav, respectively, were >256 µg/mL and 32 µg/mL.

Similar MICs were also performed with each synthesized compound (RMA) with two-fold serial dilutions from 64-0.5 µg/mL and bacteria. All MICs were >64 µg/mL, indicating that the compounds had no inhibitory effects on their own.

Next, the MRC, or minimum re-sensitizing concentration, was measured; this was to assess the compound’s bioactivity in combination with the antibiotic on the bacteria. Compounds maintained the same serial dilutions as before (64-0.5 µg/mL), while the concentration of each antibiotic was held constant at 8 µg/mL for cefazolin and 4 µg/mL for amox/clav. Similarly as before, bacteria were added so the final OD of bacteria added to each well was 0.001. The plates were then incubated in a shaker at 37°C for 18 hours. Each assay was performed in triplicate to confirm the results. Throughout the MRCs, Of1 was used as a control.

1.6.6 Time-kill Experiments

Time-kill experiments are used to monitor the growth of bacteria under various conditions; in our case, the experiment is used to monitor the activity of RMAs and antimicrobial activity. It helps with analysis by providing information such as “the rate of kill, time from addition of antibiotic to initiation of killing,
and...degree of killing observed”\textsuperscript{21}. By constructing a best-fit curve, we can also see the degree and process of synergism between the RMA and antibiotic, as compared to the antibiotic alone by comparing the curves. This testing is still in progress, but has been run with BAA-44 and different concentrations of amox/clav (Figure 1.13).

**Figure 1.13. BAA44 growth over 24 hours with no antibiotics, $\frac{1}{2}x$, 1x, 2x, and 4x MIC of amox/clav.**

Aliquots of 500 mL Mueller-Hinton II broth cation adjusted (CAMHB) were put in sterile culture tubes. Amox/clav was added to equal [0, 8, 16, 32, and 64] $\mu$g/mL, which correlates to no antibiotics, $\frac{1}{2}x$, 1x, 2x, and 4x the MIC of Amox/clav, respectively, with BAA44 (16 $\mu$g/mL). A primary culture that was grown overnight until near-saturation (12hr) was sub-cultured to an OD of 0.1 in each culture tube. Initial OD was measured for each culture, and the cultures were placed in a shaker at 37°C. The OD was measured again at 2, 6, and 24 hours. Using the standard curve previously calculated, OD was converted to CFUs and growth was plotted. Growth was markedly inhibited at an MIC of 2x and 4x Amox/clav.
1.7 References


2 Fleming, Alexander. Nobel lecture on penicillin. PA Norstedt & Söner, 1947


5 Natural Resources Defense Council: www.nrdc.org


CHAPTER 2: Community-Acquired Resistance and FDA Regulations

2.1 Introduction

Today, in the United States 80% of all antibiotics are used in food-producing animals\textsuperscript{12}, and there is a wide consensus that a major cause of human community-acquired resistance to antibiotics is due to antibiotic use in food-producing animals. While most deaths due to antibiotics resistance occur from clinically-acquired resistance, a result of “unnatural” selective pressure, “most antibiotic-resistant infections happen in the general community”\textsuperscript{1}. This is the result, foremost, of extraordinarily large quantities of antibiotics being used sub-therapeutically in the food chain, especially in livestock. This problem of misusing and overusing antibiotics in livestock has long awaited governmental regulation; recently FDA released the much-anticipated regulatory changes.

In December 2013 FDA asked for sponsors (e.g. the pharmaceutical companies manufacturing the antibiotics) to voluntarily commit to submitting medically important antibiotics for reclassification and relabeling within three months. If sponsors did not voluntarily submit the antibiotics requested, FDA indicated that legal action would be taken (under the FD&C Act\textsuperscript{i}) to assure that any medically important antibiotics were re-regulated. The vast majority of sponsors voluntarily submitted the paperwork for relabeling and reclassifying to FDA.

