

Strain diversity drives heterogeneous responses to tuberculosis combination therapy

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ABSTRACT Strain diversity in *Mycobacterium tuberculosis* (Mtb) underlies distinct clinical presentations and outcomes, but the range of drug susceptibility phenotypes among clinical isolates is poorly understood. We aimed to identify drug response patterns in phylogenetically diverse clinical isolates to combination treatment. We selected 13 strains out of 641 drug-sensitive clinical isolates that capture local and global phylogenetic diversity and included Erdman ATCC-35801 as a reference. We treated each strain with 10 single drugs, 45 drug pairs, and 20 three-way combinations in standard and cholesterol-rich media. Mtb clinical strains displayed a broad range of drug response phenotypes across the 65 drug combinations and 2 metabolic conditions tested, with the most effective drug pairs (based on potency and synergy) varying by strain and metabolic condition. Within our 14-strain panel, strains that were less sensitive to single drugs were also less sensitive to combination treatment, with very few exceptions. For all drug combinations tested, the variation in combination potency was driven primarily by variation among genetically related strains, rather than between strains belonging to disparate lineages. Preclinical regimen design should reflect the diversity of Mtb clinical strains; our data suggest that selecting strains based on the range of drug response phenotypes displayed, rather than by genetic diversity alone, may better account for pathogen diversity. Our findings also show that constituent drug pairs of high-order combinations can be differentially effective against Mtb adapted to different carbon sources. Selection of these pairs should likely involve multiple factors including the infecting strain, metabolic niche, and drug response metrics.

KEYWORDS drug combinations, clinical isolates, *Mycobacterium tuberculosis*

Tuberculosis (TB) remains challenging to treat, in part, because a subset of patients requires extended treatment. Hard-to-treat disease may have several origins: lesions associated with severe pathologies impede drug penetration and drug susceptibility, and genetic differences among infecting organisms—and the phenotypes they mediate—can manifest in heterogeneous disease presentations and responses to drug treatment (1–4). Though several studies demonstrate substantial heterogeneity in antibiotic susceptibility, transmission potential, and treatment outcomes among newly emerging *Mycobacterium tuberculosis* (Mtb) clinical strains (5), bacterial determinants (beyond canonical resistance mutations) associated with unfavorable clinical outcomes have only recently been characterized (3). Recent studies show that strain-to-strain variation in response to antibiotic and metabolic stress and modest differences in MIC values below clinical breakpoints underlie distinct clinical phenotypes such as treatment failure and cavitary disease (3, 6). However, despite growing evidence that subtle differences in antibiotic susceptibility can lead to divergent clinical outcomes, preclinical drug combination studies focus on a few commonly studied Mtb strains (Erdman, H37Rv, HN878), which may underrepresent the range of drug susceptibility patterns of clinical

Editor Sean Wasserman, Queen Mary University of London, London, United Kingdom

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The authors declare no conflict of interest.

See the funding table on p. 11.

Received 15 December 2025

Accepted 8 April 2026

Published 15 May 2026

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isolates (7–10). Though phenotypic responses to several first- and second-line antibiotics are well characterized across different *Mtb* phylogenetic lineages (11–14), TB treatment relies on combination therapy, and clinical outcomes likely reflect strain-dependent differences in drug combination efficacy (15). Yet, we lack a systematic evaluation and understanding of combination treatment responses across diverse clinical isolates. Here, we provide an analysis of drug responses to 65 unique drug combinations across a phylogenetically diverse panel of *Mtb* clinical strains in two *in vitro* conditions that model different cell states. Our data allow us to answer several fundamental questions about the impacts of strain variation on combination treatment outcomes: How does the drug susceptibility of clinical isolates vary in response to single drugs and drug combinations? Do genetically related isolates exhibit similar drug susceptibility patterns? Are certain isolates more tolerant to certain combinations, and if so, can we identify any drug-dependent or strain-dependent patterns? Finally, can we reasonably predict how susceptible a given isolate is to combination treatment based on its susceptibility to single drugs—that is, can we screen problematic isolates based on single drug susceptibility? The variation we highlight across strains and drug combination treatments, in different metabolic conditions, may aid future combination treatment optimization in accounting for pathogen diversity, with implications for de-risking, revamping, and accelerating drug regimen design.

RESULTS

Clinical isolates were obtained from TB patients with diverse clinical presentations including lung cavitation and treatment failure, as detailed in reference 3. We selected 13 strains that capture local and global phylogenetic diversity, including 2 strains from subclades of L1.2 (185 and 355), 2 strains from the locally expanded L1.1.1.1 (139 and 358), 2 outgroup strains in L1.1.1.1 (414 and 617), a strain each from the L4.1 Erdman clade and L4.4 H37Rv clade (082 and 084, respectively), a strain from the proto-Beijing L2.1 (245), and 4 strains from the expanded L2.2 lineage (044, 070, 478, 545) (Fig. 1A). We selected 10 antibiotics that represent a broad range of mechanisms of action (Table S1) and included Erdman ATCC 35,801, commonly used in systematic drug combination studies (7, 8), as a reference. Based on the phylogenetic tree of the 14 strains, we found that the strains bifurcated into two major groups: Lineage 1 (Fig. 1A, orange) and Lineages 2 and 4 (Fig. 1A, blue). We manually classified the strains into these two groups for ease of interpretation.

