A trimethoprim derivative impedes antibiotic resistance evolution

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The antibiotic trimethoprim (TMP) is used to treat a variety of Escherichia coli infections, but its efficacy is limited by the rapid emergence of TMP-resistant bacteria. Previous laboratory evolution experiments have identified resistance-conferring mutations in the gene encoding the TMP target, bacterial dihydrofolate reductase (DHFR), in particular mutation L28R. Here, we show that 4'-desmethyltrimethoprim (4'-DTMP) inhibits both DHFR and its L28R variant, and selects against the emergence of TMP-resistant bacteria that carry the L28R mutation in laboratory experiments. Furthermore, antibiotic-sensitive E. coli populations acquire antibiotic resistance at a substantially slower rate when grown in the presence of 4'-DTMP than in the presence of TMP. We find that 4'-DTMP impedes evolution of resistance by selecting against resistant genotypes with the L28R mutation and diverting genetic trajectories to other resistance-conferring DHFR mutations with catalytic deficiencies. Our results demonstrate how a detailed characterization of resistance-conferring mutations in a target enzyme can help identify potential drugs against antibiotic-resistant bacteria, which may ultimately increase long-term efficacy of antimicrobial therapies by modulating evolutionary trajectories that lead to resistance.
Antibiotic resistance is a burgeoning public health crisis, with a marked rise in mortality and morbidity associated with antibiotic resistance infections. With only a handful of antibiotic target proteins, bacteria are only a few genetic mutations away from becoming completely resistant to several antibiotic molecules. As such, the antibiotic resistance crisis will likely not be solved by developing the next novel antibiotic molecule. Therefore, understanding the mechanisms of antibiotic resistance and developing strategies to counteract the evolution of antibiotic resistance will be crucial to combat this public health predicament.

Evolution of antibiotic resistance has been studied at the molecular level for decades with the ultimate goal of devising targeted therapies to impede the evolution of resistance. Targeting evolutionarily common resistance-conferring mutations was previously proposed as a promising strategy to impede evolution of resistance based on computer simulations. However, to the best of our knowledge, there has been no biological validation of this strategy. We hypothesized that by comprehensively understanding the molecular evolution of antibiotic resistance in pathogenic bacteria, we would be able to identify and target common mutations and subsequently impede the evolution of antibiotic resistance. To test this hypothesis, we utilized Escherichia coli (E. coli), a common gram-negative pathogenic bacteria, and trimethoprim (TMP), an antibiotic widely used to treat a variety of E. coli infections such as urinary tract infections, but limited in its efficacy given the rapidity with which TMP resistance develops. TMP mediates its anti-bacterial effect by targeting bacterial dihydrofolate reductase (DHFR), a ubiquitous enzyme found in all organisms and thus also an important target for cancer and autoimmune disorders (Fig. 1a, b). DHFR catalyzes the reduction of 5,6-dihydrofolic acid (DHF) to 5,6,7,8-tetrahydrofolic acid (THF) by enantiospecific hydride transfer from NADPH cofactor (Fig. 1a). THF and its derivatives are essential precursors for the biosynthesis of nucleotides and amino acids.

