

A REPORT FROM THE AMERICAN ACADEMY OF MICROBIOLOGY

# MOVING TARGETS

An illustration of a field with blue waves in the foreground. In the field, there are yellow ducks and red and white targets on wooden posts. The background is a textured brown.

AMERICAN  
SOCIETY FOR  
MICROBIOLOGY

FIGHTING THE EVOLUTION OF RESISTANCE  
IN INFECTIONS, PESTS, AND CANCER



**MOVING TARGETS: FIGHTING THE EVOLUTION OF RESISTANCE IN INFECTIONS, PESTS, AND CANCER**  
**A REPORT ON AN AMERICAN ACADEMY OF MICROBIOLOGY COLLOQUIUM**  
**PHILADELPHIA, PENNSYLVANIA**  
**JULY 2012**

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The American Academy of Microbiology is the honorific branch of the American Society for Microbiology, a non-profit scientific society with almost 40,000 members. Fellows of the AAM have been elected by their peers in recognition of their outstanding contributions to the field of microbiology. Through its colloquium program, the AAM draws on the expertise of these fellows to address critical issues in microbiology.

This report is based on the deliberations of experts who gathered for two days to discuss a series of questions about the evolution of resistance across varied biological systems.

The report has been reviewed by all participants, and every effort has been made to ensure that the information is accurate and complete. The contents reflect the views of the participants and are not intended to reflect official positions of the American Academy of Microbiology or the American Society for Microbiology.

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# ⇒⇒ INTRODUCTION

Antibiotics, antivirals, herbicides, insecticides, and anti-cancer drugs may seem to have little in common, but biologically each serves a similar purpose. Whether they are used to cure a disease, control an insect that spreads disease, or reduce crop losses, the goal of their use is to weaken or eliminate a living entity that is harming humans or their enterprises. The deployment of antibiotics, insecticides, and other selective chemical weapons has had enormous health and economic benefits; not for nothing were early antibiotics proclaimed to be “miracle drugs.”

However, in every case, these ‘miracle’ treatments have come up against a hard truth. Given time and opportunity, the organisms we seek to control will evolve resistance to agents deployed against them. Resistance is not a new phenomenon, and it has not arisen solely because of human interventions in the biological world. Antibiotic resistance in bacteria evolved as a means of waging a “fitness” warfare between species during competition for ecological niches. Many of the antibiotics prescribed today are derivatives of those produced by fungi as chemical weapons against bacteria, or by one kind of bacteria against another. In nature, the bacteria targeted by these weapons often developed ways to avoid or inactivate them. Mechanisms of resistance found in nature range broadly from the degradation of toxic compounds, efficient expulsion of antibiotics from the cell, or modification of the antibiotic’s target site such that the target can go about its cellular task unhindered despite the presence of the antibiotic.

The same process — evolving the ability to survive contact with a deadly compound — is at work when pathogens evolve resistance to antibiotic and antiviral drugs, when insects evolve resistance to insecticides, and when cancer cells evolve resistance to anti-cancer agents. The development of resistance results in incurable infections, cancer recurrence, devastating crop losses, and the inability to stop the spread of insect-borne diseases. The underlying phenomenon is the same, but rarely do the scientific communities that develop and deploy such agents gather to discuss their common challenges and share lessons learned. In July 2012, the American Academy of Microbiology convened a colloquium focused on identifying the common evolutionary mechanisms driving resistance evolution across diverse biological systems, how treatment design and delivery can help avoid or minimize resistance, and effective practices of resistance management. Colloquium participants included specialists in antibiotic, antiviral, fungicide, herbicide, and insecticide resistance as well as cancer biologists, biochemists, and theoretical biologists, each of whom deal with the evolution of resistance in their own specialties.





## ⇒ HISTORY OF ANTIBIOTIC RESISTANCE

The first antibiotic, penicillin, discovered in 1928 by Alexander Fleming, was isolated from the *Penicillium* fungus and its antibiotic properties had been known since ancient times. Before the chemists Howard Florey and Ernst Chain published a protocol for penicillin purification in 1940, leading to its mass production and distribution in 1945, *Penicillium* was capable of producing its antibacterial compound to inhibit the growth of bacteria growing in its vicinity, potentially to prevent competition for food sources. Since the initial penicillin identification, medical researchers have discovered dozens of antibiotic compounds. Antibiotics, harvested by scientists and dispensed by clinicians, did

wondrous things for treating bacterial illnesses in the 1940s-60s. Infectious disease mortality dropped from close to 800 per 100,000 annually in 1900 to about 50 by 1960. Similarly, life expectancy rose 56% during the 20th century. However, even before the widespread use of penicillin, evidence suggested that some bacteria could degrade the compound. Since the 1940s, resistance to several classes of antibiotics have been reported, including: penicillins, aminoglycosides, cephalosporins, glycopeptides, quinolones, macrolides, and tetracyclines among others. Indeed, no class of antibiotics is immune to bacterial resistance.

### The participants addressed three aspects of the evolution of resistance during the colloquium:

1. What are the common evolutionary mechanisms underlying resistance evolution, and to what extent are these processes shared between many different targets?
2. How do treatment practices influence the development of resistance, and can we thoughtfully design regimens to prevent resistance emergence or manage its existence?
3. Can we apply knowledge of resistance mechanisms and evolutionary principles during drug design to minimize resistance evolution? How can we use model systems to screen drug candidates for their propensity to elicit drug resistance?

The group considered the similarities of resistance evolution across their disparate biological systems, and in certain instances were able to identify lessons learned in other fields as potentially applicable to their own. Discussion also revealed significant gaps in the current understanding of resistance evolution, primarily in the appreciation of pre-existing genetic diversity within target organisms and how that diversity contributes to resistance emergence. This summary of colloquium discussions outlines how selection pressure leads to the evolution of resistance, manifesting itself in diverse mechanisms that are often shared across biological systems. The report draws upon key examples of successful resistance prevention and management strategies to identify common themes that could inform future target selection and treatment plans. Throughout the colloquium, participants stressed the necessity of incorporating not only evolutionary principles, but also the ecological context of each system in understanding the dynamics of resistance. Most importantly, the colloquium demonstrated the value of sharing ideas across traditional disciplinary boundaries.

# ⇒⇒ 1. RESISTANCE: A UNIVERSAL EVOLUTIONARY RESPONSE

The goal of treatment regimens is to kill or prevent the reproduction of target organisms without harming the host or non-target species using compounds specifically designed to disable the particular target. Cancerous cells, for example, differ from their benign brethren in their accelerated and unregulated division cycles. Oncologists, therefore, prescribe drugs to halt the rapid growth of the cancerous cells, such as Gefitinib that acts as an inhibitor of signaling pathways leading to cell growth, proliferation, and metastasis. Antibiotics — like ampicillin — inhibit cell wall synthesis without which bacteria cannot proliferate. The first anti-HIV drug, AZT, targets the essential reverse transcriptase enzyme — effectively halting viral replication. Toxins produced by the soil bacterium *Bacillus thuringiensis* (Bt) are thought to kill susceptible insect larvae by forming holes in the midgut epithelium.

## Selection pressure ultimately drives resistance evolution

Disrupting features essential to life for pathogens or pests places enormous selection pressure on them to subvert or evade the treatments. **Selection pressure** is any force that reduces reproductive success in individuals from a population. Any individuals possessing a trait that mitigates the negative effects of a drug or pesticide are able to proliferate and become dominant. Such traits confer resistance, and frequency of the resistance trait in a population ultimately determines the point at which a treatment loses effectiveness and is no longer useful in controlling the target population. In an ideal therapeutic approach, a naïve population of pests or pathogens would be treated with a novel compound and all individuals would succumb. However, all populations have some level of pre-existing diversity, and some variants less vulnerable to the treatment agent usually exist before any exposure to the treatment. Even in a population arising clonally such as a cancerous tumor or an infection by a single virus, bacterium or fungal spore, every cell division provides an opportunity for genetic changes to occur via mutation. Normally these mutations, arising through mistakes in proof-reading during DNA replication or chromosome crossover events, might engender a **fitness cost** for the individual and its progeny compared to the rest of the population. But if the genetic mutation that confers resistance is



### SELECTION PRESSURE:

any force that reduces reproductive success in individuals from a population.

### FITNESS COST:

disadvantage in growth or survival of a resistant mutant in the absence of a drug or pesticide, which is associated with the resistance-conferring mutation.



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not lethal in the absence of treatment, resistant individuals could survive as a small minority in the population before treatment begins. Once the treatment kills a large percentage of the susceptible portion of the population, these resistant individuals and their progeny flourish.

For bacteria, viruses, and parasites, a fitness cost can mean that the resistant individual is less competitive in establishing an infection or growing within a host than its wild type counterparts. Other types of organisms, like plants, do not have such dual lifestyles, and so their fitness costs may involve reduced growth, survival, or reproduction. Although fitness costs and selection pressure manifest in individuals, the rate at which their effects become evident at the population level depends upon generation time, which varies across biological systems.

The fitness differential, then, between susceptible and resistant individuals in the different environments encountered by the organisms (e.g., with and without presence of a treatment) ultimately affect the spread of resistance. In absence of a treatment, such fitness differentials can vary greatly from species to species and for different mutations within a species, and some mutations conferring resistance may not even have costs associated with them. Fungi, for example, often seem to survive perfectly well with various chromosomal abnormalities, although survival may still be influenced by environmental characteristics. The take-home message is that unless the fitness cost is high or the population size is small, it is likely that there will be resistant variants existing in a population, and these resistant variants will be selected for when treatments are applied. Thus, the ideal scenario of a 100% vulnerable target population that can be eliminated in one treatment is unfortunately rare, especially when one considers that in most situations, many different populations of a pest or pathogen are exposed to treatments.

Sometimes, withdrawing the treatment that is generating the selective advantage for resistant individuals can allow the non-resistant population to return to dominance. Although not a universal principle, this approach worked in Malawi, when national policy dictated a removal of chloroquine to reduce malarial resistance to the drug. After a period of ten years, chloroquine was reintroduced and the parasite population was again susceptible. Success depends on the ability to withdraw the treatment completely and on the disappearance of the resistant individuals. An attempt to reduce chloroquine resistance in Latin America was unsuccessful because the drug was never fully abandoned and so selection for resistance persisted. Similar attempts with antivirals in HIV were unsuccessful as the resistant virus is never truly gone; HIV virions are able to persist in long-lived memory immune cells.

## **Common molecular mechanisms of drug resistance**

The molecular mechanisms through which resistance can arise are often shared across biological systems.

- **POINT MUTATIONS:** Resistance can occur through point mutations — discrete substitutions, deletions, or insertions in the genetic code. Such mutations can often be found in the active site of the target protein, upon which the drug is designed to act. In some instances, a single point mutation can engender the resistance phenotype, but in the case of malarial resistance



to the drug chloroquine, seven such mutations are necessary. Mutations, however, do not always occur in the drug target site, but can also be found in compensating pathways, effectively bypassing the drug's cellular effect.

- **GENE AMPLIFICATION:** Gene amplification is another common resistance mechanism frequently associated with cancers, and also found in pesticide and antifungal resistances. Through this mechanism, the organism or cells increase the overall copy number of the target gene such that sufficient amounts of the resulting protein are available to complete the biological task, despite a subset being hindered by the drug treatment. Gene amplification can also increase production of detoxifying enzymes. Amplified genes can be found scattered throughout the genome, and the mechanism underlying this mode of resistance is unknown. Further, it is unclear whether certain organisms are better able to amplify genetic material than others, or whether the trait is specific to certain genomic regions or DNA architecture.
- **METABOLIC DEGRADATION:** Pests and pathogens can also evolve mechanisms to metabolize toxic compounds, as is often seen in plants.
- **EXCLUSION OR SEQUESTRATION:** All organisms can pump toxins out of their cells through efflux channels, a common method among cancer cells and bacteria. Drug sequestration is another effective resistance mechanism; certain cancers develop resistance to the anti-angiogenic tyrosine kinase inhibitor sunitinib by accumulating the drug inside lysosomes. Both limited uptake and enhanced sequestration are mechanisms of some pesticide resistances.
- **STRESS RESPONSES:** Resistance can also emerge due to the effects of the drug itself if the method of action affects mutation rates. Similarly, resistance can arise through conditioning if the cellular response pathway activated by the drug triggers an up-regulation of compensatory changes.
- **ECOLOGICAL AVOIDANCE:** Finally, target organisms can physically avoid the drug or pesticide itself. For example, viruses and bacteria can enter latent phases of their lifecycles in which they do not replicate, and remain hidden to both the immune system and therapeutic approaches. Bacteria often grow in dense communities called biofilms, sometimes comprised of multiple species, and the interior environments of these biofilms experience reduced concentrations of antibiotics. Similarly, cancerous cells in the interior of a tumor face different challenges than their counterparts at the boundaries. Understanding three-dimensional tumor “ecology” will be an important development in oncology and the exploration of drug resistance in cancer.

