Low-cost anti-mycobacterial drug discovery using engineered *E. coli*

Nadine Bongaerts, Zainab Edoo, Ayan A. Abukar, Xiaohu Song, Sebastián Sosa-Carrillo, Sarah Haggenmueller, Juline Savigny, Sophie Gontier, Ariel B. Lindner & Edwin H. Wintermute

Whole-cell screening for *Mycobacterium tuberculosis* (*Mtb*) inhibitors is complicated by the pathogen’s slow growth and biocontainment requirements. Here we present a synthetic biology framework for assaying *Mtb* drug targets in engineered *E. coli*. We construct Target Essential Surrogate *E. coli* (TESEC) in which an essential metabolic enzyme is deleted and replaced with an *Mtb*-derived functional analog, linking bacterial growth to the activity of the target enzyme. High throughput screening of a TESEC model for *Mtb* alanine racemase (Alr) revealed benazepril as a targeted inhibitor, a result validated in whole-cell *Mtb*. In vitro biochemical assays indicated a noncompetitive mechanism unlike that of clinical Alr inhibitors. We establish the scalability of TESEC for drug discovery by characterizing TESEC strains for four additional targets.
Chemical genetics in the synthetic biology era offers new tools for long-standing challenges in antimicrobial drug discovery. With genetic modifications to targeted functional pathways, microbial strains can be sensitized to drugs of a particular mechanism. High-throughput screening of these strains may then reveal new drugs that act specifically on the modified pathway. Recent successful applications of chemical genetics have produced drug scaffolds and helped to identify drug mechanisms in a variety of microbial pathogens.

The chemical-genetic strategy seeks to combine the advantages of two classical approaches to drug discovery: whole-cell screens that test directly on live pathogenic bacterial and target-based screens that assay against purified cellular components. Whole-cell screening, it prioritizes molecules that can pass the membrane barrier and function in the rich intracellular context. Like target-based screening, it allows researchers to focus on specific biological functions of therapeutic interest and simplifies the process of determining a hit’s mechanism of action.

Unique technical challenges arise when using genetically engineered microbes for antimicrobial discovery. The relationship between a target’s activity and the host microbe’s sensitivity to a targeted inhibitor is quantitative and pathway-dependent. Empirical fine-tuning is required to maximize assay sensitivity while minimizing stress associated with gene over- or under-expression. Precise expression control is difficult to achieve in many pathogens, for which genetic tools are often limited. Finally, the conventional challenges of pathogen microbiology still apply. Long incubation times, noisy measurements, and expensive safety practices limit the scale at which these tools can be deployed.

Here we perform chemical-genetic screens for targeted inhibitors of pathogen-derived enzymes in an E. coli host. We develop TESEC strains in which an essential E. coli enzyme is deleted and replaced with a functionally equivalent target enzyme of heterologous origin. The target’s induction is quantitatively controlled to determine the highest and lowest expression levels compatible with robust growth. Target-specific inhibitors are expected to be more effective against TESEC strains under low induction, forming the basis for a differential screen.

As a proof of concept, we developed a TESEC strain for the enzyme Alr derived from the human pathogen Mtb. A screen of 1280 approved drugs revealed benazepril as an expression-dependent inhibitor. Whole-cell antibacterial activity of benazepril was confirmed in both Mycobacterium smegmatis and Mtb. Biochemical assays revealed a non-competitive mechanism of inhibition unlike that of known Alr inhibitor D-cycloserine (DCS).

Benazepril is an off-patent ACE inhibitor widely used to control hypertension. A retrospective study of the Taiwan national health insurance research database associated drugs of this class with a reduced risk of developing active tuberculosis. Our results suggest that this outcome may be the result of an antimycobacterial activity of benazepril.

The TESEC system is designed for maximum versatility and reuse with Golden Gate assembly. In principle, over 100 known conditionally essential E. coli metabolic genes could be complemented with pathogen-derived analogs and screened with TESEC. We characterize here four additional TESEC strains for diverse metabolic targets in Mtb. Low-cost and biosafe screening technologies may allow wider participation in antibiotic discovery efforts and unlock new economic models to incentivize work in this long-neglected domain.

Results

Design and characterization of a TESEC strain for Mtb Alr. Our interest in Mtb was motivated by the scope of the public health challenge it presents and by a decades-long gap in the emergence of new treatments. Alr is a well-known drug target required for cell wall biosynthesis in both Mtb and E. coli. The enzyme catalyzes the reversible conversion of L-alanine to D-alanine, supplying an essential building block for peptidoglycan cross-linking. DCS is a widely used Alr inhibitor that served as a positive control for our assays. Although an effective antibacterial, DCS is reserved as a second-line Mtb treatment due to serious neurological side effects. DCS shows no cross-resistance with first-line tuberculosis drugs, highlighting the value of targeting Alr by other means.

To produce the TESEC Host Alr- strain, endogenous E. coli alanine racemase activity was eliminated by deletion of the genes ddx and alr, conveying a growth requirement for supplemental D-alanine. To maximize host sensitivity to screened compounds, we further deleted the tolC efflux system and the entC enterobactin synthase, to rescue a growth defect associated with tolC deletion.

