Selective Small Molecule Inhibitor of the \textit{Mycobacterium tuberculosis} Fumarate Hydratase Reveals an Allosteric Regulatory Site

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Enzymes in essential metabolic pathways are attractive targets for the treatment of bacterial diseases, but in many cases, the presence of homologous human enzymes makes them impractical candidates for drug development. Fumarate hydratase, an essential enzyme in the tricarboxylic acid cycle, has been identified as one such potential therapeutic target in tuberculosis. We report the discovery of the first small molecule inhibitor of the \textit{Mycobacterium tuberculosis} fumarate hydratase. A crystal structure at 2.0 Å resolution of the compound in complex with the protein establishes the existence of a novel allosteric regulatory site. This allosteric site allows for selective inhibition with respect to the homologous human enzyme. We observe a unique binding mode in which two inhibitor molecules interact within the allosteric site, driving significant conformational changes that preclude simultaneous substrate and inhibitor binding. Our results demonstrate the selective inhibition of a highly conserved metabolic enzyme that contains identical active site residues in both the host and the pathogen.

I. INTRODUCTION

Because the TCA cycle connects many pathways of cellular metabolism, preventing the function of this cycle through enzyme inhibition is an attractive strategy for targeting infectious agents\(^1\). In \textit{Mycobacterium tuberculosis}, experimental evidence\(^2\) has suggested that fumarate hydratase, the essential enzyme responsible for the reversible conversion of fumarate to (L)-malate, is a vulnerable target. This vulnerability is in part due to the fact that, unlike other bacteria such as \textit{Escherichia coli}, \textit{M. tuberculosis} expresses only one enzyme that performs this function\(^3\). In addition to its role in metabolism under aerobic conditions, fumarate hydratase has also garnered interest because of the discovery of a flux toward the reverse TCA cycle under hypoxic conditions in non-replicating \textit{M. tuberculosis}\(^2,4,5\). Yet, in spite of these discoveries, no small molecule inhibitor of the \textit{M. tuberculosis} fumarate hydratase has been reported. The discovery of such an inhibitor would provide an important tool to begin probing the role of the TCA cycle, in both actively replicating and non-replicating bacteria.

From the standpoint of drug development, however, targeting the \textit{M. tuberculosis} fumarate hydratase poses a significant challenge because the protein is highly evolutionarily conserved. In particular, the human and \textit{M. tuberculosis} homologs share identical active site residues, as well as 53% overall sequence identity\(^6,7\). Both homologs form a stable homotetramer containing four active sites, and every active site is composed of residues from three enzyme subunits. Each dumbbell-shaped subunit within the tetramer contains three domains: an N-terminal domain, a central domain, and a C-terminal domain\(^8-10\). The N-terminal and C-terminal domains are predominantly α-helical, and are linked by the central domain that consists of five tightly-packed helices. The central domains of the four subunits pack together into a 20-helix bundle to form the tetrameric structure. Each subunit organizes in a head-to-head fashion with one subunit and a head-to-tail fashion with the remaining two subunits. These structural similarities further increase the challenge of selective inhibition.

Here we report the discovery of the first selective small molecule inhibitor of the \textit{M. tuberculosis} fumarate hydratase. The selectivity results from the binding of the inhibitor to a novel allosteric site composed of residues that are not conserved between the human and the \textit{M. tuberculosis} homologs. Using X-ray crystallography and steady-state kinetics, we define the location of this new site that confers selectivity of the inhibitor’s activity over the homologous human enzyme. Our findings illustrate the potential to target vulnerable metabolic enzymes effectively and selectively in spite of their high degree of evolutionary conservation.

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\textbf{SIGNIFICANCE STATEMENT}

The tricarboxylic acid (TCA) cycle plays a central role in the metabolism of many pathogens, but few inhibitors of this pathway currently exist. Our study describes the first small molecule inhibitor of the fumarate hydratase enzyme in the TCA cycle of \textit{Mycobacterium tuberculosis}. We also report the discovery of an allosteric regulatory site that confers selectivity of the inhibitor’s activity over the homologous human enzyme. Our findings illustrate the potential to target vulnerable metabolic enzymes effectively and selectively in spite of their high degree of evolutionary conservation.
binding pocket and assess the effect of the inhibitor on both enzyme structure and function. These results illustrate the potential for fumarate hydratase to be a tractable target for drug development against *M. tuberculosis*.

II. RESULTS

Fluorescence-based Assays to Monitor Fumarate Hydratase Enzyme Activity

In order to identify inhibitors of the *M. tuberculosis* fumarate hydratase, we developed a fluorescence-based assay to monitor the enzyme's activity. Inspired by others who have utilized enzyme kinetics to investigate the TCA cycle\(^{11}\), we modeled the assay after the natural progression of the cycle under aerobic conditions. In this assay design, fumarate hydratase requires coupling to a minimum of two enzymes. The first coupled enzyme, malate dehydrogenase (MDH), generates the fluorescent molecule NADH upon oxidizing (L)-malate to oxaloacetate; however, the equilibrium of this reaction favors (L)-malate. Therefore, a second coupled enzyme is required to obtain a reliable readout. We chose to include the enzyme diaphorase, which consumes the NADH from the MDH reaction and regenerates NAD\(^+\). The use of diaphorase and its substrate resorufin (7-hydroxy-3H-phenoxazin-3-one) as a product. Thus, we were able to monitor resorufin fluorescence at 598 nm as a means of measuring fumarate hydratase activity. The detection at a wavelength of 598 nm is preferable to commonly used shorter wavelengths such as 340 nm, where the intrinsic fluorescence of compounds in small molecule libraries results in assay interference\(^{12}\). Finally, we also incorporated the enzyme citrate synthase into the assay design, as the thermodynamically favorable cleavage of the thioester bond of acetyl-coenzyme A in this reaction significantly speeds the assay progression by shifting the equilibrium state toward the products, thereby making a high-throughput screen feasible (Fig. 1a).

A titration of the fumarate hydratase enzyme in our final assay conditions showed proportional changes in the initial rate of the fluorescence output, indicating that we were accurately monitoring the enzyme's activity (SI Appendix, Fig. S1). We further tested the reliability of the assay by measuring the initial reaction rate at varying substrate concentrations. The resulting, highly reproducible saturation curve was fit using the Michaelis-Menten model\(^{13,14}\), yielding a $K_m$ of 260 +/- 20 $\mu$M for fumarate (Fig. 1b). To evaluate enzyme activity in the presence of small molecules, we elected to measure fluorescence immediately upon initiation of the reaction and again after five minutes. Five minutes was found to be a suitable timepoint, as the reaction displayed a wide signal window of 1104 +/- 13 relative fluorescence units (RFU) between positive and negative (no fumarate hydratase enzyme) controls with a $Z^'$ factor\(^{15}\) of 0.71.