However, the FDA strategy of re-regulating antibiotic use will not succeed in solving the major problem of antibiotic misuse in food producing animals. Not only

\textsuperscript{i} The FD&C Act, or Federal Food, Drug, and Cosmetic Act, is a set of laws that allows the Food and Drug Administration (FDA) to regulate food and drugs in the United States. It was passed by congress in 1938 and is what gives FDA their authority.
did their strategy contain major loopholes to begin with, but as it rolls out, it is clear that vital components of the planned re-regulation have failed to be executed, thus is largely barren of any significant resolutions.

2. 2 The Mechanics of Community-Acquired Antibiotic Resistance

Because microscopic bacteria are everywhere, the process of resistance can build up in animals, plants, and humans. As humans eat animal meat that already has developed resistant bacteria (which has been encouraged through feeding the animal subtherapeutic doses of antibiotics), the pathogenic bacteria travels via the meat into our gut. In the human gut there is a flora of hundreds of kinds of “good” bacteria that help break down the meat and help our bodies absorb nutrients. During this process the “good” human bacteria meet the pathogenic bacteria (in the meat) and become infected with the same resistance mechanisms. When a resistance mechanism has been introduced and spread into the flora of human gut bacteria, it is unlikely that the human would take any notice. The generalized accumulation of resistance bacteria will propagate in the general population because people serve as both reservoirs and vectors for bacterial populations.

Therefore, the resistance bacteria will proliferate and be excreted through urine in feces back into the communal environment. Only later when a human becomes ill with a (unrelated) bacterial infection and seeks treatment would it become apparent that prescribed antibiotics won’t work for that patient due to unknown resistance. This can be seen by penicillin’s efficacy being virtually eradicated today.
2.3 A Short History: Legal Regulations of Antibiotic Use in Livestock

Fifty years ago, farmers were concerned with how to supply enough food to sustain a booming population. It was the golden age of antibiotic discovery, and a time that revolutionized medicine. While initially used to treat and prevent illnesses in animals, it was quickly discovered that antibiotics also helped the animals grow bigger and more quickly (producing more food for human consumption); it seemed to be a godsend. Additionally antibiotics were used in humans. What could be the danger? Because antibiotics were so promising, in the 1960s the Food and Drug Administration (FDA) approved classification and labeling for many antibiotics as over-the-counter drugs for growth promotion purposes (“production uses”) in livestock.

Nearly ten years later, the 1969 “Swann Report” came out of the United Kingdom stating: “There is ample and incontrovertible evidence to show that man...commonly ingest[s] [gut] bacteria of animal origin,” and “It is clear that there has been a dramatic increase over the years in the numbers of stains of [gut] bacteria of animal origin which shows resistance to one or more antibiotics...This resistance has resulted from the use of antibiotics for growth promotion and other purposes in livestock.” Following this report, FDA began researching more into the issue and in 1977 FDA expressed concerns over the use of antibiotics in livestock. FDA proposed to withdraw two of the most vital classes of antibiotics, penicillins and tetracyclines, from subtherapeutic uses in livestock. However, by this time, antibiotics in livestock had become an essential industry standard for farming and was highly profitable for the pharmaceutical industry. FDA met much resistance
from Congress to make any legal changes that would diminish the use of antibiotics in food-producing animals.

Over the following twenty years, research flooded out from all over the world linking antibiotic use in animals to human resistance. Agencies far and wide looked into the issue, including the World Health Organization (WHO), the World Organization for Animal Health (OIE), the Food and Agriculture Organization of the United Nations (FAO), and the Institute of Medicine (IOM) to name a few. In 2003 a joint panel of experts from FAO, OIE, and WHO convened and agreed that there was increased human resistance due to “non-human usage of antimicrobials”; the subtherapeutic use of antimicrobials “increased the occurrence of resistant bacteria”; the foodborne route was a major “pathway for resistant bacteria and resistant genes”; and “the consequences…are particularly severe when [the resistance is to antimicrobials that are] critically important in humans”\(^2\).

