Diarylcoumarins inhibit mycolic acid biosynthesis and kill *Mycobacterium tuberculosis* by targeting FadD32

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Infection with the bacterial pathogen *Mycobacterium tuberculosis* imposes an enormous burden on global public health. New antibiotics are urgently needed to combat the global tuberculosis pandemic; however, the development of new small molecules is hindered by a lack of validated drug targets. Here, we describe the identification of a 4,6-diaryl-5,7-dimethyl coumarin series that kills *M. tuberculosis* by inhibiting fatty acid degradation protein D32 (FadD32), an enzyme that is required for biosynthesis of cell-wall mycolic acids. These substituted coumarin inhibitors directly inhibit the acyl-acyl carrier protein synthetase activity of FadD32. They effectively block bacterial replication both in vitro and in animal models of tuberculosis, validating FadD32 as a target for antibiotic development that works in the same pathway as the established antibiotic isoniazid. Targeting new steps in well-validated biosynthetic pathways in antitubercular therapy is a powerful strategy that removes much of the usual uncertainty surrounding new targets and in vivo clinical efficacy, while circumventing existing resistance to established targets.

Tuberculosis is one of the leading causes of death by infectious diseases worldwide, killing an estimated 2 million people annually (1). The emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* has increased the threat that this disease poses to global public health. Despite a few recent successes (2–4), there are relatively few candidates in the drug development pipeline for tuberculosis. Although there is a substantial amount of genetic data defining essential genes in *M. tuberculosis* (5, 6), little is known about which of the approximately ~600 predicted essential proteins are possible drug targets. To meet current and future therapeutic needs, the discovery and validation of new drug targets and novel chemical structures that target these proteins is a critical priority.

Recent years have seen an enormous increase in efforts to discover new molecules with novel mechanisms using both whole-cell screening and mechanism-based biochemical approaches (7); however, progress in validating new targets has been slow. Although there are numerous reports of small molecules with activity against *M. tuberculosis*, target identification remains a significant challenge. Similarly, although many potential targets have been proposed based on genetic and biochemical experiments, chemical and biological validation that these targets can be inhibited by drug-like molecules with efficacy in vivo is for the most part lacking. There are very few reports of new molecules with new targets that are effective in vivo. Bedaquiline, a diarylquinoline that targets bacterial ATP synthase, was recently provisionally registered by the Food and Drug Administration and is the only candidate molecule in clinical trials that has both a clearly defined and novel target (4). Other compounds in clinical trials include PA824 and Delaminid, both of which have complex mechanisms and targets that have not been clearly defined, and Linezolid, a ribosomal inhibitor that has been repurposed for *M. tuberculosis* treatment (8, 9). Molecules that inhibit new targets and have demonstrated efficacy in animals, but are not yet in clinical trials, include Benzothiazinones that target decaprenylphosphoryl-β-d-ribose 2′-epimerase (DprE1) (2) and inhibitors of malate synthase, a glyoxylate shunt enzyme (10). In addition to their potential as drug candidates, these molecules are significant for having facilitated the identification of novel targets for further efforts geared toward drug discovery.

Herein, we report the identification of a small molecule that kills *M. tuberculosis* by inhibiting FadD32, an enzyme required for mycolic acid biosynthesis, using an unbiased whole-cell screening approach. Although FadD32 is not targeted by any known drug, mycolic acid biosynthesis is one of the few well-validated pathways in antituberculosis drug development. Isoniazid (INH), a central component of the more effective antituberculosis treatment regimens, similarly targets mycolic acid biosynthesis through inhibition of InhA (11). Because resistance to INH is on the rise worldwide, with an estimated 13% of tuberculosis cases exhibiting resistance to this important drug, its long-term utility may be limited. As a result, significant effort has been directed toward identifying novel inhibitors of InhA (12–14), including an effort by Glaxo-Smith Kline and the TB Alliance. Identification of a drug that targets the critical pathway of mycolic acid biosynthesis at a step that is distinct from InhA, thereby bypassing INH resistance, would have a major impact on treatment of MDR and XDR tuberculosis. Importantly, the FadD32 inhibitor we have identified has activity in animal models of tuberculosis that is comparable with that of INH.

Results

Identification of a Series of Substituted Coumarin Inhibitors with Activity Against *M. tuberculosis*. To identify novel compounds with antimycobacterial activity, we modified a reported GFP-based high-throughput assay (15) and screened a total of 20,502 small molecules for their ability to inhibit the growth of the H37Rv strain of *M. tuberculosis* (16). The whole-cell GFP-based assay was designed to identify compounds that target any pathway essential under in vitro growth conditions. From the primary screen, a series of four compounds characterized by a 4,6-diaryl-5,7-dimethyl coumarin core were found to have potent activity against actively replicating *M. tuberculosis* (*SI Appendix*, Fig. S1). Subsequent chemical optimization and the synthesis of over 40 substituted coumarin analogs of the initial screening hits