Identification of Fumarate Hydratase Inhibitors using High-throughput Screening

We conducted a screen of 479,984 small molecules in a 1536-well format\(^{16,17}\). The compound library contains a diverse set of molecules with drug-like physico-chemical properties, and also includes compounds with known biological activity\(^{18}\). Each compound was screened at four concentrations, ranging from 115 $\mu$M to 2.3 $\mu$M. We selected a group of 1,856 compounds that showed potential activity as defined by a dose-response curve class\(^{17}\) of either 1 or 2, and evaluated their reproducibility by re-screening them in the assay at eleven concentrations, ranging from 115 $\mu$M to 2.0 nM. This group of compounds was also subjected to rigorous counter-screening to eliminate those molecules affecting resorufin production through mechanisms other than fumarate hydratase inhibition. Compounds affecting MDH, citrate synthase, and diaphorase activity were identified and discarded using truncated versions of the primary assay (SI Appendix, Fig. S2). In addition to eliminating inhibitors of the coupled enzymes, the counterassays allowed for removal of molecules that affected fluorescence due to inherent properties of the compound or due to nonspecific effects in the assay conditions.

We identified two structurally similar candidate molecules that bear no overt resemblance to fumarate and (L)-malate (SI Appendix, Fig. S3). This observation prompted us to examine the more potent of the two compounds (N-(5-(azepan-1-ylsulfonyl)-2-methoxyphenyl)-2-(4-oxo-3,4-dihydrophthalazin-1-yl)acetamide) (7) in detail (Fig. 1c). The compound was resynthesized to confirm its identity and purity (SI Appendix, Fig. S4). Subsequent revalidation of 7 revealed a highly reproducible dose-response curve with an IC\(_{50}\) of 2.5 +/- 1.1 $\mu$M, a maximum inhibition of 97 +/- 3%, and notably, a Hill slope of 2.4 +/- 0.5 (Fig. 1d). This Hill slope suggests that a minimum of two inhibitor molecules must be functionally important for the inhibition of catalysis at a given active site\(^{19}\).

Validation of Binding using X-ray Crystallography

To validate the binding of 7 to fumarate hydratase, we performed X-ray crystallographic studies of the enzyme. We crystallized the protein in the absence of 7 in a solution containing 200 mM magnesium formate and determined the structure at 1.86 Å resolution (Table S1; PDB ID: 5F92). Formate ions from the solution are bound at two of the enzyme active sites in a fashion that mimics substrate binding, with the formate carboxylate ions making identical hydrogen bonding interactions as the carboxylates of (L)-malate described in a previ-
Fumarate (a) Schematic of the primary fluorescence-based high-throughput screening assay used to monitor fumarate hydratase activity. A high-throughput screen identifies inhibitors of the Mycobacterium tuberculosis fumarate hydratase. FIG. 1. (a) The reaction scheme, and (L)-malate (2), oxaloacetate (3), and citrate (4) are produced. The assay is monitored by measuring the conversion of resorufin (5) to resorufin (6). (b) The saturation curve, fit using the Michaelis-Menten model, for the fumarate hydratase enzyme, as measured by the assay shown in (a). Data are reported as an average of replicates (n = 3) and error bars indicate s.e.m. (c) The structure and dose-response profiles of the most potent compound (7) from the screen. Inhibition is observed in both the primary assay (black) and in a variation of the assay in which citrate synthase is removed (red). (d) The dose-response curve obtained for 7 (black, n = 3). The compound shows no inhibitory effect on the human fumarate hydratase (red, n = 2). Data are reported as an average of replicates and error bars indicate s.e.m.

One of the key features of the two bound inhibitor molecules is an unusual π-stacking interaction between their two core phenyl rings within the allosteric site (Fig. 2d). This interaction between the two molecules of 7 appears to be an important contributor to binding. Furthermore, each molecule of 7 makes several key interactions with surrounding amino acids. Each inhibitor molecule interacts with the nearest Arg432 (of either subunit A or C), with the residue forming a stacking interaction with the inhibitor’s quinazolinone ring and a hydrogen bond to its amide oxygen atom. In addition, the backbone oxygen atom of each Leu429 hydrogen bonds to the nitrogen atom of the 2-position of the nearest inhibitor molecule’s quinazolinone ring (Fig. 2e; SI Appendix Fig. S9). A stereoimage of the two molecules of 7 in their electron density, with a depiction of additional interactions with the surrounding amino acids and solvent molecules, can be found in the SI Appendix, Fig. S10.
FIG. 2. A crystal structure of the M. tuberculosis fumarate hydratase at 2.0 Å resolution reveals the existence of a novel allosteric site. (a) A ribbon/surface representation of the overall structure of the homotetrameric M. tuberculosis fumarate hydratase with 7 bound to an allosteric site. The dashed circles indicate the location of the four active sites, and the solid circles indicate the location of the two allosteric sites. Colors represent the subunits of the tetramer (green: subunit A, blue: subunit B, purple: subunit C, red: subunit D). (b) Fourier difference density (green mesh, contoured at 3σ) calculated before 7 was added to the model. (c) Superimposition of the fumarate hydratase enzyme bound with formate (gray) or 7 (colored). An arrow indicates the conformational change in the C-terminal domain of subunit C upon binding of 7. The solid circle indicates the location of the bound inhibitor at the allosteric site. (d) The stabilizing stacking interaction (indicated by a dashed line) of the central phenyl rings of 7 within the allosteric site. (e) Snapshot of several key interactions between 7 and surrounding amino acid residues. A hydrogen bond between the backbone oxygen atom of Leu429 and the 2-position of the quinazolinone ring of 7 is shown with a dashed line. Arg432 hydrogen bonds to the amide oxygen atom of 7, and also forms a stacking interaction with the quinazolinone ring.