Heterologous expression of Mtb-derived Alr was achieved with an arabinose-inducible, feedback-controlled circuit adapted from Daniel et al. Briefly, the system consists of a low-copy plasmid expressing AraC and a high-copy plasmid expressing Mtb Alr. Flow cytometry using GFP-tagged Mtb Alr confirmed that the circuit allowed uniform and unimodal protein expression over a wide dynamic range.

Differential screening for Mtb Alr inhibitors. The TESEC Mtb expression strain was screened against the Prestwick Chemical Library, 1280 approved small molecules suitable for drug repurposing. The screen was conducted in triplicate for both low and high Alr induction levels, using defined media with compounds at 0.1 mM and 1% DMSO. Strain growth was measured by optical density (OD) at 600 nm after 10 h. High- and low-induction treatments with the positive control compound DCS produced a differential Z-factor of 0.87, indicating a robust assay.

Hits were determined to be drugs significantly inhibiting the growth of low Alr strains (OD < 0.1) but not high Alr (OD > 0.2). The statistical significance of hits was assessed by strictly standardized mean difference (SSMD) to DMSO-only controls.

Ten compounds met differential growth inhibition and significance criteria. Of these, 7 belong to the β-lactam class of known antibiotics. Because both β-lactams and Alr act in the pathway of peptidoglycan synthesis, under-expression of Alr may fragilize the cell wall and therefore sensitize cells to β-lactam treatment, an interaction observed in other microbes. The hit compounds benazepril and amlexanox had no previously reported antibacterial properties and so were retained for closer analysis.

We next repeated the screen for a range of Alr induction levels. The result is a detailed chemical-genetic sensitivity profile for each of the 1280 screened compounds. Globally, increased Alr expression was associated with reduced growth. DCS and benazepril, but not amlexanox, produced distinct effects.
chemical-genetic profiles with improved growth under high Alr induction (Fig. 2e). Therefore, benazepril was retained for further validation.

**Target abundance affects chemical-genetic sensitivity profiles.**

TESEC screening seeks to detect a phenotypic difference in drug sensitivity caused by changing the expression level of a drug’s protein target, a strategy common to many chemical-genetic assays. However, previous work has shown that perturbations in protein expression can also induce systemic stress responses caused by protein misfolding, resource depletion, or metabolic imbalance. This could result in the identification of non-specific hit compounds with differential activity against stressed cells but not against the desired target. We therefore sought to better characterize the effect of Alr expression levels on the reliability of chemical-genetic drug discovery.

We compared TESEC screening for a range of Alr expression levels to an Alr+ wild-type control strain with intact native Alr activity and carrying expression plasmids for GFP only (Fig. 3). At low induction levels, the TESEC strain grew nearly as well as the wild-type control and responded similarly to drug treatments (Fig. 3a). Increasing Alr induction resulted in decreased overall growth and decreased correlation between TESEC and wild-type chemical-genetic profiles.

The Prestwick library includes 183 known antibiotics, many of which were effective against E. coli under our screening conditions. Higher Alr induction levels resulted in less statistical power for distinguishing growing from non-growing cells, therefore making antibiotic activities harder to detect (Fig. 3b). Interestingly, the antibiotic activity of DCS itself was not detectable in the wild-type strain because the effective concentration of the drug exceeds the 0.1 mM level commonly used in high-throughput screening. Only low-induction Alr TESEC strains could correctly re-discover this known antibiotic.

Overexpression of Alr resulted in reduced screening sensitivity to known antibiotics (Fig. 3c). The whole-cell assay conducted on the Alr+ wild-type control strain identified 314 growth-inhibiting compounds of which 94 were described antibiotics, a true positive rate of 30%. Low-induction TESEC strains performed similarly while high-induction strains were nonspecifically inhibited by many compounds, resulting in false positive rates above 70%.

**Characterization of hits in TESEC.** The TESEC system provides an in-line platform to characterize and validate hit compounds before advancing them to biochemical or live-pathogen assays (Fig. 4). We produced two-dimensional chemical-genetic profiles of TESEC growth under a range of drug treatments and Alr expression levels (Fig. 4a–c). As expected, the inhibitory dose of DCS varied quantitatively with Alr level, producing a characteristic diagonal line in the growth heatmap. Benazepril produced a similar diagonal profile, consistent with a target-specific effect of benazepril, while amlexanox did not.

At higher resolution, benazepril and DCS produced qualitatively different chemical-genetic profiles. Treatment with DCS shifted the growth curve horizontally, altering primarily the Alr induction level required to produce resistance. Benazepril treatment shifted the growth curves vertically, reflecting similarly decreased growth across a range of Alr expression levels. Unlike DCS, the effect of benazepril treatment was only evident at low Alr induction. These results suggested different mechanisms of action for DCS and benazepril.