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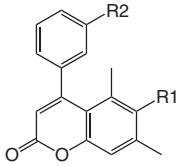
led to the development of a panel of compounds with increased potency (17–19) (*SI Appendix*, Fig. S2). The most potent analog, coumarin core analog 34 (CCA34), had a minimum inhibitory concentration (MIC) of 0.24 μM against H37Rv *M. tuberculosis* (Fig. 1). CCA34 also had good activity against clinical isolates of *M. tuberculosis*, with MICs against five independent isolates ranging from 0.33 to 0.57 μM (*SI Appendix*, Table S1) and *M. tuberculosis* strains with monoresistance to the clinically relevant drugs INH, rifampicin, streptomycin, and ciprofloxacin (*SI Appendix*, Table S1 and Fig. S3). Substituted coumarins had bactericidal activity and CCA34 decreased bacterial numbers by over four logs within 3 d of treatment (*SI Appendix*, Fig. S4*A*). The minimum bactericidal concentration (MBC) of CCA34 was determined to be 1.9 μM, which is comparable with the 0.5 μM MBC of INH.

Previous screens for antitubercular compounds have resulted in the identification of small molecules whose activity is dependent on the carbon source present in the growth medium. In particular, growth on standard glycerol containing media results in the identification of inhibitors that induce "self-poisoning" of *M. tuberculosis* by promoting the accumulation of glycerol phosphate and rapid ATP depletion (20). Inhibitors with this mechanism of action (MOA) are devoid not only of activity on other carbon sources, but notably, of in vivo efficacy. We therefore tested CCA34 activity on alternate carbon sources and found that it was equally active when acetate or valerate was used as the sole carbon source in the growth medium (*SI Appendix*, Fig. S4*B*), demonstrating that these inhibitors have carbon-source independent activity.

Currently there is interest in the identification of inhibitors with activity against *M. tuberculosis* cultured in nonreplicating states in vitro, as the bacilli are proposed to potentially enter a dormant, nonreplicating state in vivo that may contribute to latent infection (21). Carbon-starved *M. tuberculosis* is one such model (22). We tested CCA34 for activity against carbon-starved *M. tuberculosis* and found that it had low but detectable activity against nonreplicating carbon-starved *M. tuberculosis* (*SI Appendix*, Fig. S4*C*), decreasing bacterial numbers in starved cultures by one log when used at the MIC against replicating cells. Substituted coumarins therefore have only limited activity against nonreplicating cells, as is similarly observed with all current antitubercular antibiotics.

In addition to their potency, the substituted coumarins were highly species-selective. The compounds were active against *Mycobacterium bovis* bacillus Calmette–Guérin, *Mycobacterium avium*, *Mycobacterium marinum,* and *Mycobacterium intracellulare,* mycobacterial species that are closely related to *M. tuberculosis,* but were inactive against more distantly related mycobacterial species (*SI Appendix*, Table S1). In addition, these compounds exhibited no activity against any Gram-positive or Gram-negative bacterial species tested (*SI Appendix*, Table S1), indicating that the target of these compounds may be unique to mycobacteria.



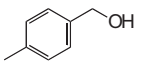
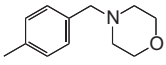
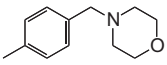
Compound	R1	R2	MIC (μM)
CCA26		H	5.6
CCA2		H	2.6
CCA31		H	0.42
CCA34		NH_2	0.24

Fig. 1. Structures of substituted coumarin analogs and MICs against *M. tuberculosis* H37Rv. The MIC was defined as the concentration at which bacterial numbers were reduced to 99% of untreated control after 14 d of treatment. Bacterial numbers were assessed by plating for cfu.

Mutation of fadD32 confers resistance to substituted coumarins. Generating resistance to an antibiotic followed by next-generation sequencing to identify the basis of resistance has been one useful approach to facilitate target identification of novel small molecules (2, 4). To identify the target of the substituted coumarins, we generated resistance to two analogs identified in the original screen, CCA1 and CCA2. Resistant mutants were generated by plating *M. tuberculosis* on solid 7H10 media containing 10× the MIC of each compound. Resistance was observed at a frequency of 1×10^{-7}, a rate that is comparable with that of other clinically used drugs including rifampicin (3×10^{-8}) and ethambutol (1×10^{-7}), and an order of magnitude lower than observed for INH (1×10^{-6}). These mutants had MICs at least 10-fold higher than the parent strain (Fig. 2*A*) and were resistant to all of the substituted coumarin analogs tested (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S5 *A* and *B*).

To identify the target of the coumarin analogs, we sequenced the genomes of four of the resistant mutants using Illumina sequencing technology and compared the genome of each mutant to the parent clone used in the individual selections (23). Point mutations in a single gene, *fadD32*, were associated with resistance to the substituted coumarins (*SI Appendix*, Table S2). Interestingly, relatively few mutations were identified in each resistant mutant, with two mutant genomes containing only a single point mutation in *fadD32*. Mutations in *fadD32* associated with resistance were found in two codons, E120 and F291, resulting in point mutants E120A, E120V, and F291L. To confirm that these mutations were responsible for conferring resistance to the coumarin inhibitors, the mutant alleles E120A and F291L were expressed in wild-type *M. tuberculosis.* Episomal expression of either mutant but not wild-type FadD32 protein was sufficient to confer resistance to substituted coumarins in the presence of endogenous wild-type FadD32, suggesting that the E120A and F291L alleles are dominant (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S5 *A* and *B*). These data suggest that substituted coumarins kill *M. tuberculosis* by inhibiting FadD32, and that FadD32 may be a good target for antitubercular activity.