**Examination of the Mode of Inhibition**

The loss of the two formate ions from the active site adjacent to 7 suggests that the binding of the two inhibitor molecules to the allosteric site competitively precludes substrate binding in the neighboring active sites. To investigate this further, we assessed the nature of the protein’s conformational change by examining the subunit that exhibits the greatest movement upon inhibitor binding (subunit C). It has been previously reported that the C-terminal domain of each subunit of the M. tuberculosis fumarate hydratase enzyme possesses both an ‘open’ and a ‘closed’ conformational state. The ‘closed’ conformational state is induced upon substrate binding at the nearest active site, and involves a 34° in-
ward rotation of the C-terminal domain from the ‘open’ unbound form. In the formate-bound structure, subunit C aligns with the ‘closed’ conformation of a subunit with (L)-malate bound to the nearest active site (subunit rmsd = 0.8 Å, PDB ID: 4ADL), because the formate ions mimic the binding mode of the carboxylates of (L)-malate (SI Appendix, Fig. S11a). Upon binding of 7, however, the C-terminal domain rotates outward to align with the substrate-free enzyme in the ‘open’ conformation (subunit rmsd = 1.3 Å, PDB ID: 3NO9) (SI Appendix, Fig. S11b). This movement suggests that inhibitor binding stabilizes the subunit’s C-terminal domain in an ‘open’-like form, thereby preventing substrate binding and catalysis in a competitive fashion (Fig. 3a). Notably, the C-terminal domain of subunit A, which also contributes to the allosteric site, remains in the ‘open’ conformation in both the formate-bound and the 7-bound structures because its adjacent active site is always unoccupied.

The effect of the inhibitor on the residues of the allosteric site and the adjacent active site is illustrated in Fig. 3b. Upon binding of 7, Leu429C and Arg432C shift to form interactions with the inhibitor within the allosteric site (Fig. 3b, top inset). In the adjacent active site, the electron density of the two formate ions is lost. Notably, the conformational change affects active site assembly by increasing the flexibility of the loop residues 316A-323A, such that these residues can no longer be modeled in the 7-bound structure (SI Appendix, Fig. S12). The loss of the electron density of this loop is significant because two of the residues, Ser318A and Ser319A, form critical hydrogen bonds with formate ions in the formate-bound structure. This change in the active site is likely an effect of the concerted motion of the C-terminal domain of subunit C induced by the binding of 7. The movement of the C-terminal domain of subunit C from the ‘closed’ conformation to the ‘open’ conformation eliminates the stabilizing interaction between Asn413C and the backbone oxygen atom of the loop residue Ile320A (SI Appendix, Fig. S13). The loss of this interaction likely contributes to the increased flexibility of the critical loop residues 316A-323A at the active site. Additional active site residues are also affected in the inhibitor-bound structure. Lys324A and Ser139D, which both form hydrogen bonds with the formate ions in the formate-bound structure, are displaced in the 7-bound structure. As a result of the movement, the γ-oxygen of Ser139D occupies the former location of a formate ion (Fig. 3b, bottom inset). Together, these changes in the active site upon binding of 7 suggest that simultaneous binding of inhibitor and substrate cannot occur and that 7 is a competitive inhibitor of enzyme activity.

In addition to the structural evidence, we also assessed the mechanism of inhibition of 7 in solution using steady-state kinetic experiments. We generated a matrix of reaction progress curves for fumarate hydratase in the presence of varying concentrations of substrate and 7, and then plotted the enzymatic saturation curves at each inhibitor concentration. Given that substrate cooperativity was not observed at the fumarate concentrations used in the assay, the kinetic data was analyzed with respect to an independent catalytic site. Furthermore, because the crystal structure suggests that the binding of two inhibitor molecules disrupts active site assembly, the model for determining mode of inhibition was modified to incorporate the equilibrium binding of two inhibitor molecules (Fig. 3c). The rate equation derived from this model represents general mixed inhibition (see equation (1) in Materials and Methods). [All standard models of inhibition (competitive, non-competitive, uncompetitive, mixed) are incorporated within this model, and upon fitting of a data set, the model may reduce to a specific case depending on the resultant K_i values.]

We fit the rate equation for the model shown in Fig. 3c to our kinetic data (Fig. 3d), and the resulting K_i values support a competitive mode of inhibition. The values for K_i1 and K_i2, which represent the affinity of the inhibitor for the enzyme without substrate bound, were found to be 6.3 +/- 4.4 µM and 3.2 +/- 0.3 µM, respectively. By contrast, K_i3 and K_i4, which represent the affinity of the inhibitor for the enzyme-substrate complex, were found to contribute negligibly to the fit ((1/K_i3) < 10^-14 and (1/K_i4) < 10^-16). This suggests that the binding of the inhibitor to the enzyme alone results in inhibition in a purely competitive fashion. The values of apparent V_max and apparent K_m for the fits shown in Fig. 3d have been calculated for each inhibitor concentration and are presented in Table S2 (SI Appendix). Consistent with competitive inhibition, apparent V_max does not change as inhibitor concentration increases, while the apparent K_m is observed to increase.

Allosteric Site Binding Confers Selectivity

Following the identification of the allosteric site, we aligned the amino acid sequences of the M. tuberculosis and the human fumarate hydratase enzymes (SI Appendix, Fig. S14a). The alignment reveals that, despite a 53% sequence identity and identical active site residues, the allosteric site residues making direct interactions with the inhibitor molecules are not conserved in the human homolog (SI Appendix, Fig. S14b). The difference in these amino acid residues suggests the potential for selectivity of 7 over the human enzyme.

