Compounds that select against the tetracycline-resistance efflux pump

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We developed a competition-based screening strategy to identify compounds that invert the selective advantage of antibiotic resistance. Using our assay, we screened over 19,000 compounds for the ability to select against the TetA tetracycline-resistance efflux pump in Escherichia coli and identified two hits, β-thujaplicin and disulfiram. Treating a tetracycline-resistant population with β-thujaplicin selects for loss of the resistance gene, enabling an effective second-phase treatment with doxycycline.

The use of antibiotics promotes the emergence and spread of resistant strains, which raises public health concerns. Since the cost of resistance is typically small, resistance alleles often remain in the population after fixation, even in the absence of antibiotics. Therefore, inverting the evolutionary advantage of resistant bacteria and driving them back to drug susceptibility requires treatments that impose substantial fitness costs to resistance alleles. Collateral sensitivity, which occurs when an allele that confers resistance to one drug simultaneously increases sensitivity to another drug, can be used to select against resistant strains in favor of drug susceptibility. In such cases, bacteria that have evolved resistance to drug A can be penalized by their increased sensitivity to drug B. Treatment with drug B can then drive the population back to drug-A susceptibility. In studies on collateral sensitivity, many drugs have been found that select against de novo resistance mutations. However, only a few drugs are known to select against specialized resistance genes and cassettes that encode major modes of clinical resistance, such as efflux pumps, drug degrading enzymes, or modified targets, and systematic screens for such selection-inverting compounds have been limited.

Focusing on tetracycline resistance, we designed a high-throughput screen to identify selection-inverting compounds, small molecules that confer a disadvantage to a resistant strain compared to its susceptible parent. Tetracycline is a broad-spectrum antibiotic whose use has dwindled in part because of widespread resistance. The TetA efflux pump, often carried by transposons, is one of the most prevalent tetracycline-resistance mechanisms. In our assay, equally fit tetracycline-susceptible (TetS) and -resistant (TetR, containing TetA) strains are differentially labeled with fluorescent proteins and competed on susceptibility. In studies on collateral sensitivity, many drugs have been found that select against de novo resistance mutations. However, only a few drugs are known to select against specialized resistance genes and cassettes that encode major modes of clinical resistance, such as efflux pumps, drug degrading enzymes, or modified targets, and systematic screens for such selection-inverting compounds have been limited.

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We evaluated the potency and selectivity of these compounds by testing them on the resistant and susceptible strains, separately and in competition. Measuring the dose–responses of disulfiram, β-thujaplicin, and the fusaric acid control alone in liquid medium further confirmed their differential ability to inhibit the growth of TetR compared to TetS strains. We performed a competition assay in liquid medium, mixing two hits from the bioactives collection retested positive in both replicates: disulfiram and β-thujaplicin (also known as hinokitiol). We decided to focus on β-thujaplicin because of its better potency and superior selectivity and the availability of chemical analogs. A kill-curve assay applying β-thujaplicin to co-cultures of fluorescently labeled TetS and TetR strains showed specific killing of the TetR over the TetS strain (>3-fold difference in the bactericidal concentration).

To understand which chemical moieties of β-thujaplicin are critical for its antibacterial and selection properties, we measured the potency and selectivity of various β-thujaplicin analogs. β-Thujaplicin far exceeded its analogs in both potency and selectivity.

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compound 1; Supplementary Figs. 5d and 6f and Supplementary Table 2). From this structure–activity relationship, we infer that the hydroxyl group is required for potency, while the presence and position of the isopropyl group affects the degree of selection (Fig. 2b).

While the hit compounds we identified preferentially inhibit the TetR strain, it remained unclear whether this cost of resistance was sufficient to evolve a resistant population back to tetracycline susceptibility. To assess this, we propagated eight replicate populations of the TetR strain in tetracycline resistance. All other cultures permanently lost tetracycline treatment with doxycycline, we next passaged them in doxycycline (Δ), Fusaric acid control). Hit compounds disulfiram and β-thujaplicin select for tetracycline susceptibility (d).