The 4,6-diaryl-5,7-dimethyl coumarins represent a unique structural class of compounds with activity against *M. tuberculosis*. Other compounds with a coumarin structural scaffold have been reported to have activity against *M. tuberculosis* (24–26), including novobiocin, an antibiotic that targets bacterial DNA gyrase. We tested whether our CCA1 and CCA2 mutants were cross-resistant to novobiocin. None of the fadD32 mutants had increased resistance to novobiocin (*SI Appendix*, Fig. S6), demonstrating that these compounds act by a mechanism that is distinct from that of novobiocin and confirming that the presence of a coumarin acts as a scaffold, with the substituents providing target specificity. Structure activity relationship (SAR) analysis also demonstrates that the coumarin analogs identified in our screen represent a unique structural series. We found that the presence of the R1 and R3 aromatic substituents is required for antimycobacterial activity (*SI Appendix*, Table S3). In addition, both the R2 and R4 methyl substituents are important contributors as substitution of either of these methyl groups with a hydrogen atom results in a >10-fold loss of activity (*SI Appendix*, Table S3). The requirement for the methyl groups may be due to the impact of the methyl groups on the torsion angles of the two biaryl substituents relative to the coumarin ring (*SI Appendix*, Fig. S7).

The 4,6-diaryl-5,7-dimethyl coumarins inhibit mycolic acid biosynthesis. FadD32 is an essential enzyme (27, 28) that plays a critical role in the biosynthesis of mycolic acids, branched fatty acids consisting of a very long meromycolate chain (C54 to C63) and a shorter alpha alkyl chain (C22 to C24) (29) that

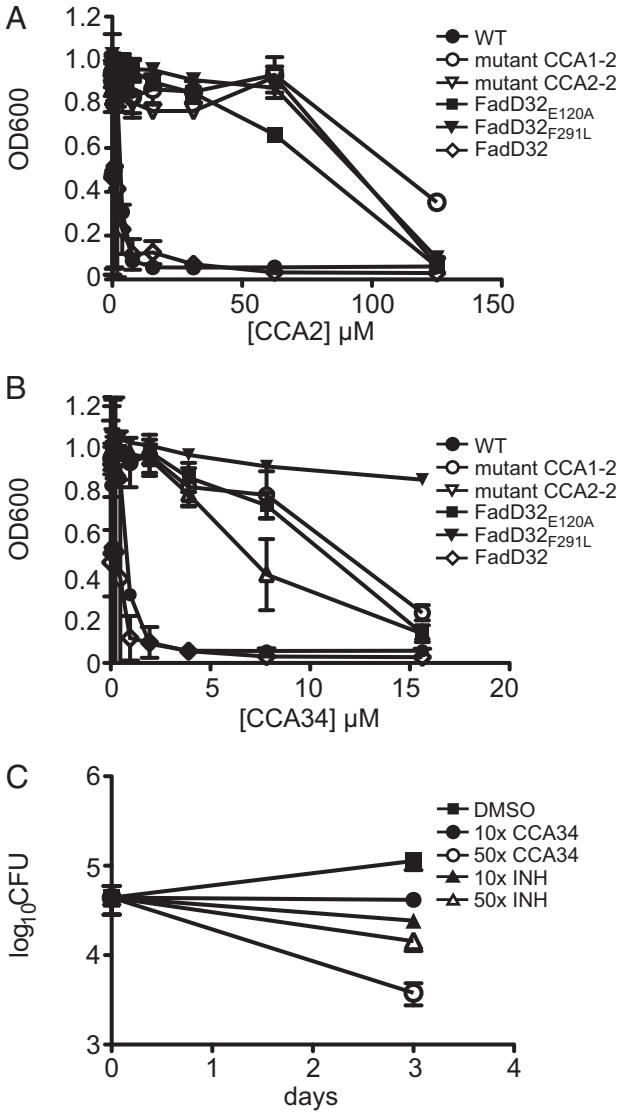


Fig. 2. Activity of substituted coumarins in vitro and in macrophages. Mutants selected on substituted coumarin inhibitors and WT *M. tuberculosis* expressing mutant FadD32 episomally are resistant to high concentrations of (*A*) CCA2 and (*B*) CCA34. (*C*) Substituted coumarin inhibitors are effective at treating infected human PBMC derived macrophages. For the macrophage infection experiment, macrophages were infected at MOI = 1 and were carried out for 3 d. All treatments relative to DMSO control gave $P < 0.02$ ($n = 3$) by the Mann–Whitney *U* test. All experiments were repeated a minimum of three times, and a representative experiment is shown. Error bars are SD.

