LETTERS

Antibiotic interactions that select against resistance

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Multidrug combinations are increasingly important in combating the spread of antibiotic-resistance in bacterial pathogens[1–3]. On a broader scale, such combinations are also important in understanding microbial ecology and evolution[4,5]. Although the effects of multidrug combinations on bacterial growth have been studied extensively, relatively little is known about their impact on the differential selection between sensitive and resistant bacterial populations[1,6,7]. Normally, the presence of a drug confers an advantage on its resistant mutants in competition with the sensitive wild-type population[1]. Here we show, by using a direct competition assay between doxycycline-resistant and doxycycline-sensitive *Escherichia coli*, that this differential selection can be inverted in a hyper-antagonistic class of drug combinations. Used in such a combination, a drug can render the combined treatment selective against the drug's own resistance allele. Further, this inversion of selection seems largely insensitive to the underlying resistance mechanism and occurs, at sublethal concentrations, while maintaining inhibition of the wild type. These seemingly paradoxical results can be rationalized in terms of a simple geometric argument. Our findings demonstrate a previously unappreciated feature of the fitness landscape for the evolution of resistance and point to a trade-off between the effect of drug interactions on absolute potency and the relative competitive selection that they impose on emerging resistant populations.

The rapid evolution of bacterial drug resistance and the alarming slowdown in development of new antibiotics is spurring attention towards multidrug treatments[2,8]. Drug combinations are classified as synergistic, additive or antagonistic, according to whether the combined effect of the drugs is larger than, equal to or smaller than the effect predicted by their individual activities (Fig. 1)[8,9]. In some cases the effect of the drug combination is even less than that of one of the drugs by itself; we refer to such hyper-antagonistic interactions as suppression (Fig. 1)[10,11]. Normally, we expect resistance to even one of the drugs in a multidrug treatment to confer an advantage on the bacteria. However, an intriguing hypothesis is that in suppressive multidrug treatments, resistance to one of the drugs could actually have the opposite effect. In such cases, although resistance would indeed diminish the burden imposed by one of the drugs, it may also remove the suppression, rendering the combined treatment more effective against the resistant mutant than against the wild type.

Motivated by this hypothesis, we examined the selective pressure imposed on a drug-resistance allele under synergistic versus suppressive drug combinations. As a model, we focused on resistance to doxycycline, a medically important tetracycline antibiotic broadly used to treat a variety of Gram-negative and Gram-positive infections[12]. Doxycycline inhibits protein synthesis by blocking aminoacyl-tRNA binding at the A-site in the 30S ribosomal subunit[13]. Resistance to tetracyclines is typically associated with mobile genetic elements and falls into three classes: efflux pump, ribosomal protection and enzymatic degradation[12,13].

To provide a simple model of the competition between resistant and sensitive strains, we constructed a pair of *Escherichia coli* strains differing only in the chromosomal presence or absence of the Tn10 transposon encoding a well-studied tetracyclines efflux pump (Methods)[14–16]. Although treatment with doxycycline alone obviously confers strong selection for the resistant strain over the sensitive strain, we examined how combinations of doxycycline with other drugs affect this selection pressure. We chose erythromycin and ciprofloxacin, representing the macrolides and quinolones[13] and showing respectively synergistic and suppressive (or antagonistic at low concentrations) interactions with doxycycline[10].

We first tested the effect of the two chosen drug pairs on the growth rate of the doxycycline-sensitive (wild-type) strain. We used a bioluminescence-based assay, which accurately measures bacterial growth rates with a detection sensitivity exceeding that of standard optical density techniques by three orders of magnitude (Methods,

