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The search for ‘evolution-proof’ antibiotics

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18 Preface

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20 The effectiveness of antibiotics has been widely compromised by the evolution of resistance
21 among pathogenic bacteria. It would be restored by the development of antibiotics to which
22 bacteria cannot evolve resistance. We first discuss two kinds of 'evolution-proof' antibiotic .
23 The first comprises literally evolution-proof antibiotics to which bacteria cannot become
24 resistant by mutation or horizontal gene transfer. The second category comprises agents to
25 which resistance may arise, but so rarely that it does not become epidemic. The likelihood that
26 resistance to a novel agent will spread is evaluated here by a simple model that includes
27 biological and therapeutic parameters governing the evolution of resistance within hosts and
28 the transmission of resistant strains between hosts. This model leads to the conclusion that
29 epidemic spread is unlikely if the frequency of mutations that confer resistance falls below a
30 defined minimum value, and it identifies potential targets for intervention to prevent the
31 evolution of resistance. Whether or not evolution-proof antibiotics are ever found, searching for
32 them is likely to improve the deployment of new and existing agents by advancing our
33 understanding of how resistance evolves.

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1. Evolution-proof antibiotics

The discovery of a new antibiotic is sometimes accompanied by the claim that it is ‘evolution-proof’. For example, teixobactin is a recently-discovered antibiotic that binds to a bacterial cell-wall precursor and kills the cell by liberating autolysins [1]. The absence of resistance was attributed to the ready availability of cell-wall precursors on the outside of Gram-positive bacteria, representing an ‘Achilles’ heel’ rendering teixobactin invulnerable to natural selection. Unfortunately, the precedents are discouraging: pathogenic bacteria have evolved resistance to all major classes of antibiotic, often soon after they have been introduced into clinical practice [2]. The very high priority that has been given to developing new antibiotics, for example by G7 Science Academies (https://www.leopoldina.org/uploads/tx_leopublication/2015_G7_Statement_Infectious_Diseases.pdf) emphasizes the need to understand the forces that drive the evolution of antibiotic resistance in pathogenic bacteria so that the next generation of antibiotics can be used more efficiently. In particular, we need to be able to evaluate ambitious claims that an evolution-proof antibiotic has been discovered, and that is the theme of this article. Several ingenious ways of minimizing the evolution of resistance have been suggested, including the use of drug combinations,[3] targeting virulence rather than growth [4] or quenching public goods [5], but our discussion is restricted to conventional antibiotics used singly.

3. The prospect of an evolution-proof antibiotic.

There are two senses in which an antibiotic might be considered evolution-proof, in the sense that resistance will fail to evolve when the agent is released for clinical use. The first is that resistance cannot evolve because there are no mutations that confer resistance to the antibiotic, and the pathogens that the antibiotic targets are unable to acquire pre-existing resistance genes by horizontal gene transfer. This is a claim based on the physiology and genetics of the bacterial cell, usually on the grounds that the antibiotic targets some highly-conserved feature of the cell that cannot be modified. The alternative is to acknowledge that resistance might arise, but will nevertheless fail to spread because of unfavourable circumstances such as a high rate of

stochastic loss, excessive fitness cost or a low rate of transmission. This is a claim based on the ecological and evolutionary properties of the bacterial population and as such it invokes a quite different set of processes and principles.

We consider first the literal interpretation of ‘evolution-proof’, meaning that resistance cannot arise through mutation and therefore does not exist in contemporary populations.

Lack of novel mutations conferring resistance. All known antibiotics target a relatively small number of essential cell functions, such as cell wall assembly and protein synthesis[6, 7].

Resistance mutations that modify these antibiotic targets compromise these essential functions, generating a fitness cost to bacteria[8-10]. It is conceivable that this cost could prevent the evolution of resistance, for example if all of the mutations that can confer resistance to an antibiotic are lethal. The reasoning seems sound in principle, but the prediction often fails in practice. For example, it has been argued that bacteria cannot evolve resistance to antimicrobial peptides because they exploit a highly conserved feature of bacterial cells, the net negative charge on the phospholipids of the cell membrane, which effectively acts as a ‘microbial Achilles’ heel’ that cannot be modified by natural selection [11, 12]. However, bacterial pathogens have evolved a complex array of inducible antimicrobial peptide resistance mechanisms[13, 14], and very high levels of constitutive antimicrobial peptide resistance can easily evolve in the lab [15]. This principle is further illustrated by resistance to rifampicin, a broad-spectrum antibiotic that binds to a domain of the β -subunit of RNA polymerase that is highly conserved across bacteria[16]. Pathogenic bacteria readily evolve resistance to rifampicin [17-20] by amino acid substitutions that alter the structure of the rifampicin binding pocket [16], trading off increased antibiotic resistance for reduced transcriptional efficiency[19, 21]. In spite of the extreme conservation of this domain, the average fitness cost of rifampicin resistance mutations across bacteria is only 20% [10] and there have been many independent evolutionary origins of rifampicin resistance in the pathogen *M. tuberculosis*[17, 18].