are the major component of the unique mycobacterial cell wall. They are primarily found as an arabinogalactan-mycolate species covalently linked to the inner peptidoglycan layer, and as a component of a free glycolipid, trehalose dimycolate (TDM), that is a major component of the outer mycomembrane (29). FadD32 is required to activate meromycolic acids to facilitate their condensation with a shorter fatty acid, resulting in the final beta-keto-alpha alkyl mycolic acid. FadD32 has two enzymatic functions. Its fatty acyl-AMP ligase (FAAL) activity first adenylates meromycolic acids generated by the fatty acid synthase (FAS) II biosynthetic pathway, and its fatty acyl ACP synthetase (FAAS) activity results in the transfer of the activated intermediate to the ACP domain of the condensing enzyme polyketide synthase 13 (PKS13) (30, 31) (Fig. 3*A*).

FadD32 is downstream of InhA in the biosynthetic pathway of mycolic acids, and an INH-resistant clinical isolate with an *inhA* promoter mutation [C(-15)T] remains susceptible to coumarin inhibitors (*SI Appendix*, Table S1), as are INH-resistant *katG* mutants (*SI Appendix*, Fig. S3). The lack of cross-resistance between INH and CCA34 resistant mutants likely explains the observation that we were unable to generate resistant mutants in the presence of both drugs. In addition, we examined the interaction of CCA34 with currently used antituberculosis (anti-TB) drugs, including INH, ethambutol, ethionamide, and rifampicin, and found no evidence of synergy or antagonism [fractional inhibitory concentration (FIC) < 4 for all drugs; *SI Appendix*]. However, as substituted coumarins and INH both inhibit targets within the same critical pathway for cell wall biosynthesis, they would be expected to result in similar phenotypic changes to *M. tuberculosis*. This prediction was confirmed in the similar transcriptional profile observed for cells treated with subinhibitory concentrations of coumarin inhibitors and INH. Many INH-responsive genes are also regulated in response to coumarin inhibitors, including *iniA-iniC*, *fas, pks13,* and *fadD32* (32) (*SI Appendix*, Table S4). Similarly, because mycolic acids are critical structural components of the mycobacterial cell wall, treatment with inhibitors of mycolic acid biosynthesis, regardless of the step that is inhibited, should result in comparable alterations in cell-wall architecture. Treatment of bacterial cells with sublethal concentrations of either INH or CCA2 resulted in strikingly similar phenotypic changes to the cell wall of *M. tuberculosis* characterized by loss of the outer electron opaque layer, a thickening of the inner electron opaque layer, and a general rounding of the cell body (*SI Appendix*, Fig. S8).

To directly determine whether 4,6-diaryl-5,7-dimethyl coumarin inhibitors affect mycolic acid biosynthesis, we treated *M. tuberculosis* with 1× or 5× the MIC of CCA2 or CCA34 and monitored production of mycolic acids by TLC using [^{14}C]acetate for labeling. Radiolabeled acetate was added concurrently with inhibitor such that mycolic acids visualized result from synthesis that occurs during the treatment period. We saw that treatment with CCA2 or CCA34 resulted in a striking decrease in the amount of mycolic acids that were synthesized during the treatment time. Treatment with substituted coumarin inhibitors blocked mycolic acid synthesis as effectively as did INH (Fig. 3*B*).

The 4,6-diaryl-5,7-dimethyl coumarins directly inhibit the FAAS activity of FadD32. To conclusively demonstrate that FadD32 is the target of these substituted coumarins, we turned to biochemical assays of FadD32 function assesing the two individual steps catalyzed by FadD32 (31). In the first assay, we tested the FAAL activity of the purified enzyme by monitoring formation of acyl-AMP from [^{14}C]myristic acid and ATP using TLC. As a control, we used the known reaction intermediate mimetic adenosine 5′-dodecylphosphate (AMPC12) (31), a substrate analog that has no activity against intact *M. tuberculosis* but as expected partially inhibits the FAAL activity of *M. tuberculosis* FadD32 (Fig. 3*C*). The FAAL reaction was not inhibited by treatment with CCA34 (Fig. 3*C*), even at the high concentration of 100 μM, indicating that these inhibitors do not affect the FAAL activity of FadD32. To assess the impact of the coumarin inhibitors on the FAAS activity of the enzyme, we next assayed the second step of FadD32, by monitoring acylation of the mycobacterial acyl carrier protein (AcpM) with [^{14}C]acyl-AMP generated from [^{14}C]myristic acid via the acyl-AMP intermediate. We found that CCA34 inhibits the formation of the [^{14}C]acyl-AcpM adduct (Fig. 3*D* and *SI Appendix*, Fig. S9), indicating that these molecules function by inhibiting the ability of FadD32 to transfer the activated intermediate to the ACP domain of PKS13. Similar to previous reports (31), we observed acylated FadD32 in this assay (Fig. 3*E* and *SI Appendix*, Fig. S9) (31), which was also inhibited by treatment with CCA34. In contrast, the substituted coumarins were unable to inhibit formation of the intermediate acylated FadD32 or the final [^{14}C]acyl-AcpM product when the FadD32F291L mutant was tested, further supporting the mechanism of action as well as validating the mechanism of resistance.