**Fig. 1 L28R is a coding mutation in E. coli DHFR with a unique resistance-conferring mechanism.** a DHFR is an essential enzyme with a central role in the biosynthesis of nucleotides and amino acids. b Trimethoprim (TMP) is a bacteriostatic antibiotic molecule that competitively inhibits DHFR activity. c Resistance-conferring DHFR mutations that reduce TMP affinity are responsible for the evolution of TMP resistance in E. coli. As TMP is a competitive inhibitor of DHFR, resistance-conferring mutations typically reduce both TMP and substrate (DHF) binding affinities. d Frequencies of resistance-conferring DHFR mutations from 40 independent E. coli populations evolved in the morbidostat under TMP selection (7 populations from this study and 33 populations from our previous studies). L28R is one of the most commonly observed DHFR mutation in laboratory evolution experiments. Different colors on the bar plot are used to represent mutated residues. Stripes with different directionalities are used to distinguish frequency of different amino acid replacements in the same residue. e Unlike other mutations, L28R indirectly increases TMP resistance (higher Ki) by increasing substrate affinity (lower Km) due to additional interactions with the glutamate tail (green) of DHF (bottom) in comparison with wild-type (top). f X-Ray crystal structure of DHFRWT (6XG5, resolution: 1.9 Å) bound to TMP drug (blue) and NADPH co-factor (green). g X-Ray crystal structure of DHFRL28R (6XG4, resolution: 2.1 Å) bound to TMP drug (blue) and NADPH co-factor (green). Omit difference electron density maps for trimethoprim in both structures are provided in Supplementary Fig. 10. h Interaction distances (Å) between DHFR residues (gray) and TMP (blue) for DHFRWT (black) DHFRL28R (magenta). i Proposed structure of L28R-specific DHFR inhibitor with modifications (colored spheres) in the hydrophobic tail of TMP while preserving its polar head (red).
for the biosynthesis of DNA bases and amino acids\textsuperscript{11}. We previously carried out several laboratory evolution experiments with E. coli and TMP using the morbidostat, an automated continuous culture device we developed to study antibiotic resistance under nearly constant selection pressure induced by antibiotics\textsuperscript{16,17}. Strikingly, we found that evolution of TMP resistance in E. coli consistently proceeds through stepwise acquisition of multiple resistance-conferring mutations in the folA gene that encodes for DHFR (Fig. 1c)\textsuperscript{16,18–20}. Antibiotic sensitive E. coli populations evolve nearly orders of magnitude higher TMP resistance by accumulating three to five mutations in folA. TMP-resistant E. coli populations acquire a promoter mutation (i.e., c-35) that increases DHFR expression, and multiple mutations in the coding region that decreases affinity (increased \(K_{i}\)) of DHFR to TMP molecules\textsuperscript{17}. Among all the mutations found in the coding region of DHFR, the most commonly mutated residues were P21, A26, D27, L28, W30, I94, and F153 (Fig. 1d)\textsuperscript{16}. As TMP is a competitive inhibitor, all of the point mutations in DHFR reduced both TMP and substrate affinities simultaneously, with the exception of the L28R mutation\textsuperscript{7,21}. Interestingly, unlike other mutations, the L28R mutation reduced TMP affinity while increasing DHF affinity. Also, in our evolution experiments, L28R was the most frequently observed mutation in the coding region of folA, with the strongest effect on TMP resistance (Fig. 1d). Moreover, L28R can compensate for the reduced catalytic activity caused by other DHFR mutations such as P21L or W30R\textsuperscript{7,22,23}. Our molecular dynamics simulations and biochemical measurements suggested that the L28R mutation leads to a unique and indirect resistance mechanism against TMP: increasing substrate affinity due to newly formed interactions between the mutated enzyme and \(p\)-aminobenzoyl glutamate tail of the substrate DHF (Fig. 1e and Supplementary Fig. 1)\textsuperscript{7,21}. Hence, we concluded that L28R would make an excellent candidate to test our hypothesis of using mutant-specific antibiotic molecules in order to modulate the evolution of antibiotic resistance. Here, we show that an L28R-specific trimethoprim derivative can impede evolution of antibiotic resistance by blocking several evolutionarily viable genetic trajectories.

**Results and discussion**

**Structure-guided design of L28R-specific DHFR inhibitor.** To gain greater mechanistic insight, we next characterized the atomic structures of wild-type DHFR (DHFR\textsuperscript{WT}) and mutant DHFR with an L28R replacement (DHFR\textsuperscript{L28R}) using X-ray crystallography. Both DHFR\textsuperscript{WT} (Fig. 1f, resolution 1.9 Å) and DHFR\textsuperscript{L28R} (Fig. 1g, resolution 2.1 Å) were individually co-crystallized with the cofactor NADPH and inhibitor TMP. Distances between the amino acid residues of the enzyme and TMP (Fig. 1h, black values are for DHFR\textsuperscript{WT} and magenta values are for DHFR\textsuperscript{L28R}) indicated that there are only subtle differences between these two structures, particularly around the enzyme’s active site. Consequently, we recognized that the 2,4-diamino pyrimidine group of the drug can equally bind to the active site of both DHFR\textsuperscript{WT} and DHFR\textsuperscript{L28R}, and should not be altered when designing an L28R-specific DHFR inhibitor. The arginine side chain at residue 28 of DHFR\textsuperscript{L28R} (purple in color, Fig. 1g) is oriented towards the tail of the TMP molecule where the 3,4,5-trimethoxy aryl ring is located. Hence, we predicted that a TMP modification with an appropriate polar group at the 3, 4, or 5 position of this aryl ring might induce additional electrostatic or hydrogen-bonding interactions and thus yield a L28R-specific drug candidate (Fig. 1i).

