Drug interactions modulate the potential for evolution of resistance

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Antimicrobial treatments increasingly rely on multidrug combinations, in part because of the emergence and spread of antibiotic resistance. The continued effectiveness of combination treatments depends crucially on the frequency with which multidrug resistance arises. Yet, it is unknown how this propensity for resistance depends on cross-resistance and on epistatic interactions—ranging from synergy to antagonism—between the drugs. Here, we analyzed how interactions between pairs of drugs affect the spontaneous emergence of resistance in the medically important pathogen Staphylococcus aureus. Resistance is selected for within a window of drug concentrations high enough to inhibit wild-type growth but low enough for some resistant mutants to grow. Introducing an experimental method for high-throughput colony imaging, we counted resistant colonies arising across a twodimensional matrix of drug concentrations for each of three drug pairs. Our data show that these different drug combinations have significantly different impacts on the size of the window of drug concentrations where resistance is selected for. We framed these results in a mathematical model in which the frequencies of resistance to single drugs, cross-resistance, and epistasis combine to determine the propensity for multidrug resistance. The theory suggests that drug pairs which interact synergistically, preferred for their immediate efficacy, may in fact favor the future evolution of resistance. This framework reveals the central role of drug epistasis in the evolution of resistance and points to new strategies for combating the emergence of drug-resistant bacteria.

antibiotic resistance | drug combinations | epistasis | Staphylococcus aureus | mutant selection window

The widespread use of antibiotics pits clinical need against the reality of evolution (1–3). The clinical goal is to kill as many pathogenic bacteria as possible, or inhibit their growth to allow the immune system to gain the upper hand; but a drug that kills or inhibits the growth of susceptible pathogens confers a dramatic selective advantage to resistant lineages, eventually making the drug ineffective. Although major advances have been made in describing the impact of single drugs on bacterial resistance (3), it is still unclear how drugs in combination affect the evolution of resistance. Combinations of drugs may inhibit bacterial growth in complex ways, deviating from the neutral situation expected when the drugs do not interact (4–6). Compared with this null situation, drug combinations that interact to increase each other’s effects are termed “synergistic”; drugs whose combined effect is smaller than expected are termed “antagonistic” (4–7, 39) (Fig. 1D). We have previously shown that these epistatic drug interactions profoundly affect the selective advantage of a single horizontally transferred resistance allele (8). Here, we focus on the more complex scenario of the evolution of multidrug resistance by spontaneously occurring mutations.

In many infectious and noninfectious diseases, including HIV (9), tuberculosis (10), malaria (11, 12), and cancer (13), high rates of mutation confer resistance to individual drugs. Combination therapies are therefore used to increase the killing of single-drug-resistant strains or mutants. Unfortunately, multidrug resistance still arises: multiple mutations conferring resistance may accumulate, or a single mutation may confer resistance to several drugs (cross-resistance). We address here the frequency of such spontaneous resistant mutations in Staphylococcus aureus, one of the most worrisome multidrug-resistant bacteria (14). Although a major mode of resistance in S. aureus is horizontal gene transfer, resistance acquired vertically by spontaneous mutations is another concern and combination therapies aimed at preventing their emergence are frequently used (15, 16). The approach we developed using S. aureus as a model system is general in scope and can be applied to pathogens such as Mycobacterium tuberculosis, where resistance acquired during treatment by spontaneous mutations is critical.

Antibiotics impose a strong selection pressure on bacterial populations (17, 18): susceptible cells do not grow, and resistant cells already present in the population are selectively enriched. A commonly used measure of the potential to evolve resistance by spontaneous mutations is the size of the mutant selection window (MSW)—the range of drug concentrations where resistance is selectively enriched (19, 20). The MSW ranges from the minimum inhibitory concentration (MIC) that inhibits wild-type growth, to the mutant prevention concentration (MPC) where even very rare mutants are unlikely to grow (Fig. 1A). The frequency of resistance and the MSW of several clinically important individual drugs have been well characterized (21–26), and evidence suggests that the MSW of drug combinations can be smaller than the MSW of any of their individual drug constituents (27). Yet, a general relationship between the frequencies of cells resistant to combinations of drugs and the frequencies of resistance to each drug alone has not been established, and the effect of drug–drug interactions (epistasis and cross-resistance) on this relationship is not known.