Supplementation of the growth medium with 5 mM D-alanine, the metabolic product of Alr, was able to rescue TESEC strain growth under treatment with both 0.25 mM DCS and 1 mM benazepril (Fig. 4d). This is consistent with a target-specific activity for the drugs.

Restoring activity of the efflux pump TolC eliminated benazepril sensitivity, indicating that the drug can be effluxed.
Fig. 2 A screen for targeted Mtb Alr inhibitors identifies benazepril. a The TESEC Mtb Alr expression strain was induced with arabinose at high (10^7 nM) or low (10^2 nM) levels and treated with the 1280-compound Prestwick library at 0.1 mM. Growth was measured by OD after 8 h and the median of three biological replicates is plotted for each drug. Hit compounds inhibit the growth of low-Alr strains but not high-Alr strains, occupying the upper-left quadrant. b SSMD scores were used to assess the statistical significance of the high- and low-induction growth levels. Hits were selected with an OD differential of 0.2 and an SSMD greater than 5. c Growth measurements for selected hits in high Alr (colored bars) and low Alr (gray bars) expression. Data are presented as the mean and individual data points for three biological replicates. d Chemical-genetic growth profiles of the TESEC Mtb Alr expression strain treated with each drug of the Prestwick library at 0.1 mM and a range of arabinose induction levels. e Selected chemical-genetic growth profiles for candidate hit compounds. Both DCS and benazepril showed growth inhibition only at low induction levels. Amlexanox, in contrast, did not show reproducible induction-specific activity. Data are presented as the mean and the standard deviation of three replicates. Source data are provided as a Source Data file.

Fig. 3 Chemical-genetic drug sensitivity is significantly altered by target overexpression. a The growth effects of 1280 drugs from the Prestwick library on the TESEC Mtb Alr expression strain induced with varying levels of arabinose and the TESEC Alr+ wild-type control. Points are colored to indicate point density and r^2 is the Pearson correlation coefficient. b A violin plot of SSMD values comparing triplicate measurements of growth inhibition under drug treatment with growth measured in DMSO-only controls. High induction levels were associated with lower growth signal and higher noise resulting in less robust differentiation between inhibiting and non-inhibiting compounds. c Sensitivity of the TESEC assay in detecting the 183 known antibiotics in the Prestwick library. Predicted antibiotics were compounds showing more than 50% growth inhibition relative to DMSO controls. High induction levels were associated with lower growth, weaker signal, and more false positives. Source data are provided as a Source Data file.
Whole-cell activity of benazepril in *M. smegmatis* and *M. tuberculosis*. We next sought to characterize the effect of benazepril as an anti-mycobacterial. Benazepril showed inhibitory activity against the non-pathogenic model mycobacterium *M. smegmatis* mc²155, producing an IC₅₀ of 1 mM (Fig. 4f). The IC₅₀ for DCS was 0.1 mM under the same conditions. Spot plating assays confirmed this activity to be bactericidal as well as growth inhibitory (Supplementary Fig. 2).

Within the human body benazepril undergoes hydrolysis to produce the active ACE inhibitor benazeprilat. This form did not show activity against either mycobacterial strain up to the limit of solubility, 0.5 mM. Supplementation with 5 mM D-alanine was able to rescue the growth of *M. smegmatis* in the presence of 0.25 mM DCS. The effect of 1 mM benazepril, in contrast, was not rescued (Fig. 4g). This may indicate that benazepril interacts with additional mycobacterial targets beyond Alr.

The H₃⁷Rv strain of *Mtb* was growth-inhibited by benazepril at concentrations between 2.25 and 4.5 mM, as compared to 125 μM for DCS (Table 1). As with *M. smegmatis*, D-alanine supplementation did not rescue this effect.

Benazepril is a noncompetitive inhibitor of Alr. The in vitro kinetics of benazepril inhibition were examined with a two-step biochemical reaction (Fig. 5). Purified Alr converts D-alanine to L-alanine which, in turn, is used by L-alanine dehydrogenase to reduce NAD⁺ to fluorescence-measurable NADH. Control assays confirmed that benazepril does not inhibit the L-alanine dehydrogenase reporter enzyme, making the assay suitable for characterizing the benazepril-Alr interaction (Supplementary Fig. 3).

The activity of known Mtb-inhibitor DCS was rescued by the addition of D-alanine, consistent with targeted inhibition of Alr. The first-line Mtb drug isoniazid served as a positive control. Observed values were consistent across five biological replicates.

**TESEC screening extends to other metabolic targets.** In developing TESEC, we sought to create a flexible chemical-genetic assay able to accommodate many potential targets. We tested the antibiotic activity of benazepril against *Mtb* *M. tuberculosis* H₃⁷Rv was cultured in 7H9 medium with the indicated treatment.

