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Compounds that select against the tetracycline resistance efflux pump

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Abstract

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 *E. 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, raising public health concerns[1]. Since the cost of resistance is typically small[2,3], resistance alleles often remain in the population after fixation, even in the absence of antibiotics[2]. Inverting the evolutionary advantage of resistant bacteria and driving them back to drug susceptibility therefore requires treatments that impose substantial fitness costs to resistance alleles[4–7].

Collateral sensitivity can be used to select against resistant strains in favor of drug susceptibility[8]. Collateral sensitivity occurs when an allele that confers resistance to one drug simultaneously increases sensitivity to another drug[9]. In such cases, bacteria that have

Accession codes

The sequences reported in this article have been deposited in the National Center for Biotechnology Information Sequence Read Archive database, www.ncbi.nlm.nih.gov/sra (accession no. SRP073071, BioProject No. PRJNA317940).

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 have no competing financial interests.

evolved resistance to drug A, can be penalized by their increased sensitivity to drug B. Treating with drug B can then drive the population back to drug A susceptibility[8]. In studying collateral sensitivity, many drugs have been found that select against *de novo* resistance mutations[8–15]. However, only a few drugs are known to select against specialized resistance genes and cassettes[16–18], which encode major modes of clinical resistance such as efflux pumps, drug degrading enzymes, or modified targets[19], and systematic screens for such selection-inverting compounds have been limited[20].

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, due to widespread resistance[21]. The TetA efflux pump, often carried by transposons, is one of the most prevalent tetracycline resistance mechanisms[21]. In our assay, equally-fit tetracycline susceptible (Tet^S) and resistant (Tet^R, containing TetA) strains are differentially labeled with fluorescent proteins and competed on diffusion-generated gradients of test compounds[20] (Fig. 1a). This competition may be altered by a compound that preferentially inhibits one of the strains (Fig. 1b). As control compounds, we used doxycycline, a tetracycline analog that selects for tetracycline resistance (Fig. 1c); fusaric acid, a known molecule that selects against the TetA efflux pump but is of limited utility due to toxicity[16,22] (Fig. 1d); ciprofloxacin, an antibiotic eliciting no or very mild selection for tetracycline resistance (Supplementary Results, Supplementary Fig. 1a); and a DMSO vehicle control (Supplementary Fig. 1b, 2). The media was supplemented with anhydrous tetracycline (ATC) at concentrations that induce expression of TetA but have no detectable effects on growth. We developed custom 48-well plates and an imaging platform for high throughput automation of the assay (Supplementary Fig. 1b, 3, Online Methods).

Screening 19,769 compounds, we identified two compounds that can select against the tetracycline resistance efflux pump. The primary screen identified 38 hits from three libraries: 30 known bioactives (0.34% of 8,752 screened), 8 natural product extracts (0.12% of 6,441), and 0 commercial library compounds (of 4,576, Supplementary Table 1). We verified positive hits by retesting them in our assay, in duplicate with a dye swap to control for autofluorescence. Of our 38 initial hits, two hits from the bioactives collection retested positive in both replicates: disulfiram and β-thujaplicin (also known as hinokitiol, Fig. 1d, Supplementary Fig. 4). Disulfiram is an FDA-approved drug (Antabuse) for treating alcoholism and acts synergistically with tetracycline in susceptible strains[23]. β-thujaplicin has widespread antifungal and antibacterial activity[24]. While these compounds were known to have antibacterial activity[24,25], their ability to select against tetracycline resistance is a newly discovered property.

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 media further confirmed their differential ability to inhibit Tet^R compared to Tet^S (Supplementary Fig. 5; IC50 of Tet^R, Tet^S; disulfiram: 91 ± 3 μM, 133 ± 4 μM, $p < 10^{-5}$; β-thujaplicin: 30 ± 1 μM, 39 ± 4 μM, $p < 10^{-3}$; fusaric acid 106 ± 6 μM, 133 ± 14 μM, $p = 10^{-3}$; mean ± s. d., N = 6, student's t-test). We define potency as the IC50 against the sensitive strain. To

determine selectivity, we performed a competition assay in liquid media, mixing fluorescently labeled Tet^S and Tet^R strains 1:1, growing them in a linear dilution series of each compound for 24 h, and using flow cytometry to measure their final ratio (N^S/N^R, Supplementary Fig. 6a). Disulfiram and β-thujaplicin, as well as the fusaric acid control, gave a competitive advantage to the Tet^S over the Tet^R strain (Fig. 2a–b, Supplementary Fig. 6b–e). For each compound, we then defined selectivity as the average selection (average of $\log_{10}(N^S/N^R)$ over the range where at least one of the strains can grow, Fig. 6c–e). Its better potency, superior selectivity, and availability of chemical analogs led us to focus on β-thujaplicin. A kill curve assay applying β-thujaplicin to co-cultures of fluorescently-labeled Tet^S and Tet^R strains showed specific killing of the Tet^R over the Tet^S strain (>3-fold difference in the bactericidal concentration; Supplementary Fig. 7).