**Antibacterial activity of L28R-specific DHFR inhibitor.** Indeed, we ultimately identified a candidate molecule, 4′-desmethyl-trimethoprim (4′-DTMP, Fig. 2a, bottom), which exhibited

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**Fig. 2** 4′-DTMP has enhanced and selective antimicrobial activity against E. coli with the L28R mutation in DHFR. A Structure of Trimethoprim (TMP) and 4′-desmethyltrimethoprim (4′-DTMP). B Representative drug dose response curves for TMP (gray) and 4′-DTMP (teal) against E. coli harboring wildtype DHFR (wild-type, left) or DHFR with the L28R mutation (right). All DHFR mutant strains were constructed in MG1655 attTn7::pRNA1-tdCherry (NDL47, see Methods’ section) by replacing the chromosomal folA gene with the corresponding chemically synthesized folA variant sandwiched between kanamycin and chloramphenicol resistance genes. C Activity of 4′-DTMP (teal) is indistinguishable from TMP (gray) activity against wildtype E. coli (\(n = 7\) replicates), whereas 4′-DTMP (teal) has -30-fold higher antimicrobial activity (\(p = 9.597 \times 10^{-4}, n = 14\) replicates) compared to TMP (gray) against E. coli with L28R mutation. D Activity of 4′-DTMP (teal) against wild-type and other frequently-observed E. coli mutants with single DHFR mutations is indistinguishable from TMP activity (gray) (\(n = 7\) replicates), except L28R (\(p = 3.5912 \times 10^{-6}\)). E Indistinguishable antimicrobial activities of TMP (gray) and 4′-DTMP (teal) against other Gram-negative and Gram-positive bacteria as well as a clinical E. coli isolate (\(n = 3\) replicates). Student’s t-test (two tailed) is used to quantify significance of IC\textsubscript{95} value differences in all panels (*\(p < 0.05\), **\(p < 0.01\), and ***\(p < 0.001\), error bars show the standard deviation, center of the error bars corresponds to the mean value of the measurements.)
of the L28R mutant with 4- DTMP was previously reported in the literature but was never used as a mutant specific DHFR inhibitor. It was shown that 4'-DTMP had similar antimicrobial activities as TMP against drug-sensitive E. coli bacteria. We characterized toxicity of 4'-DTMP since there was limited information about this molecule in previous literature. In vitro toxicity studies of these two compounds against confluent human cells (ARPE-19) indicated that 4'-DTMP has a similar toxicity profile as TMP up to 1000 μM concentration. However, we found that 4'-DTMP had significant toxicity against dividing cell lines we tested, including ARPE-19, HEK293A, and CHO-DHFR, beyond 500 μM concentration (Supplementary Fig. 6). Although significant, it is not clear whether the relatively higher toxicity of 4'-DTMP will be limiting its potential clinical use as the levels of TMP in plasma and urine samples of patients is generally reported to be around 10 μM. Further structural, genetic and metabolic studies are necessary to gain a deeper understanding of 4'-DTMP's superiority as a drug against L28R E. coli.

Elimination of L28R mutation from TMP-resistant polyclonal E. coli populations. We hypothesized that selective elimination of antibiotic resistant subpopulations of bacteria by 4'-DTMP could be an effective strategy to modulate population structures of evolving bacterial cultures. The coexistence of two or more subpopulations of bacteria, also termed as clonal interference, is often observed both in clinical settings as well as laboratory evolution experiments. Hence, we chose six polyclonal E. coli populations that we previously evolved in vitro under TMP selection. These bacterial populations exhibited high levels of TMP resistance, albeit with notable differences in DHFR mutation distribution (Fig. 3a). We first measured resistance of these populations against both TMP and 4'-DTMP (Supplementary Fig. 7). Then we created an even more diverse bacterial population by mixing the six polyclonal cultures in nearly equal ratios (Fig. 3a). We carried out a competition assay by propagating this population in 500 μM of either TMP or 4'-DTMP, as well as in the absence of drug as a control. This process was continued for 32 h using the same concentration of drugs and samples were frozen at six different time points (Fig. 3a). Finally, we calculated fitness changes in mixed populations (Fig. 3b) and quantified frequencies of DHFR mutations by amplicon sequencing of the folA gene (Fig. 3c). We found that the L28R mutation quickly increases in frequency and plateaus in the presence of TMP (Fig. 3c, middle) whereas it is eliminated from mixed populations when 4'-DTMP was used (Fig. 3c, bottom). Interestingly, when 4'-DTMP was used, the frequency of the L28R initially increased but was later outcompeted by D27E and F153S mutations. The time point at which the L28R mutation started to decrease in abundance coincided with an almost two-fold increase in the growth rate of the populations (Fig. 3b). Additionally, until this tipping point was reached, the cell density of bacterial populations growing with 4'-DTMP were always low, suggesting poor fitness of mixed populations in the presence of 4'-DTMP. Regardless of the drug used, frequencies of the promoter mutations were always constant indicating that DHFR overexpression is an effective resistance mechanism for both TMP and 4'-DTMP. Lastly, the R98P mutation gradually swept all other mutations including the promoter mutation when no drug was used (Fig. 3c, top), implying its relative fitness advantage over other mutations in the absence of drug selection.