Although the ways in which pests and pathogens develop resistance to treatment are extremely varied, there are significant commonalities across biological systems from plants to cancer to insects to viruses. Because different biological systems experience similar molecular, or proximate, causes of resistance, the means of counteracting those resistance mechanisms might also have common features, which will be explored later in this report.



## ⇒⇒ 2. FACTORS THAT AFFECT THE DYNAMICS OF RESISTANCE EVOLUTION

Given that the selection pressure imposed by treatment strategies forces target populations towards resistance emergence, prediction of resistance evolution becomes critical. Across biological systems, the molecular mechanisms underlying resistance and the general evolutionary mechanisms driving its emergence may be conserved, but systems-specific characteristics lend uniqueness to the timing, extent, and scale of resistance phenomena.

Consideration of each of these characteristics in modeling studies will better equip predictive drug and treatment development strategies. Generally, the risk and speed of resistance emergence positively correlates with the level of diversity in a population, while the spread of resistance depends on the organism's reproductive strategies as well as its ability to move across landscapes (**Figure 1**).

### Generation of genetic diversity through different reproductive strategies

Genetic diversity is generated in a population through mutation and reproduction. Reproduction can be either sexual or asexual, and some organisms such as fungi, malaria parasites, and some plant species are capable of both during their lifecycles. Asexual reproduction, such as that of viruses, bacteria, and cancer cells, generates diversity through mutations acquired during DNA (or RNA) replication. These mutations are then passed to progeny who, other than the mutations generated during replication, are genetically identical to their parents. Although asexual reproduction encompasses most of the viral and bacterial life cycle, these organisms do occasionally acquire genetic diversity through **horizontal gene transfer**, in which genetic material is passed between members of the same generation or between different species.

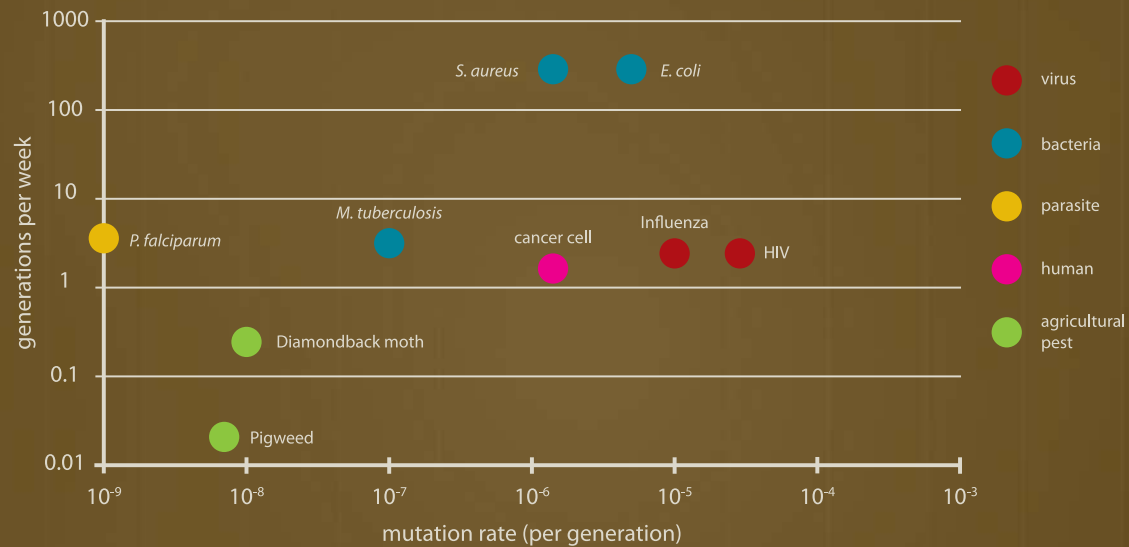
Other organisms such as fungi, parasites, plants, insects, and of course, humans reproduce sexually. Sexual reproduction allows for recombination of genes with every generation. Additionally, receiving genetic contributions from two parents means that each offspring inherits two copies of each gene that can be either dominant or recessive, further shaping the genetic landscape. Among agricultural pests, some weeds are capable of asexual reproduction, such as rooting following fragmentation of the parental plant. Others, such as dandelions, can produce seeds without flower fertilization; dissemination of these seeds via aerial routes can allow



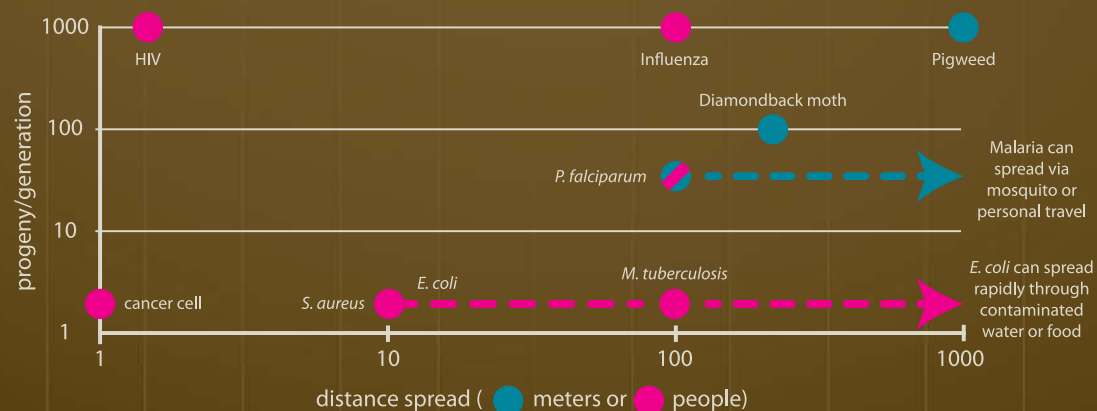
**FIGURE 1:**

The emergence and spread of resistance varies across biological systems and depends on key traits of those systems. (A) The speed at which resistance evolves positively correlates with mutation rate and rate of reproduction. A pathogen or pest with a high mutation rate that reproduces quickly, such as HIV or *E. coli*, will evolve resistance on a much swifter time scale than pigweed, for example, which mutates about three orders of magnitude less frequently and also only reproduces annually. (B) However, once resistance has evolved in a population, its spread is influenced by the number of progeny produced and how far those progeny can disperse, either in terms of distance or individuals infected. A drug-resistant cancer cannot spread beyond its immediate human host. While pigweed may develop resistance more slowly than HIV, the ability of its thousands of seeds to disperse distances of hundreds to thousands of miles makes resistance spread a real threat. Increased globalization plays a significant role in the spread of resistant pathogens; malaria-laden mosquitos may only travel a distance of 100m from where they hatched, but infected humans can bring resistant strains to naïve populations. Indeed, in cases of multi-drug resistant tuberculosis, quarantine is the most viable option for preventing resistance spread.

**(A) How quickly can resistance emerge?**



**(B) How quickly can resistance spread?**



**THE EXTENT  
OF GENETIC  
DIVERSITY IN A  
GROUP DEPENDS  
ON POPULATION  
SIZE, GENERATION  
TIME, AND  
MUTATION RATE.**

**HORIZONTAL GENE TRANSFER AND ANTIBIOTIC RESISTANCE:**

Bacterial species are not only adept at evolving antibiotic resistance *de novo* during replication, but can additionally exchange genetic material horizontally, that is, to other bacterial cells. Horizontal gene transfer, or HGT, can occur between closely related bacteria or even between members of different bacterial species. There exist a number of molecular mechanisms for this genetic exchange (described in a following box), but the result is the same — spread of resistance genes beyond clonal expansion from a single founder individual. Thus, resistance can in theory spread even more quickly than bacteria can reproduce. Because of HGT, antibiotics not only place selection pressure on pathogens, but affect all microorganisms, even those that are harmless or beneficial to humans. Thus, when one organism develops resistance, it can spread through the entire bacterial community. Horizontal gene transfer renders the effective population size virtually infinite, a frightening proposition in light of increasing instances of strains resistant to multiple antibiotics in hospital environments.

for rapid spread of clonal populations. Although some insects, such as aphids, reproduce asexually, many insect pests must reproduce sexually and therefore generate diversity through genetic recombination. Similarly, some plant species are obligate “out-crossers” either because they are comprised of unisexual individuals or their gametes are self-incompatible, and like interbreeding insects, require two individuals to colonize a new area. These different reproductive strategies affect how quickly resistance can emerge and how far resistant individuals can spread.

The extent of genetic diversity in a group depends on population size, generation time, and mutation rate. First among these traits is the population size of the target, as population size is positively correlated with diversity and therefore the probability of emerging resistance. Cancer is believed to begin with a mutation in a single somatic cell that proliferates uncontrollably, leading to tumors, and sometimes acquires the ability to grow outside of its normal body site leading to metastases. In the case of bacterial, viral, protozoal, and fungal infections, the number of pathogens required to cause disease in an individual varies significantly depending on both the pathogen and the immune status of the infected individual. Sometimes that number can be very small. A 1938 study of malaria in rhesus macaques established that inoculation with a single sporozoite could result in infection and death. Artificial inoculation with ten arthrospores of the fungal pathogen *Coccidioides immitis* was sufficient to cause infection in monkeys.

Additional factors shaping resistance spread are generation time and mutation rate, which also vary widely across organisms. Some bacterial species, such as *Staphylococcus aureus*, undergo cell division every 30 to 60 minutes, whereas mosquitos enjoy a two week lifespan. Organisms with shorter generation times can potentially develop resistance more quickly than those whose generation times are orders of magnitude longer.



## ⇒ ALTERNATIVE MECHANISMS OF GENERATING GENETIC DIVERSITY

**TRANSDUCTION:** Viruses that infect bacterial cells can transfer genetic material between hosts during viral replication cycles.

**TRANSFORMATION:** Bacteria are capable of scavenging DNA from the environment and can incorporate this DNA into their chromosomes, or maintain the DNA as extra circular plasmids.

**CONJUGATION:** Bacteria can also exchange DNA directly through conjugation, wherein one cell extends a mating pilus which contacts and connects to a neighboring cell. Once the cells are linked, DNA plasmids can be passed between them.

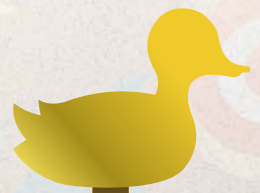
**CO-INFECTION:** Two viruses can infect the same host cell. As their genetic material is replicated and packaged into new virions within the host, exchange of coding sequence can occur leading to mixed progeny.

**REASSORTMENT:** Some viruses encode their genetic material on multiple genome segments. If co-infection occurs with two or more of these viruses, individual genome segments can reassort between the strains, leading to entirely unique virions.

Mutation rates also vary among target organisms by orders of magnitude. Viruses like HIV and influenza exhibit extremely high mutation rates on the order of  $10^{-5}$  (one event for every 100,000 base pairs replicated), which effectively translates to about one mutation per replication event because the genomes of these viruses are only on the order of  $10^4$  base pairs, much smaller than the human genome of  $10^9$  base pairs. Many viruses can also generate sequence diversity through recombination or reassortment during replication. Mutation rates among bacteria are orders of magnitude lower, and in *Plasmodium falciparum* studied *in vitro* that value drops lower still, ranging from  $10^{-11}$  to  $10^{-20}$ .

Significant debate exists in the cancer literature as to whether increased genetic instability is required to generate the multiple mutations that characterize cancer, or whether normal rates of mutation coupled with uncontrolled clonal expansion are sufficient for this process. Current evidence supports a hybrid model, in which normal mutation rates can occasionally result in a sequence discrepancy or chromosomal abnormality that itself predisposes that cell and its progeny to increased mutation frequencies.

Similar evidence exists in the microbial world that bacteria can increase their mutation rates under stress as a survival mechanism to generate additional diversity. If true, this suggests that evolutionarily, not only do antibiotics exert the selection pressure necessary to drive resistance phenotype proliferation, but may also trigger the target cells to increase their chances of developing such resistant traits. Indeed, mutation rates have been reported to increase by several orders of magnitude under starvation conditions; antibiotic stresses may also activate expression of stress-response error-prone DNA polymerases which can boost native mutation rates.