To understand the frequency of tetracycline-resistance loss and its underlying genotypic mechanisms, we performed a second selection experiment, isolating β-thujaplicin-resistant (122 μM minimum inhibitory concentration (MIC)) mutants derived from the TetR strain (91 μM MIC) and assayed their doxycycline phenotype. These mutants had the same β-thujaplicin MIC as the TetR strain (122 μM); no colonies appeared at or above this concentration. The vast majority of these isolates became doxycycline susceptible (TetS) upon β-thujaplicin selection (98 of 99; Supplementary Table 4). PCR amplification of the tetA gene showed that most had tetA deleted (77 of 99), while the rest had a 0.5–1 kb insertion in tetA (21 of 99; Supplementary Table 4 and Supplementary Fig. 12). Whole-genome sequencing of six ΔtetA isolates and four isolates with insertions in tetA confirmed these changes. Two ΔtetA isolates also had single nucleotide polymorphisms (SNPs), one in the glutathione/cysteine ABC transporter gene cydD and one upstream of the tyrosine transporter gene tyrP, but neither of these isolates displayed higher levels of β-thujaplicin resistance (Supplementary Table 4). The one isolate that became β-thujaplicin resistant without losing tetA had a frameshift mutation in marR (Supplementary Table 4), suggesting a possible rare mechanism to evolve β-thujaplicin resistance without losing the tetracycline efflux pump. While this mutant had the same β-thujaplicin MIC as other β-thujaplicin-resistant mutants, it also had somewhat higher doxycycline MIC (234 μM) compared to the TetR strain (156 μM MIC), highlighting the threat of potential cross resistance. However, the marR mutant occurred at much lower frequencies (10⁻⁸) compared to deletions or insertions in tetA (10⁻⁴), consistent with previous studies on SNP-based resistance compared to transposon loss.

Together, these data show that the vast majority of β-thujaplicin resistance appears through null insertions in or deletions of the tetA gene, while only rare cases evolve resistance to β-thujaplicin through more general resistance pathways, without loss of tetracycline resistance. Similar results appear for disulfiram selection (78 of 81 lost tetracycline resistance: 75Δ, 1 frameshift deletion in tetA, 1 insertion in tetA, and 1 insertion in tetA, and 1 insertion in tetA, Supplementary Figs. 13 and 14).

Compounds that select against resistance genes can be systemati-
cally identified through competition-based screening and can be used in a two-phase treatment regimen against resistant infections. In this strategy, a first-phase treatment with a selection-inverting compound turns the resistant population sensitive, allowing an effective second-phase treatment with the classical antibiotic. The efficacy of this
approach is enhanced by the presence of many antibiotic resistance genes on mobile elements, which can be spontaneously lost at high frequencies. This strategy can be adapted to other organisms and resistance mechanisms for counter selection in synthetic biology, microbial evolution, agriculture, and possibly therapeutics. However, bacterial populations can escape this treatment regimen through cross resistance mutations (such as the marR mutant) or mutations that provide resistance to the selection-inverting compound without losing antibiotic resistance. Resistance to the antibiotic in the second phase could further arise as a result of incomplete fixation of the antibiostatic-sensitive mutations during the first phase or because of reversal of these mutations in the second phase of treatment. The clinical application of this strategy may further be precluded by its extended treatment times. Despite these difficulties, we hope these findings will inspire future therapeutic paradigms that can reverse the evolution of resistance. Two-phase treatments beginning with selection-inverting compounds that counteract the evolutionary advantage of resistance could add valuable tools to our antimicrobial arsenal.

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Methods

Any supplementary information, chemical compound information and source data are available in the online version of the paper.

Accession codes

The sequences reported in this article have been deposited in the National Center for Biotechnology Information Sequence Read Archive database (accession number SRP073071).

References


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Author contributions

L.K.S., J.C., and R.K. designed research; L.K.S. performed experiments and analyzed data; M.B. and R.C. built the imaging setup and M.B. developed the automation; L.K.S. and M.B. performed genomic sequencing; T.D.L. analyzed genomic sequencing data; R.C. contributed the initial plate and assay design; L.K.S. and R.K. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to R.K.
ONLINE METHODS