We use both experimental and theoretical tools to explore how interactions between antibiotics impact the landscape on which selection can act. We develop a high-throughput system to measure frequencies of resistant S. aureus mutants over a matrix of concentrations of pairwise drug combinations, and apply this tool to three different types of drug pairs. Motivated by the diversity of results observed, we develop a quantitative theoretical model that makes clear the central role of drug epistasis and the impact of cross-resistance on the potential for evolution of resistance in multidrug environments. This model yields direct predictions for the impact of drug synergy on the emergence of resistance.


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Extending the MPC to Antibiotic Combinations Necessitates Consideration of Drug Epistasis. Resistant mutants may appear spontaneously because of replication errors at frequencies typically lower than 1 in 10^6 cells. The frequency of those mutants and the drug window within which they survive characterize the potential of the population to evolve resistance and is represented by the curve \( F_X(X) \)—the frequency of mutants that resist concentration \( C_X \) of drug X (Fig. 1A). This curve typically presents plateaus with sharp drops, indicating the existence of subpopulations of resistant cells. The drug concentration at which the frequency drops to nontectable levels is the MPC, defined here as \( F_X < 10^{-9} \) (see Materials and Methods). The mutant selection window (MSW) of a drug extends from the MIC to the MPC. To allow comparison between different antibiotics, we normalize drug concentrations to their MICs in different antibiotics, we normalize drug concentrations to their MICs (dashed red) above which the frequency of resistance \( f_X \) drops to nontectable values. (8) In drug combinations, these notions extend to the MIC line (blue) and the MPC line (red). Resistance frequency \( f_{XY}(C_X, C_Y) \), gray surface is a function of the two-drug dosage and depends on the frequencies of resistance to the individual drugs, cross-resistance, and epistatic interactions. “Effective drugs,” obtained by combining X and Y at fixed proportions, are geometrically represented by lines extending from the origin (black line at angle \( \theta \)), from which their MSW is defined (double-headed arrow). (C) The MSW of these effective drugs is plotted against the ratio of the drug combination (represented by \( \theta \)), the smallest of these windows characterizes the drug combination’s potential to limit the emergence of resistance. (D) The shape of the MIC line of two drugs defines their epistatic interactions: a linear line signifies no epistasis (Left), deviation below linearity signifies synergy (Center), deviation above linearity signifies antagonism (Right). The drug concentration marked by \( \theta \) allows wild-type growth in the antagonistic case (therefore, \( F_{XY} = 1 \) but not in the synergistic case \( F_{XY} < 1 \)). In both cases, frequencies of resistance to the individual drugs alone are the same \( (F_X = 0, F_Y = 0) \), illustrating that \( F_{XY} \) is not in general equal to \( F_X F_Y \) but rather depends critically on epistatic interactions.