**Table 1** Antibiotic activity of benazepril against *Mtb*.

| Compound        | Dilution range | MIC     |
|-----------------|----------------|---------|
| DCS             | 0–2.0 mM       | 0.125 mM|
| DCS + 5 mM D-ala| 0–2.0 mM       | >2 mM   |
| Benazepril      | 0–4.5 mM       | 2.25–4.5 mM|
| Benazepril + 5 mM D-ala | 0–4.5 mM | 2.25 mM
| Benazeprilat    | 0–0.5 mM       | >0.5 mM |
| Isoniazid       | 0–30 μM        | 0.3 μM  |
| DMSO            | 0–5%           | >5%     |

Metabolic viability was measured with the resazurin reduction assay after 7–9 days. The activity of known Mtb-inhibitor DCS was rescued by the addition of D-alanine, consistent with targeted inhibition of Alr. The first-line Mtb drug isoniazid served as a positive control. Observed values were consistent across five biological replicates.
modularity of our system by constructing and screening additional TESEC strains (Fig. 6). We chose 4 enzymes required for amino acid biosynthesis and proposed as anti-mycobacterial drug targets: Asd, CysH, DapB, and TrpD.

For each target, we deleted the E. coli homolog and expressed the codon-optimized enzyme from Mtb in the standard TESEC vector. The resulting strains showed diverse dose-responses to target induction and unique induction optima (Fig. 6a–d). Following the strategy used for Alr, we selected high and low arabinose induction levels for differential screening.

We performed high-throughput screening of the Prestwick library in triplicate for all five targets (Fig. 6e–h) and selected hit compounds causing differential growth (OD\text{HIGH} − OD\text{LOW} > 0.1) at significant levels (SSMD > 5).

Hits were identified for Asd and DapB (Fig. 6i, h). All hit compounds were small and hydrophilic relative to the set of known antibacterials annotated in the Prestwick library (Fig. 6k). This may reflect the role of the E. coli membrane as a selective barrier to activity in the TESEC assay. No hits were identified in the CysH and TrpD strains, which showed poor growth at all induction levels and high variance during screening. These results highlight the importance of precise control and low-burden heterologous expression for robust chemical-genetic drug discovery.

As with benazepril, we validated the hit compounds by creating chemical-genetic growth profiles varying both drug concentration and arabinose induction. Four of the five hit compounds produced characteristically diagonal growth profiles, confirming a relationship between target abundance and drug sensitivity (Fig. 6l–p). This relationship was also evident in single arabinose dose-response curves for selected drug levels in the micromolar range: arabinose induction of the TESEC strain, but not a wild-type control, significantly increased resistance to the hit compound (Fig. 6q–u). A fifth hit, diethylstilbestrol, showed no interaction with target expression in follow-up assays.

Two of the four confirmed hits have previously shown activity against whole-cell mycobacteria in the micromolar range: pentamidine and bromhexine. Both drugs are administered through inhalation and show high bioavailability in the lungs. Deoxycorticosterone has been shown to reduce mycobacterial growth within human macrophages. For each of these compounds, our results suggest a previously unknown metabolic anti-microbial mechanism of action. Riluzole has no demonstrated anti-mycobacterial activity to our knowledge, although it contains a trifluoromethoxy group rare in medicinal chemistry and found in many recent anti-TB clinical candidates including delamanid, pretomanid and, telacebec.

Of the five hit compounds, only pentamidine showed activity against M. smegmatis in the micromolar range (Supplementary Fig. 4). In contrast bromhexine, deoxycorticosterone and riluzole showed reproducible activity in the TESEC strain but not against M. smegmatis. This discrepancy may be due, in part, to membrane efflux activities present in wild-type cells but deleted in TESEC strains.

To better characterize the role of membrane efflux in TESEC screening, we tested our hit compounds against E. coli with wild-type efflux activity, as well as strains bearing deletions in the tolC system and five additional efflux deletion strains (Supplementary Fig. 5). For four compounds (Benazepril, bromhexine, diethylstilbestrol riluzole), the deletion of specifically tolC was necessary to reveal drug sensitivity. In contrast, varying the efflux phenotype had only modest effect on responses to the other two drugs, pentamidine and deoxycorticosterone, suggesting that their signal in our differential screen was produced mainly by the engineered variations in target abundance. These results are consistent with the original design motivations for the TESEC platform, in which efflux-deficient E. coli were engineered to reveal target-specific interactions rather than whole-cell activities.

**Discussion**

This study demonstrates the use of TESEC screening to identify an anti-Mtb, anti-Alr activity of benazepril. The Prestwick drug library used here has been screened extensively for antibiotic activity, including in whole-cell assays against live Mtb, and targeted assays against purified Alr. The extraction of a lead from this well-explored resource suggests that TESEC screening passes compounds through a selective filter unlike that of other discovery technologies.

The activity of benazepril against live mycobacteria appeared to differ mechanistically from that in the TESEC assay. In the E. coli model, the inhibitory effect of benazepril could be rescued by supplementation with D-ala, the enzymatic product of Alr, consistent with an activity specific to that enzyme. In M. smegmatis and Mtb, D-ala supplementation did not rescue benazepril inhibition, suggesting benazepril may act on other targets as has been
shown for cycloserine. Benazepril is also likely to be effluxed, as indicated by its substantially higher MIC in mycobacteria (~2 mM) relative to the efflux-negative TESEC strain (~100 μM). This highlights a trade-off in TESEC similar to that in purified biochemical assays: bypassing the role of the membrane to reveal target-specific interactions in early-stage screening means the membrane may reappear as a point of failure at later stages. The notoriously impermeable mycobacterial envelope represents a challenge for TESEC screening as it does for other antimycobacterial discovery strategies. Target-based screens present a challenge for TESEC screening as it does for other antibacterial discovery strategies. Target-based screens present a challenge for TESEC screening as it does for other antibacterial discovery strategies.