As the evidence grew, the topic was passed between governmental agencies in the United States. In 2004 the United States Government Accountability Office (GAO) confirmed that “antibiotic-resistant bacteria have been transferred from animals to humans”\(^4,5\) and “this transference poses significant risks to human health.” The Department of Health and Human Services (HHS) agreed saying, “we believe that there is a preponderance of evidence that the use of antimicrobials in food-producing animals has adverse human consequences…There is little evidence to the contrary.”\(^5\). All regulatory governmental bodies looking into the issue agreed that antibiotic misuse in food-producing animals is a major threat to human health.
However, as antibiotic trade is a multi-billion dollar industry, industrial giants in the pharmaceutical and farming industries heavily opposed any changes. Congress refused to acknowledge research linking antibiotic resistance in humans to antibiotic use in food-producing animals as significant and has been a major impediment to any potential solution, having thrown out twelve bills.

### U.S. Congressional Legislation Relating to Antibiotic Use, 2004–2014

#### Agricultural antibiotic use (80% of current US use by weight)
- **Preservation of Antibiotics for Medical Treatment Act (PAMTA)**
  - Comprehensive plan to limit antibiotic use for human medical purposes, would require proof of illegal human farm through resistance for animal antibiotics not deemed safe enough for human use.
- **Food, Conservation, and Energy Act**
  - Promote research and education grants for studying antibiotic-resistant bacteria in pets, farm antibiotic use, and precautions in human and veterinary medicine.
- **Food Safety Enhancement Act**
  - Would direct FDA to conduct research analyzing antibiotic resistance in food supply and evaluating methods of reducing transfer of antibiotic resistance to humans.
- **Safe Meat and Poultry Act**
  - Would direct FDA to conduct research analyzing antibiotic-resistant bacteria in meat and poultry, including on emerging pathogens and pathogens of public health significance.
- **Delivering Antimicrobial Transparency in Animals (DATA) Act**
  - Plans to document non-medical non-animal antibiotic use by requiring drug manufacturer, large-scale, and the FDA to improve use reporting with detailed data.

#### Human antibiotic use (20% of current US use by weight)
- **Infectious Diseases Research and Development Act**
  - Would establish task force to identify resistant pathogens and strategies for combating them, and direct FDA to issue guidelines on antibiotic clinical trials.
- **Medicare and Medicaid Improvements Act**
  - Would establish a minimum performance level for antibiotics used in hospitals.
- **Food and Drug Administration Amendments Act**
  - Promote research and education grants for studying antibiotic-resistant bacteria in pets, farm antibiotic use, and precautions in human and veterinary medicine.
- **Community and Healthcare-Associated Infections Act**
  - Would direct CDC to establish widespread public awareness campaign on appropriate antibiotic use. Would also conduct and fund research on bacterial infections and diagnostics.
- **Seizing Infections Through Research and Development (SIRD) Act**
  - Highlights need for new antibiotic research and rapid diagnostics for bacterial infections. Would direct HHS Secretary to establish clinical guidelines for antibiotic prescription.
- **Strategies to Address Antimicrobial Resistance (STAR) Act**
  - Comprehensive plan to combat antimicrobial resistance. Would establish Office of Antimicrobial Resistance in HHS, direct CDC to evaluate 15 surveillance efforts for resistance, establish strategic plan for stewardship, and collecter, and regional cooperatives.
- **Generating Antibiotic Incentives Now (GAIN) Act**
  - Grants approval for early clinical trials for newly approved antibiotics that treat serious infections, grants priority review for such antibiotics, establishes FDA list of “qualifying pathogens” that are public health threats.
- **Antimicrobial/Data Collection Act**
  - Would require data collection and analysis on antibiotics to improve quality and accuracy of collection and create new strategy for sales, distribution, and other practices.
- **Antibiotic Development to Advance Patient Treatment (ADAPT) Act**
  - Would accelerate FDA antibiotic approvals by allowing for smaller study populations, expedite labeling of antibiotics for use in specific populations, and provide for intensive monitoring of antimicrobials.

#### Furthest progression of bills in Congress:
- Bill signed into law (2 out of 14 bills)
- Bill passed only one house of Congress (1/14)
- Bill referred to committee (11/14)

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proposed bills addressing the issue over the last ten years⁶ (Figure 2.1). As a result, and with encouragement of the GAO, WHO, and other major agencies around the world, it falls to FDA to address the issue.