4'-DTMP impedes evolution of antibiotic resistance. In order to better understand the long-term effects of TMP and 4'-DTMP use on the evolution of E. coli antibiotic resistance, we evolved an initially isogenic, antibiotic sensitive E. coli strain (TB194, MG1655: attP21-PR-mCherry-chlorR) for 21 days (~10–15 generations per day) using TMP (Fig. 4a, gray lines, n = 7 replicates) and 4'-DTMP (Fig. 4a, teal lines, n = 8 replicates) in a morphodast. All of the populations evolved under TMP selection rapidly acquired very high levels of resistance and their MIC values were beyond the maximum solubility of TMP in growth media (~2.5 mg/mL) we used in this study. However, bacterial populations evolving under 4'-DTMP selection acquired resistance at a slower pace than the populations evolved under TMP selection (Fig. 4a–c and Supplementary Fig. 8). Furthermore, the final resistance levels of the populations evolved in 4'-DTMP were ~10 fold lower than those evolved in TMP (Fig. 4c). Bacterial populations evolved under TMP selection were also resistant to 4'-DTMP (Supplementary Fig. 9). Similarly, bacterial populations evolved under 4'-DTMP selection were found to be highly resistant to TMP (Supplementary Fig. 9). Therefore, both
TMP and 4′-DTMP select for cross-resistance to one another. However, bacterial populations evolved under 4′-DTMP selection grow slower and have significantly longer doubling times compared to the populations evolved under TMP (Supplementary Table 3, 63.7 ± 12.5 min and 49.8 ± 10 min, respectively; Student’s t-test, p = 0.04). In summary, long-term evolution of E. coli under TMP and 4′-DTMP selection revealed that 4′-DTMP impedes the evolution of antibiotic resistance.

Analysis of genetic changes in E. coli DHFR during the evolution of resistance experiments revealed that 4′-DTMP successfully blocks evolutionary trajectories leading to L28R mutation (Fig. 4). By selecting numerous samples archived from bacterial cultures evolved in the morbidostat, we observed that the populations sequentially accumulated several mutations in folA gene (as determined by amplicon sequencing and quantification of temporal changes of DHFR mutations; see “Methods” section)\(^28\). For both TMP and 4′-DTMP, we observed that the populations sequentially accumulated several mutations, and clonal interference and sweeping events were frequently observed (Fig. 4d). In both the cases, the majority of the populations acquired a promoter mutation (c-35t) early in their evolution (Fig. 4d–f). Strikingly, only one out of eight populations evolving under 4′-DTMP acquired the L28R mutations, whereas L28R was commonly observed in six out of seven populations when evolved under TMP selection (Fig. 4d–f, p = 0.017, Wilcoxon). We note that L28R mutation was observed in ~70% of our previous morbidostat experiments (n = 33) and experiments done by others\(^7,16,18,29\). The difference in final frequencies of the observed mutations under TMP and 4′-DTMP selection at the end of 21 days are displayed in Fig. 4e. The frequency of L28R mutation was drastically reduced from ~80 to ~15%. As summarized in Supplementary Table 3, populations evolved under 4′-DTMP selection also grow slower compared to populations evolved under TMP selection, further supporting the critical evolutionary role of the L28R mutation in maintaining the catalytic activity of DHFR. As summarized in Fig. 4f, elimination of L28R, the most beneficial DHFR mutation under TMP selection (Fig. 4f, left, trajectories highlighted with gray background), impedes the evolution of antibiotic resistance by diverting genetic trajectories (Fig. 4f, right, trajectories highlighted with teal background). Using 4′-DTMP instead of TMP, therefore, eliminates genetic trajectories leading to genotypes with the L28R mutation. In these genetic trajectories, we do not observe a dominant mutation that replaces L28R. Instead, we observe several DHFR mutations with
slightly elevated frequencies compared to mutations observed in the presence of TMP (Fig. 4e).