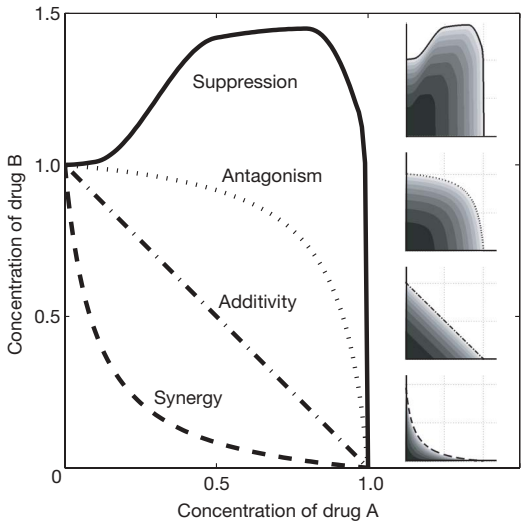

Figure 1 | Schematic representation of synergistic, additive, antagonistic and suppressive drug pairs. Lines of equal effect of the drug combination on growth rate (isoboles) are shown in the two-dimensional concentration space of the two drugs. Loewe additivity[9] assumes that two drugs do not interact if their combined outcome is that expected from a linear interpolation of their two individual outcomes (dash-dotted line). Synergistic drug pairs have a stronger than additive effect corresponding to an isobole below the additive line (dashed line). Antagonistic drug pairs have a less than additive effect (dotted). Suppression interactions (solid line) are a subclass of antagonism in which the combined treatment effect is weaker than that of at least one of the drugs alone. The insets show schematic growth rate maps (darker indicates faster growth) for each interaction type (top to bottom: suppression, antagonism, additivity, synergy). The axes of the insets are identical to those of the main figure. Growth responses to single drugs alone lie along each axis. The isoboles (black lines) shown here indicate drug pair concentrations required to just halt growth.

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and Fig. 2e)[17,18]. The response maps of the measured growth rates and the minimum inhibitory concentration (MIC) line as a function of the two-dimensional drug concentration show the strong synergy between doxycycline and erythromycin (Fig. 2a, b, green). In contrast, strong suppression is seen for doxycycline and ciprofloxacin, for which, over a range of concentrations, doxycycline relieves the inhibitory effect of ciprofloxacin (Fig. 2c, d, green).

We then repeated the measurement for the doxycycline-resistant mutant (Fig. 2a–d, red). The results indicate a roughly 100-fold increase in MIC for doxycycline. No significant changes in ciprofloxacin or erythromycin MICs and no detectable difference in growth rate in drug-free medium were observed[19,20] (Supplementary Fig. S1). Response maps for the resistant strain under both drug combinations are very similar to the corresponding maps for the wild type, except for a large (about 100-fold) rescaling along the doxycycline concentration axis. This rescaling, reflecting the increased concentration of a drug required for the same level of inhibition, is expected for resistance mechanisms that specifically decrease the intracellular concentration or activity of one of the drugs. Indeed, similar rescaling is observed for doxycycline resistances that are based on enzymatic degradation[12,21] and ribosomal protection[12,22], although it can break down at high drug concentrations (Supplementary Fig. S2).

Whereas effective rescaling of doxycycline concentrations appears under both the synergistic (doxycycline–erythromycin) and the suppressive (doxycycline–ciprofloxacin) drug pairs, its effects on selection for resistance are profoundly different (Fig. 2a, c). In the synergistic case, rescaling of the MIC line along a single axis causes the mutant growth regime to be completely inclusive of the wild type (Fig. 2a); that is, there is no combination of the drugs with which the wild type survives whereas the resistant bacteria perish. In the suppression case, however, rescaling generates just such a region of drug concentrations, at which the wild type grows but the resistant mutant does not (area marked with an asterisk in Fig. 2c; see also schematic illustration in Supplementary Fig. S3a). Similarly, resistances based on enzymatic degradation or ribosomal protection also yield drug-concentration regions in which the sensitive strain grows but the resistant mutants do not (Supplementary Fig. S2). These observations suggest that, in a competitive situation, resistant strains experience positive selection in all regions of the synergistic treatment but may actually be selected against with certain suppressive drug combinations.