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 (Fig. 2a–b, Supplementary Fig. 5–6, 8–9, Supplementary Tables 2,3). β-thujaplicin far exceeded its analogs in both potency and selectivity (Fig. 2b). The regioisomer, α-thujaplicin, had the same potency, but showed no selection between the Tet^S and Tet^R strains (Fig. 2b, compound 1; Supplementary Fig. 5d, 6f, 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 Tet^R strain, it remained unclear whether this cost of resistance was sufficient to evolve a resistant population back to tetracycline susceptibility. To answer this question, we propagated 8 replicate populations of the Tet^R strain in β-thujaplicin gradients for 7 days (each day propagating the population from the highest drug concentration that shows growth). In all 8 populations, the frequency of tetracycline resistant cells rapidly decreased, quickly falling below detection levels (10^{-6}, Fig. 2c, Supplementary Fig. 10). To see if these populations, now tetracycline sensitive, would become tetracycline resistant upon treatment with doxycycline, we next passaged them in doxycycline gradients for 3 days. Only one of the eight populations regained tetracycline resistance. All other cultures permanently lost tetracycline resistance upon β-thujaplicin treatment through deletion of *tetA* (Supplementary Fig. 11; Sanger sequencing revealed no mutations in *marR*), enabling effective doxycyline treatment. This suggests a two-phase treatment protocol in which a selection-inverting compound converts a resistant population to susceptibility and a traditional antibiotic clears any remaining bacteria.

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 MIC) mutants derived from the Tet^R strain (91 μM MIC) and assayed their doxycycline phenotype. These mutants had the same β-thujaplicin MIC as the Tet^S strain (122 μM); no colonies appeared at or above this concentration. The vast majority of these isolates became doxycycline susceptible (Tet^S) upon β-thujaplicin selection (98/99, Fig. 2d). PCR amplification of the *tetA* gene showed that most had deleted *tetA* (77/99), while the rest had a 0.5–1 kb insertion in *tetA* (21/99, Fig. 2d, Supplementary Fig. 12). Whole genome sequencing of 6 *ΔtetA* isolates and 4 isolates with insertions in *tetA* confirmed these

changes. Two *ΔtetA* isolates also had single nucleotide polymorphisms (SNPs): one in *cydD* and one upstream of *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*, the repressor of the multiple antibiotic resistance operon[26] (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 a somewhat higher doxycycline MIC (234 μM) compared to the Tet^R strain (156 μM MIC), highlighting the threat of potential cross-resistance. However, the *marR* mutant occurred at much lower frequencies (10^{-6}) compared to deletions or insertions of *tetA* (10^{-4}), consistent with previous studies on SNP-based resistance versus transposon loss[18]. 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 (78/81 lost tetracycline resistance: 75 Δ*tetA*, 1 frameshift deletion in *tetA*, 1 insertion in *tetA*, and 1 insertion in *tetR*, Fig. 2d, Supplementary Fig. 13, 14).

Compounds that select against resistance genes can be systematically 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[21,26,27], which can be spontaneously lost at high frequency[16,27]. 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 due to incomplete fixation of the antibiotic sensitive mutations during the first phase or due to reversal of these mutations in the second phase of treatment. The clinical application of this strategy may further be prohibited by its extended treatment times. Despite these difficulties, we hope these findings will inspire future therapeutic paradigms that can reverse the evolution of resistance[4]. Two-phase treatments beginning with selection-inverting compounds that counteract the evolutionary advantage of resistance could add valuable tools to our antimicrobial arsenal.