Experiments Show That Multidrug Resistance Varies Dramatically Between Drug Combinations. To explore how resistance to combinations of drugs depends on resistance to each drug alone and interactions between the drugs (epistasis and cross-resistance), we designed an experimental setup to systematically measure the frequencies of resistance to each of the individual drugs and combinations on growth inhibition can deviate from the null situation expected by Loewe additivity (Fig. 1D Left) (5), defining synergistic or antagonistic epistasis [Fig. 1D and supporting information (SI) Fig. S1]. Consider, for example, an environment containing \( 0.75 \) MIC of drug X and of 0.75 MIC of Y (Fig. 1D, \( \times \)). In the antagonistic case, the wild type is able to grow and therefore the frequency of resistance is effectively \( 1 [F_{XY}(0.75, 0.75) = 1] \). In contrast, if the drugs interact synergistically, the wild type cannot grow and therefore \( F_{XY}(0.75, 0.75) < 1 \). The frequency of resistance to each drug alone is the same in both cases \( [F_{XY}(0.75) = F_Y(0.75) = 1] \), but epistatic interactions dramatically affect resistance to the combination. This simple example illustrates that even in the absence of cross-resistance, the frequency of cells resistant to the combination is not trivially the product of the frequencies of cells resistant to each of the single drugs.
leads to effective drugs whose MSWs can be up to one order of magnitude smaller than the MSW of FUS or ERY alone (Fig. 4 Insets). Thus, combinations of FUS and ERY can be found that significantly narrow the drug regime that selects for resistance. This effect is not observed for FUS-AMI or CPR-AMP. We find that the epistatic interaction is not observed for FUS-AMI or CPR-AMP. We find that the epistasis is not observed for FUS-AMI or CPR-AMP.

**Resistance Frequencies to Individual Drugs, Cross-Resistance, and Epistasis Determine the Frequency of Resistance to Multidrug Combinations.** The frequency of resistance $F_X$ to a single antibiotic X derives from the makeup of the bacterial population at the time the antibiotic is introduced. We define $p_X(x)$, the probabilistic density of cells whose MIC of drug X is exactly $x$ (Fig. 3A); it derives from the frequency of resistance as $p_X(x) = -dF_X(x)/dx$. Because the frequency of resistance to $C_X$ of X is, by definition, the frequency of cells whose MIC is greater than $C_X$ (Fig. 3B), we have

$$F_X(C_X) = \int_{x > C_X} p_X(x)dx = \int_{x > 0} \eta_X(x/C_X)p_X(x)dx,$$  \hspace{1cm} [1]

where $\eta_X(x) = 1$ if $z < 1$ and is 0 otherwise. In this equation, $\eta_X$ characterizes the growth of one individual cell: for simplicity we consider only step functions, but $\eta_X$ could also be smooth and account for natural variations in the response of isogenic cells.

In the case of two drugs X and Y, the growth region of a bacterial cell is a function of the two-drug concentration, and is delimited by its MIC line [$\eta_{XY}(C_X, C_Y) = 1$ if the cell can grow in $(C_X, C_Y)$, zero otherwise]. Earlier work (8) has shown that growth regions of wild type ($\eta_{XY}$) and resistant mutants ($\eta$) tend to have a common shape, which characterizes the epistatic interactions between the drugs. We therefore approximate the region of growth of mutant cells in the population by a linear scaling of the wild type’s growth region (Fig. 3C Inset; blue, wild type; red, a mutant). This approximation cannot be tested directly within our experimental data, but the fit of the resulting model to measured resistance frequencies supports its validity. The growth region of a bacterial cell whose MIC of drug X is $x$ and whose MIC of Y is $y$ is approximated by $\eta_{XY}(C_X, C_Y) = \eta_{XY}(x/C_X, y/C_Y)$.

Next, by analogy to the single drug case, $p_{XY}(x, y)$ is the density of cells in the population whose MIC of drug X alone is $x$ and whose MIC of drug Y alone is $y$ (Fig. 3C)—note that it does not contain information on the ability of cells to grow in combinations of these drugs, which is carried by $\eta_{XY}$. With these definitions, the frequency of resistance to a given combination $(C_X, C_Y)$ of drugs X and Y is given by (Fig. 3D)

$$F_{XY}(C_X, C_Y) = \int_{x, y > 0} \eta_{XY}(C_X/C_X, C_Y/C_Y)p_{XY}(x, y)dxdy.$$  \hspace{1cm} [2]