To query this differential selection for resistance directly, we set up a competition assay between the sensitive and resistant strains. We used the method developed in ref. 23 to measure the change in ratio of the wild-type and resistant populations by differential labelling with cyan (CFP) and yellow (YFP) fluorescent proteins (Methods). The competition was performed in a 12×16 array of mixed concentrations of the two drugs (Fig. 3). We observed a neutral or very minor fitness cost of doxycycline resistance in drug-free medium, and in ciprofloxacin or erythromycin alone (yellow along the *y* axis in Fig. 3a, b; see also Supplementary Fig. S1 and Supplementary Tables 1 and 2). As expected, the doxycycline-resistant mutant outcompeted the wild type under doxycycline treatment alone and when doxycycline was synergistically paired with erythromycin (Fig. 3a). Indeed, selection for the resistance allele was even stronger in the synergistic multidrug treatment than under doxycycline alone (for example, adding 50 $\mu g\,ml^{-1}$ erythromycin to 0.1 $\mu g\,ml^{-1}$ doxycycline increased selection for doxycycline resistance). A possible intuitive explanation is that resistance to doxycycline effectively reduces

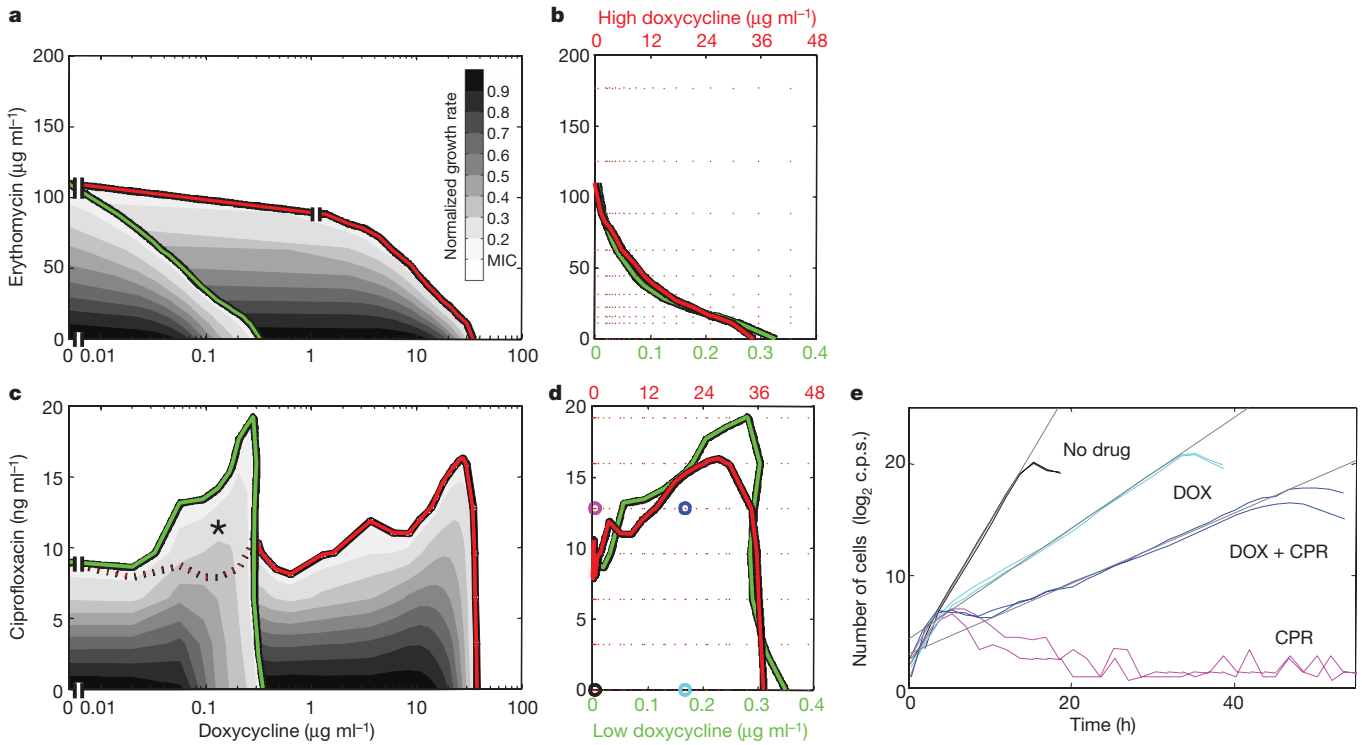

Figure 2 | Rescaling of effective drug concentrations by resistance generates a region exclusive to growth of sensitive bacteria in a suppressive drug combination. a–d, MIC lines (green, sensitive strain Wyl; red, doxycycline-resistant strain t17yl) and higher growth rate isoboles (grey scale) in synergistic (doxycycline–erythromycin (**a**, **b**)) and suppressive (doxycycline–ciprofloxacin (**c**, **d**)) drug combinations. The MIC lines of the sensitive and resistant strains are similar, except for a rescaling in doxycycline concentrations (linear scales in **b** and **d**). In the