Online Methods

Strains and Media

All experiments were conducted in low salt LB broth (RPI, catalog #L24065), supplemented with bactoagar (BD Falcon) when noted. Drug solutions were made from powder stocks (anhydrotetracycline hydrochloride (ATC), catalogue no. 37919 (Sigma, analytical standard); doxycycline hyclate, catalogue no. D9891 (Sigma, ≥ 98.0%); ciprofloxacin,

catalogue no. 17850 (Fluka, ≥ 98.0%); fusaric acid, catalogue no. AC19896 (Fisher, 99%); β-thujaplicin, catalogue no. 469521 (Sigma, 99%); disulfiram, catalogue no. 86720 (Sigma, ≥ 97.0%); α-thujaplicin (Fig. 2b, compound **1**), catalogue no. 088-08701, (Wako, ≥ 98.0%); tropolone (compound **2**), catalogue no. T89702 (Sigma, 98%); 2-Chloro-2,4,6-cycloheptatrien-1-one (compound **3**, chlorotropone), catalogue no. 669571, (Sigma, ≥ 98.0%); 2-Methoxy-2,4,6-cycloheptatrien-1-one (compound **4**, methoxytropone), catalogue no. 137-15711 (Wako, ≥ 98.0%); tropone (compound **5**), catalogue 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 promoter[28] were constructed from the pZ vector system[29]. Assay strains were grown from single colonies to saturation in low salt LB. Cell concentrations were measured by OD600 and plate count. Aliquots were stored in 15% glycerol at −80°C. Fresh aliquots were used for each experiment.

Custom Assay Plate

We tailored a design for 48 well plates composed of 2 rows of 24 lanes[20] to be compatible with high throughput screening robots by lowering the plate's skirt and adding spacing bars so that air can escape as the agar cools while the plates are stacked (Supplementary Fig. 3).

Screen for Selection Inverters

Custom 48 well screening plates were filled with 352 μL/well low salt LB 1.5% bactoagar containing 80 ng/mL ATC using a Wellmate Stacker (ThermoScientific). Approximately 1 μL/well test compounds were pinned onto the top of each lane using AFIX384FP6 (V&P Scientific) with FP6S pins (V&P Scientific) in rows A and I by the Seiko robot. Approximately 1 μL/well control compounds were pinned onto the top of each lane by hand using AFIX384FP (V&P Scientific) with FP6S pins (V&P Scientific) in appropriate wells. Each screening plate had at least one of each control: 15 mg/mL doxycycline, 15 mg/mL fusaric acid, 15 mg/mL ciprofloxacin, and DMSO vehicle control. Plates were stored at 4°C for 24 hours to allow the compounds to diffuse, creating concentration gradients down the length of the wells. Plates were then inoculated using a Wellmate Stacker with 112 μL/well frozen cell aliquots diluted 1:100 in low salt LB 0.75% bactoagar containing 80 ng/mL ATC. Each test compound is tested twice, with the fluorescent markers switched between the tetracycline susceptible and resistant strains to identify autofluorescent compounds. One replicate is inoculated with a 1:1 ratio of pY:t17pC and the other is inoculated with a 1:1 ratio of pC:t17pY. Plates were incubated at 30°C and 70% humidity for 16–18 h. Finally, plates are automatically imaged in three channels, brightfield, CFP (436/20ex, 480/40em), and YFP (500/20ex, 530/20em) with a Canon T3i using a custom-built robotic fluorescent imaging 'Macroscope' device[20].

As seen in Figure 1, the selection against resistance is concentration dependent—too much compound will kill both strains, while too little will allow both strains to grow. When screening for novel compounds that select against resistance, the concentration range in which they select is unknown. Employing diffusion gradients on agar allows rapid

assessment of a continuous range of concentrations using small amounts of library compounds. Allowing the compounds to diffuse for 24 h permits the compound gradient to somewhat stabilize before adding bacteria (the diffusion rate decreases with time as the compounds equilibrate), thus somewhat decoupling compound diffusion and bacterial growth. Because, the diffusion constants of most library compounds are unknown, their active range of concentration must be determined in follow-up studies using a defined dilution series of drug.