In order to investigate this hypothesis, we modified the enzymatic assay shown in Fig. 1a to report on human fumarate hydratase activity (SI Appendix, Fig. S14c-d). We then treated the human enzyme with 7 at concentrations up to 122 µM. We observed no inhibition of the human enzyme, indicating that 7 is highly selective (SI Appendix, Fig. S14e). The striking difference in the effect of 7 on the activity of the two enzymes is illustrated in Fig. 1d.
FIG. 3. Structural data and enzyme kinetics support a competitive mode of inhibition. (a) Cartoon representation of fumarate hydratase in the presence of substrate and the allosteric inhibitor, 7. In the absence of substrate or substrate mimic, the C-terminal domain of subunit C adopts the ‘open’ conformation (left). When (L)-malate or formate is bound to the active site, the C-terminal domain of subunit C adopts the ‘closed’ conformation (center). In the presence of the allosteric inhibitor, subunit C shifts to the ‘open’ conformation (right). The electron density for the formate ions is lost, suggesting that substrate and inhibitor cannot be simultaneously bound. (b) A depiction of the fumarate hydratase active site and allosteric site when formate is bound (gray structure, formate in yellow) or 7 is bound (colored structure). Upon binding of 7, the C-terminal domain of subunit C (purple) rotates into the ‘open’ conformation. At the allosteric site, Leu429C and Arg432C shift toward the inhibitor (top inset). At the active site, the binding of 7 results in the loss of electron density of the loop residues 316A-323A, as well as the movement of active site residues Lys324A and Ser139D (bottom inset). These active site residues form critical hydrogen bonds (dashed lines) to the substrate-mimicking formate ions in the formate-bound structure. (c) The equilibrium binding model that accounts for two molecules of inhibitor (I) binding to the enzyme (E) or enzyme-substrate complex (ES) in the presence of substrate (S) and product (P). (d) The saturation curves of the fumarate hydratase enzyme in the presence of different concentrations of 7, fit using the model shown in (c). Data are reported as an average of replicates (n = 2) and error bars indicate s.e.m.
Control cultures were treated with DMSO (black column), and GFP was measured 12 days after dosing. Cultures were grown under aerobic conditions results in a decrease in bacterial growth rate under aerobic conditions. Treatment of *M. tuberculosis* H37Rv with the fumarate hydratase inhibitor 7 under aerobic conditions resulted in a dose-dependent effect on bacterial growth rate (Fig. 4). Twelve days after treatment with 7 at a concentration of 250 µM, bacterial cells exhibited 35 +/- 6% growth relative to a vehicle-treated control culture. The growth rate could not be monitored in a similar fashion under anaerobic conditions as these cells are non-replicating.

**Effect of the Inhibitor on M. tuberculosis H37Rv**

In order to evaluate the potential of 7 in a biological context, we examined the effect of our hit compound on the survival and growth of *M. tuberculosis* H37Rv. When evaluating survival via detection of colony-forming units, we did not observe any bactericidal effect when cells were treated with 7 under either aerobic or anaerobic conditions (SI Appendix, Fig. S15 and SI Methods). However, treatment of H37Rv with 7 under aerobic conditions resulted in a dose-dependent effect on bacterial growth rate (Fig. 4). Cells constitutively expressing green fluorescent protein (GFP) were used to monitor the growth rate of the bacteria. Twelve days after treatment with 7 at a concentration of 250 µM, bacterial cells exhibited 35 +/- 6% growth relative to a vehicle-treated control culture. The growth rate could not be monitored in a similar fashion under anaerobic conditions as these cells are non-replicating.

While we were not able to measure a dose-dependent change in bacterial growth rate under aerobic conditions in the presence of 7, the magnitude of this effect is small. The observed effect could be limited by the hit compound's potency and cell permeability, or the effect could be the result of an off-target mechanism within the cell. To investigate these possibilities, a medicinal chemistry campaign to optimize the aforementioned properties of the hit compound will be required.

**III. DISCUSSION**

Although the properties of fumarate hydratase enzymes have been studied extensively since the 1950s, reports of targeted small molecule modulators of their activity have been scarce. Pyromellitic acid (benzene-1,2,4,5-tetracarboxylic acid) has been previously reported as a weak inhibitor of some fumarate hydratase homologs, but it shows no inhibitory activity with respect to the *M. tuberculosis* enzyme. We have identified the first effective and selective small molecule inhibitor of the *M. tuberculosis* fumarate hydratase. The structural features of our hit compound immediately suggest a potential allosteric effect. The compound lacks acidic functional groups to mimic substrate binding at the active site, and its molecular weight is more than triple that of the enzyme’s natural substrates. A crystal structure of our hit bound to fumarate hydratase has identified the location of an allosteric site as well as provided a rationale for the observed selectivity over the homologous human enzyme.

In addition to the location of an allosteric site, the crystal structure has also revealed an unusual compound binding mode, with two molecules bound at the interface of two protein subunits. While this structural information provides a guide for the rational design of additional inhibitors, modifications to the compound structure that preserve potency may be challenging to identify. Compound analogs must not only preserve interactions with the enzyme but also maintain the stacking interactions between the two inhibitor molecules. One potential design strategy would be to explore an array of linkers to join the two inhibitor molecules. Importantly, design strategies should take advantage of the knowledge that the allosteric inhibitor appears to prevent catalysis by locking the enzyme subunits in an ‘open’ conformation, in which the substrate cannot bind. With this in mind, any small molecule or peptidomimetic that stabilizes the open conformation of the enzyme could potentially be an effective inhibitor.

The discovery of an allosteric regulatory site raises the possibility that a natural biomolecule binds to fumarate hydratase at this location in vivo. Although we were unable to identify such a biomolecule, it is certainly possible that the allosteric site serves a yet unknown biological function. This function could be limited to simple regulation of enzymatic activity, or it could involve a second, unrelated process. Dual functions of several metabolic enzymes have recently been reported, and, in particular, the human fumarate hydratase has been implicated in the DNA damage response. The existence of these dual functions of metabolic enzymes suggests that potential biological functions of this novel allosteric site deserve further exploration.

While many inhibitors exist for *M. tuberculosis* enzymes in metabolic pathways ranging from glycolysis to sulfur metabolism, there are, to our knowledge, no reported inhibitors that target the TCA cycle in this or-
ganism. This dearth of inhibitors is likely due to both the redundancy as well as the evolutionary conservation of many enzymes in the pathway. Our work, however, exposes a vulnerability in this pathway in the form of an allosteric site that could be exploited to target these bacteria. When treating H37Rv cells with the allosteric inhibitor under aerobic conditions, we observe a decrease in bacterial growth rate. Although we cannot rule out the possibility that this result is due to an off-target mechanism, the presence of a dose-dependent effect on growth in a biological context supports further investigation to optimize the potency, solubility, and cell permeability of this molecule for greater biological activity. This optimization would facilitate studies to verify the mechanism of the compound’s biological effect and to assess the role of fumarate hydratase in various environments. In identifying the first small molecule inhibitor of the \( M. \) \( t \)uberculosis fumarate hydratase and characterizing its binding to a novel allosteric site, we illustrate the feasibility of selectively inhibiting this highly conserved metabolic enzyme, while providing a starting point for the development of these more potent probes.