Mtb occupies metabolically distinct niches *in vivo* (2). Cholesterol is a key carbon source in lipid-rich environments within granulomas (16, 17), and studies demonstrate substantial heterogeneity in drug response between *Mtb* acclimated to different carbon sources (2, 18, 19). Both glycerol- (called standard here) and lipid-based *in vitro* models (including cholesterol) have been validated as predictive of treatment outcomes *in vivo* (7, 8). To account for condition-dependent variation in drug response phenotypes, we conducted all drug combination assays in standard conditions and in a medium with cholesterol as the sole carbon source (called cholesterol here). We note that a subset of strains—245, 358, and 478—arrested growth in cholesterol and were excluded from analysis in this condition.

We observed substantial variation in doubling times across clinical isolates in both standard (14 to 21 h) and cholesterol (51 to 104 h) conditions. To compare drug and drug combination efficacy across different strains and conditions, we used GR metrics, which normalize drug treatment response to the untreated growth rate of each strain. Specifically, we used GR_{max} , the maximum growth rate inhibition, to compare drug and drug combination responses across 14 *Mtb* strains and 2 *in vitro* conditions. We also computed commonly used drug response metrics such as inhibitory concentrations (ICs) and drug interaction scores (fractional inhibitory concentrations, FICs, Fig. 1B, top). A subset of drugs failed to achieve at least 90% growth inhibition in several strains; we, therefore, used IC_{50} values to interpret phenotypic responses to single drugs and to