Screen Analysis

The images were processed using a custom MATLAB script. The blue channel from the CFP image and the green channel from the YFP image are reduced to grayscale images. A shading correction is employed to address nonuniformity in the field of illumination. The images are 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 is 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 is isolated for each well and the median is taken across the width of the well for the red and green channels. Wells with no inhibition of either strain are filtered out using a minimum intensity threshold. The remaining wells are scored by subtracting the distance to the half-max growth of the resistant strain from the distance to the half-max growth of the susceptible strain down the length of the well (Δd). Overlay images were evaluated by eye in addition to the automated ranking of hits. Using the Δd 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 ~10^4 cells/well WT (Tet^S) or ~10^4 cells/well t17 (Tet^R) cells. Experiments are run in parallel with 4 replicates. The plates are incubated for 24 h at 30°C, 70% humidity with shaking. Growth was measured every 12 minutes by optical density at 600 nm (OD600) on an Envision plate reader (Perkin Elmer).

Petri Competition Assay

Petri dishes (100mm × 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 (3μL 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 phosphate buffered saline (PBS). One replicate is inoculated with a 1:1 ratio of pY:t17pC and the other is inoculated with a 1:1 ratio of pC:t17pY. Plates were incubated at 30°C and 70% humidity for 16–18 h, then imaged in brightfield, CFP, and YFP with the 'Macroscope' device[20].

Kill Curve Assay

Frozen aliquots of fluorescently-labeled Tet^R and Tet^S 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 OD600 at 30°C, 250 rpm. Tet^S and Tet^R were then mixed 1:1 according to OD600 measurement (two dye swaps: pY:t17pC and pC:t17pY). A 10-fold dilution series of these initial cell mixtures (t = 0) was plated onto LB-agar petri dishes to count initial CFU. These two co-cultures were then aliquoted 1mL/well into a row of a 96 deep well plate and β-thujaplicin was added to each well at a series of concentrations (indicted in Supplementary Fig. 6). To measure surviving cells as a function of time, 50 μL aliquots were taken at 10, 20, 30, 45, 60, 90 and 180 min timepoints and an 8-step, 10-fold dilution series of each well at each timepoint 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 timepoints × 8 dilutions = 1344 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[20] and counted using MATLAB scripts and visual inspection. CFU/mL of each strain was calculated based on the most dilute drop with ≥ 10 colonies of that strain at each drug concentration, at each timepoint.

IC50 Measurements

Clear, flat-bottomed 96-well plates (Corning 3370) were filled with 150 μL/well low salt LB containing linear dilution series of drug, ~10^4 cells/well WT (Tet^S), and ~10^4 cells/well t17 (Tet^R) cells. Experiments are run in parallel: at least 6 replicates with and 6 replicates without 80 ng/mL ATC to show that fitness differences are dependent on the expression of the TetA pump. The plates are sealed with Aeraseal (EXCEL) to limit evaporation and incubated for 24 h at 30°C with shaking at 900 rpm on Titramax 1000 (Heidolph). Growth was measured by optical density at 600 nm (OD600) on a Victor3 plate reader (Perkin Elmer). The dose responses are fit to a 4 parameter logistic function $c + (d - c)/(1 + (x/a)^b)$ where a is the IC50, 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 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 ~10^4 cells/well fluorescently-labeled Tet^S and ~10^4 cells/well fluorescently-labeled Tet^R cells. Experiments are 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 ng/mL ATC to show that fitness differences are dependent on the expression of the TetA pump. The plates are sealed with Aeraseal (EXCEL) to limit evaporation and incubated for 24 h at 30°C with shaking at 900 rpm 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 405nm, emission detected through 505LP and 525/550nm filters; YFP excited at 488nm, emission also detected through 505LP and 525/550nm filters). The ratio of Tet^S to Tet^R cells (N^S/N^R) was

normalized to the mean N^S/N^R of eight no drug wells on each plate. Results in Figure 3 are the average of 5 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 for 7 days, then in doxycycline for 3 days (β-thujaplicin evolved strains). In addition, 3 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 ~10^4 cells/well. The plates are sealed with Aeraseal (EXCEL) to limit evaporation and incubated for 22 h at 30°C with shaking at 900 rpm on Titramax 1000 (Heidolph). Growth was measured by optical density at 600 nm (OD600) on a Victor3 plate reader (Perkin Elmer). For each evolved strain, the well containing the highest drug concentration with OD600 ≥ 0.4 was diluted and propagated daily into fresh drug plates with ~10^4 cells/well. The remainder of the well was stored in 15% glycerol at −80°C. The wells with WT and t17 ancestral controls and no drug were also stored in 15% glycerol at −80°C to serve as controls for later follow up. One of the nine β-thujaplicin lineages was contaminated in storage and is excluded from Fig. 4.