IV. MATERIALS AND METHODS

High-throughput Screen and Counterscreens

The \( M. \) \( t \)uberculosis fumarate hydratase was expressed and purified as described in the SI Appendix. The high-throughput screens were run in 1,536-well plates with a final well volume of 4 \( \mu \)L. All measurements were performed in 50 mM Tris pH 8, 5 mM MgCl\(_2\), 0.01% Brij 35. Final concentrations of reagents were as follows: 10 nM \( M. \) \( t \)uberculosis fumarate hydratase, 10 units/mL MDH, 1 unit/mL citrate synthase, 150 \( \mu \)M NAD\(^+\), 200 \( \mu \)M acetyl-CoA, 0.05 mM resazurin, and 400 \( \mu \)M fumaric acid. All reagents with the exception of the purified fumarate hydratase were purchased from Sigma-Aldrich. Detailed protocols for the high-throughput screen and counterscreens can be found in the SI Appendix.

Synthesis and Compound Characterization of 7

The synthetic scheme for the preparation of 7 (Fig. S4) and the detailed compound characterization (SI Methods) can be found in the SI Appendix.

Crystallization, Data Processing, and Structure Determination

For the formate-bound structure, crystals were grown by sitting drop vapor diffusion at 20\(^\circ\)C in 17.0% [w/v] PEG3350, 5% [v/v] DMSO, and 200 mM magnesium formate, by combining 2 \( \mu \)L of a 14 mg/mL protein solution (10 mM Tris pH 8.0, 150 mM NaCl, and 0.5 mM TCEP) with 1 \( \mu \)L of reservoir.

The crystals with 7 bound were obtained via soaking of the formate-bound crystals. Crystals (reservoir: 17.0% [w/v] PEG3350, 5% [v/v] DMSO, and 200 mM magnesium formate) were soaked for 16 hours by adding 2 \( \mu \)L containing a saturated solution of 7 in 19% PEG3350 [v/w], 7.5% [v/v] DMSO, and 200 mM magnesium formate to the sitting drop. Prior to data collection, crystals were cryo-cooled in liquid nitrogen. Detailed methods on crystallization trials, data processing, and structure determination can be found in the SI Appendix. Coordinates and structure factors for the formate-bound structure and the complex with 7 have been deposited in the Protein Data Bank under accession numbers 5F92 and 5F91, respectively.

Mode of Inhibition Steady-state Kinetic Assay

The mode of inhibition assay was run in 1,536-well plates under the same conditions as the primary high-throughput screen described above, with the following modifications: (1) a new batch of enzyme was purified for this study, resulting in the fumarate hydratase concentration being lowered to 1.25 nM and (2) to prevent pH changes at high substrate concentrations, sodium fumarate dibasic was used in place of fumaric acid to initiate the reaction. The inhibitor was tested in a seven-point dilution ranging from 28.6 \( \mu \)M to 447 nM. Nine concentrations of sodium fumarate dibasic, ranging from 0 mM to 6.4 mM were tested. Each plate was read in kinetic mode on a ViewLux High-throughput CCD imager (Perkin-Elmer) equipped with standard UV fluorescence optics (525 nm excitation, 598 nm emission) for 10 minutes. The change in fluorescence intensity over the 10-minute reaction period was normalized against no-substrate controls and the resulting initial reaction rates were plotted. The enzymatic saturation curves were fit using the following rate equation derived using the steady-state approximation from the model of general mixed inhibition pictured in Fig. 3c:

\[
v_0 = \frac{V_{\text{max}}[S]}{K_m \left(1 + \frac{[I]}{K_i1} + \frac{[I]^2}{K_i2}\right) + [S]\left(1 + \frac{[I]}{K_i3} + \frac{[I]^2}{K_i4}\right)},
\]

Human Fumarate Hydratase Selectivity

The selectivity assay was conducted in 384-well black Greiner plates with a final well volume of 40 \( \mu \)L. All measurements were performed in 50 mM Tris pH 8, 5 mM MgCl\(_2\), 0.01% Brij 35. Final concentrations of reagents were as follows: 10 nM human fumarate hydratase (Sigma-Aldrich), 10 units/mL MDH, 1 unit/mL citrate synthase, 150 \( \mu \)M NAD\(^+\), 200 \( \mu \)M acetyl-CoA, 0.05 mg/mL diaphorase, 0.05 mM resazurin,
and 400 μM fumaric acid. A detailed assay protocol can be found in the SI Appendix.

Growth Rate of H37Rv under Aerobic Conditions

An M. tuberculosis strain H37Rv containing a plasmid with the gfp gene encoding GFP under control of the constitutive msrp12 promoter of M. marinum was used to monitor growth. As the plasmid contains a selectable kanamycin marker, cultures were maintained in 25 μg/mL kanamycin in 7H9 medium (Difco) supplemented with 4 g/L glucose and 0.3 g/L casitone (Difco). Experiments were conducted in black, clear-bottom 96-well plates (Greiner). Early log phase cultures were diluted 125-fold, and 50 μL was plated into each well. 50 μL of a 2x compound solution was added to the cells. Compound 7 was dosed at concentrations ranging from 16 μM to 250 μM. Each plate contained an isoniazid control, dosed at concentrations ranging from 0.07 μM to 19 μM. Plates were incubated at 37°C and read every 2 days for 12 days on an OPTIMA microplate reader (BMG Labtech) using the bottom-read setting with excitation at 485 nm and emission at 520 nm.

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REFERENCES

SI APPENDIX

“Selective small molecule inhibitor of the Mycobacterium tuberculosis fumarate hydratase reveals an allosteric regulatory site”

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I. SI METHODS

Protein Expression and Purification

A pNAN plasmid containing N-terminal His$_6$-tagged M. tuberculosis fumarate hydratase (obtained from Dr. David Garboczi, National Institute of Allergy and Infectious Diseases) was transformed into E. coli Bl21(DE3) cells. The transformed host was grown for 16 h at 37 °C on lysogeny broth (LB) agar supplemented with 100 µg/mL ampicillin.

A single freshly transformed cell colony was grown in a starter culture (10 mL of LB media containing 100 µg/mL ampicillin) at 37 °C with shaking for 5 h. The starter culture was used to inoculate 1 L of LB media (100 µg/mL ampicillin), and the culture was grown at 37 °C with shaking to an OD$_{600}$ of ~0.6 before induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The temperature of the system was decreased to 15 °C, and the culture was grown for an additional 16 h. The cells were harvested by centrifugation (4,000 rpm, 20 min, 4 °C) using a Beckman Coulter Avanti J-20 XP centrifuge.