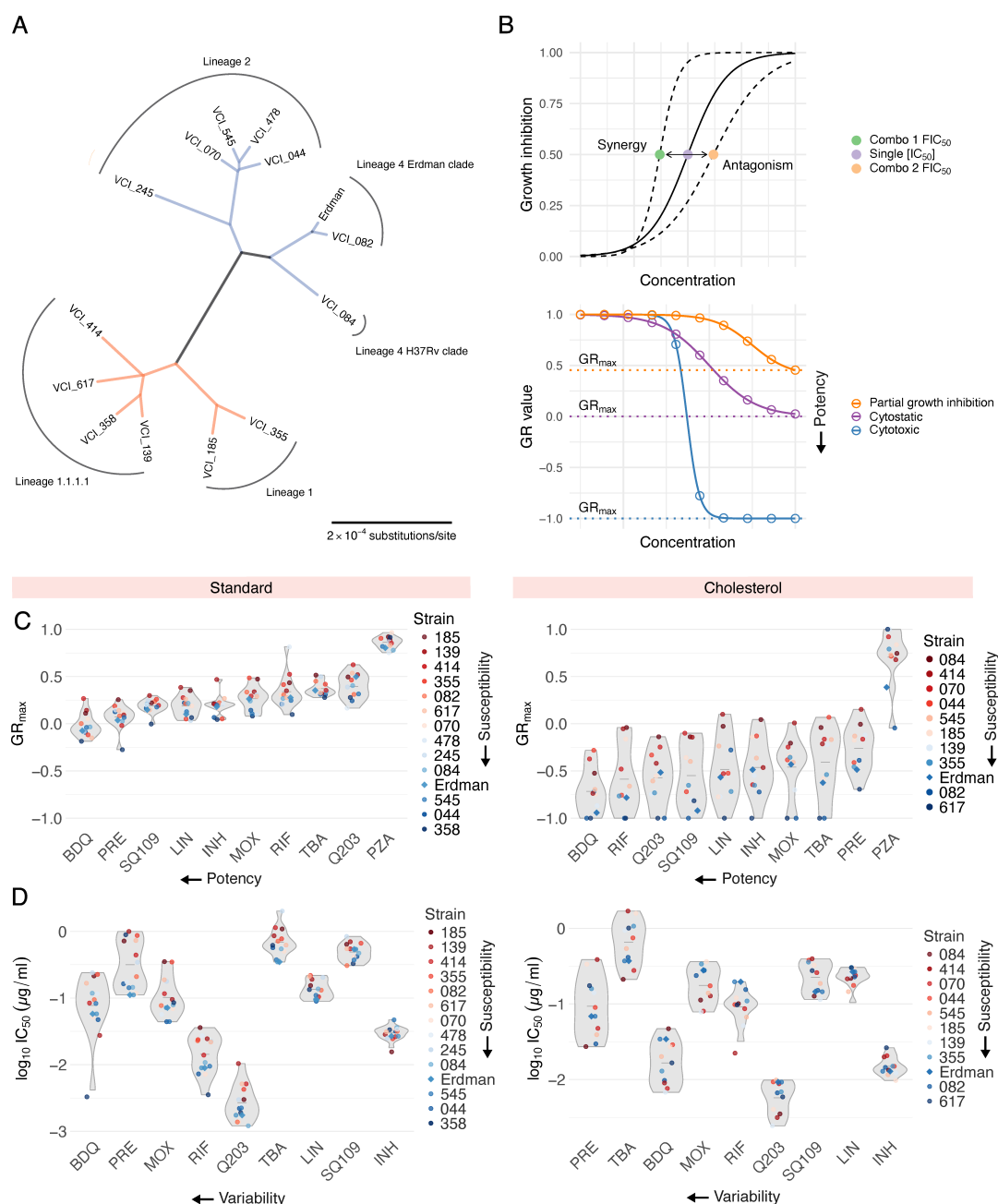


FIG 1 Susceptibility profiles of phylogenetically diverse *Mtb* clinical isolates to 10 antibiotics. (A) Unrooted maximum-likelihood phylogenetic tree of the 14 *Mtb* strains studied in this work, as determined by core gene alignments. Scale bar represents substitutions per nucleotide. Colors indicate strain groups defined for the interpretation of this work. (B) Schematics of drug interaction (top) and potency (bottom) metrics computed via DiaMOND. (Top) The solid-line dose response curve represents the inhibitory effect of a single drug. Shifts in the left and right directions indicate synergy and antagonism, respectively, when combined with another drug. (Bottom) Dose response curves indicate a cytotoxic response (blue), cytostatic response (purple), and partial growth inhibition (orange). (C) GR_{max} values of 10 antibiotic treatments across 14 strains in standard (left) and 11 strains in cholesterol (right). Strains are colored from least drug-susceptible (red), to most drug-susceptible (blue) (Fig. S1). Antibiotics are ordered from most potent to least potent, from left to right, respectively. Each point represents the median GR_{max} value across six biological replicates in standard and three biological replicates in cholesterol. (D) Half-maximal inhibitory concentrations (IC₅₀) of 9 antibiotic treatments across 14 strains in standard (left) and 11 strains in cholesterol (right). PZA is excluded due to inactivity as a single agent. Strains are colored from least drug-susceptible (red) to most drug-susceptible (blue). Antibiotics are ordered from most variable (highest coefficient of variation, CV) to least variable (lowest CV), from left to right, respectively. Each point represents the median log₁₀IC₅₀ value across six biological replicates in standard and three biological replicates in cholesterol.

compute drug interaction scores at the IC_{50} (FIC_{50}). Negative, zero, and positive $\log_2 FIC_{50}$ values denote synergy, additivity, and antagonism, respectively.

To characterize drug response patterns across our panel of 14 *Mtb* strains, we treated each strain with 10 antibiotics in standard and cholesterol conditions. For each drug treatment, we generated dose response curves from which we calculated GR_{max} and IC_{50} values, resulting in 280 metrics in standard and 220 metrics in cholesterol. In both conditions, we observed strain-to-strain differences in GR_{max} for all 10 drugs tested (Fig. 1C). For select drugs such as bedaquiline, pretomanid, and moxifloxacin in standard, and bedaquiline, pretomanid, and TBA-3731 in cholesterol, IC_{50} values varied by nearly 10-fold between strains (Fig. 1D). We observed that most clinical isolates are more drug tolerant than Erdman in both standard and cholesterol (Fig. S1). Notably, bedaquiline and pretomanid displayed the greatest variability in IC_{50} values across all strains in both conditions (Fig. 1D), suggesting that despite their use in new regimens, their efficacy may be inconsistent across clinical strains.

We next asked whether treating *Mtb* with drug pairs improves treatment efficacy relative to single drugs based on changes in GR_{max} values. To quantify GR_{max} improvement, we calculated the difference between the GR_{max} of a drug pair and the GR_{max} of its constituent singles, with negative values indicating improvement. Across all strains in standard medium, the addition of bedaquiline or pretomanid to another drug improved the GR_{max} of the other drug, with improvement observed in 216/224 (96.5%) drug pairs containing either bedaquiline or pretomanid (Fig. 2A, left; Fig. S3, top). Given that bedaquiline and pretomanid are the two most potent single drugs in standard medium, we hypothesized that combining any drug with another drug that is less potent than itself will invariably improve the GR_{max} of the less potent drug. We found that this was generally the case, but not to the same extent as bedaquiline or pretomanid. For instance, SQ109, linezolid, and isoniazid also improved the GR_{max} of drugs less potent than itself in a majority of drug pairs (84/98 drug pairs or 85.7%, 69/84 drug pairs or 82.1%, 67/70 or 95.7%, respectively), but they improved the GR_{max} of more potent drugs (such as bedaquiline and pretomanid) in only a subset of strains (Fig. S2, top). However, we did not observe this pattern in other drugs such as moxifloxacin or rifampicin—in these drugs, GR_{max} improvement was less predictable (Fig. S2, top). These findings suggest that (i) in some cases, GR_{max} improvement of drug pairs can be predicted based on the potency of the individual drugs being combined, and (ii) bedaquiline and pretomanid may have intrinsic properties that allow them to optimize drug combination efficacy in diverse strain backgrounds beyond what would be expected based on potency alone. In cholesterol, however, neither BDQ nor PRE improved GR_{max} across all combinations and strains (Fig. 2A, right; Fig. S2, bottom; Fig. S3). Instead, the magnitude of GR_{max} improvement for drug pairs containing BDQ or PRE varied by strain, suggesting that combination efficacy in cholesterol depends more on strain-specific susceptibility patterns to drug pairs than by the intrinsic properties of individual drugs.

The drug susceptibility profile of a specific *Mtb* strain is typically defined based on its minimum inhibitory concentration (MIC) values to select single drugs even though TB patients are treated with drug combinations. We, therefore, asked whether drug response phenotypes to single drugs provide any predictive insight into strain-specific phenotypic responses to combination treatment. We compared GR_{max} values across three different treatment modalities—single drugs (10 drugs), drug pairs (45 pairs), and three-way combinations (20 combinations)—for each strain (Fig. 2B; Fig. S4; Table S2 and Table S3). This comparison addressed two related questions: (i) within our 14-strain subset, are strains that are more difficult to treat with single drugs also more difficult to treat with drug combinations and (2) between different treatment modalities, does GR_{max} change in a strain-dependent manner, or does it follow a generalizable trend? In both standard and cholesterol, we found that strains that are less sensitive to single drugs are also less sensitive to drug pairs and three-way combinations on average (Fig. 2B and Fig. S4), as these strains (represented in red) occupy the upper range in GR_{max} across nearly all drugs in the three treatment modalities tested (Fig. 2B and Fig.