The Tet^R frequency was measured by creating a 10-fold dilution series of stored evolved strains and ancestral controls in PBS. Using VP407, ~1.5 μL/well of these dilution series and the storage wells were pinned onto one non-treated omnitray (Thermo Scientific) containing 30 mL low salt LB 1.5% bactoagar and one non-treated omnitray containing 30 mL low salt LB 1.5% bactoagar and 20 μg/mL doxycycline. The plates were incubated at 30°C for 22 h then imaged. Growth was then measured by eye with positive growth meaning that at least one colony grew from the spot. The cfu/mL of the dilution series was calibrated by plating 50 μL of select wells onto petri dishes containing 20 mL low salt LB 1.5% bactoagar. The data from pinning is precise to one order of magnitude. The accuracy of this method was confirmed by plating 50 μL/well of a subset of samples onto petri dishes containing 20 mL low salt LB 1.5% bactoagar and onto petri dishes containing 20 mL low salt LB 1.5% bactoagar and 20 μg/mL doxycycline.

Resistant Mutant Selection

The t17 strain was streaked on a low salt LB 1.5% bactoagar petri dish and grown overnight at 30°C. A single colony was picked and grown overnight in low salt LB to saturation (~2 × 10^9 cells/mL). This culture was spun down at 3000 rpm & 4°C and the supernatants decanted. The culture was re-suspended in PBS, spun down again, the supernatants decanted, and re-suspended in PBS to a density of ~10^7 cells/mL. Petri dishes containing 20 mL low salt LB 1.5% bactoagar, 80 ng/mL ATC, and either 40 μg/mL disulfiram or 15 μg/mL β-thujaplicin were inoculated with ~10^6 t17 cells/plate. The plates were incubated at 30°C, 70% humidity, protected from light. Disulfiram plates were incubated for 3 days and β-thujaplicin plates were incubated for 6 days.

One hundred colonies from the β-thujaplicin and disulfiram selection plates were streaked onto 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 Aeraseals and incubated at 30°C with 600 rpm 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 20 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 Fig. 9–11). The bands were compared to a 1 kb DNA ladder (New Englad Biolabs) and their size was determined within 0.5 kb. The expected band size is 1086 bp for *tetA*, 1045 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 (DsfRP1C10), 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 Tet^R t17 control were purified (QIAGEN) and Sanger sequenced at Genewiz, Boston, MA. Sanger sequencing revealed an 11 bp (frameshift) deletion in *tetA* in disulfiram resistant colony 19 (DsfRP1C10).