From a legal standpoint, it is within FDA’s power to ban the use of previously-approved drugs if the drugs later are shown to risk human health through the FD&C Act. However, in that scenario, FDA must also prove that the drug use is harming humans. Since there are many types of antibiotics and combination therapies being produced, this task would be particularly tedious, time consuming, and difficult. Additionally, there are other factors weighing into the issue of regulation. Notably, animal rights; sick animals should be allowed treatment. And of course, with antibiotics reaching 14.6 million kilograms sold and distributed to farms in 2012 alone⁷, there is a clear point of contention economically from vested industries: a battle not for the faint at heart. Nonetheless, FDA proposed a plan.

2.4 FDA’s Plan for the Re-regulation of Antibiotic Use in Livestock

Starting in 2003, FDA ranked each antibiotic as medically important (to human health) or not. This ranking was based upon the probability of transference of resistant bacteria from animal to human, and the subsequent consequences if a human were to be exposed to resistant bacteria⁸. FDA’s plan proceeded based around reclassifying and relabeling ‘medically important’ antibiotics. After reclassification, antibiotics currently available over-the-counter (OTC), or without a prescription, will require a prescription (Rx) from a veterinarian. This will limit access to antibiotics. In relabeling, “subtherapeutic” or “nontherapeutic” labeling
indications (e.g. “production uses”)\textsuperscript{5} will be removed. Production uses include helping with feed efficiency and weight gain. Since medicated feed (including water) accounts for 94\% of antibiotic administration in livestock\textsuperscript{7} and can only be administered based on its labeled uses, relabeling would essentially illegalize large-scale distribution of medically important antibiotics to livestock.

Since FDA’s request from sponsors for the ‘voluntary’ submission of medically important antibiotics for reclassification, FDA has been re-evaluating and re-labeling previously approved antibiotics under these new guidelines. The question then becomes: are these new guidelines sufficient?

### 2.4.1 Labeling: From Clearly Wrong to Vaguely Obscure

According to FDA, the purpose of relabeling medically important\textsuperscript{\textit{ii}} antibiotics is to eliminate production uses such as “increased rate of weight gain” and “improved feed efficiency”\textsuperscript{9}. As it is illegal to distribute antibiotics for reasons not explicit on the label\textsuperscript{9}, this would outlaw using antibiotics for said ‘production uses’.

Some of the relabeled antibiotics include ‘medically important’ tetracyclines and sulfas, which respectively account for 41\% and 3\% of the antibiotics used in livestock\textsuperscript{7}. The highest levels of antibiotic resistance in retail livestock (based on a poultry study) are also tied to these two antibiotics: 65\% containing bacteria resistant to tetracyclines and 45\% resistant to sulfas\textsuperscript{10}.

\textsuperscript{ii} Medically important to human health; this ranking was based upon the probability of transference of resistant bacteria from animal to human, and the subsequent consequences if a human were to be exposed to resistant bacteria\textsuperscript{8}. 
Chlortetracycline (a tetracycline) is classified by FDA as “highly important” to human health, meaning it is the “sole therapy or one of a few alternatives to treat [typhus and anthrax]”⁸. Sulfamethazine (a sulfa) is classified as “critically important”, meaning it is (1) an antimicrobial used to treat food-borne diseases, (2) the “sole therapy or one of a few alternatives to treat serious human disease” (in this case pneumocystis pneumonia), and (3) an antibiotic that is used to treat subsequent diseases caused by food-borne pathogens (such as neonatal meningitis)⁸. Clearly, these two classes of drugs are highly important in human health and are already at dangerous levels of resistance in livestock.

In an excerpt from the newly revised and approved labeling for combination therapy drug ‘558.140 chlortetracycline and sulfamethazine’ (for cattle and pigs), there are clear production use indications including, “aid[s] in the maintenance of weight gains” (Figure 2.2).

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(d) Conditions of use- (1) Cattle. It is used in feed for beef cattle as follows:
   (i) Amount. 350 milligram per head per day each, chlortetracycline and sulfamethazine
   (ii) Indications for use. Aid in the maintenance of weight gains in the presence of respiratory disease such as shipping fever.
   (iii) Limitations. Feed 28 days; withdraw 7 day prior to slaughter...
(2) Swine. It is used in swine feed as follows:
   (i) Amount. 100g/ton each, chlortetracycline and sulfamethazine
   (ii) Indications for use. For reduction of the incidence of cervical abscesses; treatment of bacterial swine enteritis.; prevention of these diseases during times of stress; and the maintenance of weight gains in the presence of atrophic rhinitis.