## Ability of resistance to spread

While reproductive strategies, population size, generation times, and mutation rates play significant roles in generating genetic diversity and thus resistance emergence, transmission of the infection or spread of pests will shape the diversity profile over time. Cancers exhibit the lowest degree of resistance spread, as each case is confined to one individual and depends on the host's genetic code and mutational events arising with each somatic cell division. Thus, cancers, and therefore resistance (with the exception of a few contagious diseases in the animal world) cannot spread from individual to individual. In long-lasting infections like HIV and tuberculosis, resistant strains can emerge in response to antiviral or antibacterial treatment of a single individual, who can then transmit the resistant pathogen to others. The ability of bacteria to exchange genes through horizontal gene transfer, even between different species, means that resistance need not arise *de novo*, nor be spread only from patient to patient, but can also be acquired from the environment. Viruses that infect bacteria, called bacteriophages, can also act as carriers of genetic information throughout microbial communities. Genetic exchange can occur within a patient, such as in an established biofilm in the lung of a cystic fibrosis patient, or attached to an implanted device, or externally in soil, water, barns or hospitals. Sexual reproduction among malarial parasites, fungi, insects, and weeds can also generate substantial diversity and allow resistance to spread quickly. Malarial parasites exist in both haploid and diploid forms, so recombination occurs within the female mosquito host. When the mosquito takes her next blood meal, diverse sporozoites are released into the human bloodstream. Resistance genes and diversity spread quickly through human populations because the malaria parasites carried by mosquitos are polyclonal and additionally, humans can be bitten by thousands of mosquitos across their lifespan. Resistance traits are likely to have greater mobility among plants and insects whose life cycles often require sexual reproduction and who enjoy dispersal mechanisms of both gametes and adults.





# ⇒⇒ 3. CASE STUDIES IN RESISTANCE MANAGEMENT

## Failures and successes

Unfortunately, when drug resistance does occur, the results can be disastrous. Failures can result for a variety of reasons, from treatment strategy to drug design, but all suffer from the common feature of immense selection pressure driving resistance evolution.

### **FAILURE DUE TO TREATMENT PLAN part 1:**

In 1987, initial efforts to treat HIV were unsuccessful, as the virus routinely developed resistance to the only available drug, AZT. Following the emergence of AZT resistance in a patient, doctors would introduce a second-line therapy, which would be continued until secondary resistance occurred. This sequential treatment strategy was an artifact of history, reflecting the order of antiretroviral drug discoveries, and sadly neglected to consider evolutionary principles. By 1993, monotherapy was proven ineffective in the treatment of HIV and combination therapy, by which several drugs are administered together, became standard practice. Sequential treatment plans are still used in the treatment of many cancers where it also leads to sequential resistance evolution.

### **FAILURE DUE TO TREATMENT PLAN part 2:**

While resistance to insecticide-treated mosquito netting is not yet rampant, this method of malaria control is feared to be facing imminent failure. Currently all bednets use similar active ingredients — pyrethroid insecticides. These insecticides are also used in agriculture and indoor sprays, thereby increasing the selection pressure on the insects.


### **FAILURE DUE TO DRUG DESIGN:**

Failures can also occur due to drug or treatment compound design. Many of the older herbicides targeted the D1 protein of photosystem II (PSII). The structures of most of these drugs, namely the triazines, were such that changes in the D1 protein could easily occur to resist the compound's action. However, the structures of other PSII inhibitors (e.g. benzaton) have been much more difficult for target sites to evolve resistance.

### **FAILURE DUE TO TARGET SELECTION:**

The herbicide endothall, while effective at controlling certain weeds, does not function well for all crops. Further, endothall targets serine-threonine protein phosphatases that are important in humans as well; thus, endothall is considered a toxin and is tightly regulated.





Despite these examples of failed drug regimens due to evolved resistance, there are cases in which resistance has either been avoided or managed successfully.

#### **SUCCESS DUE TO TARGET DESIGN:**

The anti-cancer agent imatinib has been used to treat chronic myeloid leukemia successfully in patients by targeting the bcr-abl fusion protein generated from the specific chromosomal translocation that defines the disease. Imatinib therapy works for months, although resistance does develop eventually from site-specific mutations. However, the mechanism of imatinib resistance is consistent and known, and so secondary drugs have been developed that can be employed once resistance is detected.

2,4-dichlorophenoxyacetic acid (2,4-D), an herbicide mimicking the essential plant signaling molecule auxin, has been used continually and widely for over 70 years. This synthetic drug owes its success to the extensive networks and binding partners of auxin; because auxin interacts with several cellular targets, resistance to its agonist 2,4-D often disrupts its other interactions and therefore confers a fitness cost. Cases of resistance to 2,4-D have been relatively rare.

#### **SUCCESS DUE TO TREATMENT PLAN:**

Success can also be due to treatment plan, and perhaps no approach better highlights the value of using evolutionary principles to design and implement a novel pest control strategy than the development of resistance management strategies for transgenic crops producing Bt toxins (**Figure 2**). Bt toxins are not harmful to humans and most other non-target organisms, but kill many key insect pests. Corn and cotton plants have been genetically engineered to produce Bt toxins. The insect pests ingest the toxins when feeding on such Bt crops, which reduces the need for insecticide sprays.

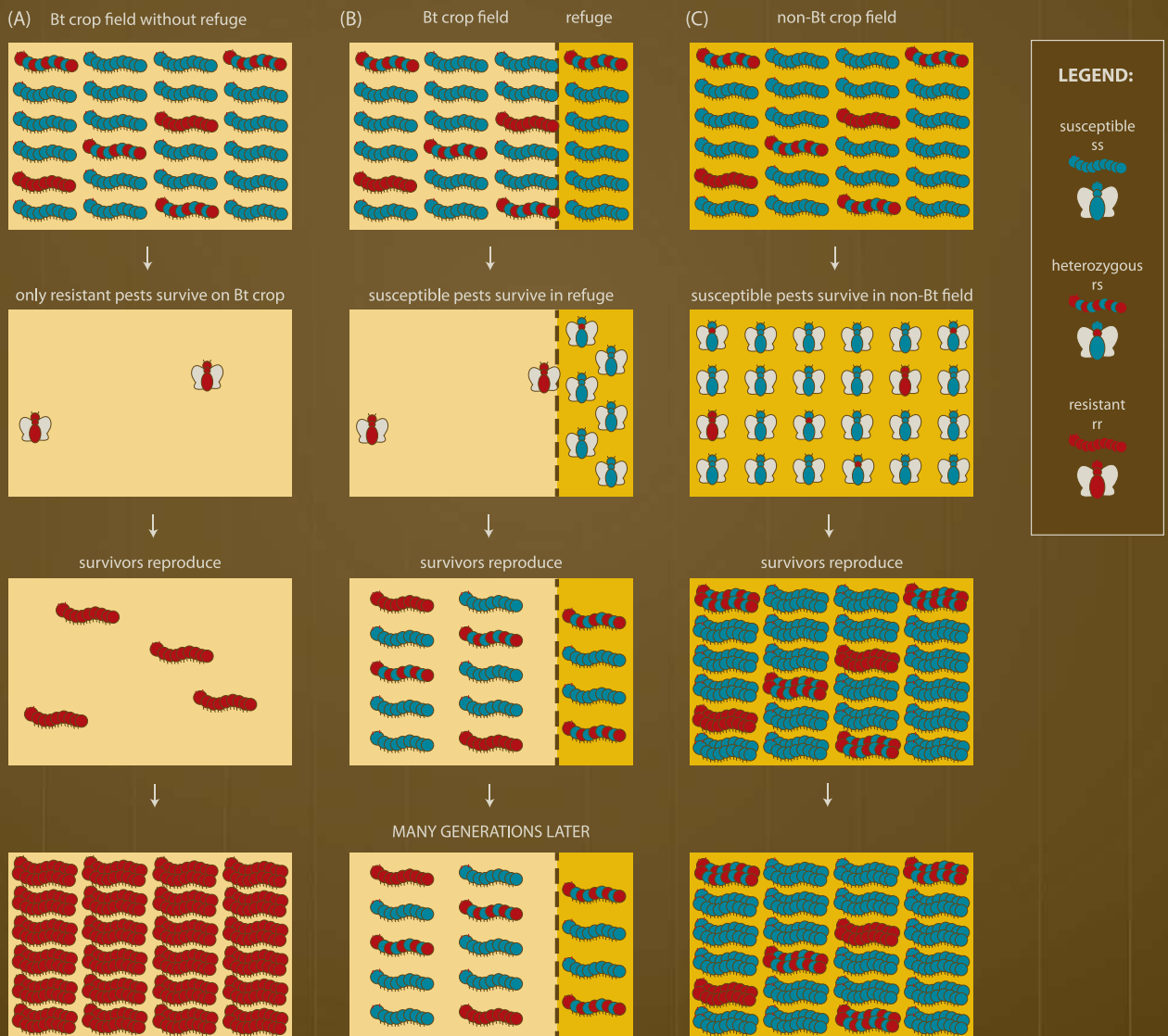
Proactively recognizing the threat of pest resistance to Bt crops, scientists used evolutionary principles to design resistance management strategies that have been implemented successfully in many cases. From the beginning, this process entailed collaboration among farmers, regulators, and scientists in academia and industry. The key element of most strategies for delaying resistance to Bt crops is a “refuge” of host plants that do not produce Bt toxins and thereby enable survival of susceptible pests. When Bt corn and cotton were first commercialized in the U.S. in 1996, EPA regulations required farmers to plant refuges of non-Bt corn and non-Bt cotton. The idea underlying the refuge strategy is that rare resistant adults emerging from Bt crops will be most likely to mate with the more abundant susceptible adults emerging from nearby refuges. If inheritance of resistance is recessive, the progeny of such matings between resistant and susceptible adults will die on Bt plants and resistance will be delayed substantially. Inheritance of resistance is recessive if the Bt plants produce a sufficiently high concentration of Bt toxins to kill heterozygous progeny produced by matings between homozygous resistant and homozygous susceptible adults. The strategy is sometimes called the “refuge/high dose strategy.” Therefore, one of the goals has been to manipulate gene expression so that the engineered plants produce high concentrations of Bt toxins.

As part of a regional program to eradicate pink bollworm, the U.S. state of Arizona has used a related strategy since 2006 to replace the host plant refuge with the release of sterile insects. The idea here is that the rare resistant insects surviving





**FIGURE 2:**

In order to protect agricultural interests from insect pests, key crops such as corn and cotton have been genetically engineered to express Bt toxins which target the digestive tract of those pests. (C) Without the Bt toxin, over time the insects would quickly reproduce and establish widespread and costly crop damage. (A) However, if the entire field is planted with Bt plants, enormous selective pressure on the insects will favor increased resistance.\* If any resistant individuals are present, they will withstand the toxin and reproduce, eventually enacting crop damage on par with untreated fields. Because the genetic mutations conferring resistance are often recessive, resistant individuals need to mate with one another or heterozygous individuals to produce resistant progeny. (B) If a wild-type population can be maintained nearby with which the resistant insects can mate, the selective pressure is reduced and the number of resistant insects increases at a slower rate. This can be accomplished by the planting of refuges, or fields of non-Bt crops fed upon by target pests, near the engineered crops. While a few resistant insects may remain, the crop losses will ultimately be minimal compared to the alternative scenarios and the spread of resistance will be reduced. The refuge strategy works best with recessive resistance, as illustrated here.




\*Initial resistance allele frequencies typically are one in one thousand, although they are represented at a much higher frequency here to illustrate the refuge concept.






on Bt cotton will mate primarily with sterile insects, resulting in no fertile progeny. Under this plan, the EPA allows farmers to plant up to 100% Bt cotton and refuges of non-Bt cotton have become rare statewide. Billions of sterile pink bollworm moths have been released from airplanes over cotton fields each year, resistance has not evolved, and this insect that had plagued farmers for a century is now difficult to find in Arizona.




First-generation Bt plants that each produce a single Bt toxin are being largely replaced by second-generation Bt plants that produce two or more toxins. Compared with resistance to a single toxin, resistance is expected to evolve slower when two or more distinct toxins target the same pest. Another recent development is “refuge-in-a-bag,” where non-Bt refuge seeds are mixed in the seed bag with Bt seeds to ensure that farmers sow the desired percentage of refuge seeds in each field. Despite some striking successes, several cases of resistance to Bt crops have emerged in the U.S. and abroad, sometimes reflecting failure to implement the refuge strategy.


### **SUCCESS DUE TO COMBINATION THERAPY:**



Another huge success has been the development of combination therapy which emerged with a paradigm shift in HIV infection management. Reports surfaced of patients prescribed just one antiviral drug, but taking another on the side. These individuals exhibited undetectable viral loads for much longer intervals than patients taking AZT alone. Despite promising stories of HIV treatment surfacing in the mid-1980s, institutional problems prevented combination therapy from rapid adoption. For a combination treatment to be considered successful, scientists were required to demonstrate that each component added benefit. Demonstrating that benefit required re-defining what was meant by benefit — in this case, the acceptance by the FDA of viral load as an indicator of HIV disease status in the early 1990s. Current best practice involves combining at least three drugs to reduce viral load and prevent resistance from developing to any single drug. Despite increasing evidence of the efficacy of combination drug therapies, the practice has not been implemented widely outside of the HIV community. Among scientists studying and treating malaria, there is agreement that artemisinin should be used in combination with other anti-malarials and implementation is increasing worldwide, but the cost and logistical challenges of providing and monitoring compliance with combination therapies are substantial.