Cell pellets were resuspended in 10 mM imidazole in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl). Cells were lysed using a French press and centrifuged (16,000 rpm, 1 h, 4 °C). Chromatography was conducted using an Akta FPLC (GE Healthcare). The lysate was loaded onto a HisTrap$^\text{TM}$FF column (GE Healthcare) equilibrated with 10 mM imidazole in lysis buffer. The column was washed using 12 column volumes of 10 mM imidazole in lysis buffer, and the protein was eluted using a 20 column volume gradient of 10 mM to 1 M imidazole in lysis buffer. Fractions containing the fumarate hydratase protein were pooled and further purified on a Superdex 200 16/600 column (GE Healthcare), equilibrated with 10 mM Tris pH 8.0, 150 mM NaCl, and 0.5 mM TCEP. A centrifugal concentrator was used to yield purified protein, and the concentration was determined by amino acid analysis. Aliquots were flash-frozen in liquid nitrogen and stored at -80 °C until further use.
High-throughput Screen and Counterscreens

For the primary high-throughput screen, 3 \( \mu \text{L} \) of a (4/3)x solution containing all reagents except fumaric acid were dispensed into a 1,536-well Greiner black plate via a BioRAPTR dispenser (Beckman Coulter). 23 nL of compounds were single-pin transferred via a pin tool (Kalypsys) equipped with 1536 slotted pins (V&P Scientific). The plates were incubated for 10 min at room temperature. 1 \( \mu \text{L} \) of a 4x solution of fumaric acid was dispensed into the plate via the BioRAPTR dispenser. The plate was centrifuged for 15 s at 1,000 rpm and read immediately on a ViewLux High-throughput CCD imager (Perkin-Elmer) using a filter set with excitation at 525 nm and emission at 598 nm. The plate was read again after 5 min. The change in fluorescence intensity between the two reads was normalized against no-inhibitor and no-enzyme controls and the resulting percent inhibition data were plotted. Compounds were plated as four-point titrations as previously described [1, 2].

The citrate synthase counterscreen modified the primary high-throughput screening protocol in the following ways: (1) the citrate synthase and acetyl-CoA components were removed from the reagent mixture and (2) plates were imaged at a 20 min interval. The malate dehydrogenase counterscreen modified the primary protocol in the following ways: (1) the fumarate hydratase, citrate synthase, and acetyl-CoA components were removed from the reagent mixture and (2) in place of a 4x solution of fumaric acid, the reaction was initiated with a 4x solution of malic acid, to yield a final concentration of 40 \( \mu \text{M} \). The diaphorase counterscreen modified the primary protocol in the following ways: (1) the fumarate hydratase, malate dehydrogenase, NAD\(^+\), citrate synthase, and acetyl-CoA components were removed from the reagent mixture and (2) in place of a 4x solution of fumaric acid, the reaction was initiated with a 4x solution of NADH, to yield a final concentration of 15 \( \mu \text{M} \).
Compound Characterization of N-(5-(azepan-1-ylsulfonyl)-2-methoxyphenyl)-2-
(4-oxo-3,4-dihydropthalazin-1-yl)acetamide (7)

LCMS analysis was performed using a Waters Aquity UPLC HClass LCMS. A 3.5 min
runtime with a 2 min gradient of 5% to 95% acetonitrile in water containing 0.1% (v/v)
formic acid was used. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker DPX 400
MHz spectrometer. Chemical shifts are reported in ppm relative to residual undeuterated
DMSO from the DMSO-d$_6$ solvent. High-resolution mass spectrometry was recorded using
a Waters LCT Premier high-resolution mass spectrometer using electrospray ionization in
the positive mode.

Compound characterization of 7: LCMS retention time: 1.90 min; $^1$H NMR (400 MHz,
DMSO-d$_6$): $\delta$ 12.62 (s, 1H), 9.86 (s, 1H), 8.46 (d, J = 2.2 Hz, 1H), 8.29 – 8.26 (m, 1H),
7.96-7.94 (m, 2H) 7.88-7.84 (m, 1H), 7.49 (dd, J = 8.7, 2.4 Hz, 1H), 7.24 (d, J = 8.7 Hz,
1H), 4.22 (s, 2H), 3.97 (s, 3H), 3.16-3.08 (m, 4H), 1.63-1.52 (m, 4H), 1.48-1.43 (m, 4H);
$^{13}$C NMR (125 MHz, DMSO-d$_6$) $\delta$ 168.3, 159.4, 152.0, 142.0, 133.5, 131.5, 130.4, 129.8,
127.6, 127.5, 125.9, 125.8, 123.4, 119.11, 119.10, 111.2, 56.3, 47.6 (2), 28.4 (2), 26.3 (2);
HRMS (ESI) m/z (M+H)$^+$ calcd. for C$_{23}$H$_{27}$O$_3$N$_4$S 471.1697, found 471.1691.

Crystallization, Data Processing, and Structure Determination

For the formate-bound structure, crystallization trials were performed using a Crystal
Phoenix crystallization robot (Art Robbins Instruments LLC) with the commercial screens
JCSG+ (Qiagen), PEGS I (Qiagen), and Wizard (Emerald BioStructures). After screening
and optimization of crystallization conditions, diffraction-quality crystals were exclusively
obtained in crystallization conditions containing magnesium formate. Crystals were grown
by sitting drop vapor diffusion at 20°C in 17.0% [w/v] PEG3350, 5% [v/v] DMSO, and
200 mM magnesium formate, by combining 2 $\mu$L of a 14 mg/mL protein solution with 1 $\mu$L
of reservoir. The buffer composition of the protein solution was 10 mM Tris pH 8.0, 150
mM NaCl, and 0.5 mM TCEP.

For the 7-bound crystals, both co-crystallization trials and soaking were attempted. Co-
crystallization screens were also performed using a Crystal Phoenix crystallization robot
(Art Robbins Instruments LLC) with the commercial screens JCSG+ (Qiagen), PEGS I
(Qiagen), and Wizard (Emerald BioStructures). Co-crystallization screening and optimiza-
tion trials did not yield diffraction-quality crystals. Soaking experiments were conducted with the formate-bound crystals. Diffraction-quality crystals were obtained from soaking of the formate-bound crystals, by adding 2 µL containing a saturated solution of 7 in 19% PEG3350 [w/v], 7.5% [v/v] DMSO, and 200 mM magnesium formate to the sitting drop.