Fig. 6 TESEC supports screening for diverse metabolic targets. a–d TESEC strains for the indicated metabolic targets grown on defined medium with the essential enzyme induced with a range of arabinose concentrations. The indicated low (L) and high (H) arabinose concentrations were selected for differential screening. Data are presented as the mean and CI95 of six biological replicates. e–h Differential screening of the Prestwick library was performed at both low and high arabinose concentrations in triplicate. Hits were selected as compounds with median differential OD measurements of 0.1 and SSMD scores >5. i–j Hit compounds identified for targets Asd, and DapB. No significant hits were identified for CysH or TrpD. Colored bars indicate mean growth under high induction, gray bars at low induction. Individual data points are shown for three biological replicates. k Molecular weight and hydrophobicity scores for identified hit compounds (Asd, blue; DapB, purple) and for all annotated antibacterials in the Prestwick library (beige). Hydrophobicity scores were estimated with the alvaMolecule software package. l–p Chemical-genetic validation profiles show TESEC grows as a function of both drug dose and TESEC target induction level. With the exception of diethylstilbestrol, hit compounds showed the characteristic diagonal pattern indicating that drug sensitivity depended on target expression level. q–u Individual dose–response curves extracted from the chemical-genetic profiles for a range of arabinose concentrations and the indicated drug dose. Except diethylstilbestrol, drug-treated TESEC strains showed improved growth at higher arabinose induction (green lines). Wild-type controls, in which target expression was not controlled by arabinose, did not show improved growth (gray lines). Data are presented as the mean and CI95 for three biological replicates. Source data are provided as a Source Data file.
includes a bacterial membrane but not a mycobacterial membrane. How might this substitution impact screening results? As with other Gram-negative bacteria, the E. coli outer membrane is coated with a stiff layer of lipopolysaccharides that excludes most large molecules. Small molecules must be sufficiently hydrophilic to pass through outer membrane porins, then sufficiently hydrophobic to cross the inner lipid bilayer by solubility-diffusion. This triple filtration means that most Gram-negative antibiotics occupy a narrow physico-chemical space.

Although mycobacteria are classified as Gram-positive, their envelope resembles that of Gram-negative strains in some respects, notably in the presence of an outer membrane bridged by porins. The mycobacterial envelope is exceptionally rich in lipids and includes a layer of waxy long-chain mycolic acids. These properties may explain why anti-Mtb actives tend to be smaller and more lipophilic than antibiotics in general.

Any compound identified by TESEC screening then confirmed for anti-Mtb activity will need to pass through both bacterial membranes. Benazepril’s small size (MW 424) compares to that of many known antibiotics. By hydrophobicity (logP 1.3), benazepril is at the high end for Gram-negative penetration and the low end for Mtb.

Once entering the cell a would-be antibiotic must contend with a battery of efflux pumps that are numerous and diverse in both E. coli and Mtb. We did not attempt to use E. coli as a model for efflux in Mtb. Instead, we eliminated major efflux activities by deleting tolC, making TESEC screening a mostly efflux-agnostic assay. This choice was motivated by the complexity and context-dependence of efflux in Mtb, which varies among natural isolates and during the course of an infection. For example, a study of 21 clinical isolates of Mtb found 10 strains with increased expression of at least one putative efflux pump relative to the H37Rv reference strain, with 6 different pumps showing natural variation. In this context, even a whole-cell screen with live Mtb cannot fully capture the clinical diversity of the Mtb barrier. The lack of a good laboratory model for Mtb efflux remains a bottleneck for antibiotic development at all stages.

Why perform a drug screen using an E. coli model rather than directly in the pathogen of interest? The TESEC approach offers low material costs, technical simplicity and base-level biosafety requirements. Synthetic biology tools developed for E. coli allow precise gene expression control, which our results indicate is important to minimize non-specific physiological stress. Efforts to make non-model microbes more genetically tractable may enable TESEC-like screening in other hosts. Alternately, genetic alterations of E. coli may allow it to more closely emulate the physiology or membrane structure of other microbes, leading to the convergence of the two approaches.

Beyond the scientific challenges, low-cost technologies like TESEC may help to alleviate the economic pressures that limit antibiotic development. Early-stage screening represents a significant fraction of total development costs due to high failure rates that require many screens to be launched for each advancing compound. While traditional pharma companies are increasingly unwilling to assume all of the financial risks emerging economic models propose to distribute the costs and risks among academic laboratories, small- and medium-sized enterprises, charitable organizations and national research institutes in high-burden countries. TESEC strains can be freely replicated and exchanged, supporting highly multilateral projects characteristic of open science and open drug discovery.