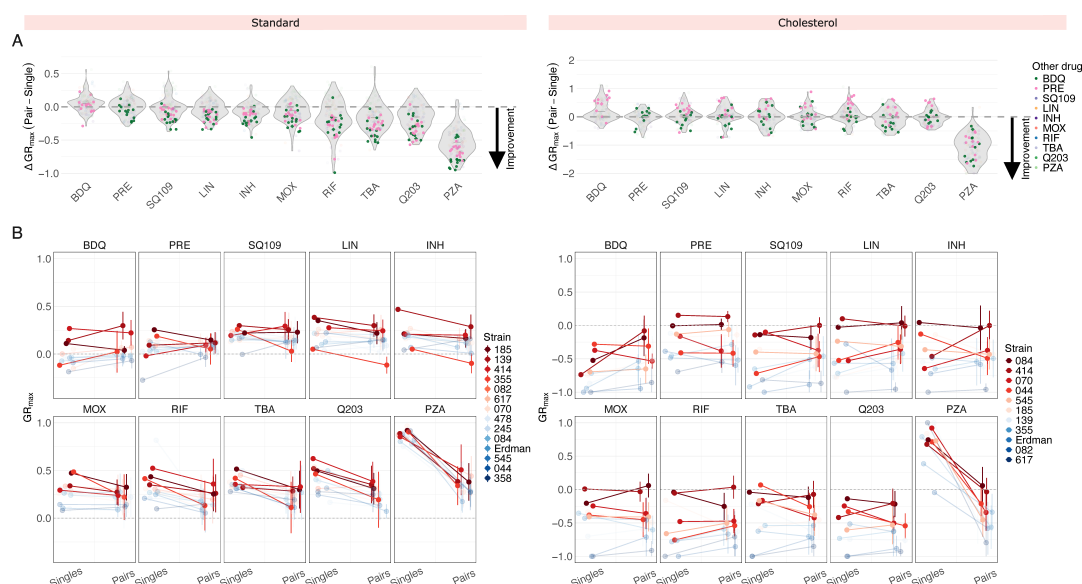


FIG 2 Comparison of GR_{max} values between different drug modalities. (A) Change in GR_{max} between single drug and pairwise treatments in standard (left) and cholesterol (right) conditions. Each point denotes a ΔGR_{max} value of a drug pair for an individual isolate and represents the median GR_{max} value across three biological replicates. Drug pairs containing bedaquiline and pretomanid are highlighted in green and pink, respectively. A ΔGR_{max} value less than zero indicates an improvement in GR_{max} when a single drug is combined with a second drug (e.g., when BDQ is combined with LIN, or when INH is combined with MOX). (B) Comparison of GR_{max} values between single drugs and their nine corresponding drug pairs (Table S2) in standard (left) and cholesterol (right) conditions. Each panel represents a single drug on the left side and the mean GR_{max} value of its nine corresponding drug pairs on the right side. Vertical bars represent the standard deviation of the mean GR_{max} value. Each point represents the median GR_{max} value across three biological replicates and corresponds to an individual strain. The four least susceptible strains for each condition are highlighted in red. (Note: opacity adjustments are not reflected in the legend.)

S4). We conclude that the stratification of strains by single-drug susceptibility is largely preserved between treatment modalities. An exception was isolate 355 in standard medium; although more tolerant to single drugs than most other strains, its GR_{max} was greatly improved by treatment with drug pairs, especially those containing SQ109, moxifloxacin, rifampin, and TBA-3731 (Fig. 2B, left).

We then evaluated whether three-way combinations provide further improvement in treatment efficacy over drug pairs. Due to experimental constraints, we restricted the three-way panel to six drugs with major clinical relevance to contemporary TB treatment and regimen design (BDQ, PRE, LIN, INH, MOX, and RIF) (20–23). Given that only 6 drugs were tested in three-way combinations, as opposed to 10 drugs in drug pairs, comparisons were limited to shared compounds. In both standard and cholesterol, there was no improvement in GR_{max} in three-way combinations over drug pairs, suggesting that high-order combinations do not provide additional improvement over drug pairs in the conditions tested (Fig. S4).

To improve the resolution of strain-specific phenotypic responses to drug combination treatment, we decomposed averaged drug combination data into all individual drug pairs (Fig. 3A) and three-way combinations (Fig. 3B) tested. This allowed us to ask several questions: (i) are hard-to-treat strains (defined by low susceptibility to single drugs) more tolerant to all drug combinations, or do specific combinations sensitize them, and (ii) do some drug combinations display larger variability in GR_{max} than others, and if so, do certain strains drive this variability? We found that in both standard and cholesterol conditions, hard-to-treat strains are more tolerant to nearly all drug pairs and three-way combinations tested (Fig. 3A and B, violin plots), suggesting that strains that are tolerant to single drugs are expected to be more difficult to treat with drug combinations. Regardless, a small subset of drug combinations was effective against select hard-to-treat strains. For example, of all 45 drug pairs and 20 three-way combinations tested, pretomanid+rifampin (PRE+RIF), pretomanid+telacebec (PRE+Q203),