synergistic case (**a**), this scaling leaves the growth region of the sensitive strain fully enclosed by that of the resistant strain. In contrast, in the suppressive case, the scaling generates a region in which only the sensitive strain grows (**c**, asterisk). Growth rates were measured at an array of drug concentrations indicated in **b** and **d** (green points, sensitive; red points, resistant) by c.p.s. of bacterial luminescence versus time (see Methods). **e**, Sample growth curves of the sensitive strain at four conditions indicated in **d**: no drug (black), doxycycline only (cyan), ciprofloxacin only (magenta) and the combination (blue). Two replicates and their linear fit (grey lines) are shown.

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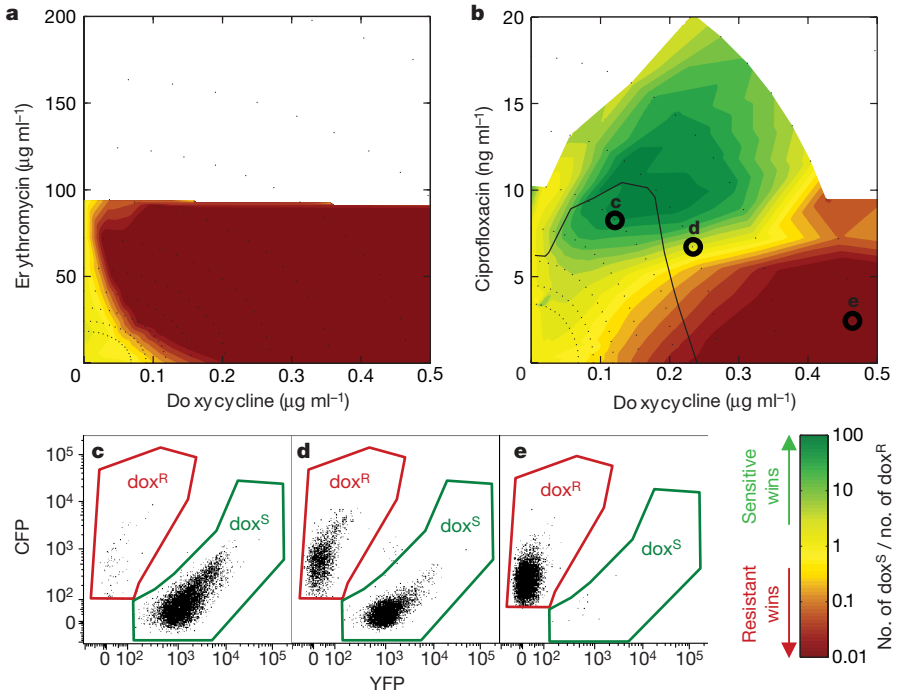

Figure 3 | Competitive selection against resistance in a suppressive drug combination. Doxycycline-sensitive (dox^S) and doxycycline-resistant (dox^R) strains, differentially tagged with CFP and YFP, were inoculated at a 1:1 ratio into an array of drug combinations (**a**, **b**, black dots). Final ratios, reflecting fitness differences (growth and death) between the strains, measured by FACS after 24 h, are shown for combination treatments of doxycycline–erythromycin (synergy, **a**) and doxycycline–ciprofloxacin (suppression, **b**) as indicated in the colour scale at the bottom right. Blank regions indicate no growth. Along a line of constant wild-type inhibition as measured in Fig. 2 (for example 70% inhibition, solid black line in **b**), increasing the doxycycline concentration can select against the resistant mutant even while maintaining inhibition of the wild type. **c**–**e**, Sample FACS data at points indicated in **b**. The data are consistent across experimental replicates and CFP/YFP marker swaps (data not shown).