Methods

Construction of the TESEC host strain. The TESEC host was derived from BW25113. Deletions of the genes araC, tolC, entC, alr, ddx, dapB, ald, cysH, and trpD were introduced by phage transduction of kanamycin resistance cassettes from the Keio collection, with cassettes subsequently removed by transient expression of Flp recombinase. A complete list of strains used in this study is provided in Supplementary Table 1.

Construction of the TESEC expression system. The plasmids pRD123 and pRD131 were modified for compatibility with Golden Gate assembly. Type IIIS restriction sites were removed and a LacI cassette was introduced at the cloning site to eliminate blue/white screening.

Coding sequences for expressed proteins were ordered as synthetic DNA (Integrated DNA Technologies) and codon-optimized for expression in E. coli. The Mtb Alr sequence was obtained from the Mtb H37Rv genome as gene ID Rv3432c. Four additional Mtb target sequences of interest dapB (Rv2773c), ked (Rv5708c), cysH (Rv2392), trpD (Rv2192c) were taken from the same Mtb genome.

A complete list of plasmids created for this study is provided in Supplementary Table 2. Plasmid DNA sequences are available as a Supplementary Data file.

E. coli culture conditions. Conventional strain manipulations used standard Luria-Bertani (LB) media and 1.5% agar LB plates. Experiments were performed using defined medium using an M9 base: 11.26 g/L M9 Minimal Salts (BD 248510), 2 mM MgSO4, 0.1 mM CaCl2, and 4 g/L D-fructose as the primary carbon source. To support robust growth, the base media was supplemented with 1 g/L complete amino acids mix (USBiological D9516) and 30 mg/L vitamin B1.

Plasmids carrying the KanR and AmpR markers were maintained with 50 μg/mL kanamycin and 100 μg/mL ampicillin, respectively. Growth media for strains bearing the alr and ddx deletions were supplemented with 100 μg/mL D-alanine except when performing Alr-dependent growth assays. All incubations were performed at 37 °C with shaking. All OD measurements were collected at 600 nm.

Mycobacterial culture conditions. M. smegmatis mc2155 was grown at 37 °C in Tryptic Soy Broth (TSB, Sigma 22092) supplemented with 0.5% Tween 80 (Sigma P9480). M. tuberculosis H37Rv was grown at 37 °C in Middlebrook 7H9 broth (Sigma M0178) containing 0.2% glycerol and 10% OADC supplement (BD 211886).

Fluorescence measurements of GFP-tagged Mtb Alr. The expression of GFP-tagged Alr was measured in defined medium supplemented with D-alanine to eliminate the growth requirement of Alr expression. An arabinose dilution series was prepared beginning with 1 M arabinose and proceeded in 22 steps of two-fold dilution with a final well receiving no arabinose. A Tecan Freedom Evo liquid-handling robot was used to dispense 30 μL of each solution into a 384-well deep-well plate (Corning 3342).

The TESEC-GFP-tagged Mtb Alr strain was grown overnight in defined medium without arabinose. The following morning cells were resuspended in defined medium at an OD of 0.05. 30 μL of cell suspension was added to each well of the arabinose dilution series. Cells were incubated for one hour. GFP fluorescence was measured from a minimum of 10,000 cells using the FITC-A channel of a flow cytometer (BD LSRFortessa).

High-throughput screening. Drug screens were performed in two formats: 96-well and 384-well. The initial drug screen was performed in 96-well format using the TESEC Mtb Alr expression strain under high- and low-induction conditions. Subsequent screens were miniaturized to 384-well format and applied a wider range of induction conditions.

For 96-well format screens, the 1280 compound Prestwick Chemical Library was screened with each compound at 10 mM concentration in DMSO. 1.5 μL samples were aliquoted to microplates with clear flat bottoms (Greiner 655090) using a Tecan Freedom Evo liquid-handling robot. The leftmost and rightmost columns of each plate were reserved for DMSO-only control wells.

Cells were grown overnight in defined medium supplemented with D-alanine. The 96-well plates were washed three times by centrifugation and resuspension in phosphate buffered saline (PBS), then diluted to an OD of 0.05 in defined medium lacking D-alanine but supplemented with arabinose at 0.1 μM for low Alr induction or 10 μM for high Alr induction.

Screening plates were filled with the dilute cell suspension at a volume of 150 μL, producing final drug concentrations of 0.1 mM and 1% DMSO. One OD reading was collected prior to incubation to establish the background absorbance of drugs alone. Plates were sealed with aluminum foil and incubated for 10 h. Aluminum foil was removed prior to taking post-incubation OD measurements with a microplate reader (Tecan Infinite). 384-well format screens were performed with small-volume microplates (Greiner 784101). An acoustic liquid handler (Labcyte Echo 500) was used to dispense 150 nL droplets of library drugs at 10 mM in DMSO. Cells were washed as above and diluted into defined media at an OD of 0.05. The diluted cell suspension was dispensed to the screening plates at a 15 μL volume to produce a final drug concentration of 0.1 mM and 1% DMSO. Plates were sealed with aluminum foil and incubated with shaking for 10 h. Foil was removed prior to final OD measurements.