Unlike the MIC line ($\eta_{XY}$), which is experimentally measured in liquid media, the density of population $p_{XY}$ cannot easily be measured. We have built a simple model where $p_{XY}$ depends only on $p_X$ and $p_Y$ (measured as the derivatives of $F_X$ and $F_Y$), and on cross-resistance between the drugs. When cross-resistance is absent, mutations conferring resistance to X or Y are independent: the density of population is $p_{XY}^{indep}(x, y) = p_X(x)p_Y(y)$. Conversely, the mechanisms of resistance could be extremely correlated, as would
be the case if the “two” drugs were in fact the same: this corresponds to a density $p_X^{\text{Corr}}$ that depends only on $p_X$ and $p_Y$ (mathematically defined as the density that maximizes the correlation between resistance to $X$ and $Y$ under the constraints $p_X(x) = f_{x>0} p_X(x,y)dy$ and $p_Y(y) = \int_{y<0} p_X(x,y)dx$; see SI Text). In general, some mutations confer resistance to only one of the drugs, and others confer resistance to both drugs at once: we model $p_{XY}$ as a linear combination between the two extreme cases, $p_{XY} = \xi p_{XY}^{\text{Indep}} + (1 - \xi)p_{XY}^{\text{Corr}}$. The parameter $\xi$ reflects cross-resistance, absent when $\xi = 0$ and maximal when $\xi = 1$.

This constitutes a model that quantifies exactly how drug epistasis, cross-resistance between drugs, and single-drug resistance determine the frequencies of cells resistant to any combination of the drugs $X$ and $Y$. The densities $p_X$ and $p_Y$ are estimated by the derivatives of the measured frequencies of resistance to the individual drugs $F_X, F_Y$ (Eq. 1). They enable the construction of $p_{XY}^{\text{Indep}}$ and $p_{XY}^{\text{Corr}}$, which, tuned by the cross-resistance $\xi$, produce $p_{XY}$. The MIC line of the wild-type is a direct measurement of $\eta_Y$. Finally, $p_{XY}$ and $\eta_Y$ predict the frequency of resistance $F_{XY}$ to any combined concentration of the drugs $X$ and $Y$ (Eq. 2).

The Theoretical Model Captures the Experimental Results. We next compared the predictions of this model with our experimental results. Frequencies of resistance to the single drugs alone ($F_X, F_Y$) were measured together with the entire population. The MIC line of each drug pair ($\eta_{XY}$) was directly measured in liquid media. Cross-resistance ($\xi$) is the only free parameter and was estimated for each drug pair by a least-squares fitting of predicted to experimental frequencies of resistance. The drug pair FUS-AMI shows strong cross-resistance ($\xi = 0.3$), whereas the drug pairs CPR-AMP and FUS-ERY show almost no cross-resistance ($\xi < 10^{-2}$). Our experimental results on resistance to combinations of drugs are well captured by the model (Fig. 4). This framework successfully accounts for very different behaviors in terms of epistatic interactions and cross-resistance.

Theory Predicts That Synergistic Epistasis Favors Resistance. We use this framework to weigh the impact of drug interactions on the evolution of resistance. Cross-resistance increases the MSW of drug combinations; with increased cross-resistance, mutants resistant to both drugs individually occur at frequencies high enough to appear in the population and contribute to the MSW of drug combinations (Fig. S3). The impact of epistasis, however, is less obvious. We present here the simplest model exhibiting the mechanisms by which epistasis affects the potential to evolve resistance to a combinations of drugs. For simplicity, we assume no cross-resistance; the combined impact of cross-resistance and epistasis is presented in Fig. S3.