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 eluting with water instead of elution buffer. Purified DNA was quantified using the Quant-iT™ High-Sensitivity DNA Assay Kit (Life Technologies). Sequencing libraries were prepared using the Nextera XT DNA Sample Preparation Kit (Illumina), using a previously described protocol[30]. Samples were sequenced using 100bp paired-end reads on the HiSeq platform at Axeq Technologies, Seoul, South Korea. Adaptors were removed using cutadapt[31], 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[32]. Over 99.7% of reads aligned to the reference genome, and average coverage across a sample ranged between 33× and 134× (median 65x). SNPs were identified using SAMtools[33] 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[34]. 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[35] (FL score of 6 or 8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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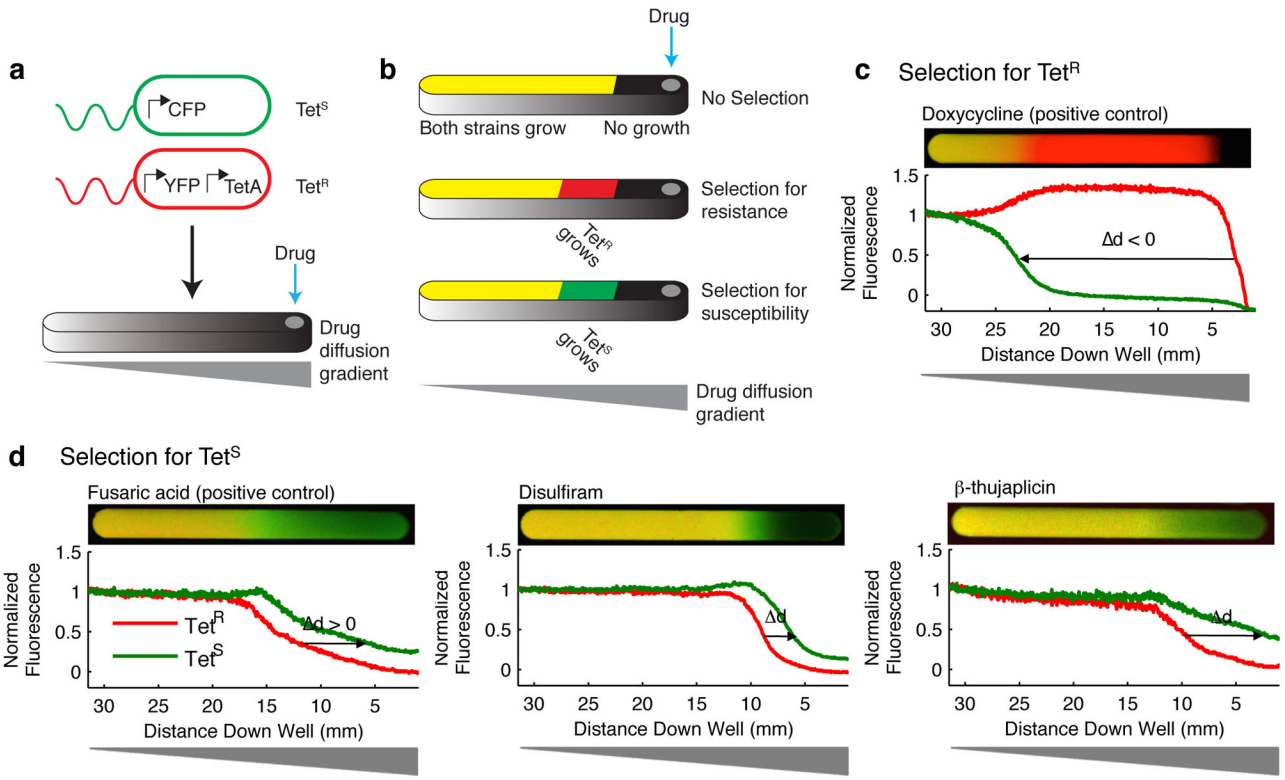

Figure 1. A high throughput diffusion-based screen identifies compounds that select against tetracycline resistance

(**a**) Tetracycline susceptible and resistant strains (Tet^S: CFP, shown in green; Tet^R: TetA, YFP, shown in red; a dye swap control is also performed) are mixed 1:1 and plated on agar lanes with diffusion gradients of locally spotted drugs. (**b**) Fluorescent imaging reveals regions of selection along the drug gradient. Areas where both strains can grow maintain a 1:1 ratio and appear yellow; areas where neither strain can grow appear dark, while areas selecting for resistance or susceptibility appear red or green, respectively. (**c–d**) Automated image analysis identifies the distance from the drug spot where each strain can grow (defined by half-maximal fluorescence). The difference between these points (Δd) is used to score hits: Δd < 0 indicates selection for resistance (**c**, doxycycline control) and Δd > 0 indicates selection against resistance (**d**, fusaric acid control). (**d**) Hit compounds disulfiram and β-thujaplicin select for tetracycline susceptibility.