By comprehensively understanding the molecular evolution of TMP resistance in E. coli, we identified and targeted a common mutation (L28R with 4′-DTMP) and subsequently effectively slowed down evolution of antibiotic resistance. Use of 4′-DTMP eliminates genetic trajectories that accumulate the L28R mutation, the most beneficial resistance-conferring DHFR mutation against TMP. The L28R mutation has positive epistatic interactions with other resistance-conferring DHFR mutations, as L28R compensates for catalytic deficiencies caused by these mutations7,16,21–23. Therefore, blocking the L28R mutation diverts evolutionary trajectories towards genotypes with different DHFR mutations. Whether the use of 4′-DTMP will also slow down evolution of...
antibiotic resistance in clinical strains of *E. coli* or other pathogenic bacterial species is currently unknown to us, and addressing this question requires further laboratory evolution experiments and other experiments in animal models. Our study is an important demonstration of how information from laboratory evolution experiments and structural analysis of resistance-conferring mutations could guide efforts for developing novel antibiotic molecules and improving existing antibiotics. Furthermore, for drug targets known to exhibit evolutionary plasticity (e.g., DHFR), close monitoring of bacterial evolution and developing mutant-specific antibiotic molecules may not only eliminate resistant bacteria, but may also increase long term efficacy of antibiotic therapies by blocking evolutionary trajectories that lead to resistance genotypes.

**Methods**

**Data collection, structure determination and refinement, final model validation, and uncertainty** X-ray diffraction data sets for both crystals were collected at the Advanced Photon Source (APS), Structural Biology Center at Sector 19-ID. HKL3000 was used to process both wild-type and L28R data sets. Computational corrections for absorption in a crystal and imprecise calculations of the Lorentz factor resulting from a minor misalignment of the goniostat were applied. Anisotropic diffraction was corrected to adjust the error model and to compensate for a radiation-induced increase of non-isomorphism within the crystal. The results of refinement against the individual R-free sets showed discrepancies expected at high resolution.

**Data processing and refinement statistics for 6XG5 and 6XG4.**

| Data collection* | Wild-type (6XG5) | L28R (6XG4) |
|------------------|-----------------|-------------|
| Space group      | P3_21           | P3_21       |
| Cell dimensions  | a, b, c (Å)     | a = b = 61.81, c = 104.63 | a = b = 61.55, c = 104.66 |
| Resolution (Å)   | R(merge)        | 52.00-1.90 (193-1.90) | 52.00-2.10 (212-2.10) |
|                  | R(merging)      | 2.25         | 2.65         |
|                  | R(Free)         | 1.90         | 2.10         |
|                  | R(Free)*        | 0.026 (NA)   | 0.025 (NA)   |
|                  | R(merging)*     | 0.058 (NA)   | 0.076 (NA)   |
|                  | CC (2)          | 0.982 (0.552) | 1.000 (0.672) |
|                  | Rmerge_Rfree*   | 33.4 (37.5)  | 37.5 (37.5)  |
|                  | Completeness (%)| 99.2 (87.3)  | 99.9 (100.0) |
|                  | Multiplicity of observations | 6.1 (4.3) | 9.3 (8.8) |
|                  | Number of refinements | 18,771 (790) | 13,880 (312) |

*Values in the parentheses represent the last resolution shell. Please note that data sets were processed and refined with different shell divisions due to automatic procedures splitting data sets so that about equal numbers of reflections are present in each shell.

The resolution is reported in two different directions because of the strong anisotropy. The high crystallographic symmetry and high redundancy result in the values of Rmerge and Rfree exceeding 1.000. Such values do not have statistical meaning; therefore, we report them as NA. (Please note that we used complete cross-validation as described in the Experimental methods section.) Please note that the nominal data completeness is very high i.e., we collected and processed complete data sets. However, we used empirical truncation before refinement and that kept only 25% of reflections at high resolution.

**Bacterial strains**

Wild-type *E. coli*. The wild-type *E. coli* strain we used for the measurements is a derivative of the NDL47 strain (MG1655 attT7:pRNA1-tdCherry, a gift from Johan Paulsson Lab at Harvard Medical School) that has the wild-type DHFR (folA) gene sandwiched between the kanamycin and chloramphenicol resistance genes. We used the chromosomal integration method described by Danskeno and Wanner (PNAS, 2000) for generating the DHFR mutation library.