not only the doxycycline burden but also the additional burden imposed by the erythromycin–doxycycline synergy. Conversely, under the suppressive drug combination (doxycycline–ciprofloxacin), we found diminished selection for the resistant mutant in comparison with doxycycline alone. Furthermore, there was a clear region of concentrations of ciprofloxacin and doxycycline at which the drug combination actually selected against the resistant genotype (Fig. 3b, green region). Note that in the presence of ciprofloxacin, adding doxycycline generates selection against its own resistant mutant (for example, adding 0.1 μg ml^{-1} doxycycline to 7.5 ng ml^{-1} ciprofloxacin). This relative selection against resistance could be achieved without reducing the absolute level of inhibition of the wild type. At constant wild-type inhibition (Fig. 3b, solid line), the suppressive combination exhibited substantial relative selection against resistance over a broad range of concentrations, whereas ciprofloxacin alone had a neutral or very small effect. These seemingly counterintuitive findings can be explained by our simple geometrical scaling model: when suppression curves such as in Fig. 1 are scaled along the horizontal axis by resistance, they necessarily generate a region of disadvantage for the resistant strain (Supplementary Fig. S3a). In contrast with the phenomenon of resistance–antagonism (specific mutations generate resistance to one drug coupled to hypersusceptibility to another)[24,25], this selection inversion works on uncoupled resistances.

Our data show that in suppressing drug combinations, a drug can be used to exert competitive selection against its own resistance allele. In contrast, synergistic interactions, while increasing absolute potency against both sensitive and resistant strains, also increase relative selection in favour of resistance. These findings point to an inherent trade-off, where antagonistic combinations, which require a higher dosage and have therefore typically been shunned in clinical therapy[2,3], may have the benefit of reducing and even inverting selection for resistance. Although the molecular mechanisms underlying drug interactions may be complex[3,26,27], suppression between antibiotics is not uncommon[10] (Supplementary Fig. S4). Our simple geometrical approximation anticipates a region of competitive selection against resistance in such suppressive drug combinations when the targeted resistance mechanism works specifically (uniaxially) on one of the drugs. Indeed, for doxycycline–ciprofloxacin, a region of drug concentrations permitting the growth of doxycycline-sensitive but not resistant strains appears for three very distinct mechanisms of resistance to tetracyclines. It is important to note that the effect observed in the doxycycline–ciprofloxacin combination is unidirectional. Although advantaging the sensitive wild type over a doxycycline-resistant strain, one would not expect it to confer the same benefit over a ciprofloxacin-resistant strain. It would therefore be of considerable interest to employ new multidrug screens[8] to search for reciprocally suppressing drug combinations in which each of the drugs suppresses the effect of the other (Supplementary Fig. S3b). Such drug combinations may block the two single-step mutational paths[28] to complete resistance by imposing selection against resistance to each of the drugs. We emphasize that our work is limited to sublethal drug concentrations, in a controlled environment *in vitro* and that any possible therapeutic implications from these findings are beyond its scope. However, we do hope that these findings may suggest avenues of research into new treatment strategies employing antimicrobial combinations with improved selection against resistance.

METHODS

Media and strains. All experiments were conducted in M63 minimal medium (2 g l^{-1} $(NH_4)_2SO_4$, 13.6 g l^{-1} KH_2PO_4, 0.5 mg l^{-1} $FeSO_4 \bullet 7H_2O$) supplemented with 0.2% glucose, 0.01% casamino acids, 1 mM $MgSO_4$ and 1.5 μM thiamine. Drug solutions were freshly made from powder stocks (doxycycline hyclate, catalogue no. D-9891 (Sigma); ciprofloxacin, catalogue no. 17850 (Fluka); erythromycin, catalogue no. 45673 (Fluka)) and filter-sterilized before each experiment. Strain construction and designations are given in Supplementary Table 3. Assay

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strains were grown from single colonies to saturation in supplemented M63 minimal medium. Cell concentrations were measured by plate count, and aliquots were stored in 15% glycerol at −80 °C. Fresh aliquots were used for each experiment.