We consider the case where only two mutations exist, one conferring resistance to drug $X$, the other to drug $Y$; small mutation frequencies preclude the appearance of double mutants, and the MSWs for drug $X$ and for drug $Y$ have the same size $M$. Only epistasis between the two drugs $X$ and $Y$ varies, and is quantified by the real number $\epsilon$, which parameterizes the MIC line as $x^{\epsilon Y} + y^{\epsilon Y} = 1$. Absence of epistatic interactions is represented by $\epsilon = 0$, while antagonism and synergy correspond to $\epsilon > 0$ and $\epsilon < 0$, respectively. Fig. 5 A and B shows the frequencies of resistance to combinations of $X$ and $Y$ for synergistic or antagonistic epistasis. In both cases, $1:1$ ($\theta = 45^\circ$) is the ratio of $X$ and $Y$ for which the effect of the lowest MSW, defined MSW$_{XY}$. Analytical expressions for the corresponding MIC$_{XY}$ (dashed line) and MPC$_{XY}$ (solid line) of this “best” effective drug are geometrically derived and a closed form of the smallest MSW, MSW$_{XY} = (\text{MPC}_{XY} - \text{MIC}_{XY})/\text{MIC}_{XY}$, is obtained:

$$MSW_{XY} = \frac{2}{(1 + (M + 1)^{-\epsilon Y})} - 1.$$
is not possible for combinations of CPR and AMP, or FUS and AMI (arrows).

"drugs" obtained by mixing the two drugs at different proportions; experi-
frequencies of resistance. Although the MPC of that effective drug (solid line) growing more slowly than its MIC (dashed line). As the epistatic interactions vary from synergy to antagonism, the MSW shrinks and eventually vanishes.

Although the MSW of the combination increases with the MSW of the single drugs, it decreases as the drugs become less synergistic (Fig. 5C). In other words, the more antagonistic the epistasis, the smaller the potential to evolve resistance to the drug combination. This signifies that antagonistic drug combinations can be more efficient than synergistic combinations at reducing the range of drug concentrations that select for resistance. Although the MPC_{XY} and the MIC_{XY} of the "best" effective drug both increase in absolute value with the degree of epistasis, the MPC_{XY} increases at a slower pace than the MIC_{XY}; relative to the MIC_{XY}, the value of the MPC_{XY} decreases (Fig. 5C Inset). Therefore, as epistasis goes from synergy to antagonism, the MSW of the combination shrinks and eventually vanishes when the drugs completely buffer or even suppress one another (Fig. S4).

**Discussion**

Current clinical practice emphasizes the use of multidrug treatments primarily to increase the spectrum of activity (29–33), to increase efficacy (34), and, in some pathogens, to decrease the likelihood of the emergence of resistance (29). Clinicians generally prefer synergistic drug pairs when prescribing combination treatments to broaden the spectrum but usually do not consider the effect of drug epistasis on resistance (29). Our results imply that synergistic drug pairs may favor the evolution of resistance. In contrast, largely overlooked antagonistic drug combinations may suppress the emergence of resistance. This study, designed to explore the mostly uncharted territory of resistance to combinations of drugs, comprises drug pairs with different epistasis, different degrees of cross-resistance, and different frequencies of resistance to single drugs. Our theoretical prediction above can be validated experimentally by comparing pairs of drugs with different epistasis but with similar degree of cross-resistance, and similar single-drug MSW. Future screens focusing on finding such pairs could be designed in light of this model.

We focused on the frequency of spontaneous mutations conferring resistance in multidrug environments and emphasized the drug window selective for resistance. We note that this measure of propensity for evolution of resistance does not include other factors that may affect the emergence and spread of drug-resistant pathogens, such as variation in the drug concentration, natural variability in the response of isogenic cells, or the pharmacodynamics of the particular antibiotics (35). The dynamics of antibiotic treatment can lead to rounds of selection and adaptation: resistance may be acquired during the course of treatment. Temporal variations in drug dosage and nongenetic phenotypic tolerance may substantially