X-ray data were collected at a wavelength of 0.969 Å at beamline I24 and at 0.979 Å at beamline I04 of the Diamond Light Source synchrotron (Oxfordshire, United Kingdom). Both structures were solved by molecular replacement using PHASER [3]. The unbound M. tuberculosis fumarate hydratase (PDB ID: 3NO9) was used as a search model for the formate-bound structure, and the formate-bound structure was used as a search model for the 7-bound structure. For the formate-bound structure, automated model rebuilding was performed using Buccaneer [4] followed by manual rebuilding and refinement in Coot [5]. The 7-bound structure was manually rebuilt in Coot, based on the molecular replacement solution from PHASER. Structures were refined using Refmac5 [6] in the CCP4 program suite [7] and the Phenix suite [8], with 5% of the reflections excluded from refinement for Rfree calculations.

Some of the residues from the N- and C-termini of each subunit and from the loop of residues 315 – 323 were omitted from the models due to missing electron density. For the formate-bound structure, missing residues are as follows – subunit A: -20 – 9 and 473 – 474; subunit B: -20 – 9, 15 – 16, and 473 – 474; subunit C: -20 – 6, 317 – 322, and 467 – 474; subunit D: -20 – 7, 317 – 323, and 470 – 474. For the 7-bound structure, missing residues are as follows – subunit A: -20 – 8, 15 – 18, 316 – 323, and 468 – 474; subunit B: -20 – 9, 14 – 19, and 468 – 474; subunit C: -20 – 8, 315 – 324, and 467 – 474; subunit D: -20 – 17, 315 – 323, and 467 – 474. Although the N-termini of both structures are disordered, they do not appear to have any influence on the observed conformational changes. Ligand densities for 7 are clearly observed, except for the distal side of the cycloheptanyl substituent, which is disordered. Ramachandran statistics calculated with Molprobity [9] show no outliers, with 2.6% in allowed and 97.4% in Ramachandran favored regions for the formate-bound structure and no outliers, with 2.2% in allowed and 97.8% in favored regions for the 7-bound structure. Coordinates and structure factors for the formate-bound structure and the complex with 7 have been deposited in the Protein Data Bank under accession numbers 5F92 and 5F91, respectively.
Human Fumarate Hydratase Selectivity

30 µL of a (4/3)x solution containing all reagents except fumaric acid was added to a 384-well plate. 1 µL of compound or vehicle was added, and the plate was incubated at room temperature for 10 minutes. 10 µL of a 4x solution of fumaric acid was added to the plate. The plate was centrifuged for 15 s at 1,000 rpm and read every 15 seconds in kinetic mode on a PHERAstar microplate reader (BMG Labtech) for 30 minutes using a filter set with excitation at 550 nm and emission at 590 nm.

Assessment of Colony-Forming Units of *M. tuberculosis* H37Rv under Anaerobic Conditions

Non-replicating persistence (NRP)-2 cultures of *M. tuberculosis* strain H37Rv were obtained by diluting early log phase cultures 100-fold in Dubos medium (Difco) and placing them in wax-sealed bottles with a head:space ratio of 0.5 for three weeks as previously described [10]. Cultures were opened and further manipulation was performed in an anaerobic chamber (MACS MG 1000 Anaerobic Workstations, Don Whitley Scientific) maintained under an atmosphere of 10:10:80 H₂:CO₂:N₂. In a 24-well plate, 980 µL of culture was added to 20 µL of drug or vehicle. The fumarate hydratase inhibitor was tested at concentrations of 200 µM, 50 µM and 12.5 µM. The metronidazole control was dosed at 100 µM, and the isoniazid control was dosed at 10 µg/mL. The plate was incubated in the anaerobic chamber at 37°C for 7 days. Cells were diluted by 10³ and 10⁴, and 100 µL was plated onto 10 cm 7H11-agar containing plates. The plates were incubated at 37°C for 5 weeks and then viable colonies were counted.
II. SI TABLES

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*Values for highest resolution shell are shown in parentheses