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-31570-3 | www.nature.com/naturecommunications
The reactions were started with the addition of 5 μL of D-alanine solution (0–20 mM) and followed by the evolution of fluorescence (340 nm excitation, 460 nm emission) using a TECAN Spark plate reader. Final DMSO concentrations were kept constant at 1% for all reactions.

Initial reaction rates were determined using the linear portion of the fluorescence evolution curves. Alr activity is reported as a percentage relative to the initial velocity in 20 mM D-alanine and no inhibitor. Best-fit kinetic parameters were determined by nonlinear regression in Python.

**Culture conditions for additional TESEC strains.** TESEC strains for trpD and cysH were cultured in the same defined M9-base medium used for TESEC Mtb Alr. To convey growth dependence on the Mb enzymes, the complete amino acid supplement was replaced with tryptophan dropout powder (USBiological D95360) for trpD or cysteine and methionine dropout powder (USBiological D9537–08) for cysH.

TESEC strains for asd and dapB were cultured in M9-base medium supplemented with 1 g/L D-fructose, 1 g/L cysteine and methionine dropout powder, and 20 μg/mL diaminopimelic acid (DAP, Sigma D1377). Screening was performed in LB medium supplemented with 1 g/L D-Fructose and 1 g/L complete amino acid supplement (USBiological D9516). DAP was omitted during growth curves and drug screening to convey growth dependence on enzyme expression.

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

**Data availability** Source data are provided with this paper. The data generated in this study have also been deposited in the Zenodo database (https://doi.org/10.5281/zenodo.6597306).

Received: 13 September 2021; Accepted: 23 June 2022; Published online: 07 July 2022.

**References**

1. Cacace, E., Kritikos, G. & Typas, A. Chemical genetics in drug discovery. *Curr. Opin. Syst. Biol.* 4, 35–42 (2017).
2. Krishnamurthy, M., Moore, R. T., Rajamani, S. & Panchal, R. G. Bacterial genome engineering and synthetic biology: Combating pathogens. *BMCMicrobiol.* 16, 258 (2016).
3. Johnson, E. O. et al. Large-scale chemical-genetics yields new *M. tuberculosis* inhibitor classes. *Nature* 571, 72–78 (2019).
4. Peters, J. M. et al. A comprehensive, CRISPR-based functional analysis of essential genes in bacteria. *Cell* 165, 1493–1506 (2016).
5. DeVito, J. A. et al. An array of target-specific screening strains for antibacterial discovery. *Nat. Biotechnol.* 20, 478–483 (2002).
6. Donald, R. G. K. et al. A staphylococcus aureus essential gene amplification reveals an abundance of promiscuous resistance determinants in Escherichia coli. *Proc. Natl Acad. Sci. USA* 108, 1484–1489 (2011).
7. Melief, E. et al. Construction of an overexpression library for *Mycobacterium tuberculosis*. *Biol. Methods Protoc.* 3, bp009 (2008).
8. Abrahams, G. L. et al. Pathway-selective sensitization of *Mycobacterium tuberculosis* for target-based whole-cell screening. *Chem. Biol.* 19, 844–854 (2012).
9. Franzblau, S. G. et al. Comprehensive analysis of methods used for the evaluation of compounds against *Mycobacterium tuberculosis*. *Tuberculosis* 92, 488–498 (2012).
10. Allison, L. E. & Dunnam, P. M. Bacterial Pathogenesis (eds DeLeo, F. R. & Otto, M.) 271–283 (Humana Press, 2008).
11. Palmer, A. C. & Kishony, R. Opposing effects of target overexpression reveal drug mechanisms. *Nat. Commun.* 5, 4296 (2014).
12. Moriya, H. Quantitative nature of overexpression experiments. *MBio* 26, e00393–e03939 (2015).
13. Chhotaray, C. et al. Advances in the development of molecular genetic tools for Mycobacterium tuberculosis. *J. Genet. Genomics* 45, 281–297 (2018).
14. Häscheid, T. & Grobusch, M. P. Biosafety and tuberculosis laboratories in Africa. *Lancet Infect. Dis.* 10, 582–583 (2010).
15. Ballou, J. A. & Goa, K. L. Benazepril. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in hypertension and congestive heart failure. *Drugs* 42, 511–539 (1991).
16. Wu, J.-Y. et al. Angiotensin-converting enzyme inhibitors and active tuberculosis: A population-based study. *Medicine* 95, e5379 (2016).
26. Cycloserine.

20. Årdal, C. et al. Antibiotic development—economic, regulatory and societal challenges. *Nat. Rev. Microbiol.* 18, 267–274 (2020).

29. Cho, H., Uehara, T. & Bernhardt, T. G. Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell 159*, 1300–1311 (2014).

28. Daniel, R., Rubens, J. R., Sarpeshkar, R. & Lu, T. K. Synthetic analog computation in living cells. *Nat. Methods* 13, 726–729 (2016).