How, then, can we estimate the potential for bacteria to evolve resistance to an antibiotic by spontaneous mutation? The classic method to approach this problem is to use a fluctuation test [22] (Figure 1A). In this method, a large number of independent cultures that were seeded with a mutant-free inoculum of a parental strain are screened for resistance by plating on agar plates containing a high concentration of antibiotic. By counting the resistant colonies that appear after a brief period of incubation, typically 1-2 days, it is possible to estimate the rate of high level resistance mutations, and sequencing can be used to identify these mutations.

An alternative method for testing the ability of bacteria to evolve resistance to an antibiotic by spontaneous mutation is to use a serial passage or ‘antibiotic ramp’ experiment (Figure 1B). In

this experimental design, replicate populations are each regularly transferred to media containing increasing doses of antibiotic. Mutations that increase resistance tend to also increase fitness at low doses of antibiotic [23-25]; therefore, selection at relatively low doses effectively enriches bacterial populations for mutations that increase resistance at higher doses. Because of this enrichment, it is easier for bacterial populations to evolve resistance at the critical point at which antibiotic concentrations exceed the lethal limit for the ancestor. In particular, stepwise evolution can lead to high levels of resistance based on several mutations, as is the case for colistin[26] and ciprofloxacin[27].

But what if no resistant bacteria are identified using these methods? On the one hand, this result may mean that it is impossible for the pathogen to evolve resistance to the antibiotic by mutation. Alternatively, it is possible that the experiment simply did not detect any resistance because resistance mutations are too rare. The power of these experiments to detect resistance is determined by the number of mutations at each site in the genome are effectively screened by selection. In the case of a fluctuation test, this is the mutation rate per nucleotide (μ) multiplied by the number of cells screened (N), i.e. $N\mu$. For a serial passage experiment, the number of mutations screened that are effectively screened is approximately $N_t\mu gr$, where N_t is the number of cells that are transferred to fresh media in each cycle, r is the number of replicate populations, and g is the number of generations that elapsed during the experiment. Serial passage experiments are therefore more powerful by a factor $(N_t/N)gr$.

Because mutation is a stochastic process, the number of mutation events per site in an expanding population of bacteria follows a Poisson distribution [22]. Put simply, some sites in the genome of an expanding bacterial population will experience more mutations than others due to chance alone. The implication of this is that the average number of mutations per site in the population of bacteria that is being screened for resistance must be considerably greater than 1 in order to ensure that at least one mutation occurs at every site in the genome. For example, the number of mutations per site should be at least 7 in order to ensure that at least one mutation will occur at 99.9% of sites in the genome according to the Poisson distribution. Ideally, the number of mutations at each site in the genome should be greater than 1 in an experiment to test for the evolution of resistance, because chance events may eliminate the lineage founded by a single resistant cell before it is able to form a visible colony during a fluctuation test or sweep to fixation during a serial passage experiment. This stochastic loss of resistant lineages is expected to be most common when resistance carries a large fitness cost, or when mutations confer only partial resistance to the antibiotic being used for selection. Therefore, we suggest that a good benchmark may be to screen enough cells to ensure that at least 3 mutations are present at 99.9% of sites in the genome, and this requires an average of 12

mutations per site. In *E.coli*, which has a mutation rate of 2×10^{-10} mutations/ nucleotide/ replication[28], this would require screening 2.4×10^{11} cells in a fluctuation test, or propagating 100 populations for 100 generations with an inoculum of 2.4×10^7 cells per population, equivalent to about 24 uL of a saturated *E. coli* culture.