Strains and media. All experiments were conducted in low-salt LB medium (RPI, catalog no. L24065), supplemented with bacto agar (BD Falcon) when noted. Drug solutions were made from powder stocks (anhidrotetracycline hydrochloride (ATC), catalog no. 37919 (Sigma, analytical standard); doxycycline hyclate, catalog no. D9891 (Sigma, 99.0%); ciprofloxacin, catalog no. 17850 (Fluka, 98.0%); fusaric acid, catalog no. AC19896 (Fisher, 99%); β-thujaplicin, catalog no. 469521 (Sigma, 99%); disulfiram, catalog no. 86720 (Sigma, 99.7%); α-thujaplicin (Fig. 2b, compound 1), catalog no. 088-08701, (Wako, 98.0%); tropolone (compound 2), catalog no. T89702 (Sigma, 98%); 2-chloro-2,4,6-cycloheptatrien-1-one (compound 3, chlorotropone, catalog no. 669571, (Sigma, 98.0%); 2-methoxy-2,4,6-cycloheptatrien-1-one (compound 4, methoxytropone), catalog no. 137-15711 (Wako, 98.0%); tropone (compound 5), catalog no. 252832 (Sigma, 97%) and filter-sterilized. All drug stocks were dissolved in DMSO at 15 mg/mL, except anhydrotetracycline hydrochloride, which was dissolved in EtOH at 1 mg/mL.

Strain construction and designations are in Supplementary Table 5. Plasmids expressing YFP or CFP under the P_R promoter26 were constructed from the pZ vector system29. Assay strains were grown from single colonies to address nonuniformity in the field of illumination. The images were further processed by subtracting the background (the median intensity of an area of no bacterial growth) and normalizing to an area of neutral selection (the median intensity of the DMSO controls). Next, an RGB overlay image was created with the susceptible strain image in the green channel and the resistant strain image in the red channel, regardless of the fluorescent proteins involved. Using this overlay, the pixel-intensity data was isolated for each well and the median was taken across the width of the well for the red and green channels. Wells with no inhibition of either strain were filtered out using a minimum intensity threshold. The remaining wells were scored by subtracting the distance to the half-maximum growth of the resistant strain from the distance to the half-maximum growth of the susceptible strain down the length of the well (Δθ). Overlay images were evaluated by eye in addition to the automated ranking of hits. Using the Δθ metric, doxycycline (selection for resistance control) and fusaric acid (selection against resistance control) were identified in comparison to ciprofloxacin (inhibition with no selection control) and DMSO (no inhibition or selection control). The Z’ factor was 0.62 for the fusaric acid control and 0.87 for the doxycycline control.

Growth curve assay. Clear, flat-bottomed 96-well plates (Corning 3370) were filled with 150 μL/well low-salt LB with 80 ng/mL ATC containing linear dilution series of DMSO and ~10^6 cells/well WT (TetS) or ~10^6 cells/well t17 (TetR) cells. Experiments were run in parallel with four replicates. The plates were incubated for 24 h at 30 °C, 70% humidity with shaking. Growth was measured every 12 min by OD_600 on an Envision plate reader (PerkinElmer).

Petri competition assay. Petri dishes (100 mm × 15 mm, BD Falcon) were filled with 20 mL low-salt LB 1.5% bactoagar containing 80 ng/mL ATC. Drug stocks were pipetted onto the plate (3 μL of 15 mg/mL fusaric acid, 1 μL of 15 mg/mL β-thujaplicin, and 6 μL of 15 mg/mL disulfiram dissolved in DMSO) and allowed to diffuse at 4 °C for 24 h. The plates were then inoculated with 100 μL of a 1:100 dilution of frozen-cell aliquots in PBS. One replicate is inoculated with a 1:1 ratio of pYt17pC and the other is inoculated with a 1:1 ratio of pCt17pY. Plates were incubated at 30 °C and 70% humidity for 16–18 h, then imaged in brightfield, CFP, and YFP with the ‘MacroScope’ device.

Kill curve assay. Frozen aliquots of fluorescently labeled TetS and TetR cells (pY, t17pC, pC, and t17pY) were diluted 1:10^4 in 20 mL LB with 80 ng/mL ATC and grown to ~0.05 OD_600 at 30 °C, 250 r.p.m. TetS and TetR were then filled with 150 μL/well low-salt LB containing linear dilution series of DMSO and ~10^6 cells/well WT (TetS) or ~10^6 cells/well t17 (TetR) cells. Each well at each time point was drop-plated (7 μL/drop) onto an omnitray filled with 30 mL LB–agar to count CFU (total of 2 dye swaps × 12 drug concentrations × 7 time points × 8 dilutions = 1,344 drops plated for CFU). Plates were incubated at 30 °C for ~12 h. Colonies were imaged in brightfield, CFP, and YFP with the ‘MacroScope’ device and counted using MATLAB scripts and visual inspection. CFU/mL of each strain was calculated based on the most dilute drop with ≥210 colonies of that strain at each drug concentration, at each time point.