The 4,6-diaryl-5,7-dimethyl coumarin inhibitors effectively block bacterial replication in vivo via inhibition of FadD32. The

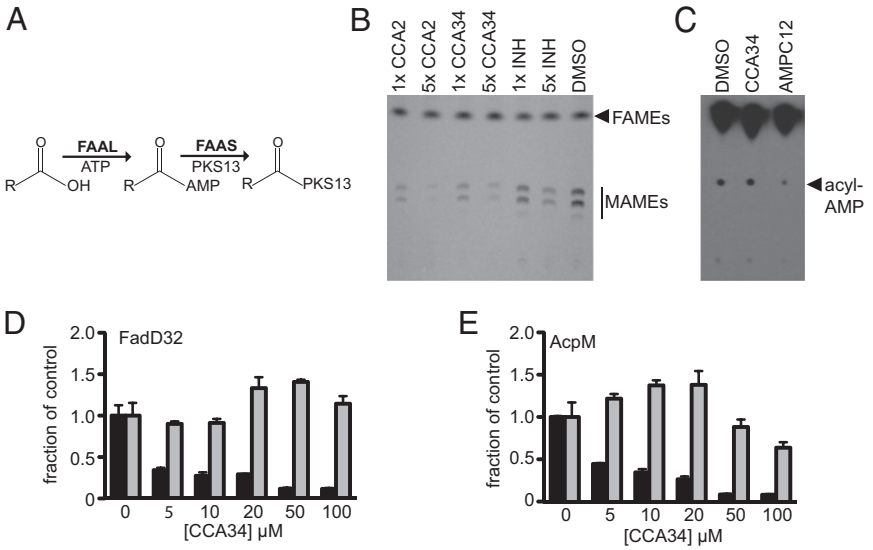


Fig. 3. Substituted coumarin inhibitors target the FAAS activity of FadD32. (*A*) FadD32 has FAAL and FAAS activity. (*B*) Treatment of *M. tuberculosis* with CCA2 or CCA34 inhibits mycolic acid biosynthesis. Cells were treated with 1× or 5× the MIC of inhibitor for 24 h, and mycolic acid synthesis was monitored by incorporation of [^{14}C]acetate. Ester derivatives of mycolic acids (MAMEs) and fatty acids (FAMEs) were visualized by TLC. (*C*) The FAAL activity of FadD32 is unaffected by 100 μM CCA34. Reaction products were separated by TLC, and [^{14}C]acyl-AMP product is indicated by the arrow. (*D*) Loading of the labeled acyl chain from [^{14}C]acyl-AMP onto AcpM is inhibited by CCA34. Reaction products were separated by SDS/PAGE, and acylated AcpM was detected by phosphphorimaging. Black bars, WT FadD32; gray bars, FadD32F291L mutant. (*E*) Loading of labeled acyl chains onto FadD32 is inhibited by CCA34. All experiments were replicated a minimum of three times. For *B* and *C*, a representative experiment is shown. For *D* and *E*, data are the average of three independent experiments, and error bars are SD.

identification of a novel target for drug development requires both chemical and biological validation. Although discovery of a small molecule inhibitor of a specific target that is able to kill the bacterium provides chemical validation, demonstration of the essentiality of that target in animal models is particularly critical in the case of *M. tuberculosis*. Because of the strong biological validation provided by INH for inhibition of mycolic acid biosynthesis in TB therapy, we next turned to investigate the activity of CCA34 against FadD32 in in vivo infection models.

To evaluate the efficacy of these inhibitors against intracellular *M. tuberculosis*, human peripheral blood mononuclear cell (PBMC)-derived macrophages were infected with *M. tuberculosis* and treated with INH or CCA34 for 3 d. CCA34 inhibited bacterial replication when used at the MIC against cells grown in axenic culture. At higher concentrations, CCA34 was able to kill intracellular bacteria, reducing the population by one log (Fig. 2*C*) while having no effect on macrophage viability (*SI Appendix*, Fig. S10). This activity profile compares favorably with that of INH and demonstrates that the FadD32 is a relevant target during intracellular growth in human macrophages.