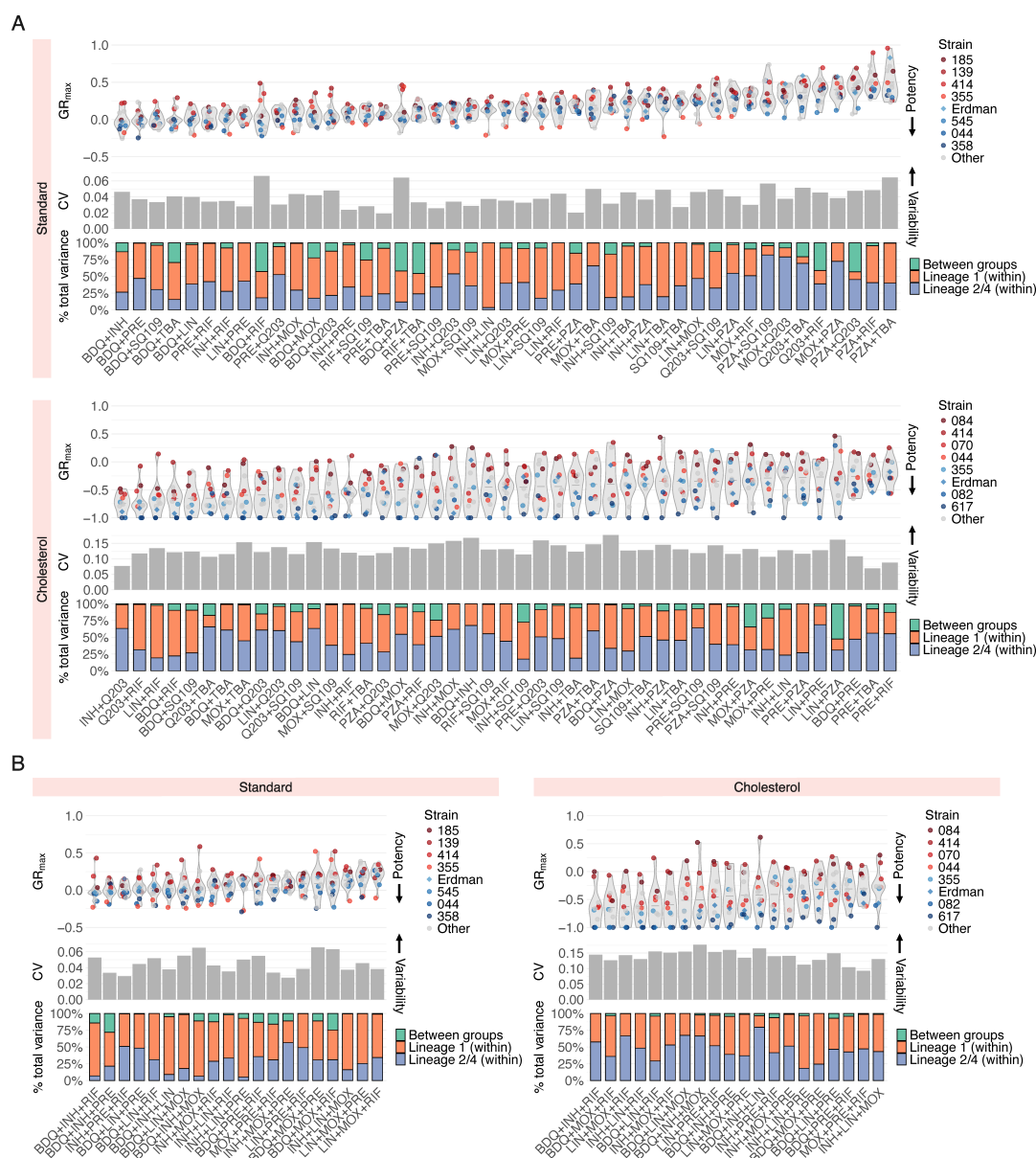
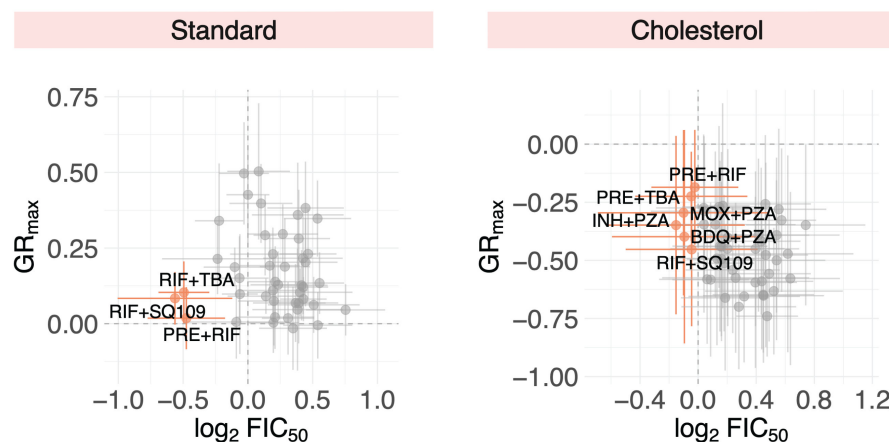