DHFR mutant *E. coli*. We generated a library of *E. coli* with various DHFR mutations by replacing the wild type folA gene of the NDL47 strain with the corresponding folA variant that is sandwiched between kanamycin and chloramphenicol resistance genes (Palmer et al.8).
BL21 E. coli cells were transformed with pET24a-foeΔ6xHisTag and were grown overnight in selective media (LB + Kan) and then diluted 100 times into TB media for further growth. Protein overexpression was induced when OD600 reached 0.6–0.8, with 1 mL of 250 mM IPTG per 1 L of the medium, and the temperature was decreased to 18 °C for further growth, with 220 rpm shaking. Recombinant protein was purified using Ni-NTA columns (Qiagen), and dialyzed overnight against 50 mM Tris-Base, pH 8.0, 0.5 M NaCl.

Crystallization of wild-type and L28R DHFR proteins. Both wild-type and L28R variant of DHFR proteins at 9 mg/mL concentration were mixed with 10 mM NADPH and 2 mM TMP and incubated overnight at 4 °C. Precipitated material was removed by centrifugation at 12,000 × g for 5 min. The supernatant was analyzed by LC-MS/MS. Crystallization of wild-type and L28R DHFR proteins was performed in glass bottles wrapped with aluminum foil to avoid light induced drug degradation.

Preparation of 4′-DTMP. Please see Supplementary Note 1 for the synthesis and characterization of 4′-DTMP.

Drug solutions. Freshly prepared drug solution of TMP and 4′-DTMP was used in every experiment. A 200 mM stock solution in DMSO was prepared, which was further diluted to required concentration using M9 minimal media supplemented with 0.4% glucose (Fisher Scientific B152-1), 0.2% ampicide (MP Biomedicals 104778), 2 mM MgSO4 (Fisher Scientific M63-500), 100 μM of CaCl2 (Fisher Scientific S2522A), and filtered. They were kept at room temperature in air.

Steady-state kinetic measurements. Reactants of DHFR reaction [DHFR (Sigma-Aldrich D7006) and NADPH (Sigma-Aldrich N7505)] has absorbance at 340 nm (at 25 °C in 1 cm cuvette containing 10 mM Tris-HCl, pH 7.5). DHFR (12.5 mM) was incubated with varying concentrations of each substrate. The reaction mixture was incubated at 37 °C and read at 562 nm.

Analytical LC-MS/MS methods. Supernatants from experiments evaluating intracellular levels of TMP or 4′-DTMP were analyzed using an AB Sciex (Framingham, MA) 4000 QTRAP mass spectrometer coupled to a Shimadzu Prominence LC to determine the amount of compound present. Standard curves were generated using blank M9 media spiked with known concentrations of compound and processed as described above. Analytes were detected with the mass spectrometer in positive MRM (multiple reaction monitoring) mode by following the precursor to fragment ion transition for two daughter ions. Only one reaction pair, indicated here, was used for quantitation for each analyte: TMP 291.092 to 230.0, 4′-DTMP 277.169 to 261.200. The transition 212.1 to 91.1 was monitored for the internal standard, n-benzylbenzamide. An Agilent C18 XBD column (5 micron, 50 × 4.6 mm) was used for chromatography with the following conditions: Buffer A: dH2O + 0.1% formic acid, Buffer B: methanol + 0.1% formic acid; flow rate 1.5 mL/min for gradient conditions: 0–1.0 min 3% B, 1.0–2.0 min gradient to 100% B, 2.0–3.5 min 100% B, 3.5–5.6 min gradient to 3% B, 3.6–4.5% B. The concentrations of drug in each time-point sample were quantified using Analyst software (Sciex). A value of 3-fold above the signal obtained from blank plasma was designated the limit of detection (LOD). The limit of quantification (LOQ) was defined as the lowest concentration, in triplicate, for 24 h. Media was removed and replaced with 50 μL of well of room temperature (RT) Cell Titer Glo 2.0 (Promega, Madison, WI) diluted 1:1 in Hanks buffered salt solution (Sigma Aldrich). Plates were mixed at RT for 5 min on an orbital shaker, incubated at RT for an additional 5 min, and luminescence was read on a Synergy 2 plate reader (10 s integration/well, BioTek, Winooski, VT). Results shown are representative of three independent experiments (Supplementary Fig. 6).