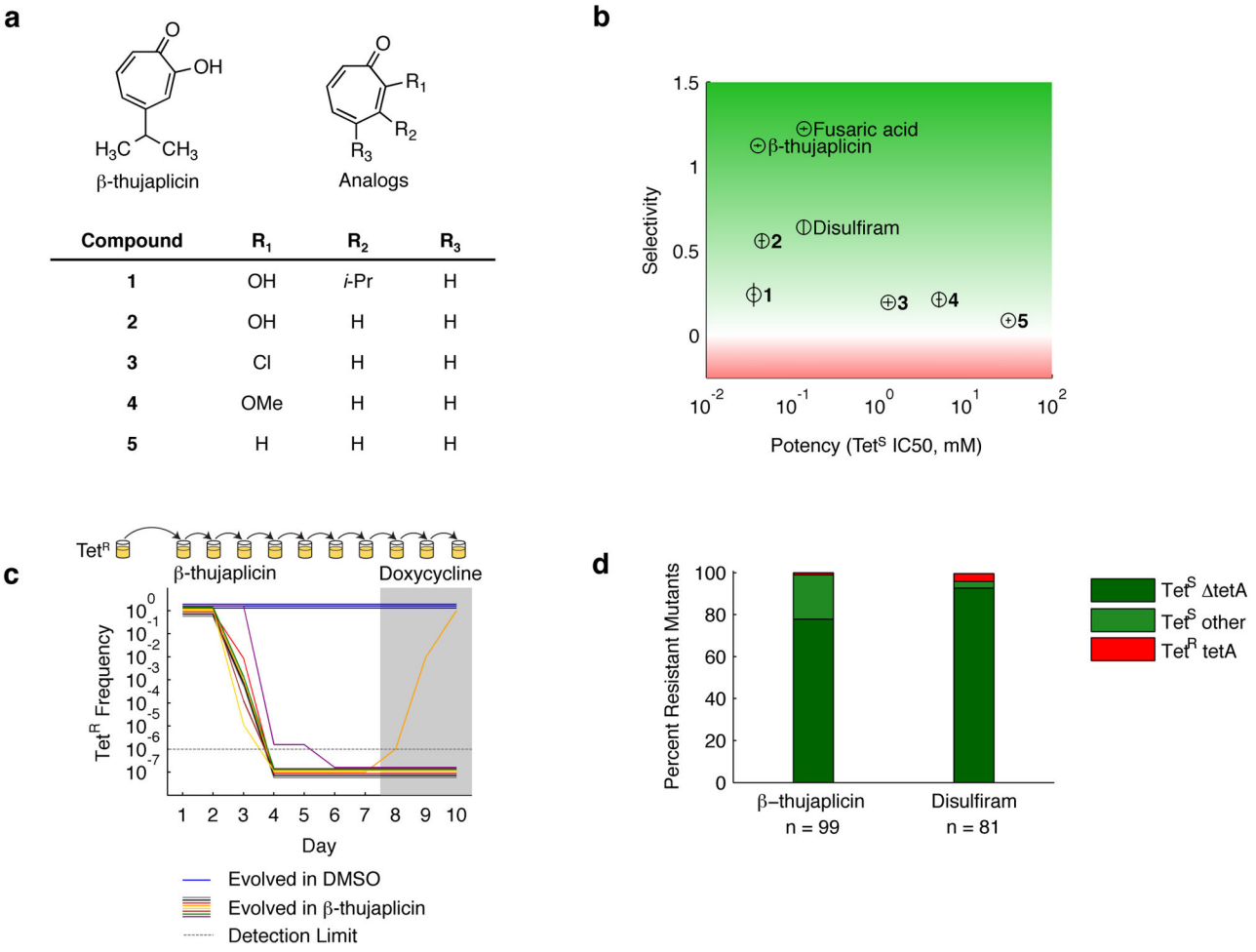

Figure 2. β-thujaplicin and disulfiram select for loss of tetracycline resistance

(**a**) β-thujaplicin and its analogs. (**b**) The advantage of the Tet^S over the Tet^R strain at each drug concentration is measured by the ratio N^S/N^R, determined by flow cytometry, and normalized to the ratio of N^S/N^R with no drug. The selectivity of the drug is represented by the average selection (normalized $\log_{10}(N^S/N^R)$) across the concentrations where at least one of the strains can grow. Examining all compounds for selectivity (mean ± s.d., n = 5) and potency (Tet^S IC50, mean ± s.d., n = 6) shows that β-thujaplicin exerts the greatest selection for Tet^S among its analogs and has better potency compared to fusaric acid. (**c**) When evolved in β-thujaplicin (days 1–7), eight parallel populations of the Tet^R strain lose their tetracycline resistance phenotype, with the frequency of resistant cells rapidly falling below the detection limit (10^{-6}). In a second selection phase (days 8–10), the lineages were evolved in doxycycline, yet the majority did not regain tetracycline resistance (7/8). In contrast, all (3/3) lineages that were evolved for 7 days in DMSO (blue) remained tetracycline resistant. Points are offset slightly to resolve overlaps. (**d**) The vast majority of β-thujaplicin and disulfiram resistant mutants selected from the Tet^R strain lost phenotypic resistance to tetracycline (Tet^S, green and light green). Most tetracycline susceptible (Tet^S) mutants completely lost *tetA* (green); others (light green) had insertions within *tetA* (21/99 β-thujaplicin mutants, 1 disulfiram mutant) or *tetR* (1 disulfiram mutant) or had an 11bp

deletion within *tetA* (1 disulfiram mutant). Only 1 β-thujaplicin resistant mutant and 3 disulfiram resistant mutants remained tetracycline resistant (Tet^R, red), with an intact *tetA* gene.