FIG 3 Susceptibility profiles of *Mtb* clinical isolates to drug pairs and high-order combinations. Drug potency and strain variability metrics of (A) 45 drug pairs and (B) 20 three-way combinations tested in standard and cholesterol. For each condition: (top) violin plots represent the GR_{max} range across all clinical strains tested. The four least drug-susceptible strains and the four most drug-susceptible strains (Fig. 1D) are highlighted in red and blue, respectively. All other strains are represented in gray. Each point represents the median GR_{max} value across three biological replicates. (Middle) Each bar represents the coefficient of variation (CV) of GR_{max} values across 14 strains in standard and 11 strains in cholesterol for each drug pair. (Bottom) Each stacked bar represents the variance decomposition of the GR_{max} values across 14 strains in standard and 11 strains in cholesterol for each drug pair. The green bar represents the percent total variance attributable to variance between lineage 1 and lineages 2 and 4; the orange bar represents the percent total variance attributable to variance within lineage 1; the blue bar represents the percent total variance attributable to variance within lineages 2 and 4.

and isoniazid+pretomanid+rifampin (INH+PRE+RIF) were the only drug combinations that induced a cytotoxic effect in isolate 139 in standard (Fig. 3A and B, violin plots for standard). Similarly, bedaquiline+isoniazid (BDQ+INH) and bedaquiline+TBA-3731 (BDQ+TBA) also showed increased potency against isolate 185 (Fig. 3A, violin plot for standard). These examples highlight the potential of targeted combinations to sensitize strains that may otherwise be tolerant to a broad swath of treatment options.

A



B

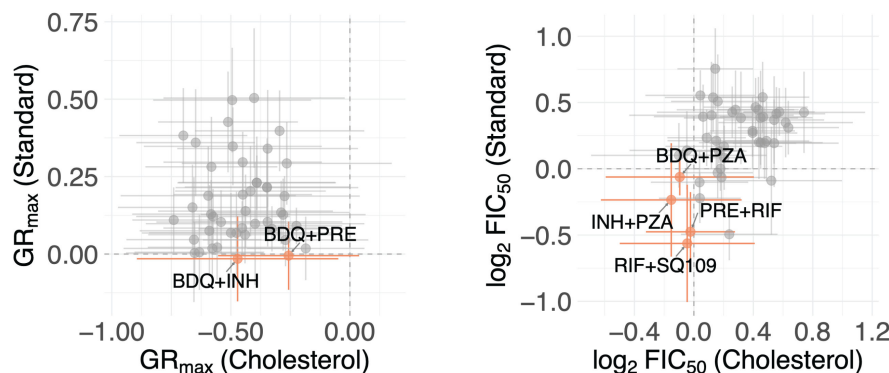


FIG 4 Comparison of drug pairs combination treatment responses across different *in vitro* conditions and dose response metrics. (A) Potent (low GR_{max} value) and synergistic (low $\log_2 FIC_{50}$ value) drug pairs identified in *in vitro* conditions. (Left) The three drug pairs with the lowest mean GR_{max} and $\log_2 FIC_{50}$ values are represented by orange points. All other drug pairs are represented by gray points. Vertical and horizontal bars denote standard deviation of GR_{max} and $\log_2 FIC_{50}$ values, respectively. (Right) Drug pairs that are both cytotoxic and synergistic, indicated by negative GR_{max} and $\log_2 FIC_{50}$ values, respectively, are represented by orange points. (B) (Left) Drug pairs that are cytotoxic (negative GR_{max} value) in both standard and cholesterol conditions are represented by orange points. Vertical and horizontal bars denote the standard deviation of GR_{max} values and standard and cholesterol, respectively. (Right) Drug pairs that are synergistic (negative $\log_2 FIC_{50}$ value) in both standard and cholesterol conditions are represented by orange points.

Across all drug pairs and three-way combinations, we sought to identify a quantitative relationship between drug combination potency, as determined by GR_{max} , and the magnitude of strain-to-strain variability, as determined by the coefficient of variation (CV) (Fig. 3A and B, gray bar plots). There was no correlation between combination potency and the magnitude of strain-to-strain variability, but a small subset of potent drug combinations—bedaquiline+rifampin (BDQ+RIF), bedaquiline+pyrazinamide (BDQ+PZA), and bedaquiline+isoniazid+moxifloxacin (BDQ+INH+MOX) in standard medium—were more variable than others in the same condition (Fig. 3A and B, gray bar plots for standard). Notably, we found that strain-to-strain variability in GR_{max} to single drugs (Fig. S5) is not correlated with variability in GR_{max} to drug pairs (Fig. 3A, gray bar plots for standard). That is, although bedaquiline and pretomanid exhibit the most strain-to-strain variability in GR_{max} (Fig. S5), combining pretomanid with another drug tended to reduce the amount of variability; in contrast, pairing bedaquiline with another drug tended to increase variability (Fig. 2B, left). Moreover, we found that three-way combinations do not reduce variability relative to drug pairs (Fig. 3A and B,

gray bar plots; Fig. S6), suggesting that increasing the number of drugs does not mitigate strain-to-strain differences in single drug response.

We next asked whether specific strains or strain groups drive the variation in GR_{max} observed in each of the drug combinations tested. We partitioned the total variance in GR_{max} for each drug combination into three components: variance within lineage 1 strains, variance within strains in lineages 2 and 4, and variance between the two strain groups (Fig. 3A and B, stacked bar plots). Strikingly, for all combinations tested, the total variance was driven primarily by variability in GR_{max} within strain groups, rather than between (Fig. 3A and B, stacked bar plots), suggesting that genetically related strains do not necessarily exhibit similar drug response phenotypes.