I_C50 measurements. Clear, flat-bottomed 96-well plates (Corning 3370) were filled with 150 μL/well low-salt LB containing linear dilution series of drug, ~10^6 cells/well WT (TetS), and ~10^6 cells/well t17 (TetR) cells. Experiments were run in parallel: six replicates with and six replicates without 80 ng/mL ATC show that fitness differences are dependent on the expression function (c (d – c)/(1 + (s/a)^b), where a is the I_C50, b is the slope parameter, c is the minimum response level, and d is the maximum response level. Normality
and homogeneity of variance confirmed by Shapiro-Wilk test and Levene’s test, respectively. Significance was determined by Student’s t-test.

Flow cytometry competition assay. Clear, flat-bottomed 96-well plates (Corning 3370) were filled with 150 μL/well low-salt LB containing linear dilution series of drug and –10° cells/well fluorescently labeled TetR- and –10° cells/well fluorescently labeled TetR+ cells. Experiments were run in parallel with a dye swap (pY & t17pC in one set of plates and pC & t17pY in another set) to show that fitness differences do not depend on the fluorescent proteins and with and without 80 μg/mL ATC to show that fitness differences are dependent on the expression of the TetA pump. The plates were sealed with Aeraset (Excel) to limit evaporation and incubated for 24 h at 30 °C with shaking at 900 r.p.m. on Titramax 1000 (Heidolph). The saturated cultures were diluted 1:100 by pinning –1.5 μL/well culture into 150 μL PBS with VP407 (V&P Scientific). Cells were counted by flow cytometry (Becton Dickinson LSRII; CFP excited at 405 nm, emission detected through 50S and 525-550 nm filters; YFP excited at 488 nm, emission also detected through 50S and 525-550 nm filters). The ratio of TetR+ to TetR- cells (N+/N-) was normalized to the mean N+/N- of eight no-drug wells on each plate. Results in Figure 2b are the average of five replicates performed with pC and t17pY cells and 80 ng/mL ATC.

Passaging experiment. Clear, flat-bottomed 96-well plates (Corning 3370) were filled to a final volume of 150 μL/well low-salt LB with 80 ng/mL ATC. Each column contained a linear dilution series of β-thujaplicin or doxycycline. Each plate included one column inoculated with WT ancestral control, one column inoculated with t17 ancestral control and one column with no bacteria to control for contamination. Nine columns contained replicate populations of t17 passaged each day into fresh β-thujaplicin or doxycycline. In addition, three replicate populations of t17 were passaged in a fixed DMSO concentration equivalent to the highest DMSO concentration used in the β-thujaplicin dilution series (DMSO evolved strains). Plates were inoculated with –10° cells/well. The plates were sealed with Aeraset (Excel) to limit evaporation and incubated for 24 h at 30 °C with shaking at 900 r.p.m. on Titramax 1000 (Heidolph). Growth was measured by OD₆₀₀ on a Victor3 plate reader (PerkinElmer). For each evolved strain, the OD₆₀₀ on a Victor3 plate reader (PerkinElmer). For each evolved strain, the OD₆₀₀ on a Victor3 plate reader (PerkinElmer). For each evolved strain, the OD₆₀₀ on a Victor3 plate reader (PerkinElmer). For each evolved strain, the OD₆₀₀ on a Victor3 plate reader (PerkinElmer). For each evolved strain, the OD₆₀₀ on a Victor3 plate reader (PerkinElmer). For each evolved strain, the OD₆₀₀ on a Victor3 plate reader (PerkinElmer). For each evolved strain, the OD₆₀₀ on a Victor3 plate reader (PerkinElmer).

Resistant mutant selection. The t17 strain was streaked on a low-salt LB 1.5% bacto agar and grown overnight at 30 °C. A single colony was picked and grown overnight in low-salt LB to saturation (~2 × 10⁹ cells/mL). This culture was spun down at 3,000 r.p.m. at 4 °C and the supernatants decanted. The culture was resuspended in PBS, spun down again, the supernatants decanted, and resuspended in PBS to a density of ~10° cells/mL. Petri dishes containing 20 mL low-salt LB 1.5% bacto agar, 80 μg/mL ATC, and either 40 μg/mL disulfiram or 15 μg/mL β-thujaplicin were inoculated with ~10° t17 cells/plate. The plates were incubated at 30 °C, 70% humidity, protected from light. Disulfiram plates were incubated for 3 d and β-thujaplicin plates were incubated for 6 d.