While combination therapy is now the standard method of care for HIV patients, in cancer treatments it remains rare. However, a recent study combined a vaccine against p53 with third line chemotherapy in treating small cell lung cancer. Neither therapy was particularly effective on its own, but patient survival was greatly increased when both were administered. This combination of immunotherapy with standard cytotoxic drugs was novel. The success of this trial comes from placing the cancer in an “evolutionary bind.” In response to the vaccine, the tumors down-regulated their p53 expression, but p53 contributes to cell survival in the face of toxic perturbations. Thus, response to one therapy resulted in sensitivity to a secondary therapy. However, the expense of clinical trials has been especially formidable for investigating combinations of cancer chemotherapies.



In exploring the successful outcomes, we can see that while in each case evolutionary principles can identify and explain mechanisms behind the success, the stories do not necessarily lend themselves to a set of predictive principles upon which to base future decisions. Further, decisions on drug development and



treatment strategies are dependent upon the ultimate goal in the particular system: eradication or containment. How success in a given system is defined will greatly affect the available choices for management of the pathogen or pest at hand.

### Defining the goal of treatment: eradicate or contain?

If the only acceptable outcome of treatment is eradication of the pathogen or pest, drug design and treatment strategy may be approached differently than if maintaining the pathogen or pest at a manageable or economically acceptable level is adequate. Choosing the goal of eradication may have consequences in terms of reducing the useful lifetime of the drug or treatment because of the higher selection pressure. In cancer or infectious disease, the priority for patients and health care practitioners is patient survival, and this is almost always equated with eradication of the infectious agent or the cancer. HIV is a notable exception — complete cures are still sought but long term management is appropriately seen as a triumph. The agricultural industry traditionally adopts a management-driven strategy; a farmer may be willing to accept slightly lower yields to extend the lifetime of an insecticide or herbicide. Despite the differences in measurements of success across systems, there is much that practitioners in disparate biological systems can learn from one another; indeed, management approaches may result in better outcomes both in terms of patient lifespan and prevention of drug resistance.

### SUCCESS AS LIFETIME OF A DRUG OR PESTICIDE:

Sometimes, the focus on individual patient outcome conflicts with population-scale issues of controlling the emergence and management of resistant pathogens. From the point of view of the global population, increasing the effective lifetime of a drug is a critical measure of success. The World Health Organization (WHO) recommends discontinuing use of a drug when 10% of pathogens have acquired resistance. However, background failure rates of 5% often already exist, leaving a window of only 5% increased failure before the drug is shelved. In contrast, the anti-malarial drug quinine, since its introduction in 1630, has enjoyed one of the longest lifespans of any drug and is still quite effective.

In the agricultural world, success is defined by economic yield and return on investment. Infestations, while detrimental to crop productivity, do not always have direct impacts on human health, and so monetary considerations often take precedence over goals of eradication. Drug companies also have economic yields to consider when developing new therapies. The total cost of drug development has increased in recent years, and the high likelihood of resistance has drastically affected the pharmaceutical industry's willingness to develop new antibiotics. Thus, the pharmaceutical industry is more likely to focus their efforts on drugs that target pathogens affecting large numbers of people, broad-spectrum drugs, or drugs that are taken for a long time. Therefore, it is in private companies' interest to postpone development of resistance as long as possible.

### SUCCESS AS ERADICATION:

As stated previously, the primary goal in infectious disease, whether dealing with viral, bacterial, fungal, or parasitic pathogens, is the clearance of infectious organisms. Eradication can also sometimes be favored in agriculture, as a single drug-resistant plant can overwhelm an entire field in a few years, given the high

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#### **TRADEOFFS WITH MANAGEMENT STRATEGIES:**

While accepting a moderate level of infestation may delay resistance by removing some selection pressure, it is important to keep in mind that remaining pests can lead to larger population sizes in future generations, thus providing a source of additional genetic diversity for evolved resistance.

reproduction rates of certain weeds. Certainly, as evidenced by the multitude of “Cure for Cancer” and “War on Cancer” campaigns, eradication of cancerous cells from the body is also the aim of oncologists. However, eradication is not always the best goal because pursuing that goal means maximizing selection pressures and may not be the best for the overall quality of life of the patient. From a social context, setting the goal of eradication and failing to meet it can lead to discouragement and can further reduce enthusiasm for continued funding.

Characteristics of the pathogen itself may make the goal of eradication unrealistic. Smallpox is a brilliant example of a virulent pathogen completely removed from the face of the Earth, but this success was due not simply to coordinated worldwide efforts by health care practitioners and scientists, but also to the pathogen having no animal reservoir or insect carrier, the fact that isolation is effective in breaking the chain of transmission, and that immunity is conferred through a single dose of a heat-stable vaccine. For pathogens that infect multiple hosts, even intensive vaccination programs may not result in total eradication. If a pathogen is certain to persist in the environment, is eliminating the pathogen from a single individual the most appropriate goal? Management of a disease or infestation, rather than eradication, is quite possibly more effective at slowing the emergence and spread of resistance, promoting patient survival, and protecting economic investments.

Already, HIV is viewed from a perspective of disease management; with effective combination therapy, viral loads can be driven below detectable levels in some cases and for all patients living with HIV infections, their disease is viewed as something to be managed and lived with, rather than cured. Influenza treatments can also be designed not to actively kill the virus but to prevent its spread. An antiviral drug targeting influenza’s neuraminidase protein does not prevent infection or inactivate the viral particles, but does prohibit new virions from detaching from host cells, thus short-circuiting the infection process, limiting the severity of the disease, and reducing the likelihood of spreading the virus to other people.

Treatment of malarial infections typically attempts to eliminate all the parasites from the patient, but this practice drives proliferation of resistant phenotypes. To keep resistance levels low in mice, lower dose drug treatments at intermittent intervals were shown to be effective, although implementation of such a strategy in real world situations would be difficult.

The key question to be addressed for each system is: can drug therapies be used to drive pathogen or cancerous cell populations sufficiently low such that either symptoms are abated or the immune system can manage the rest? For instance, in the case of bacterial infections, is the full course of an antibiotic treatment necessary for both curative purposes and resistance prevention, or could lessening the duration of drug treatments and allowing the immune system to effect the final clearance be a viable therapy? The answer to these questions will depend greatly on the immune state of the patient, as the plan of action could backfire if the immune system proves incapable of dealing with the remaining infectious agents.



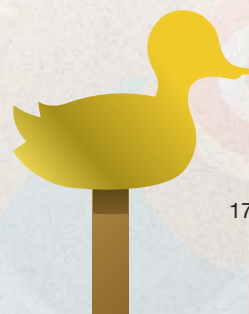
Still, the prevailing option of maintaining a low level of disease or infestation with pests in agriculture may, in some instances, be useful in a public health context not only for improving patient health but also preventing the emergence of drug resistance. Acceptance of a management-driven strategy would require drastic paradigm shifts, particularly among the cancer community, but the results could be more positive in overall outcome.

## Lessons learned from successful strategies

From an evolutionary standpoint, it is realistic to assume that resistance will always occur and so in any biological system, we must consider evolutionary principles for development of resistance prevention or management strategies. No matter how careful the drug design or target selection, enormous selection pressure will likely drive resistance eventually. From several success stories, the merits of drug combinations have been shown in diverse fields. Proper dosing is important because varied strategies can favor different types of resistance, such as dominant or recessive, and contribute to the speed at which resistance develops. Success stories also illustrate the importance of carefully designed and implemented treatment plans which account for evolutionary principles in the prevention of resistance evolution. Lessons from successful strategies, however, do not necessarily transcend boundaries between biological systems as each is defined by unique attributes that affect the frequency of resistant mutations and the potential spread of those mutations. Therefore, establishing standard guidelines for resistance prevention and management across all fields is not yet possible, but the principles discussed here may enable the formulation of system-specific recommendations and spark new approaches based on cross-system similarities.

## A key question: is resistance already there?

The prevention of *de novo* resistance emergence and management of pre-existing resistance were the two key questions posed to the colloquium participants. The group agreed that once a resistance mutation is present in a target population, continued widespread application of the drug or pesticide will encourage proliferation of resistant individuals. Potential fitness costs engendered by the resistance mechanism could be negated if most environments encountered by the pathogens or pests are treated, thus shifting the evolutionary balance in favor of resistant individuals. Given the (1) frequency of resistance alleles in a population, the (2) selection coefficient describing the relative fitness of the mutant to the wild-type in treated and untreated environments, the (3) relative abundance of treated and untreated environments, and the (4) rates of movement between those environments, classical genetics modeling can predict the rate of spread of the resistance mutation. If the resistant population grows beyond that which makes its extinction improbable, that population's continued success will be determined chiefly by selection. In some cases, the resistant population can completely overwhelm any remaining susceptible individuals, which is precisely the scenario that we wish to avoid. Therefore, if resistance pre-exists in a population, any treatment plan must preserve a drug-sensitive population to prevent complete takeover by the resistant strain.



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IN MEDICINE AND  
AGRICULTURE  
DRIVES THE  
SPREAD OF  
RESISTANCE  
THROUGH  
HOSPITALS AND  
COMMUNITIES.**

Maintenance of a drug sensitive cohort can be achieved through a variety of means. Back-mutation, in which the same mutation rates that gave rise to the initial resistance phenotype reverse the process, can lead to revertants more fit than their resistant brethren under non-selective conditions. An influx of sensitive individuals from alternative sources or inclusion of drug-free sanctuary sites or refuges as previously discussed can bolster sensitive populations. Additionally, the resistance mutations may cause a decreased fitness of the resistant individuals once the frequency of the mutation becomes too great in the total population.

In the case of antibiotics, resistance mechanisms are already widespread in the environment and have been so for millions of years. The selection pressure of antibiotic use in medicine and agriculture drives the spread of resistance through hospitals and communities. Resistance traits to drugs in other biological systems may not be so well-established or diverse, but nevertheless selection pressure will drive resistance evolution and spread in those systems as well.

Because resistant traits can arise through many different mechanisms, resistance prevention and management strategies need to incorporate evolutionary principles and accommodate the unique ecological attributes of each system. To effectively prevent resistance emergence or manage its presence, we can control features such as treatment plans, target selection, drug design and to some extent, transfer of resistance information.



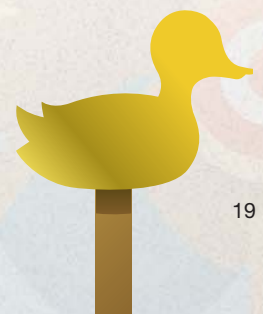


## ⇒⇒ 4. TREATMENT PRACTICES AND THE DEVELOPMENT OF RESISTANCE

### **Monotherapy versus combination therapy**

Historically, across all biological systems, treatment with a single agent has been the norm. When a virus, cancer, or crop pest proved resistant to that agent, a secondary treatment was applied if available. This approach can be successful if resistance does not develop too quickly and if multiple treatments are available. However, HIV and cancer are particularly problematic because sequential application of drugs merely leads to sequential development of resistance to all drugs applied. Still, such a strategy can lengthen the patient's lifespan overall if the development of resistance is slow.

In the human health domain, the emergence of combination therapy as a means of HIV treatment has begun to alert other fields to the potential benefits of the practice. Already, tuberculosis infections are routinely treated with a spectrum of antibiotics and the World Health Organization recommends the highly effective 3-7 day Artimisinin Combination Therapy (ACT) for treatment of malarial infections. From an evolutionary perspective, *de novo* acquisition of resistance could be less likely through combination therapy because pathogens would need multiple mutations to overcome applied therapies. The probability of two independent mutations arising in the same organism is theoretically the product of the probability of each mutation arising individually. The multiplicative rather than additive effects greatly reduces the likelihood of dual resistance and increases the likelihood that the target population will be controlled by the drugs before resistance evolves. The second drug ensures that resistance to the first drug is essentially useless because the primary resistance trait cannot help in overcoming the effects of the secondary drug. Ultimately, resistance to the first drug should be lost from the population over time, unless its rate of loss is outpaced by the rate of acquisition of resistance to the second drug. Then the evolutionary advantage disappears and treatment with two drugs may only hasten resistance evolution to the second. Horizontal gene flow complicates combination therapy for bacterial infections, as several drug resistance mechanisms may be



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transferred at once into the target population from other species. Thus, combination therapy works best when frequencies of resistance are low or absent.