For example, the paper reporting the discovery of teixobactin screened approximately 10^{10} cells of *S.aureus* for resistance. Assuming a mutation rate of roughly 1×10^{-3} mutations/ genome/ generation [29, 30] the average number of mutations per site in the *S. aureus* genome was 3.7 and at least one mutation occurred at 97.6% of sites in the genome. Although these figures are encouraging, we suggest that the claim that *S. aureus* cannot evolve teixobactin resistance by mutation should be treated with caution. First, according to our estimate, only 70 % of sites in the genome were mutated at least 3 times in this assay. Secondly, our calculations are based on relatively coarse estimates of the mutation rate across all bacteria; it is difficult to place confidence limits on this estimate of the mutation rate for any particular bacterium, and the true mutation rate of *S. aureus* might be an order of magnitude greater or less than the figure that we have used.

Lack of standing variation. It is laborious, but straightforward, to measure the potential of pathogenic bacteria to evolve resistance by spontaneous mutations in the laboratory, because the source of genetic variation can be isolated and manipulated. However, many of the most clinically important resistance genes, such as extended-spectrum β -lactamases in enteric pathogens [31-33] and *sccMec* cassettes in *S.aureus* [34, 35], have been acquired by pathogens via horizontal gene transfer. Unfortunately, studying the evolution of novel resistance by horizontal gene transfer in the lab is inherently difficult, because this process depends on complex ecological interactions that cannot be readily reproduced under simple *in vitro* experimental conditions.

One way to circumvent this problem is to use functional metagenomics (Figure 2A). In this approach, DNA from environmental sources is cloned into bacterial model strains, and transformants are screened for resistance to antibiotics [2]. Functional metagenomics has provided important insights into the diversity of resistance genes present in natural communities[36-38]. Importantly, this approach has shown that resistance to naturally occurring antibiotics is widespread environments[39, 40], even in environmental samples that predate the clinical use of antibiotics[41], perhaps because antibiotic resistance genes have alternative metabolic functions that are not related to resistance[42] . Alternatively, pre-existing resistance genes can be detected by an amplifying bioreactor [43](Figure 2B). This device consists of a chemostat-like vessel into which runs a continuous supply of material from an environmental source such as an ocean or a hospital waste stream. The physical state of the

reactor is determined by continuous input to the vessel from a reservoir that includes a selective agent such as an antibiotic. The reactor is maintained at constant volume by an overflow, so that a fixed fraction of its volume is replaced per unit time. Any strain that is able to proliferate within the vessel at a rate exceeding this replacement rate will tend to increase in abundance to a point where it can be detected. In this case, the extent of the experiment is defined by the number of cells that have been screened by an array of ABRs. For example, a single 1L vessel receiving 250mL per day from a source containing 10^6 cells mL⁻¹ will screen about 10^{10} cells per month of operation and is likely to trap any strain with a growth rate exceeding 0.25 per day.

A negative result from both experimental evolution and an ABR survey provides evidence for a literally evolution-proof antibiotic, in the conditions tested, in proportion to their extent. This could be expressed by an index representing the number of replications screened by the experiment and the number of cells screened by the survey, such that, for example, 15/17 would indicate an experiment that screened 10^{15} replications and a survey that screened 10^{17} cells. In this way, a combination of experiment and survey would provide an objective criterion to evaluate any claim that a newly discovered agent is effectively evolution-proof.

2. Eco-evolutionary epidemiology

The spread of resistance is an evolutionary process that unfolds within some combination of environmental conditions. It is therefore driven by evolutionary and ecological principles that govern how a population responds to a toxic agent through the differential propagation of lineages. Antibiotic treatment generates natural selection for resistance, and this allows resistant strains to proliferate within hosts. Transmission provides resistant strains with the opportunity to spread between hosts, and this can allow resistance to spread at an epidemiological level. Understanding the spread of resistance in pathogens therefore requires integrating processes that operate within and between hosts. Although the toxic nature of antibiotics guarantees that antibiotic use will generate selection for resistance within hosts, the epidemic transmission of resistance is far from guaranteed. In theory, it might be unlikely that resistance would spread epidemically in pathogen populations, even if bacteria readily evolved resistance during infections. In this case, the antibiotic would be effectively evolution-proof, even if it were not literally evolution-proof. In practice, the literature on within-host proliferation and the literature on between-host transmission are almost entirely separate from one another, the former stressing pharmacokinetics and genetics, whereas the latter stresses ecology. Synthetic treatments that include both, even at the level of simple models, are still quite rare [44-46]. This represents a very important gap in our understanding of the evolutionary biology of antibiotic resistance, and one of the main themes of this article is that

jointly considering within-host and between-host processes can provide novel important insights into how to prevent the evolutionary emergence of resistance.