Though we demonstrate that three-way combinations do not improve drug potency over drug pairs in any one condition, TB treatment still necessitates high-order combinations since its constituent drug pairs may target different metabolic and physical niches. We sought to identify candidate drug pairs, based on their GR_{max} and FIC_{50} values, that could potentially be effective across diverse strain backgrounds and different *in vitro* conditions. A few drug pairs were both potent and synergistic in standard and cholesterol (Fig. 4A, orange), but only a small subset of these pairs—pretomanid+rifampin (PRE+RIF) and rifampin+SQ109 (RIF+SQ109)—were shared between the two conditions (Fig. 4A, orange). For all drug pairs, the standard deviation in potency (horizontal error bars) and synergy (vertical error bars) varied greatly, suggesting that drug pairs that satisfy both criteria (potent and synergistic) vary by individual strain. We further demonstrate limited correlation between potency and synergy, as drug pairs that are potent in both conditions (Fig. 4B, left) and synergistic in both conditions (Fig. 4B, right) vary. Altogether, these data further support our understanding that different drug pairs target metabolically heterogeneous *Mtb*, and the selection of these pairs relies on a multitude of factors including genetic background, metabolic state, and a consideration of several drug response metrics.

DISCUSSION

Altogether, we demonstrate substantial heterogeneity in drug response to combination treatment across our 14-strain panel, with certain strains exhibiting attenuated sensitivity to nearly all drugs and drug combinations tested. Importantly, many clinical isolates were less sensitive to combination treatment than commonly studied strains such as Erdman, and treatment efficacy of drug combinations varied greatly by strain, especially those that included drugs that are relevant in the clinic and in preclinical studies. Our findings highlight the importance of studying the effects of drug combination treatments in phenotypically diverse *Mtb* strains, particularly to define the upper bounds of drug tolerance.

While identifying genetic resistance markers remains essential in understanding the causes of severe disease pathologies and treatment failure, an increasing body of work shows that physiological adaptations such as altered respiration and energy metabolism, metabolic reprogramming, and upregulation of multidrug efflux pumps can broadly reduce drug susceptibility independent of genotype (24–27). Our results support the understanding that such mechanisms, not readily identified via genomic analyses alone, may underlie broad-spectrum drug tolerance to a larger extent than expected: strains that are less susceptible to single agents often remain recalcitrant to high-order drug combinations, even when those combinations include drugs with distinct mechanisms of action. That is, increasing the number of drugs in a combination failed to mitigate relative drug tolerance or reduce the magnitude of strain-to-strain variability in drug response. These findings suggest that high-order combinations alone are insufficient to rescue certain forms of intrinsic or acquired drug tolerance.

Strikingly, strain-to-strain variation in drug response was driven primarily by the susceptibility profiles of individual strains, rather than by lineage identity. Strain phenotypes are likely driven by mutations arising on terminal branches, or other contemporaneous changes that are heritable yet appear randomly distributed across the

phylogenetic space due to our limited resolution ($n = 14$ strains). Additionally, adaptations shaped by various facets of the host environment that modulate drug response may have been selected for during infection and retained *in vitro*. Our results also have implications in designing strain panels for preclinical combination design studies; we propose that selecting strains based on phenotypic, in addition to genetic, diversity may more effectively capture strain diversity. Overall, our findings advocate for a holistic approach to studying the effects of combination therapy—one that probes both the upper and lower bounds of drug susceptibility across phenotypically diverse strains and contextualizes these phenotypes in several host-relevant metabolic conditions.

There are several limitations to our study. First, although our 14-strain panel spans both local and global phylogenetic diversity, it is derived from a single geographic cohort and, thus, represents a limited fraction of the global Mtb population. Further expanding the strain panel in scope and size may provide new insight into drug response traits underlying heterogeneous treatment outcomes. Second, though our cholesterol model reflects host-relevant carbon sources, it does not fully recapitulate the lesion microenvironment such as immune pressure, hypoxic stress, and nutrient restriction. Additionally, the cholesterol condition alone only represents a fraction of the metabolic diversity of Mtb during host infection. Future combination studies should include multiple host-like *in vitro* conditions to account for this diversity. Finally, a subset of strains arrested growth in cholesterol medium and were, thus, excluded from further analysis in that condition, limiting direct comparisons across the complete strain panel.

The current first-line regimen and several other drug combinations used in the clinic vary in treatment efficacy among TB patients (28, 29). To mitigate this variability, it is crucial for key processes in drug combination design and optimization to account for strain-to-strain differences in drug susceptibility. Our study demonstrates that strain variation in drug combination responses may explain some of the differences in drug responses observed in the clinic, which suggests that introducing strain diversity into these preclinical studies has major implications in optimizing and derisking drug regimen design.

MATERIALS AND METHODS

Antimicrobials, dispensing, and drug combination design

A complete list of antibiotics used in this study is provided in Table S1. 50% inhibitory concentrations (IC_{50}) were calculated for all antibiotics in two different *in vitro* models with different carbon sources: standard (sugar-rich) and cholesterol (lipid-rich). Acclimation procedures are detailed in the supplemental material. For drug combinations, equipotent mixtures of antibiotics were created by aligning the IC_{50} value of each constituent drug to the same dose level (30). Initial IC_{50} measurements were made using 14 doses with 2-fold spacing, whereas subsequent experiments used a 1.5-fold dose resolution across 10 doses.

Strains and culturing

All strains used in this study were prepared and cultured in a 7H9 broth supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% Middlebrook OADC (referred to as standard medium in the main text) and stored in -80°C until use. Frozen 1 mL stocks of Mtb were added to 9 mL of standard medium and grown in a 37°C shaking incubator to mid-log phase (optical density at 600 nm, or OD_{600} , between 0.4 and 0.8).