One hundred colonies from the β-thujaplicin and disulfiram selection plates were streaked on LB agar petri dishes and incubated overnight at 30 °C. One colony from each streak was transferred to deep 96-well plates containing 1 mL/well low-salt LB. A subset of the plate was inoculated with WT or t17 ancestral controls or left empty to control for cross contamination. The plates were sealed with Aeraset and incubated at 30 °C with 600 r.p.m. shaking on a Titramax. Using VP407, ~1.5 μL/well was transferred from these overnight plates onto omnitrays containing low-salt LB 1.5% bactoagar, 80 ng/mL ATC, and drug (5, 10, 15, 20, 25, 30 μg/mL β-thujaplicin; 10, 20, 30, 40, 50, 60 μg/mL disulfiram; 0, 0.1, 1, 5, 10, 20, 40, 60, 80, 100 μg/mL doxycycline). Omnitrays were incubated at 30 °C for 2 h. Plates were imaged and growth was recorded (single colonies or films were recorded as no growth). The minimum inhibitory concentration (MIC) was determined as the lowest concentration at which the strains did not grow.

Detection of tetA, tetR, and marR by PCR. The tetA, tetR, and marR genes were amplified with the primers (Supplementary Table 6) in 25 μL reactions using 0.2 μL OneTaq (New England BioLabs) according to the supplier’s protocol. Reactions were cycled 30 times, with an annealing temperature of 57 °C for tetA and marR and 59 °C for tetR. PCR product size was determined by gel electrophoresis on a 1% agarose gel (Supplementary Figs. 9–11). The bands were compared to a 1 kb DNA ladder (New England Biolabs) and their size was determined within 0.5 kb. The expected band size is 1,086 bp for tetA, 1,045 bp for tetR, and 611 bp for marR if the genes are present and uninterrupted. The marR PCR product was sent for Sanger sequencing (Genewiz, Boston, MA).

One mutant, disulfiram-resistant colony 19 (DisR1C10) was tetracycline susceptible despite having the expected length PCR products for both tetA and tetR. The tetA and tetR PCR products of this colony and the TetR t17 control were purified (QIAGEN) and Sanger sequenced at Genewiz, Boston, MA. Sanger sequencing revealed an 11 bp (frameshift) deletion in tetR in disulfiram-resistant colony 19 (DisR1C10).

Genomic sequencing of β-thujaplicin-resistant colonies. Genomic DNA was extracted from 1 mL cultures of eleven colonies and the ancestral t17 control using illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare) following the supplier’s protocol, except for eluting with water instead of elution buffer. Purified DNA was quantified using the Quanti-IT High-Sensitivity DNA Assay Kit (Life Technologies). Sequencing libraries were prepared with the Nextera XT DNA Sample Preparation Kit (Illumina), using a previously described protocol. Samples were sequenced using 100-bp paired-end reads on the HiSeq platform at Axeq Technologies, Seoul, South Korea. Adaptors were removed using cutadapt, reads were trimmed using Sickle, and trimmed reads were aligned to both the E. coli MC4100 reference genome and Tn10 (Genbank accession numbers NC_012759.1 and AF162223.1, respectively) using Bowtie2 (ref. 32). Over 99.7% of reads aligned to the reference genome, and average coverage across a sample ranged between 33× and 134× (median 65×). SNPs were identified using SAMtools and consensus quality (FQ score) cutoff of less than 55 for inclusion. At each variant position that met this cutoff in at least one strain, a best call was made based on the aligned reads for each strain, and positions where all strains supported a variant were discarded. Small insertions and deletions (indels) were called using Dindel. Candidate indels found in one strain were explicitly tested for in all strains; indels with at least 70% of reads in the region supporting the indel and at positions with an average of at least 10× coverage across isolates were accepted. Tn10 deletions were identified by the absence of reads aligning to the coding section of AF162223.1. Insertion elements were identified using RetroSeq (FL score of 6 or 8).