Toxicity assay. To test for potential cytotoxicity, ARPE-19 cells (CRL-2302, STR verified, University of Arizona Genetics Core), CHO-DHFR (CRL-9906, ATCC, Manassas, VA) or HEK293A (R70507, Life Technologies, Carlsbad, CA) cells were plated in black, clear-bottom 96-well plates (Corning, Corning, NY) at a density of 5000 and 10,000 cells/well, respectively, and allowed to attach overnight. The next day, cells were treated with DMSO, TMP, or 4′-DTMP (31.25–2000 μM), final concentration, in triplicate) for 24 h. Media was removed and replaced with 50 μL of well of room temperature (RT) Cell Titer Glo 2.0 (Promega, Madison, WI) diluted 1:1 in Hanks buffered salt solution (Sigma Aldrich). Plates were mixed at RT for 5 min on an orbital shaker, incubated at RT for an additional 5 min, and luminescence was read on a Synergy 2 plate reader (10 s integration/well, BioTek, Winooski, VT). Results shown are representative of three independent experiments (Supplementary Fig. 6).

Propagation of targeted MR populations. From our previous evolution experiments, six TMP-resistant bacterial populations with c-35s/D27F/E153S, g-31a/R98P, c-35s/L28R, c-35s/W30R, c-35s/W30R15F, and c-35s/94M mutations were chosen. These mutations were found by Sanger sequencing of randomly selected colonies isolated from populations. The individual minimum inhibition concentration (MIC) values of these populations were determined (Supplementary Fig. 7). From the drug dose response curves, a sub-inhibitory drug concentration of 500 μM was chosen such that the sweeping events would be observed in a large time window. All six populations were individually grown overnight, adjusted to the same OD, plated in B:A mixtures containing four drug concentrations, and incubated for 19 h at similar conditions. Three replicate populations were used for each drug concentration. The maximum number of sweeas was calculated as the lowest concentration at which back cal-

Initial drug concentration measurement. Wild-type, L28R, BW25113, and BW25113-DTcEcE. coli strains were incubated overnight at 37 °C in M9 media. Two hundred micromolar DMSO stock was prepared for the drugs TMP and 4′-DTMP. The drug solution was diluted with M9 media to adjust the concentration of drug to 6 μM. 2.5 μL of drug solution was added to a 15 mL conical tube (two time points (1 and 24 h) and three replicates for each set of conditions). OD was calculated for the E. coli strains. Required volume (~500 μL) of E. coli cells were added to the conical tube such that t = 0, concentration of the drug is 5 μM and the starting OD value is ~0.30 [1 OD = 5 × 10⁶ cells]. The cultures were incubated at 37 °C. After 1 h, OD values of one set of cultures were recorded, the cultures were transferred to 5 mL centrifuge vials and centrifuged at 4680 rpm for 3 min. The supernatant was removed and stored at -4 °C. The pellets were resuspended in 200 μL of ice-cold water, centrifuged at 4680 rpm for 3 min. The OD values were recorded and same steps were followed. The data were plotted using Prism (Supplementary Fig. 5).
containing 500 µM of drug (three replicates with no drug, seven replicates with 4′-DTMP, and seven replicates with 4′-DTMP-M). These cultures were grown at 37 °C, 200 r.p.m. so that they would normally be growing at an exponential rate if no antibiotics were added. At the end of each growth cycle, cell densities were recorded for all cultures, a small fraction of the populations were frozen for sequencing and the remaining cultures were further diluted to 0.01 OD600 with fresh media (20 mL) containing 500 µM of drug. This process was repeated till 32 h using successive concentration of drugs and the samples were frozen at different time points. Finally, we calculated fitness changes in mixed populations (Fig. 3b) and quantified frequencies of DHFR mutations by amplicon sequencing of the folA gene (Fig. 3c).