Figure 2.2. An excerpt from new label for 558.140 Chlortetracycline and sulfamethazine. Food and Drug Administration, HHS. “New Animal Drugs for Use in Animal Feeds; Chlortetracycline and Sulfamethazine; Chlortetracycline; Procaine Penicillin; and Sulfamethazine;” Federal Register 79.127 (2014): 37622.
theory production purposes such as “increased rate of weight gain” and “improved feed efficiency” were supposed to be removed in FDA’s plan, in fact they’ve merely been relocated under conditional situations such as “times of stress” (Figure 2.2). Vague conditions are open to interpretation and ultimately to abuse. For instance, many pigs (swine) are raised in tight quarters that prevent movement; it would be reasonable to assume that they are constantly stressed. Under this new labeling it would be reasonable to constantly prescribe pigs a daily regimen of chlorotetracycline and sulfamethazine because “times of stress” can be considered likely all of the time. In effect, the labeling has not remotely changed the legality of constantly distributing medically important antibiotics to pigs.

Furthermore, the new labeling begs the question: why would an antibiotic be prescribed to “aid in the maintenance of weight gain in the presence of [a] disease” (Figure 2.2) as opposed to being prescribed to treat the disease itself? If the antibiotic is not being prescribed to treat the disease, then it is being prescribed to promote weight gain and/or feed efficiency, which is exactly what FDA was suppose to be removing from the labeling to begin with. The labeling “changes” are more of a rearrangement of words, and still permit production uses such as increasing the weight of livestock.

2.4.2 A Loophole: Veterinary Oversight and Judicious Use

The major issue in FDA’s plan lies in their concept of veterinary oversight and judicious use. Switching antibiotics from over-the-counter (OTC) to prescription (Rx) was designed to restrict antibiotic use because a licensed veterinarian would
have to prescribe the antibiotics. According to FDA “judicious use involves accurately identifying a bacterial disease that is present or likely to be present...”\textsuperscript{9}. By “likely to be present” FDA is referring to preventative medicine, which leaves much open to interpretation given that the conditions the majority of food-producing animals are raised in are also ideal spawning grounds for bacterial diseases.

FDA says there are important factors for veterinarians to consider in the case of prevention, such as “no reasonable alternatives for intervention exist” and factors that are known to “increase the susceptibility of bacterial disease, including environmental factors (such as temperature extremes and inadequate ventilation)...and other factors (such as stress...).”\textsuperscript{9}. In essence then, if a veterinarian could come up with a reasonable alternative intervention, then prescribing preventative antibiotics would be injudicious. A layperson might come up with the reasonable alternative of removing the animals from the stressful conditions to more humane conditions that are less susceptible to bacterial disease, but would the veterinarian? Perhaps not, depending on the moral and ethical beliefs of the veterinarian.

A veterinarian could also prescribe preventative antibiotics “...based on the client’s production practices and herd health history”\textsuperscript{9}. This FDA guideline alone leaves a huge loophole for veterinarians to 'judiciously' prescribe antibiotics. Essentially any poor ‘production practices,’ such as dirty and inhumane conditions, would immediately qualify the entire herd to be prescribed “preventative” antibiotics for the length of those conditions remaining unchanged. To some extent
it even encourages production practices to remain poor, because it gives producers a way to still use antibiotics that otherwise would not be permitted if conditions improved. To note, the “preventative antibiotics” are no different than previously administered antibiotics for growth purposes. The same antibiotics would still be administered in feed on a regular bases, and thus no changes are actually necessary except that the job market is opening up for unethical veterinarians.