Furthermore, simply combining any two drugs does not always help unless the drugs act by sufficiently different mechanisms. If both drugs act on the same biochemical or regulatory pathway in the target organism, a single mutation could potentially arise bypassing that pathway and confer simultaneous resistance. In insect control, success is more often proportional to the difference in the ways that the insects are killed.

In practice, the preference for monotherapy over combination therapy across biological systems involves a trade-off between evolutionary and practical considerations. For herbicides, ideally the agricultural industry would want to use drug combinations to better manage infestations, but the expense and potential environmental impacts of such a practice may prohibit consistent use. Herbicides, therefore, are often rotated through fields in some cropping systems to prevent a single dominant phenotype from emerging. Such a practice temporarily reduces exposure to single compounds and can delay resistance evolution under some circumstances. Among the antifungal community, true monotherapy is typically sufficient to quell infections where clonal inoculation of the patient is the norm. Therefore, if the genotype of the fungus is known prior to treatment, application of a single drug can be the best approach. Any observed resistance is more likely to have been acquired from another individual or the environment than evolved within the current host.

### **What makes an ideal treatment combination?**

Although this is still an open research question, treatment combinations are often proposed to exhibit additive effects, wherein the dual effects of the treatments are greater than either one acting alone. To best prevent resistance from developing, the mechanisms of action and the processes targeted should be different from one another. Ideally, treatment combinations would target different pathways in the pathogen or pest, thus requiring multiple resistance mechanisms to bypass the treatments. Cross-resistance can be predictable in cancer, particularly if both drugs target the same pathway. Colloquium participants displayed optimism for the design of successful anti-fungal combinations in treating human infections. Although there are very few anti-fungal drugs available, there is depth of knowledge in what the drugs do and how they work.

Combinations that place the pathogen in an “**evolutionary trap**” are ideal, wherein resistance to one drug renders the pathogen susceptible to the action of the second, as was previously illustrated by the combination of the p53 vaccine and standard chemotherapy treatment of breast cancer. Combination HIV therapy using AZT and 3TC has also been effective in preventing dual resistance, as the mutation conferring 3TC resistance renders the virus more susceptible to AZT. Dual resistance can eventually develop, but multiple mutations become necessary to resist AZT when delivered in combination, and so overall resistance is delayed. According to a recent study, combination therapy “controls [HIV] viral replication because of steep, upwardly inflected dose-response curves for some drugs and synergies reflecting independent action for other[s]” (Jilek, et al., 2012).





### EVOLUTIONARY TRAPS:

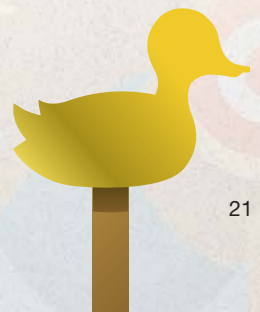
Abrupt changes to a species' environment can lead to evolutionary traps — adaptations or ecological preferences which had established themselves through generations of positive selective forces can suddenly lead that species to maladaptive choices. Such evolutionary traps are often highlighted by examples of animals pursuing disastrous life choices in the face of sudden habitat disruptions because their natural instincts compel them to maintain their responses to original cues, despite an altered situational context. Sea turtle hatchlings, for example, are drawn to the ocean by the bright light of the moon over the water. However, along more developed beaches, light pollution from beachfront structures can actually entice the young turtles up, away from the water to their deaths. Humans already use this strategy in insect control by mixing pheromones with insecticides, thus exploiting aspects of insect reproduction for their own demise. In combatting drug resistance, we can take this one step further by designing combinations where evolved resistance to drug A places the organism in a position of increased susceptibility to drug B.

For a successful combination therapy, both techniques need not be cytotoxic on their own if the addition of one compound increases the effectiveness of the other. For example, the anti-tuberculosis effects of weak acids, such as the antibiotic front-line drug pyrazinamide (PZA), can be enhanced by inhibitors of energy metabolism under aerobic conditions. However this enhancement is not observed under anaerobic conditions, highlighting that consideration of environmental variability is key during drug development and treatment design. Similarly, phage therapy could enhance the activity of antibiotics against bacterial infections. Indeed, both drugs might not need to be inhibitory to be maximally effective. One drug might be able to complement the host's immune system such that the target pathogen population, driven low, by another compound, can be managed by the host. In applying the "evolutionary trap" concept to the host immune systems, it has been hypothesized by HIV modeling that any drug to which a resistance phenotype elicits a potent immune response would be very difficult for the virus to combat.

Drug combinations can also be designed to boost the beneficial microbial background of the host. If developing resistance imposes a fitness cost on the pathogen, probiotics could be used to out-compete the resistant population. Such techniques could also be applied to plants, increasing their fitness against pathogens or insect pests. In humans, antibiotic combinations with the anti-diarrheal loperamide also have increased effectiveness.

Additionally, the two drugs need not necessarily be delivered simultaneously to be considered working in combination. Pairs can target different stages of the life cycle, such as drugs that target larval and adult insects. Combinations can also target different aspects of the pathogen's life cycle. In the case of malaria, drugs that act within the human could be combined with strategies to disrupt parasite-mosquito interactions.

An additional important feature of successful combinations is similar half-lives. The evolutionary goal is to force the pathogens to develop solutions to both treatments simultaneously. If one active ingredient decays much faster than the other, the pathogen or pest encounters a sequential monotherapy approach rather than a combination.





## How should common evolutionary principles inform the development of optimal dosing strategies?

Once a treatment strategy has been determined, the dosing regimen assumes critical importance. The longstanding paradigm in many systems is to treat at the highest possible level. In cancer, oncologists begin with the maximally tolerated dose, with the goal being to kill as many cancerous cells as possible as quickly as possible. Such a strategy is also favored in agriculture, as farmers are advised to use high doses to discourage proliferation of low level resistance genes. Unfortunately, prohibitive herbicide costs often lead farmers to dilute these products and the lower doses encourage resistance evolution. Additionally, older and larger plants are generally less susceptible to treatment than younger seedlings, again highlighting the necessity for early and intense dosing. Similarly, because fungal infections are typically clonal in nature, if resistance is not detected upon diagnosis, physicians will proscribe high monotherapy doses. Evolutionarily, a high dose strategy is favored when high doses kill the target population with enough speed and efficiency to out-run mutation rates. Suboptimal doses have been shown to facilitate acquisition of resistance in diverse pathogens, including HIV, bacteria, the fungal pathogen *Candida albicans*, and malaria. Under reduced selection pressure, slightly resistant individuals are able to survive and accumulate even greater resistance capabilities. Low dose regimens also permit the survival of susceptible parental strains who have additional generations to develop resistance. This may account for the very common development of resistance in immunocompromised patients who are routinely given low-dose prophylactic antifungals. The level of dosing can also contribute to the speed at which resistance develops. For example, very low insecticide rates may hasten the evolution of resistance, as sub-lethal doses may induce stress pathways leading to enhanced mutation rates.

Short, high dose pulses of insecticides are favored by modeling algorithms. The short duration of the treatments kills all but a few susceptible individuals, whose population is then allowed to rebound slightly before the next treatment. Between treatments, the preserved susceptible population should then be able to out-compete any resistant individuals if there is a resistance-related fitness cost.

While low dose regimens may allow the survival of weaker resistant phenotypes, maximum doses can also lead to resistance evolution; if the high doses fail to eliminate the target, the enormous selection pressure may allow resistance to emerge. Recent studies in malaria and cancer suggest that a “middle ground” approach may be successful at both managing the diseases and prolonging life. Intermittent, lower doses of malarial medication in mice were shown to be effective in keeping resistance down. Similarly, breast cancer cell lines were kept alive indefinitely through lower chemotherapy doses and no resistance was detected. Proponents of this idea cite evidence that decreased chemotherapy doses simultaneously decrease hypoxia and increase blood flow, thus allowing for better drug delivery than if higher doses were administered. The high toxicity of cancer drugs makes “metronomic therapy,” or periodic rather than continual dosing, additionally attractive because lower doses are tolerated at more frequent intervals. However, such malaria treatments *in situ* are impractical — while mouse-malaria models are encouraging from a resistance perspective, antimalarial drugs are not considered practical for distribution in developing nations if they must be taken for a course greater than three days, as



compliance and cost issues negate the treatment benefits, as well as risk resistance emergence through incomplete treatment regimens.

Acceptance of the “lower, extended dose” or “maintenance” model will also require a dramatic paradigm shift among oncologists and the public at large, who strongly view cancer as a disease to be cured. While a cure may be a feasible outcome for certain cancers, many could potentially be managed to an asymptomatic background level through continuous low dose, combination therapy treatment. Such a scheme would not necessarily cure the cancer, but could relieve the overall disease burden, prevent resistance evolution, and most importantly, prolong life.

A “maintenance” model relies on tolerance of some level of target population which begs the question for each biological system: how much control is necessary to reduce the negative effects to an acceptable level? In cancer, this is a radical idea but laboratory experiments are promising. Even after a “cure,” one can often detect residual cells by molecular tests such as PCR. There does seem to be a correlation between the remaining cancerous cells detected and disease recurrence frequency, but quantification of cancerous cells could be used to establish treatment guidelines, much like viral load in HIV. However, in treating fungal infections, elimination is necessary once the fungus reaches the bloodstream. The same issue arises with bacterial sepsis although other types of infections offer more “wiggle room;” urinary tract infections, for example, characterized by fewer than  $10^3$  cells/mL do not warrant treatment. Treatment of malarial infections also aims for complete clearance, except in cases of asymptomatic adults living in high risk areas, which occurs after more than 10 infections in the patient’s lifetime. In plant pathogens, however, farmers have room to accept a moderate level of disease, and the same is often true for weed control and insect pests.

If the dosing of treatment is important either for elimination of the pest or management of its population, the timing of treatment is also critical; waiting until there’s a heavy parasite, cancer cell, or viral load to begin treatment increases the potential for a higher degree of variation, and thus a higher likelihood of resistance evolution. However, treatment cannot commence until a diagnosis is made, which relies both on the timing of symptom development and confirmation by a health care professional. In some cases, the illness may not manifest until the target population has already reached high levels or the patient may delay in consulting with a doctor. To catch infections or infestations in their infancy, vigilance and surveillance must be maintained.

Consideration of the lifecycle of the target in treatment timing will increase the effectiveness of those treatments. Therapies should ideally be timed to apply treatment when its effect will be maximal. For example, dandelion weeds are at their weakest right after blooming, when food reserves in the roots are at their lowest. Tender young leaves are also most susceptible to herbicides in the spring. The early fall month of September can additionally be an appropriate time for herbicide treatment, because as the dandelion prepares for the upcoming winter months, it moves carbohydrates from the leaves to the roots, providing a chemical traffic flow that any applied herbicides can hijack to reach all of the plant body.

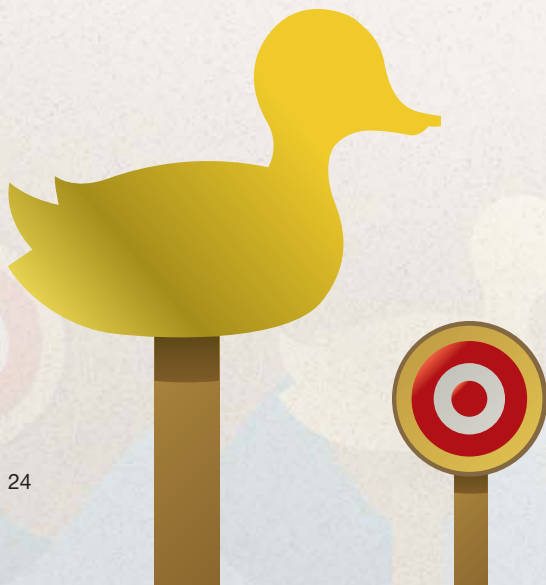
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## **Contingency planning**

Because resistance can arise through treatment mis-use and despite the most carefully-designed treatment strategies, contingency plans need to be established. When the fall armyworm *Spodoptera frugiperda* developed resistance in only four years to Bt toxin-producing corn in Puerto Rico, the Bt crop was voluntarily withdrawn from the marketplace. Such withdrawal was in line with EPA recommendations stipulating that actions must be taken to thwart resistance to Bt crops. Education is effective and important for resistance prevention. For example, Bt cotton producing a single toxin remained effective for control of pink bollworm in Arizona but evolution of resistance to single-toxin Bt cotton has been observed in India. Arizona farmers complied with the refuge strategy mandated by the EPA to manage resistance in this pest; in contrast, refuges of non-Bt cotton were required in India but compliance was low. The lack of refuges likely promoted faster evolution of resistance in India compared with the U.S. Patient compliance and drug resistance also go hand in hand; individuals need to be aware of how their actions affect their own risk of developing a resistant population, but also how that resistance can spread to the community as a whole. Education about resistance risks and cancer progression may also lead to acceptance of cancer treatments as a life-long and life-prolonging route to halt growth of the disease, rather than looking for curative measures.