Drug treatment assays

Dose response assays for drug combinations were designed using the DiaMOND (diagonal measurement of n -way drug interactions) method, which optimizes traditional checkerboard assays to measure equipotent mixtures of drugs (31). This allows efficient

measurements of drug interactions in a large number of drug combinations that would otherwise be impractical with traditional methods.

Fifty microliters of Mtb grown in either standard or cholesterol medium was back-diluted to an OD₆₀₀ of 0.05, added to drug-treated 384-well plates, and incubated at 37°C in humidified bags to prevent evaporation. Edge wells (top and bottom rows, left- and right-most columns) contained only media and were excluded from analysis. OD₆₀₀ was measured at two time points for each strain using a Synergy Neo2 Hybrid Multi-Mode Reader. For both standard and cholesterol conditions, OD₆₀₀ was measured immediately following inoculation (day 0) and at the terminal time point, which corresponds to 5 days post-inoculation (day 5) for standard and 12 days post-inoculation (day 12) for cholesterol. The difference in incubation time between these two conditions reflects the slower growth kinetics of Mtb in cholesterol medium. These time points were identified as being predictive of treatment outcomes *in vivo* and were selected in prior DiaMOND-based studies to allow consistent drug exposure time relative to condition-dependent doubling times (8).

Data processing and DiaMOND metric calculation

OD₆₀₀ data were processed using custom MATLAB analysis scripts. Raw data were first derandomized and then background-subtracted using the median OD₆₀₀ value of the media-only edge wells. Drug-treated wells were then normalized to the mean OD₆₀₀ value of the untreated controls on the same plate. To generate dose response curves ranging from 0 (no growth inhibition) to 1 (complete growth inhibition), normalized OD₆₀₀ values were subtracted from 1. Each resulting dose-response curve (single drug or drug combination) was fit to a three-parameter Hill function, and the resulting Hill curve parameters were used to calculate inhibitory concentrations (ICs). Calculation of growth rate inhibition (GR) and drug interaction metrics are detailed in the supplemental material.

Phylogenetic tree construction

A core genome phylogenetic tree was constructed by downloading complete genomes for the relevant bacterial strains from NCBI. The Erdman ATCC 35,801 strain was used as the reference genome. Paired-end FASTQ files for each strain were aligned to the reference genome, and variants were identified for each strain using the “bcftools” package in Python. Core genome alignments were generated, and evolutionary relationships between each strain and the reference genome were summarized using “parsnp” and “harvesttools” packages. A maximum-likelihood phylogenetic tree was then constructed using the “IQ-TREE” package, which evaluates multiple nucleotide substitution models and selects the best-fitting model. Scripts were written in Python 3 and Bash, and the final phylogenetic tree (Fig. 1A) was visualized using the “tree” package in R.

Statistics and data reproducibility

All experiments included in this study were performed in three biological replicates excluding single drug dose response experiments in standard (Fig. 1), where six biological replicates were used instead. Single-drug dose-response curves in standard medium were measured in two independent experiments ($n = 3$ biological replicates each) and pooled to improve the precision and reproducibility of fitted single drug metrics (32, 33). For each strain and drug treatment, the median log₁₀IC₅₀ (for single drugs), log₂FIC₅₀ (for drug combinations), and GR_{max} (for drug combinations) value across three biological replicates (for drug combinations and single drugs in cholesterol) and six biological replicates (for single drugs in standard) were measured and represented in the figures.

ACKNOWLEDGMENTS

This study was funded by the National Institutes of Health (P01AI143575 [S.M.F.], 1F32AI174653 [P.H.C.], UM1AI179699 [B.B.A.]), the Wellcome Intermediate Fellowship (2026724/Z/17/Z [N.T.T.T.]), and the Gates Foundation (INV-027276 [B.B.A.]). The conclusions and opinions expressed in this work are those of the author(s) alone and shall not be attributed to the Gates Foundation. The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript.

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M.H.Y., P.H.C., S.M.F., and B.B.A. were responsible for conceptualization of the study. M.H.Y. and B.B.A. conducted the literature search, developed the figures, and performed formal analysis and writing of the original manuscript. M.H.Y., M.P.M., and H.N. were responsible for data curation. M.H.Y., P.H.C., S.M.F., and B.B.A. contributed to data interpretation. P.H.C., N.T.T.T., S.M.F., and B.B.A. were responsible for funding acquisition and supervision of the study. All authors had access to the study data and contributed to the review and editing of the manuscript.

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FUNDING

Funder	Grant(s)	Author(s)
National Institutes of Health	1F32AI174653	Peter H. Culviner
National Institutes of Health	P01AI143575	Sarah M. Fortune
National Institutes of Health	UM1AI179699	Bree B. Aldridge
Wellcome Trust	2026724/Z/17/Z	Nguyen Thuy Thuong Thuong
Bill and Melinda Gates Foundation	INV-027276	Bree B. Aldridge

DATA AVAILABILITY

Whole-genome sequences of all strains used in this study are available through the National Center for Biotechnology Information Sequence Read Archive (SRA) under the accession number [PRJNA950969](https://www.ncbi.nlm.nih.gov/sra/PRJNA950969). Accession numbers of individual strains are listed in the supplemental material (Table S4). All equations used to quantify drug response in this study are listed in Materials and Methods and the supplemental material. The *in vitro* data used in this study and the scripts required to generate the figures are available on Figshare (34–36).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental material (AAC01849-25-S0001.pdf). Supplemental methods; Tables S1 to S4; Fig. S1 to S6.

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