Infection of zebrafish embryos (*Danio rerio)* with *M. marinum* is a useful model for studying mycobacterial infection, with many important characteristics that parallel *M. tuberculosis* infection in humans (33). Recently, it has been used to demonstrate the activity of antitubercular compounds with *M. marinum* activity (34) and potentially provides a facile means to perform initial in vivo testing of novel compounds. The *M. marinum* ortholog of FadD32 is highly conserved relative to *M. tuberculosis* FadD32 (94% identical over 627 amino acids). We therefore used the zebrafish model for preliminary evaluation of the efficacy of the substituted coumarin inhibitors in an in vivo infection model. Zebrafish embryos were infected 50 h post fertilization (hpf) with *M. marinum* expressing GFP and then immediately immersed in water containing inhibitor for 72 h, at which time bacterial growth was assessed by imaging. The minimum concentration that prevented bacterial replication was 15 μM, ~20× the MIC of CCA34 against *M. marinum* grown in axenic culture, which was used in all subsequent experiments. For comparison, we used 250 μM INH (~30× the MIC in axenic culture), a concentration that was previously reported to effect maximal bacterial killing (34). Embryos treated with CCA34 had no observable bacteria as detected by fluorescence (Fig. 4*A*). To determine whether the inhibition of bacterial proliferation by the coumarin inhibitors could prolong survival of infected embryos, infected embryos were maintained for an additional 8 d after treatment with CCA34. Infected fish treated for 3 d with CCA34 had a statistically significant survival advantage over fish left untreated ($P < 0.0001$; log-rank test).

Although this observed efficacy suggests that FadD32 is essential during infection, the observed efficacy could be due, at least in part, to off-target effects in vivo. One advantage of the zebrafish embryo model is that it allows for the maintenance of constant designated CCA34 concentrations that facilitates the ability to distinguish between sensitive and resistant alleles. To demonstrate that bacterial clearance and embryo survival were due to an on-target effect of FadD32 inhibition, we generated an *M. marinum* strain that is resistant to substituted coumarin

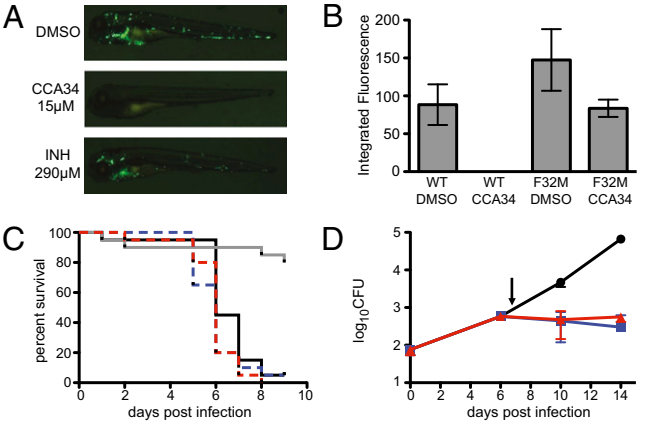


Fig. 4. Validation of FadD32 as a targetable and essential protein for in vivo infection. (*A*) CCA34 prevents replication of *M. marinum* in zebrafish. Treatment of infected zebrafish with 15 μM CCA34 inhibits bacterial proliferation. (*B*) Mutation of FadD32 confers resistance to CCA34 in vivo. Fish were infected with WT or FadD32 mutant (F32M) *M. marinum*, and day 3 bacterial loads were quantified from fluorescent images ($n = 5$ for each condition). (*C*) Treatment of infected zebrafish with CCA34 prologs survival for embryos infected with WT but not F32M. Solid black line, WT/DMSO; solid gray line, WT/15 μM CCA34; dashed red line, F32M/DMSO; dashed blue line, F32M/15 μM CCA34. For all conditions vs. WT infected DMSO treated fish, $P < 0.0001$ by the log rank test ($n = 20$). For all zebrafish figures a representative experiment of three is shown. (*D*) Efficacy of CCA34 in a mouse model of tuberculosis. BALB/C mice infected with *M. tuberculosis* via aerosol were treated with INH (25 mg/kg) or CCA34 (35 mg/kg) beginning on day 7 after infection (indicated by the black arrow) for a total of 7 d. Error bars are SD. *$P < 0.02$ ($n = 5$) using the Mann–Whitney *U* test. A representative experiment of two is shown.

inhibitors and repeated the infection experiment. This resistant mutant possesses a mutation in *fadD32* that corresponds to an amino acid change Y513H (*SI Appendix*, Fig. S11). We infected embryos with wild-type *M. marinum* or the resistant FadD32 mutant. We observed that, in contrast to infection with wild-type *M. marinum*, bacterial numbers of the mutant are not significantly decreased upon CCA34 treatment (Fig. 4*B*). Moreover, embryos infected with the FadD32 resistant mutant succumbed to infection with the same kinetics as the untreated wild-type. ($P < 0.0001$; log-rank test) (Fig. 4*C*). FadD32 is therefore required for *M. marinum* growth in zebrafish embryos, and coumarin inhibitors are able to penetrate host tissues and effectively inhibit proliferation of bacteria via inhibition of FadD32.