Growth rate assay and MIC line. We used a previously developed luminescence-based assay for measuring exponential growth rates[10,17,18]. Cells containing bacterial luciferase constitutively expressed from the pCS-λ plasmid were grown at 30 °C (for growth at 37 °C see Supplementary Fig. S5) in sealed black 96-well microtitre plates (Costar 3792; Corning) in 100 μl of supplemented M63 medium starting from about 100 bacteria per well. For each strain and drug pair, two-dimensional drug concentration gradients were set up in two replicates on 12×16 or 12×30 matrices at concentrations indicated in Supplementary Fig. S6. Emitted light from each well in counts per second (c.p.s.) was recorded by a Topcount NXT plate reader (Perkin-Elmer) at intervals of about 40 min for several days. Growth rates were measured as slopes of log(c.p.s.) over time and thus were insensitive to the absolute intensity per cell. Growth rates were obtained by automatically selecting best least-squares linear fits over sliding intervals within the exponential growth regime (see example in Fig. 2e, and the complete data set in Supplementary Fig. S6). Negative slopes, very low final light levels (c.p.s. < 0.0004 of the assay maximum) and poor fits (root-mean-square > 0.35) were annotated as no data (Supplementary Fig. S6). A response surface was fitted to the average of the replicates by using a smoothing cubic spline, and linearly interpolated isoboles were plotted (with the Matlab functions csaps and contourc). The cubic spline surface reduced noise and did not deviate appreciably from the raw growth data (Supplementary Fig. S7).

The MIC line was defined as the drug concentrations suppressing growth rate to a fixed threshold. To allow comparison with the growth region of the competition assay (comparison between Fig. 2 and Fig. 3), this threshold was defined as the slowest growth rate detectable by optical density at the end of the competition assay. Optical density (absorbance; A) detection in the competition assay requires a roughly 20-fold increase in cell count over 24 h, corresponding to a doubling rate of about $0.17\ h^{-1}$, or 14% of the maximal growth rate in drug-free medium ($1.24\ h^{-1}$). It should still be noted that the values for growth rates and MICs depend on culture conditions, which differ somewhat between the growth (Fig. 2) and competition (Fig. 3) assays.

Competition assay. Our competition assay was derived from that of ref. 23. Sensitive (Wyl and Wcl) and resistant (t17yl and t17cl) strains were labelled with either YFP or CFP on the chromosome, under a strong, constitutive P_{lac} promoter[29,30]. Each strain was labelled with each colour individually for dye-swap control experiments. Cells were introduced in 1:1 ratio, at about 10,000 cells per well, to clear, flat-bottomed, 96-well plates (Costar 3595; Corning) carrying a 12×16 matrix of drug–drug concentrations. Drug concentration ratios were varied along one axis of the matrix, and dilutions ($3^{1/4}$-fold) were performed serially along the other. The plates were covered and then incubated in the dark at 30 °C for 24 h on a Titramax 1000 shaker (Heidolph) at 600 r.p.m. Numbers of cells expressing each label were determined for wells exhibiting growth ($A_{500} > 0.02$) with a Victor III (Perkin-Elmer) fluorescence plate reader (filters: YFP, HQ500/20x and HQ535/30m; CFP, D436/20x and D480/30m), and additionally by fluorescence-activated cell sorting (FACS) with an LSRII (Becton Dickinson) (CFP, excitation at 405 nm and emission at 450 nm; YFP, excitation at 488 nm and emission at 530 nm; CFP, YFP lower threshold, 100–150 relative light units (RLU)). A surface representing the logarithmic ratio of YFP to CFP cells was plotted over the drug gradient with the use of a linear interpolation between neighbouring data points (Fig. 3 and Supplementary Tables 1 and 2).

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