Long-term Evolution of E. coli under TMP or 4′-DTMP selection using morbidostats:
We started morbidostat experiments with wild type (drug-sensitive) isogenic E. coli cells (TB194; atpD2-Pr-mCherry-chloroR, a gift from Tobias Bergmiller lab) that were frozen at –80 °C. We thawed and diluted the cells 1:1000 in M9 media and transferred ~15 mL of the solution into autoclaved morbidostat culture tubes. We assigned seven tubes for TMP and eight tubes for 4′-DTMP. The optical density of the starting culture was calculated after subtracting the average voltage value within the first 30 s. We did not make any injections of drug solutions or fresh media into the culture until the OD of the culture exceeds 0.015 in order to allow cells to adapt their environment. This waiting time is usually around 2 h. After this waiting time, there are injections of fresh media or drug solutions every 18 min. Each injection takes 1 min and is followed by 17 min of growth cycle. Waste pump periodically runs during growth cycle but is turned off when injections are made into the culture to avoid suction of the injected liquid. The total volume inside the culture tube is ~15 mL and injection pumps are operated at ~2.16 mL/min flow rate. Each injection generates ~12.6% dilution of the growing culture. We calculated the growth rate of the cell population, initial and final OD values during the growth cycle, and the drug concentration. The cultures were transferred to a new set of tubes every day, to reduce the chances of forming biofilms. The experiment was continued for 21 days and the drug concentrations used were gradually increased by 5-folds as the populations were acquiring resistance. Samples from each tube were frozen every day. After 21 days, IOD values of TMP and 4′-DTMP were calculated for all the samples and plotted the fitness changes against time (Fig. 4a–c and Supplementary Fig. 8). Finally, we quantified the frequencies of DHFR mutations in those populations by deep amplicon sequencing of the folA gene (Fig. 4d–f).

Deep sequencing of folA gene using MiSeq:
The frozen glycerol stocks of the bacterial populations were warmed to RT. 25 µL of each population were individually diluted to 1.0 mL using M9 media and grown overnight at 37 °C, to make sure that all the populations have similar OD values. A lysis buffer was prepared by adding 10 µL of Triton X-100 (Sigma Aldrich, T8787) to 10 mL of Tris EDTA buffer. Ten microliter of overnight culture was mixed with 30 µL of lysis buffer, heated at 95 °C for 5 min, and centrifuged at 13,000×g for 10 min. The supernatant was used as DNA template in PCR. The folA gene was amplified in two portions using the primers listed in Supplementary Table 4.

PCR mixture (15 µL total volume) was prepared by mixing 3.0 µL of DNA template, 0.75 µL of each forward and reverse primer, 7.5 µL of Q5 High-Fidelity 2× Master Mix (BioLabs) and 3.9 µL of H2O. Thermocycler was set to 98 °C for 3 min, [98 °C for 15 s, 56 °C for 30 s, 72 °C for 1 min] × 25 cycles, 72 °C for 5 min then 4 °C. All the PCR products were purified using Nucleospin® Gel purification kit (Macherey-Nagel). Library preparation for MiSeq was done using Illumina MiSeq 2 × 250 bp kit, following the standard protocol provided by the vendor. Concentrations of the amplicons were measured using Qubit® assay. Sequencing was done in house using an Illumina MiSeq instrument. Results were analyzed and plotted using custom Python scripts (Figs. 1d, 3c, and 4d–f).

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Data supporting the findings of this study are available within the paper and its Supplementary Information. The X-Ray crystal structures have been deposited to PDBe with PDB codes 6XGS/6XGT/6BGX (pdb) and 6XGC/6XGD (wild-type). Corresponding raw data for each figure and table used in the manuscript and the supplementary files are accessible free of cost from GitHub (https://github.com/erdaltoprak-zz/NatureCommunication2021_Manna.git). The raw sequencing data are deposited to NCBI with accession code PRJNA717019. Link to the source data files are listed in the “Source Data” file provided with this paper. Source data are provided with this paper.

Code availability
All the codes used to plot the figures of this manuscript and the supplementary information of this paper are available from Zenodo (https://doi.org/10.5281/zenodo.4630929).

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Author contributions

The project was designed by E.T., U.T., M.S.M., Y.T.T., A.R.A., and C.A. Purification and crystallization of wild-type and L28R DHFR proteins for the determination of their IC50 were done by L.G. Crystallography data collection, structure determination and refinement were done by D.B. All the compounds were synthesized and their IC50 were measured by M.S.M. Steady-state kinetic measurements were performed by N.P. Intracellular drug concentrations measurement experiments were done by M.S.M., X.W., and N.S.W. Toxicity assay was performed by D.R.W. and J.D.H. Propagation of mixed TMP-resistant populations were studied by M.S.M. Morbidostat code was written by F.C.R.T. and E.T. Long-term evolution of *E. coli* using morbidostat was performed by M.S.M. and A.A. Library preparation and deep sequencing of *folA* gene using MiSeq was done by M.S.M. and Y.T.T. The data were analyzed by Y.T.T., M.S.M., and E.T. The manuscript was written by M.S.M. and E.T., which was further edited by U.T., A.Y.K., N.S.W., D.B., and J.D.H.

Competition interests

The authors declare no competing interests.

Additional information

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