Mice are the most commonly used animal model for studying *M. tuberculosis* infection and for testing new small molecule inhibitors. To further validate FadD32 as a promising target for the development of antitubercular therapeutics and to provide a preliminary evaluation of the potential of substituted coumarins for in vivo efficacy, we tested CCA34 in the mouse tuberculosis model of infection. Preliminary studies in mice showed that CCA34 was relatively nontoxic and well tolerated, and an appropriate dosing regimen was developed (*SI Appendix*, *Discussion* and Fig. S12). We next tested CCA34 in BALB/C mice infected with the Erdman strain of *M. tuberculosis* via inhalation of aerosolized bacteria. CCA34 or INH was administered by i.p. injection beginning 1 wk after initial infection. Four doses of CCA34 (35 mg/kg) were administered over a 1-wk period with every-other-day i.p. injection. After 4 d of treatment, there was a fourfold reduction in bacterial numbers in the lungs of CCA34-treated mice compared with DMSO-treated control mice, and, after 8 d, a 30-fold reduction was observed, demonstrating that CCA34 inhibits bacterial proliferation during infection of mice (Fig. 4*D*). The efficacy of CCA34 observed in this short-course experiment was comparable with that of INH, further supporting the idea that targeting FadD32 is a valid and potentially promising therapeutic strategy.

Discussion

In this study, we have identified a series of unique 4,6-diaryl-5,7-dimethyl coumarins that target a step in mycolic acid synthesis that is distinct from that targeted by the well-established TB drug INH and have demonstrated that targeting FadD32 is effective at treating infection in vivo. FadD32 is a member of the long chain fatty acyl-AMP ligase (FAAL) family in *M. tuberculosis* that functions to adenylate a long-chain fatty acid and then transfer this activated intermediate to the polyketide synthase PKS13. Transfer of the activated fatty acid to a PKS is a novel function for FAAL proteins recently described for a subfamily of these enzymes found in *M. tuberculosis* (30, 35), as most members of this family form acyl-CoA thioesters. The mechanistic basis for the different enzymatic activity of FadD32 and related family members is as yet incompletely understood. Notably, these coumarins specifically inhibit the unique step of these enzymes, the acylation of an ACP domain of a PKS, suggesting that these inhibitors will be valuable tools for the investigation of the enzymatic function of this important family of enzymes.

This work establishes FadD32 as a validated in vivo target for *M. tuberculosis* drug discovery. Using both *M. marinum* infection of zebrafish embryos and *M. tuberculosis* infection of mice, we find that CCA34 is able to effectively inhibit bacterial proliferation. Further, zebrafish embryos provide a facile model that allowed us to demonstrate that CCA34 is on-target during in vivo infection.

Our mouse data demonstrate that inhibition of FadD32 by CCA34 can effectively prevent *M. tuberculosis* replication in a mammalian model of tuberculosis. Although CCA34 has yet to be maximally optimized for in vivo efficacy, these initial proof-of-principle studies provide important validation of Fad32 as a valuable in vivo target and motivation for further studies. Optimization of the therapeutic properties of 4,6-diaryl-5,7-dimethyl coumarins, coupled with investigation of these inhibitors in additional in vivo models, may result in exciting new leads for drug development. In addition, the validation of FadD32 as a "druggable" target should spur research into novel compound series that also target this important enzyme. A recent report of the development of a high-throughput screening assay against *Mycobacterium smegmatis* FadD32 suggests the possibility that additional inhibitors that target *M. tuberculosis* FadD32 could be identified using an enzymatic assay (36). Early testing against intact *M. tuberculosis* is important given known difficulties of translating hits from enzymatic assays into leads with activity against whole cells (37). Finally, our study confirms the concept that targeting unique steps in a biosynthetic pathway that is already targeted by a current antibiotic is an effective strategy for antituberculosis drug discovery. In the current debate surrounding antibiotic discovery of whether efforts should be focused on "old" targets that are well validated by current antibiotics or "new" targets that would circumvent existing antibiotic resistance, this work demonstrates that an effective compromise can be found by targeting unique steps in validated pathways, thereby taking advantage of prior validation without preexisting resistance. In that not all essential steps in a biosynthetic pathway can be easily or equally inhibited with a small molecule, this work demonstrates that FadD32 can in fact be targeted efficiently for bactericidal effect.

Inhibition of cell-wall synthesis is a cornerstone of antimicrobial therapy, with the inhibition of mycolic acid biosynthesis by INH historically forming a central component of most standard antitubercular regimens. Although there is much current interest in targeting nonreplicating states of *M. tuberculosis* in the context of shortening TB therapy and managing latent infection (38), it is notable that, despite limited nonreplicating activity in vitro, INH has been important in reducing transmission due to its early bactericidal activity and rapid clearing of sputum (39), and its effective use in treating latent infection (40). Thus, the loss of INH to rising resistance rates creates a major gap in the management of TB, increasing the urgency for new drugs that can quickly fill its role. Strategically, targeting novel steps in validated pathways has the clear advantages of a high likelihood of therapeutic efficacy, while bypassing existing resistance to current pathway inhibitors. Our work demonstrates that FadD32 is a unique target in a validated pathway with significant promise. Thus, the chemical and biological validation of FadD32 as an essential protein and therapeutic target opens up an important avenue for the development of therapeutics.