Within-host processes: natural selection and competitive release. Bacteria infecting a host may all be killed by antibiotic therapy, ending the infection. Alternatively, they may continue to proliferate if resistant types arise and spread through natural selection, thereby establishing a persistent infection within the host. If no resistant strain is present, the susceptible strains are unable to grow if the concentration of antibiotic in host tissue exceeds some threshold value (the minimum inhibitory concentration, or MIC). If a resistant strain is present, it has a higher growth rate above some threshold value below the MIC, and as therapy continues it spreads within the pathogen population. The outcome is a persistent infection unless the antibiotic concentration becomes so high that even the resistant strain is unable to grow (the mutant prevention concentration, or MPC) or until the immune system clears the infection. At very high concentrations selection ceases because both susceptible and resistant strains are killed, or rendered unable to grow. Thus, there is a range of antibiotic concentrations between MIC and MPC within which natural selection drives the spread of resistant strains within the host. This is the ‘mutant selection window’ hypothesis that is the basis of the conventional interpretation of selection for resistance within hosts[47].

Day, Huijben & Read have recently offered a different interpretation of pathogen dynamics within the host that is based on considering the absolute growth rate of resistant and sensitive strains[48]. The dose of antibiotic that is administered will normally be sufficient to kill all the susceptible bacteria, otherwise the infection will persist whether or not resistant types are present. Host resources that would otherwise be monopolized by susceptible bacteria are now available to be exploited by resistant strains. Whether or not the resistant bacteria proliferate does not depend on their growth rate relative to the susceptible types, but only on their absolute growth rate: if their rate of increase is positive they will increase in abundance, renewing the infection, and otherwise not. In the broadest sense, this is an evolutionary process, involving the replacement of one type by another through differential reproduction. It does not follow the usual population genetics paradigm, however, of the sequential substitution of viable types differing in relative fitness. It more closely resembles the ecological process of competitive release, whereby a species is enabled to expand into a niche through the removal of a competitor which previously excluded it.

Transmission between hosts. Resistant strains that can be transmitted to new host individuals, such as *E. coli* ST131[49] or *S. aureus* ST22 [50] give rise to epidemics. The classical account of pathogen transmission is the SIR model that categorizes host individuals as being susceptible, infected or recovered, and treats infection rates as being proportional to the

product of host and pathogen density [51, 52]. Quite unlike the literature on within-host processes, most accounts of transmission are based on absolute fitness, usually calculated as the ratio of the rate of infection of new hosts to the rate of death or recovery of old hosts. The pathogen will spread, causing an epidemic, if the rate of infection exceeds the rate of death or recovery, that is, if its absolute fitness is greater than unity.

Quite apart from its role in eliciting the evolution of resistance, antibiotic therapy increases the risk that disease will become epidemic. This effect can be explained with a simple analogy to the well-known role that the immune system plays in combating infectious diseases caused by viruses. Once infected, a host which mounts a successful immune response to a viral infection, eliminating the pathogen, cannot subsequently be re-infected. A nascent epidemic might therefore be smothered by the rising frequency of immune hosts. An infected host which has been cleared of a bacterial infection by antibiotic therapy, on the other hand, is vulnerable to re-infection. Antibiotic treatment therefore provides an ecological opportunity to pathogenic bacteria by increasing the density of hosts that are susceptible to infection. It provides this opportunity to susceptible and resistant strains, but their success relative to one another is beside the point: if the transmission of the resistant strain is enhanced by antibiotic therapy such that its absolute rate of increase exceeds unity, then an epidemic will follow.

3. An effectively evolution-proof antibiotic.

For a literally evolution-proof antibiotic, the frequency of mutations that confer resistance must be zero. A less severe condition is that the frequency of mutations that confer resistance should be less than threshold for its spread in given circumstances. To address this problem, we developed a simple evo-epidemiological model to investigate how bacterial proliferation and transmission impact the spread of resistance into a pathogen population. Our model considers a new antibiotic, and we therefore assume that the pathogen population lacks resistance to the antibiotic. In our model, individual hosts are infected with a single strain of bacteria that grows until it reaches a threshold population size where the host is treated with antibiotic. Treatment can lead to either of two outcomes, the elimination of the sensitive strain or the evolution of resistance (Figure 3A). Infected hosts contribute cells to a reservoir of pathogen cells that can then go on to infect susceptible hosts (Figure 3B).