2.5 Conclusion: The Danger Lies Dormant

The danger in antibiotic resistance is that most people will have no idea that they are resistant until they are ill and in need of antibiotics. An apparently healthy child could scrape his knee on a rusty piece of playground equipment and the doctors may find themselves battling to save the child’s life from a minor wound. Because there is a degree of separation between the pathogenic (resistant) bacteria infecting humans and the consequences of antibiotic resistance becoming apparent (dependent on a human contracting an illness), it’s difficult to perceive the scope of this problem in our daily lives. And in truth, we shouldn’t have to. Our government established FDA with a specialized role to “protect and promote [our] health.”11 (www.fda.gov). Not only is FDA being negligent in their duties to protect the American people, but FDA is further taking advantage of the trust the American people put into FDA.
2.6 References


6 The Center for Disease Dynamics, Economics & Policy: www.cddep.org


10 Seattle-King County Study 1984. “Surveillance of the Flow of Salmonella and Campylobacter in a Community.”

11 The U.S. Food and Drug Administration: www.fda.gov

12 Natural Resources Defense Council: www.nrdc.org
Summary and Conclusion

Strides in new antimicrobial drug development largely revolve around making old antibiotics usable again; this is due to economic advantages and the low feasibility of developing novel antibiotics. Resistance-Modifying Agents (RMAs) act to re-sensitize resistant bacteria to antibiotics through a variety of mechanisms, although currently most target bacterial resistance mechanisms themselves, such as β-lactamases. Foreseeably, while these compounds have shown efficacy and certainly are of value in the present crisis, it is a short-term solution in light of the evidently rapid and dynamic capability of bacteria to respond evolutionarily.

Nonetheless, a new class of RMAs, currently being researched and developed at Wang lab, hope to extend the lifespan of RMAs through making 1) synthetic compounds that 2) target gene expression; this model has the advantage of being unfamiliar to bacteria and would act upstream of current RMA targets, reducing the very of the expression of an array of resistance mechanisms.

While there are hopeful strides being made in new drug development and there is perpetual promise in the vastness of biochemical space, ultimately, humans cannot outrun bacteria in bacterial evolution. Rapid bacterial reproduction (which correlates to spontaneous mutations) aided by horizontal gene transfer makes bacteria an evolutionary hare compared to the human turtle. Furthermore, by placing selective pressure on bacteria we are inadvertently forcing bacteria into expressing and propagating genes conferring high levels of resistance. The most dangerous multi-drug resistant bacteria are nosocomial in origin (clinically-acquired); yet the majority of resistance is in the general population and is due to
extensive subtherapeutic antibiotic use in food-producing animals (community-acquired).

Both clinically and community-acquired resistance contribute to the demolition of a critical building block (antibiotics) of modern medicine. Arguably the most nonsensical piece of the puzzle is subtherapeutic antibiotic use in livestock, which accounts for 80% of all antibiotic use in the United States\(^1\). Not only are medically valuable (and currently at-risk) drugs being used for none-curative purposes, but also this misuse has empirically shown to have negative consequences for human health and safety. Furthermore, arguably the continued allowance of antibiotic abuse is a major factor in the perpetuation of inhumane and disease-causing production practices in agriculture. FDA regulations are seemingly the only feasible way to fix the problem, as other governmental branches, such as congress, have balked at intervening for many years. Likewise, it is unlikely the industry giants involved in antibiotic abuse, such as the agriculture and pharmaceutical industries, would take it upon themselves to reduce antibiotic usage. FDA, much to their credit, unveiled re-regulatory measures. And yet, as the ten-year process finally shows some tangibility through re-labeled antibiotics, it has been hugely disappointing and is arguably barren of any significant regulatory changes.

Although science is a beacon of hope in the situation, the problem of antibiotic resistance cannot be fixed by science alone. This is a multifaceted issue that also must be addressed by regulatory agencies, individuals, and clinicians to come to a resolve. The development of new antimicrobials is arguably futile if there are no regulatory changes or changes of usage practices. And yet, in its futility, we
have no choice because modern medicine is inherently dependent on antibiotics.

Finally, while it will be difficult to find a resolve, unlikely it will come by waging a war with bacteria, as we will inevitably lose, thus more likely resolve will be found at some level of symbiosis. As Stephen Jay Gould said, “...we live in the Age of Bacteria (as it was in the beginning, is now, and ever shall be, until the world ends)...”

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