Materials and Methods

Bacterial Strains and Growth Conditions. TB strains used were H37Rv, Erdman, mc^26020 (lysine/pantothenate auxotroph), or specified clinical isolates obtained from the Massachusetts Supranational TB Reference library. The following strains were also used: bacillus Calmette–Guérin Pasteur, *M. smegmatis* MC^2155, *M. marinum* strain M, *Escherichia coli* K12, *Vibrio cholerae* strain 0395, *Pseudomonas aeruginosa* PA14. For *Staphylococcus aureus* and *Enterococcus faecalis*, clinical isolates were used. *M. tuberculosis* H37Rv strains expressing GFP, WT FadD32, or mutant FadD32 were constructed using a pUV15tetORm derivative (41) with the tet repressor deleted for constitutive expression. Mycobacterial strains were cultured in Middlebrook 7H9 medium with 0.05% Tween 80 and oleic acid/albumin/dextrose/catalase (OADC) supplement. Cultures were starved for 5 wk in starvation medium (7H9 medium with no added glycerol and 0.05% tyloxapol).

Determination of IC_{90}s, MICs, and Small Molecule Inhibition Assays. For determination of IC_{90}, and for assessing resistance, OD_{600}-based assays were used. Bacteria were grown to midlog phase and plated in 96-well plates at OD_{600} = 0.025 in the presence of small molecule inhibitors for indicated time periods, and growth was assessed by reading OD_{600}. The IC_{90} value was determined as the lowest concentration that inhibited growth by 90% relative to the DMSO control. For MIC determinations, cultures were set up as described above; however, plates were incubated for a total of 14 d, at which time bacteria were plated on agar plates for enumeration of cfu. The MIC was determined as the concentration of inhibitor that resulted in 99% inhibition of growth relative to the DMSO control. The IC_{90} and MIC values given are the average from a minimum of two and up to five experiments.

Macrophage Infection Assays. Human peripheral blood monocytes were isolated and differentiated in 25 ng/mL human macrophage colony stimulating factor (MCSF). After 5 d, the cells were washed with PBS and infected with *M. tuberculosis* at a multiplicity of infection (MOI) = 1 in DMEM plus 5% (vol/vol) FBS/5% (vol/vol) horse serum. The infection was carried out for 4 h, at which time the monolayer was washed once and RPMI with DMSO or inhibitor was added. After 3 d, the cells were washed with PBS and lysed in water plus 0.5% Triton X-100. Dilutions of lysate were prepared in PBS plus 0.05% Tween 80 and were plated onto agar 7H10 plates for enumeration.

In Vitro Assays of FadD32 Function. His-tagged FadD32 WT and mutant proteins were expressed in Tuner DE3 cells (EMD Chemicals), which were were grown at 30 °C overnight with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were lysed in 50 mM Tris, pH 7.5, 500 mM NaCl, 10 mM imidazole, and 0.75 mg/mL lysozyme by sonication. Lysate was purified over nickel nitrilotriacetic acid (Ni-NTA) agarose with 250 mM imidazole elution followed by desalting with 10G desalting column (Bio-Rad), and protein was eluted into 50 mM Tris, pH 7.5, 500 mM NaCl. AcpM was purified as described for FadD32 with 3 h IPTG induction at 37 °C. FAAL assays were performed as previously described (31). FAAS assays were performed as described (31) with the following modifications. The reaction mixture consisted of 100 μM [1-^{14}C] myristic acid, 2 mM DTT, 10 mM $MgCl_2$, 5 mM ATP in 20 mM Hepes, 5 μM FadD32, and 5–10 μM AcpM. Reactions were incubated for 16 h at 30 °C and analyzed by SDS/PAGE using a 10–20% gel. Quantitation of the transfer of the acyl-AMP onto AcpM was achieved using phosphorimaging.

Infections of Zebrafish and Mice. Infection of zebrafish with *M. marinum/GFP* was carried out as described previously (34, 42). Zebrafish embryos from the AB line staged 50 hpf were microinjected with *M. marinum/GFP* into the yolk circulation valley. Infected embryos were immediately immersed in embryo medium (E3) containing inhibitor or DMSO control and incubated at 29 °C. Fluorescent images were acquired using a Zeiss DiscoveryV.12 stereomicroscope and analyzed using CellProfiler. For time-to-death experiments, infected embryos were exposed to inhibitor for a period of 3 d, after which time embryos were maintained in media without inhibitor for the duration of the experiment. Embryos were observed every day for survival with the scoring of living versus dead being ascertained by the presence of a heartbeat and circulating blood under a stereomicroscope. For mouse infections, bacteria were grown to midlog phase and sonicated to remove clumps. BALB/C mice were infected with ∼80 cfu of Erdman *M. tuberculosis* via the aerosol route using a Madison chamber (University of Wisconsin, Madison, WI). Compounds were administered by i.p. injection in a total volume of 100 μL of DMSO. Organs were homogenized in PBS plus 0.05% Tween 80 and plated onto 7H10 agar enriched with 10% OADC (Difco) for enumeration of cfu. All experiments performed using mice were approved by the Harvard Medical Area Standing Committee on Animals. All zebrafish experiments were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

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