This is a simple model that lacks many of the details of the biology of bacterial pathogens, and our justification for using a very general model is that this approach is required to generate general insights into the drivers of resistance. We focus on epidemic spread because this represents a worst-case scenario for resistance management, and the spread of antibiotic resistance in many pathogenic bacteria has been driven by the epidemic spread of a small number of resistant lineages. Our model focuses on the forces that drive the spread of new resistant strains, because an antibiotic is plainly not evolution-proof once resistance has spread to an appreciable frequency.

A full description of the model is provided in Box 1, but a simple verbal model for the epidemic spread of resistance is as follows:

$$\text{Finite rate of increase} = \text{Number of treated hosts} \times \text{Probability of fixation of resistant strain per host} \times \text{Number of new hosts infected with resistant strain}$$

Each of these components can in turn be broken down into separate terms each representing a state or a rate, enabling us to identify parameters that influence the evolution of resistance. According to our model, the crucial parameter for designating an antibiotic as ‘evolution-proof’ is f , the fraction of mutations that confer resistance. A literally resistless antibiotic has $f = 0$. More interestingly, our model shows that antibiotics can be evolution-proof if $f > 0$, and it identifies 8 parameters, equally divided between within-host proliferation and between-host transmission, that offer potential opportunities for intervention to prevent the evolution of an epidemic strain resistant to the new antibiotic. They are summarized in Box 2. This is intended to provide a framework for assessing the utility of measures that might be taken to prolong the life of a new antibiotic by shielding it from natural selection.

4. Discussion

The antibiotics that were discovered in the 20th century are rapidly becoming obsolete as resistance to them evolves and becomes widespread, and this will lead to a truly global crisis in healthcare unless new antibiotics are discovered. We are cautiously optimistic that novel approaches to antibiotic discovery will yield the next generation of antibiotics, and it will be

vitaly important to use these antibiotics in a way that minimizes the rate of evolution of resistance. In this paper, we have addressed two related issues: first, how can estimate the evolutionary potential for antibiotic resistance? And secondly, how can we use antibiotics to minimize the risk of resistance actually evolving?

We have cast this paper in terms of the prospect of an evolution-proof antibiotic, but a literally evolution-proof antibiotic is something of a straw man: a useful heuristic concept, but a concept that in practice is likely to be flawed. The vast metabolic diversity of bacteria suggests that some forms, at least, will evolve resistance to any antibiotic, given enough time and ecological opportunity. At the same time, the evo-epidemiological model of resistance that we have put forward predicts that it should be possible for any antibiotic to be effectively evolution-proof, as long as the antibiotic is administered in a way that prevents the epidemic spread of resistant lineages. This discrepancy is nicely illustrated by the contrast between vaccination, which almost never leads to the evolution of vaccine resistance[53], and antibiotic use, which effectively selects for antibiotic resistance. We speculate that this difference exists because invading pathogens are detected and destroyed almost immediately by the immune system in vaccinated hosts, and are thus eliminated before vaccine resistance can evolve. Antibiotic therapy, conversely, is delayed and systemic, which provides an opportunity for the evolution of resistance. Serial passage experiments imitate a therapeutic situation and thereby demonstrate the potential for the evolution of resistance

The threshold frequency for resistance mutations in our combined model is set by an expression that provides a catalogue of the different kinds of intervention that might be used to prevent or contain the evolution of resistance to a newly introduced antibiotic. This expression contains a logarithmic term (as numerator) and an arithmetic term (as denominator). The numerator $\ln(1 - X)$ includes terms related to the transmission of pathogens between hosts whereas the denominator $2P_0Us$ is related to the evolution of resistance within hosts. In general, it will be more effective to modify processes that are directly related to f^* , since this will elicit a directly proportional response, whereas modifying a logarithmic term will elicit a much less than proportional response. This suggests that suppressing the initial evolution of resistance within hosts is more likely to be effective than controlling transmission between hosts. In particular, early treatment (minimizing P_0) with a low dose (minimizing s) may an effective strategy.

The combined model of within-host proliferation and between-host transmission that we have described provides a consistent framework for identifying the possible kinds of intervention, the magnitude of effects and the likelihood of success. We emphasize that the model we propose here is a first step rather than a firm and final conclusion and we fully expect that it will

be replaced by more concrete and more rigorous models. The search for an evolution-proof antibiotic might be likened to the search for a perpetual-motion machine: the search was eventually found to be fruitless, but understanding the reasons for its failure led to the development of a sound theory of mechanics and thermodynamics. We need a corresponding theory of the ecology and evolution of resistance to guide the development and deployment of the next generation of antibiotics.

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