23. Strych, U., Penland, R. L., Jimenez, M., Krause, K. L. & Benedik, M. J. Characterization of the alanine racemases from two mycobacteria. *FEBS Lett.* 516, 93–98 (2001).

27. Li, Y. et al. Cycloserine for treatment of multidrug-resistant tuberculosis: A retrospective cohort study in China. *IDR* 12, 721–731 (2019).

26. Gallagher, L. A. et al. Cell wall peptidoglycan in Mycobacterium tuberculosis: An Achilles’ heel for the TB-causing pathogen. *FEBS Microbiol. Lett.* 43, 548–575 (1999).

24. Wild, J., Hennig, J., Lobocha, M., Walczak, W. & Klepoutowski, T. Identification of the dnaX gene coding for the predominant isoform of alanine racemase in Escherichia coli K12. *Molec. Gen. Genet.* 198, 315–322 (1985).

25. Maitra, A. Cell Wall peptidoglycan in Mycobacterium tuberculosis: An Achilles’ heel for the TB-causing pathogen. *FEBS Microbiol. Lett.* 43, 100–101 https://doi.org/10.1016/S1472-9792(08)70411-0 (2008).

21. Engler, C., Kandzia, R. & Marillonnet, S. A one pot, one step, precision rational drug design approach. *Antimicrob. Agents Chemother.* 44, 45–50 (2000).

10. Gengo, F. M. & Brady, E. The pharmacokinetics of benazepril relative to other ACE inhibitors. *Clin. Cardiol.* 24, 31–35 (2001).

19. Lambert, M. P. & Neuhaus, F. C. Mechanism of D-cycloserine action: Aalanine racemase from *Escherichia coli* W. J. Biol. Chem. 110, 978–987 (1927).

18. Balasegaram, M. et al. An open source pharma roadmap. *Expert Opin. Drug Metab. Toxicol.* 12, 619–629 (2016).

17. Shaw, D. L. Is open science the future of drug development? *Yale J. Biol. Med.* 81, 43–48 (2008).

16. Tükenmez, H. et al. Corticosteroids protect infected cells against mycobacterial killing in vitro. *Biochem. Biophys. Res. Commun.* 511, 117–121 (2019).

15. Raman, K., Yeturu, K. & Chandra, N. targetTB: A target identification pipeline. *PLoS One* 6, e1002544 (2011).

14. Towse, A. et al. Time for a change in how new antibiotics are reimbursed: Development of an insurance framework for funding new antibiotics based on a policy of risk mitigation. *Health Policy 121*, 1025–1030 (2017).

13. Projan, S. J. Why is big Pharma getting out of antibiotic drug discovery? *Curr. Opin. Microbiol.* 6, 427–430 (2003).

12. Pai, M. Time for high-burden countries to lead the tuberculosis research agenda. *PLoS Med.* 15, e1002544 (2018).

11. Bhardwaj, A. et al. Open source drug discovery for tuberculosis drug development. *Future Med. Chem.* 9, 147–158 (2017).

10. Balasegaram, M. et al. An open source pharma roadmap. *PLoS Med.* 14, e1002276 (2017).

9. Shaw, D. L. Is open science the future of drug development? *Yale J. Biol. Med.* 90, 147–151 (2017).

8. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad. Sci. USA* 97, 6640–6645 (2000).

7. Baba, T. et al. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Mol. Syst. Biol.* 6, 726 (2010).

6. Towse, A. et al. Time for a change in how new antibiotics are reimbursed: Development of an insurance framework for funding new antibiotics based on a policy of risk mitigation. *Health Policy 121*, 1025–1030 (2017).

5. Projan, S. J. Why is big Pharma getting out of antibiotic drug discovery? *Curr. Opin. Microbiol.* 6, 427–430 (2003).

4. Pai, M. Time for high-burden countries to lead the tuberculosis research agenda. *PLoS Med.* 15, e1002544 (2018).

3. Bhardwaj, A. et al. Open source drug discovery for tuberculosis drug development. *Future Med. Chem.* 9, 147–158 (2017).

2. Aardal, C. et al. Antibiotic development—economic, regulatory and societal challenges. *Nat. Rev. Microbiol.* 18, 267–274 (2020).

1. Årdal, C. et al. Antibiotic development—economic, regulatory and societal challenges. *Nat. Rev. Microbiol.* 18, 267–274 (2020).
Author contributions
N.B., A.B.L., and E.H.W. conceived and planned the experiments. Z.E. performed the Mtb inhibition assays. A.A.A. and S.S.-C. contributed to strain development and high throughput screening for Alr. X.S. contributed to the data analysis. S.G., S.H., and J.S. developed and screened additional strains. N.B. performed the in vitro Alr biochemical assays. N.B., A.B.L., and E.H.W. developed the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-31570-3.

Correspondence and requests for materials should be addressed to Ariel B. Lindner or Edwin H. Wintermute.

Peer review information Nature Communications thanks Cesar de la Fuente and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2022