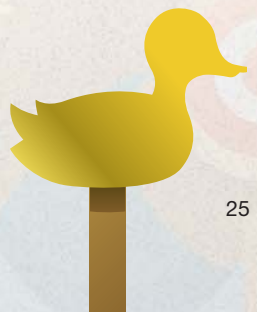


# ⇒⇒ 5. DRUG DESIGN AND RESISTANCE EMERGENCE

## Principles of drug design for direct targets

For decades, identification of therapeutic agents relied on broad screening of chemical compounds for inhibitory or cytotoxic effects. With significant advances in biochemistry and molecular biology, drug design today takes a more targeted approach. Treatment target sites are selected because they are critical to the survival of the pathogen or pest. Finding such targets in bacteria or viruses is relatively less complicated than identifying targets in eukaryotic cells of organisms such as fungi, as the latter are more closely related to humans and thus have more similarity amongst their essential proteins. Thus, the compounds designed could elicit deleterious effects in the patient either through direct treatment applications or through environmental exposure in the case of herbicides and insecticides. Intelligent anti-cancer drug design is even more challenging because the genetic makeup of the cancerous cells is almost identical to that of the host; drug targets in these instances are often the mutated forms of normal human proteins which gave rise to the cancer in the first place.

Target-based approaches require knowledge of biological processes. Small molecules are sought that mimic the actual binding partners or protein receptors of the target, and either activate or inhibit its activity thus providing therapeutic benefit to the patient. Sometimes libraries of small molecules are screened for the desired effects but increasingly small molecules are designed in combination with computational modeling of the three-dimensional structure of the target and the target's structure bound to biologically-relevant interacting partners. The goal is to isolate a compound that competitively binds tightly to the target to the exclusion of the native binding partner or prevents a key conformational change. Such designed inhibitors are used in all of the disease targets that have been described, targeting among others: proteases, transcriptases, kinases, and synthases. Ideally, the chemical interactions between the compound and the corresponding target should be very specific, eliciting a much higher binding affinity than the native partner. To avoid drug resistance, the inhibitors should be designed to be as evolutionarily constrained as



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possible; this could involve mimicking an enzymatic transition state, as is often done, or interfering with a target that has multiple necessary binding partners. Targets with multiple binding partners are more evolutionarily constrained as co-evolution would have to occur within each of the partners in the complex for resistance to occur. Thus, the large number of primary and compensatory mutations needed to occur simultaneously would greatly decrease the probability of evolved resistance.

When much is known about the target enzyme or molecule, intelligent combinations of drugs can be designed against the same target, wherein development of resistance to one drug renders the second more potent through “negative cross-resistance” or an “evolutionary trap.” The two antagonists could even be within the same molecule as a bifunctional compound that attaches to the target protein in two places. In such case the protein would be forced to make multiple simultaneous changes to avoid the toxin. For proteases, active sites are commonly targeted, although targeting of allosteric sites, that is, sites other than the active site, could be considered as this could elicit broader effects on activity, such as inhibiting polymer formation. While the most commonly targeted molecules are proteins, membrane constituents vary enough between species that it could be possible to target lipids or other membrane components specific to target organisms. This strategy might be less prone to driving resistance than targeting proteins as membranes are more difficult to modify and linked to many diverse systems within the cell.

Drugs that affect multiple cellular targets can also stymie resistance evolution. The antimalarial artemisinin, derived from a Chinese herb *qinghao*, has been used for over two millennia in the treatment of fevers. The continued success of this drug could be attributed to its method of action, which relies in part on the generation of free radicals to disrupt multiple cellular targets and alter cellular redox cycling.

In selecting a host process or enzyme as a drug target, there was debate among the colloquium participants as to whether sequence conservation among species isolates was indicative of strong selection pressure and thus importance of the target to the host. When little biochemical or cell biological data is available, genomic and protein sequences may be the best available sources of information. While ideal treatment targets should be highly evolutionarily conserved, thus reducing the likelihood of compensatory resistance mutations occurring without accruing a considerable fitness cost, participants noted that both diversifying selection and directional selection can result from evolutionary force and that sequence diversity alone does not always accurately reflect selection pressures. In some cases, 100% sequence conservation could indicate an evolutionary constraint, or that the target itself has never been acted upon by the selection pressure of the immune system or other forces.





## Indirect targeting

### HOST PROCESSES:

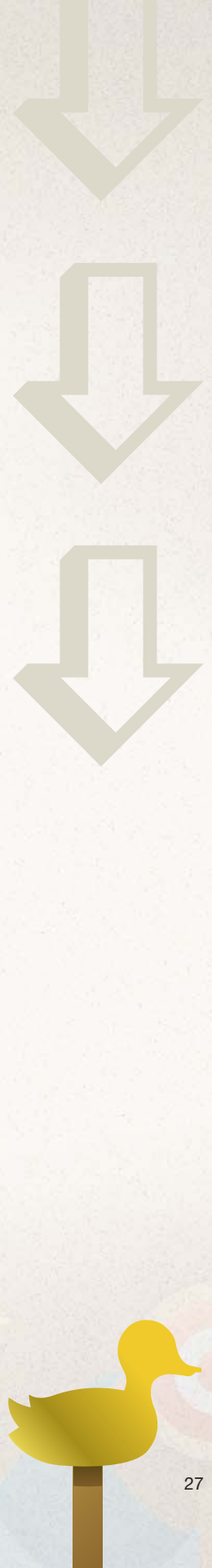
An alternative approach is to target host processes that the pathogen or pest cannot control. Because the target species lacks genetic control over the host, evolutionary evasion should become more difficult. Indeed, HIV research is trending towards interrupting cellular targets that are essential to the viral life cycle. Evidence already suggests that drug targeting of host chaperone proteins, essential for protein folding in several classes of RNA viruses, can not only impair viral replication in cell culture but also prevent the emergence of drug-resistant viruses. A potential issue with targeting and manipulating the host environment (ie humans) is that toxicity problems will arise. However toxicity issues are dealt with continually by drug companies in treating other health problems like high cholesterol and heart disease, so applying these approaches to managing infectious disease should not pose additional difficulties. Often, however, altering the pathogen or pest's host environment is an attractive strategy. Control of malaria could be achieved by treating the mosquito vectors not to kill them, but to prevent the parasites from attaching and proliferating. Manipulation of the mosquito's preference for human blood meals could also prove to be a fruitful avenue of research. An analogous approach in plants would be to alter the expression of phytochemicals that make the plant attractive to insect pests.

### SUPPORT SERVICES:

Species being targeted for control are not isolated populations, but members of diverse ecological environments. As discussed previously, environmental manipulation can supplement and even define successful treatment strategies. Treatment goals, therefore, can also exist beyond the target organisms themselves by targeting diffusible factors and other forms of cooperation. For example, anti-angiogenic therapy, or treatment targeting blood vessel growth, in cancer is designed to prevent the growth of new blood vessels supporting a tumor by targeting the signaling molecules that lead to angiogenesis. Eliminating signaling molecules can also disrupt any protective structure the pathogen might create or exploit. Compounds might be discovered to disrupt biofilm formation by bacteria, which can protect drug sensitive individuals from the antibiotic and also serve as a reservoir for antibiotic resistance genes to spread among populations and even between species. Along similar lines, targeting siderophores and other iron chelators could prove effective in depriving pathogens of access to the iron that is essential for their survival. Indeed, quinolone drugs inhibit malaria by sequestering heme so that it is not accessible to the parasites. This type of practice eliminates free riders from the environment.

### BOOST HOST DEFENSES:

Because target organisms respond to intense selection pressure by evolving resistance mechanisms, treatments could theoretically be engineered to prevent or lessen damage, rather than affecting the undesirable organism's overall survival. For example, targeting virulence factors that specifically cause disease symptoms might relieve the severe selection pressures imposed by cytotoxic drugs. In the case of cancer, virulence factors are not at play, but perhaps cytostatic drugs that inhibit cell growth rather than killing them would be more effective in the long-term than cytotoxic ones. If compounds can slow down proliferation, the cancerous cell population will slowly decline. Such treatments have been shown to be effective in breast cancer, although the expensive therapy has to be continued for a long time and also triggers early menopause. Drugs which select for altered generation times — either



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longer or shorter than wild-type — may be particularly effective. For insect-driven control of malaria, shortening the lifespan of the mosquito through late life-acting drugs would reduce selection pressure against insecticides. The mosquito would still be able to reproduce but would not be as effective at transmitting malaria because generally older insects spread the disease.

## **Counteracting alternate routes to resistance**

### **EFFLUX PUMPS:**

Because a common resistance strategy among target populations is increased export of the toxin through efflux pumps, it was proposed to simultaneously apply a “fake drug” to counteract this mechanism. By supplying the pathogen with similar but inert compounds in addition to the actual drug, the pathogen may spend considerable cellular energy expelling the non-toxic compound rendering it susceptible to the remaining toxins.

### **METABOLIC DEGRADATION:**

Mechanisms of resistance occur through metabolic degradation of toxins as well as gene amplification. Combating these mechanisms will require creative solutions, as treatments and combinations will need to be developed to evade multiple routes to resistance. A common mechanism of resistance among weeds is the evolved ability to metabolize the toxins themselves, which crop plants are genetically engineered to withstand. Interestingly, many crops are inherently able to metabolize commonly used herbicides without any genetic modifications. Thus, it should come as no surprise that weeds are able to evolve similar metabolic pathways. Additionally problematic in this approach is that toxins that cannot be degraded are difficult to use as herbicides and insecticides because such compounds will persist in the environment for long periods of time. To solve both of these issues, one possibility would be to amend a drug with certain functional groups such that the likelihood of its metabolic degradation is decreased. This approach would remove the focus from metabolic resistance to target site resistance.

### **GENE AMPLIFICATION:**

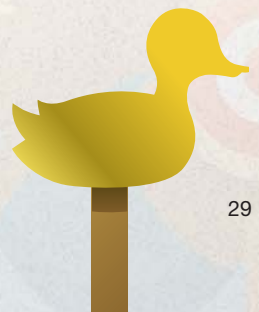
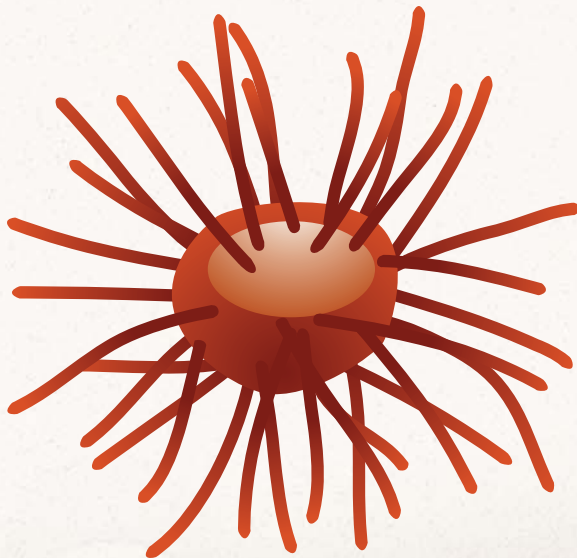
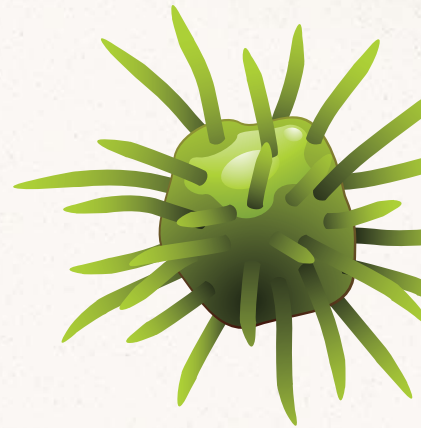
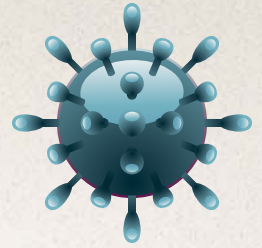
Unfortunately as of yet no hypothetical solutions exist to counteract gene amplification. The molecular mechanism underlying this phenomenon is unknown, and amplified target genes can be found scattered throughout the genome. Key questions surrounding this process are the propensity for diverse organisms, and even different types of genes, to undergo gene amplification. Once the factors leading to such amplification are known, scientists can begin devising strategies to counteract them. An ideal drug might force the pathogen into an evolutionary trap, where amplification of the gene conferring resistance would confer a fitness cost. Another type of treatment might select for deletion of the resistance gene. But realization of these scenarios will require extensive expansion of our knowledge base regarding gene amplification as a biological process.