![Fig. 4](image-url)  
**Fig. 4.** Good agreement between experimental measures and theoretical predictions. Shown are comparisons of experimental (Left) and theoretical (Right) frequencies of resistance for FUS-ERY (A), CPR-AMP (B), and FUS-AMI (C) (blue, dark red, and light red as indicated). Theoretical surfaces for each drug pair were computed based on measured frequencies of resistance to each individual drug, measured epistasis between the drugs (MIC line of the wild type), and fitted cross-resistance (see Materials and Methods). Although the behaviors of the three pairs of drugs in terms of epistasis and cross-resistance are very different, the model accounts remarkably well for the frequencies of resistance and MPC lines (red). (Insets) MSW of "effective drugs" obtained by mixing the two drugs at different proportions; experimental points are plotted with their conservative error bars, and the lines correspond to the model prediction. FUS and ERY can be combined to significantly reduce the MSW compared with that of FUS or ERY alone, whereas this is not possible for combinations of CPR and AMP, or FUS and AMI (arrows).
increase the likelihood of emergence and rate of evolution of resistance. For instance, persistor cells may remain dormant long enough for the antibiotic to decay, enabling adaptation and subsequent selection (36, 40). Furthermore, in natural and clinical settings, resistance acquired through horizontal transfer may play a major role in the evolution of resistance. Examining the impact of drug–drug interactions on these factors central to the development of drug resistance is a promising avenue for future research.

In conclusion, we present an experimental–theoretical framework that offers a quantitative, unified understanding of how resistance to individual drugs, cross-resistance, and epistatic interactions affect the frequency and efficacy of resistance to drug combinations. Importantly, our results suggest that antagonistic combinations may narrow the range of drug concentrations where resistant strains are selected for. In contrast, synergistic drug combinations, typically preferred in clinical settings, may in fact favor the evolution of resistance even though they increase killing efficiency. Our results indicate that drug interactions could be central to a tradeoff between immediate efficacy and the future prevention of resistance.

Materials and Methods

Bacteria and Antibiotics. We used a streptomycin-resistant S. aureus strain Newman NCTC 8178 (37). Growth media was liquid or agar Luria broth (LB) supplemented, as indicated, with one or two of five different antibiotics (see Table S1). A single colony (starting from a single cell) was inoculated in LB liquid and grown overnight; frozen aliquots of this culture were kept at −80°C. All experiments were initiated from a freshly thawed aliquot from this single batch.

MIC Line. The MIC line of a drug pair was measured by a standard overnight growth assay in liquid media, inoculating −10^6 wild-type cells in each of 96 wells (Costar plate; 150 μl per well) forming a 12 × 8 gradient of drug concentration (dilutions of 2/3 to 9/10). The MIC line was defined as the line separating regions of growth and no growth (practically, the contour line of optical density 0.1). The shape of the MIC line was used to define the function γ for each specific drug pair (Fig. 2 C–E Insets). The sign of the epistatic interactions was determined by fitting γ with the function x^{α,y} + y^{α,y} = 1; synergy, additivity and antagonism correspond respectively to negative, null, and positive values of α.

Frequency of Resistance. We measured the frequency of resistance with a resolution spanning nine orders of magnitude, across an 11 × 11 grid of drug concentrations. For each two-drug concentration we used one six-well plate (Becton Dickinson Multwell), and poured 7 ml of agar supplemented with the selected concentration of drugs (e.g., Fig. S5). After agar solidification, each of the six wells was inoculated with a different number of bacterial cells (approximately 10^5, 10^4, 10^5, 10^5, 10^5, and 10^6). The plates were then incubated on scanners (see below) in a controlled environmental room at 30°C and 70% relative humidity for 5 days, a duration optimized for the detection of resistant colonies by our custom software (Fig. S6: effect of the incubation time).

The sign of the epistatic interactions was determined by fitting γ with the function x^{α,y} + y^{α,y} = 1; synergy, additivity and antagonism correspond respectively to negative, null, and positive values of α.

Scanner and Imaging Platform. We built an array of 30 office scanners (Epson Perfection 3170/3490) controlled by one computer. Five plates were placed in each scanner. The scanners were programmed to take time-lapse pictures of the plates at 600 dpi every hour for 5 days. We built an image-analysis platform in MATLAB (MathWorks) to count the number of colonies arising in each plate. The platform detects single colonies larger than one-tenth of a millimeter and tracks their growth by using a custom contour-detection algorithm based on contrast gradients (Fig. 2 B).

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