**TABLE S1. Data collection and refinement statistics for the fumarate hydratase structures.**
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**TABLE S2.** Apparent \( V_{\text{max}} \) and \( K_m \) values for the curve fits shown in Fig. 3d.
FIG. S1. Validation of the high-throughput screening assay. (a) The fluorescence output of the primary high-throughput screening assay measured at different concentrations of fumarate hydratase. Fluorescence production is linear for the first seven minutes at enzyme concentrations below 20 nM. Data are reported as an average of replicates (n=64) and error bars indicate s.e.m. (b) The initial reaction rate in the assay plotted as a function of fumarate hydratase concentration. The initial rate changes proportionally with changes in enzyme concentration below 20 nM. Linearity indicates that the assay is accurately monitoring fumarate hydratase activity.
FIG. S2. Counterassays used to eliminate compounds affecting other assay components.
(a) Schematic of the fluorescence-based counterassay used to eliminate compounds affecting citrate synthase activity. The assay mimics the primary high-throughput screening assay, but lacks citrate synthase and acetyl-CoA. (b) Schematic of the fluorescence-based counterassay used to eliminate compounds affecting malate dehydrogenase activity. (L)-malate (2) is used to initiate the reaction, and oxaloacetate (3) is produced. The assay is monitored by measuring the conversion of resazurin (5) to resorufin (6). (c) Schematic of the fluorescence-based counterassay used to eliminate compounds affecting diaphorase activity. NADH is used to initiate the reaction, and the assay is monitored by measuring the conversion of resazurin (5) to resorufin (6).
FIG. S3. The structures of two compounds that were identified in the high-throughput screen. The similarities between 7 and 8 resulted in further investigation of the more potent compound, 7.
FIG. S4. The synthetic scheme for resynthesis of 7. (a) Synthetic scheme for 2-(4-oxo-3,4-dihydropthalazin-1-yl)acetic acid [11]. (b) Synthetic scheme for 5-(azepan-1-ylsulfonyl)-2-methoxyaniline [12]. (c) Amide coupling reaction to generate 7 [13]. Final compound characterization data is provided in the SI Methods.
FIG. S5. Formate ions mimic the binding mode of (L)-malate in the fumarate hydratase active site. (a) The superimposition of the formate-bound fumarate hydratase active site residues (blue) with those of the (L)-malate bound structure (gray, PDB ID: 4ADL). The formate ions are shown in magenta, and the (L)-malate is shown in green. The carboxylates of (L)-malate superimpose with the formate ions. (b) Interactions made by the carboxylates of (L)-malate with residues in an enzyme active site. (c) The interactions of the formate ions with residues in an enzyme active site. The formate ions make the same contacts as the carboxylates of (L)-malate. Residues are colored according to subunit (light green: subunit A, light purple: subunit C, pink = subunit D), and residues from three of four subunits contribute to each active site.
FIG. S6. The relative positions of the formate-bound and unoccupied active sites within the fumarate hydratase tetramer. Two of the four fumarate hydratase active sites (dashed circles) are occupied with substrate-mimicking formate ions (represented as yellow spheres). The active sites nearest to the C-terminal domains of subunits C and D contain formate ions. The remaining two active sites, nearest to the C-terminal domains of subunits A and B, are unoccupied. The binding of formate in only two of the four active sites is most likely due to crystal packing forces, as previously suggested [14]. Colors represent the subunits of the tetramer (green: subunit A, blue: subunit B, purple: subunit C, red: subunit D).
FIG. S7. Crystal contact between two symmetry-related fumarate hydratase tetramers. While the allostERIC binding site between the C-termini of subunits A (red) and C (light blue) is occupied by two molecules of 7 (yellow, stick representation), the putative allostERIC binding site between subunits B (green) and D (light orange) is occupied by the C-terminus of subunit C (dark grey) of a symmetry-related molecule (light grey), preventing the binding of 7 at this site.
FIG. S8. The active sites adjacent to each allosteric site. (a) A cartoon representation showing the location of bound formate ions in each active site in both the formate-bound and the 7-bound structure. Each subunit is represented by a different color (green: subunit A, blue: subunit B, purple: subunit C, red: subunit D), and the C-terminal and N-terminal domains are labeled with a ‘C’ or ‘N’, respectively. In the formate-bound structure, two of four active sites are occupied by formate ions. In the 7-bound structure, the active site adjacent to 7 loses the electron density for both formate ions. The active site adjacent to the crystal contact loses the electron density for one of two formate ions. The loss of this one additional formate ion near the crystal contact could be due to crystal packing effects resulting from the change in the unit cell length. (b) Superimposition of the fumarate hydratase enzyme bound with formate (gray) or 7 (colored). In the 7-bound structure, the allosteric site between the C-termini of subunits B (blue) and D (red) is occupied by a crystal contact with a symmetry-related fumarate hydratase tetramer (black). A rotation in the C-terminal domain of subunit D results in the loss of the electron density for one of two formate ions in the adjacent active site. The one remaining formate ion is depicted as a yellow sphere. Subunits A and C are depicted in green and purple, respectively.
FIG. S9. **Identical protein-ligand interactions are observed for each of the two molecules of 7.** (a) Arg432 and Leu429 on protein subunit C make stacking and hydrogen bonding interactions with one of the molecules of 7. (b) Identical interactions involving Arg432 and Leu429 on protein subunit A are observed with the other molecule of 7.
FIG. S10. **Stereogram of the inhibitors in their electron density.** A stereogram of the electron density surrounding the allosteric site, showing the bound inhibitors. Subunit A (light grey) and subunit C (dark grey) of the tetrameric complex form a new binding pocket, where two molecules of inhibitor 7 bind. Hydrogen bonds made by the inhibitor with surrounding amino acids and solvent molecules are shown as dashed lines.
FIG. S11. The ‘closed’ and ‘open’ enzyme conformations of the *M. tuberculosis* fumarate hydratase. (a) Superimposition of a fumarate hydratase subunit from a structure bound with (L)-malate (gray; PDB ID: 4ADL) or formate (colored) in the nearest active site. In both structures, the C-terminal domain adopts the ‘closed’ conformation associated with substrate binding. (b) Superimposition of a fumarate hydratase subunit from the unbound structure (gray; PDB ID: 3NO9) or with 7 bound to the adjacent allosteric site (colored). When 7 is bound, the subunit is locked in the ‘open’ conformation, unable to participate in substrate binding.
FIG. S12. **Electron density of active site loop residues is lost upon binding of 7.** (a) The electron density surrounding loop residues 314A-325A in the formate-bound structure. The portion of the $2F_o - F_c$ electron density map is depicted as a blue mesh and contoured at $1\sigma$. Ser318A, Ser319A, and Lys324A are key active site residues that form hydrogen bonds with the formate ions. (b) In the structure with 7 bound, the electron density surrounding residues 316A-323A is lost, and these residues cannot be modeled.
FIG. S13. **Hydrogen bond interaction stabilizes loop residues in the enzyme active site.**

In the ‘closed’ conformation when formate is bound (gray), a stabilizing hydrogen bond is formed between a C-terminal domain residue (Asn413C) and the backbone oxygen atom of an active site loop residue (Ile320A). This interaction is lost upon movement of the C-terminal domain into the ‘open’ conformation (purple), and the active site loop residues 316A-323A can no longer be modeled.
FIG. S14. Compound 7 demonstrates selectivity over the human fumarate hydratase.

(a) The sequence alignment of the *M. tuberculosis* fumarate hydratase (top) and the human fumarate hydratase (bottom) as determined by the Standard Protein Basic Local Alignment Search Tool developed by the National Center for Biotechnology Information. Active site residues are highlighted in yellow, and allosteric site residues are highlighted in red. (b) A superimposition of the residues in the *M. tuberculosis* fumarate hydratase (color) and the human fumarate hydratase (gray, PDB ID: 3E04) at the location of the allosteric site. (c) The fluorescence output of the high-throughput screening assay measured at different concentrations of human fumarate hydratase. Fluorescence production is linear at enzyme concentrations below 20 nM. Data are reported as an average of replicates (n=2) and error bars indicate s.e.m. (d) The initial reaction rate in the human fumarate hydratase assay plotted as a function of enzyme concentration. The initial rate changes proportionally with changes in enzyme concentration below 20 nM. Linearity indicates that the assay is accurately monitoring human fumarate hydratase activity. (e) The reaction progress curves for human fumarate hydratase in the presence of varying concentrations of inhibitor 7. The presence of 7 does not affect the progress of the reaction.
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DMSO

10 μg/mL isoniazid control

12.5 μM compound 7

50 μM compound 7

200 μM compound 7

100 μM metronidazole control

FIG. S15. Treatment of *M. tuberculosis* H37Rv cells with the fumarate hydratase inhibitor 7 under anaerobic conditions does not demonstrate a significant effect on bacterial growth. Controls include a DMSO-treated culture (black), an isoniazid-treated negative control (red), and a metronidazole-treated positive control (pink). Data are represented as an average of replicates (n=4) and error bars indicate s.e.m.
IV. SI REFERENCES


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