## Broad versus narrow spectrum treatments

When physicians are faced with the option of treating infections with either broad or narrow spectrum drugs, which is preferred from an evolutionary perspective of preventing resistance emergence? Broad-spectrum antibiotics have a clinical advantage in that they allow treatment when rapid and specific diagnosis is impractical. However, such drugs can also impose selection pressure against a wide variety of microorganisms, driving resistance more quickly than narrowly-focused drugs. Resistance that evolves in one species of bacteria can also be shared with others. The consensus among colloquium participants was that broad spectrum antibiotics should be used for as short a period of time as possible, followed by a more tailored approach as soon as the physician gains a better understanding of the pathogen at hand. An alternative option is to combine a multi-site drug with a narrow-spectrum one. This approach has shown success in fungicides. One compound hits multiple sites in the pathogen, disrupting multiple sites simultaneously, which is difficult to develop resistance against. Side effects associated with these drugs tend to discourage their use, which is why combination therapy with a very fungal-specific drug can be effective. Commercially, however, the development of broad spectrum drug therapies makes sense. Narrow spectrum drugs are a riskier venture because they are used on fewer patients and any resistance evolution negates the use of the compound. In the case of herbicides, narrow-spectrum compounds typically are not efficient to protect crops, as a single field will contain multiple weed species.





## Using model systems to screen drug candidates for their propensity to elicit drug resistance

Before predictive modeling can be used to forecast the likely course of drug resistance evolution, an understanding of how resistance has developed previously is critical. Statistical modeling can help scientists learn from the field what happened in the past, and what factors were important in determining the outcomes. For example, a long-term, large-scale field study demonstrated that refuges can delay the evolution of resistance to insecticides. The study also determined the spatial scale at which pesticide-treated fields and refuges affected the evolution of resistance, which provided critical information for development of the refuge strategy to delay resistance.

Retrospective studies drawing from clinical data following patients over time can also be extremely informative. Longitudinal biopsies from cancer patients can provide not only progressive monitoring of features known to be important for specific drug resistance events, but a rich reservoir of data to be used in future studies when new molecular mechanisms of resistance have been identified. Similarly, measurement of pathogen profiles throughout the course of HIV or tuberculosis infections could reveal important trends. In studying the course of such infections, the patients themselves become the models. Cystic fibrosis patients could provide a wealth of data over long periods of time if sputum samples could be routinely characterized and tested for antibiotic resistance.

The aforementioned studies will provide clues as to spatial and temporal patterns of resistance evolution, but a mechanistic understanding of how and why resistance evolves also plays a significant role in shaping resistance prevention strategies. Powerful tools such as transcriptomics partnered with molecular and computational biology will enable a better understanding of the pathways to diseased states. In cancer biology, over a decade of research using gene expression arrays has established certain genes and systems as being up or down-regulated in cancerous cells, but identifying the causes behind these expression changes remains elusive. Often the mutations underlying the transcriptional changes are point mutations or gene amplifications. Colloquium participants proposed performing large-scale mutagenesis studies, using a variety of mutagens to capture a full spectrum of possible mutations and combinations of mutations. The resulting mutagenic data could then be combined with the well-established transcriptomics catalog to gain a deeper understanding of the regulatory processes involved in cancer development and drug resistance. The colloquium participants also stressed that a deeper and more thorough understanding of the underlying genetic diversity within individual biological systems is critical to the success of treatment plans. Mutation rates vary among species, and DNA sequence diversity also varies across the chromosomes of any one species. Deep sequencing reveals significant genetic diversity among environmental isolates; indeed, there are 60 *var* genes in malaria contributing to immune evasion, and across this data set at least 500 sequences have been identified and none are identical.



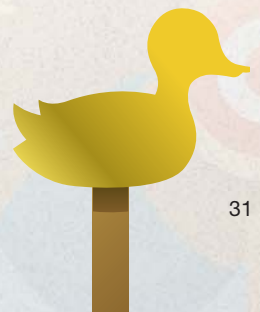
## Challenges of model systems

To simplify environmental variables, model organism systems can be established in the laboratory to predict rates and mechanisms of resistance emergence. In such laboratory studies, the actual target organism is the ideal model, but this is not always possible. Malaria parasites are notoriously difficult to grow and genetic manipulations are tricky; determining which mutations confer the resistance phenotypes and thus mechanism of action of the drug is challenging. Alternatively, model laboratory organisms can be used as proxies for the actual pathogens. For example, the yeast *Saccharomyces cerevisiae* can be used as a model system for *Candida* and other fungal pathogens, but resistance mechanisms and underlying genetic diversity may not be conserved. *Drosophila* fruit flies are also more amenable hosts to malaria than mosquitos for high throughput laboratory studies but the interspecies interactions observed may not precisely mirror the natural ecology of the parasites.

Once a model species has been chosen, laboratory studies can first test the potency of the drug. If the compound is sufficiently potent to clear a diverse population of pathogens quickly, the window for the development of resistance is drastically narrowed. The ideal treatment situation would involve a drug, or combination of drugs, that is highly lethal and also very rapidly cleared from the body or environment. Combination therapies can also be tested using model systems approaches. Biochemists can begin with an initial drug whose effects are known, and then test a library of other chemicals to find synergistic properties. Proving synergy is not simple, but if established, the doses of one or both drugs could potentially be lowered, thus reducing any side effects. A drawback of this approach is that defining synergy as the sole endpoint might miss drug combinations which have no statistically-significant impacts in terms of killing or controlling the pathogen, but might offer a drastically decreased likelihood of resistance evolution. Distinguishing between a rare event, such as resistance to monotherapy, and an extremely rare event, such as resistance to combination therapy is also challenging. However, from an evolutionary standpoint, the path and time to drug resistance should be critical considerations in the selection of treatment plans. Screening for the time to resistance requires a large, diverse starting population. With some rapidly replicating species, the necessary diversity and population size can be generated quickly. In studying resistance evolution in cancer, artificially generating diversity through carcinogens is preferred to using genetically modified mice because a vast scope of possible mutants can be more readily created.

Mutagenesis approaches are recommended for quick screening and characterization of resistant phenotypes. For organisms with smaller genomes, mutagenesis approaches can almost be guaranteed to elicit mutations spanning the entire chromosome. However, the mutations obtained through artificial mutagenesis do not always reflect those that would occur in nature, such as gene amplifications. Artificially increasing mutation rates may also generate unrealistic results. When the target site is known, site-directed mutagenesis can be a very powerful tool. Laboratory studies could drive resistance evolution in the model organism through drug dosing, rather than artificially inducing mutations through artificial processes. Such an approach might take longer to achieve significant results, but those obtained will be more environmentally relevant. Once a resistant phenotype is discovered, it becomes possible to test for fitness differences between the resistant strain and the wild-type. Among bacteria, such fitness cost evaluations should be

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AGAINST ACTUAL  
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established for communities as well. Broad spectrum antibiotics are the norm during treatment of infections, but it is important to limit the overall impact of the drugs on the body's natural microbiota. Additionally, resistance can evolve in any of the commensal community members and spread via horizontal gene transfer, so it is necessary to know how drug treatments will affect other organisms and particularly whether resistance already exists in the environment. For example, the antibiotic vancomycin was thought to be “unresistable” until the entire resistance pathway was transferred from an environmental species; thus the path to resistance was always available in the environment, just not in the pathogens that were initially targeted.

Finally, computational modeling, though not always applicable to complex environmental situations, can be used to vary multiple theoretical constraints and thereby predict the emergence of resistance under changing conditions. Such studies have a distinct time advantage over traditional laboratory work because days, months, and even years can be condensed into simple algorithms. Two types of computational studies can be conducted: the results of “truthed” studies, based on real world parameters, can be justified against actual conditions, while theoretical studies are useful to see if novel approaches would be effective and can be designed with conceptual clarity. Variables such as generation time, mutation rate, population flow, drug half-life, and dosing schedule can all be tested in computer simulations to model resistance emergence and management strategies.

Colloquium participants cautioned against placing too much trust in modeling systems, however, as results can lead to both false negatives and positives. In one cited instance, antibiotic resistance was rapidly achieved in culture, although the drug works quite well in patients with urinary tract infections without apparent emergence of resistance. Alternatively, the model plant organism *Arabidopsis thaliana* was mutagenized to search for potential resistant varieties to glyphosate and none were identified, despite isolating seedlings resistant to two other tested herbicides at a frequency of about  $10^{-5}$ . These promising results suggested that resistance to glyphosate would be extremely rare and yet many weed species have evolved resistance in the wild. Although glyphosate had been used for decades without weeds evolving resistance, the development of glyphosate-resistance crops resulted in much more intensive use of this herbicide and simplification of measures used to control weeds, thus contributing to a rapid increase in the number of glyphosate-resistance weeds. Finally, model systems cannot necessarily take into account the complexity of synergistic or negative relationships and their role in resistance. Laboratory systems are designed to minimize variables, and thus often rely on monocultures, clonal strains or inbred model organisms. For resistance prediction, these systems can greatly underestimate the strain diversity existing in nature.

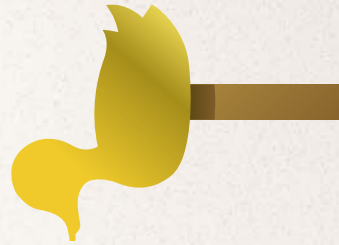
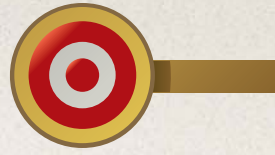


## ⇒⇒ 6. THE BIG PICTURE: RESISTANCE IN POPULATIONS AND THE ENVIRONMENT

### Measuring and monitoring resistance in target populations

Vital to the prevention of drug resistance evolution is frequent and accurate surveillance of the target populations. If the mechanism of resistance is known, testing for that trait or underlying genetic sequence should be done prior to and throughout treatment. Particular importance is placed on screening for drug resistance in bacterial, malarial, and HIV infections before treatment, as front-end diagnostics are critical to drug and treatment selection. For more than 10 years, a few drops of blood and a simple PCR test have been sufficient to type a malarial infection for resistance; if resistance is determined, more expensive second-tier therapies are used. Prolonged illnesses such as cancer and HIV syndrome should also be monitored periodically during the treatment regime. However, even when the resistance mechanism is known, colloquium participants cautioned against relying on genotype and sequence information alone to track resistance in a population. Such an approach finds only the resistance mechanisms that are already known; screening for resistance phenotypes, while ultimately “trial by ‘treatment failure,’” has the advantage of uncovering new mutations.

If the resistance mechanism is unknown, phenotype screening is the only means of detecting emerging resistance. To augment the arsenal of resistance-detection tools, drug companies should be encouraged to invest in research that will identify resistance mechanisms prior to implementation if possible, so clinicians and farmers are prepared for any real-world emergence. However, even if resistance is not observed in the lab, it may still arise *in situ* so surveillance is critical.



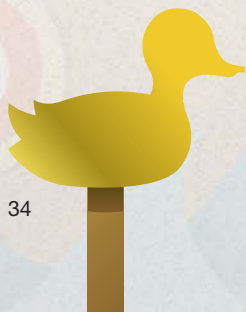
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Only through rigorous diagnostics and patient surveillance can we effectively identify the emergence of drug resistance and trace its flow across time and geography. Global networks of hospitals and other primary care facilities will need to be established, as will uniform standards of resistance identification and reporting. Similarly, agricultural stakeholders should develop cohesive plans for sharing incidence reports of resistance among pests as mobility among seeds and pollen, plant pathogens, and insects can easily cause a local problem to devolve into a regional one. Oversight of drug treatment plans and herbicide/insecticide distribution should be handled by national or perhaps even international governing bodies to ensure that adopted practices minimize risk of resistance evolution and spread.

### **Individual benefits versus the common good: the tragedy of the commons**

One final consideration is that treatment schemes can have effects beyond the individual patient, farm, or neighborhood being treated. In 1968 Garrett Hardin published an article outlining the “Tragedy of the Commons,” that is, the harm and destruction of shared community resources through seemingly rational and ultimately self-interested individual decisions. The commonly cited examples of over-grazing shared pasturelands to the point of field exhaustion or increased vehicular travel leading to gridlock highlight the concept well. Drug resistance management can also pose a ‘tragedy of the commons’ dilemma. Often, treatment strategies preferred by individuals have detrimental effects on their neighbors or even the global health community. The physical environment occupied by the pathogens and pests is shared by everyone, and the burden of managing them should also be shared.

For treatment regimes to last, they may need to be implemented such that the interests of the community are not forgotten. In agriculture, the dispersal radii of seeds and pollen, microbe propagules, and flight ranges of insects mean that resistance that evolves on one farm in response to treatment practices can potentially travel to a neighboring locale. In studying herbicide resistance, practices such as tillage, crop rotation, and periodic cessation of chemical treatments when infestation is minimal, and careful seed removal following harvest have been shown to contribute greatly to the longevity and effectiveness of the treatments. Incentives could be developed to encourage farmers to coordinate their practices with their neighbors, perhaps even executing scheduled rotation of herbicides and insecticides among them to mitigate resistance emergence after prolonged use in any one spot. In some cases, however, community benefits arise without such incentives, as seen with refuges used to delay resistance of European corn borer to Bt corn in the midwestern U.S. Most of the estimated \$7 billion in economic benefits of Bt corn over 14 years were associated with planting of non-Bt corn refuges, because Bt corn reduced pest populations on nearby non-Bt corn and non-Bt corn seed was less expensive than Bt corn seed.



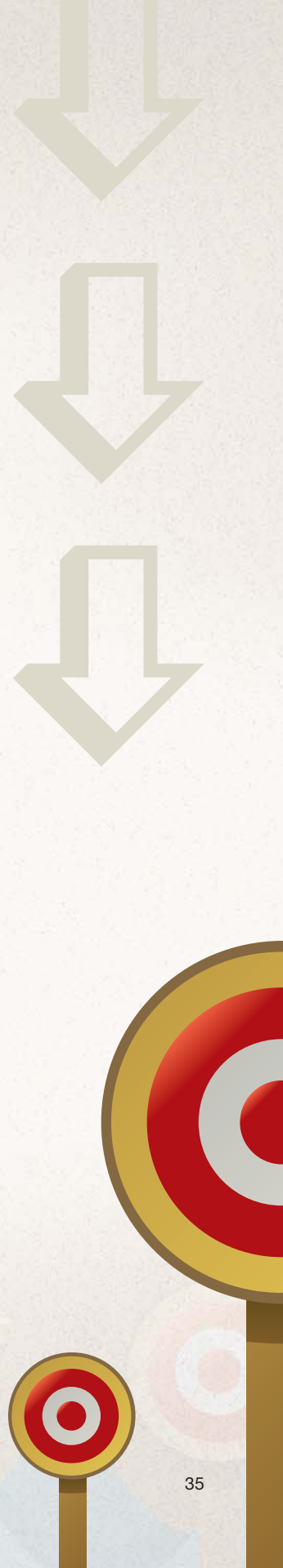


Mosquito control to combat malaria can also be seen as a community-based effort where the actions of each member either benefit or harm the group. Treatment of infectious diseases, however, will remain a constant battle for physicians, as long as they must make treatment decisions in the interest of an individual patient despite local and global issues of drug resistance. For example, the spread of antibiotic resistance genes through horizontal gene transfer is a real danger, particularly in hospital settings, and yet while drugs could potentially be designed to inhibit such gene flow through bacterial populations, only the community would be served, and not individual patients undergoing treatment; there is no financial incentive to develop such drugs, and no regulatory criterion that would favor them in clinical trials.

Encouragingly, manipulation of the target environment can also be applied directly to supplement treatment strategies. In certain cases, the environment can be altered to make drug resistance more costly for the pathogen. Temperature sensitivity is key for many resistant species, as increased temperature can render them more susceptible to treatment. This has been suggested for cancer, bacteria, fungi, and herbicide-resistant plants. Another proposed strategy includes increased use of natural enemies, as resistant genotypes could have reduced fitness relative to susceptible individuals when faced with predation. In the same vein, individuals resistant to Bt toxins could be more vulnerable to some plant defenses, and thus refuge plants could be manipulated to increase fitness costs that select against resistance.

Environmental manipulation could also be used to try to reduce movement or flow of resistance genes in a population. Through sexual education, distribution of condoms as preventative measures, and monitoring of blood banks, the spread of HIV and thus drug-resistant HIV has been lessened. With bacterial and other viral infections, the use of personal protective equipment and lifestyle modifications, along with quarantine if necessary, the spread of infectious, resistant agents can be halted. If a resistant population is truly isolated from other breeding partners, removal of the drug may result in a reemergence of the susceptible strain over time. To prevent the spread of herbicide or insecticide resistance genes in agriculture, farmers in neighboring areas should be encouraged to use different herbicides or insecticides than their neighbors, thus generating a mosaic of selection pressure.

Implementation and control are key for preventing resistance emergence and managing that resistance once it arises. However, despite the demonstrated value of certain approaches, standard practices vary significantly across the globe. In the Netherlands, “A teams” comprised of a medical microbiologist, pharmacist, and clinician monitor every hospital infection. Prophylactic treatment is always given in advance of any open gut surgery, while other surgical procedures do not receive any additional antibiotic therapy. In contrast, antibiotic use prior to surgery is fairly routine in the United States because if the patient dies from a hospital-acquired infection, many insurance agencies are not bound to pay. This practice places pressure on hospitals to prevent infections, although rampant antibiotic use is not the answer from an evolutionary standpoint. Hospitals serve as hotbeds of resistance evolution. Antibiotics are very widely used in China, and the level of resistance there is the highest ever measured; in 2001, 89% of hospital-acquired infections involving *S. aureus* were resistant compared with 16% in the United States. Antibiotics were introduced as additives in feedstock almost as soon as they were discovered. This



practice has broadly affected human health, as vancomycin resistance has flourished and pathogens like methicillin resistance *S. aureus* (MRSA) have emerged; both events seem to be products of the addition of antibiotics to livestock feed. Farm use of antibiotics is significantly less controlled than hospital use, and yet the drugs' mechanisms of actions are identical regardless of setting and yield the same paths to resistance.

A recent report in *Science* suggests a link between antibiotic resistance genes carried by soil microbiota and clinical isolates. In some instances, clusters of antibiotic resistance genes with >99% sequence identity between the two groups of microorganisms were identified, and were sometimes co-located with integrases or transposases for rapid horizontal gene transfer. Further, some soil isolates were taken from farmland fertilized with manure from antibiotic-treated livestock. Although the study does not prove directionality of gene flow, either movement of antibiotic resistance genes from soil to the clinic or vice versa is a troubling phenomenon, as the former establishes soil microbes as a direct source for pathogenic resistance genes while the latter raises the potential for transforming innocuous environmental species into pathogens.





## ⇒⇒ 7. CONCLUSION

Discussions at the colloquium were rich and exciting as scientists, clinicians, biochemists and theoreticians realized how much they could learn from each other by discussing their individual challenges within a common evolutionary framework. In a very real sense, the most important outcome of the meeting was this realization that there is much to be gained by continuing such discussions.

The group did not generate a set of recommendations, but did identify two overarching opportunities that emerged as particularly promising applications of the insights gained during the colloquium:

### **Evolutionary opportunities**

All resistance, regardless of the biological system, must be recognized as an evolutionary phenomenon. Our ability to combat cancer, in particular, would benefit from a shift towards viewing cancer as an evolutionary disease. A recent analysis of publications on cancer resistance and relapse since the 1980s showed that evolution terms were mentioned in only 1% of the abstracts. This finding is significant, as resistance to chemotherapies is often found in tumor samples taken before therapy and is driven by evolutionary pressures that kill the susceptible cells and allow the resistant ones to proliferate. Evolutionary thinking might prevent some of the pitfalls that arise when selection pressure is not considered during initial treatment plans. Re-conceptualizing cancer as a disease not to be cured, but to be managed, may also help in implementing treatments to minimize drug resistance and prolong life. Each biological system stands to improve treatment strategies by incorporating elements from others; the gift of “management” versus “eradication” from the herbicide and insecticide worlds may prove particularly valuable to other fields of study, as may a paradigm shift away from sequential drug treatment plans towards combination therapy approaches.

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## **Ecological opportunities**

While molecular and genetic techniques have enabled in-depth understanding of pathogens and identified specific means of combatting them, expanding our focus by taking into account the ecological roles of those species will greatly enhance the available tool kit. Drug developers will benefit from looking beyond the isolated laboratory setting, the cell, and the individual patient to consider the different types of environments encountered by pathogens or pests and how these can be manipulated or exploited to control outbreaks or infestations. In particular, malaria research could benefit if viewed as an ecological problem. Because of the multifaceted nature of the infection cycle, ecological approaches will reveal new places for therapies to intervene. Indeed, points of intervention exist throughout the parasite's life from its proliferation in the human host to its passage through the mosquito, which itself can be targeted at a multitude of life stages.

Additional opportunities for therapeutic inroads exist in the consideration of the local spatial dynamics of the target organisms. For drugs to be maximally effective, they need to encounter their targets equally; differences in doses experienced by the targets can determine whether the target is killed, slowed, or unharmed — which in turn affects resistance evolution in the survivors. Developing a greater understanding of biofilm dynamics and organization, for example, will enable more intelligent drug delivery systems so that individual cells are not shielded by their neighbors. Cancer cells in the center of tumors are likewise protected from chemotherapies and incorporation of tumor architecture into treatment plans is necessary.

Attention must also be given to the “ecology” of the patient as a whole. Immune and nutritional status and prior exposure to the pathogen may dictate specific courses of treatment. For example, data suggests that in Africa, 3 day quinine treatments were sufficient to cure malaria infections, while 7 days were required to achieve cures in Thailand. A high level of pre-existing immunity was thought to be present in the African patients, and that perhaps even vaccines which are not entirely preventative may still present an advantage in lessening the amount of drugs needed to fully fight infections. Such a practice could also lessen overall drug exposure, and thus the development of resistance.

In each of the above examples, drug resistance will clearly continue to be a prevalent problem unless steps are taken to include ecological characteristics and evolutionary pressures in the target selection, drug design, and treatment plan. Successful implementation of these plans will require cooperation from health and regulatory agencies, drug companies, farming communities, hospitals and physicians, and individuals who understand that their actions have the potential to contribute to better outcomes for us all.



## Management can prolong treatment effectiveness; every new treatment should have a management plan

After the initial rush of antibiotic discoveries, no new classes of antibiotics have been discovered since the 1970s. As resistance spreads across pathogens and environments, the development of new compounds becomes more urgent. To avoid the mistakes that led to emergent resistance among older drugs, colloquium participants stressed the need to make sure that access to any new drug is tightly controlled. For example, the group proposed that new drugs be given first to hospitals and placed under the control of the chief medical officer. Use of the drug should be restricted to prescriptions and only prescribed when accompanied with an accurate diagnosis. It was additionally advocated that the highest possible dose be given to patients to prevent resistance from developing.

## What do we still need to know?

A common theme throughout the colloquium discussion addressed the need to better understand what specific drugs are available and how they work. Many old drugs have been abandoned because of toxicity risks, cost in production, or insufficient knowledge about the mechanism of action. By revisiting these drugs, experiments could uncover new target pathways or effective drug combinations. Further, if older drugs have been out of commission due to resistance concerns, sufficient time may have passed for new generations of targets to have regained susceptibility. These studies, along with continued efforts at identifying new drugs, will increase our arsenal of weapons against increasingly resistant pathogens and pests.

The mechanism underlying gene amplification is still an unknown, and yet represents a significant means of resistance evolution across multiple biological systems. Colloquium participants advocated further study of this phenomenon.

The propensity and speed of resistance evolution to different drugs should also be investigated through laboratory and computational studies; diversity of both domesticated lab strains of model organisms as well as environmental populations should be ascertained and incorporated into those experiments.





## ⇒⇒ 8. TAKE-HOME MESSAGES

- Drug and pesticide treatments universally select for drug resistance.
- All pathogens and pests should be viewed as evolutionarily dynamic, not static.
- Treatment plans that incorporate evolutionary and ecological principles can deter or delay resistance.
  - Multiple drugs applied at once can slow resistance evolution, particularly if they force the target into an evolutionary trap.
  - High doses are ideal when killing all of the targets can be guaranteed, but if not, a maintenance approach involving lower doses might be a better approach for some systems.
- Treatment design against essential targets is the typical approach.
  - Attention should be made to ensure the inhibitors are as evolutionarily constrained as possible.
  - Additional drugs or treatments can boost host or environmental fitness, thus providing synergy.
- How one tackles a disease or infestation depends greatly on whether resistance is pre-existing or absent.
  - If resistance is present, population-level control is needed to prevent its spread. Thus, management, detection, and surveillance are key.
  - If resistance is unknown or undetectable, an understanding of the path to resistance is key so resistance can be monitored and combatted when observed.
- Creative solutions drawing from ecological attributes of each system could expand our arsenal of available therapies.





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